

# Informe de la reunión de la Comisión de Normas Biológicas de la OMSA

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5 a 9 de febrero de 2024

París

## Introducción y contribución de los Miembros

Este informe presenta el trabajo de la Comisión de Normas Biológicas de la OMSA (denominada en adelante "la Comisión"), que se reunió en París, Francia, del 5 al 9 de febrero del 2024.

Durante la reunión, se aprobaron 13 capítulos del *Manual de las Pruebas de Diagnóstico y de las Vacunas para los Animales Terrestres* de la OMSA (*Manual Terrestre*), que se distribuirán para una segunda ronda de comentarios por parte de los Miembros y se propondrán para adopción en la Sesión General de mayo de 2024. La Comisión desea agradecer a los siguientes Miembros por sus comentarios sobre los borradores de los textos para el *Manual Terrestre* de la OMSA distribuidos con el informe de septiembre de 2023 de la Comisión: Canadá, China (Rep. Pop. de), Estados Unidos de América (EE.UU.), Japón, Nueva Zelanda, Suiza, Reino Unido (RU) y los Estados miembros de la Unión Europea (UE). La Comisión también desea agradecer el valioso asesoramiento y las contribuciones de numerosos expertos de la red científica de la OMSA.

La Comisión revisó todos los comentarios que se presentaron antes de la fecha límite y que estaban respaldados por una justificación. En los casos en que las enmiendas eran de carácter editorial, no se ha facilitado ningún texto explicativo. La Comisión desea señalar que, cuando no se aceptaron los textos propuestos por los Miembros para mejorar la claridad, consideró que el texto era claro tal como estaba redactado. La Comisión ha introducido modificaciones en los borradores de los textos, en su caso, de la forma habitual mediante "doble subrayado" y "tachado". En los anexos pertinentes, las enmiendas propuestas en esta reunión se destacan en amarillo para distinguirlas de las realizadas anteriormente.

Valoramos su participación en el proceso normativo de la OMSA. Gracias por su compromiso con el proceso.

Durante la reunión también se evaluaron diez solicitudes de candidatura a designación como centro de referencia y diez candidaturas a designación como expertos sustitutos.

## Anexos

Los textos de los **Anexos 4 a 16** se propondrán para su aprobación en la 91ª Sesión General, que tendrá lugar en mayo de 2024.

## Para enviar los comentarios

La Comisión de Normas Biológicas anima encarecidamente a los Miembros de la OMSA y a las organizaciones internacionales con un Acuerdo de Cooperación con la OMSA a participar en la elaboración de las Normas Internacionales de la OMSA mediante la presentación de comentarios sobre los anexos pertinentes de este informe.

La participación de los Miembros y de las organizaciones internacionales en el proceso de elaboración de normas mediante la presentación de comentarios es fundamental para garantizar que el trabajo de la Comisión tenga una base científica y tenga en cuenta los diferentes contextos de los Miembros y las partes interesadas, y permita la aplicación de las normas. Para que las observaciones sean tenidas en cuenta, deberán presentarse dentro del plazo y en el formato descritos en los documentos [guía](#) y sobre [POE](#) que pueden consultarse en la página web de los Delegados y en el sitio web público de la OMSA.

Los comentarios que no tengan el formato correcto descrito en la [guía](#), podrían no ser tenidos en cuenta por la Comisión. Toda pregunta sobre los requisitos de formato y presentación de observaciones debe enviarse a [BSC.Secretariat@woah.org](mailto:BSC.Secretariat@woah.org)

La Comisión de Normas Biológicas desea subrayar que, cuando un debate de la Comisión se basa en las aportaciones de un Grupo *ad hoc*, se anima a los Miembros a examinar el informe del Grupo *ad hoc* pertinente junto con el informe de la Comisión. Los informes del Grupo *ad hoc* pueden consultarse en las páginas web específicas del sitio web de la OMSA: [Grupos ad hoc - OMSA - Organización Mundial de Sanidad Animal \(woah.org\)](#).



**Plazo para enviar los comentarios**

Para que puedan ser considerados por la Comisión de Normas Biológicas, los comentarios a los borradores de los capítulos deben enviarse a la Sede antes del [30 de abril de 2024](#).

**Destinatario de los comentarios**

Todos los comentarios deberán enviarse al Departamento Científico: [BSC.Secretariat@woah.org](mailto:BSC.Secretariat@woah.org)

**Fecha de la próxima reunión**

La Comisión de Normas Biológicas propuso las fechas de su próxima reunión, que serán confirmadas tras la elección de la Comisión en la 91ª Sesión General, en mayo de 2024.

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## 1. Bienvenida de las Directoras

### 1.1. Directora General

La Dra. Monique Eloit, Directora General de la OMSA, se reunió con la Comisión de Normas Biológicas el 6 de febrero y agradeció a sus integrantes por su apoyo y compromiso para lograr los objetivos de la OMSA.

La Dra. Eloit subrayó que esta reunión marcaba la conclusión del actual mandato de la Comisión y expresó su gratitud a los miembros por sus constantes esfuerzos a lo largo de sus años de colaboración. A punto de concluir el mandato, el pasado mes de agosto se publicó una convocatoria de candidaturas para los miembros. La lista de candidatos se presentará al Consejo en su reunión de marzo, a la que seguirán debates y negociaciones entre las regiones. La elección de las cuatro Comisiones de la OMSA está prevista para la próxima Sesión General.

La Dra. Eloit informó a la Comisión sobre el proyecto de consultoría en curso de la OMSA destinado a evaluar los *Textos Fundamentales* de la organización desde una perspectiva tanto técnica como jurídica. Esta revisión pretende mejorar los sistemas internos de la OMSA, reforzar su credibilidad y fortalecer su posición mundial. La consultoría se centra en tres pilares principales: los asuntos institucionales; el sistema científico, que abarca los TdR<sup>1</sup> tanto de las Comisiones como de los centros de referencia; y el modelo empresarial de la organización. El objetivo del análisis de los *Textos Fundamentales* es facilitar una revisión exhaustiva y presentar las conclusiones a la Asamblea. La Dra. Eloit también señaló que los miembros seleccionados de las cuatro Comisiones desempeñarán un papel importante en el proceso de revisión de los *Textos Fundamentales*.

En su discurso de clausura, la Dra. Eloit ofreció información actualizada sobre los progresos realizados en el Tratado de Pandemia con la OMS<sup>2</sup>. Destacó que este tratado reconocerá formalmente la importancia de la prevención de enfermedades, incluida la sanidad animal. Además, se prestará más atención a la investigación en el sector animal, haciendo hincapié en el papel crucial de las vacunas. La Dra. Eloit también subrayó la necesidad no solo de promover el uso de las vacunas existentes, sino también de invertir significativamente en el desarrollo de nuevas vacunas. Este planteamiento subraya una estrategia proactiva en la gestión y prevención de enfermedades, especialmente en el sector animal, en consonancia con los objetivos más amplios de la salud y la seguridad mundiales.

La Comisión agradeció a la Dra. Eloit estas actualizaciones.

### 1.2. Directora General Adjunta, Normas Internacionales y Ciencia

La Dra. Montserrat Arroyo, Directora General Adjunta de “Normas Internacionales y Ciencia” de la OMSA, dio la bienvenida a los miembros de la Comisión y les agradeció sus continuos esfuerzos y contribuciones a lo largo de los últimos 3 años. Destacó la importancia de seguir aumentando el impacto y la visibilidad de la Comisión.

La Dra. Arroyo informó a la Comisión de las actividades normativas de la OMSA. Destacó la armonización de los procesos en las cuatro Comisiones, que incluye la nueva iniciativa de publicar los comentarios de los Miembros sobre los proyectos de normas de los *Manuales y Códigos* de la OMSA. Esta iniciativa refleja el compromiso de la OMSA con la transparencia y la participación de los Miembros. La Dra. Arroyo también informó a la Comisión del calendario de reuniones de la Mesa de este año, que incluye colaboraciones entre las Comisiones de Normas Sanitarias para los Animales Acuáticos y de Normas Biológicas, así como entre la Comisión de Normas Sanitarias para los Animales Terrestres y la Comisión Científica para las Enfermedades de los Animales, destacando el enfoque colaborativo de la organización.

La Dra. Arroyo presentó una actualización de los progresos realizados con la herramienta de navegación para la consulta en línea de las normas, anunciando que se han logrado avances significativos. Esta herramienta se presentará a la Asamblea durante la Sesión General y se espera que esté operativa en julio de 2024.

En cuanto a los próximos eventos de la OMSA, la Dra. Arroyo anunció que el seminario web previo a la Sesión General de la Comisión está programado para el martes 16 de abril de 2024 de 12.00 a 14.00 CET.

Para concluir su discurso, la Dra. Arroyo expresó su agradecimiento por los logros de la Comisión durante el mandato de 3 años, que incluían la adopción de 68 capítulos, con capítulos adicionales que se espera sean adoptados este año, la implementación de las tablas de justificación para las puntuaciones de las pruebas que figuran en la *Tabla 1. Métodos analíticos disponibles y su finalidad* de los capítulos específicos de las enfermedades, y una estrategia para evaluar los centros de referencia.

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1 TdR: Términos de referencia

2 OMS: Organización Mundial de la Salud

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Los miembros de la Comisión agradecieron a la Dra. Arroyo el excelente apoyo prestado por la Secretaría de la OMSA.

### **1.3. Actualizaciones de la Sede de la OMSA**

#### **1.3.1. Transparencia del proceso de la OMSA para la elaboración de normas**

La Secretaría informó a la Comisión de los progresos realizados para mejorar la transparencia del proceso de elaboración de normas de la OMSA, en particular la publicación de los comentarios presentados por los Miembros y socios.

La Secretaría informó a la Comisión de que la Directora General había comunicado esta iniciativa a los Miembros en diciembre de 2023 y de que se habían elaborado unos POE<sup>3</sup> para la presentación de comentarios durante el proceso de elaboración de las normas internacionales de la OMSA, así como una guía sobre cómo presentar y enviar comentarios, y que estos documentos se habían publicado en el sitio web de la OMSA y en el de los Delegados.

La Secretaría recordó a la Comisión que se trata de un proceso progresivo que se iniciará en marzo/abril del 2024 con la publicación, en el sitio web de los Delegados, de los comentarios considerados sobre las normas nuevas y revisadas durante las reuniones de febrero de 2024 de la Comisión, al mismo tiempo que la publicación del respectivo informe de febrero de 2024 de la Comisión. Este proceso adopta un enfoque gradual e incluye una evolución de los informes de la Comisión hacia la transparencia de los comentarios considerados y las respuestas de la Comisión, lo que dará lugar a una mejor documentación y trazabilidad del proceso de la OMSA para la elaboración de normas.

## **2. Aprobación del orden del día**

Se presentó y aprobó el orden del día propuesto. El Dr. Emmanuel Couacy-Hymann presidió la reunión, y la Secretaría de la OMSA actuó como ponente. El orden del día y la lista de participantes se encuentran en los [Anexos 1](#) y [2](#), respectivamente.

## **3. Colaboración con otras comisiones especializadas**

### **3.1. Aspectos horizontales entre comisiones especializadas**

#### **3.1.1. Definiciones de caso: tularemia, infección por metaneumovirus aviar (rinotraqueítis del pavo)**

La Comisión de Normas Biológicas debatió las definiciones de caso de tularemia y de infección por metaneumovirus aviar (rinotraqueítis del pavo) y formuló recomendaciones a la Comisión Científica para las Enfermedades de los Animales (véase el punto 8.3.2 del informe de la reunión de la Comisión Científica para las Enfermedades de los Animales, 12-16 de febrero de 2024).

### **3.2. Comisión Científica para las Enfermedades Animales**

Nada para esta reunión.

### **3.3. Comisión de Normas Sanitarias para los Animales Terrestres**

Asuntos entre la Comisión de Normas Sanitarias para los Animales Terrestres y la Comisión de Normas Biológicas.

#### **3.3.1. Actualizaciones de la reunión de septiembre de 2023 de la Comisión del Código**

La Comisión de Normas Biológicas recibió información actualizada de la Secretaría de la Comisión del Código sobre los temas que está revisando actualmente la Comisión del Código para garantizar la complementariedad y la armonización de los respectivos programas de trabajo de ambas Comisiones.

En febrero de 2021, la Comisión del Código acordó desarrollar un marco para las normas del *Código Terrestre* que sirviera de guía útil para garantizar la normalización del contenido del *Código Terrestre*. Tomando nota de las diferencias en los objetivos y la estructura de los capítulos dentro del Volumen I y el Volumen II del

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3 POE: Procedimientos operativos estándar

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*Código Terrestre*, y dentro de las diferentes secciones del Volumen I, la Comisión pidió a la Secretaría que empezara por trabajar en el contenido de los capítulos específicos de enfermedad, es decir, el Volumen II.

Desde entonces, la Comisión del Código ha trabajado en estrecha colaboración con la Secretaría, en consulta con la Comisión Científica, y basándose en debates y acuerdos previos entre la Comisión del Código, la Comisión Científica y, en su caso, con la Comisión de Normas Biológicas, para elaborar un documento que ofrezca una descripción detallada de la estructura y el contenido de un capítulo específico de enfermedad, incluidas las referencias clave a otras partes del *Código Terrestre* y a otras normas de la OMSA, así como las convenciones relativas al uso de términos, redacción y estructura.

La Comisión del Código reconoció que el marco sería un documento vivo y que debería servir de referencia a quienes emprendieran la elaboración de capítulos nuevos o revisados. La Comisión también convino en que el marco podría ayudar a los Miembros a conocer mejor los capítulos específicos de enfermedad del *Código Terrestre* y que, en último término, el marco podría ponerse a disposición de los Miembros en una etapa posterior.

En septiembre de 2023, la Comisión del Código revisó el documento y pidió a la Secretaría que finalizase una primera edición para su reunión de febrero de 2024, y solicitó que se compartiese al mismo tiempo con la Comisión Científica y la Comisión de Normas Biológicas. Además, la Comisión del Código pidió a la Secretaría que utilizara el marco en las próximas revisiones de los capítulos específicos de enfermedad y que aportara comentarios al respecto.

### **3.3.2. Recomendaciones de la Comisión de Normas Biológicas a la Comisión de Normas Sanitarias para los Animales Terrestres**

Véase el punto 5.7. de este informe.

### **3.3.3. Información actualizada de la Comisión de Normas Biológicas sobre la solicitud del Código relativa al Capítulo 6.10 del Código Terrestre Uso responsable y prudente de agentes antimicrobianos en veterinaria**

Véase el punto 5.8. de este informe.

### **3.3.4. Cuestión sobre el capítulo sobre la diarrea viral bovina**

Se solicitó el dictamen de la Comisión de Normas Biológicas sobre la taxonomía de los agentes causantes de la diarrea viral bovina. La Comisión de Normas Biológicas informó que la taxonomía había sido actualizada y adoptada por el Comité Internacional de Taxonomía de Virus (ICTV). La nueva nomenclatura se ha introducido en el capítulo del *Manual Terrestre* (véase el punto 5.2 del orden del día) y debería aplicarse al capítulo del *Código Terrestre*:

## **3.4. Comisión de Normas Sanitarias para los Animales Acuáticos**

Reunión de las Mesas de la Comisión (véase el punto 3 de la Reunión de la Comisión de Normas Sanitarias para los Animales Acuáticos, 14-21 de febrero de 2024).

## **4. Programa de trabajo**

Se aprobó el programa de trabajo actualizado, que puede consultarse en el [Anexo 3](#).

## **5. Manual de las Pruebas de Diagnóstico y de las Vacunas para los Animales Terrestres**

Para este punto del orden del día, la Comisión contó con la presencia del Dr. Steven Edwards, redactor consultor del *Manual Terrestre* de OMSA.

### **5.1. Formato del informe y sistema de presentación y publicación de los comentarios**

A la luz de la aplicación del nuevo sistema de presentación y publicación de los comentarios de los Miembros, la Comisión revisó su sistema de notificación. Para informar mejor de las modificaciones del *Manual Terrestre*, la Comisión decidió adoptar el formato de tabla que utiliza actualmente la Comisión para los Animales Acuáticos. Los Miembros pueden ver y conocer más fácilmente las decisiones de la Comisión en respuesta a los comentarios.



## 5.2. Examen de los comentarios recibidos de los Miembros sobre los borradores de capítulos y su aprobación para su distribución para una segunda ronda de comentarios y propuesta de adopción en mayo de 2024

La Comisión examinó 15 borradores de capítulo y aprobó 13 para su distribución, algunos sujetos a la aclaración de ciertos puntos por parte de los expertos, para una segunda ronda de comentarios por parte de los Miembros antes de presentarlos para su adopción por la Asamblea en mayo de 2024.

Capítulo 1.1.5. Gestión de la calidad en los laboratorios de pruebas veterinarias.

Sección/Párrafo	Comentario	Decisión
A.2. Normas, guías y referencias, párrafo 3	Trasladar la última frase a la sección A.7.3 <i>Validación del método analítico</i> .	De acuerdo, el texto encaja mejor en esta sección
A.3. <i>Acreditación</i> , punto iii)	Suprimir el requisito de que los equipos se verifiquen y gestionen de acuerdo con el programa de mantenimiento y calibración pertinente, ya que no todos los equipos necesitarán ser verificados.	En desacuerdo, los equipos deben mantenerse y calibrarse siguiendo un calendario definido
A.6. Garantía de calidad, control de calidad y pruebas de competencia, párrafo 2	Reinsertar la palabra "prueba" en la frase: "el control de calidad se orienta a la prueba y garantiza la detección de posibles problemas"	En desacuerdo, la frase enmendada es correcta: el control de calidad está orientado a los resultados
A.7.3.1 Actividades que podría incluir la validación	Trasladar los pasos i) y ii) al final de la lista, ya que los pasos iii) a viii) se realizarían primero como parte de un proceso de validación.	De acuerdo

El Capítulo 1.1.5. "Gestión de la calidad en los laboratorios de pruebas veterinarias" revisado se presenta como [Anexo 4](#) y se propondrá su adopción en la 91ª Sesión General, en mayo de 2024.

Capítulo 1.1.9. "Pruebas de esterilidad y ausencia de contaminación en los materiales biológicos de uso veterinario":

Sección/Párrafo	Comentario	Decisión
Comentario general	Incluir una sección para vacunas contra la coccidiosis: el capítulo tiene secciones para vacunas víricas vivas, vacunas víricas o bacterianas inactivadas, y vacunas bacterianas vivas, pero no una sección para vacunas vivas que contengan una preparación de ooquistes esporulados de una línea adecuada de especies de parásitos coccidianos.	De acuerdo: este comentario se abordará en el próximo ciclo de revisión (2024/2025)
B. Vacunas víricas vivas para administración por inyección, o a través de agua potable, pulverización o escarificación cutánea, punto 3	Añadir la Administración de Medicamentos Veterinarios de China (Rep. Pop. de) a la lista de métodos publicados aceptables para comprobar que los lotes de vacunas están libres de agentes extraños.	De acuerdo
C. Vacunas víricas o bacterianas inactivadas, punto 2	Añadir "pre" antes de "inactivada" en la frase: "Si se requieren estudios sobre agentes extraños representativos, entonces añadir a la vacuna inactivada agentes representativos vivos y a continuación..." porque los agentes representativos deben añadirse a la vacuna pre-inactivada para ser inactivada para las pruebas.	En desacuerdo: dependiendo de la vacuna, puede ser más seguro trabajar con una vacuna inactivada para esta prueba que con una que contenga un agente patógeno infeccioso vivo.

Sección/Párrafo	Comentario	Decisión
G. Ejemplos de protocolo, Tabla 1	Los Miembros propusieron algunos cambios editoriales menores	De acuerdo
G.3.2 Pruebas generales para la exclusión de <i>Mycoplasma</i> sp.	El enlace a la Agencia Europea del Medicamento no funciona	Actualizado el enlace a la Agencia Europea del Medicamento
H. Información que debe presentarse al solicitar una licencia de importación, párrafo 1	Reinstaurar el requisito de que las Autoridades Veterinarias sigan el <i>Manual Terrestre</i> al realizar análisis de riesgos para productos biológicos	De acuerdo, pero aclarando que es el <i>Código Terrestre</i> el que debe seguirse
H. Información que debe presentarse al solicitar una licencia de importación, párrafo 2	Añadir el Ministerio de Agricultura y Asuntos Rurales de China (Rep. Pop. de) a la lista de ejemplos de evaluación basada en el riesgo de los productos biológicos veterinarios para su importación en un país.	De acuerdo

El capítulo 1.1.9. "Pruebas de esterilidad y ausencia de contaminación en los materiales biológicos de uso veterinario" revisado se presenta como [Anexo 5](#) y se propondrá su adopción en la 91ª Sesión General, en mayo de 2024.

#### Capítulo 2.2.4. "Incertidumbre de la medición"

Sección/Párrafo	Comentario	Decisión
<i>Introducción</i> , párrafo 2	Por coherencia, sustituir "punto de corte" por "umbral de diagnóstico".	De acuerdo.
A. La necesidad de determinar la incertidumbre de la medición (IM), apartado 1	Sustituir el término "intervalo de confianza" por "intervalo de referencia", ya que es el término correcto utilizado por la Guía ISO/CEI 98-3.	De acuerdo y se aplicó esta enmienda a todo el capítulo
A. La necesidad de determinar la IM, apartado 1	Añadir una frase que aclare que existen métodos alternativos que dependen menos de los supuestos de distribución y manejan mejor la presencia de valores atípicos.	De acuerdo.
A.2.1 Método de expresión de la IM	Cambiar el subíndice de 'L' a 'W', ya que 'bajo' se ha cambiado a control positivo 'débil'	De acuerdo y se aplicó esta enmienda a todo el capítulo
A.2.1 Método de expresión de la IM	Definir "X" en la ecuación y aclarar qué se entiende por resultado transformado.	De acuerdo: se añadió que X representa el conjunto de réplicas, y se dieron ejemplos de un resultado adecuadamente transformado.
A.2.3 Cálculo de la incertidumbre		Se añadió una declaración sobre la necesidad de transformar datos no distribuidos normalmente
A.2.4 Interpretación de los resultados	Sustituir la primera frase por la afirmación de que una muestra con un PI de entre el 36% y el 64% se encuentra dentro de la IM que rodea al valor umbral y, por lo tanto, su diagnóstico es menos seguro que el de las muestras con resultados más alejados de dicho umbral.	De acuerdo, la interpretación original era demasiado precisa dadas las múltiples aproximaciones realizadas y los matices de la interpretación de un intervalo de referencia

Sección/Párrafo	Comentario	Decisión
A.3.3 Interpretación de los resultados	Sustituir la frase por la afirmación de que una muestra con un umbral del número de ciclos (Ct, por la siglas en inglés de <i>cycle threshold</i> ) de entre un 36% y un 37% se encuentra dentro de la IM que rodea al valor umbral y, por tanto, su diagnóstico es menos seguro que el de las muestras con resultados más alejados de dicho umbral.	En parte de acuerdo, la interpretación original era demasiado precisa dadas las múltiples aproximaciones realizadas y los matices de la interpretación de un intervalo de referencia. Sin embargo, el umbral es 37, el límite superior de la IM es 38 y el límite inferior es 36; los valores se refieren a valores Ct y, por lo tanto, se ha suprimido el signo de porcentaje.

El Capítulo 2.2.4. "Incertidumbre de la medición" revisado se presenta como [Anexo 6](#) y se propondrá para su adopción en la 91ª Sesión General, en mayo de 2024.

Capítulo 2.2.6. "Elección y uso de tipos y grupos de muestras re referencia".

Sección/Párrafo	Comentario	Decisión
Figura 2	<p>Añadir "Resultado de la infección/enfermedad" y "Tiempo tras la infección experimental" a la columna "Datos de la fase de infección".</p> <p>El resultado de la infección/enfermedad es importante: aunque un animal puede (o no) tener síntomas de infección o signos clínicos de enfermedad, puede recuperarse. Incluso si la enfermedad tiene una alta tasa de mortalidad, algunos animales se recuperarán con distintos niveles de infección o signos clínicos, lo que puede crear un sesgo dependiendo del resultado que se busque en la prueba de diagnóstico.</p> <p>Periodo posterior a la infección experimental: es fundamental si se utilizan muestras de referencia tomadas de modelos experimentales de infección, ya que es probable que el análisis cambie con el tiempo. También permite recrear las muestras si el modelo experimental es repetible.</p>	De acuerdo.
F.1 Animales cuyo estado respecto a la infección se desconoce – especificidad y sensibilidad diagnóstica	Añadir "análisis" antes de "bayesiano" porque la clase latente es un modelo y el bayesiano es un método de análisis.	De acuerdo.

El capítulo 2.2.6. " Elección y uso de tipos y grupos de muestras re referencia" revisado se presenta como [Anexo 7](#) y se propondrá su adopción en la 91ª Sesión General, en mayo de 2024.

Capítulo 3.1.5 'Fiebre hemorrágica de Crimea-Congo'

Sección/Párrafo	Comentario	Decisión
Tabla 1. Formato de las pruebas de diagnóstico de las infecciones por el virus de la fiebre	Suprimir la palabra "muy" de la clave "+ = adecuado en muy pocos casos".	En desacuerdo: texto estándar de la Tabla 1 en todo el <i>Manual Terrestre</i> .

Sección/Párrafo	Comentario	Decisión
hemorrágica de Crimea-Congo en animales, Clave		
Tabla 1, RT-PCR en tiempo real, para determinar si un animal está libre de infección antes de su traslado	Cambiar la calificación de '+++' a '++' debido a la naturaleza transitoria de la viremia.	De acuerdo. Spengler <i>et al.</i> (2016) revisaron la investigación sobre el VFHCC y confirman la viremia transitoria.
Tabla 1, todos los métodos para la confirmación de casos clínicos en animales	Cambiar la calificación de todas las pruebas de esta columna a "-" porque los animales, incluidos los rumiantes, suelen ser asintomáticos respecto a la infección, aunque podrían ser virémicos de forma transitoria.	En desacuerdo: en casos de pirexia, estas pruebas pueden detectar viremia.
Tabla 1, ELISA de IgM, para determinar la Prevalencia de la infección - vigilancia	Cambiar la calificación de "-" a "++" debido a la corta persistencia de los anticuerpos IgM en respuesta a infecciones agudas, pero la prueba tiene limitaciones ya que puede no detectarse cuando la IgM disminuye.	En desacuerdo: la respuesta de IgM es débil, y la incidencia de una respuesta de IgM detectable puede ser muy baja en una población, dado que no dura mucho tiempo. Además, el ELISA de IgM no está diseñado para su uso en animales, por lo que debe adaptarse antes de su uso (véase la sección 2, <i>Pruebas serológicas</i> ).

El Capítulo 3.1.5. 'Fiebre hemorrágica de Crimea-Congo' revisado se presenta como [Anexo 8](#) y se propondrá su adopción en la 91ª Sesión General, en mayo de 2024.

#### Capítulo 3.3.6. "Tuberculosis aviar"

Sección/Párrafo	Comentario	Decisión
Comentarios generales	Cambiar el título del capítulo por micobacteriosis aviar, ya que se trata de una enfermedad no tuberculosa.	En desacuerdo: el título del capítulo se basa en la patogénesis de la enfermedad en las aves.
<i>Resumen</i> , párrafo 3	Añadir después de 'propietarios de aves de compañía' 'o cuidadores de aves cautivas'	De acuerdo.
<i>Resumen</i> , párrafo 4	Sustituir "segmentos génicos" por "secuencias de inserción", ya que es más correcto, explica la convención de nomenclatura y también porque algunas de las inserciones no son "segmentos" génicos: pueden contener genes enteros, múltiples genes, elementos repetitivos adicionales, ningún ORF, etc.	De acuerdo.
<i>Resumen</i> , párrafo 4	Incluir una mención a la espectrometría de masas por ionización de desorción láser asistida por matriz en tiempo de vuelo (MALDI-TOF MS), ya que también es una herramienta valiosa.	De acuerdo.
A. <i>Introducción</i> , párrafo 2	Esto parece confuso o posiblemente contiene un error. Se indican tres especies en la frase inicial ( <i>M. avium</i> subesp. <i>avium</i> , <i>M. avium</i> subesp. <i>silvaticum</i> y <i>M. avium</i> subesp. <i>avium</i> ).	Existe consenso en que muchos de estos cambios no afectan al tratamiento de las enfermedades. Los nuevos nombres y

Sección/Párrafo	Comentario	Decisión
	paratuberculosis,) y de nuevo más adelante tres especies, pero no las mismas tres: <i>M. avium</i> subsp. <i>avium</i> , <i>M. avium</i> subsp. <i>paratuberculosis</i> y <i>M. avium</i> subsp. <i>lepraemurium</i> ; y después tres subespecies de <i>M. avium</i> subespecie <i>avium</i> . Además, la nomenclatura utilizada en la sección de diagnóstico no parece incluir este enfoque en algunos lugares, y también hace referencia a clasificaciones adicionales que no se mencionan aquí, como los serotipos 1, 2 y 3 de <i>M. a. avium</i> .	clasificaciones tardan un tiempo en llegar a las clasificaciones formales según las normas de nomenclatura. En este apartado se mencionan las especies aprobadas formalmente junto con los resultados de estudios recientes. En otras secciones figuran los nombres tradicionales con los que están familiarizados la mayoría de los veterinarios y las limitaciones de la tipificación en zonas con recursos escasos.
Tabla 1. Métodos analíticos disponibles para el diagnóstico de la tuberculosis aviar y su finalidad	La clasificación de la tinción de Ziehl-Neelsen para la confirmación de casos clínicos (++) es correcta para las muestras de órganos, pero no para los frotis fecales.	El texto no hace referencia a frotis fecales sino solo a órganos.
B.1 Identificación del agente	Añadir una frase y una referencia a MALDI-TOF MS como valiosa herramienta de diagnóstico	De acuerdo.
B.1 Identificación del agente	Aclarar que, aunque tradicionalmente <i>M. a. avium</i> se separa de los microorganismos comunes no cromogénicos de crecimiento lento por su capacidad de crecer a 42°C, el método tiene un valor limitado, ya que existen otras especies que también son capaces de crecer a 42°C.	De acuerdo.
B.1.1 <i>Cultivo</i> , párrafo 1	Eliminar los nombres comerciales de los productos	De acuerdo.
B.1.1 <i>Cultivo</i> , párrafo 4	Sustituir la palabra "mascota" por "cautivas" después de "aves".	De acuerdo.
B.1.2 Métodos de reconocimiento de ácido nucleico, párrafo 1	Corregir la presentación de los segmentos genéticos utilizando cursiva.	De acuerdo.
B.2.1 <i>Prueba de la tuberculina</i> , párrafo 2	Añadir el nombre científico " <i>Phasianus colchicus</i> " después de "faisán común" para evitar confusiones entre los dos nombres comunes diferentes para la misma especie de ave.	De acuerdo.
C.2.2.4, iii) <i>Inocuidad</i> , párrafo 1	El diseño del estudio explicado en este párrafo es mucho menos específico en cuanto al número de animales necesarios, el tamaño mínimo del animal y el volumen de inyección por animal, a diferencia de lo que ocurre en otras partes del texto.	De acuerdo y eliminadas las últimas tres frases del párrafo
C.2.2.4, iv) <i>Potencia del lote</i>	Para mayor claridad, añadir "desplumar un área lo suficientemente grande" entre "flancos" y "proporcionar espacio para tres o cuatro inyecciones en cada lado)".	De acuerdo

El Capítulo 3.3.6. "Tuberculosis aviar" revisado se presenta como [Anexo 9](#) y se propondrá para adopción en la 91ª Sesión General, en mayo de 2024.

Capítulo 3.4.1. "Anaplasmosis bovina".

Sección/Párrafo	Comentario	Decisión
Comentario general	Sustituir "cuerpos iniciales" por "cuerpos de inclusión" en todo el capítulo.	De acuerdo.
B.1.1 <i>Examen microscópico</i> , párrafos 1 y 8	Sustituir "parásitos" por "bacterias".	De acuerdo.
Tabla 1. Métodos analíticos disponibles para el diagnóstico de la anaplasmosis bovina y su finalidad	Los expertos habían preparado tablas justificativas de las puntuaciones dadas en la Tabla 1 para las distintas pruebas y finalidades. Los enlaces a estas tablas de justificación se habían incluido en los títulos de las tablas, pero los Miembros no los tuvieron en cuenta durante la primera ronda de comentarios.	Estas tablas de justificación se han añadido como apéndices al capítulo y se han indicado como referencia cruzada en la Tabla 1.
Tabla 1. <i>Cuerpos de inclusión de Anaplasma marginale</i>	Sin comentarios, decisión de la Comisión.	Solicitar una ilustración más clara de los órganos de inclusión
Tabla 2. Oligonucleótidos utilizados en las PCR para detectar <i>A. marginale</i> y <i>A. centrale</i>	Eliminar el guion de las secuencias de oligonucleótidos.	En desacuerdo: este es el estilo del <i>Manual Terrestre</i> .
B.2.2.3 <i>Análisis de datos</i> , última frase	Sustituir la palabra "reproducibilidad" por "repetibilidad", ya que la reproducibilidad suele referirse a la precisión entre laboratorios.	De acuerdo.

El Capítulo 3.4.1. "Anaplasmosis bovina" revisado se presenta como [Anexo 10](#) y se propondrá para adopción en la 91ª Sesión General, en mayo de 2024.

Capítulo 3.4.7. "Diarrea viral bovina".

Sección/Párrafo	Comentario	Decisión
Comentario general	Se ha actualizado la taxonomía del agente patógeno. La nueva taxonomía debe utilizarse y aplicarse de manera uniforme en todo el capítulo: <i>Pestivirus bovis</i> (conocido comúnmente como VDVB tipo 1), <i>Pestivirus tauri</i> (VDVB tipo 2), y <i>Pestivirus brazilense</i> (VDVB tipo 3 o pestivirus de tipo Hobi).	De acuerdo y se ha aplicado este cambio.
<i>Resumen</i> , párrafo 1	Aclarar que los toros pueden tener una infección testicular prolongada y persistente durante periodos largos, ya que la duración de la presencia del virus en el tejido testicular puede variar significativamente, desde 28 días después de la infección aguda hasta 5 años después de la infección.	De acuerdo.
<i>Resumen</i> , párrafo 2	Añadir "o pestivirus A, B, C, D o H", según proceda, a los agentes patógenos.	En desacuerdo, la propuesta no se ajusta a la taxonomía adoptada.
A.1 Impacto de la enfermedad, párrafo 2	Aclarar que los toros pueden tener una infección testicular larga y persistente e incluir una referencia.	De acuerdo.

Sección/Párrafo	Comentario	Decisión
Tabla 1. Métodos analíticos disponibles para el diagnóstico de la diarrea viral bovina y su finalidad.	Los expertos habían preparado tablas justificativas de las puntuaciones dadas en la Tabla 1 para las distintas pruebas y finalidades. Los enlaces a estas tablas de justificación se habían incluido en los títulos de las tablas, pero los Miembros los pasaron por alto durante la primera ronda de comentarios.	Estas tablas de justificación se han añadido como anexos al capítulo y se han añadido como referencias cruzadas en la Tabla 1.
B.1.1.1 Método de inmunoperoxidasa en microplaca para el cribado masivo para la detección del virus en muestras de suero, Acetona, d)	Añadir "antiviral" después de "anticuerpo" por motivos de coherencia con la descripción del método anterior.	De acuerdo.

El Capítulo 3.4.7. "Diarrea viral bovina" revisado se presenta como [Anexo 11](#) y se propondrá para adopción en la 91ª Sesión General, en mayo de 2024.

Capítulo 3.4.12. "Dermatosis nodular contagiosa" (solo la sección sobre vacunas).

Sección/Párrafo	Comentario	Decisión
A. Introducción, párrafo 2	Sustituir la subfamilia del agente patógeno, pasando de <i>Chordopoxvirinae</i> a <i>Chordopoxviridae</i> .	En desacuerdo, la taxonomía adoptada es <i>Chordopoxvirinae</i> .
B.1.3. Reacción en cadena de la polimerasa (PCR)	Añadir dos PCR en tiempo real adicionales y referencias.	En desacuerdo, solo la sección de vacunas fue enviada para comentarios. Estas propuestas pueden abordarse cuando se actualice la sección de pruebas de diagnóstico.

El Capítulo 3.4.12. "Dermatosis nodular contagiosa" revisado (solo el apartado sobre vacunas) se presenta como [Anexo 12](#) y se propondrá para adopción en la 91ª Sesión General, en mayo de 2024.

Capítulo 3.6.9. "Rinoneumonitis equina" (infección por *Varicellovirus equidalpha1*).

Sección/Párrafo	Comentario	Decisión
Comentario general	Actualizar la taxonomía del agente patógeno. Aclarar la supresión del HVE-4 del título.	La nomenclatura del virus ha cambiado de herpesvirus-1 equino (HVE -1) a <i>Varicellovirus equidalpha1</i> . El título del capítulo ha sido modificado y la Comisión del Código ha sido informada del cambio. Se ha añadido una frase para subrayar que el capítulo cubre el HVE-1.  La segunda parte del título entre paréntesis se refiere al título del capítulo correspondiente del <i>Código Terrestre</i> , ya que solo figura el HVE -1, este capítulo solo cubre el HVE -1.
	Sustituir "ml" por "mL", ya que es el símbolo del SI correcto.	En desacuerdo, tanto 'mL' como 'ml' son aceptables, este último se

Sección/Párrafo	Comentario	Decisión
		utiliza en todo el <i>Manual Terrestre</i> .
Resumen	Los Miembros proponen algunos cambios editoriales menores.	De acuerdo.
A. <i>Introducción</i> , párrafo 1	Los nombres taxonómicos actuales de los virus son: Varicellovirus equidalpha1 y Varicellovirus equidalpha4	De acuerdo: a efectos del capítulo, se seguirán utilizando las siglas HVE -1 y HVE -4.
A. <i>Introducción</i> , párrafo 2	Eliminar las referencias al HVE-4 en todo el capítulo en consonancia con el título.	En desacuerdo: es un aspecto diferencial importante, y el potencial patógeno relativo de los dos virus es importante para el diagnóstico.
B. Pruebas de diagnóstico, párrafo	Un Miembro propuso algunos cambios editoriales menores para mayor claridad.	De acuerdo
Tabla 1. Métodos analíticos disponibles para el diagnóstico de la infección por HVE-1 y su finalidad.	Suprimir "rinoneumonitis equina" del título de la Tabla y sustituirlo por "infección por HVE - 1".	De acuerdo
Tabla 1	Los expertos habían preparado tablas que justificaban las puntuaciones dadas en la Tabla 1 para las distintas pruebas y finalidades. Los enlaces a estas tablas de justificación se habían incluido en los títulos de las tablas, pero los Miembros los pasaron por alto durante la primera ronda de comentarios.	Estas tablas de justificación se han añadido como apéndices al capítulo y se indican como referencias cruzadas en la Tabla 1.
Tabla 1	<p>Modificar la puntuación para el ELISA:</p> <p>de "+" a "++" para Población libre de infección;</p> <p>de "-" a "++" para Ausencia de infección en un animal antes de su desplazamiento;</p> <p>de "+" a "++" para Confirmación de casos clínicos;</p> <p>de '+' a '++' para Prevalencia de la infección– vigilancia;</p> <p>de "+" a "++" para Estado inmunitario en animales individuales o poblaciones tras la vacunación</p> <p>Modificar la puntuación para la fijación del complemento (FC):</p> <p>de "+++" a "+" para Confirmación de casos clínicos;</p> <p>de "+++" a "++" para el Estado inmunitario en animales individuales o poblaciones tras la vacunación.</p> <p>La FC es más complicada y difícil de mantener que el ELISA, por lo que no debería considerarse una prueba más adecuada: Hartley <i>et al.</i> (2005). <i>Comparison of antibody detection assays for the diagnosis of equine</i></p>	<p>De acuerdo en parte.</p> <p>El trabajo de Hartley <i>et al.</i> se refiere a una comparación de pruebas de detección de anticuerpos utilizando 33 muestras de suero de fase aguda y de fase convaleciente, es decir, no es un estudio de seroprevalencia. El estudio de seroprevalencia de El Brini <i>et al.</i> (2021) indica que el ELISA es menos sensible para la detección de anticuerpos contra el HVE-1 que la neutralización del virus (VN).</p> <p>Se invita a los Miembros a revisar la explicación de la puntuación de las pruebas en las tablas anexas al capítulo.</p>



Sección/Párrafo	Comentario	Decisión
	<i>herpesvirus 1 and 4 infections in horses. Am. J. Vet. Res.</i> , 66, 921–928.	
Tabla 2	Suprimir el primer conjunto de cebadores y sonda debido a problemas con su especificidad.	De acuerdo.
B.1.2 Detección de virus mediante reacción en cadena de la polimerasa. Pruebas moleculares en el punto de atención (POC, por las siglas en inglés de <i>point-of-care</i> ).	Suprimir este párrafo, ya que no es habitual hacer referencia a métodos no totalmente validados o incluidos en la Tabla 1.	En desacuerdo: se supone que el capítulo es un punto de entrada a la bibliografía, las pruebas han demostrado ser útiles, solo se mencionan brevemente. Las pruebas no se incluyen en la Tabla 1 porque no están totalmente validadas.
B.1.2 Detección de virus mediante reacción en cadena de la polimerasa. Caracterización molecular.	Suprimir este párrafo: aunque es correcto, el punto principal se expuso en el párrafo anterior y el resto es genérico. El análisis molecular puede utilizarse en todos los brotes para apoyar la epidemiología.	En desacuerdo, es importante señalar que la secuenciación no puede predecir de forma fiable las cepas neuropatógenas. La última frase se proporciona en el contexto de las dos primeras frases, por lo que es apropiada.
B.2 <i>Pruebas serológicas</i> , apartado 1	Sustituir "sin embargo" por "a pesar de".	En desacuerdo, el término "a pesar de" no es de uso común y será confuso para algunos lectores; "sin embargo" es más claro.
B.2 <i>Pruebas serológicas</i> , apartado 4	Una vacuna viva modificada contra el HVE-1 que carece del gen de la glicoproteína E está autorizada en Japón, y se utiliza un ELISA que utiliza un péptido sintético para la glicoproteína E como antígeno (Andoh <i>et al.</i> , 2013) como estrategia DIVA <sup>4</sup> para los caballos vacunados con esta vacuna. Modificar el texto para tener en cuenta este hecho.	De acuerdo, se ha sustituido la última frase por un texto nuevo y una referencia que refleja el comentario.
C.2.1.3 Validación como cepa vacunal	Incluir una medida cuantitativa sobre el límite superior del título obtenido mediante VN (estado serológico) de los caballos utilizados para confirmar la inmunogenicidad del inóculo vírico primario en las vacunas. La justificación es que será muy difícil, si no imposible, encontrar, para esta prueba, caballos nunca antes expuestos al virus.	De acuerdo en incluir una referencia.
C.2.3.4 <i>Duración de la inmunidad</i> , párrafo 2	Se reconoce que el HVE-1 y el HVE -4 dan reacción cruzada, pero resulta confuso dar a entender que se está hablando de dos agentes denominados HVE -1 y HVE 1/4. Sería más claro decir o bien HVE -1 o bien HVE 1/4. Sería más claro decir "HVE -1 o HVE -4" o eliminar por completo la mención del HVE -4.	De acuerdo en eliminar la mención del HVE -4 aquí.

El Capítulo 3.6.9. "Rinoneumonitis equina (infección por Varicellovirus equidalph1)" revisado se presenta como [Anexo 13](#) y se propondrá para adopción en la 91ª Sesión General, en mayo de 2024.

4 DIVA: Estrategia que permite diferenciar entre animales infectados y animales vacunados.

Capítulo 3.8.1. “Enfermedad de la frontera”.

Sección/Párrafo	Comentario	Decisión
<i>Resumen</i> , párrafo 3	Añadir la mención de los pestivirus A, B, C, D o H, según proceda, a los agentes patógenos.	En desacuerdo, la propuesta no se ajusta a la taxonomía adoptada.
A. <i>Introducción</i> , párrafo 1	Actualizar la información sobre los genotipos y añadir más detalles y una referencia.	De acuerdo, el detalle es necesario ya que el VDVB requiere un diagnóstico diferencial respecto al del VPPC.
B.2.1.1 <i>Procedimiento analítico</i> , iii)	No está claro por qué se han modificado los límites de aceptación. Deberían ser coherentes respecto al capítulo actual sobre la DVB.	De acuerdo, se restablece el intervalo original de 30-300 DICT <sub>50</sub> : los intervalos aceptables se calculan por los métodos de Reed y Muench o Spearman y Kärber.
C.1.1 Características de un perfil de producto objetivo, párrafo 1	Sustituir "permitir" por "proporcionar" e "infección fetal" por "protección fetal".	De acuerdo

El Capítulo 3.8.1. “Enfermedad de la frontera” revisado se presenta como [Anexo 14](#) y se propondrá para adopción en la 91ª Sesión General, en mayo de 2024.

Capítulo 3.8.12. “Viruela ovina y viruela caprina”:

Sección/Párrafo	Comentario	Decisión
A. <i>Introducción</i> , párrafo 1	Sustituir "totalmente susceptible" por "nunca antes expuesto al virus".	De acuerdo
A. <i>Introducción</i> , párrafo 7	Añadir una frase que diga que no hay pruebas de animales persistentemente infectados, y más detalles sobre las características del virus, es decir, la resistencia a las acciones físicas y químicas.	De acuerdo
B.1.1 Obtención y envío de muestras, párrafos 1 y 3	Suprimir la detección de antígenos por coherencia respecto a la Tabla 1.	De acuerdo
B.1.1 Obtención y envío de muestras, párrafo 1	Añadir una frase que indique que también se pueden tomar hisopos nasales y bucales porque el virus estará presente en las secreciones nasales y salivales	De acuerdo
B.1.1 Obtención y envío de muestras, párrafo 2	Suprimir la afirmación de que los tejidos en formol no tienen requisitos especiales de transporte, ya que es vaga y engañosa. El envío de muestras debe describirse en el capítulo introductorio.	De acuerdo
B.1.2 Aislamiento del virus	Sustituir "detección de antígenos" por "detección del genoma".	De acuerdo
B.1.4 Histopatología	Suprimir "y montaje del material de biopsia fijado en formol" de la segunda frase: la secuencia es incorrecta, incompleta e innecesaria, ya que se trata de un	De acuerdo

Sección/Párrafo	Comentario	Decisión
	procedimiento rutinario y no específico de la viruela ovina. La frase anterior es suficiente.	
B.1.6 Métodos de reconocimiento de ácido nucleico, párrafo 1	Añadir sangre y semen a los tipos de muestras.	De acuerdo, y se ha añadido una frase para aclarar que los métodos de extracción de ácidos nucleicos y de amplificación mediante PCR deben validarse para la matriz de muestra que se está analizando.
B.1.6.2 <i>Métodos de PCR en tiempo real</i> , párrafo 1	Añadir una referencia a la lista de PCR en tiempo real para cualquier virus de la viruela caprina: la prueba es utilizada por el EURL para virus de la viruela caprina y otros varios laboratorios nacionales de referencia de Europa, y toda la información de validación sobre esta prueba se puede encontrar en la publicación.	De acuerdo.
B.1.6.2 <i>Métodos de PCR en tiempo real</i> , párrafo 2	Aclarar que es un método para la detección de ADN genómico.	De acuerdo.
B.1.6.2 <i>Métodos de PCR en tiempo real</i> , extracción de ADN de sangre y tejidos	Aclarar que los kits comercializados son para la extracción, no el aislamiento, del ADN, y que deben seguirse las instrucciones del fabricante.	De acuerdo.
B.1.6.2 <i>Métodos de PCR en tiempo real</i> , PCR en tiempo real, iii) y iv)	Aclarar que se puede utilizar cualquier kit comercial de PCR en tiempo real de elección.	En desacuerdo, no añade valor alguno a la descripción de la prueba
B.1.6.3 Amplificación isotérmica del genoma	Aclarar que se ha comprobado que la LAMP permite diferenciar el virus de la viruela ovina (VVO) del virus de la viruela caprina (VVC).	De acuerdo
B.2 Pruebas serológicas	Añadir una frase que diga que la sangre para la detección de anticuerpos debe obtenerse en tubos sin anticoagulante	En desacuerdo: es lo bastante evidente
B.2 Pruebas serológicas	Añadir una mención a que los niveles detectables de anticuerpos se generan 1 semana después de que el animal muestre signos clínicos. Los niveles más altos de anticuerpos se detectan entre 1 y 2 meses después de detectarse la infección	De acuerdo
C.1.1 Fundamento y uso previsto del producto	Añadir información sobre las vacunas vivas atenuadas	En desacuerdo, solo la sección de pruebas diagnósticas fue enviada para comentarios. Esta propuesta puede abordarse cuando se actualice la sección de vacunas

El Capítulo 3.8.12. "Viruela ovina y viruela caprina" revisado se presenta como [Anexo 15](#) y se propondrá para adopción en la 91ª Sesión General, en mayo de 2024.

Capítulo 3.9.1. "Peste porcina africana (infección por el virus de la peste porcina africana)" (solo la sección sobre vacunas).

Se recibió un gran número de comentarios sobre la nueva sección propuesta relativa a las vacunas. Dado que en algunos Miembros se utilizan vacunas vivas modificadas, la Comisión cree que es importante disponer de una norma

mínima en el *Manual Terrestre* de la OMSA, con el compromiso de revisarla periódicamente a medida que se disponga de pruebas científicas.

Sección/Párrafo	Comentario	Decisión
Comentario general	Algunos Miembros tienen reservas sobre la inclusión de normas sobre vacunas en el <i>Manual Terrestre</i> por cuestiones de seguridad sobre el terreno.	La Comisión es consciente de estas cuestiones y del hecho de que las vacunas están actualmente autorizadas por algunas autoridades nacionales de reglamentación y se utilizan sobre el terreno. La sección propuesta ha sido redactada por expertos en colaboración con fabricantes de vacunas y expertos en reglamentación veterinaria. Aborda estas cuestiones lo mejor posible de acuerdo con las pruebas científicas actuales. La Comisión tiene la firme convicción de que es mejor ofrecer a las autoridades nacionales y a los fabricantes de vacunas recomendaciones basadas en datos científicos sobre las vacunas contra la PPA que no ofrecer ninguna
Resumen	Algunos Miembros han formulado comentarios sobre el <i>Resumen</i>	Solo se ha enviado para comentarios la sección de vacunas. Estas propuestas pueden abordarse cuando se actualice la sección de pruebas de diagnóstico
A. Introducción	Algunos Miembros han aportado comentarios sobre el texto no modificado de la <i>Introducción</i>	Solo se ha enviado para comentarios la sección de vacunas. Estas propuestas pueden abordarse cuando se actualice la sección de pruebas de diagnóstico
A. <i>Introducción</i> , párrafo 9	Enmendar el texto modificado para incluir información sobre mutantes y recombinantes que han surgido con una prevalencia potencialmente creciente, junto con dos referencias	De acuerdo, texto añadido
A. <i>Introducción</i> , párrafo 9	Añadir una frase que indique que no siempre es necesario seguir los principios expuestos en el Capítulo 1.1.8 <i>Principios de producción de vacunas veterinarias</i> cuando existen razones científicamente justificables para utilizar enfoques alternativos	En desacuerdo, el Capítulo 1.1.8 es una norma adoptada y no un ejemplo
A. <i>Introducción</i> , párrafo 9	Añadir un párrafo en el que se indique que es crucial confirmar la ausencia de cepas circulantes de otros genotipos del virus de la peste porcina africana antes de utilizar la vacuna, debido a las características del virus de la peste porcina africana, en el que se producen frecuentes recombinaciones entre cepas diferentes. Y añadir la afirmación de que es esencial establecer un sistema robusto de seguimiento de la vigilancia para detectar y	De acuerdo en que es importante confirmar qué genotipos del virus de la peste porcina africana circulan en una población antes de la vacunación: se ha añadido una frase a C.1. Antecedentes, párrafo 16. También de acuerdo en que es importante disponer de un sistema de vigilancia sólido.

Sección/Párrafo	Comentario	Decisión
	notificar rápidamente los acontecimientos inesperados resultantes de tales recombinaciones	Texto modificado en consecuencia
A. <i>Introducción</i> , párrafo 9	Reformular la frase sobre la validación de las vacunas vivas modificadas (MLV) para eliminar la no transmisibilidad, ya que, de acuerdo con la norma mínima, podría permitirse la transmisión de algún virus vacunal	De acuerdo
A. <i>Introducción</i> , párrafo 10	Incluir la inocuidad y la eficacia en diferentes grupos de edad de los cerdos, incluidos los verracos reproductores y las cerdas gestantes	La postura de la Comisión es que tales pruebas son preferibles, pero no se exigen en la norma mínima. En la actualidad, no se utiliza ninguna vacuna en cerdas gestantes
A. <i>Introducción</i> , párrafo 10	Incluir la duración de la inmunidad y el inicio de la inmunidad en la norma mínima	De acuerdo, se ha añadido una declaración de que el inicio y la duración de la inmunidad también son necesarios para cumplir las normas mínimas
C.1. <i>Antecedentes</i> , párrafo 1	Añadir una frase sobre la prevalencia de otros genotipos y recombinantes.	De acuerdo, y se ha modificado el texto propuesto
C.1. <i>Antecedentes</i> , párrafo 3	Aclarar que el nivel de bioseguridad apropiado debe basarse en la virulencia y las características del virus.	En desacuerdo, el concepto está cubierto por la palabra "apropiado"
C.1. <i>Antecedentes</i> , Inocuidad	Añadir una referencia a la definición de fiebre.	De acuerdo
C.1. <i>Antecedentes</i> , Inocuidad y eficacia	Los requisitos mínimos para las vacunas MLV deben incluir la inocuidad para cerdas gestantes, cerdos en diferentes etapas de crecimiento (lechones lactantes, cerdos destetados hasta los 4,5 meses y cerdos de engorde), verracos reproductores y protección cruzada contra otras cepas naturales que circulan actualmente.	La Comisión reiteró que su postura es que tales pruebas son preferibles, pero no necesarias en la norma mínima. La prueba sería necesaria si las MLV se autorizan para esas subpoblaciones
C.1. <i>Antecedentes</i> , Eficacia	Añadir "inducida por la PPA" después de "mortalidad".	En desacuerdo, el significado está implícito
C.1. <i>Antecedentes</i> , Calidad – potencia	Sustituir "potente" por "estabilidad".	De acuerdo
C.1. <i>Antecedentes</i> , Calidad – Identidad	Sustituir "identidad" por "cobertura vacunal".	De acuerdo
C.1 <i>Antecedentes</i> , párrafo 9	Incluir una declaración de que se necesita más investigación para determinar si estas MLV específicas del genotipo 2 pueden proteger eficazmente contra las nuevas variantes circulantes del genotipo II y las cepas recombinantes.	De acuerdo
C.1 <i>Antecedentes</i> , párrafo 10	Incluir las especies de destino de las vacunas que han sido autorizadas.	De acuerdo

Sección/Párrafo	Comentario	Decisión
C.1 <i>Antecedentes</i> , párrafo 11, quinto punto	Añadir una referencia a una nueva cepa candidata a vacuna que haya demostrado proporcionar una fuerte supresión de la viremia, etc., por lo que se espera que presente una mayor inocuidad en comparación con el virus de estudios anteriores.	De acuerdo
C.1 <i>Antecedentes</i> , párrafo 11 y todos los puntos	Existe la posibilidad de que un animal que haya recibido inadvertidamente dos cepas vacunales diferentes (con diferentes delecciones de un solo gen) pueda llegar a regenerar un VPPA totalmente virulento por recombinación. Considerar la posibilidad de exigir que todas las vacunas MLV contra el VPPA tengan al menos una delección atenuante en común para que esto no pueda ocurrir. Considerar también la posibilidad de combinar estos virus: todos fueron diseñados por recombinación homóloga y son cepas mutantes de delección con un número diferente de genes eliminados.	Es una posibilidad, pero el riesgo de reversión por recombinación a través de la coinfección por una vacuna y una cepa de tipo salvaje es significativamente mayor. Exigir que las vacunas tengan un único gen en común sería un reto técnico, teniendo en cuenta que la base biológica de la atenuación sigue siendo poco conocida
C.1 <i>Antecedentes</i> , párrafo 13	El texto da a entender que la próxima generación de vacunas serán las MLV; es probable que otra tecnología sea mejor. Esta redacción creará confusión en la sección de vacunas.	En desacuerdo, el texto existente es claro
C.1 <i>Antecedentes</i> , párrafo 13	Añadir una declaración de que no hay ninguna vacuna inactivada con ningún nivel de protección que pueda ser aceptable.	En desacuerdo, el texto existente es claro
C.1 <i>Antecedentes</i> , párrafo 15	Suprimir MLV, ya que debería aplicarse a la nueva tecnología de vacunas desarrollada.	De acuerdo
C.1 <i>Antecedentes</i> , párrafo 15	Añadir una descripción que haga hincapié en la importancia de la farmacovigilancia para la vacuna contra la PPA.	De acuerdo
C.1 <i>Antecedentes</i> , párrafo 16	Aclarar la definición de "circunstancias excepcionales".	De acuerdo
C.2.1.2 Criterios de calidad (esterilidad, pureza, ausencia de agentes extraños) párrafo 1	Suprimir la última frase, ya que no se encuentra en el lugar adecuado. Los requisitos de inocuidad se explican en otra parte.	De acuerdo
C.2.1.2 Criterios de calidad (esterilidad, pureza, ausencia de agentes extraños) párrafo 3	Incluir una explicación más detallada de la razón por la que debe demostrarse la estabilidad genética hasta al menos el inóculo vírico primario (MSV, por las siglas en inglés de <i>master seed virus</i> ) +10 cuando el MSV+8 es el paso máximo para su uso en la fabricación del producto final.	De acuerdo, se aclaró que, si los rendimientos del producto final son bajos, se requiere la demostración de estabilidad para el paso máximo para su uso en la fabricación del producto final según lo definido por el productor
C.2.2.4 <i>Pruebas por lotes de producto final</i> , ii) identidad	Aclarar el objetivo del paréntesis (por ejemplo, PCR en tiempo real diferencial específica).	De acuerdo, se añadió que los métodos de detección también deberían diferenciar el virus de la vacuna respecto al de la cepa madre como posible contaminante

Sección/Párrafo	Comentario	Decisión
C.2.2.4 <i>Pruebas por lotes de producto final</i> , vi) Humedad residual	Suprimir la mención de la vía de administración, ya que la prueba será necesaria para cualquier vacuna independientemente de la vía de administración.	De acuerdo
C.2.3.1 Proceso de fabricación	Modificar la frase, ya que no es necesario proporcionar información sobre lotes consecutivos de vacunas y es preferible obtener información de tres o más lotes.	De acuerdo, se ha borrado el texto
C.2.3.2 Requisitos de inocuidad	Esta sección es muy detallada y prescriptiva. ¿Puede acortarse o añadirse a otro anexo? Se sugiere centrarse en los principios más que en la naturaleza exacta de cómo llevar a cabo el estudio.	En desacuerdo, los principios generales se exponen en el Capítulo 1.1.8; los detalles que se ofrecen aquí son específicos de la PPA
C.2.3.2 Requisitos de inocuidad	Se prefiere una demostración adicional de la inocuidad de las MLV en cerdas jóvenes en edad reproductiva y cerdas gestantes, pero no se exige como norma mínima.	De acuerdo, como antes. Las vacunas actuales no están autorizadas en animales reproductores. Debe exigirse la demostración de la inocuidad en cerdas jóvenes en edad reproductiva y cerdas gestantes si se pretende utilizar la vacuna en estas subpoblaciones. Esta norma se revisará periódicamente a medida que se disponga de más datos sobre el uso de estas vacunas
C.2.3.2 <i>Requisitos de inocuidad</i> , i) Inocuidad en animales jóvenes	A menos que se considere que la categoría más sensible para las pruebas de inocuidad son los cerdos de 6-10 semanas de edad, sería preferible una redacción más flexible.	De acuerdo, modificado a un mínimo de 4 semanas y no más de 10 semanas de acuerdo con la evidencia actual
C.2.3.2 <i>Requisitos de inocuidad</i> , i) Inocuidad en animales jóvenes, párrafo 5	El periodo de observación propuesto es mucho más largo que el propuesto en la VICH GL44 para la inocuidad en especies de destino de vacunas veterinarias vivas e inactivadas. Cuando hay reacciones adversas en el lugar de la inyección al final de los 14 días de observación, el periodo de observación debería ampliarse hasta que se haya producido una resolución clínicamente aceptable de la lesión o, si procede, hasta que se practique la eutanasia al animal y se realice un examen histopatológico.	En desacuerdo, el periodo dado aquí está pensado para cubrir los signos clínicos crónicos que pueden aparecer muchas semanas después de la vacunación. Esto no está relacionado con las reacciones adversas en el lugar de la inyección
C.2.3.2 <i>Requisitos de inocuidad</i> , i) Inocuidad en animales jóvenes, párrafo 5	Para detectar una posible excreción del virus, añadir controles de virus vacunales en secreciones orales, nasales y fecales, cada 7 días durante al menos 60 días, y controles de virus vacunales en tejidos a los 28 días.	En desacuerdo porque puede haber una excreción limitada asociada al uso de vacunas MLV. La introducción de un requisito de no excreción impedirá el uso de la vacuna. A pesar de ser considerada inocua, se puede esperar un nivel mínimo de transmisión horizontal para las MLV
C.2.3.2 <i>Requisitos de inocuidad</i> , i) Inocuidad en	Aclarar que ningún lechón muestre signos notables de enfermedad: la redacción actual	De acuerdo

Sección/Párrafo	Comentario	Decisión
animales jóvenes, párrafo 7, primer punto	podría interpretarse como que los lechones vacunados que muestren signos notables de enfermedad pero que no alcancen el punto final humanitario predeterminado superarían la prueba.	
C.2.3.2 <i>Requisitos de inocuidad</i> , i) Inocuidad en animales jóvenes, párrafo 7, segundo punto	Aclarar qué se entiende por aumento "medio" de la temperatura corporal.	De acuerdo, este punto se ha reescrito
C.2.3.2 <i>Requisitos de inocuidad</i> , i) Prueba de inocuidad en cerdas gestantes y prueba de transmisión transplacentaria, párrafo 1	Dado que las MLV pueden infectar a las cerdas por transmisión horizontal y que el virus puede transmitirse verticalmente a los fetos, suprimir el texto actual y sustituirlo por el requisito de analizar las cerdas y los partos para detectar la posible excreción del virus.	En desacuerdo: como en el caso anterior, el texto actual es correcto; solo deberían exigirse pruebas adicionales si el fabricante recomienda el uso de la vacuna en cerdas en edad reproductiva y cerdas gestantes
C.2.3.2 <i>Requisitos de inocuidad</i> , ii) Prueba de inocuidad en cerdas gestantes y prueba de transmisión transplacentaria, párrafo 1	Corregir la primera frase, ya que se han realizado estudios experimentales sobre la transmisión del genotipo II del virus de la peste porcina africana de las cerdas gestantes a los fetos.	De acuerdo
C.2.3.2 <i>Requisitos de inocuidad</i> , iii) Transmisión horizontal, párrafo 1	Modificar "no menos de 12 lechones sanos" por "lechones sanos en número suficiente para confirmar la presencia o ausencia de transmisión horizontal entre animales vacunados y animales nunca antes expuestos" ya que no hay base científica para utilizar 12 lechones.	En desacuerdo, este texto es coherente con el capítulo sobre la peste porcina clásica y es aplicable a la peste porcina africana
C.2.3.2 <i>Requisitos de inocuidad</i> , iii) Transmisión horizontal, párrafo 1	Considerar la posibilidad de no mezclar directamente. Si se consideran las vacunas orales, la contaminación ambiental por el virus vacunal podría llevar a la "vacunación" de los contactos nunca antes expuestos.	Este punto es válido y se someterá a consideración cuando las vacunas orales estén listas para ser probadas
C.2.3.2 <i>Requisitos de inocuidad</i> , iii) Transmisión horizontal, párrafo 4	Sin comentarios, surgió del debate con expertos.	Aclaración de lo que se entiende por aumento de la temperatura corporal aquí y en todo el capítulo, cuando proceda
C.2.3.2 <i>Requisitos de inocuidad</i> , iii) Transmisión horizontal, párrafo 5	Suprimir el requisito de determinar los títulos de virus infecciosos mediante aislamiento cuantitativo del virus.	En desacuerdo: el genoma del virus persiste durante mucho más tiempo que el virus infeccioso, por lo que el uso de la PCR por sí sola puede dar resultados engañosos. Es importante medir el virus infeccioso (la PCR puede utilizarse para identificar muestras que puedan llegar a tener virus infeccioso). Los resultados positivos de la PCR también deben comprobarse mediante aislamiento cualitativo/cuantitativo del virus



Sección/Párrafo	Comentario	Decisión
C.2.3.2 <i>Requisitos de inocuidad</i> , iii) Transmisión horizontal, párrafo 8, tercer punto	Aclarar los criterios de aceptación de la vacuna.	De acuerdo y aclarados los puntos: la Comisión reconoce que, de acuerdo con las pruebas actuales, podría esperarse una transmisión horizontal mínima con la MLV y, sin embargo, la vacuna podría considerarse inocua
C.2.3.2 <i>Requisitos de inocuidad</i> , iv) Estudio de la cinética de replicación vírica tras la vacunación (diseminación sanguínea y tisular de la MLV), párrafo 8	Ampliar el intervalo de días en los que eutanasiar a los lechones y determinar los títulos del virus para incluir los días 1, 3 y 5.	Parcialmente de acuerdo: incluirá el día 5. El día 3 es posible con virus muy virulentos, pero si están atenuados podría ser más tarde; los días 1 y 3 probablemente sean negativos
C.2.3.2 <i>Requisitos de inocuidad</i> , v) Reversión a la virulencia, Primer pase (p1), párrafo 1	Aclarar los parámetros de observación.	De acuerdo, se ha modificado para que sea coherente con el texto estándar acordado aquí y en todo el capítulo, cuando proceda
C.2.3.2 <i>Requisitos de inocuidad</i> , v) Reversión a la virulencia, Primer pase (p1), párrafo 3	Ampliar los días en los que eutanasiar a los lechones y determinar los títulos de virus para incluir los días 1, 3 y 5.	Como antes, parcialmente de acuerdo: incluirá el día 5. El día 3 es posible con virus muy virulentos, pero si están atenuados podría ser más tarde; los días 1 y 3 probablemente sean negativos
C.2.3.2 <i>Requisitos de inocuidad</i> , v) Reversión a la virulencia, segundo pase (p2), párrafo 2	Por coherencia, cambiar "administración intramuscular" por "vía prevista".	De acuerdo
C.2.3.2 <i>Requisitos de inocuidad</i> , v) Reversión a la virulencia, quinto pase (p5), párrafo 3, segundo punto	Aclarar qué se entiende por "signos clínicos crónicos mínimos".	De acuerdo, se ha modificado el texto para hacer referencia a los signos clínicos leves
C.2.3.2 <i>Requisitos de inocuidad</i> , v) Reversión a la virulencia, quinto pase (p5), párrafo 4	Suprimir el párrafo, los requisitos pueden no ser viables para los países donde la enfermedad no es endémica.	En desacuerdo, mala interpretación del texto. Las pruebas de campo son muy importantes y pueden realizarse en un país endémico. No es necesario que se realicen en todos los países que deseen utilizar la vacuna, siempre que se disponga de los datos pertinentes de los países en los que ya se hayan realizado pruebas de campo
C.2.3.3 <i>Requisitos de eficacia</i> , i) Dosis protectora, párrafo 3	Proponer una sustitución de la descripción, que no se ajusta a las directrices VICH, por una descripción más factible. En las directrices de la VICH no se especifica la configuración de la prueba para la dosis protectora, como el número de cerdos, la edad, la consistencia de origen, la composición, etc.	En desacuerdo: La VICH no proporciona un protocolo específico para determinar la dosis mínima de protección. Sin embargo, el texto está en consonancia con las directrices generales para las pruebas de inocuidad descritas en la VICH GL 44 y otros documentos

Sección/Párrafo	Comentario	Decisión
		prescriptivos. La dosis protectora es una de las características definitorias de las vacunas y será exigida por la mayoría de las Autoridades Reguladoras, si no todas
C.2.3.3 <i>Requisitos de eficacia</i> , i) Dosis protectora, párrafo 5	Modificar el texto para que las pruebas de desafío en animales se realicen utilizando todas las cepas naturales circulantes.	En desacuerdo, no es adecuado para una norma mínima
C.2.3.3 <i>Requisitos de eficacia</i> , i) Dosis protectora, párrafo 5	Suprimir "o virus sin HAD"; HAD <sub>50</sub> y DICT <sub>50</sub> son indistinguibles.	En desacuerdo, se mantiene el texto original
C.2.3.3 <i>Requisitos de eficacia</i> , i) Dosis protectora, párrafo 6	Añadir los adjetivos oral, nasal y anal a las muestras a obtener de los lechones vacunados expuestos y analizar cada 7 días durante 60 días	Parcialmente de acuerdo: se añadieron las muestras, pero se limitó el periodo de observación a un mínimo de 45 días y preferiblemente 60 días después de la exposición
C.2.3.3 <i>Requisitos de eficacia</i> , i) Dosis protectora, párrafo 8	Añadir la histopatología después de la anatomopatología macroscópica.	De acuerdo
C.2.3.3 <i>Requisitos de eficacia</i> , i) Dosis protectora, párrafo 9	El 100% de mortalidad y morbilidad puede no ser posible dependiendo de la cepa utilizada para la infección experimental, es decir, no todas las cepas causarán un 100% de mortalidad en los cerdos control. Es mejor diseñar el estudio con cierta flexibilidad para que: a) la infección experimental pueda repetirse, y b) se utilice un número suficiente de animales para garantizar que se obtienen resultados estadísticamente relevantes sobre si la vacuna proporciona protección.	De acuerdo, se ha modificado el texto
C.2.3.3 <i>Requisitos de eficacia</i> , i) Dosis protectora, párrafo 10, segundo punto	Aclarar qué se entiende por aumento medio de la temperatura corporal.	De acuerdo y modificado aquí y en todo el capítulo, cuando proceda
C.2.3.3 <i>Requisitos de eficacia</i> , ii) Evaluación de la transmisión horizontal (estudio de excreción y propagación del virus de desafío), párrafo 10	Añadir los días 7 y 14 a los días en los que se tomarán muestras de sangre de los cerdos de contacto nunca antes expuestos, y ampliar el periodo de observación a 2 meses debido a la probable baja dosis de infección en los cerdos de contacto nunca antes expuestos.	Parcialmente de acuerdo, puede no haber anticuerpos en los cerdos de contacto en los días 7 y 14, post-contacto, por lo que es mejor que las muestras de sangre se analicen en busca de anticuerpos en los días 21 y 28 y al final del periodo de estudio. De acuerdo en ampliar el periodo de observación a, al menos, 60 días, y preferiblemente 2 meses
C.2.3.3 <i>Requisitos de eficacia</i> , ii) Evaluación de la transmisión horizontal (estudio de propagación del virus del desafío), párrafo 12	Añadir el requisito de realizar cortes histopatológicos y comprobar la situación citopática, así como de realizar pruebas inmunohistoquímicas del virus de la peste porcina africana para seguir rastreando su distribución en los órganos.	En desacuerdo, el objetivo no es caracterizar el virus, la PCR de los tejidos es suficiente

Sección/Párrafo	Comentario	Decisión
C.2.3.4 Duración de la inmunidad	Añadir una sección sobre la recombinación vacunal: la MLV puede tener recombinación genética con las cepas naturales circulantes y con otras cepas vacunales. Por lo tanto, se recomienda llevar a cabo estudios de determinación de una posible recombinación para evaluar el riesgo de recombinación vacunal.	Parcialmente de acuerdo, ya que está documentado que puede producirse recombinación, pero, debido a la dificultad de llevar a cabo este tipo de estudios de "recombinación" en el laboratorio, esto no debería ser una norma mínima, aunque sí podría ser una recomendación. Texto añadido a C. Antecedentes, párrafo 16

La nueva sección redactada del Capítulo 3.9.1. "Peste porcina africana" (infección por el virus de la peste porcina africana) (solo la sección sobre vacunas) se presenta como [Anexo 16](#) y se propondrá para adopción en la 91ª Sesión General, en mayo de 2024.

	Anexo	Capítulo	
1.	4	1.1.5.	Gestión de la calidad en los laboratorios de pruebas veterinarias
2.	5	1.1.9.	Pruebas de esterilidad y ausencia de contaminación en los materiales biológicos de uso veterinario
3.	6	2.2.4.	Incertidumbre de la medición
4.	7	2.2.6.	Elección y uso de tipos y grupos de muestras de referencia
5.	8	3.1.5.	Fiebre hemorrágica de Crimea-Congo
6.	9	3.3.6.	Tuberculosis aviar
7.	10	3.4.1.	Anaplasmosis bovina
8.	11	3.4.7.	Diarrea viral bovina
9.	12	3.4.12.	Dermatosis nodular contagiosa (solo sección sobre vacunas)
10.	13	3.6.9.	Rinoneumonitis equina (infección por <i>Varicellovirus equidalpha1</i> )
11.	14	3.8.1.	Enfermedad de la frontera
12.	15	3.8.12.	Viruela ovina y viruela caprina
13.	16	3.9.1.	Peste porcina africana (solo sección sobre vacunas)

### 5.3. Revisión rápida del capítulo sobre la influenza aviar: seguimiento del Foro de Sanidad Animal y de la Resolución adoptada sobre la influenza aviar

En la reunión de septiembre de 2023, la Comisión convino en la necesidad de una revisión rápida del capítulo del *Manual Terrestre* sobre la influenza aviar para asegurarse de que la información está al día respecto a los últimos avances científicos y es adecuada para su finalidad. Con este fin, se pidió a los laboratorios de referencia de la OMSA que actualizaran el capítulo para incluir inmediatamente las modificaciones importantes que fueran necesarias. El objetivo era someter el capítulo a una ronda de revisión con el informe de febrero de 2024 y proponer su adopción en mayo de 2024.

La Comisión tomó nota de que la actualización presentada por los laboratorios de referencia había sido ampliamente modificada. La idea de la revisión rápida era incluir las revisiones esenciales mínimas, como las secuencias de cebadores y sondas, que se requieren urgentemente para mantener la validez del capítulo en el contexto actual de la situación de la influenza aviar y que podrían proponerse para su adopción tras una sola ronda de comentarios. La Comisión decidió que, para un capítulo que había sido revisado sustancialmente, era necesaria más de una ronda de comentarios antes de presentarlo a la Asamblea, por lo que acordó incluir la actualización en el ciclo de revisión 2024/2025, en el que seguiría el procedimiento de revisión normal (dos rondas de comentarios, en octubre y en marzo, antes de proponer su adopción en mayo de 2025). Los laboratorios de referencia tendrían la oportunidad de modificarla antes de volver a presentarla a la OMSA en julio de 2024.

Entretanto, se informó a la Comisión de la existencia de un folleto titulado "Protocolos y directrices sobre la PPA", que había sido elaborado por la red de la PPA y que estaría disponible en el sitio web de la OMSA en un futuro próximo. La Comisión convino en que se trata de un método excelente para publicar rápidamente las actualizaciones

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esenciales de los protocolos, incluidas las secuencias de cebadores y sondas, con una amplia difusión de información esencial. Se preguntará a la red OFFLU si puede elaborar una publicación similar para la influenza aviar.

#### **5.4. Actualización del Capítulo 2.3.1 La aplicación de la biotecnología al desarrollo de vacunas de uso veterinario**

La Comisión identificó un centro colaborador y un experto que podrían ayudar en la revisión de este capítulo. A la Comisión le gustaría incluir futuros métodos de investigación en el desarrollo de vacunas, conservando al mismo tiempo la información sobre el desarrollo clásico de vacunas. Su propuesta es que el capítulo se centre en las vacunas contra las enfermedades de la lista de la OMSA, entre ellas:

1. Vacunas clásicas
2. Vacunas de nueva generación
3. Futuros métodos de investigación para el desarrollo de vacunas.

#### **5.5. Actualización del borrador de capítulo sobre la validación diagnóstica de las pruebas en el punto de atención para las enfermedades víricas de la lista de la OMSA utilizando muestras de campo**

Desde la última reunión, se ha pedido a las redes de laboratorios de referencia para la peste porcina africana (PPA), la rabia y la peste de pequeños rumiantes (PPR) que comenten el borrador de un nuevo capítulo sobre la validación diagnóstica de las pruebas en el punto de atención para las enfermedades víricas de la lista de la OMSA utilizando muestras de campo. Las redes están de acuerdo con el principio de publicar información sobre la validación de las pruebas en el punto de atención, ya sea como un capítulo independiente o como parte del Capítulo 1.1.6 o de los capítulos específicos de enfermedad, pero consideran que el texto necesita un mayor desarrollo para mejorar su practicidad y aplicabilidad. Los comentarios se remitirán al experto del centro colaborador que redactó el texto para que decida cuál es la mejor manera de avanzar.

#### **5.6. Avances en la elaboración de un formulario de informe de validación para las pruebas recomendadas en el *Manual Terrestre***

El [modelo de informe de validación](#) ya se ha finalizado y está disponible en la página web de la Comisión para que quienes contribuyan al *Manual Terrestre* aporten datos sobre las pruebas que recomiendan.

#### **5.7. Aplicación de los criterios para mantener los capítulos del *Manual Terrestre* sobre las enfermedades que no figuran en la lista de enfermedades de la OMSA.**

El *Manual Terrestre* contiene actualmente 26 capítulos sobre enfermedades que no figuran en la lista. Algunos de estos capítulos se refieren a enfermedades suprimidas de la lista que ya no cumplen los criterios de inclusión (por ejemplo, la leptospirosis), y otros, a enfermedades, a menudo zoonosis, que nunca figuraron en la lista pero sobre las que se consideró importante facilitar a los Miembros información a efectos del diagnóstico (por ejemplo, la toxoplasmosis). La Comisión es consciente de que mantener estos capítulos puede no ser la mejor manera de utilizar los recursos, y observó que para algunas no existen laboratorios de referencia designados, lo cual plantea dificultades para mantener los capítulos actualizados. La Comisión convino en que se aplicasen los siguientes criterios basados en pruebas a la hora de decidir el mantenimiento de un capítulo del *Manual Terrestre* sobre enfermedades que no figuran en la lista:

1. Existe un diagnóstico diferencial importante respecto a una enfermedad listada
2. Existe un laboratorio de referencia para la enfermedad que puede proporcionar apoyo científico
3. Existe un capítulo en el *Código Terrestre*

La Comisión aplicó estos criterios a los actuales 26 capítulos del *Manual Terrestre* sobre enfermedades que no figuran en la lista.

Se mantuvieron los siguientes capítulos:

1.	Leptospirosis	2.	Enfermedad por el virus Hendra
3.	Estomatitis vesicular	4.	Enfermedad de Marek

5.	Enfermedad de la frontera	5.	Melioidosis
7.	Virus de la influenza A porcina	8.	Enfermedad vesicular porcina
9.	<i>Escherichia coli</i> verocitotoxigénica	10.	Enfermedades bunivirales de los animales (excluidas la fiebre del Valle del Rift y la fiebre hemorrágica de Crimea-Congo)
11.	Zoonosis transmisibles por primates no humanos		

Los siguientes capítulos se eliminarán de la próxima edición tras la Sesión General de mayo de 2024. Estos capítulos seguirán estando disponibles en la secretaría de la BSC ([BSC.Secretariat@woah.org](mailto:BSC.Secretariat@woah.org)) previa petición:

1.	Nosemosis de las abejas melíferas	2.	Tuberculosis aviar*
3.	Enteritis viral del pato	4.	Cólera aviar
5.	Viruela aviar	6.	Fiebre catarral maligna
7.	Linfangitis epizoótica	8.	Adenocarcinoma pulmonar ovino (adenomatosis)
9.	Rinitis atrófica del cerdo	10.	Encefalomiелitis por teschovirus
11.	Criptosporidiosis	12.	Infección por <i>Campylobacter jejuni</i> y <i>C. coli</i>
13.	<i>Listeria monocytogenes</i>	14.	Sarna
15.	Toxoplasmosis		

\*una vez adoptado el capítulo en mayo de 2024, la información sobre la tuberculina aviar se trasladará al capítulo sobre la tuberculosis de los mamíferos y se suprimirá este capítulo

La Comisión también acordó que, una vez suprimidos estos capítulos del *Manual Terrestre* en mayo, dejaría de aceptar solicitudes de candidatura a designación como laboratorio de referencia para enfermedades que no figuren en la lista.

#### 5.8. Examen del dictamen presentado por los expertos de siete capítulos del *Manual Terrestre* actualizados y distribuidos en octubre de 2023 sobre si la actualización tuvo repercusiones en el capítulo correspondiente del *Código Terrestre*

En la reunión de septiembre de 2022 de las Mesas de las Comisiones del Código y de Normas Biológicas, se acordó que se pediría a los expertos que revisaron un capítulo del *Manual Terrestre* que asesoraran a la Comisión de Normas Biológicas sobre si la revisión propuesta podría tener repercusiones en el capítulo correspondiente del *Código Terrestre*. Se identificaron seis capítulos del *Manual Terrestre* en el actual ciclo de revisión que podían tener repercusiones en el *Código Terrestre*. Las Comisiones de Normas Biológicas examinaron el asesoramiento recibido de los expertos que habían emprendido las actualizaciones y acordaron presentar las siguientes recomendaciones a la Comisión del Código:

Capítulo del Código	Recomendaciones de la Comisión de Normas Biológicas a la Comisión del Código
Capítulo 11.1 Anaplasmosis bovina	La Comisión está de acuerdo en que el Artículo 11.1.2 del capítulo del <i>Código Terrestre</i> podría actualizarse para tener en cuenta la mejora de los métodos analíticos para el diagnóstico y los métodos de tratamiento efectivos.

Capítulo del Código	Recomendaciones de la Comisión de Normas Biológicas a la Comisión del Código
Capítulo 11.X Diarrea viral bovina	La Comisión está de acuerdo en que se actualice la taxonomía del agente en el <i>Código Terrestre</i> para armonizarla respecto al <i>Manual Terrestre</i> .
Capítulo 11.9 Dermatitis nodular contagiosa	La Comisión está de acuerdo en que la actualización del <i>Manual Terrestre</i> no repercute en el capítulo del <i>Código Terrestre</i> .
Capítulo 12.8 Rinoneumonitis equina	La Comisión está de acuerdo en que se actualice la taxonomía del agente en el <i>Código Terrestre</i> para armonizarla respecto al <i>Manual Terrestre</i> . También sería útil añadir una definición de caso al <i>Código Terrestre</i> .
Capítulo 14.9 Viruela ovina y viruela caprina	La Comisión está de acuerdo en que la actualización del <i>Manual Terrestre</i> no tiene ningún impacto en el capítulo del <i>Código Terrestre</i> .
Capítulo 15.1 Infección por el virus de la peste porcina africana	La Comisión está de acuerdo en que el capítulo del <i>Código Terrestre</i> debe actualizarse debido a la inclusión de la vacunación en el <i>Manual Terrestre</i> .

#### 5.9. Actualización de la solicitud de la Comisión del Código relativa al Capítulo 2.1.1 Metodologías de laboratorio para los antibiogramas

Se informó a la Comisión de los progresos realizados desde la reunión de septiembre de 2023 sobre la solicitud de la Comisión del Código de revisar el Capítulo 2.1.1. del *Manual Terrestre*, titulado *Metodologías de laboratorio para los antibiogramas* con el fin de determinar si el capítulo proporciona información suficiente y actualizada sobre el establecimiento de umbrales clínicos o si es necesario revisarlo.

Para responder a esta petición, la Comisión de Normas Biológicas consultó al Grupo de Trabajo de la OMSA sobre la Resistencia a los Antimicrobianos (RAM). El Grupo aconsejó que la experiencia en las metodologías de laboratorio actuales y futuras para la RAM, incluido el establecimiento de umbrales clínicos, recae en los centros colaboradores de la OMSA. En octubre de 2023, se pidió a tres centros colaboradores pertinentes de la OMSA que revisaran el capítulo actual y presentaran un esquema detallado de lo que hay que hacer para actualizarlo y abordar las preocupaciones de los Miembros.

La Comisión examinó el "mapa" presentado por los centros y acordó el esquema previsto para la revisión del capítulo. Se pedirá a los centros que apliquen su plan y presenten el capítulo actualizado para su revisión en la reunión de septiembre de 2024. El objetivo es proponer el capítulo para su adopción en mayo de 2025.

#### 5.10. Solicitud de reconsideración de la inclusión de partículas similares al virus de la fiebre aftosa en el *Manual Terrestre* de la OMSA

Un grupo de investigadores que había desarrollado una nueva vacuna contra la fiebre aftosa con partículas similares a virus (VLP) expresadas de manera recombinante solicitó que la Comisión reconsiderase su decisión de no incluir tales vacunas VLP en el *Manual Terrestre* hasta que hayan recibido la autorización de comercialización (cf: Informe de la reunión de la Comisión de Normas Biológicas/Septiembre de 2022). La Comisión consultó de nuevo a los laboratorios de referencia de la OMSA para la fiebre aftosa y volvió a concluir que es demasiado pronto para una inclusión de este tipo en el *Manual Terrestre*, que no incluye las vacunas que aún no están en uso. Dada la importancia de estas vacunas, incluido su impacto en el *Código Terrestre*, la Comisión acogería con satisfacción los informes de los desarrolladores sobre los avances en el proceso de registro, así como toda posible información revisada por expertos sobre su uso. Una vez que las vacunas estén disponibles y en uso, la Comisión podría aplicar el procedimiento rápido para incluirlas en el *Manual Terrestre* si la red de expertos del laboratorio de referencia lo considera oportuno.

#### 5.11. Seguimiento de la Sesión General: propuesta de incluir una vacuna en el capítulo sobre la loque americana de las abejas melíferas

En la Sesión General, un Miembro había informado a la Asamblea de que se había autorizado el uso de una nueva vacuna contra *Paenibacillus larvae* en el país y había solicitado que se incluyera dicha vacuna en el Capítulo 3.2.2. *Loque americana de las abejas melíferas* (infección de las abejas melíferas por *Paenibacillus larvae*). Los

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laboratorios de referencia de la OMSA informaron a la Comisión de que la vacuna sigue en estudio y, por lo tanto, aún no cuenta con el respaldo científico necesario para justificar una recomendación de inclusión en el *Manual Terrestre*. La Comisión pedirá a los expertos que supervisen los ensayos de campo y le informen si la vacuna puede incluirse en el futuro.

## **5.12. Situación del *Manual Terrestre*: actualización de los capítulos elegidos para el ciclo de revisión 2024/2025**

La Comisión ha animado a los laboratorios de referencia que tienen capítulos pendientes a que los entreguen dentro de plazo. Se han identificado los siguientes capítulos para su actualización en 2024/2025 (año de la última adopción entre paréntesis después del título).

- 1.1.2. Recogida, presentación y almacenamiento de muestras para el diagnóstico (2013)
- 1.1.3. Transporte de material biológico (2018)
- 1.1.4. Bioseguridad y bioprotección: Norma para la gestión del riesgo biológico en el laboratorio veterinario y en las instalaciones de los animales (2015)
- 1.1.7. Normas aplicables a la secuenciación de alto rendimiento, la bioinformática y la genómica computacional (2016)
- 2.1.3. Gestión del riesgo biológico: ejemplos de asignación de las estrategias de gestión del riesgo a los riesgos biológicos detectados (2014)
- 2.1.1. Métodos de laboratorio para las pruebas de sensibilidad de las bacterias frente a los antimicrobianos (2019)
- 2.2.1. Desarrollo y optimización de pruebas de detección de anticuerpos (2014)
- 2.2.2. Desarrollo y optimización de pruebas de detección de antígenos (2014)
- 2.2.3. Desarrollo y optimización de pruebas de detección de ácidos nucleicos (2014)
- 2.2.5. Enfoques estadísticos de la validación (2014)
- 2.2.7 Principios y métodos para la validación de pruebas de diagnóstico de enfermedades infecciosas aplicables a la fauna salvaje (2014)
- 2.2.8. Comparabilidad entre pruebas tras realizar cambios menores en un método analítico validado (2016)
- 2.3.2. El papel de los organismos oficiales en la regulación internacional de los productos biológicos de uso veterinario (2018)
- 2.3.3. Requisitos mínimos para la organización y la gestión de un centro de fabricación de vacunas (2016)
- 2.3.5. Requisitos mínimos para la producción aséptica en la fabricación de vacunas (2016)
- 3.1.2. Enfermedad de Aujeszky (infección por el virus de la enfermedad de Aujeszky) (2018)
- 3.1.8. Fiebre aftosa (infección por el virus de la fiebre aftosa) (2021)
- 3.1.9. Cowdriosis (2018)
- 3.1.14. Miasis por *Cochliomyia hominivorax* y miasis por *Chrysomya bezziana* (2019)
- 3.1.17. Fiebre Q (2018)
- 3.1.20. Peste bovina (infección por el virus de la peste bovina) (2018)
- 3.1.25. Fiebre del Nilo Occidental (2018)
- Sección 3.2. Nota preliminar sobre las enfermedades de las abejas (2013)

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- 3.2.5. Infestación por *Aethina tumida* (escarabajo de las colmenas) (2018)
  - 3.2.6. Infestación de las abejas melíferas por *Tropilaelaps* spp. (2018)
  - 3.3.1. Clamidiosis aviar (2018)
  - 3.3.2. Bronquitis infecciosa aviar (2018)
  - 3.3.4. Influenza aviar (incluida la infección por virus de la influenza aviar altamente patógenos) (2021)
  - 3.3.8. Hepatitis viral del pato (2017)
  - 3.3.11. Púlorosis y tífosis aviar (2018)
  - 3.3.12. Bursitis infecciosa (enfermedad de Gumboro) (2016)
  - 3.4.9. Leucosis bovina enzoótica (2018)
  - 3.4.11. Rinotraqueítis infecciosa bovina/vulvovaginitis pustular infecciosa (2017)
  - 3.4.15. Teileriosis bovina (infección por *Theileria annulata*, *T. orientalis* y *T. parva*) (2018)
  - 3.4.16. Tricomonosis (2018)
  - 3.6.1. Peste equina (infección por el virus de la peste equina) (2019)
  - 3.6.6. Anemia infecciosa equina (2019)
  - 3.6.7. Gripe equina (infección por el virus de la gripe equina) (2019)
  - 3.6.10. Arteritis viral equina (infección por el virus de la arteritis equina) (2013)
  - 3.6.11. Muermo y melioidosis (2018)
  - 3.8.2. Artritis/encefalitis caprina y Maedi-visna (2017)
  - 3.8.3. Agalaxia contagiosa (2018)
  - 3.8.5. Aborto enzoótico de las ovejas (clamidiosis ovina) (infección por *Chlamydia abortus*) (2018)
  - 3.8.7. Epididimitis ovina (*Brucella ovis*) (2015)
  - 3.8.11. Prurigo lumbar (2022)
  - 3.8.12. Viruela ovina y viruela caprina (2017) (sección sobre vacunas)
  - 3.9.3. Peste porcina clásica (infección por el virus de la peste porcina clásica) (2022: sección sobre pruebas de diagnóstico)
  - 3.9.8. Enfermedad vesicular porcina (2018)
  - 3.9.10. Gastroenteritis transmisible (2008)
  - 3.10.9. *Escherichia coli* verocitotoxigénica (2008)

### **15.13. Actualización del proyecto de herramienta de navegación para la consulta en línea de normas de la OMSA**

La Comisión recibió información actualizada sobre la herramienta de navegación para la consulta en línea de las normas de la OMSA, que es una iniciativa destinada a simplificar el acceso y la navegación para la consulta de las normas de la OMSA.



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El proyecto ofrecerá tres interfaces de usuario:

- Herramienta de navegación y búsqueda: una interfaz estructurada e intuitiva proporcionará una experiencia de navegación continua y guiada, permitiendo a los usuarios navegar sin esfuerzo por los *Códigos y Manuales Terrestres y Acuáticos* de la OMSA.
- Recomendaciones para un comercio internacional seguro por mercancía: esta fase permitirá a los usuarios visualizar las recomendaciones para un comercio internacional seguro por mercancía a través de un exhaustivo sistema de filtrado.
- Gestión de Normas: esta interfaz permitirá al personal de la OMSA gestionar y actualizar eficazmente las normas internacionales de la OMSA, tras la adopción de texto nuevo o revisado en la Asamblea General de la OMSA.

La herramienta se demostrará en un expositor durante la 91ª Sesión General, en mayo de 2024, y está previsto que entre en funcionamiento en julio de 2024.

Este proyecto representa un hito importante en el compromiso de la OMSA para mejorar el acceso y la utilización de las normas de la OMSA y contribuye a los objetivos del 7º Plan Estratégico para implementar la transformación digital, responder a las necesidades de los Miembros y mejorar la eficiencia y agilidad de la OMSA.

## 6. Centros de referencia de la OMSA

### 6.1. Actualización del sistema de evaluación de los informes anuales

Durante la última reunión de la Comisión, en septiembre de 2023, se introdujo un método basado en el riesgo en el sistema de evaluación de los informes anuales para aumentar su eficiencia y reducir al mismo tiempo la carga de trabajo de la Comisión. Este sistema es un método semiautomatizado destinado a crear un medio eficaz de evaluación del rendimiento, capaz de detectar con gran sensibilidad los laboratorios de referencia (LR) que tienen un rendimiento insuficiente. El objetivo era crear un sistema capaz de evaluar digital y automáticamente los informes.

El sistema emplea una metodología basada en el riesgo para el análisis inicial de los informes anuales, clasificando los LR como de bajo riesgo o de alto riesgo de presentar bajo rendimiento. Esta categorización se basa en los criterios de riesgo identificados durante la reunión de la Comisión de septiembre de 2023, como respuestas negativas a preguntas "esenciales" (las preguntas 1, 18, 19, 20 y 27 del modelo de informe), ser un LR nuevo, u obtener una puntuación inferior al 50% de media en todas las preguntas. Esta estrategia garantiza un examen uniforme de todos los informes, señalando aquellos que requieren una evaluación individual más exhaustiva por parte de los miembros de la Comisión, con especial atención a los informes potencialmente deficientes. Esto reduce el número de informes que cada miembro de la BSC debe evaluar, optimizando así sus esfuerzos.

En cuanto a las preguntas 25 y 27, la Comisión acordó que el requisito de organizar o participar en pruebas de competencia inter-laboratorios podría satisfacerse si la respuesta a cualquiera de las preguntas es afirmativa, es decir, si las pruebas de competencia se realizan con laboratorios de referencia de la OMSA o con laboratorios ajenos a la OMSA.

La Comisión acordó aplicar este sistema por primera vez en la revisión de los informes de 2022. La Secretaría distribuyó los informes identificando el sistema de forma equitativa entre los miembros de la Comisión, reduciendo el número de aproximadamente 40 informes anuales por miembro de la BSC a entre 20 y 23 informes, aproximadamente la mitad del número anterior. Se convocó una reunión extraordinaria en noviembre de 2023 para finalizar las evaluaciones de los informes anuales de 2022, valorar el rendimiento del nuevo sistema y comunicar las conclusiones dentro de la red.

Tras esta aplicación inicial del sistema, se etiquetaron 130 informes anuales en función de diversos criterios de riesgo: 90 por cuestiones esenciales, 12 por rendimiento insuficiente, ocho como laboratorios nuevos y 13 fueron seleccionados al azar. Una evaluación detallada de estos informes reveló que 49 de los 90 laboratorios con una cuestión esencial fueron confirmados como problemáticos y recibieron cartas de notificación. De los 12 laboratorios con una puntuación media inferior al 50% en todas las preguntas, se contactó con cuatro.

La Comisión convino en que el sistema minimizaba eficazmente su carga de trabajo y orientaba estratégicamente sus esfuerzos hacia los laboratorios de referencia que más atención requerían. La Comisión también convino en que el sistema demostraba una gran sensibilidad a la hora de identificar los informes anuales con riesgos significativos de bajo rendimiento. Sin embargo, hubo un debate sobre situaciones específicas en las que los laboratorios se dedican a enfermedades con baja incidencia epidemiológica, trabajan con enfermedades erradicadas o son laboratorios únicos para enfermedades específicas. En tales casos, estos laboratorios pueden tener dificultades para cumplir todos los términos de referencia y, por lo tanto, deben recibir una consideración especial. Por otra parte, la Comisión reconoce la necesidad de normalizar los criterios para la emisión de cartas de notificación de bajo rendimiento. De cara al futuro, la Comisión se compromete a comprobar y mejorar continuamente el sistema.

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## 6.2. Solicitudes de candidatura a designación como centro de referencia de la OMSA

La Comisión recomendó la aceptación de las siguientes solicitudes de candidatura a designación como centro de referencia de la OMSA:

*Laboratorio de referencia de la OMSA para la viruela ovina y la viruela caprina*  
Sciensano, Groeselenberg, 99 1180 Uccle  
BÉLGICA  
Tel.: + 32-2 379.05.14 / 379.06.27  
E mail: [nick.deregge@sciensano.be](mailto:nick.deregge@sciensano.be)  
Sitio web: <https://www.sciensano.be/en> <https://www.eurl-capripox.be/homepage>  
Experto designado: Dr. Nick De Regge

*Laboratorio de referencia de la OMSA para la rabia*  
Veterinary Research Institute, Ministry of Agriculture  
No.376, Zhongzheng Rd., Tamsui Dist., New Taipei City 251018  
TAIPEI CHINO  
Tel.: +886-2 26.21.21.11 Annex 602  
E-mail: [aphsu@mail.nvri.gov.tw](mailto:aphsu@mail.nvri.gov.tw)  
Sitio web: <https://eng.nvri.gov.tw>  
Experto designado: Dr. Ai-Ping Hsu

*Laboratorio de referencia de la OMSA para la leptospirosis*  
ICAR-National Institute of Veterinary Epidemiology and Disease Informatics (ICAR-NIVEDI),  
Post Box No. 6450, Yelahanka, Bengaluru 560064, Karnataka  
INDIA  
Tel.: +91-80 23.09.31.36 / 31.00  
E mail: [b.vinayagamurthy@icar.gov.in](mailto:b.vinayagamurthy@icar.gov.in); [director.nivedi@icar.gov.in](mailto:director.nivedi@icar.gov.in);  
Sitio web: <https://www.nivedi.res.in/>  
Experto designado: Dr. Vinayagamurthy Balamurugan

*Laboratorio de referencia de la OMSA para la peste de pequeños rumiantes*  
ICAR-National Institute of Veterinary Epidemiology and Disease Informatics (ICAR-NIVEDI),  
Post Box No. 6450, Yelahanka, Bengaluru- 560064, Karnataka  
INDIA  
Tel.: +91-80 23.09.31.36 /31.00  
E mail: [b.vinayagamurthy@icar.gov.in](mailto:b.vinayagamurthy@icar.gov.in); [director.nivedi@icar.gov.in](mailto:director.nivedi@icar.gov.in);  
Sitio web: <https://www.nivedi.res.in>  
Experto designado: Dr. Vinayagamurthy Balamurugan

*Laboratorio de referencia de la OMSA para la salmonelosis*  
Central Veterinary Laboratory,  
Ministry of Agriculture, Water and Land Reform  
24 Goethe Street, P-Bag 13187, Windhoek  
NAMIBIA  
Tel.: +264-61 23.76.84  
E-mail: [Siegfried.Khaiseb@mawlr.gov.na](mailto:Siegfried.Khaiseb@mawlr.gov.na)  
Experto designado: Dr. Siegfried Khaiseb

*Centro de referencia de la OMSA para Epidemiología de Campo*  
Centre National de Veille Zoosanitaire (CNVZ)  
38, Avenue Charles Nicolle, Cite Mahrajène, 1082 Túnez  
TÚNEZ  
Tel.: (+216) 71849790 - (+216) 71849812  
E-mail: [bo.cnvz@iresa.agrinet.tn](mailto:bo.cnvz@iresa.agrinet.tn); [baccar.vet@gmail.com](mailto:baccar.vet@gmail.com);  
Sitio web: [www.cnvz.agrinet.tn](http://www.cnvz.agrinet.tn)  
Contacto: Dra. Sana Kalthoum

Se había recibido una solicitud de un condado de África de candidatura a designación como laboratorio de referencia para la influenza aviar. La Comisión quedó plenamente satisfecha con la calidad y capacidad de la institución solicitante y con los servicios que podía prestar a los Miembros de la OMSA. Sin embargo, la Comisión cuestionó la elección del experto designado. La Comisión pedirá que se aclare la experiencia del designado en diagnóstico e investigación, así como su función en el laboratorio. Se pedirá al candidato que facilite información más detallada sobre su experiencia en normalización y validación de pruebas de diagnóstico, así como en publicaciones revisadas por expertos sobre la influenza aviar. Aunque el laboratorio tenía claramente una gran experiencia en la enfermedad,

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el experto propuesto no cumplía las expectativas de un experto de la OMSA. Por lo tanto, la Comisión no aceptó la solicitud, por el momento.

Se había recibido otra solicitud de un país de la región de Asia-Pacífico de candidatura a designación como laboratorio de referencia para la fiebre aftosa. Hace algunos años, la Comisión tuvo conocimiento de una serie de problemas de calidad y seguridad en este laboratorio. Se identificaron tres áreas de preocupación: el nivel de conocimientos del experto designado; la falta de confianza en la elección y eficacia de las pruebas realizadas por el laboratorio y en la inocuidad de los reactivos que produce y suministra a otros laboratorios; y la preocupación por el insuficiente nivel de bioseguridad. El laboratorio fue retirado de la lista de laboratorios de referencia de la OMSA mientras se sometía a un plan de supervisión del rendimiento (PMS, por las siglas en inglés de *performance monitoring scheme*) con otros laboratorios de referencia de la OMSA independientes para resolver estos problemas. La Comisión cuestionó el calendario de la solicitud, ya que el PMS no se había completado y el laboratorio sigue en construcción. La Comisión también tenía dudas sobre el nivel de bioseguridad con el que funciona actualmente el laboratorio, dada la naturaleza del trabajo que allí se realiza. La Comisión detectó lagunas en la información facilitada. Como aspecto positivo, el experto designado propuesto presentó un excelente currículum vitae y cumple las expectativas de un experto de la OMSA. Sin embargo, en conjunto, la Comisión consideró prematuro solicitar la candidatura a designación como laboratorio de referencia y no aceptó la solicitud.

Se había recibido otra solicitud de un país de la región Asia-Pacífico de candidatura a designación como laboratorio de referencia para la piroplasmosis equina. La Comisión se mostró plenamente satisfecha con la excelencia del centro para las enfermedades equinas, reconociendo en particular la excelencia científica de la institución solicitante y su potencial para contribuir significativamente a la OMSA, así como la pericia del experto designado. A pesar de estos puntos fuertes, la Comisión tiene dos grandes preocupaciones: la escasa gama de métodos de diagnóstico empleados habitualmente y la proyección internacional del laboratorio, por ejemplo, la organización y participación en pruebas de competencia internacionales. La Comisión no acepta la solicitud en este momento, pero anima al solicitante a abordar estas importantes cuestiones. La Comisión evaluará atentamente toda posible información complementaria que se presente.

Por último, se había recibido una solicitud de candidatura a designación como centro colaborador para Materiales de Referencia para Técnicas de Diagnóstico Molecular de Enfermedades de los Animales Acuáticos y Terrestres. La Comisión se mostró satisfecha con la excelencia científica del experto y consideró que el Centro sería una aportación útil a la red de la OMSA. Dado que la solicitud se centraba más en las enfermedades de los animales acuáticos, la Comisión de Normas Biológicas acordó que la responsabilidad de la decisión final sobre la aprobación de la solicitud recaía en la Comisión para los Animales Acuáticos (véase el punto 13.1 del informe de la reunión de febrero de 2024 de la Comisión para los Animales Acuáticos).

### **6.3. Cambios de expertos en los centros de referencia de la OMSA**

Los Delegados de los Miembros interesados habían presentado a la OMSA las siguientes candidaturas para los cambios de experto en los laboratorios de referencia de la OMSA. La Comisión recomendó su aceptación:

*Brucelosis (Brucella abortus, B. melitensis, B. suis):*

El Dr. Liangquan Zhu para sustituir al Prof. Jiabo Ding en el *China Institute of Veterinary Drug Control (IVDC)*, CHINA (REP. POP. DE)

*Bursitis infecciosa (enfermedad de Gumboro):*

El Dr. Yulong Gao para sustituir a la Dra. Xiaomei Wang en la *Division of Avian Immunosuppressive Disease, Harbin Veterinary Research Institute (HVRI), Chinese Academy of Agricultural Sciences (CAAS)*, CHINA (REP. POP. DE)

*Viruela ovina y viruela caprina:*

El Dr. Mohammand Hassan Ebrahimi-jam para sustituir al Dr. Hamid Reza Varshovi en el *RAZI Vaccine & Serum Research Institute*, IRÁN

*Influenza porcina:*

El Dr. Junki Mine para sustituir al Dr. Takehiko Saito en la *Viral Disease and Epidemiology Research Division, National Institute of Animal Health, National Agriculture and Food Research Organization*, JAPÓN

*Influenza aviar:*

La Dra. Eun Kyoung Lee para sustituir a la Dra. YOUN-JEONG LEE en el *Animal and Plant Quarantine Agency Ministry of Agriculture, Forest and Rural Affairs*, COREA (REP. DE)

*Rabia:*

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El Dr. Juan Antonio Montaña Hirose para sustituir al Dr. José Álvaro Aguilar Setién en el *National Centre for Animal Health Diagnostic Services*, MÉXICO

*Leptospirosis:*

La Dra. Paula Ristow para sustituir a la Dra. Marga Goris at the Academic Medical Centre, Department of Medical Microbiology and Infection Prevention University of Amsterdam, PAÍSES BAJOS

*Fiebre Q:*

La Dra. Agnieszka Jodelko para sustituir al Dr. Krzysztof Niemczuk at the National Veterinary Research Institute, Department of Cattle and Sheep Diseases, POLONIA

*Dermatosis nodular contagiosa:*

La Dra. Antoinette Van Schalkwyk para sustituir al Dr. David Wallace at the Onderstepoort Veterinary Institute, SOUTH ÁFRICA

La Comisión examinó otra candidatura para un cambio de experto y, basándose en la información facilitada, consideró que el candidato no cumplía las expectativas de un experto de la OMSA. Se pedirá al Miembro que vuelva a presentar un currículum vitae reforzado o que proponga a otro experto.

#### **6.4. Examen de solicitudes nuevas y pendientes de hermanamiento de laboratorios**

En febrero de 2024, se habían completado 90 proyectos y 16 estaban en curso. De los proyectos finalizados, 15 laboratorios de referencia y cuatro centros colaboradores han conseguido la designación de la OMSA.

Se presentaron seis propuestas de proyectos de hermanamiento de laboratorios para su examen por parte de la Comisión:

1. **Jordania - Reino Unido** para la fiebre aftosa: la Comisión apoyó el contenido técnico de esta propuesta de proyecto
2. **Sudáfrica - Turquía** para la fiebre del Valle del Rift: la Comisión apoyó el contenido técnico de esta propuesta de proyecto
3. **Estados Unidos de América - Rumanía** para la gestión de riesgos biológicos: la Comisión apoyó el contenido técnico de esta propuesta.
4. **Alemania y Camerún** para la enfermedad de Newcastle: la Comisión apoyó el contenido técnico de esta propuesta.
5. **Estados Unidos de América - Vietnam** para la rabia: la Comisión apoyó el contenido técnico de esta propuesta.
6. **Sudáfrica - Tanzania** para el desarrollo de capacidades en materia de métodos normalizados de diagnóstico de las enfermedades de los pequeños rumiantes: la Comisión apoyó el contenido técnico de esta propuesta con algunas modificaciones.

#### **6.5. Comentarios de los Laboratorios que no cumplen los TdR (términos de referencia) esenciales**

La Comisión examinó las observaciones recibidas de 28 laboratorios de referencia que no cumplían los criterios de desempeño (o términos de referencia) esenciales según sus informes anuales de 2022. La mayoría de estos LR respondieron con justificaciones aceptables de su incumplimiento de los TdR, y la Comisión aceptó sus explicaciones. Sin embargo, aunque se aceptaron las respuestas, todos los LR se incluirán en una lista de vigilancia. Esto implica que su informe anual se someterá a una revisión más exhaustiva durante la próxima ronda de evaluaciones para garantizar el cumplimiento y los avances.

Algunos LR informaron de que no recibieron ninguna solicitud de pruebas de diagnóstico porque están situados en regiones libres de la enfermedad. La Comisión estudiará cómo evaluar a los laboratorios en situaciones en las que la enfermedad está bien controlada o no está muy extendida. Del mismo modo, el hecho de ser el único LR para una enfermedad específica supuso que algunos laboratorios no pudieran unirse a otros o formar redes, lo cual fue señalado por la Comisión. Sin embargo, en estos casos, los TdR indican que también pueden formarse redes con instituciones que no sean LR de la OMSA, y la Comisión anima a los LR a establecer tales redes.

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## 6.6. Revisión de la plantilla de currículum vitae para las candidaturas a designación como experto sustituto

Al revisar las candidaturas a designación como expertos sustitutos, la Comisión detectó un problema recurrente con la información facilitada, que a menudo está incompleta o no cumple las normas de evaluación exigidas. Para promover la uniformidad en las candidaturas y evitar retrasos causados por datos insuficientes en el *currículum vitae* (CV), la Comisión acordó revisar la plantilla de CV para los nuevos candidatos y las candidaturas a designación como expertos sustitutos en los centros de referencia.

En primer lugar, la Comisión añadió más campos obligatorios para información básica, como una dirección de correo electrónico y el nombre de la enfermedad. Para evaluar mejor su idoneidad, se pedirá a los candidatos que faciliten una lista más completa de sus cualificaciones académicas y profesionales, incluido el año de obtención de cada título. En una sección aparte, se pide a los expertos que faciliten información sobre sus funciones, duración y responsabilidades en el pasado.

Dada la necesidad de determinar el nivel de sus conocimientos especializados, ahora se pide a los candidatos que faciliten detalles sobre su reconocimiento y prestigio internacional, incluidos nombramientos, premios, pertenencia a asociaciones, participación en grupos de trabajo y actividades relevantes. Por último, la sección sobre publicaciones revisadas por expertos se ha modificado para garantizar su relevancia en el campo, exigiendo a los expertos que destaquen su nombre en negrita en el título de la publicación, junto con la enfermedad o agente patógeno en cuestión. Las publicaciones deben enumerarse cronológicamente, y se debe demostrar la contribución continua del experto y su posición actual en su campo de especialización.

Esta plantilla también fue aprobada por la Comisión para los Animales Acuáticos (véase el punto 3.1 del informe de la reunión de febrero de 2024 de la Comisión para los Animales Acuáticos).

La plantilla modificada se puede consultar en el [Anexo 17](#).

## 6.7. Comentarios de los centros que no cumplen los TdR esenciales

La Comisión examinó los comentarios de siete centros colaboradores que no cumplían los criterios de desempeño esenciales según sus informes anuales de 2022. Se citaron comúnmente dos razones para no cumplir los TdR esenciales. 1) La colaboración o las actividades con otros centros no suelen tener lugar anualmente. La Comisión entiende y acepta que los esfuerzos y los recursos se destinen a estas actividades cada dos años. 2) El impacto del SARS-CoV-2: la Comisión aceptó esta respuesta para los informes de 2022, pero subrayó que no espera que la pandemia Covid-19 figure entre los motivos de incumplimiento de los TdR en los informes anuales de 2023.

La Comisión aceptó las propuestas ofrecidas por los siete centros para mejorar su rendimiento y los incluyó en una lista de vigilancia para una revisión de seguimiento durante el próximo ciclo de revisión de los informes anuales.

## 6.8. Revisión del procedimiento propuesto para evaluar los centros al final de su mandato de 5 años

Los centros colaboradores son designados por un periodo de 5 años, durante el cual se adhieren a un plan de trabajo quinquenal que se presenta al principio del periodo de designación. Al final de este periodo, la Directora General envía una carta solicitando un informe de los logros de los 5 años, tal y como se describen en el plan de trabajo. La Comisión evalúa este informe y decide si la designación del centro colaborador debe renovarse o no en función de sus resultados y de la necesidad de mantener un centro para el tema en cuestión.

Este sistema de designación de centros colaboradores por un periodo de 5 años se introdujo en 2020 con la adopción de los POE ([Centro Colaborador – Procedimientos para la Designación](#)). Los primeros centros que alcancen el final de su designación de 5 años lo harán a finales de 2024.

En su reunión de septiembre de 2023, la Comisión acordó que a finales del segundo trimestre del quinto año de la designación se enviaría una carta solicitando un informe final de sus actividades durante los últimos 5 años en relación con el plan de trabajo quinquenal presentado inicialmente. También se pedirá a los centros que presenten el informe anual ordinario, y ambos serán evaluados por la Comisión.

La Comisión ha revisado y actualizado la plantilla para este informe final, incluidos los criterios específicos de desempeño. La plantilla está diseñada para recopilar pruebas exhaustivas de los impactos y logros del centro a lo largo del periodo de 5 años, así como los beneficios aportados al territorio, la región o, incluso, mundialmente. La plantilla revisada está diseñada para recopilar la información necesaria para evaluar el cumplimiento por parte del centro de su plan de trabajo quinquenal. Incluye secciones para detallar las metas y objetivos de la presentación original, indicando su estado actual como "alcanzados", "en curso", "modificados" o "no iniciados", justificando siempre cada respuesta. Además, la plantilla incluye una tabla para resumir las actividades completadas, centrándose en los beneficios esperados y los conseguidos. Para concluir, se ha añadido una sección de

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"Renovación", en la que los centros colaboradores pueden expresar su interés en que se considere su renovación. Se les pedirá que esbocen su estrategia para contribuir al mandato de la OMSA y mejorar la visibilidad de los centros. También se les pedirá que describan en viñetas cómo pueden ayudar a los miembros de la OMSA. Por último, la Comisión evaluará la pertinencia del ámbito de actividad de cada centro colaborador en relación con el Plan Estratégico de la OMSA.

La Comisión llevará a cabo una revisión preliminar de estos informes finales, cuyos resultados iniciales se anunciarán en la siguiente reunión de febrero. Los centros con informes finales aprobados y con una visión clara para contribuir al Plan Estratégico de la OMSA serán informados tras las reuniones de febrero de la Comisión de su elegibilidad para la renovación y serán invitados a presentar un nuevo plan quinquenal. Los centros cuyos resultados se consideren insatisfactorios o los que no presenten el informe dispondrán de un periodo de apelación de 6 meses, hasta la próxima reunión de la Comisión, en septiembre. En esta reunión se reevaluará el estado de su designación, lo cual puede dar lugar a su retirada de la lista.

#### **6.9. Revisión de las formas de mejorar los resultados de los centros colaboradores en beneficio de la OMSA y de sus Miembros**

La Comisión debatió las formas de mejorar los resultados de los centros colaboradores en beneficio de los propios Centros, de la OMSA y de sus Miembros. Una posibilidad que se barajó fue la de revisar los TdR para garantizar que sigan siendo pertinentes y eficaces. Reconociendo el amplio abanico de temas tratados por los centros como un recurso valioso, la Comisión se preguntó si la red actual cubre lo suficiente todas las necesidades de los Miembros y de la OMSA. La Comisión acordó centrarse en la evaluación de las posibles lagunas entre los ámbitos de especialización existentes, sobre todo en relación con el mantenimiento de los conocimientos especializados en medio de una tecnología en constante evolución. Un importante punto de debate fue cómo lograr que los Miembros aprovechen mejor este recurso, lo que podría conseguirse aumentando la comunicación con los Miembros y facilitando un uso más eficaz de los centros. Para aumentar su visibilidad, la Comisión propuso pedir a los centros que presenten de tres a cinco viñetas sobre los servicios que ofrecen, que se añadirán a la entrada específica del centro que puede consultarse en el sitio web de la OMSA a través de un enlace titulado "Cómo podemos ayudarle". Por último, la Comisión señaló que un criterio importante para el apoyo consiste en mantener el contacto con los laboratorios de referencia, garantizando una red de colaboración e información.

#### **6.10. Actualización de la red de tres laboratorios de referencia (PPA, PPR<sup>5</sup> y rabia)**

##### **Peste porcina africana**

La red de laboratorios de referencia de la OMSA para la PPA celebró reuniones virtuales periódicas para intercambiar conocimientos científicos y técnicos, incluidos los últimos avances en vacunas contra la PPA, y debatió las actividades relativas al desarrollo de programas de formación para ayudar a los países en riesgo, incluida la organización de pruebas de competencia.

La red está finalizando un manual de laboratorio, que incluye algoritmos de diagnóstico para detectar variantes poco virulentas y nuevas variantes emergentes del virus de la PPA, para explorar los requisitos de los usuarios en una plataforma de intercambio de información de libre acceso para los datos de la secuencia del genoma del virus de la PPA y la detección de virus recombinantes circulantes.

##### **Peste de los pequeños rumiantes**

La red de laboratorios de referencia de la OMSA para la PPR sigue actualizando periódicamente su [sitio web](#) y organiza actividades en apoyo de sus Miembros. En noviembre de 2023, se celebró en Bengaluru (India) la sexta reunión de la Red Mundial de Investigadores y Expertos sobre la PPR, que se centró en las innovaciones derivadas de la investigación de la PPR para apoyar la segunda y tercera fases de erradicación del plan de acción (*blueprint*) para la PPR. También en noviembre de 2023, en la región africana, se celebró en Grand Bassam (Costa de Marfil) un importante taller de armonización transfronteriza y una reunión del Grupo Asesor Regional para la erradicación de la PPR. Este taller se centró en estrategias de colaboración para la gestión del riesgo y las actividades para la erradicación de la PPR. En diciembre de 2023, la red de laboratorios de referencia de la PPR de la OMSA llevó a cabo un taller centrado en aspectos críticos de la gestión de la PPR.

El desarrollo en curso de los módulos electrónicos de formación sobre la Herramienta de Seguimiento y Evaluación de la situación de la PPR (PMAT, por las siglas en inglés de *PPR Monitoring and Assessment Tool*) se está gestionando en el Centro de Formación Virtual de la FAO<sup>6</sup>. Paralelamente, avanza la digitalización de las PMAT, lo que supone un gran paso adelante en la modernización de estas herramientas. En una reunión virtual de las partes interesadas se presentaron las nuevas directrices del Episistema para la PPR. Se espera su aprobación final en

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<sup>5</sup> PPR: Peste de los pequeños rumiantes

<sup>6</sup> FAO: Organización de las Naciones Unidas para la Alimentación y la Agricultura.

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breve. Por último, se elaboró una plantilla revisada para el desarrollo de Planes Estratégicos Nacionales (PEN) para la PPR, que se presentó a los países y a las partes interesadas para su adopción. Esta plantilla actualizada será utilizada ahora por los países para alinear sus PEN con los *blueprints* (o planes de acción) para la PPR, garantizando un enfoque más cohesivo y eficaz de la gestión y los esfuerzos para la erradicación de la PPR.

## Rabia

La red de laboratorios de referencia de la OMSA para la rabia (RABLAB) continuó reuniéndose cada dos meses para compartir información y alinear las actividades con el fin de mejorar el apoyo mundial para el diagnóstico de la rabia, así como la vigilancia, el desarrollo de capacidades y la implementación de actividades de control de la rabia. Una segunda reunión presencial de la red se celebró el 8 de noviembre de 2023, en Roma, Italia, para revisar los avances e identificar las prioridades clave para 2024.

Continúan los esfuerzos para mejorar la promoción y la transparencia de las actividades de la RABLAB, incluido un próximo boletín anual que mostrará los principales resultados y actualizaciones de la red. La BSC volvió a señalar la necesidad de destacar mejor las actividades de la RABLAB en el sitio web de la OMSA.

La RABLAB sigue apoyando a los Miembros de la OMSA a través de varios proyectos de hermanamiento para crear capacidad de laboratorio para el diagnóstico de la rabia, y apoyará al *Foro Unidos Contra la Rabia* en la aplicación de los tres pilotos iniciales del *Country Partnership Programme*, que tiene como objetivo proporcionar un apoyo más amplio, a través del concepto "Una sola salud", a los países donde la rabia es endémica. Los expertos de la RABLAB también han contribuido al desarrollo de las recomendaciones indicadas en el documento '[Oral vaccination of dogs against rabies: Recommendations for field application and integration into dog rabies control programmes](#)'.

La red RABLAB prosigue las conversaciones con los fabricantes pertinentes para estudiar cómo mejorar los protocolos de los dispositivos de flujo lateral (LFD) con el fin de apoyar la vigilancia de la rabia. En la actualidad, la [declaración de la red RABLAB](#) sobre el uso de LFD sigue sin modificarse.

En 2024, la red RABLAB seguirá prestando apoyo directo a los países donde la rabia es endémica en cuanto a la elaboración y aplicación de sus Planes Estratégicos Nacionales y, cuando proceda, les ayudará a solicitar la aprobación por parte de la OMSA; apoyará a la OMSA en el seguimiento de las normas internacionales para garantizar que sigan siendo adecuadas para su finalidad; mejorará la colaboración entre los miembros de la red RABLAB; y difundirá información científica entre los Miembros de la OMSA y la comunidad involucrada en general.

### 6.11. Sistema de informes anuales para los centros colaboradores y los laboratorios de referencia de la OMSA

En diciembre de 2022, se puso en marcha un sistema electrónico para recopilar los informes anuales de los centros de referencia de la OMSA. Lamentablemente, varios centros de referencia tuvieron dificultades para cumplimentar y enviar sus informes debido a fallos del sistema.

Para abordar los problemas identificados y mejorar la facilidad de uso, la OMSA contrató a un proveedor de servicios en noviembre de 2023 para actualizar y evolucionar el sistema actual en función de los problemas identificados durante su uso inicial. Esta renovación del sistema pretende mejorar y desarrollar funcionalidades adicionales para el actual sistema de información de los laboratorios de referencia y centros colaboradores (LR y CC) de la OMSA. El sistema de información de los LR y CC debe recopilar, guardar, procesar y presentar eficazmente los informes de las actividades de los LR y CC de la OMSA, apoyando la toma de decisiones, la coordinación, el control, el análisis y la visualización de los informes finales. Está diseñado para automatizar y agilizar los procesos empresariales, reduciendo así el esfuerzo manual, mitigando los posibles riesgos y mejorando la eficacia operativa tanto de la OMSA como de la red de CC y LR.

La evolución del sistema implantará el uso de una dirección de correo electrónico para acceder a las plantillas de los CC y los LR, lo cual facilitará el trabajo de quienes se ocupan de ambas. Esta mejora permitirá a los usuarios de los LR y los CC acceder a múltiples informes sin necesidad de conectarse y desconectarse manualmente al cambiar de informe. Además, permitirá a los LR y CC añadir múltiples usuarios para rellenar y editar informes simultáneamente. Por otra parte, el sistema mejorará las funcionalidades existentes, como el diseño de la experiencia del usuario (UX), modificará las plantillas de formularios existentes tanto para los LR como para los CC y corregirá los errores existentes en el sistema.

El plan actual del proyecto prevé poner en marcha el nuevo sistema en marzo de 2024. La Comisión insistió en que, si el sistema no cumple los altos estándares exigidos en el plazo previsto, habrá que posponer su despliegue hasta que satisfaga plenamente todos los criterios de calidad necesarios.

La Comisión ha expresado su preocupación por garantizar que el sistema alcance el nivel de excelencia de la Organización y que los centros de referencia puedan utilizar un sistema que responda a sus necesidades. La

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Comisión ha agradecido a los centros de referencia su comprensión con respecto al aplazamiento de la presentación de los informes anuales y ha subrayado que sus informes anuales se evaluarán en septiembre de 2024.

#### **6.12. Uso fraudulento del emblema/logotipo de la OMSA**

La Comisión tuvo conocimiento de un laboratorio de referencia de la OMSA que está utilizando el emblema de la OMSA en vacunas que vende a los Miembros. Se trata de un uso fraudulento del emblema/logotipo de la OMSA, que se describe claramente como tal en las [Directrices sobre el Uso del Emblema de Centro de referencia de la OMSA](#). La OMSA está tratando este asunto con la institución implicada, que ha retirado los productos del mercado. Se recuerda a los Centros de referencia que deben seguir las Directrices o preguntar a la Sede de la OMSA si tienen alguna duda sobre cómo pueden utilizar el emblema de la OMSA.

### **7. Grupos *ad hoc*: Actualización de las actividades de los pasados Grupos *ad hoc***

#### **7.1. Grupo *ad hoc* sobre la sustitución del estándar internacional de tuberculina bovina (ISBT) y de tuberculina aviar (ISAT)**

Se informó a la Comisión de que el tercer ensayo clínico se había completado en octubre de 2023 y, basándose en los resultados, el Grupo *ad hoc* recomendó seguir realizando una última serie de ensayos con el candidato B afinando los parámetros del estudio. El Grupo *ad hoc* también debatió los resultados de todas las series de ensayos, que habían indicado que dos de los cuatro ensayos no eran válidos. Un ensayo, sin embargo, se había acercado a la aceptabilidad según los criterios de la Farmacopea de la UE, que sugerían una potencia de entre el 50% y el 200%, y el estándar interno proporcionado por el fabricante estaba cerca del intervalo aceptable de potencia de 30 000 unidades. El Grupo *ad hoc* recomendó revisar los datos originales del fabricante para conocer mejor los factores que contribuyen a las estimaciones de potencia más bajas. En la última serie de ensayos, la duración de la infección y la dosis de inoculación se aumentaron secuencialmente para minimizar toda posible variable. Este cuarto y último ensayo está actualmente en curso, y los resultados se esperan para mediados de marzo de 2024.

Si los ensayos son favorables, la Comisión consultará a distancia para decidir si identifica al candidato B como sustituto de la ISBT en la próxima Sesión General. Además, la Comisión recomendó que, en caso de que los ensayos sean desfavorables, la OMSA siga buscando un nuevo candidato y reinicie los ensayos. La Comisión recomienda que la OMSA siga movilizando recursos para encontrar financiación que permita mantener el proyecto, ya que, sin una norma aceptada universalmente, los Miembros tendrían que basarse en la norma del fabricante, lo cual podría dar lugar a variabilidad en los resultados.

En cuanto a la tuberculina aviar, se informó a la Comisión de que en diciembre de 2023 se lanzó una convocatoria de donaciones de una tuberculina aviar candidata. La fecha límite para recibir las solicitudes de los fabricantes era el 16 de febrero de 2024. La Comisión recomendó que el Grupo *ad hoc* revisase y recomendase a la Comisión los candidatos preseleccionados.

#### **7.2. Grupo *ad hoc* para la revisión del Capítulo 4.7. “Obtención y tratamiento de semen de bovinos, pequeños rumiantes y verracos” del Código Terrestre**

Se informó a la Comisión de que se celebraría virtualmente una consulta de expertos para elaborar un plan de acción para los trabajos de este Grupo *ad hoc*. Se designó a un miembro de la Comisión de Normas Biológicas para participar en las reuniones del Grupo.

#### **7.3. Grupo *ad hoc* sobre enfermedades emergentes (incluidas las reemergentes) y los desencadenantes de emergencia de enfermedades animales**

La Comisión fue informada de las actividades de este Grupo y tomó nota de las recomendaciones pertinentes.

### **8. Normalización/armonización internacional**

#### **8.1. Registro de la OMSA de kits de diagnóstico – actualización y revisión de solicitudes nuevas renovaciones de solicitud**

La Secretaría para el Registro de Kits de Diagnóstico (SRDK) informó a la Comisión de la situación de las solicitudes en curso. En la actualidad, en el Registro de Kits de Diagnóstico de la OMSA, hay 16 kits de pruebas de diagnóstico.



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#### **8.1.1. Incorporación de un nuevo kit de diagnóstico al registro de la OMSA: Kit de PCR en tiempo real Genelix™ para la detección del VPPA**

Se está evaluando la solicitud del kit de PCR en tiempo real Genelix™ para la detección del VPPA (Sanigen). La revisión y aprobación de las conclusiones, de las recomendaciones del informe final del grupo de revisión y del resumen de los estudios de validación (VAS, por las siglas en inglés de *Validation Studies Abstract*) se tramitarán por procedimiento escrito. Si tiene lugar la aprobación, está prevista una Resolución: añadir un nuevo kit de diagnóstico al registro de la OMSA para su adopción durante la 91ª Sesión General, en 2024.

Finalidad prevista del kit: el kit de PCR en tiempo real Genelix™ para la detección del VPPA es un producto que detecta y confirma cualitativamente el diagnóstico del virus de la peste porcina africana mediante un sistema de detección por PCR en tiempo real en sangre total, suero o tejidos de cerdos sospechosos de estar infectados por el VPPA.

El resumen de los estudios de validación - Datos suplementarios, redactado por el fabricante y aprobado por el Panel de Expertos para la Revisión, fue aprobado por la Comisión (véase el [Anexo 18](#)).

#### **8.1.2. Incorporación de un nuevo kit de diagnóstico al registro de la OMSA: Prueba rápida Sentinel® de detección de anticuerpos contra el VPPA**

Se informó a la Comisión de que había concluido la evaluación del expediente de la prueba rápida Sentinel® de detección de anticuerpos contra el VPPA (fabricante: *Excelsior Bio-System Incorporation*). Teniendo en cuenta el informe final del Panel de Expertos para la Revisión, la Comisión respaldó la recomendación del Panel de aprobar la "idoneidad para la finalidad" del kit, tal como se describe en el Resumen de los Estudios de Validación y en el Manual del Usuario (Instrucciones para los Usuarios).

La prueba rápida Sentinel® de detección de anticuerpos contra el VPPA es un ensayo inmunocromatográfico de flujo lateral (LFA) destinado a la detección de anticuerpos contra el VPPA en muestras de suero porcino. La prueba está diseñada para su uso en el diagnóstico de la infección por el VPPA, junto con otras pruebas o procedimientos de diagnóstico, y para la evaluación de la generación de anticuerpos contra la infección.

El Resumen de los Estudios de Validación redactado por el fabricante y aprobado por el Panel de Expertos para la Revisión fue aprobado por la Comisión (véase el [Anexo 19](#)).

Se preparará una Resolución en consecuencia para añadir un nuevo kit de diagnóstico al registro de la OMSA para su adopción durante la 91ª Sesión General en 2024.

#### **8.1.3. Decisión de la renovación por 5 años y una Resolución: Kit de detección de anticuerpos contra la influenza aviar (número de registro 20080203) de BioChek (Reino Unido) Ltd**

La Comisión aprobó la recomendación de renovación por 5 años con una Resolución para el kit de detección de anticuerpos contra la influenza aviar (número de registro 20080203) de BioChek (UK) Ltd, a partir de la información facilitada y de conformidad con el procedimiento acordado.

#### **8.1.4. Decisión de la renovación por 5 años y una Resolución: Kit de detección de anticuerpos contra la enfermedad de Newcastle (CK116; número de registro 20140109) de BioChek (Reino Unido) Ltd**

La Comisión aprobó la recomendación de renovación por 5 años con una Resolución para el kit de detección de anticuerpos contra la enfermedad de Newcastle (número de registro 20140109) de BioChek (Reino Unido) Ltd a partir de la información facilitada y de conformidad con el procedimiento acordado.

#### **8.1.5. Actualización del registro de la OMSA de kits de diagnóstico**

Teniendo en cuenta la información facilitada a la Comisión en [febrero de 2023 \(punto 8.1.7 del orden del día\)](#) sobre la futura Secretaría para el Registro de Kits de Diagnóstico (SRDK), se informó a la Comisión de que, de acuerdo con la Directora General y la Directora General Adjunta para Normas Internacionales y Ciencia, la SRDK procederá a la congelación completa de las actividades del Registro de Kits de Diagnóstico y de todos los procedimientos relacionados a partir de la 91ª Sesión General, por un periodo renovado de 24 meses más, es decir, hasta mayo de 2026. Esto significará que:

- Los kits validados y aprobados mantendrán su certificación;
- No habrá tramitación de renovaciones, aunque lleguen a la fecha de vencimiento de 5 años;

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- Se retirarán todas las solicitudes incompletas, con devolución de las tasas a los solicitantes;
  - No se llevará a cabo ninguna revisión de los posibles procedimientos de recurso;
  - No se llevará a cabo ninguna revisión ni validación de nuevas solicitudes;
  - Se considerarán los casos excepcionales, relacionados con una situación zoonositaria de emergencia, a petición de los Miembros.

## 8.2. Programa de normalización

### 8.2.1. Proyecto de ampliación de la lista de reactivos de referencia aprobados por la OMSA: revisión de las directrices

En la última reunión, de septiembre de 2023, la Comisión decidió enviar las directrices actuales (para los patrones de anticuerpos<sup>7</sup>, los patrones de antígenos<sup>8</sup> y las PCR<sup>9</sup>) a las redes para enfermedades específicas, a saber, la PPA, la fiebre aftosa, la rabia y la PPR, con la petición de que establecieran criterios mínimos para la elaboración de reactivos de referencia, de modo que las directrices resultaran más asequibles manteniendo al mismo tiempo la calidad de los reactivos producidos.

La red para la PPR respondió a la petición y presentó unas directrices mínimas para la preparación y validación de materiales de referencia para los métodos de diagnóstico de la PPR. La Comisión revisó las directrices, introdujo modificaciones para hacerlas genéricas y propuso que se facilitaran a las demás redes para que las comentaran y aprobaran antes de que estuvieran disponibles en el sitio web de la OMSA. Se espera que estas directrices animen a más laboratorios a solicitar que sus reactivos sean aprobados por la OMSA como reactivos de referencia.

### 8.2.2. *Association française de normalisation*: seguimiento desde septiembre de 2023

Tras la reunión de septiembre de 2023, la Comisión deliberó sobre la situación actual de la AFNOR, señalando que tienen un Acuerdo de Enlace con la OMSA. La Comisión observó que la situación actual no aclara si la AFNOR tiene jurisdicción para comentar de forma independiente las normas de la OMSA. El acuerdo establecido permite a la OMSA actuar como organización de enlace, participando en las actividades del CEN/TC. La Comisión observó que recibir comentarios en estos términos podría sentar un precedente inadvertido, permitiendo a múltiples organizaciones ofrecer comentarios sobre las normas, lo cual podría conducir a una situación insostenible.

En vista de ello, la Comisión decidió que era necesario que la Unidad de Asuntos Jurídicos de la OMSA examinara a fondo el acuerdo de enlace para aclarar su alcance.

Por último, la Comisión reiteró su recomendación de que la AFNOR envíe sus comentarios a través de un Miembro, utilizando la representación de un Delegado oficial designado.

## 9. Resoluciones para la Sesión General

La Comisión tomó nota de que se propondrían las siguientes resoluciones para su adopción durante la Sesión General, en mayo de 2024:

Una resolución que propone la adopción de 13 borradores de capítulos para el *Manual Terrestre*;

Una resolución que propone los nuevos centros colaboradores de la OMSA.

Las siguientes resoluciones se propondrían para su adopción mediante el procedimiento alternativo desarrollado en mayo de 2020 en respuesta a la pandemia de Covid-19, en el que los Delegados presentan sus votos a través de un mecanismo en línea disponible antes de la Sesión General de mayo de 2024:

- Una resolución por la que se proponen los nuevos laboratorios de referencia de la OMSA para las enfermedades de los animales terrestres;
- Una resolución sobre el Registro de la OMSA de kits de diagnóstico.

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7 <https://www.woah.org/app/uploads/2021/03/a-guideline-antibody-standards.pdf>

8 <https://www.woah.org/app/uploads/2021/03/a-guideline-antigen-standards.pdf>

9 <https://www.woah.org/app/uploads/2021/03/a-guideline-pcr-standards.pdf>

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## 10. Conferencias, talleres y reuniones

### 10.1. Actualización sobre el seminario de la OMSA que se celebrará durante el Simposio WAVLD en Calgary, Canadá, en 2025

La Asociación mundial de Veterinarios de Laboratorios de Diagnóstico tiene como misión mejorar la sanidad animal, la salud humana y el concepto "Una sola salud" facilitando la accesibilidad a pruebas de laboratorio de calidad proporcionadas por laboratorios de diagnóstico veterinario de todo el mundo. Como parte de su misión, celebran un simposio internacional cada dos años. Este simposio reúne a veterinarios especialistas en diagnóstico y a otras personas implicadas en el diagnóstico de laboratorio veterinario. El próximo ISWAVLD se celebrará en Calgary, Canadá, del 12 al 14 de junio de 2025, y adoptará el tema del concepto "Una sola salud: de la detección a la prevención de enfermedades", centrándose en el concepto "Una sola salud", la resistencia a los antimicrobianos, la detección de enfermedades y la respuesta a los brotes, poniendo en común la veterinaria, la medicina humana y la industria.

Clásicamente, la Comisión de Normas Biológicas organiza en paralelo un seminario de un día durante el Simposio, que se celebrará el 13 de junio de 2025. La Comisión debatió varios temas que podrían ser de interés para el próximo Seminario y sugirió invitar presentaciones por parte de las redes de la OMSA específicas de enfermedades, como la peste porcina africana, la peste de pequeños ruminantes, la rabia, la fiebre aftosa o la influenza aviar, sobre las últimas tecnologías para el diagnóstico de enfermedades, estudios de casos de enfermedades transmisibles recientes, como la propagación de la encefalitis japonesa en Australia, la aparición de la encefalitis equina occidental en Sudamérica, un resumen sobre los pros y los contras de las pruebas en el punto de atención (POCT, por las siglas en inglés de *point-of-care tests*) y cómo integrarlas en el diagnóstico de campo, información sobre técnicas de validación, secuenciación del genoma completo y metagenómica, inteligencia artificial, bioinformática, impacto del Protocolo de Nagoya en la sanidad animal, etc. La Secretaría se pondrá en contacto con diversos ponentes especialistas en los temas sugeridos para redactar un orden del día provisional que se debatirá en la reunión de septiembre.

### 10.2. Vacunación y vigilancia dirigida a la IAAP en aves de corral: Situación actual y perspectivas de futuro

Un taller titulado "Vacunación y vigilancia de la IAAP en aves de corral: Situación actual y perspectivas de futuro" organizado por la IABS (*International Alliance for Biological Standardization*) en colaboración con la OMSA se celebrará en la sede de la OMSA los días 22 y 23 de octubre de 2024. El objetivo del taller es debatir cómo implementar la vigilancia en poblaciones de aves de corral vacunadas junto con otros aspectos de la vacunación contra la IAAP. Se espera la participación de una gran variedad de partes interesadas, como delegados, científicos, organizaciones internacionales, empresas de cría de aves de corral y biológicas, y organizaciones relacionadas con el bienestar animal y con la salud humana. Las recomendaciones serán preparadas y presentadas por un panel designado.

La organización eximirá del pago de las tasas de inscripción a los delegados de la OMSA y a los expertos del laboratorio de referencia designado por la OMSA.

## 11. Asuntos de interés para información o consideración

### 11.1. Actualización sobre la OFFLU<sup>10</sup>

La Comisión fue informada de las actividades de la OFFLU y la OMSA sobre la influenza aviar. Durante el periodo del informe, la epidemia de influenza aviar continuó con un elevado número de detecciones notificadas en todo el mundo en aves de corral y no de corral, incluidas aves silvestres, y la primera incursión del virus H5 de la influenza aviar de alta patogenicidad en la región subantártica se detectó en octubre de 2023 en la isla de Georgia del Sur. Los expertos de la OFFLU señalan que el [impacto de virus H5 de la IAAP en la fauna silvestre de la Antártida](#) podría ser inmenso y podría dar lugar a una mortalidad alta.

En diciembre de 2023, la OMSA publicó un [informe normativo sobre el uso de la vacunación contra la influenza aviar](#): "Vacunación contra la influenza aviar: Por qué no debe suponer ningún obstáculo a un comercio seguro". El propósito de este documento es recordar a las autoridades nacionales que la vacunación, cuando se utiliza de acuerdo con las normas internacionales de la OMSA, es compatible con el comercio seguro de aves domésticas y sus productos.

Para la [reunión de la OMS sobre la composición de la vacuna, celebrada en septiembre de 2023](#), laboratorios de sanidad animal de países de África, América, Asia, Europa y Oceanía aportaron datos de 1 368 secuencias genéticas de virus H5 de influenza aviar de alta patogenicidad y 117 de virus H9 de influenza aviar. Además, se analizaron y enviaron datos de 191 secuencias de H1 porcina y 49 secuencias de H3 porcina. Los laboratorios colaboradores de la OFFLU llevaron a cabo caracterizaciones antigénicas y, posteriormente, se actualizaron las recomendaciones de

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10 OFFLU: Red conjunta OMSA-FAO de expertos en influenza animal

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la OMS para el desarrollo de nuevos virus candidatos a ser incluidos en las vacunas con fines de preparación ante posibles pandemias.

La OFFLU se ha embarcado en un proyecto denominado *Avian Influenza Matching* (AIM) para proporcionar en tiempo real las características antigénicas de los virus de la influenza aviar circulantes en diferentes regiones, con el fin de respaldar la vacunación de las aves de corral. Se ha estado llevando a cabo un proyecto piloto preliminar en el que participan determinados centros de referencia y expertos de la OFFLU. En octubre de 2023, se publicó [el informe](#) en el que se presentaban los resultados de este proyecto para apoyar a las partes interesadas y a los países en sus decisiones sobre la elección de vacunas y la cobertura vacunal (grado de similitud entre las cepas circulantes y las de la vacuna).

La Comisión de Normas Biológicas, con el apoyo de los expertos en influenza aviar de los laboratorios de referencia de la OMSA, está revisando en profundidad el capítulo actual del *Manual Terrestre* sobre la influenza aviar con vistas a su adopción en mayo de 2025.

La aplicación del marco de resolución sobre la influenza aviar (junio de 2023-mayo de 2025) avanza gracias a una herramienta específica de seguimiento y evolución que recopila, rastrea y evalúa trimestralmente la ejecución de las actividades en consonancia con el mandato esbozado en el documento [Resolución No. 28](#) para combatir la influenza aviar.

El desarrollo de la nueva estrategia del Programa Global de Enfermedades Animales Transfronterizas (GF-TADs, por las siglas en inglés de *Global Framework – Transboundary Animal Diseases*) sobre la IAAP para 2024-2033 está en curso y el borrador de la estrategia se someterá a consultas y a una ronda de comentarios con diferentes partes interesadas, incluidos los Miembros, en marzo de 2024, con el objetivo de que se lance en mayo de 2024.

## 11.2. Actualización sobre la peste bovina

La Comisión recibió información actualizada sobre las actividades posteriores a la erradicación de la peste bovina. La OMSA sigue trabajando en colaboración con la FAO para reducir las existencias de materiales que contienen virus de la peste bovina (RVCM, por las siglas en inglés de *rinderpest virus-containing materials*)<sup>11</sup> en todo el mundo, a excepción de los materiales para el diagnóstico y las vacunas, como parte de la "segunda fase" de la era posterior a la erradicación. Este esfuerzo conducirá a una reducción del número de instalaciones que conservan el virus de la peste bovina (RHF, por las siglas en inglés de *rinderpest holding facilities*) designadas por la FAO y la OMSA<sup>12</sup> como de la Categoría A, además de una reducción de los RVCM mantenidos por los Miembros de la OMSA en centros no autorizados.

Lamentablemente, no se ha producido ningún avance en el secuestro o destrucción de RVCM en los cinco Miembros que poseen estos materiales fuera de los RHF designados por la FAO-OMSA, a pesar de que se han mantenido varios debates presenciales y virtuales. En cuanto a la preparación, el *Ethiopia National Veterinary Institute* ha recibido un permiso excepcional para producir dos millones de dosis de vacuna RBOK para reponer la reserva de la AU-PANVAC<sup>13</sup> tras una inspección minuciosa y una revisión de los procedimientos. La OMSA organizó una reunión el 25 de octubre de 2023 para revisar los procedimientos operativos estándar de inspección de depósitos junto con representantes de las secretarías para la viruela y la poliomielitis y la EuFMD<sup>14</sup>. Se han examinado los resultados de las evaluaciones de las inspecciones de 2022 y las recomendaciones de la reunión se aplicarán en las inspecciones de 2024.

La reunión bienal de la Red de RHF designada por la FAO-OMSA tuvo lugar en París los días 6 y 7 de diciembre de 2023. Los miembros de la red actualizaron sus términos de referencia y redactaron un plan de trabajo para el periodo 2024-2026. Los miembros de la red destacaron la necesidad de ejercicios de simulación más frecuentes para poner a prueba el Plan de Acción Mundial contra la peste bovina y el mecanismo de despliegue de vacunas. Las RHF también animaron a la FAO y a la OMSA a fomentar una cooperación más estrecha con la PPR GREN<sup>15</sup>.

Los nuevos miembros del Comité Asesor Conjunto (CAC) de la FAO-OMSA para la peste bovina fueron invitados en enero de 2024. La próxima reunión del CAC se celebrará virtualmente en el segundo trimestre de 2024 y se centrará en la reducción mundial sobre RVCM, el apoyo a través de países destacados y la preparación frente a posibles emergencias

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11 RVCM: materiales que contienen el virus de la peste bovina

12 RHF: instalaciones que almacenan virus de la peste bovina

13 AU-PANVAC: Centre Centro Panafricano de Vacunas Veterinarias de la Unión Africana

14 EuFMD: Comisión Europea para la Lucha contra la Fiebre Aftosa

15 PPR GREN: Red mundial de investigación y pericia en materia de PPR

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### 11.3. Actualización del programa sobre el impacto global de las enfermedades animales

El año 2024 es de transición en cuanto al papel de la OMSA en el Programa GBAD (por las siglas en inglés de *Global Burden of Animal Diseases*). Este programa sigue en fase de descubrimiento científico y se necesita más tiempo para establecer métodos analíticos sólidos y sistemáticos. Las instituciones académicas y de investigación del consorcio del GBAD están aportando la experiencia necesaria en esta fase. Por ello, la OMSA ha decidido reorientar su participación en el GBAD y abandonar su papel de colíder y principal beneficiario. La OMSA debería seguir asumiendo un papel de asesoramiento y dirección para contribuir a evaluar la solidez científica del GBAD desde una perspectiva de idoneidad para los fines de los Miembros de la OMSA, y asesorar sobre la dirección del programa para garantizar su coherencia y utilidad para las necesidades políticas de los Miembros de la OMSA. Este cambio no es inmediato, ya que la OMSA seguirá desempeñando su papel de principal beneficiario de las subvenciones activas durante sus respectivos periodos de vigencia hasta que se cierre la última subvención (en 2025). No obstante, a partir de mayo de 2024, la OMSA dejará de ser colíder del consorcio del GBAD. Una vez que las fases centradas en la investigación hayan concluido y las metodologías hayan demostrado su utilidad para los Miembros de la OMSA y los Servicios Veterinarios nacionales, la OMSA podrá reconsiderar su participación en el GBAD: Ello puede incluir facilitar el despliegue sostenible o la institucionalización del GBAD mediante las metodologías del GBAD para informar las directrices de la OMSA en materia de economía de la sanidad animal, posibles normas de la OMSA y materiales de formación para los Miembros.

### 11.4. Actualización sobre las vacunas DIVA contra la peste de los pequeños rumiantes

Las actuales vacunas vivas atenuadas contra la PPR son inocuas, baratas y efectivas y proporcionan una inmunidad duradera tras una única dosis. Sin embargo, estas vacunas tienen inconvenientes: en primer lugar, son termolábiles y, por lo tanto, su administración es cara debido a la necesidad de una cadena de frío; en segundo lugar, la reacción inmunitaria es idéntica a la de la infección natural, por lo que no es posible diferenciar los animales infectados de los vacunados. Se trata de una cuestión importante porque los estudios serológicos llevarían a confusión a la hora de determinar si el virus ha sido eliminado por la vacunación.

Existen varias tecnologías para alcanzar los objetivos de la estrategia DIVA que señalan que las vacunas recombinantes y basadas en vectores que expresan subunidades víricas pueden constituir una alternativa a las vacunas convencionales, ya que pueden emparejarse fácilmente con las herramientas de diagnóstico de la estrategia DIVA. Esto será útil durante la fase de erradicación de la PPR para demostrar, mediante el empleo de pruebas DIVA, que la población animal previamente libre de PPR, pero vacunada con vacunas DIVA, está libre de infección.

#### *Vacunas vectorizadas con virus de la viruela*

También se han desarrollado vacunas contra la PPR vectorizadas con virus de la viruela caprina que actúan como vacuna dual para proteger tanto contra la PPR como contra la viruela ovina y caprina.

La vacuna vectorizada con virus de la viruela caprina fue descrita por Fakri et al. (2018), ha sido adoptada por una empresa comercial de África y fue identificada como candidata para la producción bajo el nombre comercial de "Combivax POX-PPR". Esta vacuna resultó ser relativamente termoestable, aunque no provocó una reacción óptima de anticuerpos probablemente debido a la inmunidad preexistente contra el vector.

Aún no se conocen los avances en el registro y la producción de la vacuna.

#### *Vacuna vectorizada con adenovirus*

El adenovirus 5 de replicación deficiente (Ad5) se considera un buen vector recombinante para su uso en pequeños rumiantes porque carecen de inmunidad preexistente contra este vector (Thacker *et al.*, 2009). Se ha observado que la vacunación de cabras contra la PPR solo con Ad-H o con Ad-F induce una potente reacción inmunitaria tanto humoral como celular, aunque la combinación de Ad-H y Ad-F indujo una mejor protección. Varios informes han descrito la tecnología recombinante de la vacuna contra la PPR vectorizada con Ad5 y la posibilidad de capacidades DIVA.

Una empresa comercial de África también ha identificado una vacuna contra la PPR vectorizada con Adenovirus como candidata a la producción bajo el nombre comercial de "Adeno-PPRH". Sin embargo, no se dispone de datos sobre los avances en el registro y la producción.

#### *Vacuna vectorizada con el virus de la enfermedad de Newcastle*

Se ha demostrado que la vacuna vectorizada con el virus de la enfermedad de Newcastle (VEN) protege contra la PPR y tiene aplicabilidad DIVA y una alta tolerancia térmica.

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Una empresa comercial de África también ha identificado la vacuna contra la PPR vectorizada con adenovirus como candidata a la producción bajo el nombre comercial de "Combivax ND-PPR". Sin embargo, no se dispone de los avances en materia de registro y producción.

#### *Vacunas vectorizadas con herpesvirus bovino*

Se ha demostrado que la vacuna vectorizada con herpesvirus bovino que contiene hemaglutinina del virus de la PPR induce anticuerpos neutralizantes y reacciones celulares. Esta vacuna se considera candidata DIVA para proteger contra la infección de rebaños por el VPPR y se puede llegar a aplicar en los programas de erradicación.

No hay información sobre ensayos de campo, registro ni producción.

### **11.5. Actualización sobre las actividades de la VICH<sup>16</sup>: la 42ª reunión del Comité Directivo de la VICH y la 16ª reunión del Foro de la VICH, que tuvieron lugar en Tokio del 13 al 16 de noviembre de 2023**

Se informó a la Comisión sobre la 42ª reunión del Comité Directivo de la VICH y la 16ª reunión del Foro de la VICH, que tuvieron lugar en Tokio del 13 al 16 de noviembre de 2023. Se destacó que el Comité Directivo (CD) de la VICH acordó los criterios para que los países progresen en las categorías de miembros de la VICH como consecuencia de la reestructuración de la VICH. Este esfuerzo fue una continuación del trabajo para modernizar la estructura de la organización y alinear mejor el Foro de la VICH con las diversas expectativas de los miembros. Además, Suiza se convirtió en nuevo miembro observador de la VICH.

El Comité Directivo también inició dos nuevas actividades relacionadas con:

- (1) El Marco reglamentario mundial para los medicamentos veterinarios; y
- (2) Principios de orientación técnica para la transición a métodos *in vitro* para las pruebas de potencia por lotes de productos inmunológicos de uso veterinario

El Grupo de Trabajo de Expertos en Productos Biológicos avanzó en relación con la "Prueba para determinar posibles virus extraños en las vacunas de uso veterinario". Se ha preparado el primer borrador de la Directriz. La Directriz se compartirá durante la fase de consulta con la Comisión y los Delegados de la OMSA y sus respectivos Puntos Focales para Productos Veterinarios. El subgrupo ha finalizado sus tareas en lo relativo a tres Directrices (GL 50, 55 y 59) sobre la armonización de los criterios para la exención de las pruebas de inocuidad en lotes de animales para la fase de aplicación.

El material formativo fue elaborado por el JMAFF (Ministerio de Agricultura, Silvicultura y Pesca de Japón) y está disponible en el sitio web de la VICH: <https://www.vichsec.org/en/training.html> sobre las GL 50, 55 y 59).

GL 50: Armonización de los criterios para la exención de las pruebas de inocuidad en animales de las especies de destino por lotes de vacunas inactivadas de uso veterinario.

GL 55: Armonización de los criterios para la exención de las pruebas de inocuidad en animales de las especies de destino por lotes de vacunas vivas de uso veterinario.

GL 59: Armonización de los criterios para la exención de las pruebas de inocuidad en animales de laboratorio por lotes de vacunas de uso veterinario. <https://www.vichsec.org/en/guidelines/biologicals/bio-safety/target-animal-batch-safety.html>

### **11.6. Actualización sobre el Proyecto Biobanco Virtual**

La Comisión recibió información actualizada sobre el Proyecto Biobanco Virtual. El proyecto está gestionado por el centro de referencia de la OMSA para el *Veterinary Biological Biobank*, albergado por el *Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna* (IZSLER), Italia, y la OMSA. Este proyecto consiste en un catálogo web de recursos biológicos conservados en biobancos, que constituye una fuente de información para buscar, localizar y recuperar muestras, especialmente reactivos de diagnóstico y reactivos de referencia, junto con los metadatos asociados.

Tras la reactivación del proyecto en abril de 2023, el centro colaborador mantuvo reuniones mensuales con la OMSA para su desarrollo. La Comisión fue informada de que, desde la última reunión, se han desarrollado funcionalidades del sistema, como la búsqueda en el catálogo, la gestión de contenido y el panel de peticiones. Actualmente se está

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<sup>16</sup> La VICH constituye un programa trilateral (UE-Japón-EE.UU.) destinado a armonizar los requisitos técnicos para el registro de productos veterinarios. Su título completo es Cooperación Internacional para la Armonización de los Requisitos Técnicos para el Registro de Medicamentos Veterinarios.

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desarrollando el sitio web para añadir funciones como el acceso a las normas de la OMSA, una sección de noticias y capacidades multilingües.

La Comisión asistió a una demostración de los últimos avances de la plataforma web. Durante esta presentación, se hizo hincapié en que el sistema no está diseñado como una compra directa. Por el contrario, su función principal es ofrecer un catálogo de recursos biológicos y servir de intermediario facilitador, poniendo en contacto a los laboratorios que poseen recursos biológicos con los posibles compradores. Con este planteamiento se pretende agilizar el proceso de acceso a estos recursos, garantizando una comunicación eficiente y eficaz entre los laboratorios y las partes interesadas.

La Comisión felicitó al centro colaborador por los avances y el desarrollo del proyecto. No obstante, subrayaron la necesidad crítica de mantener altos niveles de calidad tanto para los laboratorios proveedores como para los productos ofrecidos. La Comisión hizo hincapié en la importancia de cumplir las normas de calidad ISO 17025 para este sistema. Además, se expresaron inquietudes sobre el mantenimiento permanente de las normas de la OMSA y las posibles implicaciones de la responsabilidad de la OMSA en relación con estos productos. También se planteó la cuestión de restringir este sistema a los centros de referencia para garantizar la calidad y la inocuidad de los productos del biobanco. La Comisión decidió presentar esta sugerencia para evaluarla más detenidamente durante la próxima presentación del sistema.

Aunque la Comisión está interesada en el correcto desarrollo de este sistema, sus miembros desean supervisarlos de cerca para asegurarse de que garantiza la calidad de los laboratorios, sus productos y el mantenimiento de las normas de la OMSA, al tiempo que sigue siendo sostenible.

### **11.7. Actualizaciones sobre WAHIAD<sup>17</sup> y la plataforma WAHIS<sup>18</sup>**

La Comisión recibió información actualizada sobre la situación y el calendario de desarrollo y evolución de la plataforma en 2023, que incluía la optimización de los módulos de alerta temprana e informe semestral, y el desarrollo del módulo para el informe anual.

Se informó a la Comisión de que, en 2023, se organizaron sesiones con miembros de las comisiones para mostrarles cómo utilizar las funcionalidades de WAHIS y recabar información sobre sus necesidades. En 2024 se celebrarán sesiones similares y se animó a la Comisión a participar en ellas. Se informó a la Comisión de las actualizaciones pertinentes de las Tablas de Referencia de WAHIS realizadas en diciembre de 2023. El objetivo de este trabajo era alinearse con los cambios adoptados en los *Códigos y Manuales Sanitarios para los Animales Terrestres y Acuáticos* en la Sesión General de 2023. La Comisión elogió este trabajo y convino en que una buena comunicación entre la Secretaría y WAHIAD en relación con el trabajo que puede dar lugar a cambios en los *Códigos/Manuales*, que deberán reflejarse en el comportamiento o la funcionalidad de WAHIS, permitirá a WAHIAD avisar de toda posible limitación o restricción que pueda existir desde el punto de vista de la plataforma de notificación. Por último, se informó a la Comisión de que WAHIAD colaborará con el Departamento de Normas. Este trabajo permitirá a WAHIAD participar activamente en el proceso de elaboración de normas, realizando aportaciones a la Comisión pertinente. Esta colaboración se iniciará con la Comisión de Normas Sanitarias para los Animales Terrestres, pero el objetivo es extenderla progresivamente a las demás Comisiones.

### **11.8. Herramienta PVS**

La Comisión recibió información actualizada sobre los avances en el desarrollo del Sistema de Información del Proceso para la Evaluación del Desempeño de los Servicios Veterinarios (PVS, por las siglas en inglés de *Performance of Veterinary Services*). El Sistema de Información (IS, por las siglas en inglés de *Information System*) del PVS (IS del PVS) va dirigido a las partes interesadas directas del Proceso PVS, que incluyen los Delegados y Puntos Focales Nacionales, socios institucionales y donantes, y Expertos en el proceso PVS que aportan su experiencia y llevan a cabo misiones PVS a petición de los Miembros de la OMSA. Los Delegados y Puntos Focales Nacionales tendrán a su disposición una gran cantidad de datos a través de visuales interactivos y gráficos que muestran las fortalezas, debilidades y recomendaciones a los responsables de la toma de decisiones para el desarrollo de casos de inversión más impactantes para los Servicios Veterinarios. El IS del PVS tiene por objeto responder a las necesidades cambiantes de los Servicios Veterinarios y facilitar la mejora del desempeño ofreciendo más información que los informes PVS descriptivos. El Informe PVS, que ofrece una documentación completa de las prestaciones de los Servicios Veterinarios, contiene perspectivas desbloqueadas por la OMSA para que los gobiernos, los inversores y los socios puedan acceder a sus recomendaciones, utilizarlas y actuar en consecuencia con mayor facilidad.

La innovación que subyace al IS del PVS libera el poder de los datos históricos y de los conocimientos contenidos en los Informes PVS. Centrándose en los puntos fuertes y débiles y en las recomendaciones para cada competencia

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17 WAHIAD: *World Animal Health Information and Analysis Department*

18 WAHIS: *World Animal Health Information System*

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crítica del PVS, la OMSA ha migrado toda la información esencial a su base de datos. Esto permite un análisis rápido y sistemático de las tendencias del proceso PVS. Por primera vez, la OMSA está utilizando el procesamiento del lenguaje natural y el aprendizaje automático. Un resultado clave de este novedoso enfoque es un mayor conocimiento de las recomendaciones más comunes y persistentes, así como de los puntos fuertes y débiles de los Servicios Veterinarios de todo el mundo. Los Miembros pueden acceder a este análisis a través de paneles interactivos con los principales indicadores actualizados en tiempo real a medida que se dispone de nuevos datos. El Sistema de Información se irá presentando progresivamente a la red de la OMSA (su personal, Miembros, expertos PVS, socios y donantes) antes de culminar con su lanzamiento mundial en mayo de 2024.

### 11.9. Actualización sobre el Gran Desafío para laboratorios sostenibles

Durante más de 10 años, la OMSA ha trabajado con *Global Affairs Canada* (GAC), el *International Biosecurity Programme* del Reino Unido, Chatham House y la OMS para mejorar la sostenibilidad de los laboratorios (especialmente en entornos con pocos recursos). Una de las líneas de este programa de trabajo se ha centrado en explorar el uso de la innovación abierta para encontrar soluciones que mejoren la sostenibilidad de los laboratorios. El año pasado, la OMSA dirigió un estudio (subcontratado a *Grand Challenges Canada*) para evaluar la viabilidad de poner en marcha una iniciativa de innovación abierta. El informe final se entregó en julio de 2023.

La OMSA, el GAC<sup>19</sup> y la OMS no podrían llevar a cabo con éxito una iniciativa de innovación abierta por sí solos porque necesitan recursos adicionales (más allá de lo que pueden ofrecer los socios inversores existentes); experiencia adicional (recaudación de fondos, participación del sector privado, especialistas en innovación) y representación de los sectores del desarrollo y la filantropía, y en noviembre de 2023, la OMSA celebró una reunión en Wilton Park, Reino Unido, para involucrar a las principales partes interesadas en un consorcio para llevar adelante una iniciativa de innovación abierta para mejorar la sostenibilidad de los laboratorios de diagnóstico.

Se había invitado a 40 participantes, entre ellos, posibles socios inversores y expertos técnicos (laboratorios e innovación). La reunión fue un éxito y logró sus objetivos: 1. Todo el grupo estuvo de acuerdo en que la sostenibilidad de los laboratorios era un problema que había que abordar. 2. Un núcleo de representantes de alto nivel (de sectores clave) mostró un gran interés y aceptó formar parte de un grupo de trabajo encargado de desarrollar un plan de trabajo para llevar adelante la iniciativa, que incluiría la recaudación de fondos, la promoción y la innovación técnica. Este grupo incluía a la Casa Blanca/Estados Unidos (General de División Paul Friedrichs); la Comisión Europea (Anne Sophie Lequarre); la Unión Africana (Aggrey Ambali); el Fondo Mundial para la Seguridad Sanitaria (Andrew Nerlinger); *Effective Giving* (Joshua Monrad); la Fundación Gates (David Blazes); el Gobierno australiano (Phoebe Readford), y los líderes existentes (la OMSA, el Reino Unido, Canadá y la OMS).

Desde entonces, la OMSA ha desarrollado un discurso de presentación y la iniciativa se ha bautizado como BIO-PREVAİL, siglas de *Biological Preparedness and Resilience through Evolution and Innovation of Laboratories* (preparación y resiliencia biológicas a través de la evolución y la innovación de los laboratorios).

El grupo de trabajo informal creado en la reunión de Wilton Park elaborará un plan de trabajo y una estructura de gobernanza, y buscará oportunidades de participación y promoción, incluida la posibilidad de un acto paralelo en la Asamblea General de la ONU.

### 11.10. Hoja de ruta de la investigación en bioseguridad

Tras reunirse periódicamente durante dos años, el Grupo de Trabajo Técnico de la OMSA presentó seis artículos científicos para respaldar la aplicación de la gestión de riesgos biológicos de laboratorio basada en la evidencia<sup>20</sup>. Tras la revisión por expertos, los artículos se publicaron en acceso abierto en *Applied Biosafety*. En uno de ellos, se ofrece una visión general del proyecto, y en los otros cinco se revisa la base de evidencias que sustenta las medidas de bioseguridad de uso común para determinados agentes patógenos (*Bacillus anthracis*, *Brucella melitensis*, SARS-CoV-2, virus Mpox, influenza aviar, *Mycobacterium tuberculosis*, *Shigella* spp., virus de la fiebre aftosa).

Este proyecto también ha dado lugar a una revisión de 20 años de accidentes y escapes de laboratorio en laboratorios de salud humana y animal. El propio estudio y un artículo de opinión se publicaron en *The Lancet Microbe* en diciembre de 2023<sup>21</sup>. Los documentos piden más transparencia en torno a los accidentes de laboratorio

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19 GAC: Global Affairs Canada

20 <https://www.liebertpub.com/doi/10.1089/apb.2022.0040>

<https://www.liebertpub.com/doi/10.1089/apb.2022.0042>

<https://www.liebertpub.com/doi/10.1089/apb.2022.0039>

<https://www.liebertpub.com/doi/10.1089/apb.2022.0045>

<https://www.liebertpub.com/doi/10.1089/apb.2022.0038>

<https://www.liebertpub.com/doi/10.1089/apb.2022.0046>

21 [https://www.thelancet.com/journals/lanmic/article/PIIS2666-5247\(23\)00288-4/fulltext](https://www.thelancet.com/journals/lanmic/article/PIIS2666-5247(23)00288-4/fulltext)



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para apoyar la gestión del riesgo biológico y, en última instancia, mitigar futuros accidentes, así como una mayor inversión en profesionales de la bioseguridad.

En un taller conjunto de la OMSA, la OMS y Chatham House también se elaboró un documento dirigido a responsables de alto nivel y financiadores. Se ha publicado como documento de Chatham House<sup>22</sup>.

La hoja de ruta de la investigación en bioseguridad también se debatió en una mesa redonda paralela a la Conferencia del Premio Príncipe Mahidol 2024, celebrada en Tailandia. En esta mesa redonda también se debatió la necesidad de gestionar el riesgo a lo largo de toda la cadena de valor de los agentes patógenos, desde la toma de muestras hasta la destrucción o inactivación de los agentes patógenos. Clásicamente, la gestión del riesgo biológico se ha centrado en determinados puntos críticos de control a lo largo de la cadena de valor de los agentes patógenos, como el envío de muestras o la manipulación de muestras y agentes patógenos en el laboratorio. Sin embargo, cada vez se reconoce más que la gestión del riesgo biológico debe aplicarse a lo largo de toda la cadena. La OMSA sugirió que puede haber lagunas en las normas de la OMSA (que se centran en los laboratorios y el envío) y que podría ser útil que la OMSA elaborara algunas normas para gestionar los riesgos a lo largo de toda la cadena de valor de los agentes patógenos. La Comisión convino en que sería una buena idea y que se podría empezar a trabajar en este ámbito.

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.../Anexos

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<sup>22</sup> <https://www.chathamhouse.org/laboratory-accidents-and-biocontainment-breaches/issues-need-be-addressed>

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## Anexo 1. Orden del día aprobado

### REUNIÓN DE LA COMISIÓN DE NORMAS BIOLÓGICAS

París, 5–9 de febrero de 2024

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1. Bienvenida
  - 1.1. Directora General
  - 1.2. Directora General Adjunta, Normas Internacionales y Ciencia
  - 1.3. Actualizaciones de la Sede de la OMSA
2. Aprobación del orden del día
3. Colaboración con otras comisiones
  - 3.1. Temas horizontales entre Comisiones Especializadas
    - 3.1.1. Revisión de las definiciones de caso: tularemia, infección por metapneumovirus aviar (rinotraqueítis del pavo)
  - 3.2. Comisión Científica para las Enfermedades de los Animales
    - 3.1.1. Nada para esta reunión.
  - 3.3. Comisión de Normas Sanitarias para los Animales Terrestres
    - 3.3.1. Actualizaciones de la reunión de la Comisión del Código de septiembre de 2023
    - 3.3.2. Recomendaciones de la Comisión de Normas Biológicas a la Comisión de Normas Sanitarias para los Animales Terrestres
    - 3.3.3. Actualización de la Comisión de Normas Biológicas sobre la petición del Código relativa al Capítulo 6.10 del *Código Terrestre*: Uso responsable y prudente de los agentes antimicrobianos en medicina veterinaria
    - 3.3.4. Cuestión sobre el capítulo de la diarrea viral bovina
    - 3.3.5. Marco de trabajo para las normas del *Código Terrestre* (capítulos específicos de enfermedad)
  - 3.4. Comisión de Normas Sanitarias para los Animales Acuáticos
    - 3.4.1. Nada para esta reunión.
4. Programa de trabajo
5. Manual de las Pruebas de Diagnóstico y de las Vacunas para los Animales Terrestres
  - 5.1. Formato del informe y sistema de presentación y publicación de los comentarios
  - 5.2. Revisión de los comentarios de los Miembros sobre los borradores de capítulos y su aprobación para distribuirlos para una segunda ronda de comentarios y propuesta para adopción en mayo de 2024
  - 5.3. Revisión rápida del capítulo sobre la influenza aviar: Seguimiento del Foro de Sanidad Animal y Resolución adoptada sobre la influenza aviar
  - 5.4. Actualización del Capítulo 2.3.1 La aplicación de la biotecnología al desarrollo de vacunas de uso veterinario
  - 5.5. Actualización del borrador de capítulo sobre la validación diagnóstica de las pruebas en el punto de atención para enfermedades víricas de la lista de la OMSA empleando muestras de campo
  - 5.6. Seguimiento desde septiembre de 2023: conclusiones y recomendaciones de la *Revista Científica y Técnica* de la OMSA sobre la base científica para la validación de las pruebas de diagnóstico
    - 5.6.1. Avances en el desarrollo de un modelo de informe de validación de las pruebas recomendadas en el *Manual Terrestre*
    - 5.6.2. Avances en el desarrollo de un modelo para una nueva sección del *Manual Terrestre* sobre los motivos de la elección de las pruebas que figuran en la Tabla 1. *Métodos analíticos disponibles y su finalidad*.
  - 5.7. Aplicación de los criterios de mantenimiento de capítulos en el *Manual Terrestre* sobre enfermedades no incluidas en la lista

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- 5.8. Revisión de los consejos de los expertos sobre siete capítulos del *Manual Terrestre* actualizados y distribuidos en octubre de 2023, acerca de si la actualización tuvo impacto en el capítulo correspondiente del *Código Terrestre*
  - 5.9. Actualización sobre la petición de la Comisión del Código relativa al Capítulo 2.1.1. Métodos de laboratorio para las pruebas de sensibilidad de las bacterias frente a los antimicrobianos
  - 5.10. Petición para reconsiderar la inclusión de partículas de tipo vírico de la fiebre aftosa en el *Manual Terrestre* de la OMSA
  - 5.11. Seguimiento desde la Sesión General: propuesta de incluir una vacuna en el capítulo sobre la loque americana de las abejas melíferas
  - 5.12. Situación del *Manual Terrestre*: elección de capítulos para su actualización en el ciclo de revisión 2024/2025
  - 5.13. Actualización sobre la herramienta de navegación para la consulta en línea de las normas de la OMSA

## 6. Centros de referencia de la OMSA

- 6.1. Actualización sobre el sistema de informe anual
- 6.2. Solicitudes de candidatura a la designación como centro de referencia de la OMSA
- 6.3. Cambios de expertos en centros de referencia de la OMSA
- 6.4. Examen de solicitudes nuevas y pendientes para el hermanamiento entre laboratorios  
*Laboratorios de referencia – Implementación de los POE*
- 6.5. Comentarios de los laboratorios que no cumplan con los TdR esenciales
- 6.6. Revisión de la plantilla para que los candidatos a designación como expertos en laboratorios de referencia presenten su *curriculum vitae*  
*Centros colaboradores – Implementación de los POE*
- 6.7. Comentarios de los centros que no cumplan con los TdR esenciales
- 6.8. Revisión de la propuesta de procedimiento para la evaluación de centros al final su mandato quinquenal
- 6.9. Revisión de las formas de mejorar el rendimiento de los centros colaboradores en beneficio de la OMSA y sus Miembros  
*Redes de centros de referencia*
- 6.10. Actualización de las tres redes de laboratorios de referencia (peste porcina africana, peste de pequeños rumiantes y rabia)
- 6.11. Sistema de informe para los centros colaboradores y los laboratorios de referencia

## 7. Grupos *ad hoc*: actualización de las actividades de los pasados grupos *ad hoc*

- 7.1. Grupos *ad hoc* sobre el reemplazo de las tuberculinas de referencia internacionales bovina (ISBT) y aviar (ISAT): actualización sobre el reemplazo de la ISBT y la ISAT
- 7.2. Grupo *ad hoc* sobre estrategias alternativas para el control de la infección por el complejo *Mycobacterium tuberculosis* (CMTB) y la tuberculosis bovina (TBb) en especies ganaderas
- 7.3. Grupo *ad hoc* para la revisión del Capítulo 4.7. del *Código Terrestre*: Toma y tratamiento de semen de bovinos, pequeños rumiantes y verracos
- 7.4. Grupo *ad hoc* sobre enfermedades emergentes

## 8. Normalización y armonización internacional

- 8.1. Registro de los kits de diagnóstico de la OMSA: actualización y examen de solicitudes nuevas o renovadas
  - 8.1.1. Inclusión de un nuevo kit de diagnóstico al registro de la OMSA: kit de PCR en tiempo real Genelix™ para la detección del VPPA
  - 8.1.2. Inclusión de un nuevo kit de diagnóstico al registro de la OMSA: prueba rápida Sentinel® de detección de anticuerpos contra el VPPA
  - 8.1.3. Decisión de la renovación quinquenal y una Resolución: kit de detección de anticuerpos contra la influenza aviar (número de registro 20080203) de BioChek Ltd.
  - 8.1.4. Decisión de la renovación quinquenal y de una Resolución: kit de detección de anticuerpos contra la enfermedad de Newcastle
- 8.2. Programa de normalización
  - 8.2.1. Proyecto para ampliar la lista de reactivos de referencia aprobados por la OMSA: examen de las directrices
  - 8.2.2. Asociación francesa de normalización: seguimiento desde septiembre de 2023

## 9. Resoluciones para proponer en la Sesión General

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## 10. Conferencias, talleres y reuniones

- 10.1. Actualización del seminario de la OMSA para celebrar durante el Simposio WAVLD, en Calgary, Canadá, en 2025
- 10.2. Vacunación y vigilancia de la IAAP en aves de corral: Situación actual y perspectivas de futuro; semana del 21 de octubre de 2024 en la Sede de la OMSA. Reunión de 2-3 días, organizada por la IABS en colaboración con la OMSA.

## 11. Asuntos de interés para información o consideración

- 11.1. Actualización sobre la OFFLU
- 11.2. Actualización sobre la peste bovina
- 11.3. Actualización del programa sobre el Impacto Global de las Enfermedades Animales
- 11.4. Actualización sobre las vacunas DIVA<sup>23</sup> contra la peste de pequeños rumiantes
- 11.5. Actualización sobre las actividades de la VICH: la 42ª reunión del Comité Directivo de la VICH y la 16ª reunión del Foro de la VICH, celebradas en Tokio del 13 al 16 de noviembre de 2023
- 11.6. Actualización: Sanidad para los animales
- 11.7. Actualización sobre el proyecto Biobanco Virtual
- 11.8. Actualizaciones de WAHIAD y la plataforma WAHIS
- 11.9. La herramienta PVS
- 11.10. Actualización sobre el Gran Desafío para laboratorios sostenibles
- 11.11. Hoja de ruta de la investigación en bioseguridad
- 11.12. Actualización sobre las actividades del acuerdo de colaboración entre la IHSC<sup>24</sup> y la OMSA y del proyecto de asesoría en Asia (asuntos relacionados con los caballos: proyectos de asesoría en Asia y Sudamérica)

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<sup>23</sup> DIVA: Detección de infección en animales vacunados

<sup>24</sup> IHSC: *International Horse Sports Confederation*

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## Anexo 2. Lista de participantes

### REUNIÓN DE LA COMISIÓN DE NORMAS BIOLÓGICAS

París, 5–9 de febrero de 2024

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#### MIEMBROS DE LA COMISIÓN

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**Prof. Emmanuel Couacy-Hymann**

(Presidente)  
Profesor de virología  
CNRA/LIRED  
Abidjan  
CÔTE D'IVOIRE

**Prof. Ann Cullinane**

(Vicepresidenta)  
Jefe de la Unidad de Virología  
*Irish Equine Centre*  
Naas  
IRLANDA

**Dr. John Pasick**

(Vicepresidente)  
Antiguo *National Centre for Foreign Animal Diseases*  
Winnipeg  
CANADÁ

**Dr. Joseph S. O'Keefe**

(Miembro)  
Jefe del Laboratorio de Sanidad Animal  
Ministerio de Industrias Primarias  
Upper Hutt  
NUEVA ZELANDA

**Dr. Satoko Kawaji**

(Miembro)  
Científico Principal  
*National Institute of Animal Health*  
Naro  
JAPÓN

**Prof. Chris Oura**

(Miembro)  
Profesor de Virología Veterinaria  
Universidad de las Indias Occidentales  
St-Augustine  
TRINIDAD Y TOBAGO

#### REDACTOR CONSULTOR DEL MANUAL TERRESTRE

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**Dr. Steven Edwards**

c/o OMSA, París, FRANCIA

#### SEDE DE LA OMSA

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**Dr. Gregorio Torres**

Jefe del  
Departamento Científico

**Sra. Sara Linnane**

Oficial Científico Sénior  
Departamento Científico

**Dr. Gounalan Pavade**

Coordinador Científico Sénior  
Departamento Científico

**Dra. Charmaine Chng**

Jefa Adjunta del  
Departamento Científico

**Dra. Mariana Delgado**

Oficial de la Secretaría Científica  
Departamento Científico

Anexo 3. Programa de trabajo para la Comisión de Normas Biológicas de la OMSA

REUNIÓN DE LA COMISIÓN DE NORMAS BIOLÓGICAS

París, 5–9 de febrero de 2024

Actividad	Tema	Situación y acción
<b>Actualización del <i>Manual Terrestre</i></b>	1) Distribuir los capítulos aprobados por la BSC entre los Miembros para una segunda ronda de comentarios y propuesta de adopción en mayo de 2024.	Marzo de 2024
	2) Enviar recordatorio a los autores de los capítulos identificados previamente para su actualización pero que aún no se han recibido, e invitar a los autores de los capítulos identificados recientemente para su actualización.	En curso
	3) Cargar y publicar la base de datos de informes de validación que se publicarán en el sitio web de la OMSA para las pruebas recomendadas en el <i>Manual Terrestre</i> , e informar a los laboratorios de referencia.	En curso
	4) Incluir como apéndices, al final de los capítulos específicos de cada enfermedad, las tablas que explican las puntuaciones otorgadas a las pruebas de la Tabla 1 <i>Métodos analíticos disponibles y su finalidad</i> . Añadir enlaces a los informes de validación cuando los haya (punto 3 anterior).	Terminado
	5) Pedir a los centros de referencia que proporcionen enlaces a vídeos didácticos adecuados para añadirlos al final de los capítulos específicos de enfermedad. Los vídeos serán revisados por la Comisión cuando se revise el capítulo.	En curso
	6) Fijar criterios para eliminar capítulos de enfermedades no incluidas en la lista y evaluar dichos capítulos según los criterios.	Terminado
	7) Revisar los nuevos avances en enfermedades que causan un impacto mundial significativo (por ejemplo, la influenza aviar o la peste porcina africana) y dar prioridad a estos capítulos.	En curso
	8) Iniciar el proceso de tramitación de la solicitud de acceso a las versiones anteriores y a la evolución del <i>Manual Terrestre</i> como se hizo con el <i>Código Terrestre</i> .	En curso
<b>Centros colaboradores</b>	1) Implementación de los POE adoptados:	
	a) Crear una plantilla para el informe de evaluación del rendimiento quinquenal de los centros colaboradores, que se comparará con su plan de trabajo quinquenal presentado inicialmente.	Terminado

Actividad	Tema	Situación y acción
	b) Enviar la plantilla de evaluación del plan de trabajo quinquenal a los centros colaboradores correspondientes.	Julio de 2024
	2) Evaluar los comentarios de los centros que han cumplido los 5 años de trabajo y valorar la pertinencia actual del alcance de sus actividades con vistas a la renovación.	Febrero de 2025
	3) Aumentar la visibilidad de los centros actuales: pedir que presenten un máximo de 5 viñetas para añadirlas a la entrada de su sitio web bajo el título "Cómo podemos ayudar"?"	Septiembre de 2024
	4) Explorar mecanismos para mejorar la colaboración reuniendo a los Centros que tengan la misma área de interés principal (actualmente seis): implicación de la industria u otros socios para la financiación.	En curso
	5) Elaborar un cuestionario para recabar la opinión de los centros colaboradores sobre sus experiencias como CC de la OMSA, similar al de los laboratorios de referencia.	Septiembre de 2024
<b>Laboratorios de referencia</b>	1) Incluir a los laboratorios con peores resultados en una lista de vigilancia y supervisar su rendimiento	En curso
	2) Aplicar el nuevo sistema de evaluación de los informes anuales y facilitar a los miembros de la Comisión la lista de los informes asignados.	Terminado
	3) Enviar comentarios a la red de laboratorios de referencia sobre el cuestionario	Terminado
	4) Explorar mejoras en el proceso de elaboración del informe anual: posibilidad de cumplimentar la plantilla del informe anual a lo largo del año.	May 2024
<b>Redes de centros de referencia</b>	1) Seguimiento con las tres redes de laboratorios de referencia (PPA, PPR y rabia).	En curso
<b>Normalización/armonización</b>	1) Proyecto para ampliar la lista de reactivos de referencia aprobados por la OMSA	
	a) Preguntar a las demás redes si aceptan el documento de normas mínimas propuesto por la red para la PPR. A continuación, cargar el documento para su aplicación.	Para septiembre de 2024
	2) Proyecto de desarrollo de las tuberculinas de referencia internacionales bovina y aviar de reemplazo: finalizar el informe y proponerlo para su aprobación	En curso
<b>Grupos ad hoc</b>	1) Grupo <i>ad hoc</i> sobre laboratorios sostenibles	En curso
	2) Contribuir a la revisión del Capítulo 4.7 del <i>Código Terrestre</i> : Toma y tratamiento de semen de bovinos, pequeños rumiantes y verracos	En curso
	3) Contribuir al Grupo <i>ad hoc</i> sobre enfermedades emergentes y desencadenantes de emergencia de enfermedades de los animales	En curso
<b>Proyectos</b>	1) Biobanco veterinario (proyecto)	En curso

Actividad	Tema	Situación y acción
<b>Conferencias, talleres y reuniones con la participación de Miembros de la BSC</b>	1) Hoja de ruta de la investigación en bioseguridad	Terminado
	2) Seminario de la OMSA que se celebrará durante el ISWAVLD, en junio de 2025, en Canadá: desarrollar un tema y el programa, y especificar los ponentes	Septiembre de 2024
<b>Desempeño</b>	1) Participar en los procesos en curso sobre problemas de desempeño con los laboratorios de referencia	En curso
<b>Desarrollo de normas de laboratorio para enfermedades emergentes</b>	1) Comentar el capítulo del <i>Código Terrestre</i> una vez adoptado y considerar la introducción de un capítulo correspondiente para el <i>Manual Terrestre</i>	A partir de mayo de 2024
<b>Definiciones de caso</b>	1) Seguimiento de la aplicación de los POE para las definiciones de caso	En curso



REUNIÓN DE LA COMISIÓN DE NORMAS BIOLÓGICAS

París, 5–9 de febrero de 2024

CHAPTER 1.1.5.

QUALITY MANAGEMENT IN VETERINARY TESTING LABORATORIES

SUMMARY

*Valid laboratory results are essential for diagnosis, surveillance, and trade. Such results are achieved by the use of assured through implementation of good management practices, valid system that supports accurate and consistent test and calibration methods, proper techniques, quality control and quality assurance, all working together within a quality management system. Laboratory quality management includes technical, managerial, and operational elements of testing, performing, interpreting and the interpretation of reporting test results. A quality management system enables the laboratory to demonstrate both competency and an ability to generate consistent technically valid results that meet the needs of its customers. The need for Mutual recognition and acceptance of test results for international trade, and the acceptance accreditation of tests to international standards such as ISO/IEC<sup>1</sup> 17025:2005 (General Requirements for the Competence of Testing and Calibration Laboratories) (ISO/IEC, 2005-2017b) requires good suitable laboratory quality management systems. This chapter is not intended to reiterate the requirements of ISO/IEC 17025, nor has it been endorsed by accreditation bodies. Rather, it outlines the important issues and considerations a laboratory should address in the design and maintenance of its quality management system, whether or not it has been formally accredited regardless of formal accreditation status. Chapter 1.1.1 Management of veterinary diagnostic laboratories gives an introduction to veterinary diagnostic laboratories introduces the components of governance and management of veterinary laboratories that are necessary for the effective delivery of diagnostic services, and highlights the critical elements that should be established as minimum requirements.*

A. KEY CONSIDERATIONS FOR THE DESIGN AND MAINTENANCE OF A LABORATORY QUALITY MANAGEMENT SYSTEM

To ensure that the quality management system is appropriate and effective, the design must be carefully thought out planned and, where accreditation is sought, must address all criteria of the appropriate quality standard. The major categories of considerations and the their associated key issues and activities within each of these categories are outlined in the following eight sections of this chapter.

<sup>1</sup> ISO/IEC: International Organization for Standardization/International Electrochemical Commission.

30 **1. The work, responsibilities, and goals of the laboratory**

31 Many factors affect the necessary elements and requirements of a quality management system. ~~These factors~~  
32 ~~include, including:~~

- 33 i) Type of testing ~~done performed~~, e.g. research versus diagnostic work;
- 34 ii) Purpose and requirements of ~~the test results~~, e.g. ~~for import or /export quarantine~~ testing, surveillance,  
35 emergency disease exclusion, declaration of freedom from disease post-outbreak;
- 36 iii) Potential impact of a questionable ~~or~~, erroneous or unfavourable result, e.g. detection of foot and mouth  
37 disease (FMD) in an FMD-free country;
- 38 iv) ~~The tolerance level of~~ Risk and liability tolerance, e.g. vaccination ~~vs versus~~ culling ~~or /~~slaughter;
- 39 v) Customer ~~needs (requirements~~, e.g. sensitivity and specificity ~~of the test method~~, cost, turnaround time, strain  
40 or genotype level of characterisation), ~~e.g. for surveillance, or declaration of freedom after outbreak;~~
- 41 vi) ~~The role of the laboratory~~ Role in legal work or in regulatory programmes, e.g. for disease eradication and  
42 declaration of disease freedom to the WOAAH;
- 43 vii) ~~The role of the laboratory~~ Role in assisting with, confirming, or overseeing the work of other laboratories (e.g.  
44 as a reference laboratory);
- 45 viii) Business goals ~~of the laboratory~~, including the need for any third-party recognition or accreditation.

46 **2. Standards, guides, and references**

47 The laboratory should ~~choose reputable and accepted~~ follow globally recognised standards and guides to assist in  
48 designing the quality management system. For laboratories seeking ~~accreditation formal recognition~~ of testing  
49 competency, and for all WOAAH Reference Laboratories, the use of ISO/IEC 17025 (ISO/IEC, ~~2005-2017b~~) or  
50 equivalent ~~will be is~~ essential. This standard ~~includes specifies~~ managerial and technical requirements and  
51 accredited laboratories ~~that are compliant~~ are regarded as competent. Further information on standards may be  
52 obtained from the national standards body of each country, from the International Laboratory Accreditation  
53 Cooperation (ILAC)<sup>2</sup>, and from accreditation bodies, ~~e.g. the National Association of Testing Authorities (NATA),~~  
54 ~~Australia, the United Kingdom Accreditation Service (UKAS), the American Association for Laboratory Accreditation~~  
55 ~~(A2LA), etc.~~ Technical and international organisations such as AOAC International (The Scientific Association  
56 Dedicated to Analytical Excellence; formerly the Association of Official Analytical Chemists) and the International  
57 Organization for Standardization (ISO) publish useful references, guides, application documents and standards that  
58 supplement the general requirements of ISO/IEC 17025. Other relevant documents may include guide  
59 <https://nata.com.au/files/2021/05/Animal-Health-ISO-IEC-17025-Appendix-effective-March2021.pdf>; Newberry &  
60 Colling, 2021.

61 The ISO International Standard 9001 (ISO, 2015), ~~is a certification standard~~ specifies the requirements for quality  
62 management systems and while it may be a useful ~~supplement framework to a underpin~~ a laboratory quality system,  
63 fulfilment of its requirements does not necessarily ensure or imply assure technical competence (in the areas listed  
64 in Section 3 Accreditation). Conformance to the requirements of ISO 9001 is assessed by a certification body that  
65 is accredited ~~to undertake such assessments~~ by the national accreditation body to undertake such assessments.  
66 When a laboratory meets the requirements of ISO 9001, the term *registration* or *certification* is used to indicate  
67 conformity, not *accreditation*.

68 With the advent of stronger alliances between medical and veterinary diagnostic testing under initiatives such as  
69 “One Health”, some laboratories may ~~wish to choose to follow~~ other ISO standards such as ISO 15189 Medical  
70 Laboratories – Requirements for Quality and Competence (ISO/IEC, 2012), which include 2022, for testing of  
71 human samples, e.g. for zoonotic diseases. It should be noted that for veterinary laboratories, limited availability of  
72 suitable material may render validation difficult; under these circumstances it is necessary to highlight the limited  
73 validation status when reporting results and their interpretation (Stevenson et al., 2021).

74 **3. Accreditation**

75 If ~~the laboratory decides to proceed with~~ formal recognition of ~~its a laboratory's~~ quality management system and  
76 testing, ~~then is sought~~, third party verification of its conformity with the selected standard(s) ~~will be is~~ necessary.  
77 ILAC has published specific requirements and guides for laboratories and accreditation bodies. Under the ILAC  
78 system, ISO/IEC 17025 is to be used for laboratory accreditation of testing or calibration activities. Definitions  
79 regarding laboratory accreditation may be found in ISO/IEC International Standard 17000: Conformity Assessment

<sup>2</sup> ILAC: The ILAC Secretariat, PO Box 7507, Silverwater, NSW 2128, Australia; <http://ilac.org/>

80 – Vocabulary and General Principles (ISO/IEC, 2004a-2020). Accreditation is ~~not~~ dependent on demonstrated  
81 competence, which ~~is~~ encompasses significantly more than having and following documented procedures.  
82 Providing a competent and customer-oriented service also ~~means that the laboratory requires:~~

83 i) Adequate facilities and environmental controls;

84 ~~ii) Has~~ Appropriately qualified and trained personnel with a depth of technical knowledge commensurate with  
85 appropriate level of authority;

86 ~~iii) Has appropriate~~ Equipment ~~with planned~~ that is appropriately verified and managed in accordance with the  
87 relevant maintenance and calibration schedule;

88 ~~iv) Has adequate facilities and environmental control;~~

89 ~~v) Has procedures and specifications that ensure accurate and reliable results;~~

90 ~~vi) Implements continual improvements in testing and quality management;~~

91 ~~vii) Can assess the need for and implement appropriate corrective or preventive actions, e.g. customer~~  
92 ~~satisfaction;~~

93 ~~viii) Accurately assesses and controls uncertainty in testing;~~

94 iv) Appropriate sample and materials management processes;

95 v) Has Technically valid and validated test methods, procedures and specifications ~~that are,~~ documented in  
96 accordance with the requirements of the applicable standard or guidelines, e.g. Chapter 1.1.6 *Principles and*  
97 *methods of validation of diagnostic assays for infectious diseases* ~~and,~~ chapters 2.2.1 to 2.2.8  
98 *Recommendations for validation of diagnostic tests* and Special Issue of the *Scientific and Technical Review*  
99 (2021)<sup>3</sup>;

100 ~~vi) Demonstrates~~ Demonstrable proficiency in the applicable test methods ~~used~~ (e.g. by regular participation in  
101 proficiency ~~tests on a regular basis~~ testing schemes);

102 vii) Accurate assessment and control of the measurement of uncertainty in testing;

103 viii) Good documentation practices, e.g. ALCOA+ principles (i.e. Attributable, Legible, Contemporaneous, Original,  
104 Accurate, Complete, Consistent, Enduring, Available);

105 ix) Non-conformance management process, including detection, reporting, risk-assessment and implementation  
106 of effective corrective and preventive actions;

107 x) Complaints management;

108 xi) Adequate control of data and information;

109 xii) Appropriate reporting and approval process;

110 xiii) Culture of continual improvement.

111 xiv) Has demonstrable competence to generate technically valid results.

#### 112 **4. Selection of an accreditation body**

113 To facilitate the acceptance of the laboratory's test results for trade, the accreditation standard used must be  
114 recognised by the international community and the accreditation body recognised as competent to accredit  
115 laboratories. Programmes for the recognition of accreditation bodies are, in the ILAC scheme, based on the  
116 requirements of ISO/IEC International Standard 17011: Conformity Assessment – General Requirements for  
117 Accreditation Bodies Accrediting Conformity Assessment Bodies (ISO/IEC, 2004b–2017a). Information on  
118 recognised accreditation bodies may be obtained from the organisations that recognise them, such as the Asia-  
119 Pacific Accreditation Cooperation (APAC), the Inter-American Accreditation Cooperation (IAAC), and the European  
120 Co-operation for Accreditation (EA).

121 Accreditation bodies may also be signatory to the ILAC and regional (e.g. APAC) mutual recognition arrangements  
122 (MRAs). These MRAs are designed to reduce technical barriers to trade and further facilitate the acceptance of a  
123 laboratory's test results in foreign markets. Further information on the ILAC MRA may be obtained from the  
124 <http://www.ilac.org>.

<sup>3</sup> Available at: <https://doc.woah.org/dyn/portal/index.xhtml?page=alo&aloid=41245>

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## 125 5. Determination of the scope of the quality management system or of the laboratory's 126 accreditation

127 The scope of the quality management system should ~~cover all areas of activity affecting all~~ include all activities that  
128 impact testing that is done at ~~performed by~~ the laboratory. Whilst only accredited laboratories are obliged to meet  
129 the requirements of the relevant standard as detailed below, ~~these~~, the guiding principles should be considered  
130 best practise and are relevant to all testing laboratories.

131 ~~Laboratories accredited~~ A laboratory's accreditation to ISO/IEC 17025 ~~have~~ includes a specific list of these  
132 accredited tests that are accredited, called, referred to as the schedule or scope of accreditation or the scope.  
133 ~~Veterinary testing facilities include government and private facilities, veterinary practices, university veterinary~~  
134 ~~schools, and other laboratories for the testing of animals and animal products for the diagnosis, monitoring and~~  
135 ~~treatment of disease.~~ In principle, if new testing methods are introduced these must be assessed and accredited  
136 before they can be added to the scope, however a flexible scope can be implemented that assesses the laboratory  
137 as competent to add tests to scope, which are then formally added at the next accreditation visit. ~~The quality~~  
138 ~~management system should ideally cover all areas of activity affecting all testing that is done at the laboratory.~~  
139 However, it is up to the laboratory to decide which tests are to be accredited and included in the scope. If an  
140 accredited laboratory also offers unaccredited non-accredited tests, these must be clearly indicated as such on any  
141 reports that claim or make reference to accreditation. ~~Factors~~ It is ultimately the decision of the laboratory to decide  
142 which tests require inclusion in the scope of accreditation, and factors that might affect ~~the laboratory's choice of~~  
143 ~~tests for scope of accreditation~~ this decision include:

- 144 i) ~~The impact of initial accreditation on resources within a given deadline;~~  
145 i) Associated risks and opportunities;  
146 ii) Initial investment required (e.g. time, resources);  
147 ~~iii) A~~ Contractual requirement for accredited testing (e.g. for international trade, research projects);  
148 ~~iv) The~~ Importance of the test and the potential impact of an incorrect result;  
149 ~~v) The cost of maintaining an accredited test~~ versus frequency of use;  
150 ~~vi) Availability of personnel, facilities and equipment;~~  
151 ~~vii) Availability of appropriate materials and reference standards (e.g. standardised reagents, internal quality~~  
152 ~~control samples controls, reference cultures) and~~  
153 viii) Access to proficiency testing schemes;  
154 ~~ix) The quality assurance control processes~~ necessary for materials, reagents and media;  
155 ~~x) The validation status, e.g. access to field samples from infected and non-infected animals,~~ technical  
156 complexity and reliability of the test method;  
157 ~~xi) The~~ Potential for subcontracting of accredited tests.

## 158 6. Quality assurance, quality control and proficiency testing

159 Quality assurance (QA) is the part element of quality management focused on providing confidence that quality  
160 defined requirements ~~will be~~ are fulfilled. The requirements may be internal or defined in an accreditation or  
161 certification standard. QA is process-oriented and ensures provides ~~the right things are being done in the right way~~  
162 appropriate inputs to prevent problems arising.

163 Quality control (QC) is the systematic and planned monitoring of outputs to ensure the minimum levels of quality  
164 requirements have been met. For a testing laboratory, this is to ensure test processes ensures tests are working  
165 correctly performing consistently and reliably, and results are within the expected acceptable parameters and limits.  
166 QC is ~~test orientated and ensures the results are as expected~~ oriented and ensures detection of any problems that  
167 arise.

168 Proficiency testing (PT), sometimes referred to as external quality assurance or (EQA), is the determination  
169 assessment of a laboratory's performance by when testing a standardised panel of specimens of undisclosed  
170 content. Ideally, PT schemes should be run managed by an external independent provider. Participation in  
171 proficiency testing schemes enables the laboratory to assess and demonstrate the their testing reliability of results  
172 by in comparison with these from other participating laboratories.

173 All laboratories should, where possible, participate in external proficiency testing schemes appropriate to their  
174 testing. ~~Participation~~ the suite of tests provided; participation in such schemes is a requirement for accredited

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175 laboratories. This provides an independent assessment of the testing methods used and as well as the level of staff  
176 competence. If such schemes are not available, valid alternatives may be used, such as ring trials organised by  
177 reference laboratories, inter-laboratory testing, use of certified reference materials or internal quality control  
178 samples, replicate testing using the same or different methods, retesting of retained items, and or correlation of  
179 results for different characteristics of a specimen.

180 Providers and operators of proficiency testing programmes should be accredited to ISO/IEC 17043 – Conformity  
181 Assessment – General Requirements for Proficiency Testing (ISO/IEC, 2010).

182 Proficiency testing material from accredited providers ~~has been~~ is well characterised and any spare material, once  
183 the proficiency testing has been completed, can be useful to demonstrate staff competence or for test validation.  
184 Information about selection and use of reference samples and panels is available in Chapter 2.2.6 *Selection and*  
185 *use of reference samples and panels*. Proficiency testing and reproducibility scenarios are described by Johnson &  
186 Cabuang (2021) and Waugh & Clark (2021), respectively.

## 187 **7. Test methods**

188 ISO/IEC 17025 requires the use of appropriate test methods and has requirements for their selection, development,  
189 and validation to ~~show~~ demonstrate fitness for purpose.

190 This *Terrestrial Manual* provides recommendations on the selection of test methods for trade, diagnostic and  
191 surveillance purposes in the chapters on specific diseases. Disease-specific chapters include, or will include in the  
192 near future, a table of the tests available for the disease, graded against the test's fitness for purpose; these  
193 purposes are defined in the WOAHP Validation Template (chapter 1.1.6), which identifies six main purposes for which  
194 diagnostic tests may be carried out. The table is intended to be as a general guide to test application; the fact that  
195 a test is recommended does not necessarily mean that a laboratory is competent to perform it. The laboratory  
196 quality system should incorporate provision of evidence of competency.

197 In ~~the~~ the veterinary ~~profession laboratories~~, other standard methods (published in international, regional, or national  
198 standards) or fully validated methods (having undergone a full collaborative study and that are published or issued  
199 by an authoritative technical body such as the AOAC International) may be preferable to use, but ~~may not be~~  
200 available. Many veterinary laboratories develop or modify methods, and most laboratories have test systems that  
201 use non-standard methods, or a combination of standard and non-standard methods. In veterinary laboratories,  
202 even with the use of standard methods, some in-house evaluation, optimisation, or validation is generally ~~must be~~  
203 ~~done~~ required to ensure valid results.

204 Customers and laboratory staff must have a clear understanding of the performance characteristics of the test, and  
205 customers should be informed if the method is non-standard. Many veterinary testing laboratories will therefore  
206 need to demonstrate competence in the development, adaptation, verification and validation of test methods.

207 This *Terrestrial Manual* provides more detailed and specific guidance on test selection, optimisation,  
208 standardisation, and validation in chapter 1.1.6. ~~Chapter 1.1.6 refers to chapters 2.2.1–2.2.8~~ Recommendations for  
209 validation of diagnostic tests that deal with the development and optimisation of fundamentally different assays such  
210 as antibody, antigen and nucleic acid detections tests, measurement uncertainty, statistical approaches to test  
211 validation, selection and use of reference samples and panels, validation of diagnostic tests for wildlife, and  
212 comparability experiments after changes in a validated test method.

213 The following are key test method issues for those involved in the quality management of the laboratory.

### 214 **7.1. Selection of the test method**

215 Valid results begin with the selection of a test method that meets the needs of the laboratory's customers  
216 in addressing their specific requirements (fitness for purpose). Some issues relate directly to the laboratory,  
217 others to the customer.

#### 218 **7.1.1. Considerations for the selection of a test method**

- 219 i) International acceptance;
- 220 ii) Scientific acceptance;
- 221 iii) Appropriate or current technology;

- 
- 222 iv) Suitable performance characteristics (e.g. analytical and diagnostic sensitivity and specificity,  
223 repeatability, reproducibility, isolation rate, limits of detection, precision, trueness, and  
224 uncertainty);
- 225 v) Suitability of the test in the species and population of interest;
- 226 vi) Sample type (e.g. serum, tissue, milk) and its expected quality or state on arrival at the  
227 laboratory;
- 228 vii) Test target (e.g. antibody, antigen, live pathogen, nucleic acid sequence);
- 229 viii) Test turnaround time;
- 230 ix) Resources and time available for development, adaptation, evaluation;
- 231 x) Intended use (e.g. export, import, surveillance, screening, diagnostic, confirmatory);
- 232 xi) Safety factors and biocontainment requirements;
- 233 xii) Customer expectations;
- 234 xiii) Throughput of test Sample numbers and required throughput (automation, robot);
- 235 xiv) Cost of test, per sample;
- 236 xv) Availability of reference standards, reference materials and proficiency testing schemes. (See  
237 also chapter 2.2.6.).

## 238 7.2. Optimisation and standardisation of the test method

239 Once the method has been selected, it must be set up at the laboratory. Additional optimisation is  
240 necessary, whether the method was developed in-house (validation) or imported from an outside source  
241 (verification). Optimisation establishes critical specifications and performance standards for the test  
242 process as used in a specific laboratory.

### 243 7.2.1. Determinants of optimisation

- 244 i) Critical specifications for equipment, ~~instruments consumables,~~ and reagents (e.g. chemicals,  
245 biologicals), reference standards, reference materials, and internal controls;
- 246 ii) Robustness – critical control points and acceptable ranges, attributes or behaviour at critical  
247 control points, using statistically acceptable procedures;
- 248 iii) Quality control activities necessary to monitor critical control points;
- 249 iv) The type, number, range, frequency, and arrangement of test run controls;
- 250 v) Criteria for ~~non-subjective~~ objective acceptance or rejection of ~~a batch of~~ test results;
- 251 vi) Criteria for ~~the~~ interpretation and reporting of test results;
- 252 vii) ~~A-Documented test method and reporting procedure for use by laboratory staff;~~
- 253 viii) Evidence of technical competence for those ~~who performing~~ the test processes-methods,  
254 authorising test results and interpreting results.

## 255 7.3. Validation of the test method

256 Test method validation evaluates the test for its fitness for ~~a given use purpose~~ by establishing test  
257 performance characteristics such as sensitivity, specificity, and isolation rate; and diagnostic parameters  
258 such as positive or negative cut-off, repeatability, reproducibility and titre of interest or significance.  
259 Validation should be ~~done performed~~ using an optimised, documented, and fixed procedure. The extent  
260 and depth of the validation process will depend on logistical and risk factors-~~It and~~ may involve any number  
261 of activities and amount of data, with subsequent data analysis using appropriate statistical methods  
262 (Chapter 1.1.6.). Acknowledging diagnostic test validation science as a key element in the effective  
263 detection of infectious diseases, WOAHA recently published a Special Issue representing an up-to-date  
264 compilation of the relevant standards (WOAH and non-WOAH) and guidance documents for all stages of  
265 diagnostic test validation and proficiency testing, including design and analysis, as well as clear, complete  
266 and transparent reporting of validation studies in the peer-reviewed literature (Colling & Gardner, 2021). It  
267 is important to note that the current version of ISO 17025:2017 specifies that personnel must be authorised  
268 to perform validation and related activities, which means that training in validation and verification methods,  
269 including results interpretation, is likely to become more important to prove competence (Colling &

270 Gardner, 2021). It should also be noted that for veterinary laboratories, limited availability of suitable  
271 material may render validation difficult; under these circumstances it is necessary to highlight the limited  
272 validation status when reporting results and their interpretation (Stevenson *et al.*, 2021).

### 273 7.3.1. Activities that validation might include

- 274 ~~i) Field or epidemiological studies, including disease outbreak investigations and testing of~~  
275 ~~samples from infected and non-infected animals;~~
- 276 ~~ii) Development of testing algorithms for specific purposes, e.g. surveillance, outbreak~~  
277 ~~investigations, etc.;~~
- 278 ~~iii) Repeat testing in the same laboratory to establish the effect of variables such as operator,~~  
279 ~~reagents, equipment;~~
- 280 ~~iv) Comparison with other, preferably standard methods and with reference standards (if~~  
281 ~~available);~~
- 282 ~~iv) Collaborative studies with other laboratories using the same documented method. Ideally~~  
283 ~~organised by a reference laboratory and including testing a panel of samples of undisclosed~~  
284 ~~composition or titre with expert evaluation of results and feedback to ~~the~~ participants to~~  
285 ~~estimate reproducibility;~~
- 286 ~~iv) Reproduction of data from an accepted standard method, or from a reputable peer-reviewed~~  
287 ~~publication (verification);~~
- 288 ~~vii) Experimental infection or disease outbreak studies;~~
- 289 ~~vii) Analysis of internal quality control data.~~

290 vii) Field or epidemiological studies, including disease outbreak investigations and testing of  
291 samples from infected and non-infected animals;

292 viii) Development of testing algorithms for specific purposes, e.g. surveillance, outbreak  
293 investigations, etc.;

294 Validation is always a balance between cost, risk, and technical possibilities. There may be  
295 cases where ~~quantities such as only basic~~ accuracy and precision can ~~only be given~~  
296 ~~determined, e.g. when the disease is not present in a simplified way country or region~~. Criteria  
297 and procedures for the correlation of test results for diagnosis of disease status or for  
298 regulatory action must be developed. The criteria and procedures developed should account  
299 for screening methods, retesting and confirmatory testing.

300 Test validation is covered in chapter 1.1.6.

### 301 7.4. Uncertainty of the test method

302 Statistically relevant numbers of samples from infected and non-infected animals are discussed in chapter  
303 1.1.6. test validation and chapter 2.2.5 statistical approaches to validation.

### 304 7.4. Estimation of Measurement Uncertainty

305 Measurement of Uncertainty (MU) is “a parameter associated with the result of a measurement that  
306 characterises the dispersion of values that could reasonably be attributed to the measure” (Eurachem,  
307 2012). Uncertainty of measurement does not imply doubt about a result but rather increased confidence  
308 in its validity. It is not the equivalent to *error*, as it may be applied to all test results derived from a particular  
309 procedure.

310 Laboratories must estimate the MU for each test method resulting in a quantitative measurement included  
311 in their scope of accreditation, and for any methods used to calibrate equipment, included in their scope  
312 of accreditation (ISO/IEC 17025, 2005-2017b).

313 Tests can be broadly divided into two groups: quantitative (e.g. biochemical assays, enzyme-linked  
314 immunosorbent assays [ELISA], titrations, real-time polymerase chain reaction [PCR], pathogen  
315 enumeration, etc.); and qualitative (bacterial culture, parasite identification, virus isolation, endpoint PCR,  
316 immunofluorescence, etc.).

317 The determination of MU is well established in *quantitative* measurement sciences (ANSI, 1997). It may  
318 be given as a numeric expression of reliability and is commonly shown as a stated range. Standard  
319 deviation (SD) and **confidence-reference** interval (**C-R**) are examples of the expression of MU, for example  
320 the optical density result of an ELISA expressed as  $\pm n$  SD, where  $n$  is usually 1, 2 or 3. The confidence  
321 interval (usually 95%) gives an estimated range in which the result is likely to fall, calculated from a given  
322 set of test data. For quantitative measurements, example for a top-down or control-sample approach are  
323 provided for an antibody ELISA in chapter 2.2.4, and by the Australian government webpage<sup>4</sup>. An example  
324 for a quantitative PCR hydrolysis probe (TaqMan) assay is provided by Newberry & Colling (2021).

325 The ISO/IEC 17025 requirement for “quality control procedures for monitoring the validity of tests” implies  
326 that the laboratory must use quality control procedures that cover all major sources of uncertainty. There  
327 is no requirement to cover each component separately. Laboratories may establish acceptable  
328 specifications, criteria, ranges, etc., at critical control points for each component of the test process. The  
329 laboratory can then implement appropriate quality control measures at these critical points, or seek to  
330 reduce or eliminate the uncertainty effect of each component.

#### 331 **7.4.1. Potential sources of uncertainty include:**

- 332 i) Sampling;
- 333 ii) Contamination;
- 334 iii) Sample transport and storage conditions;
- 335 iv) Sample processing;
- 336 v) Reagent quality, preparation and storage;
- 337 vi) Type of reference material;
- 338 vii) Volumetric and weight manipulations;
- 339 viii) Environmental conditions;
- 340 ix) Equipment effects;
- 341 x) Analyst or operator bias;
- 342 xi) Biological variability;
- 343 xii) Unknown or random effects.

344 Systematic errors or bias determined by validation must be corrected by changes in the method,  
345 adjusted for mathematically, or have the bias noted as part of the report statement.

346 If an adjustment is made to a test or procedure to reduce uncertainty or correct bias then a new  
347 source of uncertainty is introduced (the uncertainty of the correction). This must be assessed as  
348 part of the MU estimate.

349 The application of the principles of MU to *qualitative* testing is less well defined. The determination  
350 and expression of MU has not been standardised for veterinary (or medical, food, or environmental)  
351 testing laboratories, but sound guidance exists and as accreditation becomes more important,  
352 applications are being developed. The ISO/IEC 17025 standard recognises that some test methods  
353 may preclude metrologically and statistically valid calculation of uncertainty of measurement. In  
354 such cases the laboratory must attempt to identify and estimate all the components of uncertainty  
355 based on knowledge of the performance of the method and making use of previous experience,  
356 validation data, internal control results, etc.

357 Many technical organisations and accreditation bodies (e.g. AOAC International, ISO, NATA, A2LA,  
358 Standards Council of Canada, UKAS, Eurachem, the Cooperation of International Traceability in  
359 Analytical Chemistry) teach courses or provide guidance on MU for laboratories seeking  
360 accreditation.

361 ~~The ISO/IEC 17025 requirement for “quality control procedures for monitoring the validity of tests”~~  
362 ~~implies that the laboratory must use quality control procedures that cover all major sources of~~  
363 ~~uncertainty. There is no requirement to cover each component separately. Laboratories may~~

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<sup>4</sup> Australian Government, Department of Agriculture, Fisheries and Forestry, Worked examples of measurement uncertainty. Measurement uncertainty in veterinary diagnostic testing – DAFF (agriculture.gov.au) (accessed 15 March 2023).



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364 establish acceptable specifications, criteria, ranges, etc., at critical control points for each  
365 component of the test process. The laboratory can then implement appropriate quality control  
366 measures at these critical points, or seek to reduce or eliminate the uncertainty effect of each  
367 component. Measurement Uncertainty is covered in chapter 2.2.4.

368 **7.4.1. Components of tests with sources of uncertainty include:**

- 369 i) Sampling;  
370 ii) Contamination;  
371 iii) Sample transport and storage conditions;  
372 iv) Sample processing;  
373 v) Reagent quality, preparation and storage;  
374 vi) Type of reference material;  
375 vii) Volumetric and weight manipulations;  
376 viii) Environmental conditions;  
377 ix) Equipment effects;  
378 x) Analyst or operator bias;  
379 xi) Biological variability;  
380 xii) Unknown or random effects.

381 Systematic errors or bias determined by validation must be corrected by changes in the method,  
382 adjusted for mathematically, or have the bias noted as part of the report statement.

383 If an adjustment is made to a test or procedure to reduce uncertainty or correct bias then a new  
384 source of uncertainty is introduced (the uncertainty of the correction). This must be assessed as  
385 part of the MU estimate.

386 Additional information on the analysis of uncertainty may be found in the Eurachem Guides to  
387 Quantifying Uncertainty in Measurement (Eurachem, 2012) and Use of uncertainty information in  
388 compliance assessment Uncertainty Information in Compliance Assessment (Eurachem, 2007).

389 **7.5. Implementation and use of the test method**

390 Training should be a planned and structured activity with steps to ensure adequate supervision is  
391 maintained while analysts are being trained. Depending on the complexity of the test and the experience  
392 of the analyst, training may include any combination of reading and understanding the documented test  
393 method, initial demonstration, performance of the test under supervision and independent performance.  
394 Analysts should be able to demonstrate proficiency in using the test method prior to producing being  
395 authorised to produce reported results, and on an ongoing basis.

396 The laboratory must be able to demonstrate traceability for all accredited tests and the principle should  
397 apply to all tests whether accredited or not. This covers all activities relating to test selection, development,  
398 optimisation, standardisation, validation, verification, implementation, reporting, personnel, quality control  
399 and quality assurance (see also Section 7.3.1. point vi). Traceability is achieved by using appropriate  
400 documented project management, record keeping, data management and archiving systems.

401 **8. Strategic planning**

402 Laboratories should have evidence of continual improvement, which is an obligatory requirement for  
403 accredited laboratories. The laboratory must be knowledgeable of and stay maintain current with knowledge  
404 of the relevant quality and technical management standards and with methods used to demonstrate laboratory  
405 competence and establish and maintain technical validity. Evidence of this may be provided by include:

- 406 i) Attendance at conferences, organisation of in-house or external meetings on diagnostics and quality  
407 management;  
408 ii) Participation in Membership of local and international organisations;

- 
- 409           iii) ~~Participation in writing~~ Contribution to national and international standards (e.g. on ILAC and ISO  
410           committees);
- 411           iv) Maintenance of current awareness of publications, writing through review of and ~~reviewing publications~~  
412           ~~about diagnostic methods~~ contribution to relevant literature;
- 413           v) Participation in training programmes, including visits to other laboratories;
- 414           vi) Conducting research;
- 415           vii) Participation in cooperative programmes (e.g. Inter-American Institute for Cooperation in Agriculture);
- 416           viii) Exchange of procedures, methods, reagents, samples, personnel, and ideas;
- 417           ix) Planned, continual professional development and technical training;
- 418           x) Management reviews;
- 419           xi) Analysis of customer feedback;
- 420           xii) Root cause analysis of anomalies and implementation of corrective, preventive and improvement  
421           actions, as well as effectiveness reviews.

422

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<sup>5</sup> NCSL: The National Conference of Standards Laboratories.

<sup>6</sup> CITAC: The Cooperation of International Traceability in Analytical Chemistry.

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452 \*  
453 \* \*

454 NB: FIRST ADOPTED IN 1996 AS *GOOD LABORATORY PRACTICE, QUALITY CONTROL AND QUALITY ASSURANCE*.  
455 MOST RECENT UPDATES ADOPTED IN 2017.

REUNIÓN DE LA COMISIÓN DE NORMAS BIOLÓGICAS

París, 5–9 de febrero de 2024

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CHAPTER 1.1.9.

TESTS FOR STERILITY AND FREEDOM FROM  
CONTAMINATION OF BIOLOGICAL MATERIALS  
INTENDED FOR VETERINARY USE

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INTRODUCTION

*The international trade-related movements of biological materials intended for veterinary use are subject to restrictions imposed to minimise the spread of animal and human pathogens. Countries may impose requirements for proof-of-freedom testing before allowing the regulated importation of materials of animal derivation and substances containing such derivatives. Where chemical or physical treatments are inappropriate or inefficient, or where evidence is lacking of the effectiveness of the treatment is lacking, there may be general or specific testing requirements imposed by authorities of countries receiving such materials. This chapter provides guidance on the approach to such regulated testing, particularly as might be applied to the movement of vaccine master seed and master cell stocks, and to related biological materials used in manufacturing processes. The term seed stocks is used when testing live products, for killed products the preferred reference is master cell stocks. While the onus for ensuring safety of a product remains with the manufacturer and may be regulated by therapeutic guidelines, this chapter provides procedures that are designed in particular to minimise the risk of undetected contaminants in veterinary therapeutics and biological reagents causing the cross-border spread of agents of concern to particular importing countries. In their review "Extraneous agent detection in vaccines" Farsang & Kulcsar, 2012 reported the following examples of contamination of vaccines with extraneous agents: a) Foamy virus (Spumaretroviridae) was identified as a contaminant of primary monkey kidney cultures used for vaccine production in the early 1950, b) In the 1960s it was shown that yellow fever live attenuated vaccines prepared in chicken embryo fibroblasts were infected with avian leukosis virus (ALV). c) Calicivirus was found in Chinese hamster ovary (CHO) cells, d) Newcastle disease vaccine strains were found in different live poultry vaccines, e) in 1990 a live attenuated multi component canine vaccine was contaminated with a serotype of Bluetongue virus causing abortions and death in pregnant bitches, f) Fetal calf serum transmitted Pestiviruses (BVDV types 1 and 2) are one of the most common extraneous agents in veterinary and human vaccines, g) RD114 is a replication-competent feline endogenous gamma retrovirus which contaminated canine corona and parvovirus vaccines, h) a notable case of human vaccine contamination may have been when in the 20th century tens of millions of people worldwide were immunised with polio vaccines containing simian virus 40 (SV40). SV40 was found to cause cancer in animals and is associated with human brain, bone and lung cancers, however, a clear connection was not found between this certain vaccine and any human tumour case, i) a porcine circovirus 1 (PCV1) was found in a rotavirus vaccine widely used worldwide for children. Farsang &*

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36 Kulcsar (2012) and WHO (2015) describe case studies of veterinary and human vaccines  
37 contaminated with extraneous agents and findings support the need of accurate and validated  
38 amplification and detection methods as key elements for effective detection and control. Further  
39 examples are given in Section G. Protocol examples below. Control of contamination with  
40 transmissible spongiform encephalopathy (TSE) agents is not covered in this chapter because  
41 standard testing and physical treatments cannot be used to ensure freedom from these agents.  
42 Detection methods are described in Chapter 3.4.5. Bovine spongiform encephalopathy.

43 Sterility is defined as the absence of viable microorganisms, which for the purpose of this chapter,  
44 includes viruses. It should be achieved using aseptic techniques and validated sterilisation methods,  
45 including heating, filtration, chemical treatments, and irradiation that fits the intended purpose.  
46 Freedom from contamination is defined as the absence of specified viable microorganisms. This may  
47 be achieved by selecting materials from sources shown to be free from specified microorganisms  
48 and by conducting subsequent procedures aseptically. Adequate assurance of sterility and freedom  
49 from contaminating microorganisms can only be achieved by proper control of the primary materials  
50 used and their subsequent processing. Tests on intermediate products are necessary throughout the  
51 production process to check that this control has been achieved.

52 Biological materials subject to contamination that cannot be sterilised before or during use in vaccine  
53 production, such as ingredients of animal origin, e.g. serum and trypsin, primary and continuous cells  
54 and cell lines, and viral or bacterial seed stocks, etc., should be tested for viable extraneous agents  
55 before use. Assays to detect viral contaminants, if present, can be achieved by various culture  
56 methods, including use of embryonated eggs, which are supported by cytopathic effects (CPE)  
57 detection/embryo death, fluorescent antibody techniques and other suitable (fit for purpose), methods  
58 such as polymerase chain reaction (PCR) and antigen detection ELISA (enzyme-linked  
59 immunosorbent assay). As is explained in more detail in this chapter care must be taken when using  
60 PCR and ELISA techniques for detection as such tests do not distinguish viable from non-viable  
61 agent detection. Specific assays to detect other contaminants, such as fungi, protozoa and bacteria  
62 (including rickettsia and mycoplasma) are also described.

63 Avian materials and vaccines are required to be inoculated on to primary avian cell cultures or eggs  
64 for the detection of avian viruses. A combination of general tests, for example to detect  
65 haemadsorbing, haemagglutinating and CPE causing viruses and specific procedures aimed at the  
66 growth and detection of specific viruses is recommended to increase the probability of detection.  
67 Assays to detect other contaminants, such as bacteria, fungi, protozoa, rickettsia and mycoplasma  
68 are also described.

69 ~~Procedures applied~~ Testing procedures should be validated and found to be “fit for purpose” following  
70 Chapter 1.1.6. Validation of diagnostic assays for infectious diseases of terrestrial animals, where  
71 possible.

72 It is a requirement of many regulators, that a laboratory testing report notes the use of validated  
73 procedures and describes the validated procedures in detail including acceptance criteria. This gives  
74 the regulator transparency in the procedures used in a testing laboratory.

75 The validation assessment of an amplification process in cell culture should include documentation  
76 of the history of permissive cell lines used, reference positive controls and culture media products  
77 used in the process of excluding adventitious agents, to ensure the process is sound and is not  
78 compromised. The validation assessment should give information (published or in-house) of the  
79 limitations that may affect test outcomes and an assessment of performance characteristics such as  
80 analytical specificity and sensitivity of each cell culture system, using well characterised, reference  
81 positive controls.

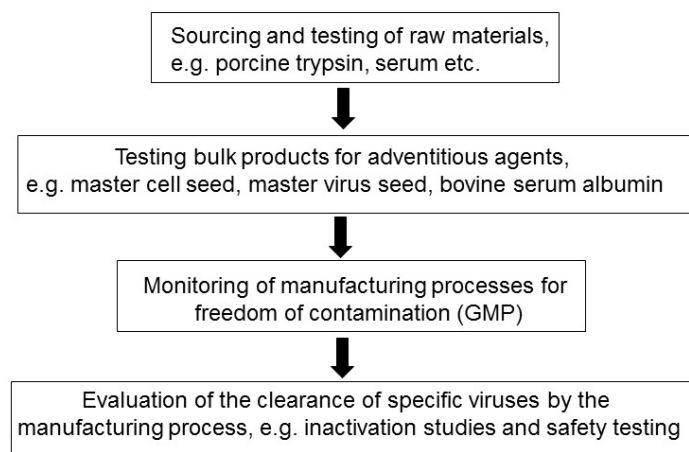
82 It is the responsibility of the submitter to assure ensure a representative selection and number of items  
83 to be tested. The principles of Appendix 1.1.2.1 Epidemiological approaches to sampling: sample size  
84 calculations of Chapter 1.1.2 Collection, submission and storage of diagnostic specimens apply  
85 describes the principles to be applied. Adequate transportation is described in Chapter 1.1.2 and  
86 Chapter 1.1.3 Transport of biological materials describe transportation requirements.

## A. AN OVERVIEW OF TESTING APPROACHES

88 Although testing is seen as a key component of biosafety in biological products intended for veterinary use, testing is not  
 89 enough to ensure a given product is free of viable infectious contaminants, and so a holistic, multifaceted approach must  
 90 be taken. Such an approach includes risk assessment, risk mitigation and management strategies (Barone *et al.*, 2020).  
 91 In general:

- 92 • Primary materials must be collected from sources shown to be free from contamination and handled in such a way  
 93 as to minimise contamination and the opportunities for any contaminants to multiply (Figure 1).
- 94 • Materials that are not sterilised and those that are to be processed further after sterilisation must be handled  
 95 aseptically. Such materials will require further assessment of freedom of contaminants at certain stages of production  
 96 to assure freedom of adventitious agents.
- 97 • Materials that can be sterilised without their biological activities being affected unduly must be sterilised by a method  
 98 effective for the pathogens ~~concerned of concern~~. The method must reduce the level of contamination to be  
 99 undetectable, as determined by an appropriate sterility test study. ~~(See Section D.1. below)~~. If a sterilisation process  
 100 is used, it shall be validated to demonstrate that it is fit for purpose. Suitable controls will be included in each  
 101 sterilisation process to monitor efficiency.
- 102 • The environment in which any aseptic handling is carried out must be maintained in a clean state, protected from  
 103 external sources of contamination, and controlled to prevent internal contamination. Rules governing aseptic  
 104 preparation of vaccines are documented in Chapter 2.3.3 *Minimum requirements for the organisation and*  
 105 *management of a vaccine manufacturing facility*.

106 **Figure 1. Testing algorithm Risk assessment flowchart for vaccine production.**



107

108 Some procedures have been properly validated and found to be “fit for purpose”, whilst others may have undergone  
 109 only limited validation studies. For example, methods for bacterial and fungal sterility may have not been formally  
 110 validated although they have been used for many years. In particular, ~~the~~ *in-vivo* and ~~cell culture~~ *in-vitro* methods have  
 111 essentially unknown sensitivity and specificity (Sheets *et al.*, 2012) though there is an accepted theoretical sensitivity,  
 112 regarding cell culture of 1 colony-plaque-forming unit (CFU-PFU). For example, an evaluation of methods to detect  
 113 bovine and porcine viruses in serum and trypsin based on United States (of America) Code of Federal Regulations,  
 114 Title 9 (9CFR) revealed gaps in sensitivity, even within virus families (Marcus-Secura *et al.*, 2011). It is therefore  
 115 important to interpret, and report results in the light of specific conditions of cultures employed and considering  
 116 sensitivity and specificity of detection systems.

117 Newer, more sensitive methods such as molecular assays may afford the ability to detect contaminants, which may  
 118 not be successfully amplified in traditional culturing systems. The detection range can be broadened by using family  
 119 specific primers and probes if designed appropriately. However, most, if not all ~~such new molecular-based~~ tests are  
 120 also able to detect evidence for non-infectious contaminants, such as traces of nucleic acid from inactivated  
 121 contaminants. ~~Follow up testing would be required to determine the nature of the contaminant, for example, non-~~  
 122 ~~infectious nucleic acid or infectious virus. Attempts at virus isolation or sequencing may remedy this.~~ Note: molecular  
 123 assays if not designed as fit for purpose may miss detection of contaminating agents or lack sensitivity to do so  
 124 (Hodinka, 2013).

125 More recently metagenomic high throughput sequencing (HTS) workflows have shown potential for quality control of  
126 biological products (van Borm *et al.*, 2013) and vaccines (Baylis *et al.*, 2011; Farsang & Kulcsar, 2012; Neverov &  
127 Chumakov, 2010; Onions & Kolman, 2010; Victoria *et al.*, 2010) in particular for the identification and characterisation of  
128 unexpected highly divergent pathogen variants (Miller *et al.*, 2010; Rosseel *et al.*, 2011) that may remain undetected using  
129 targeted diagnostic tests. Nevertheless, targeted assays, e.g. amplification in cell culture followed by polymerase chain  
130 reaction (PCR) may be superior to HTS for specific agent detection (Wang *et al.*, 2014) due to lack of sensitivity of HTS at  
131 this time. Chapter 1.1.7. gives an overview of the standards for high throughput sequencing, bioinformatics and  
132 computational genomics. Similarly, recent improvements in protein and peptide separation efficiencies and highly accurate  
133 mass spectrometry have promoted the identification and quantification of proteins in a given sample. Most of these new  
134 technologies are broad screening tools, limited by the fact that they cannot distinguish between viable and non-viable  
135 organisms.

136 Given the availability of new technologies, there will be future opportunities and challenges to determine presence of  
137 extraneous agents in biologicals intended for veterinary use for industry and regulators. Problems can arise when the  
138 presence of genome positive results are interpreted as evidence for the presence of contamination (Mackay & Kriz,  
139 2010). When using molecular technologies, it is important to understand the correlation between genome detection and  
140 detection of live virus-agent. It cannot be assumed that detection of genome corresponds to the presence of an infectious  
141 agent.

## 142 **B. LIVING VIRAL VACCINES FOR ADMINISTRATION BY INJECTION, OR THROUGH** 143 **DRINKING WATER, SPRAY, OR SKIN SCARIFICATION**

144 1. Materials of animal origin ~~shall~~ should be ~~(a) sterilised, or (b) and~~ obtained from healthy animals that, in so far as is  
145 possible, should be shown to be free from pathogens that can be transmitted from the species of origin to the species  
146 to be vaccinated, or any species in contact with them by means of extraneous agents testing.

147 2. Seed lots of virus, any continuous cell line and biologicals used for virus growth ~~shall~~ should be shown to be  
148 free from ~~viable~~ bacteria, fungi, mycoplasmas, protozoa, rickettsia, and extraneous viruses ~~and other~~  
149 ~~pathogens~~ that can be transmitted from the species of origin to the species to be vaccinated or any species  
150 in contact with them. ~~There may be some exceptions for a limited number of non pathogenic bacteria and~~  
151 ~~fungi to be present in live viral vaccines produced in eggs and administered through drinking water, spray, or~~  
152 ~~skin scarification.~~

153 For ~~the~~ production of vaccines in embryonated chicken eggs and the quality control procedures for these  
154 vaccines, it is recommended (~~required in many countries~~) that eggs from specific pathogen-free birds should  
155 be used.

156 3. Each batch of vaccine ~~shall~~ should pass tests for freedom from extraneous agents that are consistent with the  
157 importing country's requirements for accepting the vaccine for use. Some examples of published methods that  
158 document acceptable testing ~~procedures~~ processes in various countries include: (US) Code of Federal Regulations  
159 (2015); European Pharmacopoeia (2014); European Commission (2006); World Health Organization (WHO) (1998;  
160 2012) and Department of Agriculture (of Australia) (2013).

- 161 • Code of Federal Regulations, Title 9 (9CFR) (of the United States of America) (2015).
- 162 • Department of Agriculture, Forest and Fisheries (Australia) (2013).
- 163 • Department of Agriculture, Forest and Fisheries (Australia) (2021b) Live Veterinary Vaccines.
- 164 • Regulation on Veterinary Drug Administration (China [People's Republic of]) (2020).
- 165 • European Medicines Agency Sciences Medicines Health (2016).
- 166 • European Pharmacopoeia, 10th Edition (2021).
- 167 • World Health Organization (WHO) (1998; 2012).

168 4. Tests for ~~sterility~~ freedom of contamination ~~shall~~ should be appropriate to prove that the vaccine is free from viable  
169 extraneous viruses, bacteria including rickettsia and mycoplasmas, fungi, and protozoa. Each country will have  
170 ~~particular~~ requirements as to what agents are ~~necessary to exclude~~ should be tested for and ~~what by which~~  
171 ~~procedures are acceptable~~. Such tests will include amplification of ~~viable~~ extraneous agents using cell culture that is  
172 susceptible to ~~particular known~~ viruses of the species of concern, tests in embryonated eggs, bacterial, mycoplasma  
173 and fungal culturing techniques and, where ~~necessary and possible~~ there is no alternative ~~ie, tests involving~~ animal  
174 inoculation. PCR, fluorescence antibody test (FAT), presence of colonies or cytopathic effects (CPE) and antigen  
175 detection ELISA ~~will can~~ be used for detection purposes after amplification using culturing techniques to improve  
176 specificity and sensitivity. If *in-vitro* or *in-vivo* amplification of the target agent is not possible, direct PCR may be  
177 useful if validated for this purpose.

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178 **~~C. LIVING VIRAL VACCINES FOR ADMINISTRATION THROUGH DRINKING WATER,~~**  
179 **~~SPRAY, OR SKIN SCARIFICATION~~**

180 ~~1. Section B applies.~~

181 ~~2. A limited number of contaminating, non-pathogenic bacteria and fungi may be permitted (see Section I.2.2 General~~  
182 ~~Procedure for testing live viral vaccines produced in eggs and administered through drinking water, spray, or skin~~  
183 ~~scarification for the presence of bacteria and fungi).~~

184 **D-C. INACTIVATED VIRAL AND BACTERIAL VACCINES**

185 1. Each batch of vaccine shall pass a test for inactivation of the vaccinal ~~virus seed~~ or bacterial and should include  
186 inactivation studies on representative extraneous agents if the virus or bacterial seed has not already been  
187 tested and shown to be free from extraneous agents. An example of a simple inactivation study could include  
188 assessment of the titre of live vaccine before and after inactivation and assessing the log<sub>10</sub> drop in titre during  
189 the inactivation process. This would give an indication of the efficacy of the inactivation process. There is  
190 evidence that virus-titration tests may not have sufficient sensitivity to ensure complete inactivation. In these  
191 circumstances, a specific innocuity test would need to be developed and validated to be fit for increased  
192 sensitivity. To increase sensitivity more than one passage would be required depending on the virus or  
193 bacteria of concern. An example of this approach can be found at:  
194 [https://www.aphis.usda.gov/animal\\_health/vet\\_biologics/publications/memo\\_800\\_117.pdf](https://www.aphis.usda.gov/animal_health/vet_biologics/publications/memo_800_117.pdf) (accessed 25 July  
195 2023).

196 2. If studies on representative extraneous agents are required, then spiking inactivated vaccine with live representative  
197 agents and following the example of an inactivation study ~~as in D.1 above would~~ could be useful. The inactivation  
198 process and the tests used to detect live virus agent after inactivation must be validated and shown to be suitable for  
199 their intended purpose.

200 In addition, each country may have ~~particular~~ its own requirements for sourcing or tests for sterility as detailed in  
201 Section B above.

202 **E. D. LIVING BACTERIAL VACCINES**

203 1. See Section B applies.

204 2. Seed lots of bacteria shall be shown to be free from other bacteria as well as fungi and mycoplasmas, protozoa,  
205 rickettsia, and extraneous viruses. Agents required for exclusion will be dependent on the country accepting the  
206 vaccine for use. Use of antibiotics to 'inactivate' the living bacterial seed or vaccine prior to exclusion of viruses and  
207 fungi is recommended to ensure testing in culture is sensitive. ~~Interference testing is recommended to ensure that~~  
208 ~~the antibiotics used do not affect the growth of the extraneous virus or fungi that is being excluded. Sonication may~~  
209 ~~also be useful~~

210 ~~Interference testing is required to ensure that antibiotics used (or sonication) does not affect the growth of extraneous~~  
211 ~~virus or fungi being excluded, compromising the test outcome.~~

212 Due to the difficulties and reduced sensitivity in exclusion of extraneous bacteria and some mycoplasma, protozoa,  
213 and rickettsia from high-titred seed lots of bacteria, the use of narrow-range antibiotics aimed specifically at reducing  
214 seed lot bacteria is ~~recommended~~ useful if antibiotics do not affect the growth of bacteria being excluded. The optimal  
215 concentration of antibiotics can be determined in a dilution experiment such as documented in 9CFR Section  
216 113.25(d). Other methods of exclusion of extraneous bacteria from bacterial seeds may include filtering for size  
217 exclusion such as removing bacteria seed to look for mycoplasma contamination and use of selective culturing media.  
218 Such processes would require ~~validation~~ verification to ensure the process does not affect the sensitivity of exclusion  
219 of extraneous agents of concern.

220 3. Sonication of a living bacterial seed may be useful when excluding specific viral agents. Once again, the  
221 inactivation procedure would require a verification process to ensure the adventitious virus being excluded is  
222 not affected by the treatment. Use of a suitable reference virus control during the exclusion process would  
223 be required.



- 
- 224 4. Direct PCR techniques may be useful when culturing processes fail to be ~~sensitive~~ successful in detecting extraneous  
225 bacteria from live bacterial seeds or vaccines.

226 **~~F. INACTIVATED BACTERIAL VACCINES~~**

- 227 ~~1. Section D applies. It should not be necessary to test for extraneous viruses that would not grow in bacteriological~~  
228 ~~culture media as long as freedom from contamination of all starting materials can be assured. Complete inactivation~~  
229 ~~of the vaccinal bacteria should be demonstrated by means of titration and innocuity tests — in some cases general~~  
230 ~~bacterial sterility testing (Section I.2.1) may suffice.~~

231 **~~G. E. SERA, PLASMA AND DIAGNOSTIC AGENTS FOR ADMINISTRATION TO~~**  
232 **~~ANIMALS~~**

- 233 1. ~~Section B.4 applies for sera/diagnostic agents that are not inactivated. Section C applies for non-inactivated~~  
234 ~~sera/diagnostic agents.~~
- 235 2. Some countries require quarantine, health certification, and tests for specific diseases to be completed for all serum  
236 and plasma donor animals, for example, 9CFR (2015) and Australian Quarantine Policy and Requirements for the  
237 Importation of Live and Novel Veterinary Bulk and Finished Vaccines (1999). For some diseases, for example equine  
238 infectious anaemia, the product (plasma) must be stored until the seroconversion period has been exceeded and the  
239 donors tested negative.
- 240 ~~3. It is recommended that each batch of non-inactivated serum be assessed for viable extraneous agents, including~~  
241 ~~mycoplasma. Each batch of serum shall pass a test for freedom from extraneous agents. Suitable test methods have~~  
242 ~~been published for various countries, for example, European Pharmacopoeia (2014); 9 CFR (2015) and Australian~~  
243 ~~Quarantine Policy and Requirements for the Importation of Live and Novel Veterinary Bulk and Finished Vaccines~~  
244 ~~(1999) and Department of Agriculture (of Australia) (2013).~~
- 245 4. ~~Inactivated serum, Section D applies.~~
- 246 5. ~~Section B or D may apply if a virus is used in the production of the diagnostic agent; Section E or F may apply if a~~  
247 ~~bacterium is used.~~

248 **~~H. F. EMBRYOS, OVA, SEMEN~~**

249 Special precautions must be taken with relation to the use of embryos, ova, semen (Hare, 1985). Most countries will have  
250 regulatory guidelines for import of these biologicals for veterinary use. Such guidelines can be found at various websites  
251 such as the European Commission (2015), FAO and Department of Agriculture Forest and Fisheries (2021a; 2021b),  
252 though ~~many such some~~ guidelines may give more detail in regard to the food safety aspect.

253 **~~J. G. PROTOCOL EXAMPLES~~**

254 **1. General procedures Introduction to protocol examples**

255 This section provides some examples to illustrate scope and limitations of testing protocols. It is not intended to be  
256 prescriptive or exhaustive. Examples are based on standards and published methods to increase the sensitivity for  
257 exclusion of live adventitious agents, using general and specific techniques.

258 In principle, proposed testing represents ~~an~~ attempted isolation of viable agents in culturing systems normally  
259 considered supportive of the growth of each specified agent or group of general agents. After amplification,  
260 potential pathogens can be detected further, by sensitive and specific diagnostic tests such as FAT or PCR if as  
261 required. General detection systems can include haemabsorbance and CPE by immunohistochemistry staining  
262 methods. The example procedures for sterility-detection of contamination testing and general detection of viable  
263 virus, fungi, protozoa and bacteria (including rickettsia and mycoplasma, fungi and viruses) described below are  
264 derived from standards such as the 9CFR (2015), European Pharmacopoeia, ~~(2014)~~ 10th Edition (2021), European  
265 Commission (2006), WHO Medicines Agency Sciences Medicines Health (2016), Department of Agriculture, Forest  
266 and Fisheries (Australia) (2013) and World Health Organization (1998; 2012).

267 Individual countries or regions should adopt a holistic, risk-based approach to determine the appropriate testing  
 268 protocols based on their animal health status. As well as applying general testing procedures documented in  
 269 national or regional standards as mentioned above, it may be necessary to apply rigorous exclusion testing for  
 270 specific agents that are exotic to the particular country or region of concern.

271 General procedures will do not necessarily detect all extraneous agents that may be present in biological material;  
 272 however, they are useful as screening tests. Some examples of agents that may require specific methods for  
 273 detection in biologicals refer to Table 1 below. Procedures documented in the Review of Published Tests to Detect  
 274 Pathogens in Veterinary Vaccines Intended for Importation into Australia (2013) available from the Department of  
 275 Agriculture, Forest and Water Resources, Australia Fisheries are able to address such agents in offering sensitive  
 276 testing approaches based on reputable publications. A CVMP reflection paper published written by the European  
 277 Medicines Agency Sciences Medicines Health Committee of Veterinary Medicinal Products (CVMP) in (2016),  
 278 adopted in May 2017, documents lists specific test method approaches for a number of agents, listed in Table 1,  
 279 that cannot be excluded using general test procedures (Table 1).

280 Exclusion of specific agents requires procedures that maximise sensitivity by providing ideal amplification and  
 281 detection of the pathogen in question. Extraneous agents, for example, Maedi Visna virus, bovine  
 282 immunodeficiency virus, (and other retroviruses), Trypanosoma evansi and porcine respiratory coronavirus are  
 283 difficult to culture even using the most sensitive approaches. In these circumstances, application of molecular  
 284 assays directly to the biological material in question to assess, assessing for the presence of nucleic acid from  
 285 adventitious agents offers an alternative. Refer to Table 1. Consideration must be noted as described in Section  
 286 A.6 as, though detection of the presence of non-viable and host associated agents may is also be detected using  
 287 this procedure possible.

288 Table 1 gives examples of causative infectious agents that may be present in animal biologicals intended for  
 289 veterinary use, for example PCV-1 in a rotavirus vaccine (WHO, 2015). BVDV is well known for its presence in  
 290 many bovine associated biologicals, including cell culture. More recently, non-CPE pestivirus, BVD type 3 (HoBi-  
 291 like) are found in foetal calf serum and cell culture. Classical Swine fever has contaminated various porcine cell  
 292 lines used for African swine fever and FMDV diagnosis, and thus the potential for contamination of porcine based  
 293 vaccines. PEDV is linked to spray-dried porcine plasma used for feed. This is not an exhaustive list of agents of  
 294 concern or by any means required for exclusion by every country based on risk, they are just examples of infectious  
 295 agents that are not culturable using general culturing procedures and require a more use of specialised culturing  
 296 processes and specific detection process by means of the indirect fluorescent antibody test, PCR or ELISA, where  
 297 applicable processes. Notably, some subtypes of an agent type may be detectable by general methods, and some  
 298 may require specialised testing for detection. For example, bovine adenovirus subgroup 1 (serotypes 1, 2, 3 and  
 299 9) can be readily isolated using general methods (Vero cells) however bovine adenovirus subgroup 2 (serotypes  
 300 4, 5, 6, 7, 8 and 10) are not readily isolated and required specialised methods for isolation.

301 **Table 1. Some-Examples of infectious agents of veterinary importance**  
 302 **that require specialist specialised culturing and detection techniques**

Rotaviruses	Pestiviruses (non-CPE)	Turkey rhinotracheitis
Porcine epidemic diarrhoea virus	Bluetongue virus	<i>Brucella abortus</i>
Porcine circoviruses (PCV-1, 2)	Swine pox virus	Rickettsias
Swine/equine influenza, some strains	Some adenoviruses	Protozoa
Bovine respiratory syncytial virus	<u>Rhabdoviruses (e.g. rabies virus)</u>	Some fungi (e.g. <i>Histoplasma</i> )

## 303 2. Example of detection of bacteria and fungi contamination

### 304 2.1. General procedure for assessing the sterility of viable bacteria and fungi

305 Standard tests for detecting extraneous bacteria and fungi (sterility testing) in raw materials, master cell stocks,  
 306 or final product are the membrane filtration test or the direct inoculation sterility test.

307 For the membrane filtration technique, a filter having a nominal pore size not greater than 0.45 µm and a  
 308 diameter of at least 47 mm should be used. Cellulose nitrate filters should be used if the material is aqueous or  
 309 oily; cellulose acetate filters should be used if the material is strongly alcoholic, oily or oil-adjuvanted.  
 310 Immediately before the contents of the container or containers to be tested are filtered, the filter is moistened  
 311 with 20–25 ml of Diluent A or B.

312

**2.1.1. Diluent A**

313

Diluent A is for aqueous products or materials. Dissolve 1 g peptic digest of animal tissue in water to make 1 litre, filter, or centrifuge to clarify, adjust the pH to  $7.1 \pm 0.2$ , dispense into containers in 100 ml quantities, and sterilise by steam.

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316

**2.1.2. Diluent B**

317

Diluent B is for oil-adjuvanted products or materials: Add 1 ml polysorbate 80 to 1 litre Diluent A, adjust the pH to  $7.1 \pm 0.2$ , dispense into containers in 100 ml quantities, and sterilise by steam.

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If the biological being tested has antimicrobial properties, the membrane is washed three times after sample application with approximately 100 ml of the appropriate diluent (A or B). The membrane is then transferred whole to culture media, aseptically cut into equal parts and placed in media, or the media is transferred to the membrane in the filter apparatus. If the test sample contains merthiolate as a preservative, fluid thioglycolate medium (FTM) is used and the membranes are incubated at both 30–35°C and 20–25°C. If the test sample is a killed biological without merthiolate preservative, FTM is used at 30–35°C and soybean casein digest medium (SCDM) at 20–25°C. If the sample tested is a live viral biological, SCDM is used at both incubation temperatures. It has been suggested that sulfite-polymyxin-sulfadiazine agar be used to enhance the detection of *Clostridium* spp. when the membrane filtration technique is used (Tellez *et al.*, 2005).

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If direct inoculation of culture media is chosen, a sterile pipette or syringe and needle are used to aseptically transfer the biological material directly into liquid media. If the biological being tested has antimicrobial properties, the ratio of the inoculum to the volume of culture medium must be determined before the test is started, for example as explained in 9CFR 113.25(d) and detailed testing procedures can be found for example in supplemental assay method [USDA SAM 903](https://www.aphis.usda.gov/animal_health/vet_biologics/publications/sam903.pdf) [https://www.aphis.usda.gov/animal\\_health/vet\\_biologics/publications/sam903.pdf](https://www.aphis.usda.gov/animal_health/vet_biologics/publications/sam903.pdf) (accessed 24 July 2023). (SAM) 903 USDA SAM 903, See [https://www.aphis.usda.gov/animal\\_health/vet\\_biologics/publications](https://www.aphis.usda.gov/animal_health/vet_biologics/publications) (accessed 4 July 2022). To determine the correct medium volume to negate antimicrobial activity, 100 CFU of the control microorganisms listed in Table 2 are used. If the test sample contains merthiolate as a preservative, FTM is used in test vessels incubated at both 30–35°C and 20–25°C. Growth should be clearly visible after an appropriate incubation time (see Section I.2.1.3 *Growth promotion and test interference*). If the test sample is a killed biological without merthiolate, or a live bacterial biological, FTM is used at 30–35°C and SCDM at 20–25°C. If the test sample is a live viral biological, SCDM is used at both incubation temperatures. If the inactivated bacterial vaccine is a clostridial biological, or contains a clostridial component, the use of FTM with 0.5% added beef extract (FTMB) in place of FTM is preferred. It may also be desirable to use both FTM and SCDM for all tests.

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**Table 2. Some American Type Culture Collection<sup>1</sup> strains with their respective medium and incubation conditions**

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Medium	Test microorganism	Incubation	
		Temperature (°C)	Conditions
FTM	<i>Bacillus subtilis</i> ATCC # 6633	30–35	Aerobic
FTM	<i>Candida krusei</i> ATCC # 6258	20–25	Aerobic
SCDM	<i>Bacillus subtilis</i> ATCC # 6633	30–35	Aerobic
SCDM	<i>Candida krusei</i> ATCC # 6258	20–25	Aerobic
FTMB	<i>Clostridium sporogenes</i> ATCC # 11437	30–35	Anaerobic
FTMB	<i>Staphylococcus aureus</i> ATCC #6538	30–35	Aerobic

348

For both membrane filtration and direct inoculation sterility tests, all media are incubated for no fewer than 14 days. At intervals during incubation, and after 14 days' incubation, the test vessels are examined for evidence of microbial growth. Microbial growth should be confirmed by subculture and Gram stain.

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<sup>1</sup> American Type Culture Collection, 10801 University Boulevard, Manassas, Virginia 20110-2209, USA.

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351 **2.1.3. Example of growth promotion and test interference**

352 The sterility of the media should be confirmed by incubating representative containers at the  
353 appropriate temperature for the length of time specified for each test.

354 The ability of the culture media to support growth in the presence and absence of product, product  
355 components, cells, seeds, or other test material should be validated for each product to be tested,  
356 and for each new batch or lot of culture media for example as outlined in 9CFR 113.25(b). Detailed  
357 testing procedures can be found for example in USDA SAMs 900-902, See USDA APHIS |  
358 Supplemental Assay Methods - 900 Series (accessed 22 July 2023)  
359 [https://www.aphis.usda.gov/animal\\_health/vet\\_biologics/publications\\_\(accessed 4 July 2022\)](https://www.aphis.usda.gov/animal_health/vet_biologics/publications_(accessed 4 July 2022)).

360 To test for ability to support growth in the absence of the test material, media should be inoculated  
361 with 10–100 viable control organisms of the suggested ATCC strains listed in Table 2 and  
362 incubated according to the conditions specified.

363 To test for ability of the culture media to support growth in the presence of the test material,  
364 containers should be inoculated simultaneously with both the test material and 10–100 viable  
365 control organisms. The number of containers used should be at least one-half the number used  
366 to test the product or product component. The test media are satisfactory if clear evidence of  
367 growth of the control organisms appears in all inoculated media containers within 7 days. In the  
368 event that growth is evident, the organism should be identified to confirm that it is the organism  
369 originally added to the medium. The sterility test is considered invalid if any of the media show  
370 inadequate growth response, or if the organism recovered, is not the organism used to inoculate  
371 the material.

372 If the material being tested renders the medium turbid so that the presence or absence of  
373 microbial growth cannot be readily determined by visual examination, 14 days after the beginning  
374 of incubation transfer portions (each not less than 1 ml) of the medium to fresh vessels of the  
375 same medium and then incubate the original and transfer vessels for not less than 4 days.

376 **~~2.2. General procedure for testing live viral vaccines produced in eggs and administered~~**  
377 **~~through drinking water, spray, or skin scarification for the presence of bacteria and fungi~~**

378 ~~Each batch of final container biological should have an average contamination of not more than one bacterial~~  
379 ~~or fungal colony per dose for veterinary vaccines. From each container sample, each of two Petri dishes are~~  
380 ~~inoculated with vaccine equal to ten doses if the vaccine is recommended for poultry, or one dose if~~  
381 ~~recommended for other animals. To each plate 20 ml of brain heart infusion agar are added containing 0.007~~  
382 ~~IU (International Units) of penicillinase per ml. One plate should be incubated at 30–35°C for 7 days and the~~  
383 ~~other at 20–25°C for 14 days. Colony counts are made at the end of each incubation period. An average colony~~  
384 ~~count of all the plates representing a batch should be made for each incubation condition. If the average count~~  
385 ~~at either incubation condition exceeds one colony per dose in the initial test, one retest to rule out faulty~~  
386 ~~technique may be conducted using double the number of unopened final containers. If the average count at~~  
387 ~~either incubation condition of the final test for a batch exceeds one colony per dose, the batch of vaccine should~~  
388 ~~be considered unsatisfactory.~~

389 **2.32. Example of general procedure for testing seed lots of bacteria and live bacterial**  
390 **biologicals for purity**

391 Each seed lot of bacteria or batch of live bacterial biological should be tested for purity by inoculation of  
392 SCDM, which is incubated at 20–25°C for 14 days, and FTM, which is incubated at 30–35°C for 14 days.  
393 Using good practices in sterile technique to avoid laboratory contamination, a sterile pipette or syringe  
394 and needle is used to aseptically transfer the quantity of biological directly into the two types of culture  
395 medium. The minimum ratio of inoculum to culture medium is 1/15. Both positive and negative controls  
396 are set up as well.

397 If the inoculum or growth of the bacterial vaccine renders the medium turbid so that the absence of  
398 atypical microbial growth cannot be determined by visual examination, subcultures should be made from  
399 all turbid tubes on day 3 through until day 11. Subculturing is done by transferring 0.1–1.0 ml to  
400 differential broths and agar and incubating for the balance of the 14-day period. Microscopic examination  
401 by Gram stain should also be done.

402 If no atypical growth is found in any of the test vessels when compared with a positive control included  
403 in the test, the lot of biological may be considered satisfactory for purity. If atypical growth is found but

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404 it can be demonstrated by a negative control that the media or technique were faulty, then the first test  
405 ~~may-should~~ be repeated. If atypical growth is found but there is no evidence invalidating the test, then  
406 a retest ~~may-should~~ be conducted. Twice the number of biological containers and test vessels of the  
407 first test are used in the retest. If no atypical growth is found in the retest, the biological could be  
408 considered to be satisfactory for purity but the results from both the initial and retest should be reported  
409 for assessment by the individual countries relevant regulatory agency if the laboratory is sure that the  
410 first test result was not due to in-laboratory contamination. If atypical growth is found in any of the retest  
411 vessels, the biological is considered to be unsatisfactory for purity. If, however, it can be demonstrated  
412 by controls that the media or technique of the retest were faulty, then the retest ~~may-should~~ be repeated.

413 **2.43. ~~————~~ An Example of a specific test procedure for exclusion of *Brucella* sp. including  
414 *B. abortus* (where general testing is not sufficient) for detection of *Brucella abortus***

415 It should be confirmed that each batch of culture medium supports the growth of *B. abortus* by  
416 inoculating plates and flasks of biphasic medium with a known number of cells (around 100) of the  
417 fastidious *B. abortus* biovar 2. If the media supports the growth of this biotype it will support all other  
418 biovars.

419 Inoculate 1.0 ml of prepared master or working ~~viral~~ live agent or cell seed material (not containing  
420 antibiotics) by inoculating 50 µl of the test product into each of 10 flasks containing biphasic medium.  
421 At the same time 10 plates of serum dextrose agar (SDA) are inoculated with 50 µl of inoculum and  
422 spread with a sterile bent glass Pasteur pipette or hockey stick. An un-inoculated serum dextrose agar  
423 plate and a biphasic flask are also set up at the same time as negative controls.

424 For assessment of inhibitory substances 50 µl of previously prepared master or working viral or cell seed  
425 material and 10–100 CFU of *B. abortus* are inoculated on to duplicate SDA plates. Positive controls are  
426 prepared by inoculating 10–100 CFU of *B. abortus* on to duplicate SDA plates.

427 All plates and flasks are incubated at 37°C in a 5–10% CO<sub>2</sub> environment. Plates are incubated with the  
428 agar uppermost and flasks with the agar slope vertical. Flasks are incubated with the cap loose.

429 Plates are checked for growth of colonies at days 4 and 8 of incubation. The biphasic medium is  
430 examined every 4 to 7 days for 28 days. After each examination of the flasks, they are tilted so that the  
431 liquid phase runs over the solid phase, then righted and returned to the incubator.

432 During the incubation period, SDA plates with positive control and test material are visually compared  
433 with plates with the positive control only and if there is no inhibition of growth of the organism in the  
434 presence of the test material, the interference testing test is successful, and testing can be assured to  
435 be sensitive.

436 Any signs of growth of suspicious contaminating microorganisms on SDA plates, cloudiness or colonies  
437 in biphasic flasks require follow-up testing by PCR to confirm whether *B. abortus* is present.

438 **2.54. ~~————~~ An Example of a general procedure for detection of *Salmonella* contamination**

439 Each batch of ~~live virus~~ biological reagents made in eggs should be free from contamination with  
440 *Salmonella*. This testing must be done before bacteriostatic or bactericidal agents are added. Five  
441 samples of each batch should be tested; 5 ml or one-half of the container contents, whichever is the  
442 lesser, of the sample should be used to inoculate 100 ml of tryptose broth and tetrathionate broth. The  
443 inoculated broths should be incubated for 18–24 hours at 35–37°C. Transfers from these broths should  
444 be made on to MacConkey and *Salmonella-Shigella* agar, incubated for 18–24 hours, and examined. If  
445 no growth typical of *Salmonella* is noted, the agar plates should be incubated an additional 18–24 hours  
446 and again examined. If colonies typical of *Salmonella* are observed, further subculture on to suitable  
447 differential media should be made for positive identification. Sensitive PCR tests are available for the  
448 detection of *Salmonella* spp. in cultured material. If *Salmonella* is detected, the batch is determined to  
449 be unsatisfactory.

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### 450 3. Example of detection of *Mycoplasma* contamination

#### 451 3.1. An example of a general specific procedure for detection-exclusion of *Mycoplasma* 452 *mycoides* subsp. *mycoides* (where general testing is not sufficient)

453 Each batch of live viral vaccine, each lot of master seed virus (MSV), each lot of primary and master  
454 cell stock (MCS), and all ingredients of animal origin not steam-sterilised should be tested for the  
455 absence of mycoplasmas. Solid and liquid media that will support the growth of small numbers of test  
456 organisms, such as typical contaminating organisms *Acholeplasma laidlawii*, *Mycoplasma arginini*,  
457 *M. fermentans*, *M. hyorhinis*, *M. orale*, and *M. synoviae* should be used. The nutritive properties of the  
458 solid medium should be such that no fewer than 100 CFU should occur with each test organism when  
459 approximately 100–200 CFUs are inoculated per plate. An appropriate colour change should occur in  
460 the liquid media when approximately 20–40 CFUs of each test organism are inoculated. The ability of  
461 the culture media to support growth in the presence of product should be validated for each product to  
462 be tested, and for each new batch or lot of culture media.

463 One sample of each lot of vaccine, e.g. MSV or MCS, should be tested. Four plates of solid medium are  
464 inoculated with 0.25 ml of the sample being tested, and 10 ml of the sample inoculated into 100 ml of  
465 the liquid medium. An alternative is to inoculate each of the plates with 0.1 ml and to inoculate 100 ml  
466 of liquid medium with 1 ml of the sample being tested. Two plates are incubated at 35–37°C aerobically  
467 (an atmosphere of air containing 5–10% CO<sub>2</sub> and adequate humidity) and two plates are incubated  
468 anaerobically (an atmosphere of nitrogen containing 5–10% CO<sub>2</sub> and adequate humidity) for 14 days.  
469 On day 3 or day 4 after inoculation, 0.25 ml from the liquid media are subcultured on to two plates of  
470 solid media. One plate is incubated aerobically and the second anaerobically at 35–37°C for 14 days.  
471 The subculture procedure is repeated on day 6, 7, or 8 and again on day 13 or 14. An alternative method  
472 is to subculture on days 3, 5, 10, and 14 on to a plate of solid medium. All the subculture plates are  
473 incubated for 10 days except for the 14 day subculture, which is incubated for 14 days. Liquid media is  
474 observed every 2–3 days and, if any colour change occurs, has to be subcultured immediately.

#### 475 3.2. Interpretation of *Mycoplasma* test results

476 At the end of the incubation period (total 28 days), examine all the inoculated solid media microscopically  
477 for the presence of mycoplasma colonies. The test sample passes the test if the growth of mycoplasma  
478 colonies has occurred on the positive controls, and if growth has not occurred on any of the solid media  
479 inoculated with the test material. If at any stage of the test, more than one plate is contaminated with  
480 bacteria or fungi, or is broken, the test is invalid and should be repeated. If mycoplasma colonies are  
481 found on any agar plate, a suitable confirmatory test on the colonies should be conducted, such as PCR.  
482 Some mycoplasmas cannot be cultivated, in which case the MSV and MCS have to be tested using an  
483 indicator cell line such as Vero cells, DNA staining, or PCR methods.

484 Further detailed procedures can be found in Veterinary Medicinal Products, VICH GL34:  
485 [http://www.ema.europa.eu/docs/en\\_GB/document\\_library/Scientific\\_guideline/2013/03/WC500140352-](http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2013/03/WC500140352.pdf)  
486 [pdf](http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2013/03/WC500140352.pdf)

487 Prior to beginning testing it is necessary to determine that each batch of media promotes the growth of  
488 *M. mycoides* subsp. *mycoides* SC (*MmmSC*) type strain PG1. General mycoplasma broth and agar are  
489 used but contain porcine serum as a supplement. Each batch of broth and agar is inoculated with 10–  
490 100 CFU of *MmmSC*. The solid medium is suitable if adequate growth of *MmmSC* is found after 3–  
491 7 days' incubation at 37°C in 5–10% CO<sub>2</sub>. The liquid medium is suitable if the growth on the agar plates  
492 subcultured from the broth is found by at least the first subculture. If reduced growth occurs another  
493 batch of media should be obtained and retested.

494 1 ml of cell or virus seed to be tested is inoculated into 9 ml of the liquid medium and 100 µl on to solid  
495 mycoplasma agar. The volume of the product is inoculated so that it is not more than 10% of the volume  
496 of the medium. The liquid medium is incubated at 37°C in 5–10% CO<sub>2</sub> and 100 µl of broth is subcultured  
497 on to agar at days 7, 14 and 21. The agar plates are incubated at 37°C in 5–10% CO<sub>2</sub> for no fewer than  
498 14 days, except those corresponding to day 21 subculture, which are incubated for 7 days. An un-  
499 inoculated mycoplasma broth and agar plate are incubated as negative controls. For assessment of  
500 inhibitory substances, inoculate 1 ml of sample to be tested into 9 ml of the liquid medium and 100 µl on  
501 to solid medium and add 10–100 CFU of *MmmSC* to each. Prepare positive control by inoculating 9 ml  
502 of mycoplasma broth and a mycoplasma agar plate with 10–100 CFU of *MmmSC*. Incubate as for  
503 samples and negative controls.

504 During incubation time, visually compare the broth of the positive control with sample present with the  
505 positive control broth and, if there is no inhibition of the organism either the product possesses no  
506 antimicrobial activity under the conditions of the test, or such activity has been satisfactorily eliminated  
507 by dilution. If no growth or reduced growth of *MmmSC* is seen in the liquid and solid medium with test  
508 sample when compared with the positive control, the product possesses antimicrobial activity, and the  
509 test is not satisfactory. Modifications of the conditions to eliminate the antimicrobial activity and repeat  
510 test are required.

511 If antimicrobial activity is present it is necessary to dilute the test product further. Repeat the test above  
512 using 1.0 ml of sample in 39 ml of mycoplasma broth and then inoculate with 10–100 CFU of *MmmSC*  
513 and incubate as above. All broths and plates are examined for obvious evidence of growth. Evidence of  
514 growth can be determined by comparing the test culture with the negative control, the positive control,  
515 and the inhibition control.

516 If evidence of microbial growth is found in the test samples the contaminating bacterium will be identified and  
517 confirmed as *MmmSC* by specific PCR assay.

### 518 **3.2 General testing for exclusion of *Mycoplasma* sp.**

519 General testing for exclusion of *Mycoplasma* sp. that are less fastidious may require up to 28 days in  
520 culture, using general mycoplasma media. Some mycoplasmas cannot be cultivated, in which case the  
521 live biological sample will have to be tested using an indicator cell line such as Vero cells, DNA staining,  
522 or PCR methods.

523 Further detailed procedures can be found in Veterinary Medicinal Products, VICH GL34:  
524 [http://www.ema.europa.eu/docs/en\\_GB/document\\_library/Scientific\\_guideline/2013/03/WC500140352.pdf](http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2013/03/WC500140352.pdf)  
525 [https://www.ema.europa.eu/en/vich-gl34-biologicals-testing-detection-mycoplasma-contamination-scientific-](https://www.ema.europa.eu/en/vich-gl34-biologicals-testing-detection-mycoplasma-contamination-scientific-guideline)  
526 [guideline](https://www.ema.europa.eu/en/vich-gl34-biologicals-testing-detection-mycoplasma-contamination-scientific-guideline)

527 and

528 USDA SAM 910: [https://www.aphis.usda.gov/animal\\_health/vet\\_biologics/publications/910.pdf](https://www.aphis.usda.gov/animal_health/vet_biologics/publications/910.pdf), (both accessed  
529 25 July 2023).

## 530 **4. Example of detection of rickettsia and protozoa**

531 There are no general test procedures for exclusion of rickettsia or protozoa. Procedures to exclude specific agents of  
532 concern such as *Coxiella burnetti* (Q fever), *Ehrlichia canis*, *Trypanosoma evansi* and *Babesia caballi* can be found for  
533 example, in the Review of Published Tests to detect pathogens in veterinary vaccines Intended for Importation into  
534 Australia (Australian Government Department of Agriculture [of Australia], Forest and Fisheries (2013)). The review is  
535 based on the reading and interpretation of applicable published papers from reputable journals and are regarded as  
536 examples of sensitive methods for detection of specified agents.

### 537 **4.1. An Example of a specific test protocol based on published methods for exclusion of *Babesia*** 538 ***caballi* and *Theileria equi***

539 *Babesia caballi* and *Theileria equi* can be cultured *in vitro* in 10% equine red blood cells (RBC) in supportive  
540 medium supplemented with 40% horse serum and in a micro-aerophilic environment. Culture isolation of *T. equi*  
541 is more sensitive than for *B. caballi*. Giemsa-stained blood smears are prepared from cultures daily for 7 days  
542 (Avarzed *et al.*, 1997; Ikadai *et al.*, 2001). *Babesia caballi* is characterised by paired merozoites connected at  
543 one end. *Theileria equi* is characterised by a tetrad formation of merozoites or 'Maltese cross'. Confirmation of  
544 the diagnosis is by PCR (see Chapter 2.5.8 *Equine piroplasmosis*). Molecular diagnosis is recommended for  
545 the testing of biological products that do not contain whole blood or organs. Molecular diagnosis by PCR or  
546 loop-mediated isothermal amplification (LAMP) assay are the most sensitive and specific testing methods for  
547 detection of the pathogens of equine *piroplasmosis* (Alhassan *et al.*, 2007).

## 548 **5. Example of detection of virus viruses in biological materials**

549 In brief, general testing usually includes the use of continuous and primary cell lines of the source species, e.g. cells of  
550 known susceptibility to the likely viral contaminants, which are inoculated for usually a period of up to 3–4 weeks with  
551 weekly subcultures. Virus seeds also require testing on a primary cell line of the species in which the final product is  
552 intended. At Day 21 or 28, assessment of the monolayers is done using H&E-appropriate histology staining procedures to

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553 assess CPE<sub>2</sub> and haemadsorption with guinea-pig and chicken RBC to assess the presence of haemadsorbing agents.  
554 Note that general testing is useful as a screening tool though not sufficiently sensitive enough to detect all viruses of  
555 concern to all countries.

556 Specific testing requires test material to be inoculated on to sensitive, susceptible cells lines for the virus to be excluded;  
557 the amplification process in cell culture is usually up to 28 days but depending ~~of on~~ the virus<sub>2</sub> may require longer culturing  
558 times. Detection of specific viral contaminants is by recognition of CPE in conjunction with more sensitive antigen detection  
559 or molecular tests such as FAT and PCR and ELISA after the amplification process in cell culture is completed.

560 All testing using cell lines to amplify for target viruses is contingent on the sensitivity of the cells for the target agent and  
561 the ability to recognise the presence of the agent in the cells. The quality, characteristics<sub>2</sub> and virus permissibility profile of  
562 cell lines in use should be determined as fit for purpose and appropriately maintained. ~~Positive and negative controls should  
563 be used at all passages of cell culture to determine sensitivity and specificity. Interference testing should be performed at  
564 first pass to ensure that the test sample does not inhibit the growth of the virus being excluded for.~~

#### 565 **5.1. An example of general testing for the exclusion of viruses from virus and cell seed stocks** 566 **used in production of veterinary vaccines**

567 ~~If the test virus inoculum is cytopathogenic. If a virus seed is known to cause cytopathic effect (CPE) in a permissive cell  
568 line, the effect must be specifically neutralised without affecting the likelihood of isolation of the target agent. For affected  
569 cell type, 1 ml of the test master (or working) virus seed (MVS) is thawed or reconstituted and neutralised with the addition  
570 of 1 ml mono-specific antiserum. The serum must be shown to be free from antibodies against any agents for which the  
571 test is intended to detect. Antiserum ~~must should~~ be tested for nonspecific inhibiting affects. For a general test, this can be  
572 difficult to ascertain. Serum should be of sufficiently high titre to neutralise the seed virus effectively with the use of an  
573 approximately equal volume or less of serum. A microplate block titration is ~~used useful~~ to determine the titre amount of  
574 the antiserum required to neutralise ~~the MVS a known amount of concern. The antiserum CPE causing virus seed. This is  
575 allowed to neutralise the MVS at 37°C for 1 hour. The MVS and antiserum mixture is then inoculated on to a 75 cm<sup>2</sup> flask  
576 with appropriate cells. If the MVS is known to be high titred or difficult to neutralise, the blocking antiserum can be added  
577 to the growth medium at a final concentration done in the normal conditions required of 1–2% each test system (e.g. time,  
578 temperature, cell type etc.)~~.~~

579 ~~Master cell-~~If a virus seed is known to be high-titred or difficult to neutralise, antiserum can be added to the growth medium  
580 in a test system at a final concentration of 1–2%.

581 Cell seed stocks do not require a neutralisation process.

#### 582 **5.1. Example of general testing procedures for the exclusion of viruses from virus and cell seed** 583 **stocks used in production of veterinary vaccines**

##### 584 **5.1.1 Example of amplification in cell culture**

585 ~~The cells should be passaged weekly up to a 28 day period. Continuous and primary, 75 cm<sup>2</sup> area  
586 monolayers of the source species (and intended species as applicable) are infected with 1 ml of seed  
587 stocks and passaged weekly for ~~between up to 21–28 days. Depending on the procedure followed,  
588 monolayers can be subcultured between passes or freeze/thawed to disrupt cells. Negative and positive  
589 controls should be also set up at each pass using the same cell population. Certain relevant viruses may  
590 be selected as indicators for sensitivity and interference (positive controls) but these will not provide  
591 validation for the broader range of agents targeted in general testing. The final culture is examined for  
592 cytopathology and haemadsorption.~~~~

##### 593 **5.1.2 Example of general detection procedures: cytopathology**

594 May–Grünwald–Giemsa or H&E staining procedures are used to assess for cytopathological changes  
595 associated with virus growth. Monolayers must have a surface area of at least 6 cm<sup>2</sup> and can be prepared  
596 on appropriate chambered tissue culture slides and incubated for 7 days. The plastic wells of the slides  
597 are removed leaving the rubber gasket attached to the slide. The slides are rinsed in Dulbecco's  
598 phosphate buffered saline (PBS), fixed in acetone, methanol or formalin depending on the stain used  
599 and placed on a staining rack. For May–Grünwald–Giemsa staining: the slides are stained for 15 minutes  
600 at room temperature with May–Grünwald stain diluted 1/5 with absolute methanol. The May–Grünwald  
601 stain is removed by inverting the slides. The slides are then stained for 20 minutes with Giemsa stain  
602 diluted 1/15 in deionised water. The Giemsa stain is removed by inverting the slides and rinsing them in  
603 deionised water for 10–20 seconds. The slides are air-dried and mounted with a coverslip using paraffin  
604 oil. The May–Grünwald–Giemsa stain differentially stains ribonucleoprotein (RNP); DNA RNP stains red-  
605 purple, while RNA RNP stains blue. The monolayers are examined with a conventional microscope for



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606 the presence of inclusion bodies, an abnormal number of giant cells, or other cytopathology attributable  
607 to a viral contaminant of the test product. The inoculated monolayers are compared with suitable control  
608 non-inoculated monolayers. If specific cytopathology attributable to an extraneous virus is found, results  
609 are reported, and additional specific testing may be conducted.

### 610 **5.1.3 Example of general detection procedures: haemadsorption**

611 Testing for haemadsorption ~~uses~~ requires the use of 75 cm<sup>2</sup> area monolayers established in tissue  
612 culture flasks after the 28-day passage period described above. Guinea-pig, chicken, and any other  
613 blood for use in this assay is collected in an equal volume of Alsever's solution and may be stored at 4°C  
614 for up to 7 days. Immediately prior to use, the stored erythrocytes are again washed by adding 5 ml of  
615 blood in Alsever's solution to 45 ml of calcium and magnesium-free PBS (PBSA) and centrifuging in a  
616 50 ml centrifuge tube at 500 g for 10 minutes. The supernatant is aspirated, and the erythrocytes are  
617 suspended in PBSA and re-centrifuged. This washing procedure is repeated at least twice until the  
618 supernatant is clear. Erythrocytes from each species are combined by adding 0.1 ml of each type of  
619 packed blood cells to 100 ml of PBSA. The erythrocytes from different species may be kept separate or  
620 combined, as desired. To each flask, add 5 ml of the erythrocyte suspension, and incubate the flasks at  
621 4°C for 30 minutes. Monolayers are washed twice with PBSA and examined for haemadsorption. If no  
622 haemadsorption is apparent, 5 ml of ~~the fresh~~ erythrocyte suspension is added to each flask; the flasks  
623 are incubated at 20–25°C (room temperature) for 30 minutes, rinsed as before, and examined for  
624 haemadsorption. Separate flasks may be used for each incubation temperature if desired. Monolayers  
625 are examined for the presence of haemadsorption using an illuminated light box and microscopically.  
626 Non-inoculated monolayers are used as negative controls. The PBSA and fresh erythrocytes should  
627 prevent most nonspecific haemadsorption from occurring. If specific haemadsorption attributable to an  
628 extraneous agent is found, results are reported, and additional specific testing may be conducted.

629 ~~Specific testing requires specialised test procedures that are sensitive to amplifying a particular agent in culture~~  
630 ~~and then detection of that agent by means of fluorescence, antigen capture ELISA or PCR; whichever is more~~  
631 ~~sensitive. Specific testing is usually required when general procedures are not adequate for effective exclusion~~  
632 ~~of more fastidious, viruses. Some examples are listed in Table 1.~~

## 633 **5.2. An Examples of specific virus agent exclusion testing from of biologicals used in the** 634 **production of veterinary vaccines**

### 635 **5.2.1. Example of porcine epidemic diarrhoea virus (PEDV)**

636 Trypsin presence is required at inoculation and in the culture medium for isolation of porcine epidemic  
637 diarrhoea virus (PEDV) in Vero cells (CCL81, ATCC) to ensure the virus can enter host cells. Just  
638 confluent monolayers (100%) are required; ~~as~~ under confluent monolayers (≤90%) are more sensitive to  
639 the presence of trypsin ~~and will be destroyed well before the 7 days required for each passage in culture~~.  
640 An over confluent or aging monolayer will not be sensitive for growth of PEDV. Maintenance media (MM)  
641 formulation consists of Earle's MEM (minimal essential medium) (with 5.6 M HEPES [N-2-  
642 hydroxyethylpiperazine, N-2-ethanesulphonic acid] and glutamine) + 0.3% Tryptose phosphate broth,  
643 0.02% yeast extract and 4 µg/ml TPCK treated trypsin. The addition of the trypsin into ~~the~~ MM should  
644 occur on the day the media is to be used.

645 Prior to inoculation, confluent 75 cm<sup>2</sup> monolayers are washed twice with ~~the MM (with trypsin added)~~  
646 to remove growth media containing FCS. Virus or cell seed (1 ml) is added with 1 ml of MM to each  
647 monolayer; incubate at 37°C for 2 hours, then add 30 ml/flask of MM. Negative control monolayers of  
648 the same size are set up prior to inoculation of test material. Positive and interference controls are set  
649 up last, and where possible, in a separate laboratory area to avoid contamination. Assessment for  
650 sensitivity and interfering substances requires ~~assessment use~~ of PEDV reference virus of known titre.  
651 A control for interference using co-inoculation of test sample and PEDV needs only to be set up on the  
652 first pass. Positive controls ~~must~~ should be set up at every pass to ensure each monolayer used gives  
653 expected sensitivity. PEDV virus is titrated in log dilutions starting at 10<sup>-1</sup> to 10<sup>-6</sup> in MM (depending ~~of on~~  
654 the endpoint titre of reference virus) in duplicate rows of 6 wells of a 24-well tissue culture plate. For the  
655 interference test, PEDV is titrated in the same dilution series but using MM spiked with a 10% volume of  
656 test material. Decant off the growth media and discard. Wash plates to ensure no FCS is present. Two  
657 washes using approximately 400 µl/well MM (with trypsin added) are sufficient.

658 Add 100 µl of diluted virus on to each of two duplicate wells. Rock inoculated plates to distribute the  
659 inoculum evenly over the surface of the monolayer. Incubate at 37°C with 5% CO<sub>2</sub> for 2 hours then add  
660 a further 1 ml volumes/well of MM.

661 After 7 days, 75 cm<sup>2</sup> monolayers have cells disrupted using two freeze–thaw cycles at –80°C. Positive  
662 control plates are read for end-point titres, and these are compared with virus in the presence of test  
663 material to ensure titres are comparable and interference has not occurred. Freeze–thaw lysates are  
664 clarified at 2000 g for 5 minutes and re-passed on to newly formed monolayers as for the first passage.  
665 Passages are repeated until a total of four passages are completed at which point cell lysates are  
666 assessed by PCR for detection of PEDV and day 7 monolayers in 24-well plates are fixed and stained  
667 by IFA for FAT. If a seed virus is to be tested and requires neutralisation using antiserum, extra care in  
668 the isolation of PEDV needs to be considered. Trypsin is rendered inactive in the presence of serum  
669 proteins and without trypsin present, PEDV is unable to grow in cell culture grows poorly, or not at all.  
670 Washing off the inoculum with two MM washes is required after an extended adsorption time of up to 4  
671 hours to ensure acceptable sensitivity.

## 672 **J.H. INFORMATION TO BE SUBMITTED WHEN** 673 **APPLYING FOR AN IMPORT LICENCE**

674 When undertaking risk analysis for biologicals, Veterinary Authorities should follow the *Terrestrial Code Manual*, and the  
675 manufacturer should follow the requirements of the importing country. Requirements for each importing country should be  
676 accessible and published online. The manufacturer or the Veterinary Authority of the exporting country should make  
677 available detailed information, in confidence if as necessary, on the source of the materials used in the manufacture of the  
678 product (e.g. substrates). They should make available details of the method of manufacture (and where appropriate  
679 inactivation) of the substrates and component materials, the quality assurance procedures for each step in the process,  
680 final product testing regimes, and the pharmacopoeia with which the product must conform in the country of origin. They  
681 should also make available challenge organisms, their biotypes and reference sera, and other means of appropriate  
682 product testing.

683 For detailed examples of a risk-based assessment of veterinary biologicals for import into a country refer to:

- 684 • European Commission (2015). *The Rules Governing Medicinal Products in the European Union. Eudralex. Volume*  
685 6. Notice to applicants and regulatory guidelines for medicinal products for veterinary use.
- 686 • Department of Agriculture, Forest and Fisheries of Australia (2021b). *Live veterinary vaccines Summary of*  
687 information required for biosecurity risk assessment, Version 6 and *Inactivated veterinary vaccines, Version 8.*
- 688 • Outline of the Regulatory System of Veterinary Drugs in Japan (2015) *Assurance of the Quality, Efficacy, and Safety*  
689 Based on the Law for Ensuring the Quality, Efficacy, and Safety of Drugs and Medical Devices.
- 690 • Ministry of Agriculture and Rural Affairs, China (People's Rep. of), *Regulations on the Administration of Veterinary*  
691 drugs (revised in 2020).

692 When applying for an import licence other regulatory requirements may need to be addressed depending on the type of  
693 sample and if the sample needs to be shipped out of country to a testing laboratory. For example, cell seeds may come  
694 under certain requirements for permits such as the Convention for International Trade in Endangered Species of Wild  
695 Fauna and Flora (CITES), where a cell line is derived from an endangered species, e.g. the cell line and its derivatives.  
696 Applying for such a permit is time consuming and requires input from both the exporting and importing country.

697 Genetically modified organisms (GMOs) are becoming more frequent in use with changes in manufacturing technologies  
698 and specialised, time-consuming procedures need to be in place. A laboratory that accepts a GMO product for testing shall  
699 follow the procedures of the Office of the Gene Regulator (OGTR) to allow the GMO to be dealt with.

## 700 **I. RISK ANALYSIS PROCESS**

701 Risk analysis should be as objective and transparent as possible and should be performed in accordance with Section 2  
702 of the *Terrestrial Code*, and certification in line with Section 5 of the *Terrestrial Code*. Of necessity, assessment of the  
703 country and commodity factors and risk reduction measures will be based largely on manufacturers' data. These data  
704 depend on quality assurance at all stages of manufacture, rather than on testing of the final product alone.

705 Domestic exposure may be influenced by the approved usage of the product. Veterinary Authorities may place limits on  
706 usage of some products (e.g. restricting usage to institutions of appropriate biosecurity).

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## 4. BIOCONTAINMENT

707

708 Suitable biocontainment may be necessary for many forms of biologicals. In particular, the importation of exotic micro-  
709 organisms should be carried out in accordance with Chapter 1.1.4 *Biosafety and biosecurity: standard for managing*  
710 *biological risk in the veterinary laboratory and animal facilities*.

711 Laboratories using high risk agents should have well researched and documented risk assessments in place prior to  
712 working with such agents to ensure the safety of their staff and laboratory.

713

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## FURTHER READING

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**NB:** FIRST ADOPTED IN 1989. MOST RECENT UPDATES ADOPTED IN 2017.

REUNIÓN DE LA COMISIÓN DE NORMAS BIOLÓGICAS

París, 5–9 de febrero de 2024

CHAPTER 2.2.4.

MEASUREMENT UNCERTAINTY

INTRODUCTION

*The WOAH Validation Recommendations provide detailed information and examples in support of the ~~WOAH Validation Standard that is published as Chapter 1.1.6 Principles and methods of Validation of diagnostic assays for infectious diseases of terrestrial animals this Terrestrial Manual, or Chapter 1.1.2 of the Aquatic Manual. The Term “WOAH Validation Standard” in this chapter should be taken as referring to those chapters.~~*

*Estimation of measurement uncertainty (MU), ~~sometimes termed measurement imprecision,~~ is a requirement for testing laboratories based on international quality standards such as ISO/IEC 17025-2005, 2017 General requirements for the competence of testing and calibration laboratories (ISO/IEC 17025). The measurement process for detection of an analyte in a diagnostic sample is not entirely reproducible and hence there is no exact value that can be associated with the measured analyte. Therefore, the result is most accurately expressed as an estimate ~~together~~ with an associated ~~level of imprecision level~~. This imprecision is the measurement uncertainty (MU). MU is limited to the measurement process of quantitative tests. The approach described here is known as “top-down” or “control sample” because it uses a weak positive control sample and expresses the MU result at the cut-off diagnostic threshold, where it most matters. It is not a question of whether the measurement is appropriate and fit for whatever use to which it may be applied. It is not an alternative to test validation but is rightly considered a component of that process (see ~~the WOAH Validation Standard, chapter 1.1.6 Section B.1.1 Repeatability~~).*

A. THE NECESSITY OF DETERMINING MU

To assure compliance with ISO/IEC 17025-2005-2017 requirements, national accreditation bodies for diagnostic testing laboratories require laboratories to calculate MU estimates for accredited test methods that produce quantitative results, e.g. optical densities (OD), percentage of positivity or inhibition (PP, PI), titres, cycle threshold (CT) values, etc. This includes tests where numeric results are calculated and then ~~are~~ expressed as a positive or negative result at a cut-off value. For the purpose of estimating MU in serology and reverse transcriptase polymerase chain reaction (RT-PCR), suitable statistical measures are mean target values  $\pm 2$  standard deviations (SD), which is an approximately equal to a 95% confidence reference interval (C-RI), relative standard deviation (RSD = SD / mean of replicates) and coefficient of variation (CV = RSD  $\times$  100%). Examples provided below assume normal distribution of data. Alternative methods are available that are less sensitive to both that assumption and to the presence of outliers; they are not illustrated here The concept of MU does not apply to strictly binary (qualitative) results (positive or negative).

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## 32 1. Samples for use in determining MU

33 Repeatability is the level of agreement between results of replicates of a sample both within and between runs of the same  
34 test method in a given laboratory. During assay development, repeatability is estimated by evaluating variation in results  
35 of independent replicates from a minimum of three (preferably five) samples representing analyte activity within the  
36 operating range of the assay (see ~~the WOAHS Validation Standard, Chapter 1.1.6 Validation of diagnostic assays for~~  
37 ~~infectious diseases of terrestrial animals~~, Sections A.2.5 *Robustness* and B.1.1 *Repeatability*, and Chapter 2.2.6 *Selection*  
38 ~~and use of reference samples and panels~~, Section 3.4 ~~A.4.2~~). Typically, the variation in replicate results is expressed as  
39 RSD or CV. The significant feature is that repeatability studies can be used to define the expected precision of the assay  
40 in the detection of a range of analyte concentrations.

41 The use of internal quality or process controls over a range of expected results has become part of daily quality control  
42 and quality assurance operations of accredited facilities (see ~~the WOAHS Validation Standard, chapter 1.1.6~~, Sections A.2.6  
43 *Calibration of the assay to standard reagents* and B.5.1 *Monitoring the assay*, and Chapter 2.2.6, Section 4.4 ~~C.1~~). These  
44 results provide a continuous monitor relative to different aspects of repeatability, e.g. intra- and inter-assay variation, intra-  
45 and inter-operator variation and intra- and inter-batch variation, which, when subjected to statistical analysis, provide an  
46 expression of the level of robustness (precision) of a test procedure. The monitoring of assay quality control parameters  
47 for repeatability provides evidence that the assay is or is not performing as expected. For control samples to provide valid  
48 inferences about assay precision, they should be treated in exactly the same way as test samples in each run of the assay,  
49 e.g. including sample preparation such as extraction steps or dilution of serum samples for an antibody enzyme-linked  
50 immunosorbent assay (ELISA).

51 The variation of the results for control samples can also be used as an estimate of those combined sources of uncertainty  
52 and is called the “top-down” approach. This approach recognises that the components of precision will be manifest in the  
53 ultimate measurement. So monitoring the precision of the measurement over time will effectively show the combined effects  
54 of the imprecision associated with component steps.

55 The imprecision or uncertainty of the measurement process associated with a test result becomes increasingly more  
56 important the closer the test value is to the diagnostic cut-off value. This is because an interpretation is made relative to  
57 the assay threshold regarding the status of the test result as positive, negative, or inconclusive (as will be described in the  
58 following example). In this context, low-weak positive samples, like those used in repeatability studies or as the low-weak  
59 positive control, are most appropriate for estimation of MU. The rationale being that MU, which is a function of assay  
60 precision, is most critical at decision-making points (i.e. thresholds or cut-offs), which are usually near the lower limit of  
61 detection for the assay. In this chapter, the application of MU with respect to cut-off (threshold) values, whether  
62 recommended by test-kit manufacturers or determined in the diagnostic laboratory, is described.

63 MU, using the top-down approach, ideally requires long-term accumulated data from a weak positive control sample after  
64 multiple test runs over time, with multiple operators and variable conditions. The examples given below are based on 10  
65 data points but higher numbers will increase robustness.

## 66 2. Example of MU calculations in ELISA serology

67 For most antibody detection tests, it is important to remember that the majority of tests are measurements of antibody  
68 activity relative to a threshold against which a dichotomous interpretation of positive or negative is applied. This is important  
69 because it helps to decide where application of MU is appropriate. In serology, uncertainty is frequently most relevant at  
70 the threshold between positive and negative determinations. Results falling into this zone are also described as  
71 intermediate, inconclusive, suspicious or equivocal (see ~~the WOAHS Validation Standard, chapter 1.1.6~~, Section B.2.4  
72 *Selection of a cut-off (threshold) value for classification of test results*).

73 A limited data set from a competitive ELISA for antibody to avian influenza virus is used as an example of a “top-down”  
74 approach for serology. A low-weak positive control sample was used to calculate MU at the cut-off level<sup>1</sup>.

### 75 2.1. Method of expression of MU

76 As the uncertainty is to be estimated at the threshold, which is not necessarily the reaction level of the low-weak  
77 positive control serum, the relative standard deviation (RSD), or coefficient of variation (CV), if expressed as a  
78 percentage, provides a convenient transformation:

$$\text{RSD (X)} = \text{SD (X)} / \text{mean (}\bar{X}\text{)}$$

---

<sup>1</sup> The Australian Government, Department of Agriculture, Fisheries and Forestry, has compiled worked examples for a number of  
diagnostic tests Available online at: [https://www.agriculture.gov.au/agriculture-](https://www.agriculture.gov.au/agriculture-land/animal/health/laboratories/tests/measurement-uncertainty)  
land/animal/health/laboratories/tests/measurement-uncertainty (accessed 22 June 2023)

79

X represents the set of replicates

80 To simplify assessment, the a suitably transformed result (such as sample-to-positive ratio, per cent inhibition  
81 or background-corrected optical density) is regarded as the assay output result, which is then averaged across  
82 the number of replicates ( $\bar{X}$ ). In the case of this example, a competitive ELISA, results are “normalised” (as  
83 defined in the WOH Validation Standard, chapter 1.1.6, Section A.2.7 ‘Normalising’ test results to a working  
84 standard) to a working standard by forming a ratio of all optical density (OD) values to the OD result of a non-  
85 reactive (negative) control ( $OD_N$ ). This ratio is subtracted from 1 to set the level of antibody activity on a positive  
86 correlation scale; the greater the level, the greater the calculated value. This adjusted value is expressed as a  
87 per cent and referred to as the percentage inhibition or PI value. So for the low-weak positive control serum  
88 ( $OD_{LW}$ ), the transformation to obtain the per cent inhibition values for the low-weak positive control ( $PI_{LW}$ ) is:

89 
$$PI_{LW} = 100 \times [1 - \{OD_{LW} / OD_N\}]$$

90 The relative standard deviation becomes:

91 
$$RSD (PI_{LW}) = SD (PI_{LW}) / \text{mean} (PI_{LW})$$

## 92 2.2. Example

93 A limited data set for the AI competitive ELISA example is shown below. In the experiment, the operator tested  
94 the low-weak positive control serum ten times in the same run. Ideally in the application of this “top down”  
95 method, a larger data set would be used, which would enable accounting for effects on precision resulting from  
96 changes in operator and assay components (other than only the control serum).

97 Table 1. Top-down or control sample approach for an influenza antibody C-ELISA

Test	PI (%)
1	56
2	56
3	61
4	64
5	51
6	49
7	59
8	70
9	55
10	42

98 Mean PI = 56.3; Std Dev (SD) = 7.9; Assays ( $n$ ) = 10

## 99 2.3. Calculating uncertainty

100 From the limited data set,

101 
$$RSD (PI_{LW}) = SD / \text{Mean} = 7.9 / 56.3 = 0.14 \text{ (or as coefficient of variation = 14\%)}$$

102 Expanded uncertainty (U) is the statistic defining the interval within which the value of the measure and is  
103 believed to lie within a specified level of confidence, usually 95%. Expanding the uncertainty is done by  
104 multiplying the RSD ( $PI_{LW}$ ) by a factor of 2; this allows the calculation of an approximate 95% confidence  
105 reference interval around the threshold value (in this case at PI = 50%), assuming normally distributed data. If  
106 data are not normally distributed they must be transformed to fit a normal distribution using a log scale.

107 
$$U (95\% \text{ C-R}) = 2 \times RSD = 0.28$$

108 This estimate can then be applied at the threshold level



109 95% **C-R**l =  $50 \pm (50 \times 0.28) = 50 \pm 14\%$

#### 110 2.4. Interpretation **of the results**

111 Any positive result (PI > 50%) that is less than 64% is not positive with 95% confidence. Similarly, a negative  
112 result (PI < 50%) that is higher or equal to a PI of 36 is not negative at the 95% confidence level. A sample with  
113 a PI between 36% and 64% is within the MU surrounding the threshold value, and thus its diagnostic status is  
114 less certain than those of samples with results further from that threshold. This zone of lower confidence may  
115 correlate with the “grey zone” or “inconclusive/suspect zone” for interpretation that should be established for all  
116 tests (Greiner *et al.*, 1995).

### 117 **3. Example of MU calculation in molecular tests**

#### 118 **3.1. Example**

119 For real-time PCRs, replicates of positive controls with their respective cycle threshold (Ct) values can be used  
120 to estimate MU using the top-down approach (Newberry & Colling, 2021). The method of expression follows the  
121 same formula as for the ELISA example above. This example uses data from replicate runs of a weak positive  
122 control sample (10 runs) of an equine influenza hydrolysis probe assay.

123 *Table 2. Top-down or control sample approach for an equine influenza TaqMan A assay*

<u>Test</u>	<u>Ct value</u>
<u>1</u>	<u>33.60</u>
<u>2</u>	<u>33.20</u>
<u>3</u>	<u>33.96</u>
<u>4</u>	<u>33.18</u>
<u>5</u>	<u>33.96</u>
<u>6</u>	<u>32.72</u>
<u>7</u>	<u>33.57</u>
<u>8</u>	<u>33.45</u>
<u>9</u>	<u>32.80</u>
<u>10</u>	<u>33.20</u>

124 Mean = 33.36; Std Dev (SD) = 0.43; Assay n=10

#### 125 **3.2. Calculating uncertainty**

126 From the limited data set,

127  $RSD (PI_{LW}) = SD/Mean = 0.43/33.36 = 0.0128$  (or as coefficient of variation = 1.28%)

128 Expanded uncertainty (U) is the statistic defining the interval within which the value of the measure and is  
129 believed to lie within a specified level of confidence, usually 95%. Expanding the uncertainty is done by  
130 multiplying the RSD (PI<sub>LW</sub>) by a factor of 2; this allows the calculation of an approximate 95% confidence interval  
131 around the threshold value (in this case at Ct value = 37), assuming normally distributed data.

132  $U (95\% \text{ C-R}l) = 2 \times RSD = 0.0255$

133 This estimate can then be applied at the threshold level

134  $95\% \text{ C-R}l = 37 \pm (37 \times 0.0255) = 37 \pm 0.94$

135 The mean cycle threshold (Ct) value after 10 runs is 33.36 and the standard deviation is 0.43. The relative  
136 standard deviation is 0.0128. The expanded uncertainty (95% **C-R**l) is 2 × the relative standard deviation =  
137 0.0255. Measurement of uncertainty (MU) is most relevant at the cut-off (Ct = 37) and can be applied by

138 multiplication ( $37 \times 0.0255 = 0.94$ ). Subtraction from the threshold ( $37 - 0.94$ ) provides the lower 95% confidence  
139 reference limit ( $Ct = 36.06$ ) and addition ( $37 + 0.94$ ) the upper 95% confidence reference limit ( $Ct = 37.94$ ).

### 140 **3.3. Interpretation of the results**

141 Any positive result ( $Ct < 37$ ) that is higher than 36  $Ct$  is not positive with 95% confidence. Similarly, any negative  
142 result ( $Ct > 37$ ) that is less than 38  $Ct$  is not negative with 95% confidence. A sample with a  $Ct$  between 36 and  
143 38 is within the MU surrounding the threshold value, and thus its diagnostic status is less certain than those of  
144 samples with results further from that threshold.

## 145 **B. OTHER APPLICATIONS**

146 The top-down approach should be broadly applicable for a range of diagnostic tests including molecular tests. For the  
147 calculation of tests using a typical two-fold dilution series for the positive control such as virus neutralisation, complement  
148 fixation and haemagglutination inhibition tests geometric mean titre (i.e. geomean and expanded [SD] of log base 2 titre  
149 values) of the positive control serum should be calculated. Relative standard deviations based on these log scale values  
150 may then be applied at the threshold (log base 2) titre, and finally transformed (by antilog) to represent the uncertainty at  
151 the threshold. However, in all cases, the approach assumes that the variance about the positive control used to estimate  
152 the RSD is proportionally similar at the point of application of the MU, for example at the threshold. If the RSD varies  
153 significantly over the measurement scale, the positive control serum used to estimate the MU at the threshold should be  
154 selected for an activity level close to that threshold. The Australian Government, Department of Agriculture, Fisheries and  
155 Water Resources Forestry, has compiled worked examples for a number of diagnostic tests (see footnote 1). (DAFF, 2010),  
156 which are available online at:

157 <http://www.agriculture.gov.au/animal/health/laboratories/tests/worked-example-measurement>

158 For quantitative real-time PCRs (qPCR) replicates of positive controls with their respective cycle threshold (CT) values can  
159 be used to estimate MU using the top-down approach.

160 Other approaches and variations have been described, i.e. for serological tests (Dimech *et al.*, 2006; Goris *et al.*, 2009;  
161 Toussaint *et al.*, 2007). ~~Additional work and policy Central documents are available from the National Pathology  
162 Accreditation Advisory Group and Life Science. The central document to MU is the Guide to the expression of  
163 uncertainty in measurement (GUM), ISO/IEC Guide, (1995) and Eurachem/CITAC Guide, 2012 CG 4: Quantifying  
164 uncertainty in analytical measurement.~~

### 165 **Scope and limitations of the top-down approach**

166 Methods for quantifying uncertainty (addressing MU) for tests vary. When estimating MU for quantitative, biologically based  
167 diagnostic tests, where variations in the substrate or matrix have large and unpredictable effects, a top-down approach is  
168 recommended (Dimech *et al.*, 2006; Eurachem 2012; Goris *et al.*, 2009; ISO/IEC Guide 98-3:2008; Newberry & Colling,  
169 2021; Standards Council of Canada, 2021; and footnote 1). The advantage of this method is that quality control data are  
170 generated during normal test runs and can be used to estimate the precision of the assay and express it at the cut-off. The  
171 application at the cut-off depends on the performance of the test at different analyte concentrations, e.g. variation is likely  
172 to increase at higher diluted samples. The top-down approach does not identify individual contributors to measurement  
173 uncertainty but rather provides an overall estimate. Measurement uncertainty does not replace test validation; however,  
174 the validation process includes assessments of repeatability through quality control samples which facilitate calculation of  
175 MU.

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211 \* \*

212 **NB: There is a WOAHA Collaborating Centre for**  
213 **Diagnostic Test Validation Science in the Asia-Pacific Region (please consult the WOAHA Web site:**  
214 **<https://www.woah.org/en/what-we-offer/expertise-network/collaborating-centres/#ui-id-3>**  
215 **Please contact the WOAHA Collaborating Centre for any further information on validation.**

216 **NB: FIRST ADOPTED IN 2014.**

REUNIÓN DE LA COMISIÓN DE NORMAS BIOLÓGICAS

París, 5–9 de febrero de 2024

CHAPTER 2.2.6.

SELECTION AND USE OF  
REFERENCE SAMPLES AND PANELS

INTRODUCTION

The WOAH Validation Recommendations provide detailed information and examples in support of ~~the WOAH Validation Standard that is published as Chapter 1.1.6 Principles and methods of Validation of diagnostic assays for infectious diseases of terrestrial animals this Terrestrial Manual, or Chapter 1.1.2 of the Aquatic Manual. The Term “WOAH Validation Standard” in this chapter should be taken as referring to those chapters.~~

Reference samples and panels are essential from the initial proof of concept in the development laboratory through to the maintenance and monitoring of assay performance in the diagnostic laboratory and all of the stages in between. The critical importance of reference samples and panels cannot be over-emphasised. The wrong choice of reference materials can lead to bias and flawed conclusions right from development through to validation and use. Therefore, care must be exercised in selecting reference samples and designing panels.

**Fig. 1.** Reference samples and panels grouped based on similar characteristics and composition. The topics and alphanumeric subheadings (e.g. Proof of concept, A.2.1) refer to the relevant section in ~~the~~ WOAH Validation Standard, Chapter 1.1.6 Validation of diagnostic assays for infectious diseases of terrestrial animals.

<b>Group A</b>		<b>Group B</b>		<b>Group D</b>
Proof of concept, A.2.1.		Asp, B.1.2.		Standard method comparison, B.2.6.
Operating range, A.2.2-3.		Analytical accuracy, <u>ancillary tests</u> B.1.4.		Provisional recognition, B.2.6-7.
<u>ASe, B.1.3.</u>		<b>Reference samples and panels</b>		Biological modifications, B.5.2.2.
Optimisation, A.2.-3-2.		<b>Group C</b>		<b>Group E</b>
Robustness, A.2.5. <u>Preliminary repeatability, A.2.8.</u>		Repeatability B.1.1.		<b>DSp and DSe Gold standard, B.2.1.</b>
Calibration <u>and process control</u> , A.2.6.		Preliminary reproducibility, B.2.6-7.		<b>Group F</b>
Process control, A.2.6.		Reproducibility, B.3.		<b>DSp and DSe no gold standard B.2.2.</b>
<u>ASe, B.1.3.</u>		Proficiency testing, B.5.1.		
Technical modifications, B.5.2.1.				
Reagent replacement, B.5.2.3.				

20

*ASp = Analytical specificity; ASe = Analytical sensitivity; DSp = diagnostic specificity; DSe = diagnostic sensitivity*

21

~~As can be seen in Figure 1, Reference samples and/or panels are mentioned throughout the WOAHP Validation Standard, chapter 1.1.6. As defined in the glossary of the OIE Quality Standard and Guidelines for Veterinary Laboratories: Infectious Diseases, "Reference materials are "substances whose properties are sufficiently homogenous and well established to be used for the calibration of an apparatus, the assessment of a measurement method, or for assigning values to materials"<sup>1</sup>. In the context of test method validation, reference materials or samples contain the analyte of interest in varying concentrations or activities reactivities and are used in developing and evaluating the candidate assay's analytical and diagnostic performance characteristics. In our case, Analyte means the specific component of a test sample that is detected or measured by the test method, e.g. antibody, antigen or nucleic acid. These Reference samples may be sera, fluids, tissues, excreta, feed and/or environmental samples that contain the analyte of interest and are usually harvested from infected animals and their environments. However, in some cases, they may be prepared in the laboratory from an original starting material (e.g. a dilution of a high positive serum in negative serum) or perhaps created by spiking the chosen matrix with a derived analyte (e.g. a bacterial or viral culture, a recombinant/expressed protein, or a genomic construct). Whether natural or prepared, they are used in experiments throughout the development process, carry over into the validation pathway and can be used to monitor performance throughout the lifespan of the assay.~~

37

~~In Figure 1, reference samples and panels are grouped based on similar characteristics and composition and these groupings will be the basis for the following descriptions. As a cross reference, the appropriate Section of the OIE Validation Standard is indicated under each particular application of the reference sample or panel.~~

41

~~Reference samples may be used for multiple purposes from the initial stages of development and optimisation, through Stage 1 and into continual monitoring and maintenance of the assay. Wherever possible, large quantities of these reference samples should be collected or prepared and preserved for long-term use. Switching reference samples during the validation process introduces an intractable variable that can severely undermine interpretation of experimental data and therefore, the integrity of the development and validation process. For assays that may target multiple species, the samples should be representative of the primary species of interest. It is critical that these samples reflect both the target analyte and the matrix in which it is found in the population for which the assay is intended. The reference materials should appropriately represent the range of analyte concentration to be detected by the assay.~~

50

~~It is important to emphasise that, no matter whether reference samples are selected from natural sources or prepared in the laboratory, all selection criteria or and preparation procedures, as well as testing requirements, need to be fully described and put into document control. Not only is this good quality management practice, but it will provide both an enhanced level of continuity and confidence throughout the lifespan of the assay. Summaries of the data to be collected and documented for reference material can be found in Figure 2. For more detail on best practice and quality standards for the documentation of provenance of reference material refer to Watson et al. (2021).~~

57

~~**Fig. 2. Documentation of reference material should be thorough to ensure i) transparency of intended purpose during assay development; ii) the correct sample types are used in all stages of assay development and validation; iii) accurate replacement of depleted reagents; and iv) appropriate choice of reference material during assay modification and re-validation. Minimum descriptive metadata are listed for pathogen, animal host, tissue type and phase of infection.**~~

61

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<sup>1</sup> [https://www.techlab.fr/Commun/UK\\_Def\\_MRC.asp](https://www.techlab.fr/Commun/UK_Def_MRC.asp)

<u>Pathogen data</u>	<u>Animal host and sample type data</u>	<u>Phase of Infection data</u>
<ul style="list-style-type: none"> <li>• <u>Strain/isolate</u></li> <li>• <u>Serotype</u></li> <li>• <u>Genotype</u></li> <li>• <u>Lineage</u></li> <li>• <u>Tests used for characterisation</u></li> </ul>	<ul style="list-style-type: none"> <li>• <u>Natural infection</u></li> <li>• <u>Experimental infection and protocol used</u></li> <li>• <u>Species</u></li> <li>• <u>Breed</u></li> <li>• <u>Age</u></li> <li>• <u>Sex</u></li> <li>• <u>Reproductive status</u></li> <li>• <u>Vaccination history</u></li> <li>• <u>Herd history</u></li> </ul>	<ul style="list-style-type: none"> <li>• <u>Clinical signs</u></li> <li>• <u>infection/disease outcome</u></li> <li>• <u>Antibody profiles</u></li> <li>• <u>Pathogen loading and shedding</u></li> <li>• <u>Tests used to determine status of disease/infection (case definition)</u></li> <li>• <u>Time post-experimental infection</u></li> </ul>
	<ul style="list-style-type: none"> <li>• <u>Tissue type/s (matrix) used</u></li> <li>• <u>For spiked samples – detail source of analyte and diluent (matrix) used</u></li> <li>• <u>Details relating to pooling of samples</u></li> </ul>	

## A. GROUP A

62

63 The question of pooling of samples to create a reference sample is often asked. If reference material is harvested from a  
64 single animal, it is important to ascertain whether or not it is representative of a typical course and stage of infection within  
65 the context of the population to be tested. If not, this could lead to bias and flawed conclusions related to validation. Pooling  
66 is a good alternative but it is imperative to pool from animals that are in a similar phase of infection. This is particularly  
67 important for antibody detection systems. Pooling also addresses the issue of the larger quantities of reference material to  
68 be stored for long term use, especially when dealing with smaller host species. Before pooling any samples, it is preferable  
69 that they be independently tested to demonstrate that they are similar with respect to analyte concentration and/or  
70 reactivity. There should be an assessment following pooling to ensure that unforeseen interference is not introduced by  
71 the pooling of multiple samples, for example differing blood types or antibody composition within the independent samples  
72 could cross-react within the pool, thus causing the pooled sample to behave differently in the specified assay than the  
73 individual samples when tested independently.

74 It is often difficult to obtain individual samples that truly represent analyte concentrations or reactivities across the spectrum  
75 of the expected range. Given the dynamics of many infections or responses to pathogens, intermediate ranges are often  
76 very transient. In the case of antibody responses, early infection phases in individual animals often result in highly variable  
77 and heterogeneous populations of antibody isotypes and avidities. In general, these do not make good reference samples  
78 for assessing the analytical characteristics of an assay. They are nonetheless important for different types of reference  
79 panels as will be discussed later. For most applications in Group A, it is acceptable to use prepared samples that are  
80 spiked with known concentrations of analyte or a dilution series of a high positive in negative matrix to create a range of  
81 concentrations.

82 Whether natural or prepared, reference samples should represent the anticipated range of analyte concentrations, from  
83 low-weak to high-strong positive, which would be expected during a typical course of infection. A negative reference sample  
84 should be included as a background monitor. If a negative (matrix) is used as diluent for preparation of a positive reference  
85 sample (e.g. a negative serum used to dilute a high positive serum or tissue spiked with a construct), that negative should  
86 definitely be included as the negative reference sample.

87 ~~As mentioned above, all reference samples should be well characterised. This includes documentation on both the  
88 pathogen and donor host. For pathogens, this may include details related to strain, serotype, genotype, lineage, etc. The  
89 source of the host material should be well described with respect to species, breed, age, sex, reproductive status,  
90 vaccination history, herd history, etc. Wherever possible, the phase of infection should be noted. This could include details  
91 related to clinical signs, antibody profiles, pathogen load or shedding, etc. Equally important, tests that are used to  
92 determine disease/infection status need to be well documented (see Section E of this chapter for further explanation). In  
93 some cases, experimental infection/exposure may be the only viable option for the production of reference material. In this  
94 case, all of the above considerations plus the experimental protocol should be detailed.~~

95 Above all else, natural or prepared, reference materials must be unequivocal with respect to their status as representing  
96 either a true positive or a true negative sample. This may require that the status be confirmed using another test or battery  
97 of tests. For example, many antibody reference sera are characterised using multiple serological tests. This provides not  
98 only confidence but additional documented characteristics that may be required when attempting to replace or duplicate  
99 this reference material in the future.

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100 Recommendations regarding stability and storage of reference materials are available: [https://www.woah.org/en/what-we-](https://www.woah.org/en/what-we-offer/veterinary-products/#ui-id-4)  
101 [offer/veterinary-products/#ui-id-4](https://www.woah.org/en/what-we-offer/veterinary-products/#ui-id-4)

## 102 **1. Proof of concept (WOAH Validation Standard, Chapter 1.1.6, Section A.2.1)**

103 ~~The WOA Validation Standard, Chapter 1.1.6~~ states that test methods and related procedures must be appropriate for  
104 specific diagnostic applications in order for the test results to be of relevance. In other words, the assay must be 'fit for  
105 purpose'. Many assays are developed with good intentions but without a specific application in mind. At the very outset, it  
106 is critical that the diagnostic purpose(s) should be defined with respect to the population(s) to be tested. The most common  
107 purposes are listed in broad terms in Section A of ~~the WOA Validation Standard, chapter 1.1.6~~. As such, they are inclusive  
108 of more narrow and specific applications. However, these specific purpose(s) need to be clearly defined from the outset  
109 and are critically important in the context of a fully validated assay. As will be seen in the following descriptions, clearly  
110 defining the application will have impact on both the selection of reference samples and panels and the design of analytical  
111 and diagnostic evaluations.

## 112 **2. Operating range (WOAH Validation Standard, Chapter 1.1.6, Section A.2.2-3) and** 113 **analytical sensitivity (WOAH Validation Standard, Chapter 1.1.6, Section B.1.3)**

### 114 **2.1. Analytical approaches Operating range and analytical sensitivity**

115 The operating range of the assay ~~is~~ defines the lower and upper analyte detection limits and the interval of  
116 analyte concentrations (amounts) over which the method provides suitable accuracy and precision. It also  
117 defines the lower and upper detection limits the assay. To establish this range, The operating range is  
118 established by serial dilution, to extinction, of replicates of a high-strong positive reference sample is selected.  
119 This high positive sample, either natural or prepared, is serially diluted to extinction. Dilutions of the strong  
120 positive are made in a negative matrix representative of the typical sample matrix of samples type taken from  
121 animals in the population targeted by the assay. This includes antibody assays where a high-replicates of a  
122 strong positive reference serum should be diluted in a negative reference serum to create the dilution series.  
123 Analytical sensitivity (ASe) is measured by replicates of the lower limit of detection (LOD) of an analyte in an  
124 assay. The same high-strong positive reference sample may be used to determine both the operating range and  
125 the analytical LOD.

### 126 **2.2. Comparative approaches to analytical sensitivity**

127 If the intended purpose is to detect low levels of analyte or subclinical infections, it may be difficult to obtain the  
128 appropriate reference materials from early stages of the infection process. In some cases, it may be useful to  
129 determine a comparative ASe by running a panel of samples on the candidate assay and on another  
130 independent assay. Ideally this panel of samples would be serially collected from either naturally or  
131 experimentally infected animals and should represent infected animals early after infection, ~~or~~ through to the  
132 development of clinical or fulminating disease, if possible. This would provide a relative comparison of ASe  
133 between the assays, as well as, and a temporal comparison of the earliest point of detection relative to the  
134 pathogenesis of the disease.

135 An experiment like the one described above, provides a unique opportunity to collect reference samples  
136 representing a natural range of concentrations that would be useful for other validation purposes. Care must be  
137 taken to avoid use of such samples when inappropriate (consult Group D below). Wherever possible serial  
138 samples should be collected from at least five a statistically sound number of animals throughout the course of  
139 infection. In cases where sampling is lethal (e.g. requiring the harvest of internal organ tissues), the number of  
140 animals required would be a minimum depends on need and fitness of five per sampling event the experimental  
141 approach. In all cases approval from an ethics committee is required. For smaller host species, this the number  
142 may need to be increased in order to collect sufficient reference material. Given that experiments like this require  
143 a high commitment of resources, it would be wise to maximise the collection of not only the currently targeted  
144 reference samples but additional materials (e.g. multiple tissues, fluids, etc.) that may be useful as reference  
145 materials in the future.

## 146 **3. Optimisation (WOAH Validation Standard, Chapter 1.1.6, Section A.2.32) and preliminary** 147 **repeatability (WOAH Validation Standard, Chapter 1.1.6, Section A.2.68)**

148 Optimisation is the process by which the most important physical, chemical and biological parameters of an assay are  
149 evaluated and adjusted to ensure that the performance characteristics of the assay are best suited to the intended  
150 application. At least three reference samples representing negative, low-weak and high-strong positive may be chosen  
151 from either natural or prepared reference samples. Optimisation experiments are rather exhaustive especially when assays

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152 with multiple preparatory and testing steps are involved. It is very important that a sufficient quantity of each reference  
153 sample be available to complete all optimisation experiments. Changing reference samples during the course of  
154 optimisation is not recommended as this will result in the addition of an uncontrolled variable and a disruption in the  
155 continuity of optimisation evidence.

156 Assessment of repeatability should begin during assay development and optimisation stages. ~~Repeatability and~~ is further  
157 verified during Stage 1 of assay validation (Section B.1.1 of chapter 1.1.6). The same reference samples should be used  
158 ~~for both processes, again throughout~~ to provide continuity of evidence.

## 159 **4. Calibration and process controls (~~WOAH Validation Standard, Chapter 1.1.6, Section~~** 160 **A.2.6)**

### 161 **4.1. International, national or in-house analyte reference standards**

162 International reference standards are highly characterised, contain defined concentrations of analyte, and are  
163 usually prepared and held by international reference laboratories. They are the reagents to which all assays  
164 and/or other reference materials should be standardised. National reference standards are calibrated by  
165 comparison with an international standard reagent whenever possible. In the absence of an international  
166 standard, a national reference standard may be selected or prepared and it then becomes the standard of  
167 comparison for the candidate assay. In the absence of both of the above, an in-house standard should be  
168 selected or prepared by the development laboratory within the responsible organisation. In all cases, thorough  
169 documentation of reference material should be observed as summarised in Figure 2. All of the standard  
170 reagents, whether natural or prepared, must be highly characterised through extensive analysis, and preferably  
171 the methods for their characterisation, preparation, and storage have been published in peer-reviewed  
172 publications (Watson et al., 2021). These reference standards should also be both stable and innocuous.

173 Reference standards, especially antibody, are usually provided in one of two formats. They may be provided as  
174 a single positive reagent of given titre with the expectation that the candidate assay will be standardised to give  
175 an equivalent titre. This is a straight forward analytical approach but many of these 'single' standards have been  
176 prepared from highly positive samples as a pre-dilution in a negative matrix in order to maximise the number of  
177 aliquots available. The drawback here is that there is no accounting for any potential matrix effect in the  
178 candidate assay as there is no matrix control provided. The other approach is to provide a negative and a ~~low~~  
179 ~~weak~~ and ~~high-strong~~ positive set of reference standards that are of known concentrations or reactivities and  
180 are within the operating range of the standard method that was used to prepare them. The negative provided in  
181 the set must be the same as the negative diluent used to prepare the weak and strong positive reference  
182 standard, if the positive standards were diluted. This compensates for any potentially hidden matrix effect. In  
183 addition, this set of three acts as a template for the selection and/or preparation of process controls (discussed  
184 below).

185 Classically, the above standards usually have been polyclonal antibody standards and to a lesser extent,  
186 conventional antigen standards used for calibration of serological assays. However, today, reference standards  
187 could also be monoclonal antibodies or recombinant/expressed proteins or genomic constructs, if they are to  
188 be used to calibrate assays to a single performance standard.

### 189 **4.2. Working standards or process controls**

190 Working standard reagent(s), commonly known as quality or process controls, are calibrated to international,  
191 national, or in-house standard reagents. They are selected or prepared in the local matrix which is found in the  
192 population for which the assay is intended. Ideally, negative and ~~low-weak~~ and ~~high-strong~~ positive working  
193 standards should be selected or prepared. Concentrations and/or reactivities should be within the normal  
194 operating range of the assay. Large quantities should be prepared, aliquoted and stored for routine use in each  
195 diagnostic run of the assay. The intent is that these controls should mimic, as closely as possible, field samples  
196 and should be handled and tested like routine samples. They are used to establish upper and lower control  
197 limits of assay performance and to monitor random and/or systematic variability using various control charting  
198 methods. Their daily performance will determine whether or not an assay is in control and if individual runs may  
199 be accepted. As such, these working reference samples are critically important from a quality management  
200 standpoint.

## 201 **5. Technical modifications (~~WOAH Validation Standard, Chapter 1.1.6, Section B.5.2.1)~~**

202 Technical modifications to a validated assay such as changes in instrumentation, extraction protocols, and conversion of  
203 an assay to a semi-automated or fully automated system using robotics will typically not necessitate full revalidation of the  
204 assay. Rather, a methods comparison study may be done to determine if these minor modifications to the assay protocol

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205 will affect the test results. Consult See chapter 2.2.8 Comparability of assays after changes in a validated test method for  
206 description of experiments and statistical approaches to assay precision in the face of technical modifications that are  
207 appropriate for comparability testing (Bowden & Wang, 2021; Reising et al., 2021).

208 In general, these approaches require the use of three reference samples, a negative, a weak and a low and high strong  
209 positive. Again these samples to represent the entire operating range of both assays. Samples may be either natural or  
210 prepared. The important point to re-iterate here is that the same reference samples that were used in the developmental  
211 stages of the assay may be used to assess modifications after the method has been put into routine diagnostic use. This  
212 provides a higher level of confidence assessing potential impacts because the performance characteristics of these  
213 reference samples have been well characterised. At the very least, if new reference samples are to be used, they should  
214 be selected or prepared using the same criteria or preparation procedures established for previous materials. Again as this  
215 enhances the continuity of evidence.

## 216 **6. Reagent replacement (WOAH Validation Standard, Chapter 1.1.6, Section B.5.2.3)**

217 When a reagent such as a process control sample is nearing depletion, it is essential to prepare and repeatedly test a  
218 replacement before such a control is depleted. The prospective replacement should be included in multiple runs of the  
219 assay in parallel with the original control to establish their proportional relationship. It is important to change only one  
220 control reagent at a time to avoid the compound problem of evaluating more than one variable.

221 Again, it cannot be over-emphasised that any Replacement reference reagent should be selected or prepared using the  
222 same criteria or preparation procedures established for previous materials. Again as this enhances the continuity of  
223 evidence and confidence in the assay and underlines the importance of documentation of reference material data (Figure  
224 2).

## 225 **B. GROUP B**

### 226 **1. Analytical specificity (WOAH Validation Standard, Chapter 1.1.6, Section B.1.2)**

227 Analytical specificity (ASp) is the degree to which the assay distinguishes between the target analyte and other components  
228 that may be detected in the assay. This is a relatively broad definition that is often not well understood. ASp may be broken  
229 down into different elements as described below.

230 The choice of reference samples that are required to assess ASp is highly dependent on the specific intended purpose or  
231 application that was originally envisaged defined at the development stage of the assay. Assessment of ASp is a crucial  
232 element in proof of concept and verification of fitness for purpose and may be broken down into three elements: selectivity,  
233 exclusivity and inclusivity.

234 Selectivity: an important element is the extent to which a method can accurately detect and or quantify the targeted analyte  
235 in the milieu of nucleic acids, proteins and/or antibodies in the test matrix. This is sometimes termed 'selectivity'. An  
236 example is the use of reference samples for tests that are designed to differentiate infected from vaccinated animals (DIVA  
237 tests).

238 Reference samples need to be selected and tested from i) non-infected/non-vaccinated, ii) non-infected/vaccinated, iii)  
239 infected/non-vaccinated, and iv) infected/vaccinated animals. These samples may be collected under field conditions but  
240 it is important that an accurate history be collected, ideally with respect to the animals, but at least to the herds involved,  
241 including vaccination practices and disease occurrences (Figure 2). Alternatively, it may be necessary to produce this  
242 material in experiments like those described in Section A.2.2 of this chapter, but including a combination of experimentally  
243 vaccinated and challenged animals. It Application of the 3 Rs (replacement, reduction and refinement) aims to avoid or  
244 minimise the number of animals used in experiments. For enzyme-linked immunosorbent assays (ELISAs), it is important  
245 to avoid use of the vaccine as capture antigen in the assay (e.g. indirect ELISA enzyme-linked immunosorbent assay [I-  
246 ELISA]), because carrier proteins in the vaccine may stimulate non-specific antibody responses in vaccinated animals that  
247 may be detected in ELISA leading to false positives in the assay. Similarly to the comparative approach described above  
248 with respect to ASe, at least five animals in each group should be considered. For smaller host species, this number may  
249 need to be increased in order to collect sufficient reference material, leading to false positives in the assay. Depending on  
250 the DIVA test, a single experiment could be designed to assess aspects of both ASe and ASp.

251 A second element, sometimes termed 'exclusivity', Exclusivity is the capacity of the assay to detect an analyte or genomic  
252 sequence that is unique to a targeted organism, and excludes all other known organisms that are potentially cross-  
253 reactive. This is especially true in serological assays where there are many examples of antigens expressed by other  
254 organisms that are capable of eliciting cross-reacting antibody. An attempt should be made to obtain reference samples  
255 from documented cases of infections and/or organisms that may be cross-reactive. Depending on the type of assay, these

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256 reference materials may represent the organism itself, host-derived samples, or genomic sequences. A profile for the  
257 exclusivity of the assay should be established, and expanded on a continual basis as potentially cross-reactive organisms  
258 arise.

259 ~~Thirdly, a critical design consideration~~ Inclusivity relates to the capacity of an assay to detect one or several strains or  
260 serovars of a species, several species of a genus, or a similar grouping of closely related ~~organisms~~ viruses, bacteria or  
261 antibodies. This defines the scope of detection and thus the fitness for purpose. Reference samples are required to define  
262 the scope of the assay. If for example an assay is developed as a screening test to detect all known genotypes or serotypes  
263 of a virus, then reference samples from each representative type should be tested. As new lineages or serotype variants  
264 arise, they too should be tested as part of the test profile, which should be updated on an ongoing basis.

## 265 **2. Analytical accuracy of adjunct ancillary tests (WOAH Validation Standard, Chapter 1.1.6,** 266 **Section B.1.4)**

267 Some test methods or procedures are solely analytical tools ~~and are usually applied used~~ to further characterise an analyte  
268 that has been detected in a primary assay, ~~for example assays like~~ Examples are the virus neutralisation tests used to  
269 type an isolated virus or characterise an antibody response and subtyping of haemagglutinin genes by polymerase chain  
270 reaction of avian influenza virus. Such ~~adjunct ancillary~~ tests must be validated for analytical performance characteristics,  
271 ~~but and differ from to~~ routine diagnostic tests because they do not require validation for diagnostic performance  
272 characteristics. The analytical accuracy of these tests is often dependant on the use of reference ~~reagents material~~. These  
273 reagents, whether they are antibody for typing strains of organisms or reference strains of the organism, etc., should be  
274 thoroughly documented, as required for any other reference material (Figure 2), with respect to their source, identity and  
275 performance characteristics.

276

## C. GROUP C

277 Reference samples in Group C may be used for a number of purposes. In the initial development stages, they may be  
278 used in the assessment of assay repeatability and both preliminary reproducibility in Stage 1 and the more in depth  
279 assessment of reproducibility in Stage 3 of the Validation Pathway. However, these samples have a number of other  
280 potential uses once the assay is transferred to the diagnostic laboratory. They may be used as panels for training and  
281 qualifying of analysts, and for assessing laboratory proficiency in external ring testing programmes. Ideally, 20 or more  
282 individual samples should be prepared in large volumes. About a quarter (25%) should be negative samples and the  
283 remainder (75%) should represent a collection of positives spanning the operating range of the assay. They should be  
284 aliquoted into individual tubes in sufficient volumes for single use only and stored for long term use (Chapter 1.1.2  
285 Collection, submission and storage of diagnostic specimens). The number of aliquots of each that will be required will  
286 depend on how many laboratories will be using the assay on a routine diagnostic basis and how often proficiency testing  
287 is anticipated. Ideally, they should be prepared in an inexhaustible quantity, but this is seldom feasible. At a minimum,  
288 several hundred or more aliquots of each should be prepared at a time if the assay is intended for use in multiple  
289 laboratories. This allows assessment of laboratory proficiency by testing the same sample over many testing intervals – a  
290 useful means of detecting systematic error (bias) that may creep into long term use of an assay.

291 These samples may be natural or prepared from either single or pooled starting material. The intent is that they should  
292 mimic as closely as possible a true test sample. Because mass storage is always a problem, it may be necessary to store  
293 these materials in bulk and prepare working aliquots from time to time. However, if storage space is available, it is  
294 preferable to prepare and store large numbers of aliquots at one time because bulk quantities of analyte, undergoing  
295 freeze–thaw cycles to prepare a few aliquots at a time, may be subject to degradation. Because this type of reference  
296 material is consumed at a fairly high rate, they will need to be replaced or replenished on a continual basis. As potential  
297 replacement material is identified during routine testing or during outbreaks, it is advisable to work with field counterparts  
298 to obtain bulk reference material and store it for future use. Alternatively, it may be necessary to produce this material in  
299 experiments like those described in Section A.2.2 of this chapter. Similar to the comparative approach described above  
300 with respect to ASe, at least five animals in each group should be considered. For smaller host species, this number may  
301 need to be increased in order to collect sufficient reference material.

## 302 **1. Repeatability (WOAH Validation Standard, Chapter 1.1.6, Section B.1.1) and preliminary** 303 **reproducibility provisional assay recognition (WOAH Validation Standard, Chapter 1.1.6,** 304 **Section B.2.6)**

305 Repeatability is the level of agreement between results of replicates of a sample both within and between runs of the same  
306 test method in a given laboratory. Repeatability is estimated by evaluating variation in results of replicates from a minimum  
307 of three (preferably five) samples representing analyte activity within the operating range of the assay. Consult Chapter  
308 2.2.4 *Measurement uncertainty* for statistical approaches for measures of uncertainty for assessments of repeatability.

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309 Reproducibility is the ability of a test method to provide consistent results, as determined by estimates of precision, when  
310 applied to aliquots of the same samples tested in different laboratories. However, preliminary reproducibility estimates of  
311 the candidate assay should be determined during developmental stages. A small panel of three (but preferably five)  
312 representing negative, weak and ~~both low and high~~ strong positives, like those described above, would be adequate. This  
313 type of panel could also be used for a limited evaluation of reproducibility to enhance provisional acceptance status for the  
314 assay. The test method is usually assessed in ~~one~~ two or more laboratories with a high level of experience and proficiency  
315 in assays similar to the candidate assay. The panel of 'blind' samples is evaluated using the candidate assay in each of  
316 these laboratories, using the same protocol, same reagents and comparable equipment. This is a scaled-down version of  
317 Stage 3 of assay validation. ~~Consult Chapter 2.2.4 for further explanation of the topic and its application.~~

## 318 **2. Reproducibility (WOAH Validation Standard, Chapter 1.1.6, Section B.3)**

319 Reproducibility is an important measure of the precision of an assay when used in a cross-section of laboratories located  
320 in distinct or different regions or countries using the identical assay (protocol, reagents and controls). As the number of  
321 laboratories increases, so does the number of variables encountered with respect to laboratory environments, equipment  
322 differences and technical expertise. ~~These~~ An overview of the factors affecting testing reproducibility is provided in Waugh  
323 & Clark (2021). Reproducibility studies are a measure of an assay's capacity to remain unaffected by substantial changes  
324 or substitutions in test conditions anticipated in multi-laboratory use (e.g. shipping conditions, technology transfer, reagents  
325 batches, equipment, testing platforms and/or environments). ~~Each of~~ At least three laboratories should test the same panel  
326 of 'blind' samples containing a minimum of 20 samples, representing negative and a range of positive samples. If selected  
327 negative and/or positive samples ~~in the panel~~ are duplicated; in the panel then it may be possible to assess both assay  
328 reproducibility and within-laboratory repeatability ~~estimates may be augmented by replicate testing of these samples when~~  
329 used in the reproducibility studies.

## 330 **3. Proficiency testing (WOAH Validation Standard, Chapter 1.1.6, Section B.5.1)**

331 A validated assay in routine use in multiple laboratories needs to be continually monitored to ensure uniform performance  
332 and provide overall confidence in test results. This is assessed through external quality assurance programmes. Proficiency  
333 testing is one measure of laboratory competence derived by means of an inter-laboratory comparison; implied is that  
334 participating laboratories are using the same (or similar) test methods, reagents and controls. Results are usually  
335 expressed qualitatively, i.e. either negative or positive, to determine pass/fail criteria. However, ~~for single dilution assays,~~  
336 where semi-quantitative results provide are provided, additional data for assessment of analysis may assess non-random  
337 error among the participating laboratories. Refer to Johnson & Cabuang (2021) for an overview of proficiency testing and  
338 ring trials.

339 Proficiency testing programmes are varied depending on the type of assay in use. For single dilution type assays, panel  
340 sizes ~~also vary but a minimum of five samples, representing negative and both low and high positives, like those described~~  
341 ~~above, would be adequate. Proficiency testing is not unlike a continuous form of reproducibility assessment. However,~~  
342 ~~reproducibility, by definition, is a measure of the assay's performance in multiple laboratories; whereas proficiency testing~~  
343 ~~is an assessment of laboratory competence in the performance of an established and validated assay. Measurements of~~  
344 ~~precision can be estimated for both the reproducibility and repeatability data if replicates of the same reference sample are~~  
345 ~~included in this 'blind' panel. Consult Chapter 2.2.4 for further explanation of the topic and its application. vary but a~~  
346 minimum of five samples, representing negative weak and strong positives, would be adequate.

## 347 **D. GROUP D**

348 Reference samples in Group D differ from the previous Groups in that each sample in the panel should be from a different  
349 individual animal. As indicated in Chapter 2.2.8 Comparability of assays after changes in a validated test method,  
350 experimental challenge studies often include repeated sampling of individual animals to determine the progression of  
351 disease, but this is a different objective ~~than to~~ comparing performance characteristics that would be associated with  
352 diagnostic sensitivity (DSe) and diagnostic specificity (DSp) of a test method. Serially drawn samples, taken on different  
353 days from the same animal, cannot be used as representative of individual animals in populations targeted by the assay,  
354 because such samples violate the rule of independence of samples required for such studies.

355 Care must be taken in choosing the reference samples and the standard (independent) method used in this type of  
356 comparison to ensure that the analytes being detected (if different) demonstrate the same type of pathogenic profile in  
357 terms of time of appearance after exposure to the infectious agent, and relative abundance in the test samples chosen.

## 358 **1. Standard method comparison and provisional recognition (WOAH Validation Standard,** 359 **Chapter 1.1.6, Sections B.2.6-5 and B.2.6)**

360 There are situations where it is not possible or desirable to fulfil Stage 2 of the Validation Pathway because appropriate  
361 samples from the target population are scarce and animals are difficult to access (such as for exotic diseases). However,  
362 a small but select panel of highly characterised test samples representing the range of analyte concentration should be  
363 run in parallel in the candidate assay method and ~~by a~~ WOAH standard method, as published in the WOAH *Manuals*.  
364 Biobanks may be a useful resource in this context, providing well-characterised samples supported with metadata to  
365 enhance transparency and provenance of samples used in method comparisons (Watson *et al.*, 2021). If the methods are  
366 deemed to be comparable (Chapter 2.2.8), and depending on the intended application of the assay, the choice may be  
367 made that further diagnostic validation is not required. For example, if the intended application is for screening of imported  
368 animals or animal products for exotic pathogens or confirmation of clinical signs, full validation beyond a test method  
369 comparison may not be feasible or warranted.

370 Experience has shown that the greatest obstacle to continuing through Stage 2 of the Validation Pathway is the number of  
371 defined samples required to estimate diagnostic performance parameters with a high degree of certainty (~~WOAH Validation~~  
372 ~~Standard, chapter 1.1.6~~, Section B.2). In some cases, provisional recognition by international, national or local authorities  
373 may be granted for an assay that has not been completely evaluated past analytical stages. The different rationales for  
374 provisional acceptance are well explained in ~~the WOAH Validation Standard, chapter 1.1.6~~. In all cases however, sound  
375 evidence must exist for comparative estimates of DSp and DSe based on a small select panel of well-characterised  
376 samples containing the targeted analyte.

377 Ideally, for both comparison with a standard method or provisional recognition, a panel of, for example, 60 samples could  
378 be assembled to ensure sufficient sample size for statistical analysis of the resulting data. This would include 30 'true'  
379 negatives and 30 'true' positives. Wherever possible, the positives should reflect the range of analyte concentrations or  
380 activities expected in the target population. As mentioned above, each sample in this panel must represent an individual  
381 animal. Consult Chapter 2.2.5 for statistical approaches to determining methods comparability using diagnostic samples.

## 382 **2. Biological modifications (~~WOAH Validation Standard, Chapter 1.1.6~~, Section B.5.2.2)**

383 There may be situations where changes to some of the biologicals used in the assay may be necessary and/or warranted.  
384 This may include changes to reagents themselves or a change to a different type of specimen which contains the same  
385 analyte as targeted in the original validated assay (e.g. from serum to saliva). At the very least, all of the analytical criteria  
386 of the validation pathway must be re-assessed before proceeding. If the analytical requisites are met, the remaining  
387 question relates to whether or not a full diagnostic validation is required. A similar approach to the above using a panel of  
388 60 individual reference samples may be considered. However, in this case the original test method would be considered  
389 as the standard (independent) test and the modified method would be considered the candidate. Consult Chapter 2.2.5 for  
390 statistical approaches to determining methods comparability using diagnostic samples.

## 391 **E. GROUP E**

392 Reference animals and reference samples in this Group E are well described in ~~the WOAH Validation Standard, chapter~~  
393 1.1.6, Section B.2.1). However, there are a few points that are worth re-iterating here.

### 394 **1. 'Gold standard'<sup>2</sup> – diagnostic specificity and diagnostic sensitivity (~~WOAH Validation~~** 395 **~~Standard, Chapter 1.1.6~~, Section B.2.1)**

396 For conventional estimates of DSp, negative reference samples refer to true negative samples, from animals that have  
397 had no possible infection or exposure to the agent. In some situations, where the disease has never been reported in a  
398 country or limited to certain regions of a country, identification of true negative reference samples is usually not a problem.  
399 However, where the disease is endemic, samples such as these may be difficult to locate. It is often possible to obtain  
400 these samples from regions within a large country or perhaps different countries where the disease in question does not  
401 occur or has ~~either been eradicated or has never had the disease in question~~.

402 ~~Again~~ For conventional estimates of DSe, positive reference samples refer to true positives. Care must be taken to ensure  
403 that the sample population is representative of the population that will be the target of the validated assay. It is generally  
404 problematic to find sufficient numbers of true positive reference animals, as determined by isolation of the organism. It may  
405 be necessary to resort to samples from animals that have been tested by a combination of methods that unequivocally  
406 classify animals as infected/exposed as discussed in ~~the WOAH Validation Standard, chapter 1.1.6~~.

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<sup>2</sup> The term "Gold Standard" is limited to a perfect reference standard as described in ~~the WOAH Validation Standard, chapter 1.1.6~~,  
Section B.2.1.2, and Chapter 2.2.5 *Statistical approaches to validation*, Introduction and Figure 1.

407 ~~The important point here is that~~ All samples, irrespective of origin, must be documented as they would for any other  
408 reference sample ~~se as to~~ unequivocally ~~to~~ classify animals as infected or exposed, dependent on the fitness for purpose  
409 and proposed use of the test. As mentioned in Section A, and summarised in Figure 2, of this chapter, all reference samples  
410 should be well characterised. ~~This includes documentation on both the pathogen and donor host. For pathogens, this may~~  
411 ~~include details related and data documented~~ to strain, serotype, genotype, lineage, etc. The source of the host material  
412 ~~should be well described with respect to species, breed, age, sex, reproductive status, vaccination history, herd history,~~  
413 ~~etc. Wherever possible, the phase of infection should be noted. This could include details related clinical signs, antibody~~  
414 ~~profiles, pathogen load or shedding, etc. In some cases, experimental infection/exposure may be the only viable option~~  
415 ensure appropriate sample selection for the production of reference material (see the OIE Validation Standard, Section  
416 B.2.3). In this case, all of the above and the experimental protocol should be detailed intended purpose.

417 Particularly relevant to these reference samples, the tests that are used to determine their so called 'true' disease/infection  
418 status need to be well documented in order to assess potential errors in estimates that may be carried over into the  
419 estimates for the candidate assay. Indeed, when using imperfect standard assays to define reference animal or sample  
420 status, the DSe and DSp performance estimates of the candidate assay may be flawed and often overestimated. Consult  
421 Chapter 2.2.5 for statistical considerations. Situations where a perfect reference is available for either positive or negative  
422 animals, and one where the reference is perfect for both are described for diagnostic test validation by Heuer & Stevenson  
423 (2021).

424

## F. GROUP F

### 425 1. Animals of unknown status – diagnostic specificity and diagnostic sensitivity (WOAH 426 Validation Standard, Chapter 1.1.6, Section B.2.2)

427 Latent-class models are introduced in ~~the WOAH Validation Standard, chapter 1.1.6~~. They do not rely on the assumption  
428 of a perfect reference (standard or independent) test but rather estimate the accuracy of the candidate test and the  
429 reference standard with the combined test results. Because these statistical models are complex and require critical  
430 assumptions, statistical assistance should be sought to help guide the analysis and describe the sampling from the target  
431 population(s), the characteristics of other tests included in the analysis, the appropriate choice of model and the estimation  
432 methods based on peer-reviewed literature. Consult Chapter 2.2.5 for statistical considerations.

433 Reference populations, not individual reference samples, used in latent-class studies need to be well described. ~~This~~  
434 ~~includes documentation on both the pathogen and donor host. For pathogens, this may include details related to strain,~~  
435 ~~serotype, genotype, lineage, etc., that may be circulating in the population. The source of the host material should be well~~  
436 ~~described with respect to species, breed, age, sex, reproductive status, vaccination history, herd history, etc. as~~  
437 summarised in Figure 2. Wherever possible, the phase of infection in the populations should be noted with respect to  
438 morbidity or mortality events, recovery, etc.

439 As a special note, if latent class models are to be used to ascribe estimates of DSe and DSp and include multiple  
440 laboratories in the design, it is possible to incorporate an assessment of reproducibility into the assessment. ~~As stated~~  
441 ~~above, statistical advice should be sought in this respect. Bayesian analysis of latent class models are complex and require~~  
442 adherence to critical assumptions. Statistical assistance should be sought to help guide the analysis and describe the  
443 sampling from the target population(s), the characteristics of other tests included in the analysis, the appropriate choice of  
444 model and the estimation methods (based on peer-reviewed literature). See chapter 2.2.5 for details and Cheung *et al.*,  
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446

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466 **NB: There is a WOAHA Collaborating Centre for**  
467 **Diagnostic Test Validation Science in the Asia-Pacific Region (please consult the WOAHA Web site:**  
468 **<https://www.woah.org/en/what-we-offer/expertise-network/collaborating-centres/#ui-id-3>.**  
469 **Please contact the WOAHA Collaborating Centre for any further information on validation.**

470

**NB: FIRST ADOPTED IN 2014.**

471

REUNIÓN DE LA COMISIÓN DE NORMAS BIOLÓGICAS

París, 5–9 de febrero de 2024

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CHAPTER 3.1.5.

CRIMEAN–CONGO HAEMORRHAGIC FEVER

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SUMMARY

*Crimean–Congo haemorrhagic fever virus (CCHFV) of the genus Orthonairovirus of the family Nairoviridae causes a zoonotic disease in many countries of Asia, Africa, the Middle East and south-eastern Europe. As the distribution of CCHFV coincides with the distribution of its main vector, ticks of the genus Hyalomma, the spread of infected ticks into new, unaffected areas facilitates the spread of the virus. The virus circulates in a tick–vertebrate–tick cycle, but can also be transmitted horizontally and vertically within the tick population. Hyalomma ticks infest a wide spectrum of different wildlife species, e.g. deer and hares, and free-ranging livestock animals, e.g. goat, cattle, and sheep. Many birds are resistant to infection, but ostriches appear to be more susceptible. Viraemia in livestock is short-lived, and of low intensity. These animals play a crucial role in the life cycle of ticks, and in the transmission and amplification of the virus and are, therefore, in the focus of veterinary public health. As animals do not develop clinical signs, CCHFV infections have no effect on the economic burden regarding livestock animal production. In contrast to animals, infections of humans can result in the development of a severe disease, Crimean–Congo haemorrhagic fever (CCHF).*

*Every year, more than 1000 human CCHF cases are reported with case fatality rates of 5–80% depending on the virus strain and other local factors. The pathogenesis of the disease in humans is not well understood. Most people become infected by tick bites and by crushing infected ticks, but infection is also possible through contact with blood and other body fluids of viraemic animals, for example in slaughterhouses. As CCHFV also has the potential to be transmitted directly from human-to-human, nosocomial outbreaks have been reported.*

*There is no approved CCHF vaccine available and therapy is restricted to treatment of the symptoms. Health education and information on prevention and behavioural measures are most important in order to enhance public risk perception and, therefore, decrease the probability of infections. Thus the identification of endemic areas is crucial for focused and targeted implementation of public health measures. Serological screening of ruminants allows CCHFV-affected areas to be identified, as antibody prevalence in animals is a good indicator of local virus circulation. Treatment with tick repellents can be quite effective in reducing the tick infestation of animals. To protect laboratory staff, handling of CCHFV infectious materials should only be carried out at an appropriate biocontainment level.*

**Detection and identification of agent:** *Only a single virus serotype is known to date although sequencing analysis indicates considerable genetic diversity. CCHFV has morphological and physiochemical properties typical of the family Nairoviridae. The virus has a single-stranded, negative-sense RNA genome consisting of three segments: L (large), M (medium) and S (small), each of which is contained in a separate nucleocapsid within the virion. The virus can be isolated from serum or plasma samples collected during the febrile or viraemic stage of infection, or from liver of infected animals. Primary isolations are made by inoculation of several tissue cultures, commonly African green monkey kidney (Vero) cells. For identification*

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37 and characterisation of the virus, conventional and real-time reverse transcription polymerase chain reaction  
38 (PCR) can be used. As infections of animals remain clinically unapparent, the likelihood of isolating virus  
39 from a viraemic animal is very low.

40 **Serological tests:** Type-specific antibodies are demonstrable by indirect immunofluorescence test or by  
41 IgG-sandwich and IgM-capture enzyme-linked immunosorbent assay. Commercial test systems are  
42 available for animal health; in addition a few in-house systems have been published or kits are used replacing  
43 the conjugate provided in kit with one that is suitable for the animal species to be screened for CCHFV-  
44 specific antibodies.

45 **Requirements for vaccines:** There is no vaccine available for animals.

## 46 A. INTRODUCTION

47 Crimean-Congo haemorrhagic fever (CCHF) is a zoonotic disease caused by a primarily tick-borne CCHF virus (CCHFV)  
48 of the genus *Orthonairovirus* of the family *Nairoviridae*, order *Bunyvirales*. CCHFV possesses a negative-sense RNA  
49 genome consisting of three segments, L (large), M (medium) and S (small) each contained in a separate nucleocapsid  
50 within the virion. All orthonairoviruses are believed to be transmitted by either ixodid or argasid ticks, and only three are  
51 known to be pathogenic to humans, namely CCHF, Dugbe and Nairobi sheep disease viruses (Swanepoel & Burt, 2004;  
52 Swanepoel & Paweska, 2011; Whitehouse, 2004). CCHFV can be grown in several tick cell lines derived from both a  
53 natural vector (*Hyalomma anatolicum*) and other tick species not implicated in natural transmission of the virus (Bell-Sakyiet  
54 *et al.*, 2012).

55 The virus from an outbreak of “Crimean haemorrhagic fever” in the Crimean Peninsula in 1944 was not isolated or  
56 characterised until 1967. “Congo haemorrhagic fever” virus, isolated from a patient in the former Zaire (now Democratic  
57 Republic of the Congo) in 1956, was shown in 1969 to be the same virus. As a consequence the names of both countries  
58 have been used in combination to describe the disease (Hoogstraal, 1979). Distribution of the virus reflects the broad  
59 distribution of *Hyalomma* ticks, the predominant vector of the virus (Avsic-Zupanc, 2007; Grard *et al.*, 2011; Papa *et al.*,  
60 2011; Swanepoel & Paweska, 2011).

61 The natural cycle of CCHFV includes transovarial and transstadial transmission among ticks and a tick-vertebrate-tick  
62 cycle involving a variety of wild and domestic animals. Infection can also be transferred between infected and uninfected  
63 ticks during co-feeding on a host; so called ‘non-viraemic transmission’ phenomenon. *Hyalomma* ticks feed on a variety of  
64 domestic ruminants (sheep, goats, and cattle), and wild herbivores, hares, hedgehogs, and certain rodents. CCHFV  
65 infection in animals was reviewed by Nalca & Whitehouse (2007). Experimental infections of wild animals and livestock  
66 with CCHFV were reviewed by Spengler *et al.* (2016). Although animal infections are generally subclinical, the associated  
67 viraemia levels are sufficient to enable virus transmission to uninfected ticks (Swanepoel & Burt, 2004; Swanepoel &  
68 Paweska, 2011). Many birds are resistant to infection, but ostriches appear to be more susceptible than other bird species  
69 (Swanepoel *et al.*, 1998). Although they do not appear to become viraemic, ground feeding birds may act as a vehicle for  
70 spread of CCHFV infected ticks. Results from serological surveys conducted in Africa and Eurasia indicate extensive  
71 circulation of the virus in livestock and wild vertebrates (Swanepoel & Burt, 2004).

72 Humans acquire infection from tick bites, or from contact with infected blood or tissues from livestock or human patients.  
73 After incubation humans can develop a severe disease with a prehaemorrhagic phase, a haemorrhagic phase, and a  
74 convalescence period. Haemorrhagic manifestations can range from petechiae to large haematomas. Bleeding can be  
75 observed in the nose, gastrointestinal system, uterus and urinary tract, and the respiratory tract, with a case fatality rate  
76 ranging from 5% to 80% (Ergonul, 2006; Yen *et al.*, 1985; Yilmaz *et al.*, 2008). The severity of CCHF in humans highlights  
77 the impact of this zoonotic disease on public health. Although CCHFV has no economic impact on livestock animal  
78 production, the serological screening of animal serum samples for CCHFV-specific antibodies is very important. As  
79 seroprevalence in animals is a good indicator for local virus circulation, such investigations allow identification of high-risk  
80 areas for human infection (Mertens *et al.*, 2013). Slaughterhouse workers, veterinarians, stockmen and others involved  
81 with the livestock industry should be made aware of the disease. They should take practical steps to limit or avoid exposure  
82 of naked skin to fresh blood and other animal tissues, and to avoid tick bites and handling ticks. Experiences from South  
83 Africa demonstrated that the use of repellents on animals before slaughter could reduce the numbers of infected  
84 slaughterhouse workers (Swanepoel *et al.*, 1998). The treatment of livestock in general can reduce the tick density among  
85 these animals and thus reduce the risk of tick bite in animal handlers (Mertens *et al.*, 2013). Such tick control by the use  
86 of acaricides is possible to some extent, but may be difficult to implement under extensive farming conditions. Inactivated  
87 mouse brain vaccine for the prevention of human infection has been used on a limited scale in Eastern Europe and the  
88 former USSR (Swanepoel & Paweska, 2011). Progress in CCHFV vaccine development is being made with several  
89 different approaches trialled to overcome current challenges (Dowall *et al.*, 2017).

90 Infectivity of CCHFV is destroyed by boiling or autoclaving and low concentrations of formalin or beta-propiolactone. The  
91 virus is sensitive to lipid solvents. It is labile in infected tissues after death, presumably due to a fall in pH, but infectivity is



92 retained for a few days at ambient temperature in serum, and for up to 3 weeks at 4°C. Infectivity is stable at temperatures  
 93 below –60°C (Swanepoel & Paweska, 2011). CCHFV should be handled with appropriate biocontainment measures  
 94 determined by risk analysis as described in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk*  
 95 *in the veterinary laboratory and animal facilities* (Palmer, 2011; Whitehouse, 2004).

## 96 B. DIAGNOSTIC TECHNIQUES

97 **Table 1. Diagnostic test formats for Crimean-Congo haemorrhagic fever virus infections in animals**

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases in animals	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection and identification of the agent <sup>(a)</sup>						
Real-time RT-PCR	–	++ <sup>‡</sup>	–	+++ <sup>(b)</sup>	+ <sup>(c)</sup>	–
Virus isolation in cell culture	–	–	–	+ <sup>(b)</sup>	–	–
Detection of immune response						
IgG ELISA	+++	+	–	++ <sup>(d)</sup>	+++	–
Competitive ELISA	+++	+	–	++ <sup>(d)</sup>	+++	–
IgM ELISA	–	++	–	++ <sup>(e)</sup>	–	–

98 Key: +++ = recommended for this purpose; ++ recommended but has limitations;

99 + = suitable in very limited circumstances; – = not appropriate for this purpose.

100 RT-PCR = reverse-transcription polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay.

101 <sup>(a)</sup>A combination of agent identification methods applied on the same clinical sample is recommended.

102 <sup>(b)</sup>Molecular testing/isolation can be used to confirm acute infection in rare cases in animals showing  
 103 clinical signs as viraemia tends to be transient.

104 <sup>(c)</sup>RT-PCR is used for the screening of tick populations in the context of surveillance studies.

105 <sup>(d)</sup>Serological evidence of active infection with CCHFV has been demonstrated by seroconversion based on a rise in total or IgG  
 106 antibody titres on samples taken at 2–4 weeks apart.

107 <sup>(e)</sup>Serological evidence of active infection with CCHFV has been demonstrated by the detection of IgM antibodies specific to

108 CCHFV infection causes only a mild fever in domestic and wild vertebrate animals with a detectable viraemia of up to 2  
 109 weeks (Gonzalez *et al.*, 1998; Gunes *et al.*, 2011). Similarly infected ostriches develop only low and short-lived viraemia  
 110 and no clinical signs (Swanepoel & Burt, 2004). Therefore, recent infections in animals are rarely diagnosed and methods  
 111 such as polymerase chain reaction (PCR), virus isolation in cell culture and IgM detection by enzyme-linked immunosorbent  
 112 assay (ELISA) are mainly used in human CCHF diagnostics or in the special case that an animal has to be classified as  
 113 CCHFV free. For prevalence analysis and for determination of whether CCHFV is circulating in a country, methods for the  
 114 detection of IgG antibodies are preferred (Table 1). If there is any possibility or suspicion that diagnostic samples could be  
 115 contaminated with CCHFV, they should be handled under an adequate biosafety level and all persons dealing with those  
 116 samples should be aware of the possible risk and should use personal protective equipment to avoid human infections.

### 117 1. Detection and identification of the agent

118 For testing animals for viraemia, rapid diagnosis can be achieved by detection of viral nucleic acid in serum or plasma  
 119 using conventional (Burt *et al.*, 1998) or real-time reverse transcription (RT-) PCR (Drosten *et al.*, 2002; Duh *et al.*, 2006;  
 120 Koehler *et al.*, 2018; Negredo *et al.*, 2017; Sas *et al.*, 2018; Wolfel *et al.*, 2007), or by demonstration of viral antigen  
 121 (Shepherd *et al.*, 1988). Specimens to be submitted for laboratory confirmation of CCHF include blood and liver samples.  
 122 Because of the risk of laboratory-acquired infections, work with CCHFV should be conducted in appropriate biosafety  
 123 facilities.

124 The virus can be isolated from serum and organ suspensions in a wide variety of cell cultures, including Vero, LLC-MK2,  
 125 SW-13, BSR-T7/5, CER and BHK21 cells, and identified by immunofluorescence using specific antibodies. Isolation and

126 identification of virus can be achieved in 1–5 days, but cell cultures lack sensitivity and usually only detect high  
127 concentrations of virus present in the blood.

## 128 1.1. Virus isolation in cell culture

129 CCHFV can be isolated in mammalian cell cultures. Vero cells are commonly used, usually yielding an isolate  
130 between 1 and 5 days post-inoculation (p.i.). CCHFV is poorly cytopathic and thus infectivity is titrated by  
131 demonstration of immunofluorescence in infected cells (Shepherd *et al.*, 1986). SW-13 cell line has also been  
132 used extensively for virus isolation, producing plaques within 4 days (p.i.). Identification of a CCHFV isolate has  
133 to be confirmed by immunofluorescence or molecular techniques (Burt *et al.*, 1998; Shepherd *et al.*, 1986).

### 134 1.1.1. Test procedure

- 135 i) Susceptible cell lines include Vero-E6, BHK-21, LLC-MK2 and SW-13 cells. Inoculate 80%  
136 confluent monolayers of the preferred cell line with the specimen. The volume of specimen to be  
137 used depends on the size of the culture vessel (i.e. 25 cm<sup>2</sup> culture flask or 6- or 24-well tissue  
138 culture plate). The specimen volume should be sufficient to cover the cell monolayer. Samples of  
139 insufficient volumes can be diluted with tissue culture medium to prepare sufficient inoculation  
140 volume.
- 141 ii) Adsorb the specimen for 1 hour at 37°C.
- 142 iii) Remove inoculum. Add fresh tissue culture medium containing 2% fetal calf serum and other  
143 required additives, as per specific medium and cell line requirements.
- 144 iv) Incubate at 37°C and 5% CO<sub>2</sub> for 4–7 days.
- 145 v) Test supernatant for presence of CCHFV viral RNA using real-time RT-PCR as described below,  
146 or perform immunofluorescence assay on cell scrapings.
- 147 vi) Isolates of CCHFV from clinical specimens cause no microscopically recognisable cytopathic  
148 effects (CPE) in most of these cell lines.

## 149 1.2. Nucleic acid detection

150 Molecular-based diagnostic assays, such as RT-PCR, serve as the front-line tool in the diagnosis of CCHF, as well  
151 as other viral haemorrhagic fevers (Drosten *et al.*, 2003). The benefit of molecular diagnostic assays is their rapidity  
152 compared to virus culture, often allowing a presumptive diagnosis to be reported within a few hours after receiving  
153 a specimen (Burt *et al.*, 1998). The RT-PCR is a sensitive method for diagnosis, but because of the genetic diversity  
154 of CCHFV, there might be some challenges with regard to design of primers or probes that allow detection of all  
155 circulating strains of the virus. Indeed, based on geographical origin and phylogenetic analyses of the S gene  
156 segment, CCHFV has previously been classified into nine geographical clades – four predominantly diffused in  
157 Africa, three in Europe, and two in Asia. Several real-time RT-PCR assays that detect strains from different  
158 geographical locations have been evaluated (Gruber *et al.*, 2019). While some assays have been shown to be  
159 highly sensitive, detecting as little as 10 viral RNA copies per ml of plasma, it is necessary to combine at least two  
160 molecular assays to ensure detection of the different CCHFV clades (Gruber *et al.*, 2019). The best assay  
161 combination(s) with the best detection efficacy for each CCHFV clade, on the basis of all CCHFV sequences  
162 known at the time of the study, are shown in Table 2. In addition, a low-density microarray has been extensively  
163 validated in clinical specimens collected from confirmed cases of CCHF over 20 years by a WHO reference  
164 laboratory. It was shown to detect as few as 6.3 genome copies per reaction (Wolfel *et al.*, 2009).

165 **Table 2. Molecular assay combinations for the detection of CCHFV-specific nucleic acid**

Clade	Molecular assay combinations	Primer and probe names (5' → 3' sequence)
Africa 1	Real-time RT-PCR	Fwd CCRRealP1 (TCT-TYG-CHG-ATG-AYT-CHT-TYC) Rev CCRRealP2 (GGG-ATK-GTY-CCR-AAG-CA) Probe (ACA-SRA-TCT-AYA-TGC-AYC-CTG-C)
Africa 2	Real-time RT-PCR	Fwd CCRRealP1 (TCT-TYG-CHG-ATG-AYT-CHT-TYC) Rev CCRRealP2 (GGG-ATK-GTY-CCR-AAG-CA) Probe (ACA-SRA-TCT-AYA-TGC-AYC-CTG-C)
	Real-time RT-PCR	Fwd CCHF-SF2 (GGA-VTG-GTG-VAG-GGA-RTT-TG) Rev CCHF-SR2 (CAD-GGT-GGR-TTG-AAR-GC) Fwd CCHF-N2 (CAA-RGG-CAA-RTA-CAT-MAT)

Clade	Molecular assay combinations	Primer and probe names (5' → 3' sequence)
Africa 3	Nested RT-PCR	Fwd CCHF1 (CTG-CTC-TGG-TGG-AGG-CAA-CAA) Rev CCHF2_5 (TGG-GTT-GAA-GGC-CAT-GAT-GTA-T) Nested Fwd CCHFn15 (AGG-TTT-CCG-TGT-CAA-TGC-AAA) Nested Rev CCHFn25 (TTG-ACA-AAC-TCC-CTG-CAC-CAG-T)
	Nested RT-PCR	Fwd CrCon1+ (RWA-AYG-GRC-TTR-TGG-AYA-CYT-TCA-C) Rev CrCon1- (TRG-CAA-GRC-CKG-TWG-CRA-CWA-GWG-C) Nested Fwd CriCon2+ (ART-GGA-GRA-ARG-AYA-TWG-GYT-TYC-G) Nested Rev CriCon2- (CYT-TGA-YRA-AYT-CYC-TRC-ACC-ABT-C)
Africa 4	Real-time RT-PCR	Fwd CCRRealP1 (TCT-TYG-CHG-ATG-AYT-CHT-TYC) Rev CCRRealP2 (GGG-ATK-GTY-CCR-AAG-CA) Probe (ACA-SRA-TCT-AYA-TGC-AYC-CTG-C)
	Real-time RT-PCR	Fwd CCHF-III (CAA-GAG-GTA-CCA-AGA-AAA-TGA-AGA-AGG-C) Rev CCHF-III-r (GCC-ACG-GGG-ATT-GTC-CCA-AAG-CAG-AC) Probe CCHFprobe-1 (ATC-TAC-ATG-CAC-CCT-GCY-GTG-YTG-ACA) Probe CCHFprobe-2 (TTC-TTC-CCC-CAC-TTC-ATT-GGR-GTG-CTC-A)
Asia 1	Nested PCR	Fwd CCF-115F (AAR-GGA-AAT-GGA-CTT-RTG-GA) Fwd CCF-131F (TGG-AYA-CYT-TCA-CAA-ACT-CC) Rev CCF-759R (GCA-AGG-CCT-GTW-GCR-ACA-AGT-GC)
	Real-time RT-PCR	Fwd CC1a_for (GTG-CCA-CTG-ATG-ATG-CAC-AAA-AGG-ATT-CCA-TCT) Rev CC1a_rev (GTG-CCA-CTG-ATG-ATG-CAC-AAA-AGG-ATT-CCA-TCT) Probe CCHF-01 (CAA-CAG-GCT-GCT-CTC-AAG-TGG-AG)
	Real-time RT-PCR	Fwd CCHF-SF2 (GGA-VTG-GTG-VAG-GGA-RTT-TG) Rev CCHF-SR2 (CAD-GGT-GGR-TTG-AAR-GC) Probe CCHF-N2 (CAA-RGG-CAA-RTA-CAT-MAT)
Asia 2	Nested PCR	Fwd CCF-115F (AAR-GGA-AAT-GGA-CTT-RTG-GA) Fwd CCF-131F (TGG-AYA-CYT-TCA-CAA-ACT-CC) Rev CCF-759R (GCA-AGG-CCT-GTW-GCR-ACA-AGT-GC)
	Sybrgreen Real-time RT-PCR	Fwd (GAT-GAG-ATG-AAC-AAG-TGG-TTT-GAA-GA) Rev (GTA-GAT-GGA-ATC-CTT-TTG-TGC-ATC-AT)
	RT-PCR	Fwd CCS (ATG-CAG-GAA-CCA-TTA-ART-CTT-GGG-A) Rev 1 CCAS1 (CTA-ATC-ATA-TCT-GAC-AAC-ATT-TC) Rev 2 CCAS2 (CTA-ATC-ATG-TCT-GAC-AGC-ATC-TC)
Europe 1	Real-time RT-PCR	Fwd CCRRealP1 (TCT-TYG-CHG-ATG-AYT-CHT-TYC) Rev CCRRealP2 (GGG-ATK-GTY-CCR-AAG-CA) Probe (ACA-SRA-TCT-AYA-TGC-AYC-CTG-C)
	Nested RT-PCR	Fwd CCF-115F (AAR-GGA-AAT-GGA-CTT-RTG-GA) Fwd CCF-131F (TGG-AYA-CYT-TCA-CAA-ACT-CC) Rev CCF-759R (GCA-AGG-CCT-GTW-GCR-ACA-AGT-GC)
Europe 2	Nested RT-PCR	Fwd CrCon1+ (RWA-AYG-GRC-TTR-TGG-AYA-CYT-TCA-C) Rev CrCon1- (TRG-CAA-GRC-CKG-TWG-CRA-CWA-GWG-C) Fwd CriCon2+ (ART-GGA-GRA-ARG-AYA-TWG-GYT-TYC-G) Rev CriCon2- (CYT-TGA-YRA-AYT-CYC-TRC-ACC-ABT-C)
Europe 3	Real-time RT-PCR	Fwd CCRRealP1 (TCT-TYG-CHG-ATG-AYT-CHT-TYC) Rev CCRRealP2 (GGG-ATK-GTY-CCR-AAG-CA) Probe (ACA-SRA-TCT-AYA-TGC-AYC-CTG-C)

Clade	Molecular assay combinations	Primer and probe names (5' → 3' sequence)
	Real-time RT-PCR	Fwd CCRRealP1 (TCT-TYG-CHG-ATG-AYT-CHT-TYC) Rev CCRRealP2 (GGG-ATK-GTY-CCR-AAG-CA) Probe (ACA-SRA-TCT-AYA-TGC-AYC-CTG-C)
	Nested RT-PCR	Fwd CrCon1+ (RWA-AYG-GRC-TTR-TGG-AYA-CYT-TCA-C) Rev CrCon1- (TRG-CAA-GRC-CKG-TWG-CRA-CWA-GWG-C) Rev Fwd CriCon2+ (ART-GGA-GRA-ARG-AYA-TWG-GYT-TYC-G) Nested Rev CriCon2- (CYT-TGA-YRA-AYT-CYC-TRC-ACC-ABT-C)
All	Real-time RT-PCR	Fwd CCHF-SF2 (GGA-VTG-GTG-VAG-GGA-RTT-TG) Rev CCHF-SR2 (CAD-GGT-GGR-TTG-AAR-GC) Probe CCHF-N2 (CAA-RGG-CAA-RTA-CAT-MAT)
	RT-PCR	Fwd CCS (ATG-CAG-GAA-CCA-TTA-ART-CTT-GGG-A) Rev 1 CCAS1 (CTA-ATC-ATA-TCT-GAC-AAC-ATT-TC) Rev 2 CCAS2 (CTA-ATC-ATG-TCT-GAC-AGC-ATC-TC)
	Real-time RT-PCR	Fwd CC1a_for (GTG-CCA-CTG-ATG-ATG-CAC-AAA-AGG-ATT-CCA-TCT) Rev CC1a_rev (GTG-CCA-CTG-ATG-ATG-CAC-AAA-AGG-ATT-CCA-TCT) Probe CCHF-01 (CAA-CAG-GCT-GCT-CTC-AAG-TGG-AG)

166 (Data and table modified from Gruber *et al.* 2019)

## 167 2. Serological tests

168 Virus neutralisation assays, generally considered to be highly specific, are rarely used for CCHFV diagnosis. Members of  
169 the *Orthonairovirus* genus generally induce a weaker neutralising antibody response than members of other genera in the  
170 family *Nairoviridae*. Another drawback is the necessity to perform this assay in high biosafety containment because it uses  
171 live virus (Burt *et al.*, 1994; Rodriguez *et al.*, 1997).

172 Currently, there are only a few CCHFV commercial kits for IgM or IgG by ELISA or immunofluorescence (IFA). These are  
173 all designed for the human diagnostic market. However, it is possible to adapt these commercial ELISAs and IFAs for  
174 serological testing in animals. In addition, some in-house ELISAs have been published for the detection of CCHFV-specific  
175 antibodies in animals.

176 Diagnostic performance for humans have been compared between the methods using sensitivity, specificity, concordance  
177 and degree of agreement with particular focus on the phase of the infection (Emmerich *et al.*, 2021). Available serological  
178 test systems detect anti-CCHFV IgM and IgG antibodies accurately, but their diagnostic performance varies with respect  
179 to the phase of the infection. In the early and convalescent phases of infection, the sensitivity for detecting specific IgG  
180 antibodies differed for the ELISA. Both test systems based on immunofluorescence showed an identical sensitivity for  
181 detection of anti-CCHFV IgM antibodies in acute and convalescent phases of infection.

182 IgM antibodies in livestock (sheep, goat and cattle) can be detected by using an IgM-capture ELISA. IgG antibodies can  
183 be detected by an IgG-sandwich or indirect ELISA, and total antibodies can be detected by competition ELISA. The benefit  
184 of competitive ELISA is the capacity to investigate different animal species, because they are host species independent.  
185 Commercial kits for the detection of CCHFV-specific antibodies or the detection of viral antigen are available. The limiting  
186 factor for the replication of these protocols in other laboratories is the availability of antigens and (where relevant) specified  
187 monoclonal antibodies. Most of the tests described for livestock and wild animals have not undergone a formal validation  
188 process (Mertens *et al.*, 2013). One of the biggest challenges for such validation studies is the availability of an adequate  
189 number of positive well characterised control samples.

190 For information on the availability of reference reagents for use in veterinary diagnostic laboratories, contact the WOA  
191 Collaborating Centres for Zoonoses in Europe and in Asia-Pacific.

## 192 C. REQUIREMENTS FOR VACCINES

193 There is no vaccine available for animals.

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289 **NB:** At the time of publication (2023) there was no WOA Reference Laboratory for Crimean–Congo haemorrhagic fever  
290 (please consult the WOA Web site:  
291 <https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3>).

292

**NB:** FIRST ADOPTED IN 2014. MOST RECENT UPDATES ADOPTED 2023.

CHAPTER 3.3.6.

AVIAN TUBERCULOSIS

SUMMARY

**Description of the disease:** Avian tuberculosis, or avian mycobacteriosis, is ~~an important~~ a significant disease that affects companion, captive exotic, wild, and domestic birds and mammals. The disease is most often caused by Mycobacterium avium subsp. avium (M. a. avium), a member of the M. avium complex. However, more than ten other mycobacterial species have been reported to infect birds. The most significant cause of poultry disease is M. a. avium.

Clinical signs of the disease vary depending on the organs involved. The classical presentation is characterised by chronic and progressive wasting and weakness. ~~Diarrhoea is common and joint swelling are standard features in infected flocks.~~ Some birds may show respiratory signs, and occasionally, sudden death occurs. Some birds may develop granulomatous ocular lesions.

Mycobacterium tuberculosis, the agent that most commonly causes human tuberculosis (gene IS61101) is less commonly rarely the cause of infection in birds, and it is often as a the result of transmission from pet bird owners or caretakers of captive birds.

Members of M. avium complex: M. a. avium (serotypes 1–3; containing gene segments insertion sequences IS901 and IS1245), M. avium subsp. hominissuis (serotypes 4–6, 8–11, and 21; lacking gene segment IS901 and containing segment IS1245) and M. intracellulare (serotypes 7, 12–20, and 22–28; lacking both IS901 and IS1245) can also infect an extensive range of mammals such as swine, cattle, deer, sheep, goats, horses, cats, dogs, and exotic species. In humans, all members of the M. avium complex and M. genavense are capable of inducing can induce a progressive disease that is refractory to treatment, ~~mostly mainly~~ in immunocompromised patients.

~~All manipulations involving~~ Due to the contagious nature of this group of organisms, handling of open live cultures or of material from infected birds must only be carried out with after an appropriate bio risk management risk assessment and the implementation of biosafety measures designed to avoid infection.

Diagnosis of avian tuberculosis in birds depends on the demonstration of ~~the above mentioned a~~ mycobacterial species in live or dead birds or the detection of an immune response, cellular or humoral, culture examination, or gene segments ~~IS6110, IS901 and IS1245~~ by polymerase chain reaction (PCR) in the excretions or secretions of live birds.

**Detection of the agent:** Where clinical signs of avian tuberculosis are seen in the flock, or typical tuberculous lesions are present in birds at necropsy, the demonstration of acid-fast bacilli in smears or sections made from affected organs is sufficient for a quick positive diagnosis. If acid-fast bacilli are not found but typical tuberculous signs or lesions are present in the birds, a culture of the organism or PCR must be attempted. PCR could also be carried out directly on tissue samples. Any acid-fast organism isolated should be identified by nucleic-acid-based tests or chromatographical (e.g. high-performance liquid chromatography [HPLC]) criteria. Serotyping of isolates of M. avium complex members or PCR for 16S rRNA gene followed by sequencing, or the presence of an amplicon for the insertion sequences IS6110, IS901,



38 and IS1245 ~~could can~~ also be performed. Matrix-assisted laser desorption ionisation-time of flight mass  
39 spectrometry (MALDI-TOF MS) is a valuable tool as well for isolates following culture.

40 **Tuberculin test and serological tests:** These tests are ~~normally typically~~ used to determine ~~the disease~~  
41 ~~prevalence of disease in a flock or to detect infected birds.~~ When used to detect the presence of avian  
42 tuberculosis in a flock, they should be supported by the necropsy of any birds that give positive reactions.

43 In domestic fowl, the tuberculin test in the wattle is the test of choice. This test is less useful in other species  
44 of bird. A better test, especially in waterfowl, is The whole blood stained-antigen agglutination test is better,  
45 especially in waterfowl. It is more reliable and has the advantage that it will can give a result within a few  
46 minutes while the bird is still being held.

47 **Requirements for vaccines and diagnostic biologicals:** No vaccines are available for use in birds. Avian  
48 tuberculin purified protein derivative (PPD) is the standard preparation for use in the tuberculin test of  
49 domestic poultry. Avian PPD is also used as a component in the comparative intradermal tuberculin test in  
50 cattle (see Chapter 3.1.13 Mammalian tuberculosis [infection with *Mycobacterium tuberculosis* complex]).

## 51 A. INTRODUCTION

52 Several mycobacterial species can be involved in the aetiology of avian tuberculosis ~~and, also known as~~ avian  
53 mycobacteriosis. Avian tuberculosis is most commonly ~~produced caused~~ by infection with *Mycobacterium avium* subsp.  
54 *avium* (serotypes 1, 2, and 3: containing specific gene segment IS901 and nonspecific segment IS1245) and less frequently  
55 by *M. genavense* (Guerrero *et al.*, 1995; Pavlik *et al.*, 2000; Salamatian *et al.*, 2020; Sattar *et al.*, 2021; Tell *et al.*, 2001).  
56 Avian mycobacteriosis is also caused by other two members of the *M. avium* complex: *M. avium* subsp. *hominissuis*  
57 (serotypes 4–6, 8–11, and 21: lacking gene segment IS901 and containing segment IS1245 and mainly infecting humans  
58 and pigs) and *M. intracellulare* (serotypes 7, 12–20, and 22–28: lacking both gene segments IS901 and IS1245) and by  
59 ~~*M. intracellulare*, *M. scrofulaceum*, *M. fortuitum*, and~~ other potentially pathogenic mycobacterial species including  
60 *M. scrofulaceum* and *M. fortuitum*. Under some circumstances, an extensive range of mammalian species, such as swine,  
61 cattle, deer, sheep, goats, horses, cats, dogs, and exotic animals, can be infected by these mycobacterium species  
62 (Dvorska *et al.*, 2004; Kunze *et al.*, 1992; Mijs *et al.*, 2002; Shitaye *et al.*, 2009; Tell *et al.*, 2001; Thorel *et al.*, 1997; 2001).  
63 *Mycobacterium tuberculosis* and *M. bovis* are ~~less common as causal rarely the causative~~ agents of tuberculosis in birds  
64 (Hoop, 2002; Lanteri *et al.*, 2011; Peters *et al.*, 2007; Schmidt *et al.*, 2022; Tell *et al.*, 2001).

65 *Mycobacterium avium* species with standing in nomenclature as of 2023<sup>1</sup> (Arahal *et al.*, 2023) consists of ~~four three~~  
66 subspecies: *M. avium* subsp. *avium*, ~~*M. avium* subsp. *hominissuis*, *M. avium* subsp. *silvaticum*, and *M. avium* subsp.~~  
67 *paratuberculosis* (Mijs *et al.*, 2002; Thorel *et al.*, 1990). The latter is the causal agent of Johne's disease, or  
68 paratuberculosis, in ruminants and other mammalian species (see Chapter 3.1.16 *Paratuberculosis [Johne's disease]*).  
69 *Mycobacterium a. silvaticum*, which like *M. avium* subsp. *paratuberculosis* grows *in-vitro* only on media with Mycobactin,  
70 which can cause avian tuberculosis in wood pigeons (Thorel *et al.*, 1990). With the widespread use of whole genome  
71 sequencing (WGS) and bioinformatics, some studies have investigated the classification of species belonging to the genus  
72 *Mycobacterium* and have proposed that *M. avium* comprises three subspecies *M. avium* subsp. *avium*, *M. avium* subsp.  
73 *paratuberculosis*, and *M. avium* subsp. *lepraemurium*. Further subdividing *M. avium* subsp. *avium* into three variants *M.*  
74 *avium* subsp. *avium* var. *avium*, *M. avium* subsp. *avium* var. *silvaticum*, and *M. avium* subsp. *avium* var. *hominissuis* (Riojas  
75 *et al.*, 2021; Tortoli *et al.*, 2019).

76 All *M. a. avium* isolates from birds and mammals, including humans, have a multiple repetitive sequence IS901 in their  
77 genome and produce a characteristic three band pattern in IS1245 restriction fragment length polymorphism (RFLP) as  
78 described and standardised previously (Dvorska *et al.*, 2003; Ritacco *et al.*, 1998). This repetitive sequence is also present  
79 in *M. a. silvaticum* and RFLP analysis can help with identification. IS901 has only been detected in *M. avium* strains with  
80 serotypes 1, 2 and 3 (Pavlik *et al.*, 2000; Ritacco *et al.*, 1998) that are apparently more pathogenic to birds than other  
81 serotypes (Tell *et al.*, 2001). On the basis of genetic and phenotypic differences it has recently been proposed to  
82 differentiate *M. a. avium* into two subspecies based on the target organism: *M. a. hominissuis* for human and porcine  
83 isolates and *M. a. avium* for bird type isolates (Mijs *et al.*, 2002). *Mycobacterium a. hominissuis* has polymorphic multiband  
84 IS1245 RFLP patterns and is able to grow between 24 and 45°C (Mijs *et al.*, 2002; Van Soelingen *et al.*, 1998). It is worth  
85 noting that the typical features of bird isolates, the three band pattern in IS1245 RFLP and presence of IS901, have also  
86 been found in cervine and bovine isolates of *M. a. avium*.

87 Avian tuberculosis in birds is most prevalent in gallinaceous poultry and in wild birds raised in captivity. Turkeys are quite  
88 susceptible, but ducks, geese, and other water birds are comparatively resistant. The practices of allowing poultry to roam  
89 at large on the farm (free range) and of keeping the breeders for several years are conducive to the spread of the causal  
90 agent of avian tuberculosis among them. Infected individuals and contaminated environments (water and soil) are the main

<sup>1</sup> <https://psn.dsmz.de/species/mycobacterium-avium>

91 primary sources of infection. The above-mentioned mycobacterial species causing avian tuberculosis can survive for  
92 several months in the environment (Dvorska *et al.*, 2007; Kazda *et al.*, 2009; Shitaye *et al.*, 2008; Tell *et al.*, 2001).

93 ~~In most cases,~~ Infected birds usually show no clinical signs but ~~they~~ may eventually become lethargic and emaciated.  
94 Many affected birds show diarrhoea and swollen joints, and comb and wattles may regress and become pale. Affected  
95 birds, especially gallinaceous poultry, are usually older than 1 year. Some show respiratory signs ~~and, including~~ sudden  
96 death may occur, dyspnoea is less common, and granulomatous ocular lesions (Pocknell *et al.*, 1996) ~~as well as and~~ skin  
97 lesions have been reported. Under intensive husbandry conditions, sudden death may occur, often associated with severe  
98 lesions in the liver; such lesions are easily observed at post-mortem examination (Salamatian *et al.*, 2020; Tell *et al.*, 2001).

99 The primary lesions of avian tuberculosis in birds-poultry (chickens and turkeys) are nearly always in the intestinal tract.  
100 Such lesions take the form of deep ulcers filled with caseous material containing many mycobacterial cells, and these are  
101 discharged into the lumen and appear in the faeces. Before the intestinal tract is opened, the ulcerated areas appear as  
102 tumour-like masses attached to the gut wall, ~~but~~ Still, when the intestine is opened, the true nature of the mass becomes  
103 evident. Typical caseous lesions are nearly always found in the liver and spleen, ~~and~~; these organs are usually ~~are~~ greatly  
104 enlarged because of the formation of new tuberculous tissue. The lungs and other tissues are ordinarily free from lesions  
105 even in advanced cases (Salamatian *et al.*, 2020; Tell *et al.*, 2001; Thorel *et al.*, 1997).

106 ~~Among domestic animals (mammals), domestic pigs (*Sus scrofa f. domesticus*) are the most susceptible to avian~~  
107 ~~tuberculosis. Usually, no clinical manifestations are observed in such animals. Avian tuberculosis is suspected when~~  
108 ~~tuberculous lesions are found in the head and mesenteric lymph nodes on meat inspection after slaughter. Findings of~~  
109 ~~tuberculous lesions that involve other organs (liver, spleen, lungs, etc.) are rare, usually occurring at the advanced stage~~  
110 ~~of the disease. *Mycobacterium a. avium* accounted for up to 35% of the *Mycobacteria* isolated from such tuberculous~~  
111 ~~lesions (Dvorska *et al.*, 1999; Pavlik *et al.*, 2003, 2005; Shitaye *et al.*, 2006). Unlike the other species mentioned previously,~~  
112 ~~cattle are highly resistant to the causative agent of avian tuberculosis, and tuberculous lesions are detected in head lymph~~  
113 ~~nodes, or occasionally in liver lymph nodes, only on meat inspection. *Mycobacterium a. avium* can be successfully isolated~~  
114 ~~from tuberculous lesions in mesenteric lymph nodes from juvenile cattle: the isolation rate from cattle under 2 years of age~~  
115 ~~was 34.4% in contrast to 13.0% from cattle over 2 years of age (Dvorska *et al.*, 2004).~~

116 Pet and wild birds with avian mycobacteriosis have clinical presentations often exacerbated by age and viral and fungal  
117 co-infections (Schmidt *et al.*, 2022; Schmitz *et al.*, 2018b). The presence of nonspecific clinical signs and the absence of  
118 gross finds during necropsy in psittacine and passeriform birds may confound diagnosis. Furthermore, differences in body  
119 condition and gross pathology are observed, where psittacines have more severe lesions than passeriform birds. These  
120 differences could also be attributed to the fact that they are often more likely infected with *M. genavense* than *M. avium*  
121 (Schmitz *et al.*, 2018a). The advent of more affordable WGS has allowed the study of *M. avium* and *M. genavense* and  
122 their epidemiology in a large captive population of birds belonging to multiple taxa for over 22 years. In this large bird  
123 population, 68% of all birds at necropsy had isolates that were infected with *M. avium* or *M. genavense*. The WGS study  
124 of these mycobacterium isolates demonstrated strong evidence of disease clustering among those birds infected with *M.*  
125 *avium* but not among those harbouring *M. genavense* (Witte *et al.*, 2021). This works sheds light on the epidemiology of  
126 mycobacterium among captive birds, and future studies are necessary to understand these pathogens' epidemiology better  
127 and to help identify its reservoirs.

128 It is essential to bear in mind that all members of *M. avium*-~~complex~~ and *M. genavense* are capable of giving rise to a  
129 progressive disease in humans that is refractory to treatment, especially in immunocompromised individuals (Narsana *et*  
130 *al.*, 2023; Pavlik *et al.*, 2000; Tell *et al.*, 2001). ~~Members of *Mycobacterium avium* complex are classed in Risk Group 2 for~~  
131 ~~human infection and should be handled with appropriate measures~~ All *Mycobacterium* species can cause infection in  
132 people (Cowman *et al.*, 2019). Caution should be exercised by those working with birds in environments infected with  
133 *Mycobacterium*, especially those immunosuppressed. All laboratory manipulations with live cultures or potentially  
134 infected/contaminated material must be performed at an appropriate biosafety and containment level determined by  
135 conducting a thorough risk assessment as described in Chapter 1.1.4 Biosafety and biosecurity: Standard for managing  
136 biological risk in the veterinary laboratory and animal facilities. Biocontainment measures should be determined by risk  
137 analysis as described in Chapter 1.1.4. The CDC's online Manual for Biosafety in Microbiological and Biomedical  
138 Laboratories is also a good reference<sup>2</sup>.

<sup>2</sup> [https://www.cdc.gov/labs/pdf/SF\\_\\_19\\_308133-A\\_BMBL6\\_00-BOOK-WEB-final-3.pdf](https://www.cdc.gov/labs/pdf/SF__19_308133-A_BMBL6_00-BOOK-WEB-final-3.pdf)

## B. DIAGNOSTIC TECHNIQUES

**Table 1. Test methods available for the diagnosis of avian tuberculosis and their purpose**

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
<b>Detection and identification of the agent<sup>(a)</sup></b>						
Ziehl–Neelsen staining	–	–	–	++	–	–
Culture	–	–	–	++	–	–
Haemagglutination (stained antigen)	±	+++	±	–	++	–
PCR	+++	±	++	+++	±	–
<b>Detection of immune response</b>						
Haemagglutination (stained antigen)	±	+++	±	≡	++	≡
Tuberculin test	++	+++	+	–	++	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;

+ = suitable in very limited circumstances; – = not appropriate for this purpose.

PCR = polymerase chain reaction.

<sup>(a)</sup>A combination of agent identification methods applied to the same clinical sample is recommended.

### 1. Identification of the agent

If there is a characteristic history of avian tuberculosis in a flock and typical lesions are found in birds at post-mortem, the detection of acid-fast bacilli (AFB) in smears or sections from affected organs, stained by the Ziehl–Neelsen method usually is normally sufficient to establish a diagnosis. Confirmation of *M. avium* subspecies should be carried out by PCR or other molecular techniques (Kaevska *et al.*, 2010; Slana *et al.*, 2010). Occasionally a case will occur, presumably as a result of due to large infecting doses giving rise to acute overwhelming disease, in which affected organs, most obviously the liver, have a ‘morocco leather’ appearance with fine greyish or yellowish mottling. In such cases AFB may not be found in such cases, but careful inspection will reveal parallel bundles of brownish refractile bacilli. Prolongation of the hot carbol-fuchsin stage of Ziehl–Neelsen staining to 10 minutes will usually reveal that these are indeed AFB, with unusually high resistance to penetration of the stain. Recently, DNA probes and, polymerase chain reaction (PCR), and WGS techniques have been used to identify the agent at the species and subspecies level specifically. Matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF MS) is a valuable tool as well (Fernández-Esgueva *et al.*, 2021). Traditionally, *M. a. avium* is separated from common nonchromogenic slow-growing organisms by their ability to grow at 42°C (*M. a. avium*). The method has limited value, as other species are able to grow at 42°C. *Mycobacterium genavense* is particularly fastidious and has special-unique requirements for growth and identification (Shitaye *et al.*, 2010).

#### 1.1. Culture

If there is a characteristic flock history and suggestive lesions are found at necropsy, but no AFB are seen in smears or sections, an attempt must be made to isolate the causative organism from the necropsy material. The liver or spleen is usually the best organ to use, but if the carcass is decomposed, bone marrow may prove more satisfactory as it could be less contaminated. As with the culture of *M. bovis*, non-sterile specimens need to be processed with detergent, alkali, or acid to eliminate rapidly growing microorganisms before culture (see Chapter 3.1.13 *Mammalian tuberculosis [infection with Mycobacterium tuberculosis complex]*). *Mycobacterium a. avium* grows best on media such as Lowenstein–Jensen, Herrold’s medium, Middlebrook 7H10 and, 7H11, or Coletsos, with 1% sodium pyruvate added. It may occasionally be necessary to incorporate mycobactin J, as it is used for the isolation of to isolate *M. a. paratuberculosis genavense* and *M. a. silvaticum*. Growth may be confined to the edge of the condensation water. Cultures should be incubated for at least 8–12 weeks, less if using liquid media. Typically, *M. a. avium* produces ‘smooth’ colonies within 2–4 weeks; rough variants de

172 occur. Shorter incubation times can be achieved using the liquid culture **BACTEC** system or the automated  
173 fluorescent **MGIT-960** culture system. *Mycobacterium a. avium* can also be detected in massively infected tissue  
174 by a conventional PCR, which also allows acceleration of the accelerates pathogen detection and identification  
175 (Moravkova *et al.*, 2008). ~~Currently~~, Direct detection and quantification of *M. a. avium* using IS901 quantitative  
176 real-time PCR can be considered as the best fast and inexpensive method (~~despite its rather high cost per test~~)  
177 (Kaevska *et al.*, 2010; Slana *et al.*, 2010).

178 For *M. genavense*, the optimal medium is liquid media supplemented with Mycobactin J (an iron chelator) and  
179 then plated onto a solid medium is such as Middlebrook 7H11-medium acidified to pH 6 and supplemented with  
180 blood and charcoal (Realini *et al.*, 1999). The incubation period at 37°C with 5–7% CO<sub>2</sub> should be extended for  
181 at least 6 months ~~42 days~~. If samples are directly plated onto solid media, plates should be held for at least 12  
182 weeks. Bacterial growth should be prepared in a smear and stained using an acid-fast stain. All acid-fast  
183 organisms should be identified using MALDI-TOF (matrix assisted laser desorption ionisation–time of flight  
184 [mass spectrometry]) or PCR (Buckwalter *et al.*, 2016; Hall *et al.*, 2003; Shitaye *et al.*, 2010).

185 Typing of mycobacteria to the species and subspecies level requires a specialised laboratory. Conventional  
186 biochemical tests for species identification are lengthy and fail to distinguish between *M. avium* and  
187 *M. intracellulare*. Thus, a miscellaneous group of mycobacteria that includes both species is usually classified  
188 under the ~~denomination of~~ *M. avium* complex denomination. Seroagglutination, ~~which is based on the~~ sugar  
189 residue specificity of surface glycopeptidolipids, allows ~~classification the parsing of~~ *M. avium* complex organisms  
190 into 28 serovars (Wolinsky & Schaefer, 1973). More sophisticated typing methods directed at cell-wall-specific  
191 targets are currently available, such as enzyme-linked immunosorbent assays with monoclonal antibodies to  
192 major serovars, ~~and high-performance liquid chromatography (HPLC), and WGS. Based on DNA–rRNA~~  
193 hybridisation serovars 1 to 6, 8 to 11, and 21 are currently have been ascribed to *M. a. avium* and  
194 *M. a. hominissuis*, and serovars 7, 12 to 20, and 25 to *M. intracellulare*. However, no consensus was achieved  
195 on other serovars, and some isolates cannot be serotyped (Inderlied *et al.*, 1993). For final species and  
196 subspecies identification, the current methods are WGS and bioinformatic analysis of isolates obtained from  
197 sick birds. Avian tuberculosis in birds is commonly caused by *M. a. avium* types 1, 2, or 3. If the isolate is not  
198 one of these three serotypes, further molecular identification tests (IS901 PCR) must be carried out conducted  
199 in a specialised laboratory. However, it should be ~~borne in mind noted~~ that superficial-tuberculous lesions in  
200 caged pet-captive birds, especially psittacines, may be caused by *M. tuberculosis*, and IS6110 PCR should be  
201 used for precise identification should always be attempted (Hoop, 2002; Lanteri *et al.*, 2011; Peters *et al.*, 2007;  
202 Schmidt *et al.*, 2008; Tell *et al.*, 2001).

## 203 1.2. Nucleic acid recognition methods

204 Specific and reliable genetic tests for speciation ~~are currently have been~~ available (Saito *et al.*, 1990)–, including  
205 commercial nucleic acid-hybridisation probes have become a ‘gold standard’ reference method for distinction  
206 between distinguishing *M. avium*, and *M. intracellulare* cultures, and *M. genavense* can also be distinguished  
207 with these tests. A further probe that covers the whole *M. avium* complex was also developed, as genuine  
208 *M. avium* complex strains have been described that fail to react with specific *M. avium* and *M. intracellulare*  
209 probes (Soini *et al.*, 1996). Nevertheless, identification errors were reported due to the cross-reactivity, which  
210 may have serious consequences (van Ingen *et al.*, 2009). Various in-house molecular methods have been  
211 reported ~~for the identification of~~ to identify mycobacterial cultures, including MAC–members of the  
212 *Mycobacterium avium* complex. The following gene segments could be used to identify *Mycobacterium* isolates  
213 as *M. avium* in one multiplex PCR reaction: IS900, IS901, IS1245. The isolates of *M. a. avium*/*M. a. silvaticum*  
214 are IS900–, IS901+, IS1245+, the isolates of *M. a. hominissuis* are IS900–, IS901–, IS1245+, and the isolates  
215 of *M. a. paratuberculosis* are IS900+, IS901–, IS1245– (Kaevska *et al.*, 2010; Moravkova *et al.*, 2008). A  
216 multiplex–16S rRNA PCR and sequencing method for differentiating *M. avium* from *M. intracellulare* and  
217 *M. tuberculosis* complex has some advantages (Cousins *et al.*, 1996). 16S rRNA is currently commercially  
218 available. Similarly, many veterinary diagnostic laboratories commonly perform in-house PCR and sequencing  
219 (Kirschner *et al.*, 1993) may also be used. Culture-independent in-house molecular tests have been developed  
220 for the detection to detect and identification of identify species belonging to the *M. avium* complex directly from  
221 samples (Hall *et al.*, 2003; Kaevska *et al.*, 2010). WGS of isolates has recently become the go-to molecular  
222 method to identify mycobacterium isolates from birds with great accuracy. It enables, with the use of  
223 bioinformatic tools, not only an accurate identification of species and subspecies, but also helps to determine  
224 the organism relatedness within a flock or environment (Witte *et al.*, 2021). In recent years, veterinary diagnostic  
225 laboratories have extensively adopted real-time PCR methods to detect *M. a. avium* directly from different  
226 specimens (faeces, tissues, formalin-fixed tissues, and environmental samples). The technique rapidly detects  
227 fastidious and slow-growing microorganisms, such as *M. a. avium* (Tell *et al.*, 2003a; 2003b).

228 Several commercial diagnostic PCR tests for detecting *M. a. avium* are available. Still, users should consider  
229 the skill set and equipment necessary to perform such tests. Furthermore, it is important to determine the fitness

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230 for the purpose of these tests before implementation. The interpretation of the results of these molecular tests  
231 also requires veterinary expertise.

232 *Mycobacterium a. avium*, the causative agent of avian tuberculosis (Thorel *et al.*, 1990), previously designated  
233 as *M. avium* species only, is assigned to serotypes 1 to 3 within the *M. avium* complex of 28 serotypes (Wolinsky  
234 & Schaefer, 1973). As revealed by molecular biological studies, the detected insertion sequence IS901 (Kunze  
235 *et al.*, 1992) is possessed not only by the isolates of the above-named serotypes, but also by isolates, virulent  
236 for birds, that could not be typed because agglutination occurred (Pavlik *et al.*, 2000). In epidemiological studies,  
237 a standardised IS901-RFLP methods replaced serotyping (Dvorska *et al.*, 2003).

## 238 2. Immunological methods

239 Tests used for export depend on the importing requirement of individual countries. In the main, the tuberculin test or the  
240 haemagglutination (stained antigen) test are most frequently used for export testing of poultry.

### 241 2.1. Tuberculin test

242 The tuberculin test is the most widely used test ~~in-for~~ domestic fowl and the only test for which an international  
243 standard for the reagent exists. Tuberculin is the standard avian purified protein derivative (PPD). Birds are  
244 tested by intradermal inoculation in the wattle with 0.05 ml or 0.1 ml of tuberculin (containing approximately  
245 2000 International Units [IU]), using a ~~very~~ fine needle of approximately 26 gauge, 10 mm ~~long~~ x 0.5 mm. The  
246 test is read after 48 hours ~~and~~. A positive reaction is any swelling at the site, from a small firm nodule  
247 approximately 5 mm in diameter to gross oedema extending into the other wattle and down the neck. ~~With~~  
248 ~~practice~~. Even very small wattles on immature birds can be inoculated successfully. However, ~~in immature birds~~  
249 the comb may be used in immature birds, although the results are not ~~so~~ as reliable. Tuberculin testing of the  
250 wattle in turkeys is much less reliable consistent than in the domestic ~~fowl~~ chickens. Inoculation in the wing web  
251 has been recommended as ~~being~~ more efficient, but this is still not as good as ~~for domestic fowl~~ in chickens.  
252 Other birds may also be tested in the wing web, but results are not generally satisfactory. The bare ornamental  
253 skin areas on Muscovy ducks and some ~~species of~~ pheasant species can be used, but ~~reliability~~ dependability  
254 is doubtful, and interpretation is difficult. Testing in the foot web of waterfowl has also been described; the test  
255 is not very sensitive and is often complicated by infections of the inoculation site.

256 In the common pheasant (*Phasianus colchicus*), the tuberculin test can be performed in either of two ways. In  
257 the first, 0.05 ml or 0.1 ml of tuberculin is injected into the skin of the lower eyelid. A positive result is indicated  
258 by marked swelling at the injection site after 48 hours. Alternatively, 0.25 ml of tuberculin is injected into the  
259 thoracic muscles, and the birds are observed for 6–10 hours. Infected birds will show signs of depression and  
260 keep aside from the flock, and there may be cases of sudden death. No clinical signs will be provoked in  
261 uninfected birds.

### 262 2.2. Stained antigen test

263 The stained-antigen agglutination test has been used with good results, especially in domestic and ornamental  
264 waterfowl. A drop (0.05–0.1 ml) of the antigen is mixed with the same volume of fresh whole blood, obtained by  
265 venipuncture, on a white porcelain or enamel tile. The mixture is rocked for 2 minutes and examined for  
266 agglutination. The agglutination may be coarse, in which case it is obvious, or quite fine, in which case it may  
267 be most clearly seen as an accumulation of the malachite-green-stained antigen around the edge of the drop,  
268 leaving the centre a normal blood-red colour. This test is especially useful for screening large flocks for  
269 immediate culling and therefore has advantages over the tuberculin test for controlling the disease, even in  
270 domestic fowl. It has also been claimed that it is more reliable in domestic poultry than the tuberculin test.

#### 271 2.2.1. Preparation of the antigen

272 An antigen stained with 1% malachite green is used for the rapid whole blood plate agglutination test  
273 (Rozanska, 1965). The strain used to prepare the stained antigen must be smooth and not auto-  
274 agglutinate in saline suspension. It must conform to the characteristics of *M. a. avium*, preferably  
275 obtained from a culture collection, to guarantee its authenticity.

276 A strain that will detect infection with any serotype is recommended instead of the specific serotype most  
277 likely to be encountered (in Europe, serotype 2 for domestic fowl, serotype 1 for waterfowl, and birds and  
278 swine in the USA). Using a highly specific strain for the serotype is recommended. The specificity of  
279 strains can be determined only by testing them as antigens, although, in general, a serotype 2 antigen  
280 will always detect serotype 3 infection and vice versa. Serotype 1 strains detect a wide spectrum of  
281 infections and frequently detect infections with mycobactin-dependent mycobacteria or *M. a. silvaticum*.  
282 There is no reason not to use a culture containing more than one strain of *M. a. avium* if it shows the

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283 desired properties of sensitivity and specificity. Consistency of results between batches will be easier  
284 using pure cultures.

285 The organism should be grown in a suitable liquid medium, such as Middlebrook 7H9 containing 1%  
286 sodium pyruvate. Good growth should be obtained in approximately 7 days. The liquid culture is used as  
287 a seed for bulk antigen preparation.

288 Antigen for agglutination tests is best obtained on a solid medium, such as Löwenstein–Jensen or 7H11,  
289 containing 1% sodium pyruvate instead of glycerol, using Roux flasks or large bottles. Using a solid  
290 medium maximizes the chance of detecting contamination, and antigens grown in some liquid media are  
291 not agglutinated by specific antibodies. Liquid seed culture should be diluted (based on experience) to  
292 give discrete colonies on the solid medium. This will usually give the best yield increasing the chance of  
293 detecting contamination. About 10 ml of inoculum will usually allow it to wash over the whole surface and  
294 provide sufficient moisture to keep the air in the bottle near 100% humidity.

295 The bottles are incubated at 37°C, and good growth should be obtained in 14–21 days with most strains.  
296 The antigen is harvested by adding sterile glass beads and twice the volume of sterile normal saline  
297 (containing 0.3% formalin) as was used to inoculate the bottle. The bottle is then shaken gently to wash  
298 off all the growth, and the washing is collected into a sterile bottle and re-incubated at 37°C for 7 days.  
299 The killed bacilli are washed twice in sterile normal saline with 0.2% formalin by centrifugation and re-  
300 suspension. This sequence is safer than the original method in which the washing was carried out before  
301 the incubation that kills the organisms. Finally, bacilli are again centrifuged and re-suspended in sterile  
302 normal saline containing 0.2% formalin and 0.4% sodium citrate to a concentration of about 10<sup>10</sup> bacteria  
303 per ml. This corresponds to a concentration ten times that which matches tube No. 4 on McFarland's  
304 scale.

305 Cultures for antigen should be inspected for contamination daily for the first 5 days of incubation. The  
306 suspension made from the culture washings is also re-examined microscopically (for likely contaminants  
307 such as yeasts) and rechecked by culture to ensure that the formalin has killed the mycobacteria.

### 308 **2.2.2. Validation of the antigen**

309 Cultures should be checked by Gram staining for contamination by organisms other than mycobacteria.

310 One or more batches for agglutinating antigen must be tested for efficacy in using serum from naturally  
311 or artificially infected tuberculous birds by comparison with a standard preparation of known potency.  
312 When using animals for research or reagent testing, approval of the procedures and the use of animals  
313 by the institution's ethics committee should be sought before any testing occurs. The potency relative to  
314 that of the standard preparation must not differ significantly from that declared on the label. Each bottle  
315 of antigen must be tested with normal chicken serum (to detect autoagglutination) and *M. a. avium*  
316 positive chicken serum of low and high antibody content. This should be done, where possible, alongside  
317 a previous batch of stained antigens. Those bottles that give satisfactory agglutination reactions with the  
318 antisera can now be pooled and the antigen stained. This is done by adding 3 ml of 1% malachite green  
319 solution per 100 ml of suspension. The stained antigen should be checked using whole blood, just as  
320 the unstained antigen was tested with serum. The agglutinating antigen should stay in the refrigerator  
321 for at least 6 months at 4°C and much longer if frozen at –20°C or below. If a batch has not been used  
322 for several weeks, it should be rechecked, especially for autoagglutination.

323 It is critical to perform a safety test of the unwashed antigen by culture and incubation to ensure that all  
324 the bacilli are dead.

### 325 **Note on limitation of use**

326 Neither the tuberculin test with avian tuberculin nor the stained-antigen agglutination test is likely to be of any value in  
327 cases of *M. tuberculosis* infection in caged-pet birds.

## 328 **C. REQUIREMENTS FOR DIAGNOSTIC BIOLOGICALS**

### 329 **1. Background**

330 No vaccines are available.

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331 Avian tuberculin is a preparation of purified protein derivatives (PPD-A) made from the heat-treated products of growth of  
332 *M. a. avium*. It is used by intradermal injection to reveal delayed hypersensitivity as a means of identifying to identify birds  
333 infected with or sensitised to the same species of tubercle bacillus *Mycobacterium*. Importantly it is also used as an aid  
334 to differential diagnosis in the comparative intradermal tuberculin test for bovine tuberculosis (see Chapter 3.1.13). An  
335 international standard preparation of PPD-A is being developed by WAOH to replace the former WHO Standard<sup>3</sup>.

336 The general principles as given in Chapter 1.1.8 *Principles of veterinary vaccine production*, should be followed for  
337 injectable diagnostic biologicals such as tuberculin. The standards set out here and in chapter 1.1.8 are intended to be  
338 general in nature and may be supplemented by national and regional requirements.

## 339 2. Outline of production and minimum requirements for tuberculin production

### 340 2.1. Characteristics of the seed

#### 341 2.1.1. Biological characteristics of the master seed

342 Strains of *M. a. avium* used to prepare seed cultures should be purchased from a culture collection and  
343 identified as to species by appropriate tests. Several strains are recommended by for this purpose in  
344 different countries. For example, in the European Union (EU), for example, are D4ER and TB56.  
345 Reference may also be made to are recommended. The relevant national recommendations should be  
346 followed. Globally there are commercial sources for PPD-A.

#### 347 2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

348 Seed cultures should be shown to be free from contaminating organisms and to be capable of producing  
349 tuberculin with of sufficient potency. The necessary tests are described below.

### 350 2.2. Method of manufacture

#### 351 2.2.1. Procedure

352 The seed material is kept as a stock of freeze-dried cultures. If the cultures have been grown on solid  
353 media, it will be necessary to adapt the organism to grow as a floating culture. This is most easily  
354 accomplished by incorporating a piece of potato in the flasks of liquid medium (e.g. Watson Reid's  
355 medium). When the culture has been adapted to a liquid medium, it can be maintained by a passage at  
356 2–4-week intervals (Angus, 1978; Haagsma & Angus, 1995).

357 The organism is cultivated in modified Dorset-Henley's synthetic medium, then killed by heating in flowing  
358 steam and filtered to remove cells. The protein in the filtrate is precipitated chemically (ammonium  
359 sulphate or trichloroacetic acid [TCA] are used), washed, and resuspended. An antimicrobial  
360 preservative that does not give rise to false-positive reactions, such as phenol (not more than 0.5% [w/v]),  
361 may be added. Mercurial derivatives should not be used. Glycerol (not more than 10% [w/v]) or glucose  
362 (2.2% [w/v]) may be added as a stabiliser. The product is dispensed aseptically into sterile neutral glass  
363 containers, which are then sealed to prevent contamination. The product may be freeze-dried.

#### 364 2.2.2. Requirements for ingredients

365 The production culture substrate must be shown to be capable of producing produce a product that  
366 conforms to the standards of the European Pharmacopoeia (2000-2024<sup>4</sup>) standards or other international  
367 standards such the WHO (WHO, 1987). It must be free from ingredients known to cause toxic or allergic  
368 reactions.

#### 369 2.2.3. In-process controls

370 The production flasks, inoculated from suitable seed cultures, are incubated for the appropriate time  
371 period. Any flasks showing contamination or grossly abnormal growth should be discarded after  
372 autoclaving. As incubation proceeds, the surface growth of many cultures becomes moist and may sink  
373 into the medium or to the bottom of the flask. In PPD-A tuberculin, the pH of the dissolved precipitate  
374 (the so-called concentrated tuberculin) should be pH 6.6–6.7. The Kjeldahl method determines the  
375 protein level (total organic nitrogen) of the PPD-A concentrate is determined by the Kjeldahl method.  
376 Total nitrogen and trichloroacetic acid precipitable nitrogen are usually compared.

<sup>3</sup> PPD of *M. avium* tuberculin, WHO (1955) Technical Report Series, no.96, 11.

<sup>4</sup> [https://www.edqm.eu/en/d/234640?p\\_l\\_back\\_url=%2Fen%2Fsearch%3Fq%3Dpurified%2Bprotein%2Bderivative](https://www.edqm.eu/en/d/234640?p_l_back_url=%2Fen%2Fsearch%3Fq%3Dpurified%2Bprotein%2Bderivative)

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#### 2.2.4. Final product batch tests

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i) Sterility

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Sterility testing is generally performed according to the European Pharmacopoeia (2000-2024) or other guidelines (see ~~also~~ Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use*).

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ii) Identity

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One or more batches of tuberculin may be tested for specificity together with a standard preparation of bovine tuberculin by comparing the reactions produced in guinea-pigs sensitised with *M. bovis* using a procedure similar to that described in Section C.2.2.4.iv. ~~In guinea-pigs sensitised with *M. bovis*.~~ The potency of the preparation of avian tuberculin must be shown to be not more than 10% of the potency of the standard preparation of bovine tuberculin used in the potency test. The use of animals for this purpose should be reviewed and approved by your institution's ethical committee.

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iii) Safety

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Tuberculin PPD<sub>A</sub> can be examined for freedom from living mycobacteria using the culture method described previously. This culture method, which does not require the use of animals, is used in many laboratories, and its use is encouraged over the use of animals for this purpose. The following is the previously described method, using experimental animals to evaluate the safety of PPD. The use of animals for this purpose should be reviewed and approved by the institution's ethics committee. Two guinea-pigs, each weighing not less than 250 g and that have not been treated previously treated with any material that will interfere with the test, are injected subcutaneously with 0.5 ml of the tuberculin under test. No abnormal effects should occur within 7 days.

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Tests on tuberculin for living mycobacteria may be performed either on the tuberculin immediately before it is dispensed into final containers or on samples taken from the final containers themselves. A sample of at least 10 ml must be taken and ~~this must be~~ injected intraperitoneally or subcutaneously into at least two guinea-pigs, dividing the volume to be tested equally between the guinea-pigs. It is desirable to take a larger sample, 50 ml, and to concentrate any residual mycobacteria by centrifugation or membrane filtration. The guinea-pigs are observed for at least 42 days and are examined macroscopically at post-mortem. Any lesions found are examined microscopically and by culture. Each filled container must be inspected before it is labelled, and any showing abnormalities must be discarded.

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A test for the absence of toxic or irritant properties must be ~~carried out~~ conducted according to the ~~specifications of the~~ European Pharmacopoeia (2000-2024) specifications or the equivalent regulatory documents for each country or region.

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To test for lack of sensitising effect, three guinea-pigs that have not previously been treated with any material that could interfere with the test are each injected intradermally on ~~each of three~~ occasions with the equivalent of 500 ~~IU~~ International units – one IU is equal to the biological activity 0.02 µg of PPD – of the preparation under test in a 0.1 ml volume. In the USA and Canada, the potency of the tuberculin is expressed as tuberculin unit (TU) rather than IU. One TU is also defined as 0.02 µgs of PPD. Each guinea-pig, together with ~~each of the~~ three control guinea-pigs that have not been injected previously, is injected intradermally 15–21 days after the third injection with the same dose of ~~the same~~ tuberculin. The reactions of the two groups of guinea-pigs should not be significantly different when measured 24–28 hours later.

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iv) Batch potency

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The potency of avian tuberculin is determined in guinea-pigs sensitised with *M. a. avium*, ~~by comparison~~ compared with a standard preparation calibrated in IU or TU.

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Use no fewer than nine albino guinea-pigs, each weighing 400–600 g. Sensitise the guinea-pigs by administering ~~to each, by deep intramuscular injection,~~ a suitable dose of inactivated or live *M. a. avium* to each by deep intramuscular injection. The test is performed between 4 and 6 weeks later ~~as follows: Shave. Briefly, have~~ the guinea-pigs' flanks shaved (an area large enough so as to provide space for three-to-four injections on each side). Prepare at least three dilutions of the tuberculin under test and at least three dilutions of the standard preparation in an isotonic buffer solution containing 0.0005% (w/v) polysorbate 80 (Tween 80). Choose the dilutions so that the reactions produced have diameters of not less than 8 mm and not more than 25 mm. Allocate the

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431 dilutions to the injection sites randomly ~~according to~~ using a Latin square design. The dilutions  
432 correspond to 0.001, 0.0002, and 0.00004 mg of protein in a final dose of 0.2 ml, injected  
433 intradermally.

434 At 24 hours, the reactions' diameters ~~of the reactions~~ are measured, and the results are calculated  
435 using standard statistical methods, taking the diameters to be directly proportional to the logarithms  
436 of the concentrations of the tuberculin. The estimated potency must be not less than 75% and not  
437 more than 133% of the potency stated on the label. The test is not valid unless the fiducial limits of  
438 error ( $p = 0.95$ ) are not less than 50% and not more than 200% of the estimated potency. If the  
439 batch fails a potency test, the test may be repeated one or more times, provided that the final  
440 estimate of potency and ~~of~~ fiducial limits is based on the combined results of all the tests.

441 It is recommended that avian tuberculin should contain the equivalent of at least 25,000 IU/ml or  
442 approximately 0.5 mg protein per ml, giving a dose for practical use of 2500 IU/0.1 ml.

### 443 3. Requirements for authorisation/registration/licensing

#### 444 3.1. Manufacturing process

445 The manufacturing process should follow the requirements of European Pharmacopoeia (~~2000-2024~~) or other  
446 international standards.

#### 447 3.2. Safety requirements

##### 448 3.2.1. Target and non-target animal safety

449 Antimicrobial preservatives or other substances that may be added to a tuberculin must have been shown  
450 not to impair the safety and effectiveness of the product. The maximum permitted concentrations for  
451 phenol is 0.5% (w/v), and for glycerol, it is 10% (v/v). The pH should be between 6.5 and 7.5.

##### 452 3.2.2. Precautions (hazards)

453 Experience ~~both~~ in humans and animals led to the observation that appropriately diluted tuberculin  
454 injected intradermally results in a localised reaction at the injection site without generalised  
455 manifestations. Even in very sensitive persons, severe, generalised reactions are extremely rare and  
456 limited.

#### 457 3.3. Stability

458 During storage, liquid avian tuberculin should be protected from the light and held at a temperature of 5°C  
459 ( $\pm 3^\circ\text{C}$ ). Freeze-dried preparations may be stored at higher temperatures (~~but not exceeding~~ 25°C) and protected  
460 from ~~the~~ light. During use, periods of exposure to higher temperatures or to direct sunlight should be kept at a  
461 minimum.

462 ~~Provided the tuberculins are~~ Following accepted practice, tuberculin should be stored at a temperature of  
463 between 2°C and 8°C and protected from light; they may be used up to the end of the following periods  
464 subsequent to ~~after~~ the last satisfactory potency test: Liquid PPD tuberculins: 2 years; lyophilised PPD-A  
465 tuberculins: 8 years; HCSM (heat-concentrated synthetic-medium) tuberculins diluted: 2 years. Recent research  
466 on the temperature stability of human, bovine, and avian tuberculin solutions has shown that they are stable for  
467 a year at 37°C. This should be further explored as these products are used in the field in remote areas of the  
468 world where maintaining temperature control is very difficult (Maes *et al.*, 2011).

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619 **NB:** There is currently (2024) no WOAHA Reference Laboratory for avian tuberculosis  
620 (please consult the WOAHA Web site for the current list:

621 <https://www.woaha.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3http://www.oie.int/>).

622

**NB:** FIRST ADOPTED IN 1989 AS TUBERCULOSIS IN BIRDS. MOST RECENT UPDATES ADOPTED IN 2014.

REUNIÓN DE LA COMISIÓN DE NORMAS BIOLÓGICAS

París, 5–9 de febrero de 2024

SECTION 3.4.

BOVINAE

CHAPTER 3.4.1.

BOVINE ANAPLASMOSIS

SUMMARY

**Definition of the disease:** Bovine anaplasmosis results from infection with *Anaplasma marginale*. A second species, *A. centrale*, has long been recognised and usually causes benign infections. *Anaplasma marginale* is responsible for almost all outbreaks of clinical disease. *Anaplasma phagocytophilum* and *A. bovis*, which infect cattle, ~~have been recently~~ are also included within the genus but they are not reported to *Anaplasma phagocytophilum* can cause clinical self-limiting disease in cattle. There are no reports of disease associated with *A. bovis* infection. The organism is classified in the genus *Anaplasma* belonging to the family Anaplasmataceae of the order Rickettsiales.

**Description of the disease:** Anaemia, jaundice in acute, severe cases and sudden-unexpected death are characteristic signs of bovine anaplasmosis. Other signs include rapid loss of milk production and weight, but the clinical disease can only be confirmed by identifying the organism. Once infected, cattle may remain carriers for life, and identification of these animals depends on the detection of specific antibodies using serological tests, or of rickettsial DNA using molecular amplification techniques. The disease is typically transmitted by tick vectors, but mechanical transmission by biting insects or by needle can occur.

**Detection ~~Identification~~ of the agent:** Microscopic examination of blood or organ smears stained with Giemsa stain is the most common method of identifying *Anaplasma* in clinically affected animals. In these smears, *A. marginale* organisms appear as dense, rounded, intraerythrocytic bodies approximately 0.3–1.0 µm in diameter situated on or near the margin of the erythrocyte. *Anaplasma centrale* is similar in appearance, but most of the organisms are situated toward the centre of the erythrocyte. It can be difficult to differentiate *A. marginale* from *A. centrale* in a stained smear, particularly with low levels of rickettsaemia. Commercial stains that give very rapid staining of *Anaplasma* spp. are available in some countries. *Anaplasma phagocytophilum* can only be observed in infected granulocytes, mainly neutrophils and *A. bovis* can only be observed in infected monocytes ~~infecting granulocytes, mainly neutrophils~~.

It is important that smears be well prepared and free from foreign matter. Smears from live cattle should preferably be prepared from blood drawn from the jugular vein or another large vessel. For post-mortem diagnosis, smears should be prepared from internal organs (including liver, kidney, heart and lungs) and

31 from blood retained in peripheral vessels. The latter are particularly ~~desirable~~ useful if post-mortem  
32 decomposition is advanced.

33 **Serological tests:** A competitive enzyme-linked immunosorbent assay (C-ELISA) has ~~been demonstrated~~  
34 ~~to have~~ good sensitivity in detecting carrier animals. Card agglutination is the next most frequently used  
35 assay. The complement fixation test (CFT) is no longer considered a reliable test ~~for disease certification of~~  
36 ~~individual animals~~ due to variable sensitivity. Cross reactivity between *Anaplasma* spp. can complicate  
37 interpretation of serological tests. In general, the C-ELISA has the best specificity, with cross-reactivity  
38 described between *A. marginale*, *A. centrale*, *A. phagocytophilum* and *Ehrlichia* spp. Alternatively, an  
39 indirect ELISA ~~using the CFT with modifications~~ (I-ELISA) is a reliable test used in many laboratories and  
40 can be prepared in-house for routine diagnosis of anaplasmosis. Finally, a displacement double-antigen  
41 sandwich ELISA has been developed to differentiate between *A. marginale* and *A. centrale* antibodies.

42 Nucleic-acid-based tests have been used ~~are often used~~ in diagnostic laboratories and experimentally,  
43 and are capable of detecting the presence of low-level infection in carrier cattle and tick vectors. A nested  
44 conventional polymerase chain reaction (PCR) reaction is necessary ~~has been used~~ to identify low-level  
45 ~~carriers using conventional polymerase chain reaction (PCR),~~ and although nonspecific amplification can  
46 occur. Recently, Real-time PCR assays with have analytical sensitivity equivalent to nested conventional  
47 PCR have been described and are preferable in a diagnostic setting to reduce the risk of amplicon  
48 contamination.

49 **Requirements for vaccines:** Live vaccines are used in several countries to protect cattle against  
50 ~~*A. marginale* infection~~ bovine anaplasmosis. A vaccine consisting of live *A. centrale* is most widely used and  
51 gives partial protection against challenge with virulent *A. marginale*. Vaccination with *A. centrale* leads to  
52 infection and long-term persistence in many cattle. Vaccinated cattle are typically protected from disease  
53 caused by *A. marginale*, but not infection.

54 *Anaplasma centrale* vaccine is provided in chilled or frozen forms. Quality control is very important as other  
55 blood-borne agents that may be present in donor cattle can contaminate vaccines and be disseminated  
56 broadly. For this reason, frozen vaccine is recommended as it allows thorough post-production quality control,  
57 which limits the risk of contamination with other pathogens.

58 *Anaplasma centrale* vaccine is not entirely safe. A practical recommendation is to restrict its use, as far as  
59 possible, to calves, as nonspecific immunity will minimise the risk of some vaccine reactions that may require  
60 treatment with tetracycline or imidocarb. Partial immunity develops in 6–8 weeks and lasts for several years  
61 after a single vaccination. In countries where *A. centrale* is exotic, it cannot be used as a vaccine against *A.*  
62 *marginale*.

## 63 A. INTRODUCTION

64 Outbreaks of bovine anaplasmosis are due to infection with *Anaplasma marginale*. ~~*Anaplasma centrale* is capable of~~  
65 ~~producing can produce~~ a moderate degree of anaemia, but clinical outbreaks in the field are extremely rare. ~~New species~~  
66 ~~of *Anaplasma*. Other members of the family Anaplasmataceae that infect cattle include *A. phagocytophilum* and *A. bovis*~~  
67 ~~(Dumler et al., 2001), with a primary reservoir. *Anaplasma phagocytophilum* has a broad host range and causes the~~  
68 ~~diseases human granulocytic anaplasmosis (HGE), equine granulocytic anaplasmosis (EGA), and canine granulocytic~~  
69 ~~anaplasmosis (CGA), in humans, horses, and dogs, respectively (Matei et al., 2019). In northern Europe in rodents, *A.*~~  
70 ~~*phagocytophilum* causes tick-borne fever, primarily affecting lambs. In cattle, *A. phagocytophilum* infections have been~~  
71 ~~reported to infect cattle, but do not cause~~ from many geographical regions, however the association with disease is less  
72 commonly reported. Naturally occurring clinical disease as reported in Germany was characterised by fever (39.5–41.7°  
73 C), sudden reduction in milk production, lower limb oedema, and stiffness with leukopenia, erythropenia, neutropenia,  
74 lymphocytopenia and monocytopenia. The affected animals recovered without antibiotic treatment (Droher et al., 2005;  
75 Hofmann-Lehmann et al., 2004; Silaghi et al., 2018).

76 The most marked clinical signs of bovine anaplasmosis are anaemia and jaundice, the latter occurring in acute severe  
77 cases or late in the disease. Haemoglobinaemia and haemoglobinuria are not present, and this may assist in the differential  
78 diagnosis of bovine anaplasmosis from babesiosis, which is often endemic in the same regions. The disease can only be  
79 confirmed, however, by identification of the organism in erythrocytes from the affected animal. Caution must be exercised  
80 if using nucleic acid techniques alone to diagnose *A. marginale* in anaemic cattle. Persistent, low-level infection can be  
81 detected by these techniques and may lead to a misdiagnosis of bovine anaplasmosis. Visualisation of *A. marginale* bodies  
82 in erythrocytes is therefore required for confirmation.

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83 *Anaplasma marginale* occurs in most tropical and subtropical countries and is widely distributed in ~~some more~~-temperate  
84 regions. *Anaplasma centrale* was first described from South Africa. The organism has since been imported by other  
85 countries – including Australia and some countries in South America, South-East Asia and the Middle East – for use as a  
86 vaccine against *A. marginale*.

87 *Anaplasma* species were, though originally regarded ~~described~~ as protozoan parasites, but further research showed they  
88 had no significant attributes to justify this description. Since the last major accepted revision of the are obligate intracellular  
89 Gram-negative bacteria. Based on taxonomy established in 2001 (Dumler *et al.*, 2001), the Family *Anaplasmataceae*  
90 (Order *Rickettsiales*) is now composed of four five genera, *Anaplasma*, *Ehrlichia*, *Neorickettsia*, and *Wolbachia*. The genus  
91 and *Aegyptianella* is retained within the Family *Anaplasmataceae* as genus *incertae sedis*. The revised genus, The genus  
92 *Anaplasma* now contains *Anaplasma marginale* as the type species, *A. phagocytophilum* the agent of human granulocytic  
93 ehrlichiosis (formerly *Ehrlichia phagocytophila* and *E. equi*), *A. platys*, and *A. bovis* (formerly *E. bovis*). *Haemobartonella*  
94 and *Eperythrozoon* are now considered most closely related to the mycoplasmas.

95 *Anaplasma* species are transmitted either mechanically or biologically by arthropod vectors. ~~Reviews based on careful~~  
96 study-Detection of reported transmission experiments list up pathogen DNA within a tick is insufficient to 19 different ticks  
97 as capable of determine the ability of a particular tick species to transmit a pathogen. Studies demonstrating transmission  
98 of the pathogen are critical in determining the potential role of a particular tick species in pathogen transmission-transmitting  
99 *A. marginale* (Kocan *et al.*, 2004). These are: *Argas persicus*, *Ornithodoros lahorensis*, Many studies have demonstrated  
100 the transmission ability of *Dermacentor albipictus*, *D. andersoni*, *D. hunteri*, *D. occidentalis*, *D. variabilis*,  
101 *Hyalomma excavatum*, *H. rufipes*, *Ixodes ricinus*, *I. scapularis*, and *D. albipictus*. Additionally, transmission by multiple  
102 *Rhipicephalus* species is well recognised including *R. annulatus* (formerly *Boophilus annulatus*), *R. bursa*, *R. calcaratus*,  
103 *R. decoloratus*, *R. evertsi*, *R. microplus*, *R. sanguineus* and *R. simus*. However, the classification of several ticks in these  
104 reports has been questioned, and *R. sanguineus*. Other species of *Rhipicephalus* also likely serve as biological vectors  
105 of *A. marginale*. *Anaplasma marginale* DNA has been widely reported in *Hyalomma* species, and transmission has been  
106 demonstrated with *H. excavatum*. It is likely that multiple *Hyalomma* species also serve as vectors of *A. marginale* (Shkap  
107 *et al.*, 2009).

108 Intrastadial or transstadial transmission is the usual mode can occur, even in the one-host, *Rhipicephalus* species. Male  
109 ticks may be particularly important as vectors, as they can become persistently infected and serve as a reservoir are most  
110 likely to move between cattle searching for infection-female ticks. Experimental demonstration of vector competence does  
111 not necessarily imply a role in transmission in the field. However, *Rhipicephalus* species are clearly important vectors of  
112 anaplasmosis in countries such as Australia and countries in, many regions of Africa, and Latin America, and some species  
113 of *Dermacentor spp.* are efficient vectors in the United States of America (USA).

114 Various other biting arthropods have been implicated as mechanical vectors, particularly in the USA. Experimental  
115 transmission has been demonstrated with a number of species of *Tabanus* (horseflies), and with mosquitoes of the genus  
116 *Psorophora* (Kocan *et al.*, 2004). The importance of biting insects in the natural transmission of anaplasmosis appears to  
117 vary greatly from region to region. *Anaplasma marginale* also can be readily transmitted during vaccination against other  
118 diseases unless a fresh or sterilised needle is used for injecting each animal. Similar transmission by means of unsterilised  
119 surgical instruments has been described (Reinbold *et al.*, 2010a).

120 The main-only known biological vectors of *A. centrale* appear to be multihost ticks is *R. simus*, endemic in Africa, including  
121 *R. simus*. ~~The~~ Though multiple transmission studies have been done, there is no evidence that the common cattle tick  
122 (*R. microplus*) has not been shown to be can serve as a vector for *A. centrale*. This is of relevance-relevant where  
123 *A. centrale* is used as a vaccine in *R. microplus*-infested regions.

124 *Anaplasma marginale* infection has not been reported in humans. ~~Thus, There~~ is no minimal risk of field or laboratory  
125 transmission to workers and from laboratories working with *A. marginale* may operate at the lowest biosafety level,  
126 equivalent to BSL1. Nevertheless the agent should be handled with appropriate biosafety and containment procedures as  
127 determined by biorisk analysis (see Chapter 1.1.4 Biosafety and biosecurity: Standard for managing biological risk in the  
128 veterinary laboratory and animal facilities).



## B. DIAGNOSTIC TECHNIQUES

**Table 1. Test methods available for the diagnosis of bovine anaplasmosis and their purpose**

Method	Purpose					
	Population freedom from infection <sup>(a)</sup>	Individual animal freedom from infection prior to movement <sup>(b)</sup>	Contribute to eradication policies <sup>(c)</sup>	Confirmation of clinical cases <sup>(d)</sup>	Prevalence of infection – surveillance <sup>(e)</sup>	Immune status in individual animals or populations (post-vaccination) <sup>(f)</sup>
Microscopic examination	–	± <sub>≡</sub>	–	+++	–	–
Detection of the agent <sup>(g)</sup>						
PCR	–	++ +	–	+++	–	–
Detection of immune response						
CAT <sup>(h)</sup>	–	–	–	–	+	+
C-ELISA <sup>(h)</sup>	+++	+++	+++	–	+++	+++
IFAT <sup>(h)</sup>	+	–	–	–	++	++
CFT	–	–	–	–	±	–
ddasELISA	≡	≡	≡	≡	≡	±±

Key: +++ = recommended for this purpose; ++ recommended but has limitations; + = suitable in very limited circumstances; – = not appropriate for this purpose.

Agent id. = agent identification; CAT = card agglutination test; CFT = complement fixation test;

C-ELISA = competitive enzyme-linked immunosorbent assay; ddasELISA = displacement double-antigen, sandwich ELISA;

IFAT = indirect fluorescent antibody test; PCR = polymerase chain reaction.

<sup>(a)</sup>See Appendix 1 of this chapter for justification table for the scores giving to the tests for this purpose.

<sup>(b)</sup>See Appendix 2 of this chapter for justification table for the scores giving to the tests for this purpose.

<sup>(c)</sup>See Appendix 3 of this chapter for justification table for the scores giving to the tests for this purpose.

<sup>(d)</sup>See Appendix 4 of this chapter for justification table for the scores giving to the tests for this purpose.

<sup>(e)</sup>See Appendix 5 of this chapter for justification table for the scores giving to the tests for this purpose.

<sup>(f)</sup>See Appendix 6 of this chapter for justification table for the scores giving to the tests for this purpose.

<sup>(g)</sup>A combination of agent identification methods applied on the same clinical sample is recommended.

<sup>(h)</sup>These tests do not distinguish infected from vaccinated animals.

### 1. Detection of the agent

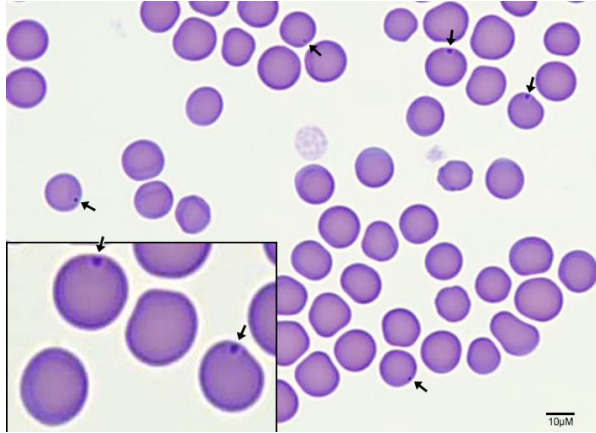
#### 1.1. Microscopic examination

Samples from live cattle should include thin blood smears and blood collected into an anticoagulant. Air-dried thin blood smears can be kept satisfactorily at room temperature for at least 1 week. The blood sample in anticoagulant should be held and transferred at 4°C, unless it can reach the laboratory within a few hours. This sample is useful for preparing fresh smears if those submitted are not satisfactory. In addition, a low packed cell volume and/or erythrocyte count can help to substantiate the involvement of *A. marginale* when only small numbers of the ~~parasites bacteria~~ are detected in smears, ~~for example particularly~~ during the recovery stage of the disease.

In contrast to *Babesia bovis*, *A. marginale* ~~does infected erythrocytes do~~ not accumulate in capillaries, so blood drawn from the jugular or other large vessel is satisfactory. *Anaplasma marginale* replicate in the erythrocytes to form small membrane-bound colonies, also termed inclusion bodies or initial inclusion bodies. ~~Because of the rather indistinctive morphology of Anaplasma~~ These initial inclusion bodies can be visualised on a blood smear, but are small and easily confused with debris or stain precipitate (see Figure 1). Thus it is essential that smears are well prepared and including ensuring slides are free from foreign matter, as specks of debris can confuse

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diagnosis and stain is recently filtered (Watman #1 filter paper). Thick blood films as are used sometimes for the diagnosis of babesiosis are not appropriate for the diagnosis of anaplasmosis, as *Anaplasma A. marginale* are difficult to identify once they become dissociated from erythrocytes.



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163 Fig. 1. *Anaplasma marginale* initial inclusion bodies. A Diff-Quick stained blood smear from a bovine experimentally  
164 infected with *A. marginale*. Arrows point to the *A. marginale* initial inclusion bodies.  
165 Photo from S. Noh.

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Samples from dead animals should include air-dried thin smears from the liver, kidney, heart and lungs and from a peripheral blood vessel. The latter is particularly recommended should there be a significant delay before post-mortem examination because, under these circumstances, bacterial contamination of organ smears often makes identification of *Anaplasma A. marginale* equivocal. Brain smears, which are useful for the diagnosis of some forms of babesiosis, are of no direct value for diagnosing anaplasmosis, but should be included for differential diagnosis where appropriate.

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Blood from organs, rather than organ tissues *per se*, is required for smear preparation, as the aim is to microscopically examine intact erythrocytes for the presence of *Anaplasma A. marginale* colonies. Organ-derived blood smears can be stored satisfactorily at room temperature for several days.

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Both blood and organ smears can be stained in 10% Giemsa stain for approximately 30 minutes after fixation in absolute methanol for 1 minute. After staining, the smears are rinsed three or four times with tap water to remove excess stain and are then air-dried. Conditions for Giemsa staining vary from laboratory to laboratory, but distilled water is not recommended for dilution of Giemsa stock. Water should be pH 7.2–7.4 to attain best resolution with Giemsa stain. Commercial stains that give very rapid staining of *Anaplasma A. marginale* are available in some countries. Smears are must be examined under oil immersion at a magnification of ×700–1000.

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*Anaplasma marginale* appear as dense, initial inclusion bodies are rounded and deeply stained intraerythrocytic bodies, and approximately 0.3–1.0 µm in diameter. Most of these bodies are located on or near the margin of the erythrocyte. This feature distinguishes *A. marginale* from *A. centrale*, as in the latter most of the organisms have a more central location in the erythrocyte. However, particularly at low levels of rickettsaemia, differentiation of these two species in smears can be difficult. Appendages associated with the *Anaplasma* body initial body have been described in some isolates of *A. marginale* (Kreier & Ristic, 1963; Stich *et al.*, 2004).

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The percentage of infected erythrocytes varies with the stage and severity of the disease. Maximum rickettsaemias in excess of 50% may occur with *A. marginale*. Multiple infections of individual erythrocytes are common during periods of high rickettsaemias.

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The infection becomes visible microscopically 2–6 weeks following transmission. During the course of clinical disease, the rickettsaemia approximately doubles each day for up to about 10 days, and then decreases at a similar rate. Severe anaemia may persist for some weeks after the parasites bacteria have become virtually undetectable in blood smears. Following recovery from initial infection, cattle remain latently infected for life.

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## 1.2. Polymerase chain reaction

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Nucleic acid-based tests to detect *A. marginale* infection in carrier infected cattle have been developed although not yet fully validated. The analytical sensitivity of polymerase chain reaction (PCR)-based methods has been

198 estimated at 0.0001% infected erythrocytes, but at this level, only a proportion of carrier cattle would be detected.  
199 A nested PCR has been used to identify *A. marginale* carrier cattle with a capability of identifying as few as 30  
200 infected erythrocytes per ml of blood, well below the lowest levels in carriers. However, nested PCR is time  
201 consuming as it requires two full PCR reactions, and poses significant quality control and specificity problems  
202 for routine use (Torioni De Echaide *et al.*, 1998). Real-time PCR assays are reported to achieve a level of  
203 analytical sensitivity equivalent to nested PCR has also been described for identification of *A. marginale* and  
204 should be considered instead of the nested PCR (Carelli *et al.*, 2007; Decaro *et al.*, 2008; Reinbold *et al.*, 2010b).  
205 Two Advantages of this technique the real-time PCR, which uses a single closed tube for amplification and  
206 analysis, are reduced opportunity for risk of amplicon contamination and a semi-quantitative assay result.  
207 Equipment and reagents needed for real-time PCR is are expensive, requires preventive maintenance, and may  
208 be beyond the capabilities of some laboratories. Real-time PCR assays may target one of several genes (Carelli  
209 *et al.*, 2007; Decaro *et al.*, 2008), or 16S rRNA (Reinbold *et al.*, 2010b), and are reported to achieve a level of  
210 analytical sensitivity equivalent to nested conventional PCR (Carelli *et al.*, 2007; Decaro *et al.*, 2008; Reinbold  
211 *et al.*, 2010b).

212 The most widely cited assays for the detection *A. marginale* in individual animals use a probe for increased  
213 specificity and are designed to detect *msp1b* (Carelli *et al.*, 2007) or *msp5* (Futse *et al.*, 2003) in genomic DNA  
214 extracted from whole blood. The assay based on detection of *msp1b* has been partially validated to detect the  
215 pathogen in individual animals and was used to define samples for the validation of a C-ELISA (Carelli *et al.*,  
216 2007; Chung *et al.*, 2014). The analytical test performance of this assay is robust, and exclusivity testing  
217 confirmed other bacterial and protozoal tick-borne pathogens of cattle were not detected. The assay, evaluated  
218 using 51 blood samples from 18 cattle herds in three regions of southern Italy, had 100% concordance with  
219 nested PCR.

220 *Msp1b* is a multigene family. Based on the annotation of the St. Maries strain of *A. marginale*, the designed  
221 primers and probe will amplify multiple members of this gene family, including *msp1b-1*, *msp1b-2*, and *msp1-*  
222 *pg3*. This may help increase diagnostic sensitivity, but may pose challenges if quantification of the pathogen is  
223 desired. Additionally, some *A. marginale* strains have single nucleotide polymorphisms in *msp1b* within the  
224 primer and probe binding regions. Thus, if *msp1b* is used as a diagnostic target, primer and probe design should  
225 consider local *A. marginale* strains. *Msp1b* has the advantage as a target in that orthologs of this gene family  
226 are absent in the related *A. phagocytophilum* and *Ehrlichia* spp., including *E. ruminantium*, thus helping ensure  
227 specificity of the test.

228 *Msp5* has also been used as a target to detect *A. marginale* in cattle in field samples and more frequently in  
229 experimental samples (Futse *et al.*, 2003). *Msp5* is highly conserved among *A. marginale* strains and is a single  
230 copy gene, thus providing some advantages as a target for ensuring detection of widely variant strains of *A.*  
231 *marginale*. However, the related *Anaplasma* spp. and *Ehrlichia* spp. all have *msp5* orthologs with 50% identity  
232 to an *E. ruminantium* gene (NCBI accession: L07385.1), thus specificity must be determined in laboratory and  
233 field samples. Additionally, little work has been done to validate an *msp5*-based real-time PCR test for diagnostic  
234 purposes.

235 A third primer-probe set is designed to detect *A. marginale* using real-time, reverse transcriptase PCR. The  
236 primers amplify a 16sRNA gene segment from *A. marginale* and *A. phagocytophilum*, while the probe  
237 differentiates between the two species (Reinbold *et al.*, 2010b). The analytical performance of this assay is  
238 robust. However, the diagnostic sensitivity, specificity, and of particular importance with 16sRNA sequence-  
239 based tests, exclusivity for other tick-borne pathogens of cattle have not been evaluated. Additionally, this assay  
240 is designed for use following RNA extraction and reverse transcription, which is more laborious and expensive  
241 than DNA extraction. Bacterial RNA is rapidly degraded, and this may ultimately reduce diagnostic sensitivity of  
242 this assay.

243 In regions that use *A. centrale* as a vaccine, it may be useful to differentiate between *A. marginale* and  
244 *A. centrale* infected/vaccinated animals. PCR is best suited for this task. The real-time PCR assay developed  
245 by Carelli *et al.* can also be used in a duplex reaction to detect and differentiate between *A. centrale* and *A.*  
246 *marginale* (Decaro *et al.*, 2008). Primers and probe have been designed to specifically amplify a region of *A.*  
247 *centrale* *groEL*, but not *A. marginale* *groEL*, despite 97% sequence identity between the two genes. The *A.*  
248 *marginale*-specific primers and probes perform similarly in the single and duplex PCR (Carelli *et al.*, 2007).  
249 Using the same 51 field samples from cattle in Italy, the *A. centrale* assay had less analytical sensitivity  
250 compared with nested PCR and discordance in 4 of 51 samples between an *A. centrale* reverse line blot test  
251 and the duplex PCR assay.

**Table 2. Oligonucleotides used in PCR assays to detect *A. marginale* and *A. centrale***

<u>Assay</u>	<u>Reference</u>	<u>Oligonucleotides<sup>(a)</sup></u>	<u>Sequence 5'–3'<sup>(b)</sup></u>	<u>Amplicon size (bp)</u>	<u>NCBI accession number</u>
<u>Real-time PCR</u>	<u>Carelli et al., 2007</u>	<u><i>Am_msp1b_F</i></u>	<u>TTG-GCA-AGG-CAG-CAG-CTT</u>	<u>95</u>	<u>M59845</u>
		<u><i>Am_msp1b_R</i></u>	<u>TTC-CGC-GAG-CAT-GTG-CAT</u>		
		<u><i>Am_msp1b_PB</i></u>	<u>TCG-GTC-TAA-CAT-CTC-CAG-GCT-TTC-AI</u>		
<u>Real-time PCR</u>	<u>Futse et al., 2003</u>	<u><i>Am_msp5_F</i></u>	<u>GCC-AAG-TGA-TGG-TGA-TAT-CGA</u>	<u>151</u>	<u>M93392</u>
		<u><i>Am_msp5_R</i></u>	<u>AGA-ATT-AAG-CAT-GTG-ACC-GCT-G</u>		
		<u><i>Am_msp5_PB</i></u>	<u>AAC-GTT-CAT-GTA-CCT-CAT-CAA</u>		
<u>Reverse-transcription real-time PCR</u>	<u>Reinbold et al., 2010</u>	<u><i>16S rRNA_F<sup>(c)</sup></i></u>	<u>CTC-AGA-ACG-AAC-GCT-GG</u>	<u>142</u>	<u>M60313</u>
		<u><i>16S rRNA_R<sup>(c)</sup></i></u>	<u>CAT-TTC-TAG-TGG-CTA-TCC-C</u>		
		<u><i>Am_16S rRNA_PB<sup>(d)</sup></i></u>	<u>CGC-AGC-TTG-CTG-CGT-GTA-TGG-T</u>		
<u>Real-time PCR<sup>(d)</sup></u>	<u>Decaro et al., 2008</u>	<u><i>Ac_groEL_F<sup>(e, f)</sup></i></u>	<u>CTA-TAC-ACG-CTT-GCA-TCT-C</u>	<u>77</u>	<u>CP001759.1</u>
		<u><i>Ac_groEL_R<sup>(e, f)</sup></i></u>	<u>CGC-TTT-ATG-ATG-TTG-ATG-C</u>		
		<u><i>Ac_groEL_PB<sup>(e, f)</sup></i></u>	<u>TCA-TCA-TTC-TTC-CCC-TTT-ACC-TCG-T</u>		

<sup>(a)</sup>*Am* denotes *A. marginale*, *Ac* denotes *A. centrale*, *Pb* denotes probe sequence.

<sup>(b)</sup>Fluorophores and quenchers not included in probe sequences.

<sup>(c)</sup>Amplifies *A. phagocytophilum* and *A. marginale* 16S rRNA gene.

<sup>(d)</sup>Probe is specific for *A. marginale* 16S rRNA gene.

<sup>(e)</sup>Can be used as a duplex PCR with *msp1b* primers and probe based on Carelli et al., 2007.

<sup>(f)</sup>Primers and probe amplify *A. centrale groEL*.

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## 259 2. Serological tests

260 In general, unless animals have been treated or are at a very early stage of infection (<14 days), serology using the  
261 competitive enzyme-linked immunosorbent assay (C-ELISA), indirect ELISA (I-ELISA) or card agglutination test (CAT)  
262 (see below) may be the preferred methods of identifying infected animals in most laboratories. *Anaplasma marginale*  
263 infections usually persist for the life of the animal. However, except for occasional small recrudescences, *Anaplasma*  
264 *A. marginale* **initial inclusion bodies** cannot readily be detected in blood smears after acute rickettsaemia and, even end-  
265 point PCR may not detect the presence of *Anaplasma* ~~the pathogen~~ in blood samples from asymptomatic carriers. Thus,  
266 a number of serological tests have been developed with the aim of detecting persistently infected animals.

267 A feature of the serological diagnosis of anaplasmosis is the highly variable results with regard to both sensitivity and  
268 specificity reported for many of the tests from different laboratories. This is due at least in part to inadequate ~~evaluation~~  
269 validation of the tests using significant numbers of known positive and negative animals. ~~Importantly, the capacity of several~~  
270 ~~assays to detect known infections of long-standing duration has been inadequately addressed.~~ An exception is a C-ELISA  
271 (see below), which ~~has been~~ was initially validated using true positive and negative animals defined by nested PCR (Torioni  
272 De Echaide et al., 1998), and the card agglutination assay, for which relative sensitivity and specificity in comparison with  
273 the C-ELISA has been evaluated (Molloy et al., 1999). ~~And updated in 2014 (Chung et al., 2014).~~ Therefore, while most of  
274 the tests described in this section are useful for obtaining broad-based epidemiological data, caution is advised on their  
275 use for disease certification. The C-ELISA, I-ELISA and CAT are described in detail below.

276 It should be noted that there is a high degree of cross-reactivity between *A. marginale* and *A. centrale*, as well as cross-  
277 reactivity with both *A. phagocytophilum* and *Ehrlichia* spp. in serological tests (Al-Adhami et al., 2011; Dreher et al., 2005).  
278 While the infecting species can sometimes be identified using antigens from homologous and heterologous species,  
279 equivocal results are obtained on many occasions. Efforts have been made to develop tests that differentiate between  
280 naturally acquired immunity to *A. marginale* and vaccine acquired immunity due to immunisation with *A. centrale* (Bellezze  
281 et al., 2023; Sarli et al., 2020).

### 282 2.1. Competitive enzyme-linked immunosorbent assay

283 A C-ELISA using a recombinant antigen termed Major surface protein 5 (MSP5) is an immunodominant protein  
284 expressed by *A. marginale*, *A. ovis*, and *A. centrale*. In *A. marginale* the gene is highly conserved making it a  
285 useful target across broad geographical regions with high *A. marginale* strain diversity (Knowles et al., 1996;

286 Torioni De Echaide et al., 1998). Thus, a C-ELISA based on recombinantly expressed (rMSP5 and MSP5-) in  
287 combination with an MSP5-specific monoclonal antibody (mAb) has proven very sensitive and specific for  
288 detection of Anaplasma-infected animals (Hofmann-Lehmann et al., 2004; Molloy et al., 1999; Reinbold et al.,  
289 2010b; Strik et al., 2007). All A. marginale strains tested, along with Additionally, A. ovis and A. centrale, express  
290 the MSP5 antigen and induce infected animals produce antibodies against the immunodominant epitope  
291 recognised by the MSP5-specific mAb. A recent report mAb used in the C-ELISA. This C-ELISA was updated  
292 in 2014 to improve performance by using glutathione S-transferase (GST) instead of maltose binding protein  
293 (MBP) as the tag on the rMSP5 (Chung et al., 2014). This assay no longer requires adsorption to remove the  
294 antibodies directed against MBP, thus it is faster and easier than the previous version of the C-ELISA. The  
295 diagnostic sensitivity is 100% and the diagnostic specificity is 99.7% using a cut-off of 30% inhibition as  
296 determined by receiver operating characteristic (ROC) plot (Chung et al., 2014). For this validation, 385 sera  
297 defined as negative were from dairy cattle maintained in tick-free facilities from farms with no clinical history of  
298 bovine anaplasmosis. The 135 positive sera were from cattle positive for A. marginale using nested PCR and  
299 serology.

300 One study suggested that antibodies from cattle experimentally infected with A. phagocytophilum will test  
301 positive in the C-ELISA (Dreher et al., 2005). However, in another study no cross-reactivity could be  
302 demonstrated, and the mAb used in the assay did not react with A. phagocytophilum MSP5 in direct binding  
303 assays (Strik et al., 2007). Cross reactivity has been demonstrated between A. marginale and Ehrlichia spp. in  
304 naturally and experimentally infected cattle (Al-Adhami et al, 2011). Earlier studies had shown that the C-ELISA  
305 was 100% specific using 261 known negative sera from a non-endemic region, detecting acutely infected cattle  
306 as early as 16 days after experimental tick or blood inoculation, and was demonstrated to detect cattle that have  
307 been experimentally infected as long as 6 years previously (Knowles et al., 1996). In detecting persistently  
308 infected cattle from an anaplasmosis-endemic region that were defined as true positive or negative using a  
309 nested PCR procedure, the rMSP5 C-ELISA had a sensitivity of 96% and a specificity of 95% (Torioni De  
310 Echaide et al., 1998) A. marginale and Ehrlichia sp. BOV2010 isolated in Canada, in naturally and  
311 experimentally infected cattle (Al-Adhami et al, 2011).

312 Test results using the rMSP5 C-ELISA are available in less than 2.5-hours. A test kit is available commercially  
313 that contains specific instructions. Users should follow the manufacturer's instructions. In general, however, it is  
314 conducted as follows.

#### 315 **2.1.1. Kit reagents**

- 316 A 96-well microtitre plate coated with rMSP5 antigen,
- 317 A 96-well coated adsorption/transfer plate for serum adsorption to reduce background binding,
- 318 100×Mab\_peroxidase conjugate,
- 319 10× wash solution and ready-to-use conjugate diluting buffer,
- 320 Ready to use substrate and stop solutions,
- 321 Positive and negative controls

#### 322 **2.1.2. Test procedure**

- 323 i) Add 70 µl of undiluted serum sample to the coated adsorption/transfer plate and incubate at room  
324 temperature for 30 minutes.
- 325 ii) Transfer 50 µl per well of the adsorbed undiluted serum to the rMSP5-coated plate and incubate at  
326 room temperature for 60 minutes.
- 327 ii) Discard the serum and wash the plate twice using diluted wash solution.
- 328 iii) Add 50 µl per well of the 1× diluted MAb\_peroxidase conjugate to the rMSP5 coated plate wells,  
329 and incubate at room temperature for 20 minutes.
- 330 iv) Discard the 1×diluted MAb\_peroxidase conjugate and wash the plate four times using diluted wash  
331 solution.
- 332 v) Add 50 µl per well of the substrate solution, cover the plate with foil, and incubate for 20 minutes at  
333 room temperature.
- 334 vi) Add 50 µl per well of stop solution to the substrate solution already in the wells and gently tap the  
335 sides of the plate to mix the wells.
- 336 vii) Immediately read the plate in the plate reader at 620, 630 or 650 nm.

337

### 2.1.3. Test validation

338

The mean average optical density (OD) of the negative control must range from 0.40 to 2.10. The average per cent inhibition of the positive control must be  $\geq 30\%$ .

339

340

### 2.1.4. Interpretation of the results

341

The % inhibition is calculated as follows:

$$100 - \frac{\text{Sample OD} \times 100}{\text{Mean negative control OD}} = \text{Per cent inhibition}$$

342

$$\% \text{ inhibition} = 100[1 - (\text{Sample OD} \div \text{Negative Control OD})]$$

343

Samples with  $< 30\%$  inhibition are negative. Samples with  $\geq 30\%$  inhibition are positive.

344

Specificity of the MSP5 C-ELISA may be increased by using a higher percentage inhibition cut-off value (Bradway *et al.*, 2001); however the effect of this change on sensitivity has not been thoroughly evaluated.

345

346

Recently, an improved MSP5 C-ELISA was developed by replacing rMBP-MSP5 with rGST-MSP5 in addition to an improvement in the antigen-coating method by using a specific catcher system. The new rMSP5-GST C-ELISA was faster, simpler, had a higher specificity and an improved resolution compared with the rMSP5-MBP C-ELISA with MBP adsorption (Chung *et al.*, 2014).

347

348

349

350

## 2.2. Indirect enzyme-linked immunosorbent assay

351

An I-ELISA was first developed using the CAT antigen, which is a crude *A. marginale* lysate (see below), and it The test can be implemented where the commercial C-ELISA is not available. Unlike the C-ELISA, most reagents, such as buffers and ready-to-dissolve substrates, are available commercially in many countries. Any laboratory can prepare the antigen using local strains of *A. marginale*, though standardised methods have not been developed. I-ELISA uses small amounts of serum and antigen that and the sensitivity and specificity of the test standardised with true positive and negative sera is as good as for the C-ELISA. As it can be prepared in each laboratory, Only the general procedure is described here (Barry *et al.*, 1986). For commercial kits, the manufacturer's instructions should be followed. In the case of in-house I-ELISA, The sensitivity and specificity of the test was 87.3% and 98.4–99.6% respectively, though this varied by laboratory (Nielsen *et al.*, 1996). For general methods, refer to Barry *et al.* (1986). Initial bodies and membranes are obtained as for the complement fixation test (Rogers *et al.*, 1964). This antigen is treated with 0.1% sodium dodecyl sulphate for 30 minutes prior to fixing the antigen to the microtitre plate. For each laboratory, the specific amount of antigen has to must be adjusted/optimised to obtain the best reading and the least expenditure.

352

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364

Alternatively, rMSP5 can be used as the antigen in this test. This eliminates the need for preparation and standardisation of antigen derived from splenectomised, *A. marginale* infected animals (Silva *et al.*, 2006). In a comparison between I-ELISA using the CAT antigen and rMSP with a histidine tag (rMSP5-HIS), these two I-ELISAs performed identically. In this comparison, IFAT was used as the gold standard test (Silva *et al.*, 2006).

365

366

367

368

Test results using the I-ELISA are available in about 4 to 5 hours. It is generally conducted as follows:

369

### 2.2.1. Test reagents

370

A 96-well microtitre plate coated with ~~crude~~ *A. marginale* antigen,

371

PBS/Tween buffer, (PBS 0.1 M, pH 7.2, Tween 20 0.05%),

372

Blocking reagent (e.g. commercial dried skim milk)

373

Tris buffer 0.1 M, MgCl<sub>2</sub> 0.1 M, NaCl 0.05 M, pH 9.8

374

Substrate *p*-Nitrophenyl phosphate disodium hexahydrate

375

Positive and negative controls.

376

### 2.2.2. Test procedure (this test is run in triplicate)

377

i) Plates can be prepared ahead of time and kept under airtight conditions at  $-20^{\circ}\text{C}$ .

378

ii) Carefully remove the plastic packaging before using plates, being careful not to touch the bottom of them as this can distort the optical density reading.

379

- 
- 380 iii) Remove the lid and deposit 200 ml PBST20 solution in each well and incubate at room temperature  
381 (RT) for 5 minutes.
- 382 iv) For one plate, dissolve 1.1 g of skim milk (blocking agent) in 22 ml of PBST20.
- 383 v) Remove the plate contents and deposit in each well 200 µl of blocking solution, put the lid on and  
384 incubate at 37°C for 60 minutes.
- 385 vi) Wash the plate three times for 5 minutes with PBST20.
- 386 vii) Dilute all serum samples including controls 1/100 in PBST20 solution.
- 387 viii) Remove the contents of the plate and deposit 200 µl of diluted serum in each of the three wells for  
388 each dilution, starting with the positive and negative and blank controls.
- 389 ix) Incubate plate at 37°C covered for 60 minutes.
- 390 x) Wash three times as described in **point-subsection vi**.
- 391 xi) Dilute 1/1000 anti-IgG bovine alkaline phosphatase conjugate in PBST20 solution. Add 200 µl of  
392 the diluted conjugate per well. Incubate the covered plate at 37°C for 60 minutes.
- 393 ~~xii) Remove the lid and wash three times as described in point vi above make three washes with  
394 PBST20.~~
- 395 xiii) Remove the contents of the plate and deposit 195 µl of 0.075% *p*-Nitrophenyl phosphate disodium  
396 hexahydrate in Tris buffer in each well and incubate at 37°C for 60 minutes.
- 397 xiv) The reaction is quantified by a microplate reader spectrophotometer, adjusted to 405 nm  
398 wavelength. The data are expressed in optical density (OD).

### 399 2.2.3. Data analysis

400 Analysis of results should take into account the following parameters.

- 401 i) The mean value of the blank wells.
- 402 ii) The mean value of the positive wells with their respective standard deviations.
- 403 iii) The mean value of negative wells with their respective standard deviations.
- 404 iv) The mean value of the blank wells is subtracted from the mean of all the other samples if not  
405 automatically subtracted by the ELISA reader.
- 406 v) Control sera are titrated to give optical density values ranging from 0.90 to 1.50 for the positive and,  
407 0.15 to 0.30 for the negative control.

408 Positive values are those above the cut-off calculated value which is the sum of the average of the  
409 negative and two times the standard deviation.

410 ~~For purposes of assessing the consistency of the test operator, the error "E" must also be estimated;  
411 this is calculated by determining the percentage represented by the standard deviation of any against  
412 their mean serum.~~

413 As with all diagnostic tests, it is important to measure **repeatability-reproducibility**. For more details see  
414 Chapter 2.2.4 Measurement uncertainty.

### 415 **2.3. Displacement double-antigen sandwich ELISA to differentiate between *A. marginale* and** 416 ***A. centrale* antibodies**

417 In regions where vaccination with *A. centrale* is used to control bovine anaplasmosis, differentiation between *A.*  
418 *centrale*-vaccinated and *A. marginale*-infected animals may be useful. Because there is often high amino acid  
419 identity between *A. marginale* and *A. centrale* surface proteins, identifying unique targets for serological assays  
420 for this purpose is difficult. Epitopes from MSP5 (aa28-210, without the transmembrane region) that are not  
421 shared between *A. marginale* and *A. centrale* were used to develop a displacement double-antigen sandwich  
422 ELISA (ddasELISA) (Bellezze *et al.*, 2023; Sari *et al.*, 2020). The recombinant MSP5 epitopes from *A. marginale*  
423 or *A. centrale* are expressed in *E. coli* with a histidine tag and purified. The ELISA plates are then coated with  
424 either the recombinant *A. marginale* MSP5 epitope, or the *A. centrale* MSP5 epitope and blocked. Serum is  
425 added to the wells and allowed to incubate. Following washing, a combination of biotinylated and non-  
426 biotinylated recombinant proteins are added to improve specificity of the reaction (see below for specifics). The  
427 protein-biotin binding to the serum antibody is detected with a peroxidase-streptavidin based detection system.

428 The optical density for the *A. marginale* MSP5-coated well (ODAm) and the OD for the *A. centrale* MSP5 (ODAc)  
429 coated well for each animal is measured. If the OD for either target is <0.2, the sample is excluded from the  
430 analysis. For the remaining samples, the ratio between the OD values (ODAm/ODAc) is calculated. If the ratio  
431 is >0.38 the sample is considered positive for anti-*A. marginale* antibodies, and a ratio ≤ 0.38 is classified as  
432 vaccinated with *A. centrale*.

433 For the detection of *A. marginale* the test has a diagnostic specificity of 98% and a diagnostic sensitivity of  
434 98.9%. For 702 field samples evaluated, 131 (19%) had an OD <0.2 in the ddasELISA and thus were excluded  
435 from the analysis. Of those animals, 52% were nested PCR positive for *A. marginale*, 23% were nested PCR  
436 positive for *A. centrale*, 4.6% were nested PCR positive for *A. marginale* and *A. centrale*, 20% were nested PCR  
437 negative for both, suggesting the ddasELISA may lack sensitivity.

438 Of the 571 ddasELISA positive field samples, the agreement between the ddasELISA and nested PCR was  
439 84% and the kappa coefficient was 0.70 (95% CI: 0.635–0.754), indicating substantial agreement between tests.  
440 There was agreement between the ddasELISA and nested PCR for 93% of the *A. marginale* ddasELISA positive  
441 samples and 86% of the *A. centrale* ddasELISA positive samples. Additionally, 36 nested PCR negative  
442 samples tested positive for antibodies against *A. marginale* (n=28) or *A. centrale* (n=8) by ddasELISA. This test  
443 could not identify animals with co-infections, meaning animals vaccinated with *A. centrale* that are then infected  
444 with *A. marginale*, which is not uncommon.

445 Test results using the ddasELISA are available in 5–6 hours. It is conducted as outlined below, see Bellezze *et*  
446 *al.*, 2023 for more details.

### 447 **2.3.1. Test reagents**

- 448 i) A 96-well microtitre plate coated with either *A. marginale* or *A. centrale* recombinant protein
- 449 ii) PBS/Tween buffer (PBS (50mM sodium phosphate, 150 mM NaCl, pH 7.2) with 0.05% Tween-20)
- 450 iii) Blocking reagent (PBS with 10% commercial dried skim milk)
- 451 iv) Purified recombinant *A. marginale* MSP5 epitopes and *A. centrale* epitopes
- 452 v) Biotinylated recombinant *A. marginale* MSP5 epitopes and *A. centrale* epitopes
- 453 vi) Streptavidin-horse radish peroxidase (HRP) detection system
- 454 vii) Chromogenic substrate (1 mM 2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium  
455 salt in 0.05 M sodium citrate, pH 4.5, 0.0025% V/V H<sub>2</sub>O<sub>2</sub> (100 µl/well).
- 456 viii) ELISA plate reader (405 nm reading)
- 457 ix) Positive and negative control sera for *A. marginale* and *A. centrale*

### 458 **2.3.2. Test procedure**

- 459 i) Plates are coated overnight.
- 460 ii) Block with blocking buffer for 1 hour at room temperature and wash three times with PBS/Tween  
461 buffer.
- 462 iii) Add undiluted serum 100 µl/well and incubate at 25°C for 1 hour at 100 rpm.
- 463 iv) Wash three times with PBS/Tween buffer.
- 464 v) Add 100 µl of *A. marginale* MSP5-biotin (1 µg/ml) plus *A. centrale* MSP5 (10 µg/ml) to *A. marginale*  
465 test wells. Add *A. centrale* MSP5-biotin (1 µg/ml) plus *A. marginale* MSP5 (10 µg/ml) in PBS/Tween  
466 buffer + 10% fat-free dried milk to *A. centrale* test wells.
- 467 vi) Incubate at 25°C for 1 hour at 100 rpm and wash the plate five times with PBS/Tween buffer.
- 468 vii) To detect the bound protein–biotin complex, add streptavidin-HRP diluted in 1/500 in PBS/Tween  
469 buffer with 10% dried milk for 1 hour at 25°C, 100 rpm.
- 470 viii) Wash five times with PBS/Tween buffer.
- 471 ix) Add chromogenic substrate based on manufacturer's instructions.
- 472 x) The reaction is measured by microplate reader spectrophotometer at 405 nm wavelength. The data  
473 are expressed in optical density (OD).
- 474 xi) OD<sub>405nm</sub> <0.2 is considered negative.



475 xii) Results are expressed as the ratio between antibodies specific for *A. marginale* MSP5 and for *A.*  
476 *centrale* MSP5 (ODAm/ODAc). If the ratio is >0.38 the sample is considered positive for anti-*A.*  
477 *marginale* antibodies, and a ratio ≤ 0.38 is classified as vaccinated with *A. centrale*.

## 478 2.4. Card agglutination test

479 ~~The advantages of the CAT are that it is sensitive. The sensitivity of the CAT is from 84% to 98% (Gonzalez *et*~~  
480 ~~*al.*, 1978; Molloy *et al.*, 1999) and the specificity is 98.6% (Molloy *et al.*, 1999). Though sometimes giving variable~~  
481 ~~results, the CAT can be useful under certain circumstances, as it may be undertaken either in the laboratory or~~  
482 ~~in the field, and it gives a result within a few minutes. Nonspecific reactions may be a problem, and subjectivity~~  
483 ~~in interpreting assay reactions can result in variability in test interpretation. In addition, the CAT antigen, which~~  
484 ~~is a suspension lysate of *A. marginale* particles isolated from erythrocytes, can be difficult to prepare and can~~  
485 ~~vary from batch to batch and laboratory to laboratory. To obtain the antigen, splenectomised calves are infected~~  
486 ~~by intravenous inoculation with blood containing *Anaplasma*–*A. marginale*-infected erythrocytes. When the~~  
487 ~~rickettsaemia exceeds 50%, the animal is exsanguinated, the infected erythrocytes are washed, lysed, and the~~  
488 ~~erythrocyte ghosts and *Anaplasma* particles–*A. marginale* are pelleted. The pellets are sonicated, washed, and~~  
489 ~~then resuspended in a stain solution to produce the antigen suspension.~~

490 A test procedure that has been slightly modified from that originally described (Amerault & Roby, 1968; Amerault  
491 *et al.*, 1972) is as follows, and is based on controlled conditions in a laboratory setting:

### 492 2.4.1. Test procedure

- 493 i) Ensure all test components are at a temperature of 25–26°C before use (this constant temperature  
494 is critical for the test).
- 495 ii) On each circle of the test card (a clear perspex/plastic or glass plate marked with circles that are  
496 18 mm in diameter), place next to each other, but not touching, 10 µl of bovine serum factor (BSF),  
497 10 µl of test serum, and 5 µl of CAT antigen<sup>1</sup>. Negative and low positive control sera must be tested  
498 on each card.
- 499 BSF is serum from a selected animal with high known conglutinin level. If the conglutinin level is  
500 unknown, fresh serum from a healthy animal known to be free from *Anaplasma* can be used. The  
501 BSF must be stored at –70°C in small aliquots, a fresh aliquot being used each time the tests are  
502 performed. The inclusion of BSF improves the sensitivity of the test.
- 503 iii) Mix well with a glass stirrer. After mixing each test, wipe the stirrer with clean tissue to prevent  
504 cross-contamination.
- 505 iv) Place the test card in a humid chamber and rock at 100–110 rpm for 7 minutes.
- 506 v) Read immediately against a backlight. Characteristic clumping of the antigen (graded from +1 to  
507 +3) is considered to be a positive result. The test is considered to give a negative result when there  
508 is no characteristic clumping.

509 A latex card agglutination test, a relatively simple and rapid test platform, has been partially validated. This test  
510 uses rMSP5-HIS rather than *A. marginale* lysate and does not require BSF. The performance of this test was  
511 compared with that of the I-ELISA using rMSP5-HIS as the antigen. The relative sensitivity was 95.2% and  
512 relative specificity was 91.86% (Ramos *et al.*, 2014).

## 513 2.4. Complement fixation test

514 ~~The complement fixation (CF) test has been used extensively for many years; however, it shows variable~~  
515 ~~sensitivity (ranging from 20 to 60%), possibly reflecting differences in techniques for antigen production, and~~  
516 ~~poor reproducibility. In addition, it has been demonstrated that the CF assay fails to detect a significant~~  
517 ~~proportion of carrier cattle (Bradway *et al.*, 2001). It is also uncertain as to whether or not the CF test can identify~~  
518 ~~antibodies in acutely infected animals prior to other assays (Coetzee *et al.*, 2007; Molloy *et al.*, 1999). Therefore,~~  
519 ~~the CF test is no longer recommended as a reliable assay for detecting infected animals.~~

---

<sup>1</sup> The test as conducted in the USA and Mexico uses larger volumes of reagents: antigen (15 µl), serum (30 µl), and bovine serum factor (30 µl), and a 4-minute reaction time (see step iv).

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520

## 2.5. Indirect fluorescent antibody test

521 Because of the limitations on the number of indirect fluorescent antibody (IFA) tests that can be performed daily  
522 by one operator, other serological tests are generally preferred to the IFA test. The IFA test is performed as  
523 described for bovine babesiosis in chapter 3.4.2, except that *A. marginale* infected blood is used for the  
524 preparation of antigen smears. A serious problem encountered with the test is nonspecific fluorescence. The  
525 reported sensitivity is 97.6% and specificity 89.6% (Gonzalez *et al.*, 1978). Antigen made from blood collected  
526 as soon as adequate rickettsaemia (5–10%) occurs is most likely to be suitable. Nonspecific fluorescence due  
527 to antibodies adhering to infected erythrocytes can be reduced by washing the erythrocytes in an acidic glycine  
528 buffer before antigen smears are prepared. Infected erythrocytes are washed twice in 0.1 M glycine buffer  
529 (pH 3.0, centrifuged at 1000 *g* for 15 minutes at 4°C) and then once in PBS, pH 7.4. Recently published data  
530 show that the IFA, like the C-ELISA, can cross react with other members of the *Anaplasmataceae* family, and  
531 specifically an *Ehrlichia* spp. identified as BOV2010 (Al-Adhami *et al.*, 2011).

532

## 2.6. Complement fixation test

533 The complement fixation test (CFT) was used extensively for many years; however, it has variable sensitivity  
534 (ranging from 20 to 60%), possibly reflecting differences in techniques for antigen production, and poor  
535 reproducibility. In addition, the CF assay fails to detect a significant proportion of carrier cattle (Bradway *et al.*,  
536 2001). It is also uncertain as to whether or not the CF test can identify antibodies in acutely infected animals  
537 prior to other assays (Coetzee *et al.*, 2007; Molloy *et al.*, 1999). Therefore, the CF test is no longer recommended  
538 as a reliable assay for detecting infected animals.

539

# C. REQUIREMENTS FOR VACCINES

540

## 1. Background

541 Several immunisation methods have been used to protect cattle against anaplasmosis in countries where the disease is  
542 endemic, but none is ideal to date (~~McHardy, 1984~~). A review of *A. marginale* vaccines and antigens has been published  
543 (Kocan *et al.*, 2003–2010; Noh *et al.*, 2012). Use of the less pathogenic *A. centrale*, which gives partial cross-protection  
544 against *A. marginale*, is the most widely accepted method, although not used in many countries ~~where the disease is~~  
545 ~~exotic~~, including north America.

546 In this section, the production of live *A. centrale* vaccine is described. It involves infection of a susceptible, splenectomised  
547 calf and the use of its blood as a vaccine. Detailed accounts of the production procedure are available and reference should  
548 be made to these publications for details of the procedures outlined here (Bock *et al.*, 2004; de Vos & Jorgensen, 1992;  
549 Pipano, 1995).

550 Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*.  
551 The guidelines given here and in Chapter 1.1.8 are intended to be general in nature and may be supplemented by national  
552 and regional requirements.

553 *Anaplasma centrale* vaccine can be provided in either frozen or chilled ~~form~~ depending on demand, transport networks,  
554 and the availability of liquid nitrogen or dry ice supplies. Frozen vaccine is recommended in most instances, as it allows  
555 for thorough post-production quality control of each batch. It is, however, more costly to produce and more difficult to  
556 transport than chilled vaccine. The risk of contamination makes post-production control essential, but may be prohibitively  
557 expensive.

558

## 2. Outline of production and minimum requirements for conventional vaccines

559

### 2.1. Characteristics of the seed

560

#### 2.1.1. Biological characteristics

561 *Anaplasma centrale* was isolated in 1911 in South Africa and has been used as a vaccine in South  
562 America, Australia, Africa, the Middle East, and South-East Asia. It affords only partial, but adequate,  
563 protection in regions where the challenging circulating strains are of moderate virulence (e.g. Australia)  
564 (Bock & de Vos, 2001). In the humid tropics where *A. marginale* appears to may be a very more virulent  
565 rickettsia, the protection afforded by *A. centrale* may be inadequate to prevent disease in some animals.

---

566 *Anaplasma centrale* usually causes benign infections, especially if used in calves under 9 months of age.  
567 Severe reactions following vaccination have been reported when adult cattle are inoculated. The  
568 suitability of an isolate of *A. centrale* as a vaccine can be determined by inoculating susceptible cattle,  
569 monitoring the subsequent reactions, and then challenging the animals and susceptible controls with a  
570 virulent local strain of *A. marginale*. Both safety and efficacy can be judged by monitoring rickettsaemias  
571 in stained blood films and the depression of packed cell volumes of inoculated cattle during the  
572 vaccination and challenge reaction periods.

573 Infective material for preparing the vaccine is readily stored as frozen stabilates of infected blood in liquid  
574 nitrogen or dry ice. Dimethyl sulphoxide (DMSO) ~~and~~ or polyvinylpyrrolidone M.W. 40,000 (Bock *et al.*,  
575 2004) are the recommended cryopreservatives, as they allow for intravenous administration after thawing  
576 of the stabilate. A detailed account of the freezing technique using DMSO is reported elsewhere (Mellors  
577 *et al.*, 1982), but briefly involves the following: infected blood is collected, chilled to 4°C, and cold  
578 cryoprotectant (4 M DMSO in PBS) is added slowly with stirring to a final blood:protectant ratio of 1:1, to  
579 give a final concentration of 2 M DMSO. The entire dilution procedure is carried out in an ice bath and  
580 the diluted blood is dispensed into suitable containers (e.g. 5 ml cryovials), and frozen, as soon as  
581 possible, in the vapour phase of a liquid nitrogen container.

## 582 2.1.2. Quality criteria

583 Evidence of purity of the *A. centrale* isolate can be determined by serological testing of paired sera from  
584 the cattle used in the safety test for possible ~~contaminants~~ pathogens that may be present (Bock *et al.*,  
585 2004; Pipano, 1997). Donor calves used to expand the seed for vaccine production should be examined  
586 for all blood-borne infections prevalent in the vaccine-producing country, including *Babesia*, *Anaplasma*,  
587 *Ehrlichia*, *Theileria* and *Trypanosoma*. This can be done by routine examination of stained blood films  
588 after splenectomy, PCR, and preferably also by serology. Any calves showing evidence of natural  
589 infections of any of these agents should be rejected. The absence of other infective agents should also  
590 be confirmed. These may include the agents of enzootic bovine leukosis, mucosal disease, infectious  
591 bovine rhinotracheitis, ephemeral fever, Akabane disease, bluetongue, and foot and mouth disease, ~~and~~  
592 ~~rinderpest~~. The testing procedures will depend on the diseases prevalent in the country and the  
593 availability of tests but should involve serology of paired sera at the very least and, in some cases, virus  
594 isolation, antigen, or DNA/RNA detection (Bock *et al.*, 2004; Pipano, 1981; 1997).

## 595 2.2. Method of manufacture

### 596 2.2.1. Procedure

#### 597 i) Production of frozen vaccine

598 Quantities of the frozen stabilate (5–10 ml) are thawed by immersing the vials in water preheated  
599 to 40°C. The thawed material is kept on ice and used as soon as possible (within 30 minutes) to  
600 infect a susceptible, splenectomised calf by intravenous inoculation.

601 The rickettsaemia of ~~the~~ this donor calf is monitored daily by examining stained films of jugular  
602 blood, and the blood is collected for vaccine production when suitable rickettsaemias are reached.  
603 A rickettsaemia of  $1 \times 10^8$ /ml (approximately 2% rickettsaemia in jugular blood) is the minimum  
604 required for production of vaccine as this is the dose to vaccinate a bovine. If a suitable  
605 rickettsaemia is not obtained, passage of the strain by subinoculation of 100–200 ml of blood to a  
606 second splenectomised calf may be necessary.

607 Blood from the donor is collected by aseptic jugular or carotid cannulation using heparin as an  
608 anticoagulant (5 International Units [IU] heparin/ml blood). The use of blood collection units for  
609 human use are also suitable and guarantee sterility and obviate the need to prepare glass flasks  
610 that make the procedure more cumbersome.

611 In the laboratory, the infective blood is mixed in equal volumes with 3 M glycerol in PBS  
612 supplemented with 5 mM glucose at 37°C (final concentration of glycerol 1.5 M). The mixture is  
613 then equilibrated at 37°C for 30 minutes and dispensed into suitable containers (e.g. 5 ml cryovials).  
614 The vials are cooled at approximately 10°C/minute in the vapour phase of liquid nitrogen and, when  
615 frozen, stored in the liquid phase (Bock *et al.*, 2004).

616 DMSO can be used as a cryoprotectant in the place of glycerol. This is done in the same way as  
617 outlined for the preparation of seed stabilate (Mellors *et al.*, 1982; Pipano, 1981).

---

618 If glycerolised vaccine is to be diluted, the diluent should consist of PBS with 1.5 M glycerol and  
619 5 mM glucose (Jorgensen *et al.*, 1989). Vaccine cryopreserved with DMSO should be diluted with  
620 diluent containing the same concentration of DMSO as in the original cryopreserved blood (Pipano  
621 *et al.*, 1986).

622 **ii) Production of chilled vaccine**

623 Infective material for chilled vaccine is prepared in the same way as for frozen vaccine, but it must  
624 be issued and used as soon as possible after collection. The infective blood can be diluted to  
625 provide  $1 \times 10^7$  parasites per dose of vaccine. A suitable diluent is 10% sterile bovine serum in a  
626 glucose/balanced salt solution containing the following quantities per litre: NaCl (7.00 g),  
627 MgCl<sub>2</sub>.6H<sub>2</sub>O (0.34 g), glucose (1.00 g), Na<sub>2</sub>HPO<sub>4</sub>(2.52 g), KH<sub>2</sub>PO<sub>4</sub>(0.90 g), and NaHCO<sub>3</sub>(0.52 g).

628 If diluent is not available, acid citrate dextrose (20% [v/v]) or citrate phosphate dextrose (20% [v/v])  
629 should be used as anticoagulant to provide the glucose necessary for survival of the organisms.

630 **iii) Use of vaccine**

631 In the case of frozen vaccine, vials should be thawed by immersion in water, preheated to 37°C to  
632 40°C, and the contents mixed with suitable diluent to the required dilution. If glycerolised vaccine is  
633 prepared, it should be kept cool and used within 8 hours (Bock *et al.*, 2004). If DMSO is used as a  
634 cryoprotectant, the prepared vaccine should be kept on ice and used within 15–30 minutes (Pipano,  
635 1981). The vaccine is most commonly administered subcutaneously.

636 **iv) Chilled vaccine should be kept refrigerated and used within 4–7 days of preparation.**

637 The strain of *A. centrale* used in the vaccine is of reduced virulence, but is not entirely safe. A  
638 practical recommendation is, therefore, to limit the use of vaccine to calves, where nonspecific  
639 immunity will minimise the risk of vaccine reactions. When older animals have to be vaccinated,  
640 there is a risk of severe reactions. These reactions occur infrequently, but valuable breeding stock  
641 or pregnant animals obviously warrant close attention, and should be observed daily for 3 weeks  
642 post-vaccination. Clinically sick animals should be treated with oxytetracycline or imidocarb at  
643 dosages recommended by the manufacturers. Protective immunity develops in 6–8 weeks and  
644 usually lasts for several years.

645 Anaplasmosis and babesiosis vaccines are often used concurrently, but it is not advisable to use  
646 any other vaccines at the same time (Bock *et al.*, 2004).

647 **2.2.2. Requirements for substrates and media**

648 *Anaplasma centrale* ~~cannot can~~ be cultured in ~~vitro~~ *Rhipicephalus appendiculatus* and *Dermacentor*  
649 *variabilis* cells lines, though antigen expression and immunogenicity of the cultured *A. centrale* need to  
650 be tested (Bell-Sakyi *et al.*, 2015). No substrates or media other than buffers and diluents are used in  
651 vaccine production. DMSO or glycerol should be purchased from reputable companies.

652 **2.2.3. In-process controls**

653 **i) Source and maintenance of vaccine donors**

654 A source of calves free from natural infections of ~~Anaplasma~~ *A. marginale* and other tick-borne  
655 diseases should be identified. If a suitable source is not available, it may be necessary to breed the  
656 calves under tick-free conditions specifically for the purpose of vaccine production.

657 The calves should be maintained under conditions that will prevent exposure to infectious diseases  
658 and to ticks and biting insects. In the absence of suitable facilities, the risk of contamination with  
659 the agents of infectious diseases present in the country involved should be estimated, and the  
660 benefits of local production of vaccine weighed against the possible adverse consequences of  
661 spreading disease (Bock *et al.*, 2004).

662 **ii) Surgery**

663 Donor calves should be splenectomised to allow maximum yield of organisms for production of  
664 vaccine. This is best carried out in young calves and under general anaesthesia.

665 **iii) Screening of vaccine donors before inoculation**

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666 As for preparation of seed stabilate, donor calves for vaccine production should be examined for all  
667 blood-borne infections prevalent in the vaccine-producing country, including *Babesia*, *Anaplasma*,  
668 *Ehrlichia*, *Theileria* and *Trypanosoma*. This can be done by routine examination of stained blood  
669 films after splenectomy, and preferably also by serology. Any calves showing evidence of natural  
670 infections of any of these agents should be rejected. The absence of other infective agents should  
671 also be confirmed. These may include the agents of enzootic bovine leukosis, bovine viral  
672 diarrhoea, infectious bovine rhinotracheitis, ephemeral fever, Akabane disease, bluetongue, and  
673 foot and mouth disease. The testing procedures will depend on the diseases prevalent in the  
674 country and the availability of tests, but should involve serology of paired sera at the very least and,  
675 in some cases, virus isolation, antigen, or DNA/RNA detection (Bock *et al.*, 2004; Pipano, 1981;  
676 1997).

677 **iv) Monitoring of rickettsaemias following inoculation**

678 It is necessary to determine the concentration of rickettsia in blood being collected for vaccine. The  
679 rickettsial concentration can be estimated from the erythrocyte count and the rickettsaemia  
680 (percentage of infected erythrocytes).

681 **v) Collection of blood for vaccine**

682 All equipment should be sterilised before use (e.g. by autoclaving). Once the required rickettsaemia  
683 is reached, the blood is collected in heparin using strict aseptic techniques. This is best done if the  
684 calf is sedated and with the use of a closed-circuit collection system.

685 Up to 3 litres of heavily infected blood can be collected from a 6-month-old calf. If the calf is to live,  
686 the transfusion of a similar amount of blood from a suitable donor is indicated. Alternatively, the calf  
687 should be killed immediately after collection of the blood.

688 **vi) Dispensing of vaccine**

689 All procedures are performed in a suitable environment, such as a laminar flow cabinet, using  
690 standard sterile techniques. Use of a mechanical or magnetic stirrer will ensure thorough mixing of  
691 blood and diluent throughout the dispensing process. Penicillin (500,000 IU/litre) and streptomycin  
692 (370,000 µg/litre) are added to the vaccine at the time of dispensing.

693 **2.2.4. Final product batch tests**

694 The potency, safety and sterility of vaccine batches cannot be determined in the case of chilled vaccine,  
695 and specifications for frozen vaccine depend on the country involved. The following are the specifications  
696 for frozen vaccine produced in Australia.

697 **i) Sterility and purity**

698 Standard tests for sterility are employed for each batch of vaccine and diluent (see Chapter 1.1.9  
699 *Tests for sterility and freedom from contamination of biological materials intended for veterinary*  
700 *use*).

701 The absence of contaminants is determined by doing appropriate serological testing of donor cattle,  
702 by inoculating donor lymphocytes into sheep and then monitoring them for evidence of viral  
703 infection, and by inoculating cattle and monitoring them serologically for infectious agents that could  
704 potentially contaminate the vaccine. Cattle inoculated during the test for potency (see Section  
705 C.2.2.4.iii) are suitable for the purpose. Depending on the country of origin of the vaccine, these  
706 agents include the causative organisms of enzootic bovine leukosis, infectious bovine  
707 rhinotracheitis, bovine viral diarrhoea, ephemeral fever, Akabane disease, Aino virus, bluetongue,  
708 parainfluenza, foot and mouth disease, lumpy skin disease, rabies, Rift Valley fever, contagious  
709 bovine pleuropneumonia, Jembrana disease, heartwater, pathogenic *Theileria* and *Trypanosoma*  
710 *spp.*, *Brucella abortus*, *Coxiella*, and *Leptospira* (Bock *et al.*, 2004; Pipano, 1981; 1997). Other  
711 pathogens to consider include the causal agents of bovine tuberculosis and brucellosis as they may  
712 spread through contaminated blood used for vaccine production. Most of these agents can be  
713 tested by means of specific PCR and there are many publications describing primers, and assay  
714 conditions for any particular disease.

715 **ii) Safety**

716 Vaccine reactions of the cattle inoculated in the test for potency (see Chapter 1.1.8 *Principles of*  
717 *veterinary vaccine production*) are monitored by measuring rickettsaemia and depression of packed

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718 cell volume. Only batches with pathogenicity levels equal to or lower than a predetermined standard  
719 are released for use.

720 **iii) Potency**

721 Vaccine is thawed and diluted 1/5 with a suitable diluent (Bock *et al.*, 2004). The diluted vaccine is  
722 then incubated for 8 hours at 4°C, and five cattle are inoculated subcutaneously with 2 ml doses.  
723 The inoculated cattle are monitored for the presence of infections by examination of stained blood  
724 smears. All should become infected for a batch to be accepted. A batch proving to be infective is  
725 recommended for use at a dilution of 1/5 with isotonic diluent.

726 **2.3. Requirements for authorisation**

727 **2.3.1. Safety**

728 The strain of *A. centrale* used in vaccine is of reduced virulence but is not entirely safe. A practical  
729 recommendation is, therefore, to limit the use of vaccine to calves, where nonspecific immunity will  
730 minimise the risk of vaccine reactions. When older animals have to be vaccinated, there is a risk of  
731 severe reactions. These reactions occur infrequently, but valuable breeding stock or pregnant animals  
732 obviously warrant close attention, and should be observed daily for 3 weeks post-vaccination. Clinically  
733 sick animals should be treated with oxytetracycline or imidocarb at dosages recommended by the  
734 manufacturers.

735 *Anaplasma centrale* is not infective to other species, and the vaccine is not considered to have other  
736 adverse environmental effects. The vaccine is not infective for humans. When the product is stored in  
737 liquid nitrogen, the usual precautions pertaining to the storage, transportation and handling of deep-  
738 frozen material applies.

739 **2.3.2. Efficacy requirements**

740 ~~Partial but long-lasting immunity results from one inoculation. There is no evidence that repeated~~  
741 ~~vaccination will have a boosting effect. Immunisation with live *A. centrale* results in long-term infection of~~  
742 ~~the vaccinee, thus repeated vaccination is unnecessary. Infection with *A. centrale* does not prevent~~  
743 ~~subsequent infection with *A. marginale*, but does at least result in protection from disease (Shkap *et al.*,~~  
744 ~~2009).~~ The vaccine is used for control of clinical anaplasmosis in endemic areas. It will not provide sterile  
745 immunity, and should not be used for eradication of *A. marginale*.

746 **2.3.3. Stability**

747 The vaccine can be kept for 5 years when stored in liquid nitrogen. Once thawed, it rapidly loses its  
748 potency. Thawed vaccine cannot be refrozen.

749 **3. Vaccines based on biotechnology**

750 There are no vaccines based on biotechnology available for anaplasmosis.

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873 **NB:** There is a WOA Reference Laboratory for anaplasmosis (please consult the WOA Web site:  
874 <https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3http://www.oie.int/>)  
875 Please contact the WOA Reference Laboratory for any further information on  
876 diagnostic tests, reagents and vaccines for bovine anaplasmosis

877 **NB:** FIRST ADOPTED IN 1991. MOST RECENT UPDATES ADOPTED IN 2015.

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**Appendix 1: Bovine anaplasmosis**  
**Intended purpose of test: population freedom from infection**

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>C-ELISA +++ Bovine</u>	<u>Serum rMSP5-GST</u>	<u>Reference tests were nested PCR and IFAT. Dsp = 99.7% Dse = 100% 30% inhibition as determined by ROC analysis.</u>	<u>1. 358 known non-infected cattle from dairy herds maintained in tick free barns and no clinical history of clinical anaplasmosis. 2. 135 known positive sera as defined by nested PCR. 3. Intra-test comparison with 163 diagnostic samples with possible false positives based on rMSP5-GST C-ELISA. Test positive confirmation done with IFAT.</u>	<u>See reference</u>	<u>1. Updated version with improved specificity. 2. High sensitivity, detects persistently infected animals. 3. Commercially available. 4. Uses a standardised antigen. 5. Target antigen is highly conserved among <i>A. marginale</i> strains, thus detects infection with all strains of <i>A. marginale</i>. 6. Rapid.</u>	<u>1. Does not differentiate between infection with <i>A. marginale</i> and <i>A. centrale</i>. 2. May cross react with anti-<i>Ehrlichia</i> antibodies. 3. May not be readily available in all countries. 4. Requires a microplate absorbance reader. 5. Low percent of false positive results.</u>	<u>Chung <i>et al.</i>, 2014.</u>
<u>IFAT+ Bovine</u>	<u>Serum Glass slides with RBCs infected with <i>A. marginale</i>.</u>	<u>Reference test was blood smear. DSe 97.6% Dsp 89.6%</u>	<u>48 cattle raised in anaplasmosis free region. 82 animals from endemic region.</u>	<u>See reference</u>	<u>1. Antigen is relatively easy to produce and store. 2. Does not require many reagents.</u>	<u>1. Low specificity. 2. Time consuming and labour intensive so not suitable for high throughput. 3. Requires fluorescent microscope and blood smears with high rickettsemia.</u>	<u>Gonzalez <i>et al.</i>, 1978</u>

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**Appendix 2: Bovine anaplasmosis**  
**Intended purpose of test: Individual animal freedom from infection prior to movement.**

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>PCR ++</u>	<u>Whole blood</u> <u>Various gene targets</u>	<u>Partial validation has been published.</u>	<u>51 cattle from 18 herds in three regions of southern Italy were tested by RLB<sup>1</sup> for <i>A. marginale</i>, <i>A. centrale</i>, <i>A. bovis</i>, <i>T. buffeli</i>, <i>B. bovis</i>, <i>A. phagocytophilum</i>, and <i>B. bigemina</i>. All cattle except 4 were positive for at least one of these pathogens.</u>	<u>See reference</u>	<u>Good reported concordance between nested PCR and real time PCR. High analytic sensitivity (10<sup>1</sup> DNA copies).</u>	<u>Must be performed in a lab equipped to extract DNA and have thermocyclers for real time PCR. Though not determined empirically, it is likely that PCR has less sensitivity than C-ELISA in detection of persistently infected cattle.</u>	<u>Carelli <i>et al.</i>, 2007.</u>
<u>C-ELISA +++</u> <u>Bovine</u>	<u>Serum</u> <u>rMSP5-GST</u>	<u>Reference tests were nested PCR and IFAT. Dsp = 99.7% Dse = 100% 30% inhibition as determined by ROC analysis.</u>	<u>1. 358 known non-infected cattle from dairy herds maintained in tick free barns and no clinical history of clinical anaplasmosis. 2. 135 known positive sera as defined by nested PCR. 3. Intra-test comparison with 163 diagnostic samples with possible false positives based on rMSP5-GST C-ELISA. Test positive confirmation done with IFAT.</u>	<u>See reference</u>	<u>1. Updated version with improved specificity. 2. High sensitivity, detects persistently infected animals. 3. Commercially available. 4. Uses a standardised antigen. 5. Target antigen is highly conserved among <i>A. marginale</i> strains, thus detects infection with all strains of <i>A. marginale</i>. 6. Rapid.</u>	<u>1. Does not differentiate between infection with <i>A. marginale</i> and <i>A. centrale</i>. 2. May cross react with anti-<i>Ehrlichia</i> antibodies. 3. May not be readily available in all countries. 4. Requires a microplate absorbance reader.</u>	<u>Chung <i>et al.</i>, 2014.</u>

5 <sup>1</sup>RLB is the reverse line blot test.

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**Appendix 3: Bovine anaplasmosis**  
**Intended purpose of test: contribute to eradication policies**

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
C-ELISA +++ Bovine	Serum rMSP5-GST	Reference tests were nested PCR and IFAT. Dsp = 99.7% Dse = 100% 30% inhibition as determined by ROC analysis.	1. 358 known non-infected cattle from dairy herds maintained in tick free barns and no clinical history of clinical anaplasmosis. 2. 135 known positive sera as defined by nested PCR. 3. Intra-test comparison with 163 diagnostic samples with possible false positives based on rMSP5-GST C-ELISA. Test positive confirmation done with IFAT.	See reference	1. Updated version with improved specificity. 2. High sensitivity, detects persistently infected animals. 3. Commercially available. 4. Uses a standardised antigen. 5. Target antigen is highly conserved among <i>A. marginale</i> strains, thus detects infection with all strains of <i>A. marginale</i> . 6. Rapid.	1. Does not differentiate between infection with <i>A. marginale</i> and <i>A. centrale</i> . 2. May cross react with anti- <i>Ehrlichia</i> antibodies. 3. May not be readily available in all countries. 4. Requires a microplate absorbance reader. 5. Low percent of false positive results.	Chung <i>et al.</i> , 2014)

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**Appendix 4: Bovine anaplasmosis**  
**Intended purpose of test: confirmation of clinical cases**

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population</u>	<u>Validation report</u>	<u>Advantages</u>	<u>Disadvantages</u>	<u>References</u>
<u>Microscopic examination +++</u>	<u>Whole blood</u>	<u>No robust validation has been published.</u>	<u>N/A</u>	<u>N/A</u>	<u>1. Most laboratories have the capacity to make and examine blood smears. 2. <i>A. marginale</i> infected erythrocytes readily visible in clinically affected animals.</u>	<u>1. <i>A. marginale</i> colonies are small and can be difficult to differentiate from debris if animal has low rickettsemia. 2. Requires experience to identify <i>A. marginale</i> colonies. 3. Difficult to differentiate between <i>A. marginale</i> and <i>A. centrale</i>.</u>	
<u>PCR +++</u>	<u>Whole blood Various gene targets</u>	<u>Partial validation has been published.</u>	<u>51 cattle from 18 herds in three regions of southern Italy were tested by RLB<sup>1</sup> for <i>A. marginale</i>, <i>A. centrale</i>, <i>A. bovis</i>, <i>T. buffeli</i>, <i>B. bovis</i>, <i>A. phagocytophilum</i>, and <i>B. bigemina</i>. All cattle except 4 were positive for at least one of these pathogens.</u>	<u>See reference</u>	<u>Good reported concordance between nested PCR and real time PCR. High analytic sensitivity (10<sup>1</sup> DNA copies).</u>	<u>1. Must be performed in a lab equipped to extract DNA and have thermocyclers for real-time PCR. 2. Important to use PCR in conjunction with diagnosis of anaemia and blood smear because PCR can detect low level rickettsemia leading to misdiagnosis.</u>	<u>Carelli <i>et al.</i>, 2007</u>

10 N/A: not available.  
11 <sup>1</sup>RLB is the reverse line blot test.

**Appendix 5: Bovine anaplasmosis**  
**Intended purpose of test: prevalence of infection – surveillance**

<b>Test with score and species</b>	<b>Sample type and target analytes</b>	<b>Accuracy</b>	<b>Test population</b>	<b>Validation report</b>	<b>Advantages</b>	<b>Disadvantages</b>	<b>References</b>
<b>CAT ±</b>	Serum Lysates of <i>A. marginale</i> isolated from red blood cells.	Reference test was blood smear. DSe 84.1 <sup>1</sup> -100 <sup>2</sup> % Dsp 97.9 <sup>1</sup> -98.6 <sup>2</sup> %	48 cattle raised in anaplasmosis free region. 82 animals from endemic region. <sup>1</sup> 86 sera from experimentally infected cattle and 183 sera from <i>A. marginale</i> free area <sup>2</sup>	See references	1. Can be done in field or in the laboratory	1. Antigen derived from infected cattle are difficult to produce and standardise. 2. May have false negative and false positive results. 3. Variation between tests depending on environmental conditions and the laboratory.	<sup>1</sup> Gonzalez <i>et al.</i> , 1978. <sup>2</sup> Molloy <i>et al.</i> , 1999.
<b>C-ELISA +++ Bovine</b>	Serum rMSP5-GST	Reference tests were nested PCR and IFAT. Dsp = 99.7% Dse = 100% 30% inhibition as determined by ROC analysis.	1. 358 known non-infected cattle from dairy herds maintained in tick free barns and no clinical history of clinical anaplasmosis. 2. 135 known positive sera as defined by nested PCR. 3. Intra-test comparison with 163 diagnostic samples with possible false positives based on rMSP5-GST C-ELISA. Test positive confirmation done with IFAT.	See reference	1. Updated version with improved specificity. 2. High sensitivity, detects persistently infected animals. 3. Commercially available. 4. Uses a standardised antigen. 5. Target antigen is highly conserved among <i>A. marginale</i> strains, thus detects infection with all strains of <i>A. marginale</i> . 6. Rapid.	1. Does not differentiate between infection with <i>A. marginale</i> and <i>A. centrale</i> . 2. May cross react with anti- <i>Ehrlichia</i> antibodies. 3. May not be readily available in all countries. 4. Requires a microplate absorbance reader. 5. Low percent of false positive results	Chung <i>et al.</i> , 2014.
<b>IFAT++ Bovine</b>	Serum Glass slides with RBCs infected with <i>A. marginale</i>	Reference test was blood. DSe 97.6% Dsp 89.6%	1. 48 cattle raised in anaplasmosis free region. 2. 82 animals from endemic region.	See references	1. Antigen is relatively easy to produce and store. 2. Does not require many reagents.	1. Relatively high false positive rate. 2. Time consuming and labour intensive so not suitable for high throughput. 3. Requires fluorescent microscope and blood smears with high rickettsemia.	Gonzalez <i>et al.</i> , 1978

**Appendix 6: Bovine viral diarrhoea**  
**Intended purpose of test: population freedom from infection**

<b>Test with score and species</b>	<b>Sample type and target analytes</b>	<b>Accuracy</b>	<b>Test population used to measure accuracy</b>	<b>Validation report</b>	<b>Advantages: expert opinion</b>	<b>Disadvantages: expert opinion</b>	<b>References</b>
NA detection by (real-time) RT-PCR +++	Ear notch (skin), blood, milk	Performance has been demonstrated under field conditions in large control programs	Whole Swiss, German and Irish cattle populations	See references	<ul style="list-style-type: none"> <li>- Very sensitive</li> <li>- Rapid</li> <li>- High-throughput</li> <li>- Well established internationally</li> <li>- Detects assay-dependent all BVDV species</li> <li>- Allows assay-dependent for differentiation of BVDV types 1 and 2</li> <li>- Detects persistent and transient infection</li> <li>- Proficiency panel of different Pestivirus strains available</li> <li>- Detection of viral RNA in skin biopsy samples unaffected by maternally-derived antibodies, therefore allows for identification of persistently infected animals early in life</li> <li>- Successfully applied in ongoing or completed control programmes</li> </ul>	<ul style="list-style-type: none"> <li>- Possibility for contamination at sample collection or in laboratory, leading to false positive results</li> <li>- Needs specialised equipment</li> <li>- Detection of viral RNA does not imply per se that infectious virus is present</li> </ul>	<ul style="list-style-type: none"> <li>- Presi &amp; Heim (2010). <i>Vet. Microbiol.</i>, <b>142</b>, 137–142</li> <li>- Schweizer <i>et al.</i> (2021) <i>Front. Vet. Sci.</i>, <b>8</b>, 702730</li> <li>- Wernike <i>et al.</i> (2017). <i>Pathogens</i>, <b>6</b> (4)</li> <li>- Graham <i>et al.</i> (2021) <i>Front. Vet. Sci.</i>, <b>8</b>, 674557</li> </ul>
Antibody detection by ELISA +++	Bulk milk, blood	DSe and DSp differs based on the ELISA used (commercial/in-house) and the antibodies being tested (e.g. antibodies against structural (E2) and non-structural (NS2-3) proteins.			<ul style="list-style-type: none"> <li>- Simple to perform and cost-effective</li> <li>- Milk collection is non-invasive method with potential for herd screening with tank/bulk milk samples</li> <li>- Bulk milk sensitive indicator for PI in herd</li> </ul>	<ul style="list-style-type: none"> <li>- Some cross-reactivity with vaccines and other pestiviruses</li> <li>- PI animal will usually be seronegative</li> <li>- Bulk milk from herd excludes males, non-lactating or young stock</li> </ul>	<ul style="list-style-type: none"> <li>Beaudeau <i>et al.</i> (2001). <i>Vet. Microbiol.</i>, <b>80</b>, 329–337</li> <li>Lanyon <i>et al.</i> (2013). <i>Aust. Vet. J.</i>, <b>91</b>, 52–56.</li> </ul>

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population used to measure accuracy</u>	<u>Validation report</u>	<u>Advantages: expert opinion</u>	<u>Disadvantages: expert opinion</u>	<u>References</u>
<u>Antigen detection by ELISA +++</u>	<u>Serum, whole blood, skin biopsy</u>	<u>DSe 67–100% and DSp 98.8–100% relative to virus isolation reported</u>			<u>Relatively simple to perform, rapid, can be cost-effective (when compared to virus isolation and PCR) and suitable for high-throughput applications. There is no need for cell culture facility.</u>	<u>Maternal antibodies in colostrum may interfere with testing for antigen in serum using ELISA in calves. Ear notch samples are less affected. PI calves <i>in utero</i> defies detection.</u>	<u>Lanyon <i>et al.</i> (2013). <i>Vet. J.</i> 199, 201–209.</u>
<u>Virus isolation +</u>	<u>Serum, whole blood</u>	<u>Considered (historically) reference test: DSe &lt;90% compared with real-time RT-PCR; DSp ~100%</u>	<u>N/A</u>	<u>Historical information with no formal validation</u>	<u>- High degree of specificity - Identifies presence of infectious virus</u>	<u>- Requires specialised cell culture capabilities and access to BVDV free materials - Reduced sensitivity in presence of maternally-derived antibodies</u>	<u>N/A</u>
<u>Virus neutralisation test +</u>	<u>Serum</u>	<u>DSe &amp; DSp both extremely high, both &gt;99%. Historical reference serological test.</u>	<u>N/A</u>	<u>Historical information with no formal validation</u>	<u>Very high specificity</u>	<u>- ASe can vary depending on virus strain used - Requires cell culture, good quality samples - Labour intensive, takes 5 days to obtain results - Expensive</u>	<u>N/A</u>

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N/A: not available



REUNIÓN DE LA COMISIÓN DE NORMAS BIOLÓGICAS

París, 5–9 de febrero de 2024

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CHAPTER 3.4.7.

BOVINE VIRAL DIARRHOEA

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SUMMARY

Cattle of all ages are susceptible to infection with bovine viral diarrhoea (BVD) viruses, including *Pestivirus bovis* (commonly known as BVDV type 1 (*Pestivirus bovis*), *Pestivirus tauri* (BVDV type 2 (*Pestivirus tauri*), and *Pestivirus brazilense* (BVDV type 3 (*Pestivirus brazilense*)) (or *Hobi-like pestiviruses* (type 3 (*Pestivirus brazilense*)). Distribution is world-wide although some countries have recently eradicated the virus. BVDV infection results in a wide variety of clinical manifestations, including enteric and respiratory disease in any class of cattle, or reproductive and fetal disease following infection of a susceptible breeding female. Infection may be subclinical or extend to severe fatal disease. Animals that survive in-utero infection in the first trimester of gestation are almost always persistently infected (PI). PI animals provide the main reservoir of the virus in a population and excrete large amounts of virus in urine, faeces, discharges, milk and semen. Identification of such PI cattle is a key element in controlling the infection. It is important to avoid the trade of such animals. They may appear clinically healthy, or weak and unthrifty. Many PI animals die before reaching maturity. They may infrequently develop mucosal disease with anorexia, gastrointestinal erosions, and profuse diarrhoea, invariably leading to death. Mucosal disease can arise only in PI animals. Latent infections generally do not occur following recovery from acute infection. However, bulls may rarely have a prolonged and persistent testicular infection and excrete virus in semen for prolonged periods, perhaps indefinitely.

**Detection of the agent:** BVDV is a pestivirus in the family Flaviviridae and is closely related to classical swine fever virus (*Pestivirus suis*) and ovine border disease viruses (*Pestivirus ovis*). BVD viruses are classified into the distinct species: *Pestivirus bovis* (commonly known as BVDV type 1), *Pestivirus tauri* (BVDV type 2) and *Pestivirus brazilense* (BVDV type 3 or *Hobi-like pestivirus*). The two genotypes (types 1 and 2) are classified as separate species in the genus *Pestivirus*. A third putative genotype, BVDV type 3, has also recently been proposed. Although both cytopathic and non-cytopathic biotypes of *Pestivirus bovis* and *P. tauri* BVDV type 1 and type 2 exist, non-cytopathic strains are usually encountered in field infections and are the main focus of diagnostic virus isolation in cell cultures. PI animals can be readily identified by a variety of methods aimed to detect viral antigens or viral RNA directly in blood and tissues. Virus can also be isolated by inoculation of specimens onto susceptible cell cultures followed by immune-labelling methods to detect the replication of the virus in the cultures. Persistence of virus infection should be confirmed by resampling after an interval of at least 3 weeks, when virus will again be detected. PI animals are usually seronegative. Viraemia in acute cases is transient and usually difficult to detect. Virus isolation in semen from bulls that are acutely or persistently infected requires special attention to specimen transport and testing. RNA detection assays are particularly useful because they are rapid, have very high sensitivity and do not depend on the use of cell cultures.

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36 **Serological tests:** Acute infection with BVDV is best confirmed by demonstrating seroconversion using  
37 sequential paired samples, ideally from several animals in the group. The testing of paired (acute and  
38 convalescent samples) should be done a minimum of 21 days apart and samples should be tested  
39 concurrently in the same assay. Enzyme-linked immunosorbent assays and the virus neutralisation test are  
40 the most widely used.

41 **Requirements for vaccines:** There is no standard vaccine for BVD, but a number of commercial  
42 preparations are available. An ideal vaccine should be able to prevent transplacental infection in pregnant  
43 cows. Modified live virus vaccine should not be administered to pregnant cattle (or to their sucking calves)  
44 due to the risk of transplacental infection. Live vaccines that contain cytopathic strains of BVDV present a  
45 risk of inducing mucosal disease in PI animals. Inactivated viral vaccines are safe and can be given to any  
46 class of animal but generally require booster vaccinations. BVDV is a particularly important hazard to the  
47 manufacture of vaccines and biological products for other diseases due to the high frequency of  
48 contamination of batches of fetal calf serum used as a culture medium supplement.

## 49 A. INTRODUCTION

### 50 1. Impact of the disease

51 Cattle of all ages are susceptible to infection with bovine viral diarrhoea viruses (BVDV). Distribution of the virus is world-  
52 wide although some countries have recently eradicated the virus. BVDV infection results in a wide variety of clinical  
53 manifestations, including enteric and respiratory disease in any class of cattle or reproductive and fetal disease following  
54 infection of a susceptible breeding female. Infection may be subclinical or extend to severe fatal disease. Clinical  
55 presentations and severity of disease may vary with different strains of virus. BVDV viruses also cause immune  
56 suppression, which can render infected animals more susceptible to infection with other viruses and bacteria. The clinical  
57 impact may be more apparent in intensively managed livestock. Animals that survive *in-utero* infection in the first trimester  
58 of gestation are almost always persistently infected (PI). PI animals provide the main reservoir of the virus in a population  
59 and excrete large amounts of virus in urine, faeces, discharges, milk and semen. The virus spreads mainly by close contact  
60 between PI animals and other cattle. Virus shedding by acutely infected animals is usually less important. This virus may  
61 also persist in the environment for short periods or be transmitted ~~with-via~~ contaminated reproductive materials. Vertical  
62 transmission plays an important role in ~~its-the~~ epidemiology and pathogenesis.

63 Infections of the breeding female may result in conception failure or embryonic and fetal infection which results in abortions,  
64 stillbirths, teratogenic abnormalities or the birth of PI calves. Persistently viraemic animals may be born as weak, unthrifty  
65 calves or may appear as normal healthy calves and be unrecognised clinically for a long time. However, PI animals have  
66 a markedly reduced life expectancy, with a high proportion dying before reaching maturity. Infrequently, some of these  
67 animals may later develop mucosal disease with anorexia, gastrointestinal erosions, and profuse diarrhoea, invariably  
68 leading to death. Mucosal disease can arise only in PI animals. It is important to avoid the trade of viraemic animals. It is  
69 generally considered that serologically positive, non-viraemic cattle are 'safe', providing that they are not pregnant.  
70 However, a small proportion of persistently viraemic animals may produce antibodies to some of the viral proteins if they  
71 are exposed to another strain of BVDV that is antigenically different to the persisting virus. Consequently, seropositivity  
72 cannot be completely equated with 'safety'. Detection of PI animals must be specifically directed at detection of the virus  
73 or its components (RNA or antigens). Latent infections generally do not occur following recovery from acute infection.  
74 However, semen collected from bulls during an acute infection is likely to contain virus during the viraemic period and often  
75 for a short time afterwards. Although extremely rare, some recovered bulls may have a **prolonged and** persistent testicular  
76 infection and excrete virus in semen, perhaps indefinitely ([Read et al., 2020](#)).

77 While BVDV strains are predominantly pathogens of cattle, interspecies transmission can occur following close contact  
78 with sheep, goats or pigs. Infection of pregnant small ruminants or pigs with BVDV can result in reproductive loss and the  
79 birth of PI animals. BVDV infections have been reported in both New World and Old World camelids. Additionally, strains  
80 of border disease virus (BDV) have infected cattle, resulting in clinical presentations indistinguishable from BVDV infection.  
81 The birth of cattle PI with BDV and the subsequent development of mucosal disease have also been described. Whilst  
82 BVDV and BDV have been reported as natural infections in pigs, the related virus of classical swine fever does not naturally  
83 infect ruminants.

84 Although ubiquitous, control of BVDV can be achieved at the herd level, and even at the national level, as evidenced by  
85 the progress towards eradication made in many European countries (Moennig et al., 2005; [Schweizer et al., 2021](#)).

## 2. The causal agent

Bovine viral diarrhoea virus (BVDV) is a single linear positive-stranded RNA virus in the genus *Pestivirus* of the family *Flaviviridae*. The genus contains a number of species including *Pestivirus bovis* the two genotypes of bovine viral diarrhoea virus (BVDV) (types 1 [*Pestivirus bovis*], and 2 [*Pestivirus tauri*] (BVDV type 2) and 3 [*Pestivirus brazilense*] (BVDV type 3) and the closely related classical swine fever (*Pestivirus suis*) and ovine border disease viruses (*Pestivirus ovis*) (Postler *et al.*, 2023). Viruses in these genotypes-pestivirus species show considerable antigenic difference from each other and, within the type 1 and type 2 species *Pestivirus bovis* and *P. tauri*, BVDV isolates exhibit considerable biological and antigenic diversity. Within the two BVDV genotypes-species *Pestivirus bovis* and *P. tauri*, further subdivisions are discernible by genetic analysis (Vilcek *et al.*, 2001). The two genotypes-species may be differentiated from each other, and from other pestiviruses, by monoclonal antibodies (MAbs) directed against the major glycoproteins E2 and ERNs or by genetic analysis. Reverse-transcription polymerase chain reaction (RT-PCR) assays enable virus typing direct from blood samples (Letellier & Kerkhofs, 2003; McGoldrick *et al.*, 1999). Type 1 viruses are generally more common although the prevalence of type 2 strains can be high in North America. BVDV of both genotypes-species (*Pestivirus bovis* and *P. tauri*) may occur in non-cytopathic and cytopathic forms (biotypes), classified according to whether or not microscopically apparent cytopathology is induced during infection of cell cultures. Usually, it is the non-cytopathic biotype that circulates freely in cattle populations. Non-cytopathic strains are most frequently responsible for disease in cattle and are associated with enteric and respiratory disease in any class of cattle or reproductive and fetal disease following infection of a susceptible breeding female. Infection may be subclinical or extend to severe fatal disease (Brownlie, 1985). Cytopathic viruses are encountered in cases of mucosal disease, a clinical syndrome that is relatively uncommon and involves the 'super-infection' of an animal that is PI with a non-cytopathic virus by a closely related cytopathic strain. The two virus biotypes found in a mucosal disease case are usually antigenically closely related if not identical. Type 2 viruses are usually non-cytopathic and have been associated with outbreaks of severe acute infection and a haemorrhagic syndrome. However some type 2 viruses have also been associated with a disease indistinguishable from that seen with the more frequently isolated type 1 viruses. Further, some type 1 isolates have been associated with particularly severe and fatal disease outbreaks in adult cattle. Clinically mild and inapparent infections are common following infection of non-pregnant animals with either genotype-virus species.

There is an increasing awareness of an "atypical" or "HoBi like" pestivirus—a putative BVDV type 3-*Pestivirus brazilense* strains are also associated with clinical disease in cattle, but they appear mainly restricted to South American and Asian cattle populations, in cattle, also associated with clinical disease (Bauermann *et al.*, 2013; Chen *et al.*, 2021), but its distribution is presently unclear. These viruses are readily detected by proven pan-reactive assays such as real-time RT-PCR. Some commercial antigen ELISAs (enzyme-linked immunosorbent assays) have been shown to detect these strains (Bauermann *et al.*, 2012); generally virus isolation, etc., follows the same principles as for *Pestivirus bovis* (BVDV type 1 (*Pestivirus bovis*) and *Pestivirus tauri* (BVDV type 2- (*Pestivirus tauri*)). It should be noted however, that antibody ELISAs vary in their ability to detect antibody to *Pestivirus brazilense* (BVDV type 3- (*Pestivirus brazilense*)) and vaccines designed to protect against BVDV type 1 and BVDV type 2 may not confer full protection against infection with these novel pestiviruses (Bauermann *et al.*, 2012; 2013).

## 3. Pathogenesis

### 3.1. Acute infections

Acute infections with BVDV are encountered more frequently in young animals, and may be clinically inapparent or associated with fever, diarrhoea (Baker 1995), respiratory disease and sometimes sudden death. The severity of disease may vary with virus strain and the involvement of other pathogens (Brownlie, 1990). In particular, outbreaks of a severe form of acute disease with haemorrhagic lesions, thrombocytopenia and high mortality have been reported sporadically from some countries (Baker, 1995; Bolin & Ridpath, 1992). Infection with type 2 viruses (*Pestivirus tauri*) in particular has been demonstrated to cause altered platelet function. During acute infections there is a brief viraemia for 7–10 days and shedding of virus can be detected in nasal and ocular discharges. There may also be a transient leukopenia, thrombocytopenia or temperature response, but these can vary greatly among animals. Affected animals may be predisposed to secondary infections with other viruses and bacteria. Although BVDV may cause a primary respiratory disease on its own, the immunosuppressive effects of the virus exacerbate the impact of this virus. BVDV is one of the major pathogens of the bovine respiratory disease complex in feedlot cattle and in other intensive management systems such as calf raising units.

Infection of breeding females immediately prior to ovulation and in the first few days after insemination can result in conception failure and early embryonic loss (McGowan & Kirkland, 1995). Cows may also suffer from infertility, associated with changes in ovarian function and secretions of gonadotropin and progesterone (Fray *et al.*, 2002). Bulls may excrete virus in semen for a short period during and immediately after infection and may suffer a temporary reduction of fertility. Although the virus level in this semen is generally low it can result in reduced conception rates and be a potential source of introduction of virus into a naive herd (McGowan & Kirkland, 1995).

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### 3.2. *In-utero* infections

144 Infection of a breeding female can result in a range of different outcomes, depending on the stage of gestation  
145 at which infection occurred. Before about 25 days of gestation, infection of the developing conceptus will usually  
146 result in embryo-fetal death, although abortion may be delayed for a considerable time (McGowan & Kirkland,  
147 1995). Surviving fetuses are normal and uninfected. However, infection of the female between about 30–90  
148 days will invariably result in fetal infection, with all surviving progeny PI and seronegative. Infection at later  
149 stages and up to about day 150 can result in a range of congenital defects including hydranencephaly, cerebellar  
150 hypoplasia, optic defects, skeletal defects such as arthrogryposis and hypotrichosis. Growth retardation may  
151 also occur, perhaps as a result of pituitary dysfunction. Fetal infection can result in abortion, stillbirth or the  
152 delivery of weak calves that may die soon after birth (Baker, 1995; Brownlie, 1990; Duffell & Harkness, 1985;  
153 Moennig & Liess, 1995). Some PI calves may appear to be normal at birth but fail to grow normally thrive. They  
154 remain PI for life and are usually seronegative, exceptions may be young calves that ingested colostrum  
155 containing antibodies. The onset of the fetal immune response and production of antibodies occurs between  
156 approximately day 90–120, with an increasing proportion of infected calves having detectable antibodies while  
157 the proportion in which virus may be detected declines rapidly. Infection of the bovine fetus after day 180 usually  
158 results in the birth of a normal seropositive calf.

159

### 3.3. Persistent infections

160 Persistently viraemic animals are a continual source of infective virus to other cattle and are the main reservoir  
161 of BVDV in a population. In a population without a rigorous BVDV control programme, approximately 1–2% of  
162 cattle are PI. During outbreaks in a naive herd or breeding group, if exposure has occurred in the first trimester  
163 of pregnancy, a very high proportion of surviving calves will be PI. If a PI animal dies, there are no  
164 pathognomonic lesions due to BVDV and the pathology is often complicated by secondary infections with other  
165 agents. Some PI animals will survive to sexual maturity and may breed successfully but their progeny of female  
166 PI animals will also always be PI. Animals being traded or used for artificial breeding should first be screened  
167 to ensure that they are not PI.

168

### 3.4. Mucosal disease

169 Persistently viraemic animals may later succumb to mucosal disease (Brownlie, 1985). However, cases are  
170 rare. This syndrome has been shown to be the outcome of the infection of a PI animal with an antigenically  
171 similar cytopathic virus, which can arise either through superinfection, recombination between non-cytopathic  
172 biotypes, or mutation of the persistent biotype (Brownlie, 1990). There is usually little need to specifically confirm  
173 that a PI animal has succumbed to mucosal disease as this is largely a scientific curiosity and of little practical  
174 significance, other than that the animal is PI with BVDV. However, cases of mucosal disease may be the first  
175 indication in a herd that BVDV infection is present and should lead to more in depth investigation and  
176 intervention.

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### 3.5. Semen and embryos

178 Bulls that are PI usually have poor quality, highly infective semen and reduced fertility (McGowan & Kirkland,  
179 1995). All bulls used for natural or artificial insemination should be screened for both acute and persistent BVDV  
180 infection. A rare event, possibly brought about by acute infection during pubescence, can result in persistent  
181 infection of the testes and thus strongly seropositive bulls that intermittently excrete virus in semen (Voges *et*  
182 *al.*, 1998). This phenomenon has also been observed following vaccination with an attenuated virus (Givens *et*  
183 *al.*, 2007). Embryo donor cows that are PI with BVDV also represent a potential source of infection, particularly  
184 as there are extremely high concentrations of BVDV in uterine and vaginal fluids. While oocysts without an intact  
185 zona pellucida have been shown to be susceptible to infection *in vitro*, the majority of oocysts remain uninfected  
186 with BVDV. Normal uninfected progeny have also been 'rescued' from PI animals by the use of extensive  
187 washing of embryos and *in-vitro* fertilisation. Female cattle used as embryo recipients should always be  
188 screened to confirm that they are not PI, and ideally, are seropositive or were vaccinated at least 4 weeks before  
189 first use.

190 Biological materials used for *in-vitro* fertilisation techniques (bovine serum, bovine cell cultures) have a high risk  
191 of contamination and should be screened for BVDV. Incidents of apparent introduction of virus via such  
192 techniques have highlighted this risk. It is considered essential that serum supplements used in media should  
193 be free of contaminants as detailed in Chapter 1.1.9 *Tests for sterility and freedom from contamination of*  
194 *biological materials intended for veterinary use*, using techniques described in Section B.3-1.1 of this chapter.

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## 195 4. Approaches to diagnosis and sample collection

196 The diagnosis of BVDV infection can sometimes be complex because of the delay between infection and clinical  
197 expression. While detection of PI animals should be readily accomplished using current diagnostic methods, the recognition  
198 of acute infections and detection of BVDV in reproductive materials can be more difficult.

### 199 4.1. Acute infections

200 Unlike PI animals, acutely infected animals excrete relatively low levels of virus and for a short period of time  
201 (usually about 7–10 days) but the clinical signs may occur during the later stages of viraemia, reducing the time  
202 to detect the virus even further. In cases of respiratory or enteric disease, samples should be collected from a  
203 number of affected animals, preferentially selecting the most recently affected. Swabs should be collected from  
204 the nares and conjunctiva of animals with respiratory disease or from rectum and faeces if there are enteric  
205 signs. Lung and spleen are preferred from dead animals. Viral RNA may be detected by real-time RT-PCR  
206 assays and have the advantages of high sensitivity and being able to detect genome from non-infectious virus.  
207 As the virus levels are very low, it is not usually practical to undertake virus isolation unless there is a need to  
208 characterise the strain of BVDV involved. Serology undertaken on paired acute and convalescent sera (collected  
209 at least 21 days after the acute sample and from 8–10 animals) is worthwhile and gives a high probability of  
210 incriminating or excluding BVDV infection.

211 Confirmation that an abortion, stillbirth or perinatal death is caused by BVDV is often difficult to establish  
212 because there can be a long delay between initial infection and death or expulsion of the fetus. Sampling should  
213 take into consideration the need to detect either viral components or antibodies. Spleen and lung are preferred  
214 samples for virus detection while pericardial or pleural fluids are ideal samples for serology. The stomach of  
215 newborn calves should be checked to confirm that sucking has not occurred. While virus may be isolated from  
216 fetal tissue in some cases, emphasis should be placed on the detection of viral antigen by ELISA or RNA by  
217 real-time RT-PCR. For serology, both ELISAs and virus neutralisation test (VNT) are suitable though sample  
218 quality and bacterial contamination may compromise the ability to detect antibodies by VNT. Maternal serology,  
219 especially on a group of animals, can be of value, with the aim of determining whether there has been recent  
220 infection in the group. A high antibody titre (>1/1000) to BVDV in maternal serum is suggestive of fetal infection  
221 and is probably due to the fetus providing the dam with an extended exposure to virus.

### 222 4.2. Persistent infections

223 In the past, identification of PI animals relied heavily on the use of virus isolation in cell cultures. However,  
224 antigen detection ELISAs and real-time RT-PCR assays, each with relatively high sensitivity, are widely used  
225 for the detection of viral antigens or RNA in both live and dead animals. Virus isolation aimed at the detection  
226 of non-cytopathic BVDV in blood is also used, while in some countries, the virus has been identified by  
227 immunohistochemistry (IHC). Skin samples have been collected from live animals while a wide range of tissues  
228 from dead animals are suitable. Both virus isolation and IHC are labour intensive and costly and can be  
229 technically demanding. Virus isolation from blood can be confounded by the presence of maternal antibodies to  
230 BVDV in calves less than 4–5 months of age (diagnostic gap). Also for antigen detection ELISAs and flow  
231 cytometry from blood or blood leukocytes, there are restrictions that limit when animals that ingested colostrum  
232 that contains antibodies to against BVDV can be reliably tested. In older animals with persistent viraemia  
233 infection, low levels of antibody may be present due to their ability to seroconvert to strains of BVDV (including  
234 vaccines) antigenically different to the persisting virus (Brownlie, 1990). Bulk (tank) or individual milk samples  
235 have been used to monitor dairy herds for the presence of a PI animal. Antigen ELISA, real-time PCR and virus  
236 isolation have all been used. To confirm a diagnosis of persistent infection, animals should be retested after an  
237 interval of at least 3 weeks by testing of blood samples for the presence of the virus and for evidence absence  
238 of seroconversion. Care should be taken with retesting of skin samples as it has been shown that, in some acute  
239 cases, viral antigen may persist for many weeks in skin (Cornish *et al.*, 2005).

### 240 4.3. Mucosal disease

241 Although not undertaken for routine diagnostic purposes, for laboratory confirmation of a diagnosis of mucosal  
242 disease it is necessary to isolate the cytopathic virus. This biotype may sometimes be isolated from blood, but  
243 it can be recovered more consistently from a variety of other tissues, in particular spleen, intestine and Peyer's  
244 patch tissue. Virus isolation is readily accomplished from spleen which is easy to collect and is seldom toxic for  
245 cell culture.

### 246 4.4. Reproductive materials

247 Semen donor bulls should be sampled for testing for freedom from BVDV infection prior to collection of semen,  
248 in accordance with the *Terrestrial Animal Health Code*. It is necessary to confirm that these bulls are not PI, are  
249 not undergoing an acute infection and to establish their serological status. This initial testing should be carried

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out on whole blood or serum samples. To establish that a seropositive bull does not have a persistent testicular infection (PTI), samples of semen should be collected on at least three separate occasions at intervals of not less than 7 days due to the possibility of intermittent low level virus excretion, especially during the early stages of infection. There is also a need to submit a number of straws from each collection, or an appropriate volume of raw semen. Particular care should be taken to ensure that sample transport recommendations are adhered to and that the laboratory documents the condition of the samples on arrival at the laboratory. Further details of collection, transport and test requirements are provided in sections that follow.

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## B. DIAGNOSTIC TECHNIQUES

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**Table 1. Test methods available for diagnosis of bovine viral diarrhoea and their purpose**

Method	Purpose					
	Population freedom from infection <sup>(a)</sup>	Individual animal freedom from infection prior to movement <sup>(a)</sup>	Contribute to eradication policies <sup>(a)</sup>	Confirmation of clinical cases <sup>(a)</sup>	Prevalence of infection – surveillance <sup>(a)</sup>	Immune status in individual animals or populations (post-vaccination) <sup>(f)</sup>
Detection of the agent <sup>(g)</sup>						
Virus isolation	+	++ ±	++	++ ±	–	–
Antigen detection by ELISA	+++ <sub>±</sub>	+++	+++	+++	+++	–
Antigen detection by IHC	–	–	–	++	–	–
NA detection by real-time RT-PCR	+++	+++	+++	+++	+++	–
Detection of immune response						
<b>Antibody detection by ELISA</b>	+++	++	+++	– <u>+(g)</u>	+++	+++
VN	+	+++±	++	–	+	+++

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Key: +++ = recommended for this purpose; ++ recommended but has limitations; + = suitable in very limited circumstances; – = not appropriate for this purpose.

ELISA = enzyme-linked immunosorbent assay; IHC = immunohistochemistry method; NA = nucleic acid; RT-PCR = reverse-transcription polymerase chain reaction; ISH = *in-situ* hybridisation; VN = virus neutralisation.

<sup>(a)</sup>See Appendix 1 of this chapter for justification table for the scores giving to the tests for this purpose.

<sup>(b)</sup>See Appendix 2 of this chapter for justification table for the scores giving to the tests for this purpose.

<sup>(c)</sup>See Appendix 3 of this chapter for justification table for the scores giving to the tests for this purpose.

<sup>(d)</sup>See Appendix 4 of this chapter for justification table for the scores giving to the tests for this purpose.

<sup>(e)</sup>See Appendix 5 of this chapter for justification table for the scores giving to the tests for this purpose.

<sup>(f)</sup>See Appendix 6 of this chapter for justification table for the scores giving to the tests for this purpose.

<sup>(g)</sup>A combination of agent detection methods applied on the same clinical sample is recommended.

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### 1. Detection of the agent

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To prevent the shipment of either animals or animal derivatives (especially semen and embryos) that are infected with BVDV, it is necessary to test for the presence of the infectious virus (virus isolation), viral antigens (antigen detection ELISA) or RNA (real-time RT-PCR) in the blood of the animal being shipped, or the donor of the germplasm (semen or embryos). The exception is for seropositive bulls where semen must be tested rather than the donor bull. Serology only plays a role for establishing that seronegative animals are not undergoing an acute infection or, to establish the serological status of donor bulls. Due to their variable sensitivity without prior virus amplification, procedures such as IHC or *in-situ* hybridisation (ISH) directly on tissues are not considered to be suitable for certification for freedom from BVDV for international trade purposes. In contrast, immune-staining is an essential component of virus isolation in cell culture to detect the presence of non-cytopathic strains of BVDV which predominate in field infections.

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280 All test methods must be extensively validated by testing on known uninfected and infected populations of cattle, including  
281 animals with low- and high-titre viraemias. Methods based on polyclonal or MAb-binding assays (ELISA or IHC), immune  
282 labelling (VI) or on nucleic acid recognition (PCR) must be shown to detect the full range of antigenic and genetic diversity  
283 found among BVD viruses. There are ~~three~~ designated WOA Reference Laboratories for BVDV that can assist with  
284 relevant information; the reference laboratories for classical swine fever could also be approached to offer some advice.

## 285 1.1. Virus isolation

286 When performed to a high standard, BVDV isolation is very reliable. However, it does have very exacting  
287 requirements to ensure that the cell cultures and medium components give a system that is very sensitive and  
288 are not compromised by the presence of either low levels of BVDV specific antibody or virus. Virus isolation only  
289 has the capacity to detect infectious virus which imposes certain limits on sample quality. Further, to detect low  
290 levels of virus that may be present in some samples, particularly semen, it may be necessary to examine larger  
291 volumes of specimen than is usual. Some of these limitations can be overcome by the use of antigen detection  
292 ELISAs with proven high analytical sensitivity, or the use of real-time RT-PCR.

293 The virus may be isolated in a number of bovine monolayer cell cultures (e.g. kidney, lung, testis or turbinate).  
294 In some instances, ovine cells are also suitable. Primary or secondary cultures can be frozen as cell suspensions  
295 in liquid nitrogen. These can then be tested over a series of passages, or seeded to other susceptible cells and  
296 checked for freedom from contaminants and to evaluate their sensitivity compared to an approved batch of cells  
297 before routine use. Such problems may be reduced by the use of continuous cell lines, which can be obtained  
298 BVD-free, however, their BVDV-free status and susceptibility must be monitored regularly. Continuous cells  
299 should be used under a 'seed lot' system where they are only used over a limited passage range, within which  
300 they have been shown to have acceptable sensitivity to BVDV infection. Although particular continuous cell lines  
301 are considered to be appropriate for use for BVDV isolation, there can be significant variation in batches of cells  
302 from different sources due to differing passage histories so their suitability must still be confirmed before routine  
303 use.

304 Non-cytopathic BVDV is a common contaminant of bovine tissues and cell cultures must be checked for freedom  
305 from adventitious virus by regular testing. Cells must be grown in proven cell culture medium components and  
306 a large area of cells must be examined. It is not appropriate to screen a few wells of a 96 well plate – examining  
307 all wells of a 96 well plate will be more convincing evidence of freedom. The fetal bovine serum that is selected  
308 for use in cell culture must also be free not only from virus, but also and of equal or perhaps even greater  
309 importance, from BVDV neutralising antibody. Heat treatment (56°C for 30–45 minutes) is inadequate for the  
310 destruction of BVDV in contaminated serum; irradiation with a dose of at least 25 kiloGrays (2.5 Mrad) is more  
311 certain. Commercial batches of fetal bovine serum mostly test positive by real-time RT-PCR even after the virus  
312 has been inactivated by irradiation. Further, most commercially collected batches of fetal bovine serum contain  
313 antibodies to pestiviruses, sometimes at levels that are barely detectable but sufficient to inhibit virus isolation.  
314 To overcome this, serum can be obtained from BVD virus and antibody free donor animals and used with  
315 confidence. Testing of donors for both virus and antibody occurs on an individual animal basis. Although horse  
316 serum has been substituted for bovine fetal serum, it is often found to have poorer cell-growth-promoting  
317 characteristics. Further there has sometimes been cross contamination with fetal bovine serum during  
318 processing, negating the objective of obtaining a BVDV-free product.

319 Buffy coat cells, whole blood, washed leukocytes or serum are suitable for isolation of the virus from live animals.  
320 Maternal antibody may interfere with isolation from serum in young calves. Tissue suspensions from post-  
321 mortem cases should be prepared by standard methods. Confirmation that a bull is not PI with BVDV is most  
322 readily achieved by testing of a blood sample. However, persistent testicular infections (PTI) have been detected  
323 in some bulls that have recovered from acute infection, are no longer viraemic and are now seropositive (Voges  
324 *et al.*, 1998). Virus may be detected in most but not all collections of semen from these bulls. Although still  
325 considered to be uncommon, to exclude the potential for a PTI it is essential to screen semen from all  
326 seropositive bulls. To be confident that a bull does not have a PTI, batches of semen collected over several  
327 weeks should be screened. Once a series of collections have been screened, further testing of semen from a  
328 seropositive bull is not warranted. Raw semen, and occasionally extended semen, is cytotoxic and must be  
329 diluted in culture medium. For these reasons, it is important to monitor the health of the cells by microscopic  
330 examination at intervals during the incubation. These problems are largely overcome by the use of real-time  
331 RT-PCR which has several advantages over virus isolation, including higher sensitivity and the potential to be  
332 completed within a few hours rather than weeks for virus isolation.

333 There are many variations of procedure in use for virus isolation. All should be optimised to give maximum  
334 sensitivity of detection of a standard virus preparation. All biological components used for cell culture should be  
335 screened and shown to be free of both BVDV and antibodies to BVDV. Cell cultures (whether primary or  
336 continuous lines) should be regularly checked to confirm that they maintain maximum susceptibility to virus  
337 infection. Depending on the specimen type and purpose for testing, virus isolation is likely to require one or more

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338 passages in cell cultures. While PI animals can be readily identified by screening blood or serum with one  
339 passage, semen should be routinely cultured for three passages and biological products such as fetal bovine  
340 serum up to five times (original inoculation plus four passages). Conventional methods for virus isolation are  
341 used, with the addition of a final immune-staining step (immunofluorescence or, more frequently, peroxidase  
342 staining) to detect growth of non-cytopathic virus. Thus, tube cultures should include flying cover-slips, while  
343 microplate cultures can be fixed and labelled directly in the plate. Examples are given below. Alternatively,  
344 culture supernatant from the final passage can be screened by real-time RT-PCR (see below).

345 **1.1.1. Microplate immunoperoxidase method for mass screening for virus detection in serum**  
346 **samples (Meyling, 1984)**

- 347 i) 10–25 µl of the serum sample is placed into each of four wells of a 96-well tissue-culture grade  
348 microplate. This is repeated for each sample. Known positive and negative controls are included.
- 349 ii) 100 µl of a cell suspension at the appropriate concentration (usually about 150,000 cells/ml) in  
350 medium without fetal calf serum (FCS) is added to all wells. *Note:* the sample itself acts as the cell-  
351 growth supplement. If testing samples other than serum, use medium with 10% FCS that is free of  
352 antibodies to ruminant pestiviruses.
- 353 iii) The plate is incubated at 37°C for 4 days, either in a 5% CO<sub>2</sub> atmosphere or with the plate sealed.
- 354 iv) Each well is examined microscopically for evidence of cytopathology (cytopathic effect or CPE), or  
355 signs of cytotoxicity.
- 356 v) The cultures are frozen briefly at approximately –80°C and 50 µl of the culture supernatant is  
357 passaged to new cell cultures, repeating steps 1.1.i to iv above.
- 358 vi) The cells are then fixed and stained by one of two methods:
- 359 • **Paraformaldehyde**
- 360 a) Add 200 µl of a 1/10 dilution of formaldehyde solution (approximately 3% concentration) to the plate  
361 and leave at room temperature for 10 minutes.
- 362 b) The contents of the plate are then discarded and the plate is washed.
- 363 c) Wash plates 5 times with 0.05% Tween 20 in water (an automatic microplate washer can be used  
364 with a low pressure and speed setting).
- 365 d) To each well add 50 µl of an antiviral antibody at the appropriate dilution (prepared in phosphate  
366 buffered saline/ PBS containing 1% gelatin) and incubate for 60–90 minutes at 37°C in a humidified  
367 chamber.
- 368 e) Wash plates five times as in step c).
- 369 f) Dilute the appropriate peroxidase conjugated antiserum to the optimum dilution in 1% gelatin/PBS (e.g.  
370 peroxidase conjugated rabbit anti-mouse immunoglobulin when the antiviral antibody is a mouse  
371 monoclonal). The optimum concentration should be determined for each batch of conjugate by  
372 “checkerboard” titration against reference positive and negative controls.
- 373 g) To each well of the microplate add 50 µl of the diluted peroxidase conjugate and incubate for 90 minutes  
374 at 37°C in a humidified chamber.
- 375 h) Wash plates five times as in step c).
- 376 i) “Develop” the plate by adding 3-amino-9-ethyl carbazole (AEC) substrate (100 µl/well) and allowing to  
377 react for 30 minutes at room temperature.
- 378 j) Add 100 µl of PBS to each well and add a lid to each plate.
- 379 k) Examine the wells by light microscopy, starting with the negative and positive control wells. There  
380 should be no or minimal staining apparent in the cells that were uninfected (negative control). The  
381 infected (positive control) cells should show a reddish- brown colour in the cytoplasm.



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- 382                           •   **Acetone**
- 383                           a)   The plate is emptied by gentle inversion and rinsed in PBS.
- 384                           b)   The cells are fixed as follows: the plate is dipped into a bath of 20% acetone in PBS, emptied  
385 immediately and then transferred to a fresh bath of 20% acetone in PBS for 10 minutes. The plate  
386 is drained thoroughly and as much fluid as possible is removed by tapping and blotting. The plate  
387 is dried thoroughly for at least 3 hours at a temperature of 25–30°C (e.g. using radiant heat from a  
388 bench lamp). *Note:* the drying is part of the fixation process.
- 389                           c)   The fixed cells are rinsed by adding PBS to all wells.
- 390                           d)   The wells are drained and the **antiviral** BVD antibody (50 µl) is added to all wells at a predetermined  
391 dilution in PBS containing 1% Tween 80 (PBST) and 5% horse serum or 1% gelatin. (Horse serum  
392 or gelatin may be added to reduce nonspecific staining.)
- 393                           e)   Incubate at 37°C for 15 minutes.
- 394                           f)   Empty the plate and wash three times in PBST.
- 395                           g)   Drain and add the appropriate anti-species serum conjugated to peroxidase at a predetermined  
396 dilution in PBST (50 µl per well) for 15 minutes at 37°C.
- 397                           h)   Empty the plate and wash three times in PBST.
- 398                           i)   Rinse the plate in distilled water. Ensure all fluid is tapped out from the plate.
- 399                           j)   Add freshly prepared hydrogen peroxide substrate with a suitable chromogen, e.g. 3-amino-9-ethyl  
400 carbazole (AEC).
- 401                           An alternative substrate can be made, consisting of 9 mg diaminobenzidine tetrahydrochloride and  
402 6 mg sodium perborate tetrahydrate dissolved in 15 ml of PBS. Though the staining is not quite so  
403 intense, these chemicals have the advantage that they can be shipped by air.
- 404                           k)   The plate is examined microscopically. Virus-positive cells show red-brown cytoplasmic staining.
- 405                           Alternative methods for fixation of the cells may be used and include the use of heat (see Chapter 3.8.3  
406 *Classical swine fever*, Section B.2.2.1.viii). These should be first evaluated to ensure that the capacity to  
407 detect viral antigen is not compromised.

#### 408                           **1.1.2. Tube method for tissue or buffy coat suspensions**

- 409                           *Note:* this method can also be conveniently adapted to 24-well plastic dishes. *Note:* a minimum of 2 and  
410 preferably 3 passages (including primary inoculation) is required.
- 411                           i)   Tissue samples are ground up and a 10% suspension in culture medium is made. This is then  
412 centrifuged to remove the debris.
- 413                           ii)   Test tube cultures with newly confluent or subconfluent monolayers of susceptible bovine cells are  
414 inoculated with 0.1 ml of the sample. The culture is left to adsorb for 1 hour at 37°C.
- 415                           iii)   The culture is washed with 1 ml of medium; this is then discarded and 1 ml of culture maintenance  
416 medium is added.
- 417                           iv)   The culture is incubated for 4–5 days at 37°C and examined microscopically for evidence of CPE  
418 or signs of cytotoxicity.
- 419                           v)   The culture should then be frozen and thawed for passage to fresh cultures for one or preferably  
420 two more passages (including the culture inoculated for the final immunostaining). At the final  
421 passage, after freeze–thaw the tissue culture fluid is harvested and passaged on to microtitre plates  
422 for culture and staining by the immunoperoxidase method (see section B.3.1.1 above) or by the  
423 immunofluorescent method. For immunofluorescence, cover-slips are included in the tubes and  
424 used to support cultured cells. At the end of the culture period, the cover slips are removed, fixed  
425 in 100% acetone and stained with an immunofluorescent conjugate to BVDV. Examine the cover  
426 slips under a fluorescent microscope for diffuse, cytoplasmic fluorescence characteristic of  
427 pestiviruses. Alternatively, culture supernatant from the final passage can be screened by real-time  
428 RT-PCR (see below).

#### 429                           **1.1.3. Virus isolation from semen**

- 430                           The samples used for the test are, typically, extended bovine semen or occasionally raw semen. Semen  
431 samples should be transported to the laboratory in liquid nitrogen, or on dry ice. The samples should be
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432 stored in liquid nitrogen or at lower than  $-70^{\circ}\text{C}$  (for long-term storage) or  $4^{\circ}\text{C}$  (for short-term storage of  
433 not more than 1–2 days). The receiving laboratory should document the condition under which samples  
434 are received. Raw semen is generally cytotoxic and should be prediluted (e.g. 1/10 in BVDV free bovine  
435 serum) before being added to cell cultures. At least 0.1 ml of raw semen should be tested with three  
436 passages in cell culture. Toxicity may also be encountered with extended semen. For extended semen,  
437 an approximation should be made to ensure that the equivalent of a minimum of 0.1 ml raw semen is  
438 examined (e.g. a minimum of 1.0 ml extended semen). If toxicity is encountered, multiple diluted samples  
439 may need to be tested to reach a volume equivalent to 0.1 ml raw semen (e.g.  $5 \times 1$  ml of a sample of  
440 extended semen that has been diluted 1/5 to reduce toxicity). A suggested method is as follows:

- 441 i) Dilute 200  $\mu\text{l}$  fresh semen in 1.8 ml bovine serum containing antibiotics. This can be the same  
442 serum as is being used for supplementing the cell cultures, and must be shown to be free from  
443 antibodies to against BVDV.
- 444 ii) Mix vigorously and leave for 30 minutes at room temperature.
- 445 iii) Inoculate 1 ml of the semen/serum mixture into a monolayer of susceptible cells (see virus isolation  
446 from tissue above) in cell culture tubes or a six-well tissue culture plate.
- 447 iv) Incubate the cultures for 1 hour at  $37^{\circ}\text{C}$ .
- 448 v) Remove the mixture, wash the monolayer several times with maintenance medium and then add  
449 new maintenance medium to the cultures.
- 450 vi) Include BVDV negative and positive controls in the test. Special caution must be taken to avoid  
451 accidental contamination of test wells by the positive control, for example always handling the  
452 positive control last.
- 453 vii) Observe plates microscopically to ensure freedom from contamination and cytotoxicity. No  
454 cytopathology is expected as a result of BVDV infection but other viruses such as BHV-1 could be  
455 inadvertently isolated.
- 456 viii) After 5–7 days, the cultures are frozen at or below approximately  $-70^{\circ}\text{C}$  and thawed, clarified by  
457 centrifugation, and the supernatant used to inoculate fresh monolayers.
- 458 ix) At the end of the second passage, the supernatant from the freeze-thaw preparation should be  
459 passaged onto cultures in a suitable system for immunoperoxidase staining or other antigen  
460 detection or by real-time RT-PCR after 5 days of culture. This is most readily achieved in 96 well  
461 microplates. The sample is considered to be negative, if there is no evidence of viral antigen or  
462 BVDV RNA detected.

## 463 1.2. Nucleic acid detection

464 Conventional gel-based RT-PCR has in the past been used for the detection of BVD viral RNA for diagnostic  
465 purposes. A multiplex RT-PCR has been used for the simultaneous amplification and typing of virus from cell  
466 culture, or direct from blood samples. However, gel-based RT-PCR has the disadvantage that it is relatively  
467 labour intensive, expensive and prone to cross contamination. These problems had been markedly reduced  
468 following the introduction of probe-based real-time or quantitative RT-PCR methods. Nevertheless, stringent  
469 precautions should still be taken to avoid nucleic acid contamination in the test system and general laboratory  
470 areas where samples are handled and prepared (see Chapter 1.1.6 *Principles and methods of validation of*  
471 *diagnostic assays for infectious diseases* and Chapter 2.2.3 *Development and optimisation of nucleic acid*  
472 *assays*). These assays have even higher sensitivity than gel-based RT-PCR and can be completed in a few  
473 hours. They are in widespread use for the diagnosis of infectious diseases, allowing the direct detection of viral  
474 RNA from a wide range of specimens including serum, whole blood, tissues, milk and semen. The high analytical  
475 sensitivity allows the adoption of strategies to screen pools of individual samples or testing of bulk tank milk. By  
476 using this approach, the presence of one or more PI animals can be identified in herds containing several  
477 hundred cows. However, it is not appropriate to pool blood samples taken from calves between day 7 and 40 of  
478 life, when colostrum that contains antibodies to against BVDV was ingested. During this time the sensitivity of  
479 PCR can be reduced and infected animals escape detection. In contrast, the detection of viral RNA in skin  
480 biopsy samples remains unaffected (Fux & Wolf, 2012). Although slightly more expensive than immunostaining  
481 methods, real-time RT-PCR is a quick and reliable method that can also be used to screen culture supernatant  
482 from the final passage of cell cultures. While real-time RT-PCR has very high sensitivity and can be applied to  
483 the screening of biological materials used for vaccine manufacture, caution is needed in the interpretation of  
484 results, as the detection of viral RNA does not imply *per se* that infective virus is present. Real-time RT-PCR  
485 assays based on fluorescent-labelled DNA probes can also be used to differentiate pestiviruses (e.g. McGoldrick  
486 *et al.*, 1999).

487 Primers for the assay should be selected in highly conserved regions of the genome, ideally the 5'-noncoding  
488 region, or the NS3 (p80 gene). There are published assays that are broadly reactive across the pestivirus genus,  
489 detecting all BVDV types (*Pestivirus bovis*, *tauri* and *brazilense*), CSFV (*Pestivirus suis*), some strains of BDV  
490 (*Pestivirus ovis*) and most of the several 'atypical' pestiviruses (e.g. Hoffman *et al.*, 2006). A sensitive broadly  
491 reactive assay is recommended for diagnostic applications because interspecies transfer of different  
492 pestiviruses is occasionally encountered. When further identification of the specific virus is required, pestivirus  
493 species-specific assays can be applied to further type the virus. It is important to thoroughly optimise all aspects  
494 of the real-time RT-PCR assay, including the nucleic acid extraction and purification. Optimal concentrations of  
495 Mg<sup>2+</sup>, primers, probe and polymerase, and the cycling parameters need to be determined. However, fully  
496 formulated and optimised 'ready to use' 'mastermixes' are now available commercially and only require addition  
497 of optimised concentrations of primers and probe. Optimised cycling conditions are often recommended for a  
498 particular mastermix.

499 A variety of commercially available nucleic acid purification systems are available in kit form, and several can  
500 be semi-automated. Systems based on the capture and purification of RNA using magnetic beads are in  
501 widespread use and allow rapid processing of large numbers of samples. Specific products should be evaluated  
502 to determine the optimal kit for a particular sample type and whether any preliminary sample processing is  
503 required. For whole blood samples, the type of anticoagulant and volume of blood in a specimen tube is  
504 important. More problems with inhibitors of the PCR reaction are encountered with samples collected into  
505 heparin treated blood than EDTA. These differences are also exacerbated if the tube does not contain the  
506 recommended volume of blood, thereby increasing the concentration of anticoagulant in the sample. To identify  
507 possible false-negative results, it is recommended to spike an exogenous ('internal control') RNA template into  
508 the specimen prior to RNA extraction (e.g. Hoffman *et al.*, 2006). By the inclusion of PCR primers and probe  
509 specific to the exogenous sequence, the efficiency of both the RNA extraction and also the presence of any  
510 PCR inhibitors can be monitored. While valuable for all sample types, the inclusion of an internal control is  
511 particularly desirable when testing semen and whole blood. When using an internal control, extensive testing is  
512 necessary to ensure that PCR amplification of the internal control does not compete with the diagnostic PCR  
513 and thus lower the analytical sensitivity (see also chapter 1.1.6).

514 When it is suspected that a sample may contain substances that are adversely affecting either the efficiency of  
515 RNA extraction or the real-time RT-PCR assay, modest dilution of the sample in saline, cell culture medium or  
516 a buffer solution (e.g. phosphate buffered gelatin saline [PBGs]) will usually overcome the problem. Dilution of  
517 a semen sample by 1/4 and whole unclotted blood at 1/10 is usually adequate. As the real-time RT-PCR has  
518 extremely high analytical sensitivity, dilution of the sample rarely has a significant impact on the capacity of the  
519 assay to detect viral RNA when present.

### 520 1.2.1. Real-time polymerase chain reaction for BVDV detection in semen

521 Real-time RT-PCR has been shown to be extremely useful to screen semen samples to demonstrate  
522 freedom from BVDV and, apart from speed, often gives superior results to virus isolation in cell culture,  
523 especially when low virus levels are present, such as may be found in bulls with a PTI. The real-time RT-  
524 PCR described here uses a pair of sequence-specific primers for amplification of target D-RNA and a 5'-  
525 nuclease oligoprobe for the detection of amplified products. The oligoprobe is a single, sequence-specific  
526 oligonucleotide, labelled with two different fluorophores. The primers and probe are available  
527 commercially and several different fluorophores options are available. This pan-pestivirus real-time RT-  
528 PCR assay is designed to detect viral D-RNA of all strains of BVDV types 1 (*Pestivirus bovis*) and BVDV,  
529 2 (*Pestivirus tauri*) and 3 (*Pestivirus brazilense*) as well as BDV, CSFV (*Pestivirus suis*), some strains of  
530 BDV (*Pestivirus ovis*) and most atypical pestiviruses. The assay selectively amplifies a 208 base pair  
531 sequence of the 5' non-translated region (5' NTR) of the pestivirus genome. Details of the primers and  
532 probes are given in the protocol outlined below.

- 533 i) Sample preparation, equipment and reagents
- 534 a) The samples used for the test are, typically, extended bovine semen or occasionally raw semen. If  
535 the samples are only being tested by real-time RT-PCR, it is acceptable for them to be submitted  
536 chilled, but they must still be cold when they reach the laboratory. Otherwise, if a cold chain cannot  
537 be assured or if virus isolation is being undertaken, the semen samples should be transported to  
538 the laboratory in liquid nitrogen or on dry ice. At the laboratory, the samples should be stored in  
539 liquid nitrogen or at lower than -70°C (for long-term storage) or 4°C (for short-term storage of up to  
540 7 days). *Note:* samples for virus isolation should not be stored at 4°C for more than 1–2 days.
- 541 b) Due to the very high analytical sensitivity of real-time RT-PCR, much smaller volumes of semen  
542 may be used. However, at least three straws (minimum 250 µl each) from each collection batch of  
543 semen should be processed. The semen in the three straws should be pooled and mixed thoroughly  
544 before taking a sample for nucleic acid extraction.

- 
- 545 c) A real-time PCR detection system, and the associated data analysis software, is required to perform  
546 the assay. A number of real-time PCR detection systems are available from various manufacturers.  
547 ~~Other equipment required for the test includes a micro-centrifuge, a chilling block, a micro-vortex,~~  
548 ~~and micropipettes.~~ As real-time RT-PCR assays are able to detect very small amounts of target  
549 nucleic acid molecules, appropriate measures are required to avoid contamination, ~~including~~  
550 ~~dedicated and physically separated 'clean' areas for reagent preparation (where no samples or~~  
551 ~~materials used for PCR are handled), a dedicated sample processing area and an isolated area for~~  
552 ~~the PCR thermocycler and associated equipment. Each area should have dedicated reagents and~~  
553 ~~equipment.~~ Furthermore, a minimum of one negative sample should be processed in parallel to  
554 monitor the possibility of low level contamination. Sources of contamination may include product  
555 carry-over from positive samples or, more commonly, from cross contamination by PCR products  
556 from earlier work.
- 557 d) The real-time RT-PCR assay involves two separate procedures.
- 558 1) Firstly, BVDV RNA is extracted from semen using an appropriate validated nucleic acid  
559 extraction method. Systems using magnetic beads for the capture and purification of the  
560 nucleic acid are recommended. It is also preferable that the beads are handled by a  
561 semi-automated magnetic particle handling system.
- 562 2) The second procedure is the RT-PCR analysis of the extracted RNA template in a real-  
563 time RT-PCR system.
- 564 ii) Extraction of RNA
- 565 RNA or total nucleic acid is extracted from the pooled (three straws collected at the same time from  
566 the same animal) semen sample. Use of a commercially available magnetic bead based extraction  
567 kit is recommended. However, the preferred kit should be one that has been evaluated to ensure  
568 optimal extraction of difficult samples (semen and whole blood). Some systems and kit protocols  
569 are sufficiently refined that it is not necessary to remove cells from the semen sample. Prior to  
570 extraction dilute the pooled semen sample 1/4 in phosphate buffered gelatin saline (PBGS) or a  
571 similar buffered solution. Complete the RNA extraction by taking 50 µl of the diluted, pooled sample  
572 and add it to the sample lysis buffer. Some commercial extraction kits may require the use of a  
573 larger volume. It has also been found that satisfactory results are obtained by adding 25 µl of  
574 undiluted pooled sample to sample lysis buffer. Complete the extraction by following the kit  
575 manufacturer's instructions.
- 576 iii) Real-time RT-PCR assay procedure
- 577 a) Reaction mixture: There are a number of commercial real-time PCR amplification kits available from  
578 various sources and the particular kits selected need to be compatible with the real-time PCR  
579 platform selected. The required primers and probes can be synthesised by various commercial  
580 companies. The WOA Reference Laboratories for BVDV can provide information on suitable  
581 suppliers.
- 582 b) Supply and storage of reagents: The real-time PCR reaction mixture is normally provided as a 2 ×  
583 concentration ready for use. The manufacturer's instructions should be followed for application and  
584 storage. Working stock solutions for primers and probe are made with nuclease-free water at the  
585 concentration of 20 µM and 3 µM, respectively. The stock solutions are stored at -20°C and the  
586 probe solution should be kept in the dark. Single-use or limited use aliquots can be prepared to limit  
587 freeze-thawing of primers and probes and extend their shelf life.
- 588 c) Primers and probe sequences
- 589 Selection of the primers and probe are outlined in Hoffmann *et al.* (2006) and summarised below.
- 590 *Forward:* BVD 190-F 5'-GRA-GTC-GTC-ART-GGT-TCG-AC  
591 *Reverse:* V326 5'-TCA-ACT-CCA-TGT-GCC-ATG-TAC  
592 *Probe:* TQ-pesti 5'-FAM-TGC-YAY-GTG-GAC-GAG-GGC-ATG-C-TAMRA-3'
- 593 d) Preparation of reaction mixtures
- 594 The PCR reaction mixtures are prepared in a separate room that is isolated from other PCR  
595 activities and sample handling. For each PCR test, appropriate controls should be included. As a  
596 minimum, a no template control (NTC), appropriate negative control (NC) and two positive controls  
597 (PC1, PC2) should be included. The positive and negative controls are included in all steps of the  
598 assay from extraction onwards while the NTC is added after completion of the extraction. The PCR  
599 amplifications are carried out in a volume of 25 µl. The protocol described is based on use of a 96
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600 well microplate based system but other options using microtubes are also suitable. Each well of the  
601 PCR plate should contain 20 µl of reaction mix and 5 µl of sample as follows:

602	12.5 µl	2× RT buffer – from a commercial kit.
603	1 µl	BVD 190-F Forward primer (20 µM)
604	1 µl	V326 Reverse primer (20 µM)
605	1 µl	TQ-pesti Probe (3 µM)
606	2 µl	tRNA (40 ng/µl)
607	1.5 µl	<u>nuclease free</u> water
608	1 µl	25× enzyme mix
609	5 µl	sample (or controls – NTC, NC, PC1, PC2)

610 e) Selection of controls

611 NTC: usually consists of nuclease free water or tRNA in nuclease free water that is added in place  
612 of a sample when the PCR reaction is set up.

613 NC: In practice, many laboratories use PBGS or a similar buffer. Ideally the controls for testing of  
614 semen samples should be negative semen, from seronegative bulls. However, as a minimum, the  
615 assay in use should have been extensively validated with negative and positive samples to confirm  
616 that it gives reliable extraction and amplification with semen.

617 PCs: There are two positive controls (PC1=moderate – [Ct 29-32] and PC2=weak [Ct 32–35]  
618 positive). Positive semen from naturally infected bulls is preferable as a positive control. However,  
619 this is likely to be difficult to obtain. Further, semen from a PI bull is not considered suitable because  
620 the virus loads are usually very high and would not give a reliable indication of any moderate  
621 reduction in extraction or assay performance. Negative semen spiked with defined quantities of  
622 BVDV virus could be used as an alternative. If other samples are used as a routine PC, as a  
623 minimum the entire extraction process and PCR assay in use must have been extensively validated  
624 using known positive semen from bulls with a PTI or from bulls undergoing an acute infection. If  
625 these samples are not available and spiked samples are used for validation purposes, a number of  
626 samples spiked with very low levels of virus should be included. On a day-to-day basis, the inclusion  
627 of an exogenous control with each test sample will largely compensate for not using positive semen  
628 as a control and will give additional benefits by monitoring the efficiency of the assay on each  
629 individual sample. Positive control samples should be prepared carefully to avoid cross  
630 contamination from high titred virus stocks and should be prepared in advance and frozen at a  
631 'ready to use' concentration and ideally 'single use' volume.

632 f) Extracted samples are added to the PCR mix in a separate room. The controls should be added  
633 last, in a consistent sequence in the following order: NTC, negative and then the two positive  
634 controls.

635 g) Real-time polymerase chain reaction

636 The PCR plate or tubes are placed in the real-time PCR detection system in a separate, designated  
637 PCR room. Some mastermixes have uniform reaction conditions that are suitable for many different  
638 assays. As an example, the PCR detection system is programmed for the test as follows:

639	1 × 48°C 10 minutes
640	1 × 95°C 10 minutes
641	45 × (95°C 15 seconds, 60°C 1 minute)

642 h) Analysis of real-time PCR data

643 The software program is usually set to automatically adjust results by compensating for any  
644 background signal and the threshold level is usually set according to the manufacturer's instructions  
645 for the selected analysis software used. In this instance, a threshold is set at 0.05.

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- 646 i) Interpretation of results
- 647 a) Test controls – all controls should give the expected results with positive controls (PC1
- 648 and PC2) falling within the designated range and both the negative control (NC) and no
- 649 template control (NTC) should have no Ct values.
- 650 b) Test samples
- 651 1) Positive result: Any sample that has a cycle threshold (Ct) value less than 40 is
- 652 regarded as positive.
- 653 2) Negative result: Any sample that shows no Ct value is regarded as negative.
- 654 However, before reporting a negative result for a sample, the performance of the
- 655 exogenous internal control should be checked and shown to give a result within the
- 656 accepted range for that control (for example, a Ct value no more than 2–3 Ct units
- 657 higher than the NTC).

### 658 1.3. Enzyme-linked immunosorbent assay for antigen detection

659 Antigen detection by ELISA has become a widely adopted method for the detection of individual PI animals.

660 These assays are not intended for the detection of acutely infected animals (though from time to time this may be

661 achieved). Importantly, these assays are not designed for screening of semen or biological materials used in

662 assays or vaccine manufacture. Several methods for the ELISA for antigen detection have been published and

663 a number of commercial kits are available. Most are based on the sandwich ELISA principle, with a capture

664 antibody bound to the solid phase, and a detector antibody conjugated to a signal system, such as peroxidase.

665 Amplification steps such as the use of biotin and streptavidin in the detection system are sometimes used to

666 increase assay sensitivity. Both monoclonal- and polyclonal-based systems are described. The test measures

667 BVD antigen (NS2-3 or ERNS) in lysates of peripheral blood leukocytes; the new generation of antigen-capture

668 ELISAs (ERNS capture ELISAs) are able to detect BVD antigen in blood as well as in plasma or serum samples.

669 The best of the methods gives a sensitivity similar to virus isolation, and may be preferred in those rare cases

670 where persistent infection is combined with seropositivity. Due to transient viraemia, the antigen ELISA is less

671 useful for virus detection in acute BVD infections.

672 The NS2-3-antigen detection ELISAs may be less effective in young calves that have had colostrum due to the

673 presence of BVDV maternal antibodies, especially when blood samples or blood leucocytes are tested (Fux &

674 Wolf, 2012). Blood or blood leucocytes should not be tested in the first month (ERNS capture ELISA) or the first

675 3 months (NS2-3 ELISA) of life due to the inhibitory effect of maternal antibodies. The real-time RT-PCR is

676 probably the most sensitive detection method for this circumstance, but the ERNS ELISA has also been shown

677 to be a sensitive and reliable test, particularly when used with skin biopsy (ear-notch) samples (Cornish *et al.*,

678 2005).

### 679 1.4. Immunohistochemistry

680 Enzyme-labelled methods are useful to detect BVDV antigen in tissue sections, particularly where suitable MAbs

681 are available. However, these assays are not appropriate to certify animals for international trade and use should

682 be limited to diagnostic investigations. It is important that the reagents and procedures used be fully validated,

683 and that nonspecific reactivity be eliminated. For PI cattle almost any tissue can be used, but particularly good

684 success has been found with lymph nodes, thyroid gland, skin, brain, abomasum and placenta. Skin biopsies,

685 such as ear-notch samples, have shown to be useful for *in-vivo* diagnosis of persistent BVDV infection.

## 686 2. Serological tests

687 Antibody to BVDV can be detected in cattle sera by a standard VNT or by ELISA, using one of several published methods

688 or with commercial kits (e.g. Edwards, 1990). Serology is used to identify levels of herd immunity, for the detection of the

689 presence of PI animals in a herd, to assist with investigation of reproductive disease and possible involvement of BVDV

690 and to establish the serological status of bulls being used for semen collection and to identify whether there has been a

691 recent infection. ELISA for antibody in bulk milk samples can give a useful indication of the BVD status of a herd (Niskanen,

692 1993). High ELISA values (0.8 or more absorbance units) in an unvaccinated herd indicates a high probability of the herd

693 having been exposed to BVDV in the recent past, most likely through one or more persistently viraemic animals being

694 present. In contrast, a very low or negative values ( $\leq 0.2$ ) indicates that it is unlikely that persistently viraemic animals are

695 present. However, ELISA values are not always a reliable indicator of the presence of PI animals on farms, due to differing

696 husbandry (Zimmer *et al.*, 2002), recent administration of vaccine and also due to the presence of viral antigen in bulk milk,

697 which may interfere with the antibody assay itself. Determination of the antibody status of a small number of young stock

698 (9–18 months) has also been utilised as an indicator of recent transmission of BVDV in the herd (Houe *et al.*, 1995), but

699 this approach is also dependent on the degree of contact between different groups of animals in the herd and the potential

700 for exposure from neighbouring herds. VN tests are more frequently used for regulatory purposes (e.g. testing of semen  
701 donors) while ELISAs (usually in the form of commercially prepared kits) are commonly used for diagnostic applications.  
702 Whether ELISA or VNT, control positive and negative standard sera must be included in every test. These should give  
703 results within predetermined limits for the test to be considered valid. In the VNT, a 'serum control' to monitor sample  
704 toxicity should also be included for each test sample.

## 705 2.1. Virus neutralisation test

706 Selection of the virus strain to include in a VNT is very important. No single strain is likely to be ideal for all  
707 circumstances, but in practice one should be selected that detects the highest proportion of serological reactions  
708 in the local cattle population. Low levels of antibody to BVDV type 2 virus (*Pestivirus tauri*) may not be detectable  
709 by a neutralisation test that uses type 1 strain of the virus, and vice versa (Fulton *et al.*, 1997). It is important  
710 that BVDV type 1 and BVDV type 2 (*Pestivirus bovis* and *P. tauri*) be used in the test and not just the one that  
711 the diagnostician thinks is present, as this can lead to under reporting. Because it makes the test easier to read,  
712 most laboratories use highly cytopathic, laboratory-adapted strains of BVDV for VN tests. Two widely used  
713 cytopathic strains are 'Oregon C24V' and 'NADL'. However immune-labelling techniques are now available that  
714 allow simple detection of the growth or neutralisation of non-cytopathic strains where this is considered  
715 desirable, especially to support the inclusion of a locally relevant virus strain. An outline protocol for a microtitre  
716 VN test is given below (Edwards, 1990):

### 717 2.1.1. Test procedure

- 718 i) The test sera are heat-inactivated for 30 minutes at 56°C.
- 719 ii) From a starting dilution of 1/4, serial twofold dilutions of the test sera are made in a cell-culture  
720 grade flat-bottomed 96-well microtitre plate, using cell culture medium as diluent. For each sample,  
721 three or four wells are used at each dilution depending on the degree of precision required. At each  
722 dilution of serum, for each sample one well is left without virus to monitor for evidence of sample  
723 toxicity that could mimic viral cytopathology or interfere with virus replication. Control positive and  
724 negative sera should also be included in each batch of tests.
- 725 iii) An equal volume (e.g. 50 µl) of a stock of cytopathic strain of BVDV containing 100 TCID<sub>50</sub> (50%  
726 tissue culture infective dose) is added to each well. A back titration of virus stock is also done in  
727 some spare wells to check the potency of the virus (acceptance limits 30–300 TCID<sub>50</sub>).
- 728 iv) The plate is incubated for 1 hour at 37°C.
- 729 v) A flask of suitable cells (e.g. bovine turbinate, bovine testis) is trypsinised and the cell concentration  
730 is adjusted to  $1.5 \times 10^5$ /ml. 100 µl of the cell suspension is added to each well of the microtitre plate.
- 731 vi) The plate is incubated at 37°C for 4–5 days, either in a 5% CO<sub>2</sub> atmosphere or with the plate sealed.
- 732 vii) The wells are examined microscopically for CPE or fixed and stained by immunoperoxidase staining  
733 using an appropriate monoclonal antibody. The VN titre for each serum is the dilution at which the  
734 virus is neutralised in 50% of the wells. This can be calculated by the Spearman–Kärber or Reed  
735 Muench methods. A seronegative animal will show no neutralisation at the lowest dilution (1/4),  
736 equivalent to a final dilution of 1/8. For accurate comparison of antibody titres, and particularly to  
737 demonstrate significant (more than fourfold) changes in titre, samples should be tested in parallel  
738 in the same test.

## 739 2.2. Enzyme-linked immunosorbent assay

740 Both indirect and blocking types of test can be used. A number of commercial kits are available. As with the  
741 virus neutralisation test, ELISAs configured using antigen from one ~~genotype-species~~ of BVDV may not  
742 efficiently detect antibody induced by another ~~genotype-virus species~~. Tests should therefore be selected for  
743 their ability to detect antibody to the spectrum of types and strains circulating in the country where the test is to  
744 be performed.

745 The chief difficulty in setting up the test lies in the preparation of a viral antigen of sufficient potency. The virus  
746 must be grown under optimal culture conditions using a highly permissive cell type. Any serum used in the  
747 medium must not inhibit growth of BVDV. The optimal time for harvest should be determined experimentally for  
748 the individual culture system. The virus can be concentrated and purified by density gradient centrifugation.  
749 Alternatively, a potent antigen can be prepared by treatment of infected cell cultures with detergents, such as  
750 Nonidet P40, N-decanoyl-N-methylglucamine (Mega 10), Triton X-100 or 1-octyl-beta-D-glucopyranoside (OGP).  
751 Some workers have used fixed, infected whole cells as antigen. ~~In the future,~~ increasing use ~~may be~~ is made of  
752 artificial antigens manufactured by expressing specific viral genes in bacterial or eukaryotic systems. Such  
753 systems should be validated by testing sera specific to a wide range of different virus strains. In the future, this

754 technology should enable the production of serological tests complementary to subunit or marker vaccines, thus  
755 enabling differentiation between vaccinated and naturally infected cattle. An example outline protocol for an  
756 indirect ELISA is given below (Edwards, 1990).

### 757 2.2.1. Test procedure

- 758 i) Roller cultures of secondary calf testis cells with a high multiplicity of infection (about one), are  
759 inoculated with BVDV strain Oregon C24V, overlaid with serum-free medium and incubated for 24  
760 hours at 37°C.
- 761 ii) The cells are scraped off and pelleted. The supernatant medium is discarded. The pellet is treated  
762 with two volumes of 2% OGP in PBS for 15 minutes at 4°C, and centrifuged to remove the cell  
763 debris. The supernatant antigen is stored in small aliquots at -70°C, or freeze-dried. Non-infected  
764 cells are processed in parallel to make a control antigen.
- 765 iii) The antigen is diluted to a predetermined dilution in 0.05 M bicarbonate buffer, pH 9.6. Alternate  
766 rows of an ELISA-grade microtitre plate are coated with virus and control antigens overnight at 4°C.  
767 The plates are then washed in PBS with 0.05% Tween 20 or Tween 80 (PBST) before use in the  
768 test.
- 769 iv) Test sera are diluted 1/50 in serum diluent (0.5 M NaCl; 0.01 M phosphate buffer; 0.05% Tween  
770 20; 0.001 M ethylene diamine tetra-acetic acid; 1% polyvinyl pyrrolidone, pH 7.2) and added to  
771 virus- and control-coated wells for 1 hour at 37°C. The plates are then washed five times in PBST.
- 772 v) Rabbit anti-bovine IgG peroxidase conjugate is added at a predetermined dilution (in serum diluent)  
773 for 1 hour at 37°C, then the plates are again washed five times in PBST.
- 774 vi) A suitable enzyme substrate is added, such as hydrogen peroxide/tetramethyl benzidine. After  
775 colour development, the reaction is stopped with sulphuric acid and the absorbance is read on an  
776 ELISA plate reader. The value obtained with control antigen is subtracted from the test reaction to  
777 give a net absorbance value for each serum.
- 778 vii) It is recommended to convert net absorbance values to sample:positive ratio (or percentage  
779 positivity) by dividing net absorbance by the net absorbance on that test of a standard positive  
780 serum that has a net absorbance of about 1.0. This normalisation procedure leads to more  
781 consistent and reproducible results.

## 782 C. REQUIREMENTS FOR VACCINES

### 783 1. Background

784 BVDV vaccines are used primarily for disease control purposes. Although they can convey production advantages  
785 especially in intensively managed cattle such as in feedlots. In some countries where BVDV eradication is being  
786 undertaken, PI animals are removed and remaining cattle are vaccinated to maintain a high level of infection-antibody  
787 positivity and prevent the generation of further PI animals. Vaccination to control BVDV infections can be challenging due  
788 in part to the antigenic variability of the virus and the occurrence of persistent infections that arise as a result of fetal  
789 infection. Ongoing maintenance of the virus in nature is predominantly sustained by PI animals that are the product of *in-*  
790 *utero* infection. The goal for a vaccine should be to prevent systemic viraemia and the virus crossing the placenta. If this  
791 is successfully achieved it is likely that the vaccine will prevent the wide range of other clinical manifestations, including  
792 reproductive, respiratory and enteric diseases and immunosuppression with its secondary sequelae. There are many  
793 different vaccines available in different countries. Traditionally, BVD vaccines fall into two classes: modified live virus or  
794 inactivated vaccines. Experimental recombinant subunit vaccines based on BVD viral glycoprotein E2 expressed with  
795 baculovirus, ~~or~~ transgenic plants or heterologous viruses and BVDV E2 DNA vaccines have been described but few, if  
796 any, are in commercial production. They offer a future prospect of 'marker vaccines' when used in connection with a  
797 complementary serological test.

#### 798 1.1. Characteristics of a target product profile

799 Traditionally, BVD vaccines fall into two classes: modified live or inactivated virus vaccines. The essential  
800 requirement for both types is to ~~afford~~ provide a high level of fetal infection-protection. Many of the live vaccines  
801 have been based on a cytopathic strain of the virus which is considered to be unable to cross the placenta.  
802 However, it is important to ensure that the vaccine virus does not cause fetal infection. In general, vaccination  
803 of breeding animals should be completed well before insemination to ensure optimal protection and avoid any  
804 risk of fetal infection. Live virus vaccine may also be immunosuppressive and precipitate other infections. On  
805 the other hand, modified live virus vaccines may only require a single dose. Use of a live product containing a



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806 cytopathic strain of BVDV may precipitate mucosal disease by superinfection of persistently viraemic animals.  
807 Properly formulated inactivated vaccines are very safe to use but, to obtain satisfactory levels of immunity, they  
808 usually require booster vaccinations, which may be inconvenient. A combined vaccination protocol using  
809 inactivated followed by live vaccine may reduce the risk of adverse reaction to the live strain. Whether live or  
810 inactivated, because of the propensity for antigenic variability, the vaccine should contain strains of BVDV that  
811 are closely matched to viruses found in the area in which they are used. For example, in countries where strains  
812 of BVDV type 2 (*Pestivirus tauri*) are found, it is important for the vaccine to contain a suitable type 2 strain. For  
813 optimal immunity against type 1 strains (*Pestivirus bovis*), antigens from the dominant subtypes (e.g. 1a and 1b)  
814 should be included. Due to the need to customise vaccines for the most commonly encountered strains within  
815 a country or region, it is not feasible to produce a vaccine antigen bank that can be drawn upon globally.

816 Guidance for the production of veterinary vaccines is given in Chapter 1.1.8 *Principles of veterinary vaccine*  
817 *production*. The guidelines given here and in chapter 1.1.8 are intended to be general in nature and may be  
818 supplemented by national and regional requirements.

## 819 2. Outline of production and minimum requirements for vaccines

### 820 2.1. Characteristics of the seed

821 For optimal efficacy, it is considered that there should be a close antigenic match between viruses included in  
822 a vaccine and those circulating in the target population. BVDV type 2 strains (*Pestivirus tauri*) should be included  
823 as appropriate. Due to the regional variations in ~~genotypes-species~~ and subtypes of BVDV, many vaccines  
824 contain more than one strain of BVDV to give acceptable protection. A good appreciation of the antigenic  
825 characteristics of individual strains can be obtained by screening with panels of MAbs (Paton *et al.*, 1995).

#### 826 2.1.1. Biological characteristics of the master seed

827 Isolates of cytopathic virus are often mixed with the noncytopathic biotype. The separation and  
828 purification of the two biotypes from an initial mixed culture is important to maintain the expected  
829 characteristics of the ~~seed~~ seed and depends on several cycles of a limiting dilution technique for the  
830 noncytopathic virus, or plaque selection for the cytopathic virus. Purity of the cytopathic virus should be  
831 confirmed by at least one additional passage at limiting dilution. When isolates have been cloned, their  
832 identity and key antigenic characteristics should be confirmed. The identity of the seed virus should be  
833 confirmed by sequencing. Where there are multiple isolates included in the vaccine, each has to be  
834 prepared separately.

835 While retaining the desirable antigenic characteristics, the strains selected for the seed should not show  
836 any signs of disease when susceptible animals are vaccinated. Live attenuated vaccines should not be  
837 transmissible to unvaccinated 'in-contact' animals and should not be able to infect the fetus. Ideally seeds  
838 prepared for the production of inactivated vaccines should grow to high titre to minimise the need to  
839 concentrate the antigens and there should be a minimal amount of protein from the cell cultures  
840 incorporated into the final product. Master stocks for either live or inactivated vaccines should be  
841 prepared under a seed lot system involving master and working stocks that can be used for production  
842 in such a manner that the number of passages can be limited and minimise antigenic drift. While there  
843 are no absolute criteria for this purpose, as a general guide, the seed used for production should not be  
844 passaged more than 20 times beyond the master seed and the master seed should be of the lowest  
845 passage from the original isolate as is practical.

#### 846 2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

847 It is crucial to ensure that all materials used in the preparation of the bulk antigens have been extensively  
848 screened to ensure freedom from extraneous agents. This should include master and working seeds,  
849 the cell cultures and all medium supplements such as bovine serum. It is particularly important to ensure  
850 that any serum used that is of bovine origin is free of both adventitious BVDV of all ~~genotypes~~ and  
851 antibodies against BVDV strains because low levels of either virus or antibody can mask the presence  
852 of the other. Materials and vaccine seeds should be tested for sterility and freedom from contamination  
853 with other agents, especially viruses as described in the chapter 1.1.8 and chapter 1.1.9.

#### 854 2.1.3. Validation as a vaccine strain

855 All vaccines should pass standard tests for efficacy. Tests should include as a minimum the  
856 demonstration of a neutralising antibody response following vaccination, a reduction in virus shedding  
857 after challenge in vaccinated cattle and ideally a prevention of viraemia. Efficacy tests of BVD vaccines  
858 by assessing clinical parameters in non-pregnant cattle can be limited by the difficulty of consistently  
859 establishing clinical signs but, when employed, clinical parameters such as a reduction in the rectal

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860 temperature response and leukopenia should be monitored. Although it can be difficult by using virus  
861 isolation in cell culture to consistently demonstrate the low levels of viraemia associated with an acute  
862 infection, real-time PCR could be considered as an alternative method to establish the levels of  
863 circulating virus.

864 If a vaccine passes basic tests, the efficacy of vaccination should ultimately be measured by the capacity  
865 to prevent transplacental transmission. If there is a substantial reduction and ideally complete prevention  
866 of fetal infection, a vaccine would be expected to be highly effective in other situations (for example  
867 prevention of respiratory disease). A suitable challenge system can be established by intranasal  
868 inoculation of noncytopathic virus into pregnant cows between 60 and 90 days of gestation (Brownlie *et*  
869 *al.*, 1995). Usually this system will reliably produce persistently viraemic offspring in non-immune cows.  
870 In countries where BVDV type 2 viruses (*Pestivirus tauri*) are commonly encountered, efficacy in  
871 protecting against BVDV type 2 infections should be measured.

## 872 **2.2. Method of manufacture**

### 873 **2.2.1. Procedure**

874 Both cytopathic and noncytopathic biotypes will grow in a variety of cell cultures of bovine origin.  
875 Standard procedures may be used, with the expectation for harvesting noncytopathic virus on days 4–7  
876 and cytopathic virus on days 2–4. The optimal yield of infectious virus will depend on several factors,  
877 including the cell culture, isolate used and the initial seeding rate of virus. These factors should be taken  
878 into consideration and virus replication kinetics investigated to establish the optimal conditions for large  
879 scale virus production. Whether a live or inactivated vaccine, the essential aim will be to produce a high-  
880 tited virus stock. This bulk antigen preparation can subsequently be prepared according to the type of  
881 vaccine being considered.

### 882 **2.2.2. Requirements for ingredients**

883 Most BVDV vaccines are grown in cell cultures of bovine origin that are frequently supplemented with  
884 medium components of animal origin. The material of greatest concern is bovine serum due to the  
885 potential for contamination with BVD viruses and antibodies to these viruses. These adventitious  
886 contaminants not only affect the efficiency of production but also may mask the presence of low levels  
887 of infectious BVDV that may have undesirable characteristics. In addition to the virus seeds, all materials  
888 should be tested for sterility and freedom from contamination with other agents, especially viruses as  
889 described in chapters 1.1.8 and 1.1.9. Further, materials of bovine or ovine origin should originate from  
890 a country with negligible risk for transmissible spongiform encephalopathies [TSEs] (see chapter 1.1.9).

### 891 **2.2.3. In-process controls**

892 In-process controls are part of the manufacturing process. Cultures should be inspected regularly to  
893 ensure that they remain free from contamination, and to monitor the health of the cells and the  
894 development or absence of CPE, as appropriate. While the basic requirement for efficacy is the capacity  
895 to induce an acceptable neutralising antibody response, during production, target concentrations of  
896 antigen required to achieve an acceptable response may be monitored indirectly by assessment of the  
897 quantity of infectious virus or antigen mass that is produced. Rapid diagnostic assays such as the ELISA  
898 are useful to monitor BVDV antigen production. Alternatively, the quality of a batch of antigen may be  
899 determined by titration of the quantity of infectious virus present, although this may underestimate the  
900 quantity of antigen. For inactivated vaccines, infectivity is evaluated before inactivation. For inactivated  
901 vaccines the inactivation kinetics should be established so that a suitable safety margin can be  
902 determined and incorporated into the routine production processes. At the end of production, *in-vitro* cell  
903 culture assays should be undertaken to confirm that inactivation has been complete. These innocuity  
904 tests should include a sufficient number of passages and volume of inoculum to ensure that very low  
905 levels of infectious virus would be detected if present.

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- 906           **2.2.4. Final product batch tests**
- 907           i)    Sterility
- 908                    Tests for sterility and freedom from contamination of biological materials intended for veterinary use  
909                    may be found in Chapter 1.1.9.
- 910           ii)   Identity
- 911                    Identity tests should demonstrate that no other strain of BVDV is present when several strains are  
912                    propagated in a facility producing multivalent vaccines.
- 913           iii)   Safety
- 914                    Safety tests shall consist of detecting any abnormal local or systemic adverse reactions to the  
915                    vaccine by all vaccination route(s). Batch-to-batch safety tests are required unless safety of the  
916                    product is demonstrated and APPROVED in the registration dossier and production is consistent  
917                    with that described in chapter 1.1.8.
- 918                    The safety test is different to the innocuity test (see above).
- 919                    Live vaccines must either be demonstrated to be safe in pregnant cattle (i.e. no transmission to the  
920                    fetus), or should be licensed with a warning not to use them in pregnant animals. Live vaccines  
921                    containing cytopathic strains should have an appropriate warning of the risk of inducing mucosal  
922                    disease in PI cattle.
- 923           iv)   Batch potency
- 924                    BVD vaccines must be demonstrated to produce adequate immune responses, when used in their  
925                    final formulation according to the manufacturer's published instructions. The minimum quantity of  
926                    infectious virus and/or antigen required to produce an acceptable immune response should be  
927                    determined. *In-vitro* assays should be used to monitor individual batches during production.

## 928           **2.3. Requirements for authorisation/registration/licensing**

### 929           **2.3.1. Manufacturing process**

930                    For registration of a vaccine, all relevant details concerning manufacture of the vaccine and quality  
931                    control testing should be submitted to the relevant authorities. Unless otherwise specified by the  
932                    authorities, information should be provided from three consecutive vaccine batches with a volume not  
933                    less than 1/3 of the typical industrial batch volume.

934                    There is no standard method for the manufacture of a BVD vaccine, but conventional laboratory  
935                    techniques with stationary, rolled or suspension (micro-carriers) cell cultures may be used. Inactivated  
936                    vaccines can be prepared by conventional methods, such as binary ethylenimine or beta-propiolactone  
937                    inactivation (Park & Bolin, 1987). A variety of adjuvants may be used.

### 938           **2.3.2. Safety requirements**

939                    *In-vivo* tests should be undertaken using a single dose, overdose (for live vaccines only) and repeat  
940                    doses (taking into account the maximum number of doses for primary vaccination and, if appropriate, the  
941                    first revaccination/booster vaccination) and contain the maximum permitted antigen load and, depending  
942                    on the formulation of the vaccine, the maximum number of vaccine strains.

#### 943           i)    Target and non-target animal safety

944                    The safety of the final product formulation of both live and inactivated vaccines should be assessed  
945                    in susceptible young calves that are free of maternally derived antibodies and in pregnant cattle.  
946                    They should be checked for any local reactions following administration, and, in pregnant cattle, for  
947                    any effects on the unborn calf. Live attenuated vaccines may contribute to immunosuppression that  
948                    might increase mortality. It may also contribute to the development of mucosal disease in PI animals  
949                    that is an animal welfare concern. Therefore vaccination of PI animals with live attenuated vaccines  
950                    containing cytopathic BVDV should be avoided. Live attenuated vaccines must not be capable of  
951                    being transmitted to other unvaccinated animals that are in close contact.

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952 ii) Reversion-to-virulence for attenuated/live vaccines and environmental considerations  
953 Virus seeds that have been passaged at least up to and preferably beyond the passage limit  
954 specified for the seed should be inoculated into young calves to confirm that there is no evidence  
955 of disease. If a live attenuated vaccine has been registered for use in pregnant animals, reversion  
956 to virulence tests should also include pregnant animals. Live attenuated vaccines should not be  
957 transmissible to unvaccinated 'in-contact' animals.

958 iii) Precautions (hazards)  
959 BVDV is not considered to be a human health hazard. Standard good microbiological practice  
960 should be adequate for handling the virus in the laboratory. A live virus vaccine should be identified  
961 as harmless for people administering the product. However adjuvants included in either live or  
962 inactivated vaccines may cause injury to people. Manufacturers should provide adequate warnings  
963 that medical advice should be sought in the case of self-injection (including for adjuvants, oil-  
964 emulsion vaccine, preservatives, etc.) with warnings included on the product label/leaflet so that  
965 the vaccinator is aware of any danger.

### 966 2.3.3. Efficacy requirements

967 The potency of the vaccine should be determined by inoculation into seronegative and virus negative  
968 calves, followed by monitoring of the antibody response. Antigen content can be assayed by ELISA and  
969 adjusted as required to a standard level for the particular vaccine. Standardised assay protocols  
970 applicable to all vaccines do not exist. Live vaccine batches may be assayed by infectivity titration. Each  
971 production batch of vaccine should undergo potency and safety testing as batch release criteria. BVD  
972 vaccines must be demonstrated to produce adequate immune responses, as outlined above, when used  
973 in their final formulation according to the manufacturer's published instructions.

### 974 2.3.4. Vaccines permitting a DIVA strategy (detection of infection in vaccinated animals)

975 To date, there are no commercially available vaccines for BVDV that support use of a true DIVA strategy.  
976 Experimental subunit vaccines based on baculovirus-expressed BVD viral glycoprotein E2 have been  
977 described but are not available commercially. They offer a future prospect of 'marker vaccines' when  
978 used in connection with a complementary serological test. Experimental BVDV E2 DNA vaccines and  
979 BVDV E2 subunit vaccines expressed using transgenic plants and alphavirus replicon or chimeric  
980 pestivirus vaccines have also been described.

### 981 2.3.5. Duration of immunity

982 There are few published data on the duration of antibody following vaccination with a commercial product.  
983 Protocols for their use usually recommend a primary course of two inoculations and boosters at yearly  
984 intervals. Only limited data are available on the antibody levels that correlate with protection against  
985 respiratory infections (Bolin & Ridpath, 1995; Howard *et al.*, 1989) or *in-utero* infection (Brownlie *et al.*,  
986 1995). However, there are many different commercial formulations and these involve a range of  
987 adjuvants that may support different periods of efficacy. Consequently, duration of immunity data must  
988 be generated separately for each commercially available product by undertaking challenge tests at the  
989 end of the period for which immunity has been claimed.

### 990 2.3.6. Stability

991 There are no accepted guidelines for the stability of BVD vaccines, but it can be assumed that attenuated  
992 virus vaccine (freeze-dried) should remain potent for at least 1 year if kept at 4°C. Inactivated virus  
993 vaccine could have a longer shelf life at 4°C. Lower temperatures could prolong shelf life for either type,  
994 but adjuvants in killed vaccine may preclude this. Bulk antigens that have not been formulated into  
995 finished vaccine can be reliably stored frozen at low temperatures but the antigen quality should be  
996 monitored with *in-vitro* assays prior to incorporation into a batch of vaccine.

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1071 persistently infected BVDV carriers in dairy herds. *Res. Vet. Sci.*, **72**, 75–82.
- 1072 \*
- 1073 \* \*
- 1074 **NB:** There are WOAHP Reference Laboratories for bovine viral diarrhoea (please consult the WOAHP Web site:  
1075 <https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3> )  
1076 Please contact the WOAHP Reference Laboratories for any further information on  
1077 diagnostic tests, reagents and vaccines for bovine viral diarrhoea
- 1078 **NB:** FIRST ADOPTED IN 1990. MOST RECENT UPDATES ADOPTED IN 2015.

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**Appendix 1: Bovine viral diarrhoea**  
**Intended purpose of test: population freedom from infection**

<b>Test with score and species</b>	<b>Sample type and target analytes</b>	<b>Accuracy</b>	<b>Test population used to measure accuracy</b>	<b>Validation report</b>	<b>Advantages: expert opinion</b>	<b>Disadvantages: expert opinion</b>	<b>References</b>
NA detection by (real-time) RT-PCR +++	Ear notch (skin), blood, milk	Performance has been demonstrated under field conditions in large control programs	Whole Swiss, German and Irish cattle populations	See references	<ul style="list-style-type: none"> <li>- Very sensitive</li> <li>- Rapid</li> <li>- High-throughput</li> <li>- Well established internationally</li> <li>- Detects assay-dependent all BVDV species</li> <li>- Allows assay-dependent for differentiation of BVDV types 1 and 2</li> <li>- Detects persistent and transient infection</li> <li>- Proficiency panel of different Pestivirus strains available</li> <li>- Detection of viral RNA in skin biopsy samples unaffected by maternally-derived antibodies, therefore allows for identification of persistently infected animals early in life</li> <li>- Successfully applied in ongoing or completed control programmes</li> </ul>	<ul style="list-style-type: none"> <li>- Possibility for contamination at sample collection or in laboratory, leading to false positive results</li> <li>- Needs specialised equipment</li> <li>- Detection of viral RNA does not imply per se that infectious virus is present</li> </ul>	<ul style="list-style-type: none"> <li>- Presi &amp; Heim (2010). <i>Vet. Microbiol.</i>, <b>142</b>, 137–142</li> <li>- Schweizer <i>et al.</i> (2021) <i>Front. Vet. Sci.</i>, <b>8</b>, 702730</li> <li>- Wernike <i>et al.</i> (2017). <i>Pathogens</i>, <b>6</b> (4)</li> <li>- Graham <i>et al.</i> (2021) <i>Front. Vet. Sci.</i>, <b>8</b>, 674557</li> </ul>
Antibody detection by ELISA +++	Bulk milk, blood	DSe and DSP differs based on the ELISA used (commercial/in-house) and the antibodies being tested (e.g. antibodies against structural (E2) and non-structural (NS2-3) proteins.			<ul style="list-style-type: none"> <li>- Simple to perform and cost-effective</li> <li>- Milk collection is non-invasive method with potential for herd screening with tank/bulk milk samples</li> <li>- Bulk milk sensitive indicator for PI in herd</li> </ul>	<ul style="list-style-type: none"> <li>- Some cross-reactivity with vaccines and other pestiviruses</li> <li>- PI animal will usually be seronegative</li> <li>- Bulk milk from herd excludes males, non-lactating or young stock</li> </ul>	<ul style="list-style-type: none"> <li>Beauveau <i>et al.</i> (2001). <i>Vet. Microbiol.</i>, <b>80</b>, 329–337</li> <li>Lanyon <i>et al.</i> (2013). <i>Aust. Vet. J.</i>, <b>91</b>, 52–56.</li> </ul>

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population used to measure accuracy</u>	<u>Validation report</u>	<u>Advantages: expert opinion</u>	<u>Disadvantages: expert opinion</u>	<u>References</u>
<u>Antigen detection by ELISA +++</u>	<u>Serum, whole blood, skin biopsy</u>	<u>DSe 67–100% and DSp 98.8–100% relative to virus isolation reported</u>			<u>Relatively simple to perform, rapid, can be cost-effective (when compared to virus isolation and PCR) and suitable for high-throughput applications. There is no need for cell culture facility.</u>	<u>Maternal antibodies in colostrum may interfere with testing for antigen in serum using ELISA in calves. Ear notch samples are less affected. PI calves <i>in utero</i> defies detection.</u>	<u>Lanyon et al. (2013). <i>Vet. J.</i> 199, 201–209.</u>
<u>Virus isolation +</u>	<u>Serum, whole blood</u>	<u>Considered (historically) reference test; DSe &lt;90% compared with real-time RT-PCR; DSp ~100%</u>	<u>N/A</u>	<u>Historical information with no formal validation</u>	<u>- High degree of specificity - Identifies presence of infectious virus</u>	<u>- Requires specialised cell culture capabilities and access to BVDV free materials - Reduced sensitivity in presence of maternally-derived antibodies</u>	<u>N/A</u>
<u>Virus neutralisation test +</u>	<u>Serum</u>	<u>DSe &amp; DSp both extremely high, both &gt;99%. Historical reference serological test.</u>	<u>N/A</u>	<u>Historical information with no formal validation</u>	<u>Very high specificity</u>	<u>- ASe can vary depending on virus strain used - Requires cell culture, good quality samples - Labour intensive, takes 5 days to obtain results - Expensive</u>	<u>N/A</u>

3 N/A: not available



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**Appendix 2: Bovine viral diarrhoea**  
**Intended purpose of test: individual animal freedom from infection prior to movement**

<b>Test with score and species</b>	<b>Sample type and target analytes</b>	<b>Accuracy</b>	<b>Test population used to measure accuracy</b>	<b>Validation Report</b>	<b>Advantages: expert opinion</b>	<b>Disadvantages: expert opinion</b>	<b>References</b>
Virus isolation ++	Serum, whole blood.	Considered reference test: DSe <90% compared with real-time RT-PCR; DSp ~100%	N/A	Historical information with no formal validation	<ul style="list-style-type: none"> <li>- High degree of specificity</li> <li>- Identifies presence of infectious virus</li> </ul>	<ul style="list-style-type: none"> <li>- Requires specialised cell culture capabilities and access to BVDV free materials</li> <li>- Reduced sensitivity in presence of MDA (diagnostic gap); takes several weeks for maximum DSe</li> </ul>	Edmonson <i>et al.</i> (2007); Toker & Yesilbag (2021)
Antigen detection by ELISA +++	Serum, whole blood, skin biopsy (e.g. ear notch)	DSe 67–100% and DSp 98.8–100% relative to virus isolation have been reported but usually DSe and DSp are extremely high			Relatively simple to perform, rapid, can be cost-effective and suitable for high-throughput applications. There is no need for cell culture facility.	Maternal antibodies in colostrum may interfere with testing for antigen in serum using ELISA in calves. Ear notch samples are less affected. PI calves <i>in utero</i> defies detection.	Zimmer <i>et al.</i> (2004). <i>Vet. Microbiol.</i> , <b>100</b> , 145–149
NA detection by (real-time) RT-PCR +++	Ear notch (skin), blood, nasal or oral swab	<ul style="list-style-type: none"> <li>- Depending on the assay analytical sensitivity of less than 10 genome copies/reaction</li> <li>- Accuracy 98.73% based on a proficiency test panel consisting of five ear notch and five serum samples</li> </ul>		See references	<ul style="list-style-type: none"> <li>- Very sensitive</li> <li>- Rapid</li> <li>- High-throughput</li> <li>- Well established internationally</li> <li>- Depending on the assay detects all BVDV species</li> <li>- Allows assay-dependent for differentiation of BVDV types 1 and 2</li> <li>- Detects persistent and transient infection</li> <li>- Proficiency panel of different Pestivirus strains available</li> <li>- Detection of viral RNA in skin biopsy samples unaffected by maternally-derived antibodies</li> </ul>	<ul style="list-style-type: none"> <li>- Possibility for contamination at sample collection or in laboratory, leading to false positive results</li> <li>- Needs specialised equipment</li> </ul>	<ul style="list-style-type: none"> <li>- Hoffmann <i>et al.</i> (2006). <i>J. Virol. Methods</i>, <b>136</b>, 200–209.</li> <li>- Wernike <i>et al.</i> (2019). <i>Vet. Microbiol.</i>, <b>239</b>, 108452.</li> </ul>

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population used to measure accuracy</u>	<u>Validation Report</u>	<u>Advantages: expert opinion</u>	<u>Disadvantages: expert opinion</u>	<u>References</u>
<u>Virus neutralisation test ++</u>	<u>Serum</u>	<u>DSe &amp; DSp both extremely high, both &gt;99%. Historical reference serological test.</u>	<u>N/A</u>	<u>Historical information with no formal validation</u>	<u>Very high specificity</u> <u>- Negative results generally indicate that an animal has not been infected provided it has been tested for virus/antigen</u>  <u>Animals that give positive results have usually cleared the infection provided that they are not pregnant. Bulls present a special situation. If they seroconvert during a period of quarantine, there is a risk that there will be virus in their semen. Further, a small proportion of seropositive bulls may have a persistent testicular infection that may last for many months. To ensure freedom from infection, semen must be tested on several occasions using real-time RT-PCR. Virus isolation is not sufficiently sensitive and the presence of virus may be masked by antibodies.</u>	<u>- ASe can vary depending on virus strain used</u> <u>- Requires cell culture, good quality samples</u> <u>- time consuming to perform, takes 5 days to obtain results</u> <u>- Labour intensive</u> <u>- Due to the biology of the virus (birth of PI calves that are not able to amount a specific immune response to the virus strain they are infected with) antibody-negative animals could be PI (in non-BVDV-free populations)</u>	<u>N/A</u>
<u>Antibody detection by ELISA ++</u>	<u>Blood, Individual milk sample</u>	<u>DSe and DSp differs based on the ELISA used (commercial/in-house) and the antibodies being tested (e.g. antibodies against structural (E2) and non-structural (NS2-3) proteins.</u>			<u>- Simple to perform and cost-effective</u> <u>- Milk collection is non-invasive method</u> <u>- Paired samples can be used to confirm acute infection.</u> <u>- Ensure milk is collected directly from the teat rather than bulk-milk tank to ensure no cross contamination/false-positives</u>	<u>- Maternal antibodies in colostrum may interfere with testing for antibodies in serum using ELISA in calves. Calves should be tested after 9 months of age after maternal antibodies have waned.</u> <u>- PI animal will be seronegative and may impact receiving herds if moved.</u> <u>- Using milk, limited to lactating cow only</u>	<u>N/A</u>

6 N/A: not available

**Appendix 3: Bovine viral diarrhoea**  
**Intended purpose of test: contribute to eradication policies**

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population used to measure accuracy</u>	<u>Validation report</u>	<u>Advantages: expert opinion</u>	<u>Disadvantages: expert opinion</u>	<u>References</u>
<u>Antigen detection by ELISA +++</u>	<u>Serum, whole blood, skin biopsy</u>	<u>DSse 67%–100% and DSp 98.8–100% reported</u>			<u>Relatively simple to perform, rapid, can be cost-effective and suitable for high-throughput applications. There is no need for cell culture facility.</u>	<u>Maternal antibodies in colostrum may interfere with testing for antigen in serum using ELISA in calves. Ear notch samples are less affected. PI calves <i>in utero</i> defy detection.</u>	<u>Zimmer <i>et al.</i> (2004). <i>Vet. Microbiol.</i>, <b>100</b>, 145–149</u>
<u>NA detection by (real-time) RT-PCR +++</u>	<u>Ear notch (skin), blood; milk; nasal or oral swab</u>	<u>Utility has been demonstrated under field conditions in large control programs</u>	<u>Whole Swiss, German and Irish cattle populations</u>	<u>See references</u>	<ul style="list-style-type: none"> <li>- <u>Very sensitive</u></li> <li>- <u>Rapid</u></li> <li>- <u>High-throughput</u></li> <li>- <u>Well established internationally</u></li> <li>- <u>Depending on assay, detects all BVDV species</u></li> <li>- <u>Allows assay-dependent differentiation of BVDV types 1 and 2</u></li> <li>- <u>Detects persistent and transient infection</u></li> <li>- <u>Proficiency panel of different Pestivirus strains available</u></li> <li>- <u>Detection of viral RNA in skin biopsy samples unaffected from maternally-derived antibodies, therefore allows for identification of persistently infected animals early in life</u></li> <li>- <u>Successfully applied in ongoing or completed control programmes (see references)</u></li> </ul>	<ul style="list-style-type: none"> <li>- <u>Possibility for contamination at sample collection or in laboratory, leading to false positive results</u></li> <li>- <u>Needs specialised equipment</u></li> </ul>	<ul style="list-style-type: none"> <li>- <u>Presi &amp; Heim (2010). <i>Vet. Microbiol.</i>, <b>142</b>, 137–142</u></li> <li>- <u>Schweizer <i>et al.</i> (2021). <i>Front. Vet. Sci.</i>, <b>8</b>, 702730</u></li> <li>- <u>Wernike <i>et al.</i> (2017). <i>Pathogens</i>, <b>6</b> (4)</u></li> <li>- <u>Graham <i>et al.</i> (2021). <i>Front. Vet. Sci.</i>, <b>8</b>, 674557</u></li> </ul>

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population used to measure accuracy</u>	<u>Validation report</u>	<u>Advantages: expert opinion</u>	<u>Disadvantages: expert opinion</u>	<u>References</u>
<u>Antibody detection by ELISA ++</u>	<u>Bulk milk, Blood</u>	<u>DSe and DSp differs based on the ELISA used (commercial/in-house) and the antibodies being tested (e.g. antibodies against structural (E2) and non-structural (NS2-3) proteins.</u>			<ul style="list-style-type: none"> <li>- <u>Simple to perform and cost-effective</u></li> <li>- <u>Milk collection is non-invasive method</u></li> <li>- <u>Can be used on a population basis to monitor freedom from infection by selection of appropriate aged animals e.g. borne after eradication achieved; for dairy herds by testing tank milk to monitor lactating animals</u></li> </ul>	<ul style="list-style-type: none"> <li>- <u>Some cross-reactivity with antibodies from vaccines and other pestiviruses</u></li> <li>- <u>PI animal will be seronegative</u></li> <li>- <u>Bulk milk from herd does not include males, non-lactating or young stock</u></li> </ul>	<u>Laureyns et al. (2010)</u>
<u>Virus isolation ++</u>	<u>Serum, whole blood</u>	<u>Considered reference test : DSe &lt;90% compared with real-time RT-PCR; DSp ~100%</u>	<u>N/A</u>	<u>Historical information with no formal validation</u>	<ul style="list-style-type: none"> <li>- <u>High degree of specificity</u></li> <li>- <u>Identifies presence of infectious virus.</u></li> <li>- <u>used to confirm the status of difficult cases and to provide isolates for intensive analysis e.g. NA sequencing</u></li> </ul>	<ul style="list-style-type: none"> <li>- <u>Requires specialised cell culture capabilities and access to BVDV free materials</u></li> <li>- <u>Reduced sensitivity in presence of MDA (diagnostic gap)</u></li> </ul>	<u>N/A</u>
<u>Virus neutralisation test ++</u>	<u>Serum</u>	<u>DSe &amp; DSp both extremely high, both &gt;99%. Historical reference serological test.</u>	<u>N/A</u>	<u>Historical information with no formal validation</u>	<ul style="list-style-type: none"> <li><u>Very high specificity</u></li> <li>- <u>Used for confirming the virus free status of a population after eradication;</u></li> <li>- <u>Used as a confirmatory test when surveillance utilises an ELISA</u></li> </ul>	<ul style="list-style-type: none"> <li>- <u>ASe can vary depending on virus strain used</u></li> <li>- <u>Requires cell culture, good quality samples</u></li> <li>- <u>Takes 5 days to obtain results</u></li> </ul>	<u>N/A</u>

9 N/A: not available

**Appendix 4: Bovine viral diarrhoea**  
**Intended purpose of test: confirmation of clinical cases**

<b>Test with score and species</b>	<b>Sample type and target analytes</b>	<b>Accuracy</b>	<b>Test population used to measure accuracy</b>	<b>Validation report</b>	<b>Advantages: expert opinion</b>	<b>Disadvantages: expert opinion</b>	<b>References</b>
Virus isolation ++	Serum, whole blood, tissue extracts	Considered reference test: DSe <90% compared with real-time RT-PCR; DSp ~100%	Not available	Historical information with no formal validation	<ul style="list-style-type: none"> <li>- High degree of specificity</li> <li>- Identifies presence of infectious virus</li> <li>- Preferred method to identify presence of cytopathogenic strains and hence confirmation of mucosal disease</li> <li>- Provides virus isolates for detailed characterisation</li> </ul>	<ul style="list-style-type: none"> <li>- Requires specialised cell culture capabilities and access to BVDV free materials</li> <li>- Reduced sensitivity in presence of maternally derived antibodies (diagnostic gap)</li> <li>- Requires high quality samples to avoid bacterial contamination</li> </ul>	- Meyling (1984)
Antigen detection by ELISA +++	Serum, whole blood, skin biopsy	DSe 67%–100% and DSp 98.8% to 100% reported			Relatively simple to perform, rapid, can be cost-effective and suitable for high-throughput applications. There is no need for cell culture facility.	Maternal antibodies in colostrum may interfere with testing for antigen in serum using ELISA in calves. Ear notch samples are less affected. PI calves <i>in utero</i> defies detection.	
NA detection by (real-time) RT-PCR +++	Blood; nasal, oral or conjunctival swab (in cases of respiratory disease) or faecal swab (enteric disease)	Depending on the assay analytical sensitivity of less than 10 genome copies/reaction		See reference	<ul style="list-style-type: none"> <li>- Very sensitive</li> <li>- Rapid</li> <li>- High-throughput</li> <li>- Well established internationally</li> <li>- Depending on the assay, detects all BVDV species</li> <li>- Allows assay-dependent for differentiation of BVDV types 1 and 2</li> <li>- Detects persistent and transient infection</li> <li>- Proficiency panel of different Pestivirus strains available</li> <li>- Detection of viral RNA in skin biopsy samples unaffected from maternally derived antibodies</li> </ul>	<ul style="list-style-type: none"> <li>- Possibility for contamination at sample collection or in laboratory, leading to false positive results</li> <li>- Needs specialised equipment</li> </ul>	- Hoffmann <i>et al.</i> (2006). <i>J. Virol. Methods</i> , <b>136</b> , 200–209.
Antigen detection by IHC ++	Fixed tissues or frozen sections for Ag detection or NA if using ISH	Lower DSe than other methods; high DSp	N/A	N/A	Allows visualisation of viral components in lesions and assessment of tissue distribution	Technically demanding, limited reagents for detection of Ag in formalin-fixed tissues	

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population used to measure accuracy</u>	<u>Validation report</u>	<u>Advantages: expert opinion</u>	<u>Disadvantages: expert opinion</u>	<u>References</u>
Antibody detection by ELISA +	Paired serum samples, fetal fluids (blood, pericardial, thoracic)	DSe and DSp may differ depending on the ELISA used (commercial/in-house) and the antibodies being tested (e.g. antibodies against structural (E2) and non-structural (NS2-3) proteins.			<ul style="list-style-type: none"> <li>- Simple to perform and cost-effective.</li> <li>- Can be used to differentiate between acute and persistent infections by demonstration of seroconversion in acute infections</li> <li>- Detection of antibodies in aborted fetuses, stillborn animals can confirm <i>in utero</i> infection in second half of gestation</li> </ul>	<ul style="list-style-type: none"> <li>- Some cross-reactivity with antibodies induced by other pestiviruses.</li> <li>- PI animals are usually seronegative (in both of the paired samples)</li> </ul>	

12 N/A: not available

**Appendix 5: Bovine viral diarrhoea**  
**Intended purpose of test: prevalence of infection – surveillance**

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population used to measure accuracy</u>	<u>Validation report</u>	<u>Advantages: expert opinion</u>	<u>Disadvantages: expert opinion</u>	<u>References</u>
Antigen detection by ELISA +++	Serum, whole blood	DSe 67–100% and DSp 98.8–100% reported			Relatively simple to perform, rapid, very cost-effective and suitable for high-throughput applications. There is no need for cell culture facility.	Maternal antibodies in colostrum may interfere with testing for antigen in serum using ELISA in calves. Ear notch samples are less affected. PI calves <i>in utero</i> defies detection.	Sarrazin <i>et al.</i> (2013). <i>Prev. Vet. Med.</i> , <b>108</b> , 28–37
NA detection by (real-time) RT-PCR +++	Ear notch (skin), blood, milk		Whole Swiss, German and Irish cattle populations	See references	<ul style="list-style-type: none"> <li>- Very sensitive</li> <li>- Rapid</li> <li>- High-throughput</li> <li>- Well established internationally</li> <li>- Depending on the assay, detects all BVDV species</li> <li>- Allows assay-dependent for differentiation of BVDV types 1 and 2</li> <li>- Detects persistent and transient infection</li> <li>- Proficiency panel of different Pestivirus strains available</li> <li>- Detection of viral RNA in skin biopsy samples unaffected from maternally-derived antibodies</li> </ul>	<ul style="list-style-type: none"> <li>- Possibility for contamination at sample collection or in laboratory, leading to false positive results</li> <li>- Needs specialised equipment</li> </ul>	<ul style="list-style-type: none"> <li>- Presi &amp; Heim (2010). <i>Vet. Microbiol.</i>, <b>142</b>, 137–142</li> <li>- Schweizer <i>et al.</i> (2021). <i>Front. Vet. Sci.</i>, <b>8</b>, 702730</li> <li>- Wernike <i>et al.</i> (2017). <i>Pathogens</i>, <b>6</b> (4)</li> <li>- Graham <i>et al.</i> (2021). <i>Front. Vet. Sci.</i>, <b>8</b>, 674557</li> </ul>
Antibody detection by ELISA +++	Bulk milk, blood	DSe and DSp may differ depending on the ELISA used (commercial/in-house) and the antibodies being tested (e.g. antibodies against structural (E2) and non-structural (NS2-3) proteins).			<ul style="list-style-type: none"> <li>- Simple to perform and cost-effective</li> <li>- Milk collection is non-invasive method</li> </ul>	<ul style="list-style-type: none"> <li>- Some cross-reactivity with antibodies induced by vaccines and other pestiviruses.</li> <li>- PI animal will be seronegative</li> <li>- Bulk milk from herd excludes males, non-lactating or young stock.</li> </ul>	Barrett <i>et al.</i> (2022) <i>BMC Vet Res.</i> , <b>18</b> , 210.
Virus neutralisation test +	Serum	DSe & DSp both extremely high, both >99%. Historical reference serological test.	N/A	Historical information with no formal validation	<ul style="list-style-type: none"> <li>- Very high specificity</li> <li>- Allows differentiation of antibodies to BVDV species</li> </ul>	<ul style="list-style-type: none"> <li>- ASe can vary depending on virus strain used</li> <li>- Requires cell culture, good quality samples</li> <li>- Takes 5 days to obtain results. Labour intensive - not</li> </ul>	N/A

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population used to measure accuracy</u>	<u>Validation report</u>	<u>Advantages: expert opinion</u>	<u>Disadvantages: expert opinion</u>	<u>References</u>
						amenable to testing very large numbers of samples. - No differentiation between infected and vaccinated animals	

15 N/A: not available



**Appendix 6: Bovine viral diarrhoea**

**Intended purpose of test: immune status in individual animals or populations (post-vaccination)**

<u>Test with score and species</u>	<u>Sample type and target analytes</u>	<u>Accuracy</u>	<u>Test population used to measure accuracy</u>	<u>Validation report</u>	<u>Advantages: expert opinion</u>	<u>Disadvantages: expert opinion</u>	<u>References</u>
<u>Antibody detection by ELISA +++</u>	<u>Individual milk, bulk milk, blood (antibodies present against structural and non-structural proteins)</u>	<u>DSe and DSp differs based on the ELISA used (commercial/in-house) and the antibodies being tested (e.g. antibodies against structural (E2) and non-structural (NS2-3) proteins.</u>			<ul style="list-style-type: none"> <li>- Simple to perform and cost-effective</li> <li>- Milk collection is non-invasive method</li> </ul>	<ul style="list-style-type: none"> <li>- Some cross-reactivity with antibodies induced by vaccines and other pestiviruses.</li> <li>While a DIVA capability is preferred, this is very difficult to achieve using the combinations of existing vaccines and serological assays. Live attenuated vaccines preclude a DIVA capability unless genetically modified to include a 'marker' gene or deletion that can be detected by a serological assay.</li> <li>- PI animal will be seronegative</li> <li>- Bulk milk from herd excludes males, non-lactating or young stock</li> </ul>	<u>Raue et al. (2011). <i>Vet. J.</i>, 187, 330–334.</u> <u>Gonzalez et al. (2014). <i>Vet. J.</i>, 199, 424–428.</u> <u>Sayers et al. (2015). <i>Vet. J.</i>, 205, 56–61.</u>
<u>Virus neutralisation test +++</u>	<u>Serum</u>	<u>DSe &amp; DSp both extremely high, both &gt;99%. Historical reference serological test.</u>	<u>N/A</u>	<u>Historical information with no formal validation</u>	<ul style="list-style-type: none"> <li>- Very high specificity</li> <li>- Good correlation with immunity</li> <li>- Can provide a measure of cross protection between BVDV species</li> </ul>	<ul style="list-style-type: none"> <li>- ASe can vary depending on virus strain used</li> <li>- Requires cell culture, good quality samples</li> <li>- Labour intensive, takes 5 days to obtain results</li> <li>- No differentiation between infected and vaccinated animals</li> </ul>	<u>N/A</u>

N/A: not available

REUNIÓN DE LA COMISIÓN DE NORMAS BIOLÓGICAS

París, 5–9 de febrero de 2024

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CHAPTER 3.4.12.

LUMPY SKIN DISEASE

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SUMMARY

**Description of the disease:** Lumpy skin disease (LSD) is a poxvirus disease of cattle characterised by fever, nodules on the skin, mucous membranes and internal organs, emaciation, enlarged lymph nodes, oedema of the skin, and sometimes death. The disease is of economic importance as it can cause a temporary reduction in milk production, temporary or permanent sterility in bulls, damage to hides and, occasionally, death. Various strains of capripoxvirus are responsible for the disease. These are antigenically indistinguishable from strains causing sheep pox and goat pox yet distinct at the genetic level. LSD has a partially different geographical distribution from sheep and goat pox, suggesting that cattle strains of capripoxvirus do not infect and transmit between sheep and goats. Transmission of LSD virus (LSDV) is thought to be predominantly by arthropods, natural contact transmission in the absence of vectors being inefficient. Lumpy skin disease is endemic in most ~~many~~ African and Middle Eastern countries. Between 2012 and 2022, LSD spread into south-east Europe, the Balkans, Russia and Asia as part of the Eurasian LSD epidemic.

**Pathology:** the nodules are firm and may extend to the underlying subcutis and muscle. Acute histological key lesions consist of epidermal vacuolar changes with intracytoplasmic inclusion bodies and dermal vasculitis. Chronic key histological lesions consist of fibrosis and necrotic sequestrae.

**Detection of the agent:** Laboratory confirmation of LSD is most rapid using a real-time or conventional polymerase chain reaction (PCR) method specific for capripoxviruses in combination with a clinical history of a generalised nodular skin disease and enlarged superficial lymph nodes in cattle. Ultrastructurally, capripoxvirus virions are distinct from those of parapoxvirus, which causes bovine papular stomatitis and pseudocowpox, but cannot be distinguished morphologically from orthopoxvirus virions, including cowpox and vaccinia viruses, both of which can cause disease in cattle, although neither causes generalised infection and both are uncommon in cattle. LSDV will grow in tissue culture of bovine, ovine or caprine origin. In cell culture, LSDV causes a characteristic cytopathic effect and intracytoplasmic inclusion bodies that is distinct from infection with Bovine herpesvirus 2, which causes pseudo-lumpy skin disease and produces syncytia and intranuclear inclusion bodies in cell culture. Capripoxvirus antigens can be demonstrated in tissue culture using immunoperoxidase or immunofluorescent staining and the virus can be neutralised using specific antisera.

A variety of conventional and real-time PCR tests as well as isothermal amplification tests using capripoxvirus-specific primers have been published for use on a variety of samples.

**Serological tests:** The virus neutralisation test (VNT) and enzyme-linked immunosorbent assays (ELISAs) are widely used and have been validated. The agar gel immunodiffusion test and indirect immunofluorescent antibody test are less specific than the VNT due to cross-reactions with antibody to other poxviruses.

36 Western blotting using the reaction between the P32 antigen of LSDV with test sera is both sensitive and  
37 specific, but is difficult and expensive to carry out.

38 **Requirements for vaccines:** All strains of capripoxvirus examined so far, whether derived from cattle,  
39 sheep or goats, are antigenically similar. Attenuated cattle strains, and strains derived from sheep and goats  
40 have been used as live vaccines against LSDV.

## 41 A. INTRODUCTION

42 Lumpy skin disease (LSD) was first seen in Zambia in 1929, spreading into Botswana by 1943 (Haig, 1957), and then into  
43 South Africa the same year, where it affected over eight million cattle causing major economic loss. In 1957 it entered  
44 Kenya, ~~at the same time as associated with~~ an outbreak of sheep pox (Weiss, 1968). In 1970 LSD spread north into the  
45 Sudan, by 1974 it had spread west as far as Nigeria, and in 1977 was reported from Mauritania, Mali, Ghana and Liberia.  
46 Another epizootic of LSD between 1981 and 1986 affected Tanzania, Kenya, Zimbabwe, Somalia and the Cameroon, with  
47 reported mortality rates in affected cattle of 20%. The occurrence of LSD north of the Sahara desert and outside the African  
48 continent was confirmed for the first time in Egypt and Israel between 1988 and 1989, and was reported again in 2006  
49 (Brenner *et al.*, 2006). In the past decade, LSD occurrences have been reported in the Middle Eastern, European and  
50 Asian regions (for up-to-date information, consult WOAAH WAHIS interface<sup>1</sup>). Lumpy skin disease outbreaks tend to be  
51 sporadic, depending upon animal movements, immune status, and wind and rainfall patterns affecting vector populations.  
52 The principal method of transmission is thought to be mechanical by various arthropod vectors (Tuppurainen *et al.*, 2015).

53 Lumpy skin disease virus (LSDV) belongs to the family *Poxviridae*, subfamily *Chordopoxvirinae-Chordopoxviridae*, and  
54 genus *Capripoxvirus*. In common with other poxviruses LSDV replicates in the cytoplasm of an infected cell, forming distinct  
55 perinuclear viral factories. The LSD virion is large and brick-shaped measuring 293–299nm (length) and 262–273nm  
56 (width). The LSDV genome structure is also similar to other poxviruses, consisting of double-stranded linear DNA that is  
57 25% GC-rich, approximately 150,000 bp in length, and encodes around 156 open reading frames (ORFs). An inverted  
58 terminal repeat sequence of 2200–2300 bp is found at each end of the linear genome. The linear ends of the genome are  
59 joined with a hairpin loop. The central region of the LSDV genome contains ORFs predicted to encode proteins required  
60 for virus replication and morphogenesis and exhibit a high degree of similarity with genomes of other mammalian  
61 poxviruses. The ORFs in the outer regions of the LSDV genome have lower similarity and likely encode proteins involved  
62 in viral virulence and host range determinants.

63 Phylogenetic analysis shows the majority of LSDV strains group into two monophyletic clusters (cluster 1.1 and 1.2)  
64 (Biswas *et al.*, 2020; Van Schalkwyk *et al.*, 2021). Cluster 1.1 consists of LSDV Neethling vaccine strains that are based  
65 on the LSDV/Neethling/LW-1959 vaccine strain (Kara *et al.*, 2003; Van Rooyen *et al.*, 1959; van Schalkwyk *et al.*, 2020)  
66 and historic wild-type strains from South Africa. Cluster 1.2 consists of wild-type strains from southern Africa, Kenya, the  
67 northern hemisphere, and the Kenyan KSGP O-240 commercial vaccine. In addition to these two clusters, there have  
68 recently been recombinant LSDV strains isolated from clinical cases of LSD in the field in Russia and central Asia (Flannery  
69 *et al.*, 2021; Sprygin *et al.*, 2018; 2020; Wang *et al.*, 2021). These recombinant viruses show unique patterns of accessory  
70 gene alleles, consisting of sections of both wild-type and “vaccine” LSDV strains.

71 The severity of the clinical signs of LSD is highly variable and depends on a number of factors, including the strain of  
72 capripoxvirus, the age of the host, immunological status and breed. *Bos taurus* is generally more susceptible to clinical disease  
73 than *Bos indicus*; the Asian buffalo (*Bubalus spp.*) has also been reported to be susceptible. Within *Bos taurus*, the fine-  
74 skinned Channel Island breeds develop more severe disease, with lactating cows appearing to be the most at risk. However,  
75 even among groups of cattle of the same breed kept together under the same conditions, there is a large variation in the  
76 clinical signs presented, ranging from subclinical infection to death (Carn & Kitching, 1995). There may be failure of the virus  
77 to infect the whole group, probably depending on the virulence of the virus isolate, immunological status of the host, host  
78 genotype, and vector prevalence. Seroprevalence studies, experimental infections and case reports have provided indications  
79 that several wildlife species (e.g. springbok, impala, giraffe, camel, banteng) are susceptible to LSDV infection (Dao *et al.*,  
80 2022; Hedger & Hamblin, 1983; Kumar *et al.*, 2023; Porco *et al.*, 2023). The scarcity of documented outbreaks in wildlife and  
81 the fact that available studies remain limited in number and mostly involve only a few animals, make it difficult to determine  
82 the role of wildlife in LSDV epidemiology. This topic deserves further study, especially given the current spread of LSDV in  
83 new geographical areas where large numbers of naïve, potentially susceptible wild bovines and other ruminants are present.

84 The incubation period under field conditions has not been reported, but following experimental inoculation is 6–9 days until  
85 the onset of fever. In the acutely infected animal, there is an initial pyrexia, which may exceed 41°C and persist for 1 week.  
86 All the superficial lymph nodes become enlarged. In lactating cattle there is a marked reduction in milk yield. Lesions  
87 develop over the body, particularly on the head, neck, udder, scrotum, vulva and perineum **between 7 and 19 days after**  
88 **virus inoculation (Coetzer, 2004)**. The characteristic integumentary lesions are multiple, well circumscribed to coalescing,  
89 0.5–5 cm in diameter, firm, flat-topped papules and nodules. The nodules involve the dermis and epidermis, and may

<sup>1</sup> <https://www.woah.org/en/what-we-do/animal-health-and-welfare/disease-data-collection/>

90 extend to the underlying subcutis and occasionally to the adjacent striated muscle. These nodules have a creamy grey to  
 91 white colour on cut section, which may initially exude serum, but over the ensuing 2 weeks a cone-shaped central core or  
 92 sequestrum of necrotic material/necrotic plug ("sit-fast") may appear within the nodule. The acute histological lesions  
 93 consist of epidermal vacuolar changes with intracytoplasmic inclusion bodies and dermal vasculitis. The inclusion bodies  
 94 are numerous, intracytoplasmic, eosinophilic, homogenous to occasionally granular and they may occur in endothelial  
 95 cells, fibroblasts, macrophages, pericytes, and keratinocytes. The dermal lesions include vasculitis with fibrinoid necrosis,  
 96 oedema, thrombosis, lymphangitis, dermal-epidermal separation, and mixed inflammatory infiltrate. The chronic lesions  
 97 are characterised by an infarcted tissue with a sequestered necrotic core, often rimmed by granulation tissue gradually  
 98 replaced by mature fibrosis. At the appearance of the nodules, the discharge from the eyes and nose becomes  
 99 mucopurulent, and keratitis may develop. Nodules may also develop in the mucous membranes of the mouth and  
 100 alimentary tract, particularly the abomasum and in the trachea and the lungs, resulting in primary and secondary  
 101 pneumonia. The nodules on the mucous membranes of the eyes, nose, mouth, rectum, udder and genitalia quickly  
 102 ulcerate, and by then all secretions, ocular and nasal discharge and saliva contain LSD virus (LSDV). The limbs may be  
 103 oedematous and the animal is reluctant to move. Pregnant cattle may abort, and there is a report of intrauterine  
 104 transmission (Rouby & Aboulsoudb, 2016). Bulls may become permanently or temporarily infertile and the virus can be  
 105 excreted in the semen for prolonged periods (Irons *et al.*, 2005). Recovery from severe infection is slow; the animal is  
 106 emaciated, may have pneumonia and mastitis, and the necrotic plugs of skin, which may have been subject to fly strike,  
 107 are shed leaving deep holes in the hide (Prozesky & Barnard, 1982).

108 The main differential diagnosis is pseudo-LSD caused by bovine herpesvirus 2 (BoHV-2). This is usually a milder clinical  
 109 condition, characterised by superficial nodules, resembling only the early stage of LSD. Intra-nuclear inclusion bodies and  
 110 viral syncytia are histopathological characteristics of BoHV-2 infection not seen in LSD. Other differential diagnoses (for  
 111 integumentary lesions) include: dermatophilosis, dermatophytosis, bovine farcy, photosensitisation, actinomycosis,  
 112 actinobacillosis, urticaria, insect bites, besnoitiosis, nocardiosis, demodicosis, onchocerciasis, pseudo-cowpox, and  
 113 cowpox. Differential diagnoses for mucosal lesions include: foot and mouth disease, bluetongue, mucosal disease,  
 114 malignant catarrhal fever, infectious bovine rhinotracheitis, and bovine papular stomatitis.

115 LSDV is not transmissible to humans. However, all laboratory manipulations must be performed at an appropriate  
 116 containment level determined using biorisk analysis (see Chapter 1.1.4 Biosafety and biosecurity: Standard for managing  
 117 biological risk in the veterinary laboratory and animal facilities).

## 118 B. DIAGNOSTIC TECHNIQUES

119 **Table 1. Test methods available for the diagnosis of LSD and their purpose**

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of the agent						
Virus isolation	+	++	+	+++	+	–
PCR	++	+++	++	+++	+	–
TEM	–	–	–	+	–	–
Detection of immune response						
VNT	++	++	++	++	++	++
IFAT	+	+	+	+	+	+
ELISA	++	++	++	++	++	++

120 Key: +++ = recommended for this purpose; ++ recommended but has limitations;  
 121 + = suitable in very limited circumstances; – = not appropriate for this purpose.

122 PCR = polymerase chain reaction; TEM = Transmission electron microscopy; VNT = virus neutralisation test;  
 123 IFAT = indirect fluorescent antibody test; ELISA = enzyme-linked immunosorbent assay.

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## 124 1. Detection of the agent

### 125 1.1. Specimen collection, submission and preparation

126 Material for virus isolation and antigen detection should be collected as biopsies or from skin nodules at post-  
127 mortem examination. Samples for virus isolation should preferably be collected within the first week of the  
128 occurrence of clinical signs, before the development of neutralising antibodies (Davies, 1991; Davies *et al.*,  
129 1971), **however virus can be isolated from skin nodules for at least 3–4 weeks thereafter**. Samples for genome  
130 detection using conventional or real-time polymerase chain reaction (PCR) may be collected when neutralising  
131 antibody is present. Following the first appearance of the skin lesions, the virus can be isolated for up to 35 days  
132 and viral nucleic acid can be demonstrated via PCR for up to 3 months (Tuppurainen *et al.*, 2005; Weiss, 1968).  
133 Buffy coat from blood collected into heparin or EDTA (ethylene diamine tetra-acetic acid) during the viraemic  
134 stage of LSD (before generalisation of lesions or within 4 days of generalisation), can also be used for virus  
135 isolation. Samples for histology should include the lesion and tissue from the surrounding (non-lesion) area, be  
136 a maximum size of 2 cm<sup>3</sup>, and be placed immediately following collection into ten times the sample volume of  
137 10% neutral buffered formal saline.

138 Tissues in formalin have no special transportation requirements in regard to biorisks. Blood samples with  
139 anticoagulant for virus isolation from the buffy coat should be placed immediately on ice after gentle mixing and  
140 processed as soon as possible. In practice, the samples may be kept at 4°C for up to 2 days prior to processing,  
141 but should not be frozen or kept at ambient temperatures. Tissues for virus isolation and antigen detection  
142 should be kept at 4°C, on ice or at –20°C. If it is necessary to transport samples over long distances without  
143 refrigeration, the medium should contain 10% glycerol; the samples should be of sufficient size (**e.g. 1 g in 10 ml**)  
144 that the transport medium does not penetrate the central part of the biopsy, which should be used for virus  
145 isolation.

146 ~~Samples for histology should include the lesion and tissue from the surrounding (non lesion) area, be a~~  
147 ~~maximum size of 2 cm<sup>3</sup>, and be placed immediately following collection into ten times the sample volume of~~  
148 ~~10% neutral buffered formaldehyde. Tissues in formalin have no special transportation requirements in regard~~  
149 ~~to biorisks. Material for histology should be prepared using standard techniques and stained with haematoxylin~~  
150 ~~and eosin (H&E) (Burdin, 1959). Lesion material for virus isolation and antigen detection is minced using a sterile~~  
151 ~~scalpel blade and forceps and then macerated in a sterile steel ball-bearing mixer mill, or ground with a pestle~~  
152 ~~in a sterile mortar with sterile sand and an equal volume of sterile phosphate buffered saline (PBS) or serum-~~  
153 ~~free modified Eagle's medium containing sodium penicillin (1000 international units [IU]/ml), streptomycin~~  
154 ~~sulphate (1 mg/ml), mycostatin (100 IU/ml) or fungizone (amphotericin, 2.5 µg/ml) and neomycin (200 IU/ml).~~  
155 ~~The suspension is freeze-thawed three times and then partially clarified using a bench centrifuge at 600 g~~  
156 ~~for 10 minutes. In cases where bacterial contamination of the sample is expected (such as when virus is isolated~~  
157 ~~from skin samples), the supernatant can be filtered through a 0.45 µm pore size filter after the centrifugation~~  
158 ~~step. Buffy coats may be prepared from unclotted blood using centrifugation at 600 g for 15 minutes, and the~~  
159 ~~buffy coat carefully removed into 5 ml of cold double-distilled water using a sterile Pasteur pipette. After~~  
160 ~~30 seconds, 5 ml of cold double-strength growth medium is added and mixed. The mixture is centrifuged at~~  
161 ~~600 g for 15 minutes, the supernatant is discarded and the cell pellet is suspended in 5 ml growth medium, such~~  
162 ~~as Glasgow's modified Eagle's medium (GMEM). After centrifugation at 600 g for a further 15 minutes, the~~  
163 ~~resulting pellet is suspended in 5 ml of fresh GMEM. Alternatively, the buffy coat may be separated from a~~  
164 ~~heparinised sample by using a Ficoll gradient.~~

### 165 1.2. Virus isolation on cell culture

166 LSDV will grow in tissue culture of bovine, ovine or caprine origin. MDBK (Madin–Darby bovine kidney) cells are  
167 often used, as they support good growth of the virus and are well characterised (Fay *et al.*, 2020). Primary cells,  
168 such as lamb testis (LT) cells also support viral growth, but care needs to be taken to ensure they are not  
169 contaminated with viruses such as bovine viral diarrhoea virus. One ml of clarified supernatant or buffy coat is  
170 inoculated onto a confluent monolayer in a 25 cm<sup>2</sup> culture flask at 37°C and allowed to adsorb for 1 hour. The  
171 culture is then washed with warm PBS and covered with 10 ml of a suitable medium, such as GMEM, containing  
172 antibiotics and 2% fetal calf serum. If available, tissue culture tubes containing appropriate cells and a flying  
173 cover-slip, or tissue culture microscope slides, are also infected.

174 The flasks/tissue culture tubes are examined daily for 7–14 days for evidence of cytopathic effects (CPE).  
175 Infected cells develop a characteristic CPE consisting of retraction of the cell membrane from surrounding cells,  
176 and eventually rounding of cells and margination of the nuclear chromatin. At first only small areas of CPE can  
177 be seen, sometimes as soon as 2 days after infection; over the following 4–6 days these expand to involve the  
178 whole cell ~~monolayer sheet~~. If no CPE is apparent by day 14, the culture should be freeze–thawed three times,  
179 and clarified supernatant inoculated on to a fresh cell monolayer. At the first sign of CPE in the flasks, or earlier  
180 if a number of infected cover-slips are being used, a cover-slip should be removed, fixed in acetone and stained

181 using H&E. Eosinophilic intracytoplasmic inclusion bodies, which are variable in size but up to half the size of  
182 the nucleus and surrounded by a clear halo, are diagnostic for poxvirus infection. PCR may be used as an  
183 alternative to H&E for confirmation of the diagnosis. The CPE can be prevented or delayed by adding specific  
184 anti-LSDV serum to the medium. In contrast, the herpesvirus that causes pseudo-LSD produces a Cowdry type  
185 A intranuclear inclusion body. It also forms syncytia.

186 An ovine testis cell line (OA3.T<sub>8</sub>) has been evaluated for the propagation of capripoxvirus isolates (Babiuk *et*  
187 *al.*, 2007), however this cell line has been found to be contaminated with pestivirus and should be used with  
188 caution.

### 189 1.3. Polymerase chain reaction (PCR)

190 The conventional gel-based PCR method described below is a simple, fast and sensitive method for the  
191 detection of capripoxvirus genome in EDTA blood, semen or tissue culture samples (Tuppurainen *et al.*, 2005).

#### 192 1.3.1. Test procedure

193 The extraction method described below can be replaced using commercially available DNA extraction  
194 kits.

195 i) Freeze and thaw 200 µl of blood in EDTA, semen or tissue culture supernatant and suspend in  
196 100 µl of lysis buffer containing 5 M guanidine thiocyanate, 50 mM potassium chloride, 10 mM  
197 Tris/HCl (pH 8); and 0.5 ml Tween 20.

198 ii) Cut skin and other tissue samples into fine pieces using a sterile scalpel blade and forceps. Grind  
199 with a pestle in a mortar. Suspend the tissue samples in 800 µl of the above mentioned lysis buffer.

200 iii) Add 2 µl of proteinase K (20 mg/ml) to blood samples and 10 µl of proteinase K (20 mg/ml) to tissue  
201 samples. Incubate at 56°C for 2 hours or overnight, followed by heating at 100°C for 10 minutes.  
202 Add phenol:chloroform:isoamylalcohol (25:24:1 [v/v]) to the samples in a 1:1 ratio. Vortex and  
203 incubate at room temperature for 10 minutes. Centrifuge the samples at 16,060 *g* for 15 minutes at  
204 4°C. Carefully collect the upper, aqueous phase (up to 200 µl) and transfer into a clean 2.0 ml tube.  
205 Add two volumes of ice cold ethanol (100%) and 1/10 volume of 3 M sodium acetate (pH 5.3). Place  
206 the samples at -20°C for 1 hour. Centrifuge again at 16,060 *g* for 15 minutes at 4°C and discard  
207 the supernatant. Wash the pellets with ice cold 70% ethanol (100 µl) and centrifuge at 16,060 *g* for  
208 1 minute at 4°C. Discard the supernatant and dry the pellets thoroughly. Suspend the pellets in  
209 30 µl of nuclease-free water and store immediately at -20°C (Tuppurainen *et al.*, 2005).  
210 Alternatively a column-based extraction kit may be used.

211 iv) The primers for this PCR assay were developed from the gene encoding the viral attachment  
212 protein. The size of the expected amplicon is 192 bp (Ireland & Binopal, 1998). The primers have  
213 the following gene sequences:

214 Forward primer 5'-TCC-GAG-CTC-TTT-CCT-GAT-TTT-TCT-TAC-TAT-3'

215 Reverse primer 5'-TAT-GGT-ACC-TAA-ATT-ATA-TAC-GTA-AAT-AAC-3'.

216 v) DNA amplification is carried out in a final volume of 50 µl containing: 5 µl of 10 × PCR buffer, 1.5 µl  
217 of MgCl<sub>2</sub> (50 mM), 1 µl of dNTP (10 mM), 1 µl of forward primer, 1 µl of reverse primer, 1 µl of DNA  
218 template (~10 ng), 0.5 µl of *Taq* DNA polymerase and 39 µl of nuclease-free water. The volume of  
219 DNA template required may vary and the volume of nuclease-free water must be adjusted to the  
220 final volume of 50 µl.

221 vi) Run the samples in a thermal cycler as follows: 2 minutes at 95°C; then 45 seconds at 95°C,  
222 50 seconds at 50°C and 1 minute at 72°C (34 cycles); 2 minutes at 72°C and hold at 4°C until  
223 analysis.

224 vii) Mix 10 µl of each sample with loading dye and load onto a 1.5% agarose gel in TAE buffer  
225 (Tris/acetate buffer containing EDTA). Load a parallel lane with a 100 bp DNA-marker ladder.  
226 Electrophoretically separate the products using approximately 8–10 V/cm for 40–60 minutes and  
227 visualise with a suitable DNA stain and transilluminator.

228 Quantitative real-time PCR methods have been described that are reported to be faster and have higher  
229 sensitivity than conventional PCRs (Balinsky *et al.*, 2008; Bowden *et al.*, 2008). A real-time PCR method that  
230 differentiates between LSDV, sheep pox virus and goat pox virus has been published (Lamien *et al.*, 2011).

231 Quantitative real-time PCR assays have been designed to differentiate between Neethling-based LSDV strains,  
232 which are often used for vaccination, and wild-type LSDV strains from cluster 1.2 (Agianniotaki *et al.*, 2017;

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233 Pestova *et al.*, 2018; Vidanovic *et al.*, 2016). These “DIVA” assays (DIVA: differentiation of infected from  
234 vaccinated animals) enable, for example, differentiation of “Neethling response” caused by vaccination with a  
235 LSDV Neethling vaccine strain from disease caused by infection with a cluster 1.2 wild-type virus. However  
236 these DIVA PCR assays cannot distinguish between a LSDV Neethling vaccine strain and the novel  
237 recombinant LSDV strains recently isolated from disease outbreaks in Asia (Byadovskaya *et al.*, 2021; Flannery  
238 *et al.*, 2021). These DIVA assays are also not capable of discriminating between LSDV Neethling vaccine strains  
239 and recently characterised (historic) wild-type viruses from South Africa belonging within cluster 1.1 (Van  
240 Schalkwyk *et al.*, 2020; 2021). Consequently, in regions where recombinant strains (currently Asia and possibly  
241 elsewhere) or wild-type cluster 1.1 strains are circulating (currently South Africa and possibly elsewhere), these  
242 DIVA assays are not suitable for distinguishing vaccine and wild-type virus. Thus, in order to overcome these  
243 constraints, whole genome sequencing is recommended.

#### 244 1.4. Transmission electron microscopy

245 The characteristic poxvirus virion can be visualised using a negative staining preparation technique followed by  
246 examination with an electron microscope. There are many different negative staining protocols, an example of  
247 which is given below.

##### 248 1.4.1. Test procedure

249 Before centrifugation, material from the original biopsy suspension is prepared for examination under the  
250 transmission electron microscope by floating a 400-mesh hexagonal electron microscope grid, with  
251 pioloform-carbon substrate activated by glow discharge in pentylamine vapour, onto a drop of the  
252 suspension placed on parafilm or a wax plate. After 1 minute, the grid is transferred to a drop of  
253 Tris/EDTA buffer, pH 7.8, for 20 seconds and then to a drop of 1% phosphotungstic acid, pH 7.2, for  
254 10 seconds. The grid is drained using filter paper, air-dried and placed in the electron microscope. The  
255 capripox virion is brick shaped, covered in short tubular elements and measures approximately 290 ×  
256 270 nm. A host-cell-derived membrane may surround some of the virions, and as many as possible  
257 should be examined to confirm their appearance (Kitching & Smale, 1986).

258 The ~~capripox~~ virions of capripoxvirus are indistinguishable from those of orthopoxvirus, but, apart from vaccinia  
259 virus and cowpox virus, which are both uncommon in cattle and do not cause generalised infection, no other  
260 orthopoxvirus is known to cause lesions in cattle. However, vaccinia virus may cause generalised infection in  
261 young immunocompromised calves. In contrast, orthopoxviruses are a common cause of skin disease in  
262 domestic buffalo (*Bubalus bubalis*) causing buffalo pox, a disease that usually manifests as pock lesions on the  
263 teats, but may cause skin lesions at other sites, such as the perineum, the medial aspects of the thighs and the  
264 head. Orthopoxviruses that cause buffalo pox cannot be readily distinguished from capripoxvirus by electron  
265 microscopy. The virions of parapoxvirus ~~virions~~ that cause bovine papular stomatitis and pseudocowpox are  
266 smaller, oval in shape and each is covered in a single continuous tubular element that appears as striations  
267 over the virion. Capripoxvirus virions are also distinct from the herpesvirus that causes pseudo-LSD (also known  
268 as “Allerton” or bovine herpes mammillitis).

#### 269 1.5. Fluorescent antibody tests

270 Capripoxvirus antigen can be identified on infected cover-slips or tissue culture slides using fluorescent antibody  
271 tests. Cover-slips or slides should be washed and air-dried and fixed in cold acetone for 10 minutes. The indirect  
272 test using immune cattle sera is subject to high background colour and nonspecific reactions. However, a direct  
273 conjugate can be prepared from sera from convalescent cattle (or from sheep or goats convalescing from  
274 capripox) or from rabbits hyperimmunised with purified capripoxvirus. Uninfected tissue culture should be  
275 included as a negative control as cross-reactions can cause problems due to antibodies to cellular components  
276 (pre-absorption of these from the immune serum helps solve this issue).

#### 277 1.6. Immunohistochemistry

278 Immunohistochemistry using F80G5 monoclonal antibody specific for capripoxvirus ORF 057 has been  
279 described for detection of LSDV antigen in the skin of experimentally infected cattle (Babiuk *et al.*, 2008).

#### 280 1.7. Isothermal genome amplification

281 Molecular tests using loop-mediated isothermal amplification to detect capripoxvirus genomes are reported to  
282 provide sensitivity and specificity similar to real-time PCR with a simpler method and lower cost (Das *et al.*,  
283 2012; Murray *et al.*, 2013). Field validation of the Das *et al.* method was reported by Omoga *et al.* (2016).

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## 284 2. Serological tests

285 All the viruses in the genus *Capripoxvirus* share a common major antigen for neutralising antibodies and it is thus not  
286 possible to distinguish strains of capripoxvirus from cattle, sheep or goats using serological techniques.

### 287 2.1. Virus neutralisation

288 A test serum can either be titrated against a constant titre of capripoxvirus (100 TCID<sub>50</sub> [50% tissue culture  
289 infective dose]) or a standard virus strain can be titrated against a constant dilution of test serum in order to  
290 calculate a neutralisation index. Because of the variable sensitivity of tissue culture to capripoxvirus, and the  
291 consequent difficulty of ensuring the accurate and repeatable seeding of 100 TCID<sub>50</sub>/well, the neutralisation  
292 index is the preferred method in most laboratories, although it does require a larger volume of test sera. The  
293 test is described using 96-well flat-bottomed tissue-culture grade microtitre plates, but it can be performed  
294 equally well in tissue culture tubes with the appropriate changes to the volumes used, although it is more difficult  
295 to read an end-point in tubes.

#### 296 2.1.1. Test procedure

- 297 i) Test sera, including a negative and a positive control, are diluted 1/5 in Eagle's/HEPES (N-2-  
298 hydroxyethylpiperazine, N-2-ethanesulphonic acid) buffer and inactivated at 56°C for 30 minutes.
  - 299 ii) Next, 50 µl of the first inactivated serum is added to columns 1 and 2, rows A to H of the microtitre  
300 plate. The second serum is placed in columns 3 and 4, the third in columns 5 and 6, the positive  
301 control serum is placed in columns 7 and 8, the negative control serum is placed in columns 9 and  
302 10, and 50 µl of Eagle's/HEPES buffer (without serum) is placed in columns 11 and 12, and to all  
303 wells in row H.
  - 304 iii) A reference strain of capripoxvirus, usually a vaccine strain known to grow well in tissue culture,  
305 with a titre of over log<sub>10</sub> 6 TCID<sub>50</sub> per ml is diluted in Eagle's/HEPES in bijoux bottles to give a log  
306 dilution series of log<sub>10</sub> 5.0, 4.0, 3.5, 3.0, 2.5, 2.0, 1.5 TCID<sub>50</sub> per ml (equivalent to log<sub>10</sub> 3.7, 2.7, 2.2,  
307 1.7, 1.2, 0.7, 0.2 TCID<sub>50</sub> per 50 µl).
  - 308 iv) Starting with row G and the most diluted virus preparation, 50 µl of virus is added to each well in  
309 that row. This is repeated with each virus dilution, the highest titre virus dilution being placed in row  
310 A.
  - 311 v) The plates are covered and incubated for 1 hour at 37°C.
  - 312 vi) An appropriate cell suspension (such as MDBK cells) is prepared from pregrown monolayers as a  
313 suspension of 10<sup>5</sup> cells/ml in Eagle's medium containing antibiotics and 2% fetal calf serum.  
314 Following incubation of the microtitre plates, 100 µl of cell suspension is added to all the wells,  
315 except wells H11 and H12, which serve as control wells for the medium. The remaining wells of  
316 row H are cell and serum controls.
  - 317 vii) The microtitre plates are covered and incubated at 37°C for 9 days.
  - 318 viii) Using an inverted microscope, the monolayers are examined daily from day 4 for evidence of CPE.  
319 There should be no CPE in the cells of row H. Using the 0240 KSGP vaccine strain of capripoxvirus,  
320 by way of example, the final reading is taken on day 9, and the titre of virus in each duplicate titration  
321 is calculated using the Kärber method. If left longer, there is invariably a 'breakthrough' of virus in  
322 which virus that was at first neutralised appears to disassociate from the antibody.
  - 323 ix) *Interpretation of the results:* The neutralisation index is the log titre difference between the titre of  
324 the virus in the negative serum and in the test serum. An index of ≥1.5 is positive. The test can be  
325 made more sensitive if serum from the same animal is examined before and after infection.  
326 Because the immunity to capripoxviruses is predominantly cell mediated, a negative result,  
327 particularly following vaccination, after which the antibody response may be low, does not imply  
328 that the animal from which the serum was taken is not protected.
- 329 Antibodies to capripoxvirus can be detected from 1 to 2 days after the onset of clinical signs. These  
330 remain detectable for about 7 months.

#### 331 2.2. Enzyme-linked immunosorbent assay

332 Enzyme-linked immunosorbent assays (ELISAs) for the detection of capripoxviral antibodies are widely used  
333 and are available in commercial kit form (Milovanovic *et al.*, 2019; Samojlovic *et al.*, 2019).



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### 2.3. Indirect fluorescent antibody test

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Capripoxvirus-infected tissue culture grown on cover-slips or tissue culture microscope slides can be used for the indirect fluorescent antibody test. Uninfected tissue culture control, and positive and negative control sera, should be included in the test. The infected and control cultures are fixed in acetone at  $-20^{\circ}\text{C}$  for 10 minutes and stored at  $4^{\circ}\text{C}$ . Dilutions of test sera are made in PBS, starting at 1/20 or 1/40, and positive samples are identified using an anti-bovine gamma-globulin conjugated with fluorescein isothiocyanate. Antibody titres may exceed 1/1000 after infection. Sera may be screened at 1/50 and 1/500. Cross-reactions can occur with orf virus (contagious pustular dermatitis virus of sheep), bovine papular stomatitis virus and perhaps other poxviruses.

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### 2.4. Western blot analysis

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Western blotting of test sera against capripoxvirus-infected cell lysate provides a sensitive and specific system for the detection of antibody to capripoxvirus structural proteins, although the test is expensive and difficult to carry out.

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Capripoxvirus-infected LT cells should be harvested when 90% CPE is observed, freeze-thawed three times, and the cellular debris pelleted using centrifugation. The supernatant should be decanted, and the proteins should be separated using SDS/PAGE (sodium dodecyl sulphate/polyacrylamide gel electrophoresis). A vertical discontinuous gel system, using a stacking gel made up of acrylamide (5%) in Tris (125 mM), pH 6.8, and SDS (0.1%), and a resolving gel made up of acrylamide (10–12.5%) in Tris (560 mM), pH 8.7, and SDS (0.1%), is recommended for use with a glycine running buffer containing Tris (250 mM), glycine (2 M), and SDS (0.1%). Samples of supernatant should be prepared by boiling for 5 minutes with an appropriate lysis buffer prior to loading. Alternatively, purified virus or recombinant antigens may replace tissue-culture-derived antigen.

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Molecular weight markers should be run concurrently with the protein samples. The separated proteins in the SDS/PAGE gel should be transferred electrophoretically to a nitrocellulose membrane (NCM). After transfer, the NCM is rinsed thoroughly in PBS and blocked in 3% bovine serum albumin (BSA) in PBS, or 5% skimmed milk powder in PBS, on a rotating shaker at  $4^{\circ}\text{C}$  overnight. The NCM can then be separated into strips by employing a commercial apparatus to allow the concurrent testing of multiple serum samples, or may be cut into strips and each strip incubated separately thereafter. The NCM is washed thoroughly with five changes of PBS for 5 minutes on a rotating shaker, and then incubated at room temperature on the shaker for 1.5 hours, with the appropriate serum at a dilution of 1/50 in blocking buffer (3% BSA and 0.05% Tween 20 in PBS; or 5% milk powder and 0.05% Tween 20 in PBS). The membrane is again thoroughly washed and incubated (in blocking buffer) with anti-species immunoglobulin horseradish-peroxidase-conjugated immunoglobulins at a dilution determined using titration. After further incubation at room temperature for 1.5 hours, the membrane is washed and a solution of diaminobenzidine tetrahydrochloride (10 mg in 50 ml of 50 mM Tris/HCl, pH 7.5, and 20  $\mu\text{l}$  of 30% [v/v] hydrogen peroxide) is added. Incubation is then undertaken for approximately 3–7 minutes at room temperature on a shaker with constant observation, and the reaction is stopped by washing the NCM in PBS before excessive background colour is seen. A positive and negative control serum should be used on each occasion.

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Positive test samples and the positive control will produce a pattern consistent with reaction to proteins of molecular weights 67, 32, 26, 19 and 17 kDa – the major structural proteins of capripoxvirus – whereas negative serum samples will not react with all these proteins. Hyperimmune serum prepared against parapoxvirus (bovine papular stomatitis or pseudocowpox virus) will react with some of the capripoxvirus proteins, but not the 32 kDa protein that is specific for capripoxvirus.

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## C. REQUIREMENTS FOR VACCINES

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### 1. Background: rationale and intended use of the product

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~~Live attenuated strains of capripoxvirus have been used as vaccines specifically for the control of LSD (Bronner *et al.*, 2006; Capstick & Coakley, 1961; Carn, 1993). Capripoxviruses are cross-reactive within the genus. Consequently, it is possible to protect cattle against LSD using strains of capripoxvirus derived from sheep or goats (Coakley & Capstick, 1961). However, it is recommended to carry out controlled trials, using the most susceptible breeds, prior to introducing a vaccine strain not usually used in cattle. The duration of protection provided by LSD vaccination is unknown.~~

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383  
384  
385

~~Capripoxvirus vaccine strains can produce a large local reaction at the site of inoculation in *Bos taurus* breeds (Davies, 1991), which some stock owners find unacceptable. This has discouraged the use of vaccine, even though the consequences of an outbreak of LSD are invariably more severe. Risk-benefit of vaccination should be assessed following stakeholder discussion.~~

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386 Vaccines are a key tool to control LSD. Different types of LSD vaccines have been developed and several are commercially  
387 available (Tuppurainen *et al.*, 2021).

388 Live attenuated vaccines (LAV) based on the Neethling LSDV strain (homologous LAV vaccines) have been shown to offer  
389 high levels of protection against LSD under experimental conditions (Haegeman *et al.*, 2021) and have been used  
390 successfully to control the disease in the field, through systematic vaccination of the entire country's cattle population for  
391 a number of consecutive years (Klement *et al.*, 2020). Homologous vaccines may induce fever, produce a local reaction  
392 at the site of inoculation, cause a temporary reduction in milk production and on rare occasions induce a 'Neethling'  
393 response (Ben-Gera *et al.*, 2015; Davies, 1991; Haegeman *et al.*, 2021). Such adverse effects, however, usually resolve  
394 within a few days and are largely outweighed by the overall benefits of vaccination with homologous vaccines. The duration  
395 of immunity induced by good quality live attenuated LSDV vaccines was shown to be at least 18 months (Haegeman *et*  
396 *al.*, 2023).

397 As capripoxviruses provide cross-reactive protection within the genus, heterologous LAVs comprising sheeppox virus or  
398 goatpox virus strains have also been tested and used to protect cattle against LSD. Sheeppox virus-based heterologous  
399 vaccines usually contain higher doses of virus than when administered to sheep. Although safe, their effectiveness in  
400 protecting cattle against LSD is inferior compared to homologous vaccines (Ben-Gera *et al.*, 2015; Zhugunisso *et al.*,  
401 2020). Heterologous vaccines containing goatpox virus strains for use in cattle against LSD have been developed more  
402 recently. One such vaccine based on the Gorgan strain provided protection under experimental conditions comparable to  
403 homologous vaccines (Gari *et al.*, 2015). On the other hand, a goat pox vaccine based on an attenuated Uttarkashi goatpox  
404 virus strain performed suboptimally under field conditions in India (Naveem *et al.*, 2023), indicating that further research is  
405 warranted before asserting that all goatpox virus-based vaccines induce protection equal to homologous vaccines in cattle  
406 against LSD.

407 In addition, homologous inactivated vaccines against LSD have been developed and tested (Haegeman *et al.*, 2023; Hamdi  
408 *et al.*, 2020; Wolff *et al.*, 2022). These vaccines are reported to be safe and efficacious. They however require a booster  
409 vaccination one month after primo-vaccination and then every 6 months thereafter, based on the fact that the duration of  
410 immunity is shorter than 1 year (Haegeman *et al.*, 2023).

411 None of the commercial vaccines currently available has practical DIVA capacity. This problem may be resolved in the  
412 future by introducing new types of vaccines (e.g. vector-vaccines, subunit vaccines, mRNA vaccines) that are at various  
413 stages of development and evaluation.

## 414 **2. Outline of production of LSD vaccines and minimum requirements for conventional** 415 **vaccines**

416 General requirements set for the facilities used for the production of vaccines and for the documentation and record keeping  
417 throughout the whole manufacturing process are described in Chapter 1.1.8 *Principles of veterinary vaccine production*.  
418 The documentation should include standard operating procedures (SOP) for the method of manufacture and each step for  
419 the testing of cells and reagents used in the process, each batch and the final product.

420 The production of vaccines, including LSD vaccines, starts within research and development (R&D) facilities where vaccine  
421 candidates are produced and tested in preclinical studies to demonstrate the quality, safety and efficacy of the product.

422 Minimum requirements for different production stages of veterinary vaccines are available in different chapters of the  
423 *Terrestrial Manual*. These are intended to be used in combination with country-specific regulatory requirements for vaccine  
424 production and release. Here we outline the most important requirements for the production of live and inactivated LSD  
425 vaccines. Full requirements are available in Chapter 1.1.8 *Principles of veterinary vaccine production*, Chapter 2.3.3  
426 *Minimum requirements for the organisation and management of a vaccine manufacturing facility* and Chapter 2.3.4  
427 *Minimum requirements for the production and quality control of vaccine*, and other regulatory documentation.

### 428 **2.1. Quality assurance**

429 Facilities for manufacturing LSD vaccines should operate in line with the concepts of good laboratory practice  
430 (GLP) and good manufacturing practice (GMP) to produce high quality products. Quality risk management and  
431 quality control with adequate documentation management, as an integral part of the production process, have  
432 to be in place. In case some activities of the production process are outsourced, those should also be  
433 appropriately defined, recorded and controlled.

434 The vaccine production process (Outline of Production) should be documented in a series of standard operating  
435 procedures (SOPs), or other documents describing the manufacturing of each batch and the final product  
436 (including starting materials to be used, manufacturing steps, in-process controls and controls on the final

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437 product). Detailed requirements for documentation management in the process of vaccine production are  
438 available in Chapter 2.3.3.

439 A completed Outline of Production is to be enclosed in a vaccine candidate dossier and used for the evaluation  
440 of the production process and product by regulatory bodies.

## 441 **2.2. Process validation**

442 The dossier with the enclosed Outline of Production for the vaccine candidate has to be submitted for regulatory  
443 approval, so it can be assessed and authorised by the competent authority to ensure compliance with local  
444 regulatory requirements. Among others, data on quality, safety, and efficacy will be assessed. The procedures  
445 necessary to obtain these data are described in the subsequent sections.

446 National regulatory authorities might also require official control authority re-testing (check testing) of final  
447 products and batches in government laboratories or an independent batch quality control by a third party.

## 448 **3. Requirements for LSD vaccine candidates and batch production**

### 449 **3.1. Requirements for starting materials**

450 Live attenuated vaccines (LAV) and inactivated vaccines (IV) for LSD are produced using the system of limited  
451 and controlled passages of master seed and working seed virus and cell banks with a specified maximum. This  
452 approach aims to prevent possible and unwanted drift of properties of seed virus and cells that might arise from  
453 repeated passaging.

#### 454 **3.1.1. Characteristics of the seed virus**

455 Each seed strain of capripoxvirus used for vaccine production must be accompanied by records clearly  
456 and accurately describing its origin, isolation and tissue culture or animal passage history. Preferably,  
457 the species and strain of capripoxvirus are characterised using PCR or DNA sequencing techniques.

458 A quantity of master seed vaccine virus should be prepared, frozen or desiccated and stored at low  
459 temperatures such as -80°C and used to produce a consistent working seed for regular vaccine  
460 production.

461 Each master seed strain must be non-transmissible, remain attenuated after further tissue culture  
462 passage, and provide complete protection against challenge with virulent field strains for a minimum of  
463 1 year. It must produce a minimal clinical reaction in cattle when given via the recommended route.

464 The necessary safety and potency tests are described in Section C.2.2.4 *Final product batch tests*.

#### 465 **2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)**

466 Each master seed must be tested to ensure its identity and shown to be free from adventitious viruses,  
467 in particular pestiviruses, such as border disease and bovine viral diarrhoea virus, and free from  
468 contamination with bacteria, fungi or mycoplasmas.

469 The general procedures for sterility or purity tests are described in Chapter 1.1.9 *Tests for sterility and*  
470 *freedom from contamination of biological materials intended for veterinary use*.

471 Master seed virus is a quantity of virus of uniform composition derived from an original isolate, passaged  
472 for a documented number of times and distributed into containers at one time and stored adequately to  
473 ensure stability (via freezing or lyophilisation). Selection of master seed viruses (MSVs) should ideally  
474 be based on their ease of growth in cell culture, virus yield, and in accordance with the regional  
475 epidemiological importance. Also, measures to minimise transmissible spongiform encephalopathies  
476 (TSE) contamination should be taken into account (see Section C.3.5.1 *Purity tests*).

477 For each seed strain selected for LSD vaccine production, the following information should be provided:

478 - Historical record: geographical origin, animal species from which the virus was recovered, isolation  
479 procedure, tissue culture or animal passage history

480 - Identity: species and strain identification using DNA sequencing

- 
- 481 - Purity: the absence of bacteria, fungi, mycoplasma, and other viruses (see Chapter 1.1.9 Tests for
  - 482 sterility and freedom from contamination of biological materials intended for veterinary use)
  - 483 - Safety (overdose, one/repeated dose tests, and reversion to virulence tests) (see Section C.3.3
  - 484 Vaccine safety)
  - 485 - Efficacy data, linked to a specified (protective) dose (see Section C.3.4 Vaccine efficacy)
  - 486 - Stability

487 Each master seed strain selected for production of live attenuated LSD vaccines must remain attenuated

488 after further passage in animals (see Section C.3.3. Vaccine safety), produce minimal clinical reaction

489 when given via the recommended route, provide complete protection against challenge with virulent field

490 strains, and is ideally not transmissible.

491 A quantity of master seed virus should be prepared and stored to be further used for the preparation of

492 working seeds and production seeds. Working seed viruses may be expanded in one or more (but,

493 limited) cell culture passages from the master seed stock and used to produce vaccine batches. This

494 approach and limitation of seed virus passaging will assist in maintaining uniformity and consistency in

495 production.

### 496 **3.1.2. Master cell stocks**

497 The production process of LSD vaccines ideally employs an established master cell stock (MCS) system

498 with defined lowest and highest cell passage to be used to grow the vaccine virus. Primary cells derived

499 from normal tissues can be used in the production process, but the use of primary cells has an inherently

500 higher risk of introducing extraneous agents compared with the use of established (well characterised)

501 cell lines and should be avoided where alternative methods of producing effective vaccines exist. For

502 each MCS, manufacturers should demonstrate:

- 503 - MCS identity
- 504 - genetic stability by subculturing from the lowest to the highest passage used for production
- 505 - stable MCS karyotype with a low level of polyploidy
- 506 - freedom from oncogenicity or tumorigenicity by using *in-vivo* studies using the highest cell passage
- 507 that may be used for production
- 508 - purity of MCSs from extraneous bacteria, fungi, mycoplasma, and viruses
- 509 - implemented measures to lower TSE contamination risk (see Section C.3.5.1 Purity tests).

## 510 **3.2. Method of vaccine manufacturing**

511 The method of manufacture should be documented as the Outline of Production.

### 512 **2.2.1. Procedure**

#### 513 **3.2.1. LSD vaccine batch production**

514 Vaccine batches are produced on an appropriate cell line such as MDBK. As already mentioned in the

515 first paragraphs of Section C, all steps undertaken in the production of vaccine batches should be

516 described and documented in the Outline of Production. The production of LAV and IV against LSD starts

517 with the inoculation of the required number of working vials of seed virus is reconstituted with GMEM or

518 ether-in appropriate medium and inoculated onto a suitable primary or continuous cell line grown in

519 suspension or monolayer. Cells should be harvested after 4–8 days when they exhibit 50–70% CPE for

520 maximum in the exponential growth phase. At the time highest viral infectivity, or earlier if CPE is

521 extensive and cells appear ready to detach. Techniques such as loads are present, sonication or

522 repeated freeze–thawing are used to release the intracellular virus from the cytoplasm. The lysate may

523 then be clarified using centrifugation to remove cellular debris (for example by use of centrifugation at

524 600 g for 20 minutes, with retention of the supernatant). A second passage of the virus may be required

525 to produce sufficient virus for a production batch.

526 An aliquot of the virus suspension is titrated to check the virus titre. For LAV, the virus-containing

527 suspension is diluted to attain the dose at which the vaccine candidate will be evaluated or to at least

528 the determined protective dose for approved vaccines and is then mixed with a suitable protectant such

529 as an equal volume of sterile, chilled 5% lactalbumin hydrolysate and 10% sucrose (dissolved in double-

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530 distilled water or appropriate balanced salt solution), and transferred to individually numbered labelled  
531 bottles or bags for storage at low temperatures such as  $-80^{\circ}\text{C}$ , or for freeze-drying. A written record of  
532 all the procedures followed must be kept for all vaccine batches.

### 533 **2.2.2. Requirements for substrates and media**

534 ~~The specification and source of all ingredients used in the manufacturing procedure should be~~  
535 ~~documented and the freedom of extraneous agents (bacteria, fungi, mycoplasma and viruses) should be~~  
536 ~~tested. The detailed testing procedure is described in Chapter 1.1.9. The use of antibiotics must meet~~  
537 ~~the requirements of the licensing authority.~~

### 538 **2.2.3. In-process control**

#### 539 i) ~~Cells~~

540 ~~Records of the source of the master cell stocks should be maintained. The highest and lowest~~  
541 ~~passage numbers of the cells that can be used for vaccine production must be indicated in the~~  
542 ~~Outline of the Production. The use of a continuous cell line (such as MDBK, etc.) is strongly~~  
543 ~~recommended, unless the virus strain only grows on primary cells. The key advantage of continuous~~  
544 ~~over primary cell lines is that there is less risk of introduction of extraneous agents.~~

#### 545 ii) ~~Serum~~

546 ~~Serum used in the growth or maintenance medium must be free from antibodies to capripoxvirus~~  
547 ~~and free from contamination with pestivirus or other viruses, extraneous bacteria, mycoplasma or~~  
548 ~~fungi.~~

#### 549 iii) ~~Medium~~

550 ~~Media must be sterile before use.~~

#### 551 iv) ~~Virus~~

552 ~~Seed virus and final vaccine must be titrated and pass the minimum release titre set by the~~  
553 ~~manufacturer. For example, the minimum recommended field dose of the South African Neethling~~  
554 ~~strain vaccines (Mathijs *et al.*, 2016) is  $\log_{10} 3.5 \text{ TCID}_{50}$ , although the minimum protective dose is~~  
555  ~~$\log_{10} 2.0 \text{ TCID}_{50}$ . Capripoxvirus is highly susceptible to inactivation by sunlight and allowance~~  
556 ~~should be made for loss of activity in the field.~~

557 ~~The recommended field dose of the Romanian sheep pox vaccine for cattle is  $\log_{10} 2.5$  sheep~~  
558 ~~infective doses ( $\text{SID}_{50}$ ), and the recommended dose for cattle of the RM65 adapted strain of~~  
559 ~~Romanian sheep pox vaccine is  $\log_{10} 3 \text{ TCID}_{50}$  (Coakley & Capstick, 1964).~~

### 560 **3.2.2. Inactivation process for inactivated LSD vaccines**

561 Unlike LAV, inactivated LSD vaccines contain inactivated antigens in combination with adjuvants to  
562 strengthen the induced immune response after administration. The vaccine evaluation process described  
563 below needs to show the amount of antigen necessary to elicit a protective immune response. Currently,  
564 literature data indicate that an inactivated vaccine originating from an LSDV virus stock with titre  $10^4$  cell  
565 culture infectious dose<sub>50</sub> ( $\text{CCID}_{50}$ )/ml before inactivation can be sufficient to induce an efficient immune  
566 response to prevent clinical disease, viremia and virus shedding after challenge of young cattle (Wolf *et*  
567 *al.*, 2022).

568 To monitor the inactivation process and the level of antigen inactivation, samples are taken at regular  
569 intervals during inactivation and titrated. Inactivation conditions and the length of initial and repeated  
570 exposure should be documented in detail since one or more factors during the process could influence  
571 the outcomes. The inactivation kinetics should reach a predefined target e.g. one remaining infectious  
572 unit per million doses ( $1 \times 10^{-6}$  infectious units/dose) as suggested by APHIS (2013). The confirmatory  
573 testing of inactivation is performed on each vaccine lot and represents an important part of the  
574 inactivation process monitoring. In addition to all the procedures mentioned above, the inactivation  
575 procedure and tests demonstrating that antigen inactivation is complete and consistent must additionally  
576 be documented in the Outline of Production.

577

### **3.3. Vaccine safety**

578 During the vaccine development process, vaccine safety must be evaluated in the target animal (target animal  
579 batch safety test –TABST) to demonstrate the safety of the dose intended for registration. The animals used in  
580 the safety testing should be representative (species, age and category [calves, heifers, bulls, cows,]) for all the  
581 animals for which the vaccine is intended. Vaccinated and control groups are appropriately acclimatised, housed  
582 and managed in line with animal welfare standards. Animal suffering has to be eliminated or reduced and  
583 euthanasia is recommended in moribund animals.

584 Essential parameters to be evaluated in safety studies are local and systemic reactions to vaccination, including  
585 local reactions at the site of administration, fever, effect on milk production, and induction of a ‘Neethling’  
586 response. The effect of the vaccine on reproduction needs to be evaluated where applicable.

587 A part of the safety evaluation of LAV and IV can be performed during the efficacy trials (see Section C.3.4  
588 Vaccine efficacy) by measuring local and systemic responses following vaccination and before challenge.

589 Guidelines for safety evaluation are provided by the European Medicine Agency (EMA) in VICH GL44: TABST  
590 for LAV and IV (EMA, 2009). Safety aspects of LAV and IV against LSD to be evaluated are:

#### **3.3.1. Overdose test for LAV**

591 Local and systemic responses should be measured following an overdose test whereby 10× the  
592 maximum vaccine titre is administered. If the maximum vaccine titre is not specified, 10× the minimum  
593 vaccine titre can be applied in multiple injection sites. Ideally, the 10× dose is dissolved in the 1× dose  
594 volume of the adjuvants or diluent. Generally, eight animals per group should be used (EMA, 2009).  
595

#### **3.3.2. One dose and repeat dose test**

596 This aims to test the safety of the vaccine dose applied in the vaccination regime intended for registration.  
597 LAV LSD vaccines require one dose per year, while inactivated LSD vaccines require a booster dose in  
598 addition to the primary dose. The minimal recommended interval between administrations is 14 days.  
599

600 Generally, eight animals per group should be used unless otherwise justified (EMA, 2009). For each  
601 target species, the most sensitive breed, age and sex proposed on the label should be used.  
602 Seronegative animals should be used. In cases where seronegative animals are not reasonably  
603 available, alternatives should be justified.

#### **3.3.3. Reversion to virulence tests**

604 Live attenuated vaccines inherently carry the risk of vaccine virus reverting to virulence when repeated  
605 passages in a host species could occur due to shedding and transmission from vaccinated animals to  
606 contact animals. LAV LSD vaccines should therefore be tested for non-reversion to virulence by means  
607 of passage studies. Vaccine virus (MSV, not the finished vaccine) is inoculated in a group of target  
608 animals of susceptible age via the natural route of infection or the route that is most likely to result in  
609 infection. The vaccine virus is subsequently recovered from tissues or excretions and is used directly to  
610 inoculate a further group of animals. After not less than four passages (see chapter 1.1.8), i.e. use of a  
611 total of five groups of animals, the re-isolate must be fully characterised, using the same procedures  
612 used to characterise the master seed virus.  
613

#### **3.3.4. Environmental consideration**

614 This includes the evaluation of the ability of LAV LSD vaccines to be shed, to spread and to infect contact  
615 target and non-target animals, and to persist in the environment.  
616

#### **2.2.4. Final product batch tests**

617 **i) — Sterility/purity**

618 Vaccine samples must be tested for sterility/purity. Tests for sterility and freedom from  
619 contamination of biological materials intended for veterinary use may be found in Chapter 1.1.9.  
620

621 **ii) — Safety and efficacy**

622 The efficacy and safety studies should be demonstrated using statistically valid vaccination-  
623 challenge studies using seronegative young LSDV susceptible dairy cattle breeds. The group  
624 numbers recommended here can be varied if statistically justified. Fifteen cattle are placed in a high

625 containment level large animal unit and serum samples are collected. Five randomly chosen vials  
626 of the freeze dried vaccine are reconstituted in sterile PBS and pooled. Two cattle are inoculated  
627 with 10 times the recommended field dose of the vaccine, and eight cattle are inoculated with the  
628 recommended field dose. The remaining five cattle are unvaccinated control animals. The animals  
629 are clinically examined daily and rectal temperatures are recorded. On day 21 after vaccination, the  
630 animals are again serum sampled and challenged with a known virulent capripoxvirus strain. The  
631 challenge virus solution should also be tested free from extraneous viruses. The clinical response  
632 is recorded during the following 14 days. Animals in the unvaccinated control group should develop  
633 the typical clinical signs of LSD, whereas there should be no local or systemic reaction in the  
634 vaccinates other than a raised area in the skin at the site of vaccination, which should disappear  
635 after 4 days. Serum samples are again collected on day 30 after vaccination. The day 21 serum  
636 samples are examined for seroconversion to selected viral diseases that could have contaminated  
637 the vaccine, and the days 0 and 30 samples are compared to confirm the absence of antibody to  
638 pestivirus. Because of the variable response in cattle to LSD challenge, generalised disease may  
639 not be seen in all of the unvaccinated control animals, although there should be a large local  
640 reaction.

641 Once the efficacy of the particular strain being used for vaccine production has been determined in  
642 terms of minimum dose required to provide immunity, it is not necessary to repeat this on the final  
643 product of each batch, provided the titre of virus present has been ascertained.

644 iii) Batch potency

645 Potency tests in cattle must be undertaken for vaccine strains of capripoxvirus if the minimum  
646 immunising dose is not known. This is usually carried out by comparing the titre of a virulent  
647 challenge virus on the flanks of vaccinated and control animals. Following vaccination, the flanks  
648 of at least three animals and three controls are shaved of hair. Log<sub>10</sub> dilutions of the challenge virus  
649 are prepared in sterile PBS and six dilutions are inoculated intradermally (0.1 ml per inoculum)  
650 along the length of the flank; four replicates of each dilution are inoculated down the flank. An  
651 oedematous swelling will develop at possibly all 24 inoculation sites on the control animals,  
652 although preferably there will be little or no reaction at the four sites of the most dilute inocula. The  
653 vaccinated animals may develop an initial hypersensitivity reaction at sites of inoculation within 24  
654 hours, which should quickly subside. Small areas of necrosis may develop at the inoculation site of  
655 the most concentrated challenge virus. The titre of the challenge virus is calculated for the  
656 vaccinated and control animals; a difference in titre >log<sub>10</sub> 2.5 is taken as evidence of protection.

657 **3.4. Vaccine efficacy**

658 Data enclosed in the vaccine candidate dossier should support the efficacy of the vaccine in each animal species  
659 for each vaccination regimen that is described in the product label recommendation. This includes studies  
660 regarding the onset of protection when claims for onset are made and for the duration of immunity. Efficacy  
661 studies should be conducted with the vaccine candidate that has been produced at the highest passage level  
662 permitted for vaccine production as specified in the Outline of Production.

663 Efficacy (and safety) should be demonstrated in vaccination–challenge studies using representative (by species,  
664 age and category) seronegative healthy animals for which the vaccine is intended and which are tested negative  
665 for standard viral pathogens.

666 An example of a vaccination–challenge test set-up is outlined here. The group numbers mentioned can be varied  
667 if statistically justified. Thirteen animals are placed in a high containment large animal unit and are divided into  
668 two groups:

669 - single/repeated dose test group (n=8) – animals inoculated with the vaccine dose and route intended for  
670 registration (in case of an IV against LSD, a booster dose should follow primary vaccination after minimum  
671 14 days).

672 - control group (n=5) – non-vaccinated animals

673 Throughout the *in-vivo* study, all animals are clinically examined and rectal temperatures recorded. Blood, serum  
674 and swab samples are regularly collected and subjected to laboratory testing. On day 21 after the vaccination  
675 with a LAV or after the booster vaccination for an IV, the animals in both groups are challenged with a known  
676 virulent LSDV strain. The challenge virus solution should be of known titre and tested free from extraneous  
677 viruses. Experience obtained from previous animal experiments indicates that a dose of challenge virus between  
678 10<sup>4.0</sup> and 10<sup>6.5</sup> TCID<sub>50</sub> produces clinical disease in about half of the susceptible experimental cattle (Tuppurainen  
679 *et al.*, 2021).

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680 The clinical response following challenge is recorded over a period of 14 days. No clinical signs should occur in  
681 the vaccinates, other than a local reaction at the site of inoculation. At least 1 animal in the unvaccinated control  
682 group should develop the typical clinical signs of LSD. Although a generalised disease with skin nodules may  
683 not be seen in all the unvaccinated control animals based on the knowledge that the outcome of a LSDV infection  
684 can range from inapparent to severe, at the very least a large local reaction is to be expected.

685 Clinical and laboratory results will enable assessment of the safety and efficacy of the LSD vaccine candidate  
686 and the induced immune responses. Serum samples collected at different time points during the trial can be  
687 examined to study seroconversion against selected viral diseases that could have contaminated the vaccine.

## 688 **2.3. Requirements for regulatory approval**

### 689 **2.3.1. Safety requirements**

#### 690 i) ~~Target and non-target animal safety~~

691 ~~The vaccine must be safe to use in all breeds of cattle for which it is intended, including young and~~  
692 ~~pregnant animals. It must also be non-transmissible and remain attenuated after further tissue~~  
693 ~~culture passage.~~

694 ~~Safety tests should be carried out on the final product of each batch as described in Section C.2.2.4.~~

#### 695 ii) ~~Reversion to virulence for attenuated/live vaccines~~

696 ~~The selected final vaccine should not revert to virulence during further passages in target animals.~~

#### 697 iii) ~~Environmental consideration~~

698 ~~Attenuated vaccine should not be able to perpetuate autonomously in a cattle population. Strains~~  
699 ~~of LSDV are not a hazard to human health.~~

### 700 **2.3.2. Efficacy requirements**

#### 701 i) ~~For animal production~~

702 ~~The efficacy of the vaccine must be demonstrated in statistically valid vaccination challenge~~  
703 ~~experiments under laboratory conditions. The group numbers recommended here can be varied if~~  
704 ~~statistically justified. Fifteen cattle are placed in a high containment level large animal unit and~~  
705 ~~serum samples are collected. Five randomly chosen vials of the freeze dried vaccine are~~  
706 ~~reconstituted in sterile PBS and pooled. Two cattle are inoculated with 10 times the field dose of~~  
707 ~~the vaccine, eight cattle are inoculated with the recommended field dose. The remaining five cattle~~  
708 ~~are unvaccinated control animals. The animals are clinically examined daily and rectal temperatures~~  
709 ~~are recorded. On day 21 after vaccination, the animals are again serum sampled and challenged~~  
710 ~~with a known virulent capripoxvirus strain using intravenous and intradermal inoculation (the~~  
711 ~~challenge virus solution should also be tested and shown to be free from extraneous viruses). The~~  
712 ~~clinical response is recorded during the following 14 days. Animals in the unvaccinated control~~  
713 ~~group should develop the typical clinical signs of LSD, whereas there should be no local or systemic~~  
714 ~~reaction in the vaccinates other than a raised area in the skin at the site of vaccination which should~~  
715 ~~disappear after 4 days. Serum samples are again collected on day 30 after vaccination. The day~~  
716 ~~21 serum samples are examined for seroconversion to selected viral diseases that could have~~  
717 ~~contaminated the vaccine, and the days 0 and 30 samples are compared to confirm the absence~~  
718 ~~of antibody to pestivirus. Because of the variable response in cattle to challenge with LSDV,~~  
719 ~~generalised disease may not be seen in all of the unvaccinated control animals, although there~~  
720 ~~should be a large local reaction.~~

721 ~~Once the potency of the particular strain being used for vaccine production has been determined~~  
722 ~~in terms of minimum dose required to provide immunity, it is not necessary to repeat this on the~~  
723 ~~final product of each batch, provided the titre of virus present has been ascertained.~~

#### 724 ii) ~~For control and eradication~~

725 ~~Vaccination is the only effective way to control LSD outbreaks in endemic countries and recent~~  
726 ~~experiences of the disease in Eastern Europe and the Balkans suggests this is also true for~~  
727 ~~outbreaks in non-endemic countries. Unfortunately, currently no marker vaccines allowing a DIVA~~  
728 ~~strategy are available, although to a limited extent PCR can be used for certain vaccines.~~



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729 The duration of immunity produced by LSDV vaccine strains is currently unknown.

730 **2.3.3. Stability**

731 All vaccines are initially given a shelf life of 24 months before expiry. Real-time stability studies are then  
732 conducted to confirm the appropriateness of the expiry date. Multiple batches of the vaccine should be  
733 re-titrated periodically throughout the shelf-life period to determine the vaccine stability.

734 Properly freeze-dried preparations of LSDV vaccine, particularly those that include a protectant, such as  
735 sucrose and lactalbumin hydrolysate, are stable for over 25 years when stored at  
736 -20°C and for 2–4 years when stored at 4°C. There is evidence that they are stable at higher  
737 temperatures, but no long-term controlled experiments have been reported. No preservatives other than  
738 a protectant, such as sucrose and lactalbumin hydrolysate, are required for the freeze-dried preparation.

739 **3.5. Batch/serial tests before release for distribution**

740 Quality tests on MSV and safety and efficacy tests on vaccine candidates are performed during the evaluation  
741 process for new LSD vaccines. Once vaccines are approved to be used in the field, it remains important to verify  
742 the quality of each vaccine batch produced. An independent batch quality control assessment may be warranted  
743 or requested by national or international regulatory authorities.

744 **3.5.1. Purity test**

745 Purity is defined by the absence of different contaminants (bacteria, fungi, mycoplasma, and other  
746 viruses; see full details in chapter 1.1.9) in the vaccine and its associated diluent/adjuvants. Virus  
747 isolation and bacterial culture tests can be used to show freedom from live competent replicating  
748 microorganisms, but molecular methods are more rapid and sensitive, but positives can be caused by  
749 genome fragments and incompetent replicating microorganisms.

750 Besides the contaminants mentioned above, manufacturers should demonstrate implemented measures  
751 to minimise the risk of TSE contamination in ingredients of animal origin such as:

- 752 - all ingredients of animal origin in production facilities are from countries recognised as having the  
753 lowest possible risk of bovine spongiform encephalopathy
- 754 - tissues or other substances used are themselves recognised as being of low or nil risk of containing  
755 TSE agents

756 **3.5.2. Identity tests**

757 In addition to identity tests performed on the MSV, the identity tests on final batches aim to demonstrate  
758 the presence of only the selected capripoxvirus species and strain in the vaccine as indicated in the  
759 Outline of Production and the absence of other strains or members of the genus and any other viral  
760 contaminant that might arise during the production process. Identity testing could be assured by using  
761 appropriate tests (e.g. PCRs, sanger sequencing, NGS).

762 **3.5.3. Potency tests**

763 Standard requirements for potency tests can be found in CFR Title 9 part 113, in the European  
764 Pharmacopoeia, and in this Terrestrial Manual.

765 **3.5.3.1. Live vaccines**

766 The potency of LAV against LSD can be measured by means of virus titration. The virus titre must,  
767 as a rule, be sufficiently greater than that shown to be protective in the efficacy test for the vaccine  
768 candidate. This will ensure that at any time prior to the expiry date, the titre will be at least equal to  
769 the evaluated protective titre. The titres of currently available commercial homologous LSD  
770 vaccines range between 10<sup>3</sup> and 10<sup>4</sup> infectious units/dose (Tuppurainen *et al.*, 2021).

771 **3.5.3.2. Inactivated LSD vaccines**

772 For inactivated LSD vaccines, potency tests are performed using vaccination–challenge efficacy  
773 studies in animal hosts (see Section C.3.4. Vaccine efficacy).

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### **3.5.4. Safety/efficacy**

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Safety and efficacy testing is undertaken during the evaluation process of the vaccine candidate, and also needs to be performed on a number of vaccine batches until robust data are generated in line with international and national regulations. Afterwards, when using a seed lot system in combination with strict implementation of GMP standards and depending on local regulations, TABST could be waived as described in VICH50 and VICH55, providing the titer has been ascertained using potency testing. Batches or serials are considered satisfactory if local and systemic reactions to vaccination are in line with those described in the dossier of the vaccine candidate and product literature.

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#### **3.5.4.1. Field safety/efficacy tests**

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Field testing of two or more batches should be performed on all animal categories for which the product is indicated before release of the product for general use (see chapter 1.1.8). The aim of these studies is to demonstrate the safety and efficacy of the product under normal field conditions of animal care and use in different geographical locations where different factors may influence product performance. A protocol for safety/efficacy testing in the field has to be developed with defined observation and recording procedures. However, it is generally more difficult to obtain statistically significant data to demonstrate efficacy under field conditions. Even when properly designed, field efficacy studies may be inconclusive due to uncontrollable outside influences.

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#### **3.5.4.2. Duration of Immunity**

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The duration of immunity (DOI) following vaccination should be demonstrated via challenge or the use of a validated serology test. Efficacy testing at the end of the claimed period of protection should be conducted in each species for which the vaccine is indicated or the manufacturer should indicate that the DOI for that species is not known. Likewise, the manufacturer should demonstrate the effectiveness of the recommended booster regime in line with these guidelines, usually by measuring the magnitude and kinetics of the serological response observed.

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### **3. Vaccines based on biotechnology**

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~~A new generation of capripox vaccines is being developed that uses the LSDV as a vector for the expression and delivery of immuno-protective proteins of other ruminant pathogens with the potential for providing dual protection (Boshra *et al.*, 2013; Wallace & Viljoen, 2005), as well as targeting putative immunomodulatory genes for inducing improved immune responses (Kara *et al.*, 2018).~~

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## **4. Post-market studies**

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### **4.1. Stability**

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Stability testing shall be carried out as specified in Annex II of Regulation (EU) 2019/6 and in the Ph. Eur. 0062: Vaccines for veterinary use, on not fewer than three representative batches providing this mimics the full-scale production described in the application. At the end of shelf-life, sterility has to be re-evaluated using sterility testing or by showing container closure integrity. Multiple batches of the vaccine should be re-titrated periodically throughout the shelf-life period to determine the vaccine stability.

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### **4.2. Post-marketing surveillance**

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After release of a vaccine, its performance under field conditions should continue to be monitored by competent authorities and by the manufacturer itself. Not all listed adverse effects may show up in the clinical trials performed to assess safety and efficacy of the vaccine candidate due to the limited number of animals used. Post-marketing surveillance studies can also provide information on vaccine efficacy when used in normal practice and husbandry conditions, on duration of induced immunity, on ecotoxicity, etc.

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First, a reliable reporting system should be in place to collect consumer complaints and notifications of adverse reactions. Secondly, post-marketing surveillance should be established to investigate whether the reported observations are related to the use of the product and to identify, at the earliest stage, any serious problem that may be encountered from its use and that may affect its future uptake. Vaccinovigilance should be an on-going and integral part of all regulatory programmes for LSD vaccines, especially for live vaccines.

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972 **NB:** There are WOAHP Reference Laboratories for lumpy skin disease (please consult the WOAHP Web site:  
973 <https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3>).  
974 Please contact WOAHP Reference Laboratories for any further information on  
975 diagnostic tests, reagents and vaccines for lumpy skin disease

976 **NB:** FIRST ADOPTED IN 1989. MOST RECENT UPDATES ADOPTED IN 2021.

REUNIÓN DE LA COMISIÓN DE NORMAS BIOLÓGICAS

París, 5–9 de febrero de 2024

CHAPTER 3.6.9.

EQUINE RHINOPNEUMONITIS (INFECTION WITH  
VARICELLOVIRUS EQUIDALPHA1 EQUID  
HERPESVIRUS 1 AND 4)

SUMMARY

Equine rhinopneumonitis (ER) is a collective term for any one of several contagious, clinical disease entities of equids that may occur as a result of infection by either of two closely related herpesviruses, formally known as equid alpha herpesvirus-1 and -4 (EHV-1 and EHV-4). Infection with EHV-1 is listed by WOA and is therefore the focus of this chapter. The classification of the virus has been reviewed and EHV-1 is now known as Varicellovirus equidalpha1. For the purposes of the chapter, the acronym EHV-1 will continue to be used. EHV-1 is and EHV-4 are endemic in most domestic equine populations worldwide.

Primary infection by ~~either EHV-1 or EHV-4~~ is characterised by upper respiratory tract disease of varying severity that is related to the age and immunological status of the infected animal. Following viraemia EHV-1 may also cause the more serious complications of abortion, perinatal foal death, or paralytic neurological disease (equine herpesvirus myeloencephalopathy). ~~EHV-4 has been associated with sporadic cases of abortion, but rarely multiple abortions and not the large outbreaks associated with EHV-1. Like other herpesviruses, EHV-1 and 4 induces~~ long-lasting latent infections and can be reactivated following stress ~~or pregnancy~~. Furthermore, most horses are likely to be re-infected multiple times during their lifetime, often mildly or subclinically. ~~Detection of viral DNA or anti-EHV antibodies should therefore be interpreted with care.~~

**Identification of the agent:** The standard method of identification of EHV-1 ~~and EHV-4~~ from appropriate clinical or necropsy material is by polymerase chain reaction (PCR), ~~followed by laboratory isolation of the virus in cell culture.~~

Positive identification of viral isolates as EHV-1 ~~or EHV-4~~ can be achieved by type-specific PCR or sequencing. Viruses can be isolated in ~~equine~~ cell culture from the following sample types: nasal or nasopharyngeal swab extracts taken from horses ~~during the febrile stage of~~ with acute respiratory tract infection, ~~from the placenta, from and~~ liver, lung, spleen, adrenal glands or thymus of aborted fetuses and early foal deaths, and from the leukocyte fraction of the blood of animals ~~with acute~~ during the febrile stage of EHV-1 infection. Unlike EHV-4, EHV-1 will also grow in various non-equine cell types such as the RK-13 cell line and this property can be used to distinguish between the two viruses.

A rapid presumptive diagnosis of abortion induced by EHV-1 ~~or (infrequently) EHV-4~~ can be achieved by direct immunofluorescent detection of viral antigen in cryostat sections of placenta and tissues from aborted fetuses, using a conjugated polyclonal antiserum.

Post-mortem demonstration of histopathological lesions of EHV-1 in placenta and tissues from aborted fetuses, cases of perinatal foal death, or in the central nervous system of neurologically affected animals complements other diagnostic techniques ~~the laboratory diagnosis~~.

37 **Serological tests:** As most horses possess some level of antibody to EHV-1/4, the demonstration of specific  
38 antibody in the serum collected from a single blood sample is therefore not confirmation of a positive  
39 diagnosis of recent infection. Paired, (acute and convalescent) sera from animals suspected of being  
40 infected with EHV-1 or EHV-4 should be tested for a four-fold or greater rise in virus-specific antibody titre  
41 by either virus neutralisation (VN) or complement fixation (CF) tests. Neither of these assays is type-specific  
42 but both have proven useful for diagnostic purposes especially since the CF antibody response to recent  
43 infection is relatively short-lived. Limited use has also been made of a type-specific enzyme-linked  
44 immunosorbent assay (Crabb et al., 1995; Hartley et al., 2005).

45 **Requirements for vaccines:** Both live attenuated and inactivated viral vaccines are available for use in  
46 assisting in the control of EHV-1/4. Vaccination is helpful in reducing the severity of respiratory infection in  
47 young horses and the incidence of abortion in mares, however current vaccines are not licenced to protect  
48 against neurological disease. Vaccination should not be considered a substitute for sound management  
49 practices known to reduce the risk of infection. Revaccination at frequent intervals is recommended in the  
50 case of each of the products, as the duration of vaccine-induced immunity is relatively short.

51 Standards for production and licensing of both attenuated and inactivated EHV-1/4-vaccines are established  
52 by appropriate veterinary regulatory agencies in the countries of vaccine manufacture and use. A single set  
53 of internationally recognised standards for EHV vaccines is not available. In each case, however, vaccine  
54 production is based on the system of a detailed outline of production employing a well characterised cell line  
55 and a master seed lot of vaccine virus that has been validated with respect to virus identity, safety, virological  
56 purity, immunogenicity and the absence of extraneous microbial agents.

## 57 A. INTRODUCTION

58 Equine rhinopneumonitis (ER) is a historically derived term that describes a constellation of several disease entities of  
59 horses that may include respiratory disease, abortion, neonatal foal pneumonitis, or myeloencephalopathy (Allen & Bryans,  
60 1986; Allen et al., 1999; Bryans & Allen, 1988; Crabb & Studdert, 1995). The disease has been recognised for over 60  
61 years as a threat to the international horse industry, and is caused by either of two members of the *Herpesviridae* family,  
62 formerly known as equid alphaherpesvirus-1 and -4 (EHV-1 and EHV-4). The viruses are now classified as *Varicellovirus*  
63 *equidalpha1* and *Varicellovirus equidalpha4*. For the purposes of the chapter, the acronyms EHV-1 and EHV-4 will continue  
64 to be used. EHV-1 and EHV-4 are closely related alphaherpesviruses of horses with nucleotide sequence identity within  
65 individual homologous genes ranging from 55% to 84%, and amino acid sequence identity from 55% to 96% (Telford et  
66 al., 1992; 1998). The two herpesviruses ~~With the exception of EHV-1 in Iceland (Thorsteinsdóttir et al., 2021), the two~~  
67 herpesviruses are considered endemic enzootic in all countries in which large populations of horses are maintained as  
68 part of the cultural tradition or agricultural economy. There is no recorded evidence that the two herpesviruses of ER pose  
69 any health risks to humans working with the agents. Infection with EHV-1 is listed by WOA and is therefore the focus of  
70 this chapter.

71 Viral transmission to cohort animals occurs by inhalation of aerosols of virus-laden respiratory secretions. Morbidity tends  
72 to be highest in young horses sharing the same air space. Aborted tissues and placental fluids from infected mares can  
73 contain extremely high levels of live virus and represent a major source of infection. Extensive use of vaccines has not  
74 eliminated EHV-1 infections, and the world-wide annual financial impact from this ~~these~~ equine pathogens is ~~immense~~  
75 considerable.

76 In horses under 3 years of age, clinical ER usually takes the form of an acute, febrile respiratory illness that spreads rapidly  
77 through the group of animals. The viruses infects and multiplies multiply in epithelial cells of the respiratory mucosa. Signs  
78 of infection become apparent 2–8 days after exposure to virus, and are characterised by fever, inappetence, depression,  
79 and nasal discharge. The severity of respiratory disease varies with the age of the horse and the level of immunity resulting  
80 from previous vaccination or natural exposure. Bi-phasic fever, viraemia and complications are more likely with EHV-1 than  
81 EHV-4. Subclinical infections with EHV-1/4 are common, even in young animals. Although mortality from uncomplicated  
82 ER is rare and complete recovery within 1–2 weeks is the normal outcome, respiratory infection is a frequent and significant  
83 cause of interrupted schedules among horses assembled for training, racing, or other equestrian events. Fully protective  
84 immunity resulting from infection is of short duration, and convalescent animals are susceptible to reinfection by EHV-1/4  
85 after several months. Although reinfections by the two herpesviruses cause less severe or clinically inapparent respiratory  
86 disease, the risks of subsequent abortion or neurological disease remain. Like other herpesviruses, EHV-1/4 causes long-  
87 lasting latent infections and latently infected horses represent a potential infection risk for other horses. Virus can be  
88 reactivated as a result of stress or pregnancy. The greatest clinical threats to individual breeding, racing, or pleasure horse  
89 operations posed by ER are the potential abortigenic and neurological sequelae of EHV-1 respiratory infection. ER  
90 abortions occur annually in horse populations worldwide and may be sporadic or multiple. Foals infected *in utero* may be  
91 born alive and die within a few days of birth. EHV-1 neurological disease is less common than abortions but has been  
92 recorded all over the world with associated fatalities. Outbreaks result in movement restrictions and, sometimes,  
93 cancellation of equestrian events (Couroucé et al., 2023; FEI, 2021).



94 Neurological disease, also known as equine herpesvirus myeloencephalopathy, remains an infrequent but serious  
 95 complication of EHV-1 infection. A single mutation in the DNA polymerase gene (ORF30) has been associated with  
 96 increased risk of neurological disease, however strains without this change can also cause paralysis (Goodman *et al.*,  
 97 2007; Nugent *et al.*, 2006). Strain typing techniques have been employed to identify viruses carrying the neuropathic  
 98 marker, and it can be helpful to be aware of an increased risk of neurological complications. However, for practical purposes  
 99 strain typing is not relevant for agent identification, or international trade. Strain typing may be beneficial for implementation  
 100 of biosecurity measures in the management of outbreaks of equine herpesvirus myeloencephalopathy.

101 Strain typing has been shown to be not reliable for predicting the clinical outcome of EHV-1 infection but can be useful in  
 102 epidemiological investigations (Garvey *et al.*, 2019; Nugent *et al.*, 2006; Sutton *et al.*, 2019).

103 EU: Strain typing has been shown to be unreliable not reliable for predicting the clinical outcome of EHV-1 infection but  
 104 can be useful in epidemiological investigations (Garvey *et al.*, 2019; Nugent *et al.*, 2006; Sutton *et al.*, 2019)."

## 105 B. DIAGNOSTIC TECHNIQUES

106 Both EHV-1 and EHV-4 is transmitted by the respiratory route and has have the potential to be highly contagious, viruses  
 107 particularly where large numbers of horses are housed in the same air space. EHV1 and the former can cause explosive  
 108 outbreaks of abortion or neurological disease. Rapid diagnostic methods are therefore essential useful for managing the  
 109 disease. Real-time polymerase chain reaction (PCR) assays are widely routinely used by diagnostic laboratories worldwide  
 110 and are both rapid and sensitive. Real-time PCR assays that allow simultaneous testing for EHV-1 and EHV-4 have been  
 111 developed for both detection of EHV-1 and quantification of viral load have been developed and have replaced virus  
 112 isolation has been replaced by real-time PCR as the frontline diagnostic test in the majority of laboratories, but Virus  
 113 isolation can also still be useful, particularly for the detection of viraemia. This is also true of for in cases of EHV-1-  
 114 associated abortions and neonatal foal deaths, when the high level of virus in the tissues usually produces a cytopathic  
 115 effect in 1–3 days. Immunohistochemical or immunofluorescent approaches are employed in some laboratories can be  
 116 extremely useful for rapid diagnosis of EHV-induced abortion from fresh or embedded tissue and are relatively  
 117 straightforward. Several other techniques based on enzyme-linked immunosorbent assay (ELISA) or nucleic acid  
 118 hybridisation probes have also been described, however their use is often restricted to specialised laboratories and they  
 119 are not included here. Virus neutralisation (VN) and complement fixation test (CFT) are the most frequently used serological  
 120 tests, and seroconversion in paired samples is considered indicative of exposure to virus by natural infection or by  
 121 vaccination.

122 **Table 1. Test methods available for the diagnosis of equine rhinopneumonitis infection with EHV-1 and their**  
 123 **purpose**

Method	Purpose					
	Population freedom from infection <sup>(a)</sup>	Individual animal freedom from infection prior to movement <sup>(b)</sup>	Contribute to eradication policies <sup>(c)</sup>	Confirmation of clinical cases <sup>(d)</sup>	Prevalence of infection - surveillance <sup>(e)</sup>	Immune status in individual animals or populations post-vaccination <sup>(f)</sup>
Identification of the agent <sup>(g)</sup>						
Virus isolation	–	+++	–	++	–	–
PCR	–	+++	–	+++	–	–
Direct immunofluorescence	≡	≡	≡	±±	≡	≡
Detection of immune response						
VN	±±	±±	≡±	++ #	+++	+++
ELISA	+	- ++	≡±	+±	+++±	+±
CFT	–	- ±±	–	+++	–	- ±±±

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Key: +++ = recommended for this purpose; ++ recommended but has limitations;  
+ = suitable in very limited circumstances; – = not appropriate for this purpose.  
PCR = polymerase chain reaction; VN = virus neutralisation;  
ELISA = enzyme-linked immunosorbent assay; CFT = complement fixation test.

<sup>(a)</sup>See Appendix 1 of this chapter for justification table for the scores giving to the tests for this purpose.

<sup>(b)</sup>See Appendix 2 of this chapter for justification table for the scores giving to the tests for this purpose.

<sup>(c)</sup>No eradication policies exist for equine rhinopneumonitis.

<sup>(d)</sup>See Appendix 4 of this chapter for justification table for the scores giving to the tests for this purpose.

<sup>(e)</sup>See Appendix 5 of this chapter for justification table for the scores giving to the tests for this purpose.

<sup>(f)</sup>See Appendix 6 of this chapter for justification table for the scores giving to the tests for this purpose.

<sup>(g)</sup>A combination of agent identification methods applied on the same clinical sample is recommended.

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## 1. Identification Detection of the agent

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### 1.1. Collection and preparation of specimens

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*Nasal/nasopharyngeal swabs:* swab extract can be used for DNA extraction and subsequent virus detection by PCR using one of a variety of published techniques or commercially available kits (see below). Virus isolation can also be attempted from the swab extracts. To increase the chances of isolating live virus, swabs are best obtained from horses during the ~~very early, febrile stages~~ acute stage of the respiratory disease, and are collected via the nares by sampling the area with a swab of an appropriate size and length for horses. After collection, the swab should be removed and transported immediately to the virology laboratory in 3 ml of cold (not frozen) virus transport medium (e.g. phosphate buffered saline [PBS] or serum-free MEM [minimal essential medium] with antibiotics). Virus infectivity can be prolonged by the addition of bovine serum albumin, fetal calf serum or gelatine to 0.1% (w/v).

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*Tissue samples:* total DNA can be extracted using a number of commercially available kits and used in PCR to detect viral DNA (described below in Section B.1.2.1). Virus isolation from placenta and fetal tissues from suspect cases of EHV-1 abortion is most successful when performed on aseptically collected samples of placenta, liver, lung, thymus, adrenal glands and spleen. Virus may be isolated from post-mortem cases of EHV-1 neurological disease by culture of samples of brain and spinal cord but such attempts ~~to isolate virus~~ are often unsuccessful; however, they these samples may be useful for PCR testing and pathological examination. Tissue samples should be transported to the laboratory and held at 4°C until inoculated into tissue culture. Samples that cannot be processed within a few hours should be stored at –70°C.

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*Blood:* for virus detection by PCR or isolation from blood leukocytes, collect a 10–20 ml sample of blood, using an aseptic technique in citrate, heparin or EDTA [ethylene diamine tetra-acetic acid] anticoagulant. EDTA is the preferred anticoagulant for PCR testing in some laboratories as heparin may inhibit DNA polymerase. The samples should be transported without delay to the laboratory on ice, but not frozen.

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Cerebrospinal fluid: the detection of EHV-1 DNA in cerebrospinal fluid has been reported in cases of neurological disease.

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### 1.2. Virus detection by polymerase chain reaction

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PCR has become the primary diagnostic method for the detection of EHV-1 and EHV-4 in clinical specimens, paraffin-embedded archival tissue, or inoculated cell cultures (Borchers & Slater, 1993; Lawrence *et al.*, 1994; O'Keefe *et al.*, 1994; Varrasso *et al.*, 2001). A variety of type specific PCR primers have been designed to distinguish between the presence of EHV-1 and EHV-4. The correlation between PCR and virus isolation techniques for diagnosis of EHV-1 or EHV-4 is high (Varrasso *et al.*, 2001). Diagnosis by PCR is rapid, sensitive, and does not depend on the presence of infectious virus in the clinical sample. For diagnosis of active infection by EHV, PCR methods are routinely used to detect EHV-1 DNA in nasopharyngeal swabs and tissue samples most reliable with tissue samples from aborted fetuses and placental tissue or from nasopharyngeal swabs of foals and yearlings. They are particularly useful in explosive outbreaks of abortion, respiratory or neurological disease, in which a rapid identification and monitoring of the virus spread is critical for guiding management strategies, including movement restrictions. PCR examination of spinal cord and brain tissue, as well as peripheral blood mononuclear cells (PBMC), are important in seeking a diagnosis on a horse with neurological signs (Pronost *et al.*, 2012).

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Several PCR assays have been published. A nested PCR procedure can be used to distinguish between EHV-1 and EHV-4. A sensitive protocol suitable for clinical or pathological specimens (nasal secretions, blood leukocytes, brain and spinal cord, fetal tissues, etc.) has been described by Borchers & Slater (1993). However, nested PCR methods have a high risk of laboratory cross-contamination, and sensitive rapid one-step PCR tests to detect EHV-1 and EHV-4 (e.g. Lawrence *et al.*, 1994) are preferred. The WOAHP Reference Laboratories use quantitative real time PCR assays such as those targeting heterologous sequences of major glycoprotein

180 genes to distinguish between EHV-1 and EHV-4. A multiplex real-time PCR targeting glycoprotein B gene of  
 181 EHV-1 and EHV-4 was described by Diallo *et al.* (2007). PCR protocols have been developed that can  
 182 differentiate between EHV-1 strains carrying the ORF30 neuropathogenic marker, using both restriction enzyme  
 183 digestion of PCR products (Fritsche & Borchers, 2011) or by quantitative real-time PCR (Allen *et al.*, 2007, Smith  
 184 *et al.*, 2012). Methods have also been developed to type strains for epidemiological purposes, based on the  
 185 ORF68 gene (Nugent *et al.*, 2006). The WOA Reference Laboratories employ in-house methods for strain  
 186 typing, however these protocols have not yet been validated between different laboratories at an international  
 187 level.

188 Real-time (or quantitative) PCR has become the method of choice for many the majority of diagnostic tests  
 189 laboratories and provides rapid and sensitive detection of viral DNA. Equine post-mortem tissues from newborn  
 190 and adult animals or equine fetal tissue from abortions (tissues containing lung, liver, spleen, thymus, adrenal  
 191 gland and placental tissues) can be used. For respiratory samples, equine nasopharyngeal swabs or deep nasal  
 192 swabs (submitted in a suitable viral transport medium), buffy coat, tracheal wash (TW) or broncho-alveolar  
 193 lavage (BAL) are all suitable. DNA should be extracted using an appropriate kit or robotic system.

194 There is no internationally standardised real-time PCR method for EHV-1 but Table 2 summarises the primer  
 195 and probe sequences for some of the most widely used assays. Type-specific PCR primers have been designed  
 196 to distinguish between the presence of EHV-1 and EHV-4. The optimised thermocycler times and temperatures  
 197 are documented in the publications cited.

198 **Table 2. Primer and probe sequences for EHV1/4 detection by real-time PCR**

Primer	Primer sequence (5' to 3')	Target	Reference
Forward	CAT-GTC-AAC-GCA-CTC-CCA	EHV-1 gB	Diallo <i>et al.</i> , 2006
Reverse	GGG-TCG-GGC-GTT-TCT-GT		
Probe	FAM-CCC-TAC-GCT-GCT-CC-MGB-NFQ		
Forward	CAT-ACG-TCC-CTG-TCC-GAC-AGA-T	EHV-1 gB	Hussey <i>et al.</i> , 2006
Reverse	GGTACTCGGCCTTTGACGAA		
Probe	FAM-TGA-GAC-CGA-AGA-TCT-CCT-CCA-CCG-A-BHQ1		
Forward	TAT-ACT-CGC-TGA-GGA-TGG-AGA-CTT-T	EHV-1 gB	Pusterla <i>et al.</i> , 2009
Reverse	TTG-GGG-CAA-GTT-CTA-GGT-GGT-T		
Probe	6FAM-ACA-CCT-GCC-CAC-CGC-CTA-CCG		
Forward	GCG-GGC-TCT-GAC-AAC-ACA-A	EHV-1 gC	ISO 17025 accredited for the detection of EHV-1 at WOA Reference Laboratory
Reverse	TTG-TGG-TTT-CAT-GGG-AGT-GTG-TA		
Probe	FAM-TAA-CGC-AAA-CGG-TAC-AGA-A-BHQ1		

199 \*This multiplex real-time PCR test has been validated to ISO 17025 and is designed for use in a 96-well format.  
 200 This can be readily combined with automatic nucleic acid extraction methods. Discrimination between EHV-1  
 201 and EHV-4 is carried out by the incorporation of type-specific dual-labelled probes based on methods published  
 202 by Hussey *et al.* (2006) and Lawrence *et al.* (1994). To establish such a real-time PCR assay for diagnostic  
 203 purposes, validation against blinded samples is required. Sensitivity and specificity for the assay should be  
 204 determined against each target. Support for development of assays and appropriate sample panels can be  
 205 obtained from the WOA Reference Laboratories. Reference material and sample panels for real-time PCR can  
 206 be obtained from the WOA Reference Laboratories.

207 • Point of care (POC) molecular tests

208 Loop-mediated isothermal amplification (LAMP) assays for the detection of EHV-1 have been  
 209 described (Nemoto *et al.*, 2011). An evaluation of a hydrolysis probe-based insulated isothermal  
 210 PCR (iiPCR) assay for the detection of EHV-1 showed it to have a high sensitivity and specificity  
 211 compared with real-time PCR (Balasuriya *et al.*, 2017). However further validation of POC tests in  
 212 the field is required.

213

214 • Molecular characterisation

215 Allelic discrimination real-time PCR assays identifying a single nucleotide polymorphism that was  
216 originally suggested to distinguish between neuropathogenic and non-neuro-pathogenic EHV-1  
217 strains have been developed (Smith *et al.*, 2012). However, investigations in many countries  
218 worldwide demonstrated that the nucleotide substitution was not a reliable predictor of enhanced  
219 neuropathogenicity. Multilocus typing and whole genome sequencing are useful for molecular  
220 epidemiological studies (Garvey *et al.*, 2019; Nugent *et al.*, 2006; Sutton *et al.*, 2019).

### 221 1.3. Virus isolation

222 Virus isolation is no longer a routine test used for EHV-1 detection in the majority of diagnostic laboratories but  
223 is more often conducted for surveillance and research purposes. A number of cell types may be used for isolation  
224 of EHV-1 (e.g. rabbit kidney [RK-13 (AATC–CCL37)], baby hamster kidney [BHK-21], Madin–Darby bovine  
225 kidney [MDBK], pig kidney [PK-15], etc.). RK13 cells are commonly used for this purpose. For efficient primary  
226 isolation of EHV-1 from horses with respiratory disease, equine-derived cell cultures must be used. Both EHV-  
227 1 and EHV 4 may be isolated from nasopharyngeal samples using primary equine fetal kidney cells or equine  
228 fibroblasts derived from dermal (E-Derm) or lung tissue. EHV-1 can be isolated on other cell types, as will be  
229 discussed later. The nasopharyngeal swab and its accompanying transport medium are transferred into the  
230 barrel of a sterile 10-ml syringe. Using the syringe plunger, the fluid is squeezed from the swab into a sterile  
231 tube. A portion of the expressed fluid can be filtered through a sterile, 0.45-µm membrane syringe filter unit into  
232 a second sterile tube if heavy bacterial contamination is expected, but this may also lower virus titre. Recently  
233 prepared cell monolayers in tissue culture flasks are inoculated with the filtered, as well as the unfiltered,  
234 nasopharyngeal swab extract. Cell monolayers in multiwell plates incubated in a 5% CO<sub>2</sub> environment may also  
235 be used. Virus is allowed to attach by incubating the inoculated monolayers at 37°C. Monolayers of uninoculated  
236 control cells should be incubated in parallel.

237 At Recently prepared cell monolayers in tissue culture flasks or plates are inoculated with nasopharyngeal swab  
238 extract or homogenised tissue: approximately 10% (w/v) pooled tissue homogenates of liver, lung, thymus,  
239 adrenal gland and spleen (from aborted fetuses/neonatal foals) or of brain and spinal cord (from cases of  
240 neurological disease). Virus is allowed to attach by incubating the end of the attachment period, inoculated  
241 monolayers at 37°C for 1 hour after which the inocula are removed and the monolayers are rinsed twice with  
242 PBS to remove virus neutralising antibody that may or maintenance medium. Monolayers of uninoculated  
243 control cells should be present in the nasopharyngeal secretions incubated in parallel. After addition of  
244 supplemented maintenance medium (MEM containing 2% fetal calf serum [FCS] and twice the standard  
245 concentrations of antibiotics/antifungals [penicillin, streptomycin, gentamicin, and amphotericin B]), the flasks  
246 are incubated at 37°C in a 5% CO<sub>2</sub> environment.

247 The use of a positive control virus samples of relatively low titre may be used to validate the isolation procedure  
248 carries the risk that this may lead but should be processed separately to eventual avoid contamination of  
249 diagnostic specimens. This risk can be minimised by using routine precautions and good laboratory technique,  
250 including the use of biosafety cabinets, inoculating positive controls after the diagnostic specimens,  
251 decontaminating the surfaces in the hood while the inoculum is adsorbing and using a positive control of  
252 relatively low titre. Inoculated flasks should be inspected daily by microscopy for the appearance of characteristic  
253 herpesvirus cytopathic effect (CPE) (focal rounding, increase in refractility, and detachment of cells). Cultures  
254 exhibiting no evidence of viral CPE after 1 week of incubation should be blind-passaged into freshly prepared  
255 monolayers of cells, using small aliquots of both media and cells as the inoculum. Further blind passage is  
256 usually not productive.

257 It can be useful to inoculate samples into both non-equine and equine cells in parallel to distinguish between  
258 EHV-1 and EHV-4, since EHV-4 can cause sporadic cases of abortion. Around 10% (w/v) pooled tissue  
259 homogenates of liver, lung, thymus, and spleen (from aborted fetuses) or of central nervous system tissue (from  
260 cases of neurological disease) are used for virus isolation. These are prepared by first mincing small samples  
261 of tissue into 1-mm cubes in a sterile Petri dish with dissecting scissors, followed by macerating the tissue cubes  
262 further in serum-free culture medium with antibiotics using a homogeniser or mechanical tissue grinder. After  
263 centrifugation at 1200 g for 10 minutes, the supernatant is removed and 0.5 ml is inoculated into duplicate cell  
264 monolayers in tissue culture flasks. Following incubation of the inoculated cells at 37°C for 1.5–2 hours, the  
265 inocula are removed and the monolayers are rinsed twice with PBS or maintenance medium. After addition of  
266 5 ml of supplemented maintenance medium, the flasks are incubated at 37°C for up to 1 week or until viral CPE  
267 is observed. Cultures exhibiting no evidence of viral CPE after 1 week of incubation should be passaged a  
268 second time into freshly prepared monolayers of cells, using small aliquots of both media and cells as the  
269 inoculum.

270 Blood samples: EHV-1 and, infrequently, EHV-4 can be isolated from PBMC. Buffy coats may be prepared from  
271 unclotted (heparinised) blood by centrifugation at 600–525 g for 45–5 minutes, and. The buffy coat is taken after

272 the plasma has been carefully removed. The buffy coat is then layered onto a PBMC separating solution (Ficoll;  
273 density 1077 g/ml, commercially available) and centrifuged at 400 g for 20 minutes. The PBMC interface  
274 (without most granulocytes) is and washed twice in PBS (300 g for 10 minutes) and resuspended in 1 ml three  
275 times in 3 ml MEM containing 2% FCS. As a quicker alternative method, PBMC may be collected by  
276 centrifugation directly from plasma: (525 g for 5 minutes). Following the third wash, the buffy coat is harvested  
277 and resuspended in 2.5 ml MEM containing 2% FCS. An aliquot of the rinsed cell suspension is added to each  
278 of the duplicate monolayers of equine fibroblast, equine fetal or RK-13 cell monolayers in 25 cm<sup>2</sup> flasks  
279 containing 8–10 ml freshly added maintenance medium. The flasks can be used for DNA extraction. For virus  
280 isolation, the resuspended cells (1 ml) are co-cultivated with freshly prepared primary equine lung or RK-13 cell  
281 suspensions (5 ml) in 25 cm<sup>2</sup> flasks. Confluent cell monolayers are not used. The flasks are incubated at 37°C  
282 in a 5% CO<sub>2</sub> environment for 3 days or until the cells have reached 90% confluence. The monolayers are then  
283 rinsed three times with 1 × PBS and supplemented with 5 ml MEM containing 2% FCS. They are incubated at  
284 37°C for 7 days; either with or without removal of the inoculum. If PBMCs are not removed prior to incubation,  
285 CPE may be difficult to detect in the presence of the massive inoculum of leukocytes: each flask of cells is  
286 freeze-thawed after 7 for a further 4 days of incubation and the contents centrifuged at 300 g for 10 minutes.  
287 Finally, 0.5 ml of the cell free, culture medium supernatant is transferred to freshly made cell monolayers that  
288 are just subconfluent. These are incubated and observed daily for viral CPE for at least 5–6 days. Again,  
289 samples. Samples exhibiting no evidence of viral CPE after 1 week of incubation should be passaged a second  
290 time before discarding as negative.

291 Virus identity may be confirmed by PCR or by immunofluorescence with specific antisera. Virus isolates from  
292 positive cultures should be submitted to a WOAHP Reference Laboratory for strain characterisation and  
293 to maintain a geographically diverse archive. Further strain characterisation for surveillance purposes or detection  
294 of the neurological marker can be provided at some laboratories.

#### 295 1.4. Virus detection by direct immunofluorescence

296 Direct immunofluorescent detection of EHV-1 antigens in samples of post-mortem tissues collected from aborted  
297 equine fetuses and the placenta provides a rapid preliminary diagnosis of herpesvirus abortion (Gunn, 1992).  
298 The diagnostic reliability of this technique approaches that of virus isolation attempts from the same tissues.

299 In the United States of America (USA), potent polyclonal antiserum to EHV-1, prepared in swine and conjugated  
300 with FITC, is available to veterinary diagnostic laboratories for this purpose from the National Veterinary Services  
301 Laboratories of the United States Department of Agriculture (USDA). The antiserum cross-reacts with EHV-4  
302 and hence is not useful for serotyping, however virus typing can be conducted on any virus positive specimens  
303 by PCR.

304 Freshly dissected samples (5 × 5 mm pieces) of fetal tissue (lung, liver, thymus, and spleen) are frozen,  
305 sectioned on a cryostat at –20°C, mounted on to microscope slides, and fixed with 100% acetone. After air-  
306 drying, the sections are incubated at 37°C in a humid atmosphere for 30 minutes with an appropriate dilution of  
307 the conjugated swine antibody to EHV-1. Unreacted antibody is removed by two washes in PBS, and the tissue  
308 sections are then covered with aqueous mounting medium and a cover-slip, and examined for fluorescent cells  
309 indicating the presence of EHV antigen. Each test should include a positive and negative control consisting of  
310 sections from known EHV-1 infected and uninfected fetal tissue.

#### 311 1.5. Virus detection by immunoperoxidase staining

312 Immunohistochemical (IH) staining methods, such as immunoperoxidase, have been developed for detecting  
313 EHV-1 antigen in fixed tissues of aborted equine fetuses, placental tissues or neurologically affected horses  
314 (Schultheiss *et al.*, 1993; Whitwell *et al.*, 1992). Such techniques can be used as an alternative to  
315 immunofluorescence described above and can also be readily applied to archival frozen or fixed tissue samples.  
316 Immunohistochemical staining for EHV-1 is particularly useful for the simultaneous evaluation of morphological  
317 lesions and the identification of the virus. Immunoperoxidase staining for EHV-1/4 may also be carried out on  
318 infected cell monolayers (van Maanen *et al.*, 2000). Adequate controls must be included with each  
319 immunoperoxidase test run for evaluation of both the method specificity and antibody specificity. In one WOAHP  
320 Reference Laboratory, this method is used routinely for frozen or fixed tissue, using If non-specific rabbit  
321 polyclonal sera is used raised against EHV-1. This staining method is not type specific and therefore the staining  
322 method needs to be combined with virus isolation or PCR to discriminate between EHV-1 and EHV-4, however  
323 it provides a useful method for rapid diagnosis of EHV-induced abortion.

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## 1.6. Histopathology

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Histopathological examination of sections of fixed placenta and lung, liver, spleen, adrenal gland and thymus from aborted fetuses and brain and spinal cord from neurologically affected horses should be carried out. In aborted fetuses, eosinophilic intranuclear inclusion bodies present within bronchiolar epithelium or in cells at the periphery of areas of hepatic necrosis are consistent with a diagnosis of herpesvirus infection. The characteristic microscopic lesion associated with EHV-1 neuropathy is a degenerative thrombotic vasculitis of small blood vessels in the brain or spinal cord (perivascular cuffing and infiltration by inflammatory cells, endothelial proliferation and necrosis, and thrombus formation).

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## 2. Serological tests

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EHV-1 and EHV-4 are endemic in most parts of the world and seroprevalence is high, however serological testing of paired sera can be useful for diagnosis of ER in horses. A positive diagnosis is based on the demonstration of significant increases (four-fold or greater) in antibody titres in paired sera taken during the acute and convalescent stages of the disease. The results of tests performed on sera from a single collection date are, in most cases, impossible to interpret with any degree of confidence. The initial (acute phase) serum sample should be taken as soon as possible after the onset of clinical signs, and the second (convalescent phase) serum sample should be taken 2–4 weeks later.

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'Acute phase' sera from mares after abortion or from horses with EHV-1 neurological disease may already contain maximal titres of EHV-1 antibody, with no increase in titres detectable in sera collected at later dates. In such cases, serological testing of paired serum samples from clinically unaffected cohort members of the herd may prove useful for retrospective diagnosis of ER within the herd.

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~~Finally, the serological detection of antibodies to EHV-1 in heart or umbilical cord blood or other fluids of equine fetuses can be of diagnostic value in cases of abortion especially when the fetus is virologically negative. The EHV-1/4 nucleic acid may be identified from these tissues by PCR.~~

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Serum antibody levels to EHV-1/4 may be determined by virus neutralisation (VN) (Thomson *et al.*, 1976), complement fixation tests (CFT) (Thomson *et al.*, 1976) or enzyme-linked immunosorbent assay (ELISA) (Crabb & Studdert, 1995). ~~There are no internationally recognised reagents or standardised techniques for performing any of the serological tests for detection of EHV-1/4 antibody; titre determinations on the same serum may differ from one laboratory to another. Furthermore,~~ The CF and VN tests detect antibodies that are cross-reactive between EHV-1 and EHV-4. Nonetheless, the demonstration of a four-fold or greater rise in antibody titre to EHV-1 or EHV-4 during the course of a clinical illness provides serological confirmation of recent infection with one of the viruses. Commercial ELISAs that distinguish EHV-1 and EHV-4 antibodies are available but less widely used than the CF and VN tests. Unlike other alphaherpesviruses, DIVA ELISAs, which have been very useful in eradication programmes for bovine rhinotracheitis and pseudorabies (Aujeszky's disease), have not been developed for EHV-1/4. An ELISA using a synthetic peptide for glycoprotein E as an antigen (Andoh *et al.*, 2013) is used as DIVA<sup>1</sup> for horses vaccinated with a modified live EHV-1 vaccine licensed in Japan, that lacks the glycoprotein E gene.

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The microneutralisation test is a VN and the CF tests are widely used and sensitive serological assays for detecting EHV-1/4 antibody and will thus be described here.

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### 2.1. Virus neutralisation test

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This test is most commonly performed in flat-bottom 96-well microtitre plates (tissue culture grade) using a constant dose of virus and doubling dilutions of equine test sera. At least ~~two~~ three replicate wells for each serum dilution are required. Heat-inactivated maintenance medium with a concentration of 2% FCS (HIMM) Serum-free MEM is used throughout as a diluent. Virus stocks of known titre are diluted just before use to contain 100 TCID<sub>50</sub> (50% tissue culture infective dose) in 25 µl. Monolayers of ~~E-Derm or~~ RK-13 cells are prepared monodispersed with EDTA/trypsin and resuspended at a concentration of 5 × 10<sup>5</sup>/ml. Note that RK-13 cells can be used with EHV-1 but do not show CPE with EHV-4. Antibody positive and negative control equine sera and controls for cell viability, virus infectivity, and test serum cytotoxicity, must be included in each assay. End-point VN titres of antibody are calculated by determining the reciprocal of the highest serum dilution that protects ≥75% ~~400%~~ of the cell monolayer from virus destruction in ~~both~~ of the replicate wells.

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Serum toxicity may be encountered in samples from horses repeatedly vaccinated with a commercial vaccine prepared from EHV-1 grown up in RK-13 cells. This can give rise to difficulties in interpretation of test reactions

<sup>1</sup> DIVA: detection of infection in vaccinated animals

373 at lower serum dilutions. The problem can be overcome using E-Derm or other non-rabbit kidney derived cell  
374 line.

### 375 2.1.1. Test procedure

376 A suitable test procedure is as follows:

- 377 i) Prepare semi-confluent monolayers in tissue culture microtitre plates.
- 378 ii) Inactivate test and control sera for 30 minutes in a water bath at 56°C.
- 379 iii) Add 40 25-µl of HIMM serum-free MEM to all wells of the microtitre assay plates.
- 380 iv) For test sample titration, pipette 25-40 µl of each test serum into duplicate-triplicate wells of both  
381 rows A and B of the plate. The first two rows serve as the dilution of the test serum and the third  
382 row serves as the serum toxicity control and the second row as the first dilution of the test. Make  
383 doubling dilutions of each serum starting with row B and proceeding to the bottom of the plate by  
384 sequential mixing and transfer of 25-40 µl to each subsequent row of wells. Six sera can be assayed  
385 in each plate. Add 40µl of HIMM to the serum control rows.
- 386 v) Add 40 25-µl of the appropriately diluted EHV-1 or EHV-4 virus stock to each-all wells  
387 (100 TCID<sub>50</sub>/well) of the test plate except those of row A, which are the serum controls wells. Note  
388 that the final serum dilutions, after addition of virus, run from a starting dilution of 1/4 to 1/256. A  
389 separate control plate should include titration of both a negative and positive (high and low) horse  
390 serum-sera of known titre, cell control (no virus), and a back titration of virus control (no serum),  
391 and a virus titration using six wells per log dilution (100 TCID<sub>50</sub> to 0.01 TCID<sub>50</sub>/well) calculate the  
392 actual amount of virus used in the test
- 393 vi) Incubate the plates for 1 hour at 37°C in 5% CO<sub>2</sub> atmosphere. Add 50 µl of the prepared E-Derm  
394 or RK-13 cell suspension (5 × 10<sup>5</sup> cells/ml) in MEM/10% FCS to each well.
- 395 vii) Transfer 50 µl from each well of the test and control plates to the tissue culture microtitre plates.
- 396 viii) Incubate the plates for 2-4-5 days at 37°C in an atmosphere of 5% CO<sub>2</sub> in air.
- 397 ix) Examine the plates microscopically for CPE and record the results on a worksheet. Confirm the  
398 validity of the test by establishing that the working dilution of stock virus is at 100 TCID<sub>50</sub>/well, that  
399 the (high and low) positive control sera are within one well of their pre-determined titre and that the  
400 negative control serum is negative at a 1/4 dilution. This takes approximately 72 hours. If at this  
401 stage the antigen is too weak the virus concentration may be increased by extending the incubation  
402 period up to 5 days. If the antigen is too strong the test must be repeated.
- 403 Wells are scored as positive for neutralisation of virus if ≥ 75% of the cell monolayer remains intact.  
404 The highest dilution of serum resulting in ≥ 75% neutralisation of virus (<25% CPE) in replicate  
405 wells is the end-point titre for that serum. Examine the plates microscopically for CPE and record  
406 the results on a worksheet.
- 407 x) Alternatively, the cell monolayers can be scored for CPE after fixing and staining as follows: after  
408 removal of the culture fluid, immerse the plates for 15 minutes in a solution containing 2 mg/ml  
409 crystal violet, 10% formalin, 45% methanol, and 45% water. Then, rinse the plates vigorously under  
410 a stream of running tap water. Wells containing intact cell monolayers stain blue, while monolayers  
411 destroyed by virus do not stain. Verify that the cell control, positive serum control, and serum  
412 cytotoxicity control wells stain blue, that the virus control and negative serum control wells are not  
413 stained, and that the actual amount of virus added to each well is between 10<sup>4-5</sup> and 10<sup>2-5</sup> TCID<sub>50</sub>.  
414 Wells are scored as positive for neutralisation of virus if 100% of the cell monolayer remains intact.  
415 The highest dilution of serum resulting in complete neutralisation of virus (no CPE) in both duplicate  
416 wells is the end-point titre for that serum.
- 417 xi) Calculate the neutralisation titre for each test serum, and compare acute and convalescent phase  
418 serum titres from each animal for a four-fold or greater increase.

### 419 2.2. Complement fixation test

420 The CFT can be used for the detection and quantification of antibodies against to EHV-1. The test determines  
421 whether an antigen and an antibody are capable of forming a complex. The presence of an immune complex is  
422 revealed by the detector system, which consists of guinea-pig complement and sensitised sheep red blood cells  
423 (SRBCs) coated with rabbit haemolytic serum (haemolysin). In the absence of antibodies against equine  
424 herpesvirus, no antibody/antigen complex is formed, the complement remains free in the solution and the  
425 sensitised SRBCs become lysed. In the presence of antibodies against equine herpesvirus, an antibody/antigen

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426 complex is formed, the complement becomes fixed and is therefore unable to lyse the SRBCs. They  
427 subsequently form a button at the bottom of the test well.

428 Guinea-pig complement, rabbit haemolytic serum, complement fixation diluent (CFD) and bovine serum albumin  
429 (BSA) can be obtained commercially. The dilution of guinea-pig complement that has activity at 3 HD  
430 (haemolytic dose) in the presence of sensitised SRBCs should be optimised. The recommended dilution of  
431 rabbit haemolytic serum (or the working dilution) is sometimes provided by the supplier. However, the optimal  
432 dilution of haemolysin should be determined with the in use reagents (complement etc.) so that the test can be  
433 performed reproducibly. The optimum concentration of antigen to be used in the test should be determined  
434 using an antigen versus antibody checkerboard technique and by testing a panel of known positive sera.

435 The test is performed in U bottomed microtitre plates. Paired sera should be assayed on the same plate. An  
436 antibody positive serum should be included as a control on each plate. All sera are tested on a second plate  
437 containing all components except virus to check for anti-complementary activity. A back titration of the working  
438 dilution (3 HD) of complement to 2 HD, 1 HD, 0.5 HD is set up in duplicate wells on the complement control  
439 plate (eight wells in total). An SRBC control is set up in eight wells.

### 440 **2.2.3. Preparation of samples**

441 i) Samples and controls are prepared by adding 4 volumes (600 µl) of CFD to 1 volume (150 µl) of  
442 test sera to give a 1/5 dilution.

443 ii) Diluted serum is inactivated for 30 minutes at 60°C to destroy the naturally occurring complement.

### 444 **2.2.4. Test procedure**

445 i) Prepare the test plate and anti-complementary plate by adding 25 µl 0.05% BSA/CFD to all wells  
446 except the first column (H).

447 ii) Add 50 µl of 0.05% BSA/CFD to the eight wells of the complement control (back titration).

448 iii) Add 75 µl of 0.05% BSA/CFD to eight wells of cell control.

449 iv) Add 50 µl of the diluted inactivated test serum and controls to the first well of each row on both the  
450 test and anti-complementary plates. Serial doubling dilutions are then made by transferring 25 µl  
451 across the plate and discarding the final 25 µl.

452 v) Place the microtitre plates on ice for addition of antigen and complement.

453 vi) Add 25 µl of antigen (diluted to working strength in 0.05% BSA/CFD) to the test plates.

454 vii) Add 25 µl of 0.05% BSA/CFD to all wells of the anti-complementary plate to compensate for lack  
455 of antigen.

456 viii) Add 25 µl of guinea-pig complement diluted in 0.05% BSA/CFD to 3 HD to all wells except the  
457 complement control and SRBC control.

458 ix) Back titrate the working dilution of 3 HD complement to 2 HD, 1 HD and 0.5 HD in 200 µl volumes.  
459 Add 25 µl of each dilution to the appropriate wells.

460 x) Incubate all plates at 4°C overnight.

### 461 **2.2.5. Preparation and addition of sheep blood**

462 i) SRBCs collected into Alsever's solution are washed twice in 0.05% BSA/PBS solution.

463 ii) Gently resuspend the SRBCs in 5–10 ml 0.05% BSA/CFD solution. Dilute to 2% SRBCS (v/v  
464 packed cells) in BSA/CFD solution.

465 iii) Mix the 2% SRBCs with an equal volume of BSA/CFD solution containing haemolysin at its optimal  
466 sensitising concentration to give a 1% SRBC solution. Prepare an appropriate volume of this  
467 solution by allowing 3 ml per microtitre plate.

468 iv) Incubate at 37°C for 10 minutes. Store the 1% sensitised SRBCs overnight at 4°C.

469 v) The following day, incubate the 1% sensitised SRBCs at 37°C for 30 minutes. During the final 20  
470 minutes of this incubation, transfer the test plates from 4°C to 37°C.

471 vi) At the end of the 30-minute incubation, add 25 ml of 1% sensitised SRBCs to all plates. Mix on a  
472 plate shaker for 30 seconds.



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- 473 vii) Incubate the plates at 37°C for 30 minutes. Shake the plates after 15 minutes and at the end of this  
474 incubation (a total of three times).
- 475 viii) Incubate the plates at 4°C for 2 hours to allow the cells to settle.
- 476 ix) Read and record the test results after 2 hours.

### **2.2.6. Reading results**

- 477
- 478 i) Confirm the validity of the test by establishing that the working dilution of complement is at 3 HD:  
479 100% lysis at 3 HD and 2 HD, and 50% lysis at 1 HD. Distinct buttons should be visible in the eight  
480 wells of the SRBC control.
- 481 ii) There must be 100% lysis observed at the 1/5 dilution for the negative control (<5). The antibody  
482 titre of the positive control serum must read within one well of its predetermined titre.
- 483 iii) Confirm that there are no buttons visible on the anti-complementary plates. Buttoning indicates  
484 either the presence of residual native complement in the sample or that there is a non-specific  
485 complement fixing effect occurring. Sera that show anti-complementary activity should be retested  
486 and treated as described below.
- 487 iv) In the test wells, buttoning indicates the presence of antibodies in the serum. The antibody titre is  
488 the dilution at which there is 50% buttoning and 50% lysis observed.

### **2.2.7. Treatment of samples showing anti-complementary activity**

- 489
- 490 i) Add 50 µl of guinea-pig complement to 150 µl of the serum showing anti-complementary activity.
- 491 ii) Incubate the sample at 37°C for 30 minutes.
- 492 iii) Add 550 µl of CFD (1:5 dilution).
- 493 iv) Heat inactivate at 60°C for 30 minutes.

## **C. REQUIREMENTS FOR VACCINES**

### **1. Background**

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496 Both live attenuated and inactivated vaccines are available for use in horses as licensed, commercially prepared products  
497 for use in reducing the impact of disease in horses caused by EHV-1/4 infection. The products contain different  
498 permutations of EHV-1 and EHV-4 and some also include equine influenza virus.

499 Clinical experience has demonstrated that vaccination can be useful for reducing clinical signs of respiratory disease and  
500 incidence of abortion, however none of the vaccines protect against neurological disease. Multiple doses repeated  
501 annually, of each of the currently marketed ER vaccines are recommended by their respective manufacturers. Vaccination  
502 schedules vary with a particular vaccine.

503 The indications stated on the product label for use of several available vaccines for ER are either as a preventative of  
504 herpesvirus-associated respiratory disease, or as an aid in the prevention of abortion, or both. A minority of ~~Only four~~  
505 vaccine products have met the regulatory requirements for claiming efficacy in providing protection from herpesvirus  
506 abortion as a result of successful vaccination and challenge experiments in pregnant mares. None of the vaccine products  
507 have been demonstrated to prevent the occurrence of neurological disease sometimes associated with EHV-1 infection.

508 Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*.  
509 The guidelines given here and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national  
510 and regional requirements.

### **2. Outline of production and minimum requirements for vaccines**

#### **2.1. Characteristics of the seed**

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513 The master seed virus (MSV) for ER vaccines must be prepared from strains of EHV-1 and/or EHV-4 that have  
514 been positively and unequivocally identified ~~by both serological and genetic tests~~. Seed virus must be  
515 propagated in a cell line approved for equine vaccine production by the appropriate regulatory agency. A  
516 complete record of original source (including isolate number, location, year of isolation), passage history,

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517 medium used for propagation, etc., shall be kept for the master seed preparations of both the virus(es) and cell  
518 stock(s) intended for use in vaccine production.

### 519 **2.1.1. Biological characteristics of the master seed**

520 Permanently stored stocks of both MSV and master cell stock (MCS) used for vaccine production must  
521 be demonstrated to be pure, safe and, in the case of MSV, also immunogenic.

522 Generally, the fifth passage from the MSV and the twentieth passage from the MCS are the highest  
523 allowed for vaccine production. Results of all quality control tests on master seeds must be recorded and  
524 made a part of the licensee's permanent records.

### 525 **2.1.2. Quality criteria**

526 Tests for master seed purity include prescribed procedures that demonstrate the virus and cell seed  
527 stocks to be free from bacteria, fungi, mycoplasmas, and extraneous viruses. Special tests must be  
528 performed to confirm the absence of equine arteritis virus, equine infectious anaemia virus, equine  
529 influenza virus, equine herpesvirus-2, -3, and -5, equine rhinitis A and B viruses, the alphaviruses of  
530 equine encephalomyelitis, bovine viral diarrhoea virus (BVDV – common contaminant of bovine serum),  
531 and porcine parvovirus (PPV – potential contaminant of porcine trypsin). The purity check should also  
532 include the exclusion of the presence of EHV-1 from EHV-4 MSV and *vice versa*.

### 533 **2.1.3. Validation as a vaccine strain**

534 Tests for immunogenicity of the EHV-1/4 MSV stocks should be performed in horses on an experimental  
535 test vaccine prepared from the highest passage level of the MSV allowed for use in vaccine production.  
536 The test for MSV immunogenicity consists of vaccination of horses with low antibody titres (< 1:24 by VN  
537 test) to EHV-1/4, with doses of the test vaccine that will be recommended on the final product label  
538 (Goodman *et al.*, 2006; Van de Walle *et al.*, 2010). Second serum samples should be obtained and tested  
539 for significant increases in neutralising antibody titre against the virus, 21 days after the final dose.

540 Samples of each lot of MSV to be used for preparation of live attenuated ER vaccines must be tested for  
541 safety in horses determined to be susceptible to the virulent wild-type virus, including pregnant mares in  
542 the last 4 months of gestation. Vaccine safety must be demonstrated in a 'safety field trial' in horses of  
543 various ages from three different geographical areas. The safety trial should be conducted by  
544 independent veterinarians using a precensuring batch of vaccine. EHV-1 vaccines making a claim for  
545 efficacy in controlling abortion must be tested for safety in a significant number of late gestation pregnant  
546 mares, using the vaccination schedule that will be recommended by the manufacturer for the final vaccine  
547 product.

## 548 **2.2. Method of manufacture**

### 549 **2.2.1. Procedure**

550 A detailed protocol of the methods of manufacture to be followed in the preparation of vaccines for ER  
551 must be compiled, approved, and filed as an Outline of Production with the appropriate licensing agency.  
552 Specifics of the methods of manufacture for ER vaccines will differ with the type (live or inactivated) and  
553 composition (EHV-1 only, EHV-1 and EHV-4, EHV-4 and equine influenza viruses, etc.) of each individual  
554 product, and also with the manufacturer.

### 555 **2.2.2. Requirements for ingredients**

556 Cells, virus, culture medium, and medium supplements of animal origin that are used for the preparation  
557 of production lots of vaccine must be derived from bulk stocks that have passed the prescribed tests for  
558 bacterial, fungal, and mycoplasma sterility; nontumorigenicity; and absence of extraneous viral agents.

### 559 **2.2.3. Final product batch tests**

#### 560 i) Sterility

561 Samples taken from each batch of completed vaccine are tested for bacteria, fungi, and  
562 mycoplasma contamination. Procedures to establish that the vaccine is free from extraneous  
563 viruses are also required; such tests should include inoculation of cell cultures that allow detection  
564 of the common equine viruses, as well as techniques for the detection of BVDV and PPV in  
565 ingredients of animal origin used in the production of the batch of vaccine.

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- 566 ii) Identity
- 567 Identity tests shall demonstrate that no other vaccine strain is present when several strains are
- 568 propagated in a laboratory used in the production of multivalent vaccines.
- 569 iii) Safety
- 570 Safety tests shall consist of detecting any abnormal local or systemic adverse reactions to the
- 571 vaccine in the host species by all vaccination route(s). Tests to assure safety of each production
- 572 batch of ER vaccine must demonstrate complete inactivation of virus (for inactivated vaccines) as
- 573 well as a level of residual virus-killing agent that does not exceed the maximal allowable limit (e.g.
- 574 0.2% for formaldehyde).
- 575 iv) Batch potency
- 576 Batch potency is examined on the final formulated product. ~~Batch control of antigenic potency for~~
- 577 ~~EHV-1 vaccines only may be tested by measuring the ability of dilutions of the vaccine to protect~~
- 578 ~~hamsters from challenge with a lethal dose of hamster adapted EHV-1 virus. Although potency~~
- 579 ~~testing on production batches of ER vaccine may also be performed by vaccination of susceptible~~
- 580 ~~horses followed by assay for seroconversion, the recent availability of virus type specific MAbs has~~
- 581 ~~permitted development of less costly and more rapid *in-vitro* immunoassays exist for antigenic~~
- 582 ~~potency. The basis for such *in-vitro* assays for ER vaccine potency is the determination, by use of~~
- 583 ~~the-specific MAb, of the presence of at least the minimal amount of viral antigen within each batch~~
- 584 ~~of vaccine that correlates with the required level of protection (or seroconversion rate) in a standard~~
- 585 ~~animal test for potency.~~

## 586 2.3. Requirements for authorisation/registration/licencing

### 587 2.3.1. Manufacturing process

588 For registration of vaccine, all relevant details concerning manufacture of the vaccine and quality control

589 testing (see Sections C.2.1 and C.2.2) should be submitted to the authorities. This information shall be

590 provided from three consecutive vaccine batches with a volume not less than 1/3 of the typical industrial

591 batch volume.

### 592 2.3.2 Safety requirements

593 Vaccine safety should be evaluated in vaccinated animals using different assays (see Section 2.2.3.iii).

### 594 2.3.3 Efficacy requirements

595 Vaccine efficacy (protection) is estimated in vaccinated animals directly by evaluating their resistance to

596 live pathogen challenge.

### 597 2.3.4 Duration of immunity

598 As part of the licensing or marketing authorisation procedure, the manufacturer may be required to

599 demonstrate the duration of immunity (DOI) of a given vaccine by either challenge or alternative test at

600 the end of the claimed period of protection.

601 Tests to establish the duration of immunity to EHV-1/4 or EHV1/4 achieved by immunisation with each

602 batch of vaccine are not required. The results of many reported observations indicate that immunity

603 induced by vaccination against EHV-1 or EHV induced immunity to EHV-1/4 is not more than a few

604 months in duration; these observations are reflected in the frequency of revaccination recommended on

605 ER vaccine product labels.

### 606 2.3.5 Stability

607 As part of the licensing or marketing authorisation procedure, the manufacturer will be required to

608 demonstrate the stability of all the vaccine's properties at the end of the claimed shelf-life period. Storage

609 temperature shall be indicated, and warnings should be given if product is damaged by freezing or

610 ambient temperature.

611 At least three production batches of vaccine should be tested for shelf life before reaching a conclusion

612 on the vaccine's stability. When stored at 4°C, inactivated vaccine products generally maintain their

613 original antigenic potency for at least 1 year. Lyophilised preparations of the live virus vaccine are also

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614 stable during storage for 1 year at 4°C. Following reconstitution, live virus vaccine is unstable and cannot  
615 be stored without loss of potency.

616 **Note:** current vaccines are authorised for prevention of respiratory disease or as an aid in the prevention of abortion.  
617 Unless the vaccine's ability to prevent neurological disease is under investigation, the virus used in the challenge  
618 experiments should not be a strain with a history of inducing neurological disease.

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775 **NB:** There are WOAHO Reference Laboratories for equine rhinopneumonitis (please consult the WOAHO Web site:  
 776 <https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3http://www.oie.int/>).

777 Please contact the WOAHO Reference Laboratories for any further information on  
 778 diagnostic tests, reagents and vaccines for equine rhinopneumonitis  
 779 and to submit strains for further characterisation.

780 **NB:** FIRST ADOPTED IN 1990. MOST RECENT UPDATES ADOPTED IN 2017.

REUNIÓN DE LA COMISIÓN DE NORMAS BIOLÓGICAS

París, 5–9 de febrero de 2024

SECTION 3.8.

~~OVIDAE AND CAPRINAE~~

CHAPTER 3.8.1.

BORDER DISEASE

SUMMARY

*Border disease (BD) is a viral disease of sheep and goats first reported in sheep in 1959 from the border region of England and Wales, and since recorded world-wide. Prevalence rates in sheep vary from 5% to 50% between countries and from region to region within countries. Clinical signs include barren ewes, abortions, stillbirths and the birth of small weak lambs. Affected lambs can show ~~and a fine~~ tremor, abnormal body conformation and hairy fleeces (so-called 'hairy-shaker' or 'fuzzy' lambs). Consequently, the disease has sometimes been referred to as 'hairy shaker disease'. Vertical transmission plays an important role in the epidemiology of the disease. Infection of fetuses can result in the birth of persistently infected (PI) lambs. These PI lambs are viraemic, antibody negative and constantly excrete virus. The virus spreads from sheep to sheep, with PI animals being the most potent source of infection. Infection in goats is less common with abortion being the main presenting sign.*

*BD is caused by the Pestivirus border disease virus (BDV), but in some parts of the world, especially where there is close contact between sheep or goats and cattle, the same clinical signs may be caused by infection with bovine viral diarrhoea virus (BVDV). Therefore the genetic and antigenic differences between BDV and BVDV need to be taken into consideration when investigating disease outbreaks or certifying animals or germplasm for international movement. It is important to identify the viraemic PI animals so that they will not be used for breeding or trading purposes. Serological testing is insufficient. However, it is generally considered that serologically positive, nonviraemic sheep ~~are 'safe', do not present a risk~~ as latent infections are not known to occur in recovered animals. Pregnant seropositive, nonviraemic animals may, however, present a risk by carrying a PI fetus that cannot be detected until after parturition.*

**Identification of the agent:** *BDV is a species of Pestivirus (Pestivirus ovis) in the family Flaviviridae and is closely related to classical swine fever virus (Pestivirus suis) and BVDV viruses, which are classified in the distinct species: Pestivirus bovis (commonly known as BVDV type 1), Pestivirus tauri (formerly BVDV type 2) and Pestivirus brazilense (BVDV type 3 or Hobi-like pestivirus). Nearly all isolates of BDV are noncytopathogenic in cell culture. There are no defined serotypes but virus isolates exhibit considerable antigenic diversity. A number of separate genotypes have been identified.*



31 Apparently healthy PI sheep resulting from congenital infection can be identified by direct detection of virus  
32 or nucleic acid in blood or tissues or by virus isolation in cell culture followed by immunostaining to detect  
33 the noncytopathogenic virus.

34 **Diagnostic methods:** The demonstration of virus by culture and antigen detection may be less reliable in  
35 lambs younger than 2 months that have received colostral antibody. Acute infection is usually subclinical  
36 and viraemia is transient and difficult to detect. The isolation of virus from tissues of aborted or stillborn  
37 lambs is often difficult but virus can be detected by sensitive reverse transcriptase polymerase chain reaction  
38 methods that are able to detect residual nucleic acid. However, tissues and blood from PI sheep more than  
39 a few months old contain high levels of virus, which can be easily identified by isolation and direct methods  
40 to detect antigens or nucleic acids. As sheep may be infected with BVDV, it is preferable to use diagnostic  
41 assays that are 'pan-pestivirus' reactive and will readily detect all strains of BDV and BVDV.

42 **Serological tests:** Acute infection with BDV is best confirmed by demonstrating seroconversion using paired  
43 or sequential samples from several animals in the group. The enzyme-linked immunosorbent assay and  
44 virus neutralisation test (VNT) are the most commonly used antibody detection methods. Due to the antigenic  
45 differences between BDV and BVDV, assays for the detection of antibodies to BDV, especially by VNT,  
46 should preferably be based on a strain of BDV.

47 **Requirements for vaccines:** There is no standard vaccine for BDV, but a commercial killed whole-virus  
48 vaccine has been produced. Ideally, such a vaccine should be suitable for administration to females before  
49 breeding for prevention of transplacental infection. The use of BVDV vaccines has been advocated, but the  
50 antigenic diversity of BD viruses must be considered. In many instances, the antigenic diversity of BDV  
51 strains is sufficiently different to BVDV that a BVDV vaccine is unlikely to provide protection.

52 BD viruses have contaminated several modified live veterinary vaccines produced in sheep cells or  
53 containing sheep serum. This potential hazard should be recognised by manufacturers of biological  
54 products.

## 55 A. INTRODUCTION

56 Border disease virus (BDV) is a *Pestivirus* of the family *Flaviviridae* and is closely related to classical swine fever virus  
57 (CSFV) and bovine viral diarrhoea virus (BVDV). There are ~~four~~ a number of officially recognised species, namely – BDV  
58 (*Pestivirus ovis*) CSFV (*Pestivirus suis*), BVDV types 1 and 2 (taxonomically known as *Pestivirus bovis* and *Pestivirus tauri*,  
59 respectively) and BDV (ICTV, 2016) BVDV 3 or Hobi-like pestivirus (*Pestivirus brazilense*) (Postler et al., 2023), but a  
60 number of other pestiviruses that are considered to be distinct species have been reported. While CSF viruses are  
61 predominantly restricted to pigs, examples of there are situations where the other ~~three~~ species have all been recovered  
62 from sheep. While the majority of isolates have been identified as BD viruses in areas where sheep or goats are raised in  
63 isolation from other species (Vilcek et al., 1997), in regions where there is close contact between small ruminants and  
64 cattle, BVDV may be frequently identified (Carlsson, 1991). Nearly all virus isolates of BDV are noncytopathogenic,  
65 although occasional cytopathic viruses have been isolated (Vantsis et al., 1976). BDV spreads naturally among sheep by  
66 the oro-nasal route and by vertical transmission. It is principally a cause of congenital disease in sheep and goats, but can  
67 also cause acute and persistent infections. Infection is less common in goats, in which persistent infection is rare as  
68 abortion is the main presenting sign. Pigs may also be infected by pestiviruses other than CSFV and antibodies to BDV in  
69 pigs may interfere with tests for the diagnosis of CSF (Oguzoglu et al., 2001). Several genotypes of BD viruses from sheep,  
70 goats and Pyrenean chamois (*Rupicapra pyrenaica pyrenaica*) have been described. Phylogenetic analysis using  
71 computer-assisted nucleotide sequence analysis suggests that genetic variability among BD viruses is greater than within  
72 each of the other *Pestivirus* species. Four distinguishable genogroups of BDV have been described as well as putative  
73 novel *Pestivirus* genotypes from Tunisian sheep and a goat At least eight BDV genotypes have been described (BDV type  
74 1 to BDV type 8). Other ovine pestiviruses have been identified that are responsible for BD-like syndromes such as Tunisian  
75 and Tunisian-like, Aydin-like (*Pestivirus I*, Turkey) *Pestivirus* genotypes from Tunisian sheep and a goat and a new  
76 emerging ovine pestivirus (OVPV) that were found to be genetically and antigenically closely related to CSFV (Becher et  
77 al., 2003; Righi et al., 2021; Vilcek & Nettleton, 2006). The chamois BDV is similar to isolates from sheep in the Iberian  
78 Peninsula (Valdazo-Gonzalez et al., 2007). This chapter describes BDV infection in sheep. Chapter 3.4.7 *Bovine viral*  
79 *diarrhoea* should also be consulted for related diagnostic methods.

### 80 1. Acute infections

81 Healthy newborn and adult sheep exposed to BDV usually experience only mild or inapparent disease. Slight fever and a  
82 mild leukopenia are associated with a short-lived viraemia detectable between days 4 and 11 post-infection, after which  
83 virus neutralising antibody appears in the serum (Thabti et al., 2002).

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84 Acute infections are best diagnosed serologically using paired sera from a representative number of sheep. Occasional  
85 BDV isolates have been shown to produce high fever, profound and prolonged leukopenia, anorexia, conjunctivitis, nasal  
86 discharge, dyspnoea and diarrhoea, and 50% mortality in young lambs. One such isolate was recovered from a severe  
87 epidemic of BD among dairy sheep in 1984 (Chappuis *et al.*, 1986). A second such isolate was a BDV contaminant of a  
88 live CSFV vaccine (Van Woort & Terpstra, 1988).

## 89 2. Fetal infection

90 The main clinical signs of BD are seen following the infection of pregnant ewes. While the initial maternal infection is  
91 subclinical or mild, the consequences for the fetus are serious. Fetal death may occur at any stage of pregnancy, but is  
92 more common in fetuses infected early in gestation. Small dead fetuses may be resorbed or their abortion may pass  
93 unnoticed as the ewes continue to feed well and show no sign of discomfort. As lambing time approaches, the abortion of  
94 larger fetuses, stillbirths and the premature births of small, weak lambs will be seen. Confirmation that an abortion or  
95 stillbirth is due to BDV is often difficult to establish, but virus may be isolated from fetal tissues in some cases. The use of  
96 an appropriate real-time reverse-transcription polymerase chain reaction (RT-PCR) assay may give a higher level of  
97 success because of the advantages of high sensitivity and the ability to detect genome from non-infectious virus. In aborted  
98 fetuses, it is also possible to detect virus by immunohistochemistry of brain, thyroid and other tissues (Thur *et al.*, 1997).  
99 Samples of fetal fluids or serum should be tested for BDV antibody.

100 During lambing, an excessive number of barren ewes will become apparent, but it is the diseased live lambs that present  
101 the main clinical features characteristic of BD. The clinical signs exhibited by BD lambs are very variable and depend on  
102 the breed of sheep, the virulence of the virus and the time at which infection was introduced into the flock. Affected lambs  
103 are usually small and weak, many being unable to stand. Nervous signs and fleece changes are often apparent. The  
104 nervous signs of BD are its most characteristic feature. The tremor can vary from violent rhythmic contractions of the  
105 muscles of the hindlegs and back, to barely detectable fine trembling of the head, ears, and tail. Fleece abnormalities are  
106 most obvious in smooth-coated breeds, which develop hairy fleeces, especially on the neck and back. Abnormal brown or  
107 black pigmentation of the fleece may also be seen in BD-affected lambs. Blood samples to be tested for the presence of  
108 BDV or antibody should be collected into anticoagulant from suspect lambs before they have received colostrum. Once  
109 lambs have ingested colostrum, it is difficult to isolate virus until they are 2 months old and maternal antibody levels have  
110 waned. However, during this period, it may be possible to detect viral antigen in skin biopsies, by immunohistochemistry,  
111 in washed leukocytes by enzyme-linked immunosorbent assay (ELISA) or by real-time RT-PCR. ELISAs directed at  
112 detection of the Erns antigen appear to be less prone to interference by maternal antibodies and can often be used to  
113 detect antigen in serum.

114 With careful nursing, a proportion of BD lambs can be reared, although deaths may occur at any age. The nervous signs  
115 gradually decline and can disappear by 3–6 months of age. Weakness, and swaying of the hind-quarters, together with  
116 fine trembling of the head, may reappear at times of stress. Affected lambs often grow slowly and under normal field  
117 conditions many will die before or around weaning time. In cases where losses at lambing time have been low and no  
118 lambs with obvious signs of BD have been born, this can be the first presenting sign of disease.

119 Some fetal infections occurring around mid-gestation can result in lambs with severe nervous signs, locomotor  
120 disturbances and abnormal skeletons. Such lambs have lesions of cerebellar hypoplasia and dysplasia, hydranencephaly  
121 and porencephaly resulting from necrotising inflammation. The severe destructive lesions appear to be immune mediated,  
122 and lambs with such lesions frequently have high titres of serum antibody to BDV. Most lambs infected in late gestation  
123 are normal and healthy and are born free from virus but with BDV antibody. Some such lambs can be weak and may die  
124 in early life (Barlow & Patterson, 1982).

## 125 3. Persistent viraemia

126 When fetuses survive an infection that occurs before the onset of immune competence, they are born with a persistent  
127 viraemia. The ovine fetus can first respond to an antigenic stimulus between approximately 60 and 85 days of its 150-day  
128 gestation period. In fetuses infected before the onset of immune competence, viral replication is uncontrolled and 50%  
129 fetal death is common. In lambs surviving infection in early gestation, virus is widespread in all organs. Such lambs appear  
130 to be tolerant of the virus and have a persistent infection, usually for life. A precolostral blood sample will be virus positive  
131 and antibody negative. Typically, there is no inflammatory reaction and the most characteristic pathological changes are  
132 in the central nervous system (CNS) and skin. Throughout the CNS, there is a deficiency of myelin, and this causes the  
133 nervous signs. In the skin, primary wool follicles increase in size and the number of secondary wool follicles decreases,  
134 causing the hairy or coarse fleece.

135 Persistently viraemic sheep can be identified by the detection of viral antigens, nucleic acids or infectious virus in a blood  
136 sample. Viraemia is readily detectable by testing of serum at any time except within the first 2 months of life, when virus  
137 may be masked by colostral antibody and, possibly, in animals older than 4 years, some of which develop low levels of  
138 anti-BDV antibody (Nettleton *et al.*, 1992). Methods other than virus isolation may be preferred to avoid interference from

139 antibodies. When the presence of colostral antibodies is suspected, the virus may be detected in washed leukocytes and  
 140 in skin by using sensitive ELISAs. Although virus detection in blood during an acute infection is difficult, persistent viraemia  
 141 should be confirmed by retesting animals after an interval of at least 3 weeks. The use of real-time RT-PCR should be  
 142 considered at all times and for any sample type due to its high analytical sensitivity and the lack of interference from  
 143 antibodies in a sample.

144 Some viraemic sheep survive to sexual maturity and are used for breeding. Lambs born to these infected dams are always  
 145 persistently viraemic. Persistently viraemic sheep are a continual source of infectious virus to other animals and their  
 146 identification is a major factor in any control programme. Sheep being traded should be screened for the absence of BDV  
 147 viraemia.

148 Usually persistently infected (PI) rams have poor quality, highly infective semen and reduced fertility. All rams used for  
 149 breeding should be screened for persistent BDV infection on a blood sample. Semen samples can also be screened for  
 150 virus, but virus isolation is much less satisfactory than from blood because of the toxicity of semen for cell cultures. Real-  
 151 time RT-PCR for detection of pestivirus nucleic acid would usually overcome toxicity problems, and thus this assay should  
 152 be useful for testing semen from rams.

#### 153 4. Late-onset disease in persistently viraemic sheep

154 Some PI sheep housed apart from other animals spontaneously develop intractable diarrhoea, wasting, excessive ocular  
 155 and nasal discharges, sometimes with respiratory distress. At necropsy such sheep have gross thickening of the distal  
 156 ileum, caecum and colon resulting from focal hyperplastic enteropathy. Cytopathic BDV can be recovered from the gut of  
 157 these lambs. With no obvious outside source of cytopathic virus, it is most likely that such virus originates from the lamb's  
 158 own virus pool, similar to what occurs with BVDV. Other PI sheep in the group ~~do~~ may not develop the disease. This  
 159 syndrome, which has been produced experimentally and recognised in occasional field outbreaks of BD, has several  
 160 similarities with bovine mucosal disease (Nettleton *et al.*, 1992).

## 161 B. DIAGNOSTIC TECHNIQUES

162 **Table 1. Test methods available for diagnosis of border disease and their purpose**

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Identification of the agent <sup>(a)</sup>						
Virus isolation	+	++	++	+++	–	–
Antigen detection by ELISA	+	++	+++	+++	–	–
NA detection by real-time RT-PCR	+++	+++	+++	+++	+++	–
NA detection by ISH	–	–	–	+	–	–
Detection of immune response						
<u>Antibody detection by ELISA</u>	++	++	++	+	++	++
VN	+++	+++	++	+++	+++	+++

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163 Key: +++ = recommended for this purpose; ++ recommended but has limitations;  
164 + = suitable in very limited circumstances; – = not appropriate for this purpose.  
165 ELISA = enzyme-linked immunosorbent assay; IHC = immunohistochemistry; NA = nucleic acid; RT-PCR = reverse-transcription  
166 polymerase chain reaction; ISH = *in-situ* hybridisation; VN = virus neutralisation.  
167 <sup>(a)</sup>A combination of agent identification methods applied on the same clinical sample is recommended.

## 168 1. Identification of the agent

169 There is no designated WOAHA Reference Laboratory for BDV, but the reference laboratories for BVDV or CSFV will be  
170 able to provide advice<sup>1</sup>. One of the most sensitive proven methods for identifying BDV remains virus isolation. However,  
171 a broadly reactive real-time RT-PCR assay (preferably pan-pestivirus reactive) will usually provide higher analytical  
172 sensitivity than virus isolation, can be used to test samples that are difficult to manage by virus isolation and can be  
173 performed in a few hours. Antigen-detection ELISA and immunohistochemical techniques on tissue sections are also  
174 valuable methods for identifying BDV-infected animals.

### 175 1.1. Virus isolation

176 It is essential that laboratories undertaking virus isolation have a guaranteed supply of pestivirus-free  
177 susceptible cells and bovine serum, or equivalent, that contain no anti-pestivirus activity and no contaminating  
178 virus. It is important that a laboratory quality assurance programme be in place. Chapter 3.4.7 provides detailed  
179 methods for virus isolation in either culture tubes or microplates for the isolation of pestiviruses from sheep or  
180 goat samples, including serum, whole blood, semen and tissues. The principles and precautions outlined in that  
181 chapter for the selection of cell cultures, medium components and reagents are equally relevant to this chapter.  
182 Provided proven pan-pestivirus reactive reagents (e.g. monoclonal antibodies [MAbs], primers and probes for  
183 real-time RT-PCR) are used for antigen or nucleic acid detection, the principal difference is the selection of  
184 appropriate cell cultures.

185 BD virus can be isolated in a number of primary or secondary ovine cell cultures (e.g. kidney, testes, lung).  
186 Ovine cell lines for BDV growth are rare. Semicontinuous cell lines derived from fetal lamb muscle (FLM), whole  
187 embryo (Thabti *et al.*, 2002) or sheep choroid plexus can be useful, but different lines vary considerably in their  
188 susceptibility to the virus. Ovine cells have been used successfully for the isolation and growth of BD viruses  
189 and BVDV types 1 and 2 from sheep. In regions where sheep may become infected with BVD viruses from  
190 cattle, a virus isolation system using both ovine and bovine cells could be optimal. However, bovine cells have  
191 lower sensitivity for the primary isolation and growth of some BD viruses, so reliance on bovine cells alone is  
192 inadvisable. Details of suitable bovine cell cultures are provided chapter 3.4.7. The precautions outlined in that  
193 chapter for the establishment of cells and medium components that are free from contamination with either  
194 pestiviruses or antibodies, and measures to ensure that the cells are susceptible to a wide range of local field  
195 strains are equally relevant to systems for detection of BDV.

196 From live animals, serum is the most frequently used sample to be tested for the presence of infectious virus.  
197 However, for difficult cases, the most sensitive way to confirm pestivirus viraemia is to wash leukocytes  
198 repeatedly (at least three times) in culture medium before co-cultivating them with susceptible cells in either cell  
199 culture tubes or microplates. After culture for 5–7 days, the cultures should be frozen and thawed once and an  
200 aliquot of diluted culture fluid passaged onto further susceptible cells grown in microplates or on chamber slides  
201 to allow antigen detection by immunocytochemistry. Staining for noncytopathic pestiviruses will usually detect  
202 virus at the end of the primary passage, but to detect slow-growing viruses in poorly permissive cells two  
203 passages are desirable. It is recommended that the culture supernatant used as inoculum for the second  
204 passage is diluted approximately 1/100 in new culture medium because some high titred field isolates will  
205 replicate poorly if passaged undiluted (i.e. at high multiplicity of infection – moi).

206 Tissues should be collected from dead animals in virus transport medium. In the laboratory, the tissues are  
207 ground to give a 10–20% (w/v) suspension, centrifuged to remove debris, and the supernatant passed through  
208 0.45 µm filters. Spleen, lung, thyroid, thymus, kidney, brain, lymph nodes and gut lesions are the best organs  
209 for virus isolation.

210 Semen can be examined for the presence of BDV, but raw semen is strongly cytotoxic and must be diluted,  
211 usually at least 1/10 in culture medium. As the major threat of BDV-infected semen is from PI rams, blood is a  
212 more reliable clinical sample than semen for identifying such animals. There are many variations in virus  
213 isolation procedures. All should be optimised for maximum sensitivity using a standard reference virus  
214 preparation and, whenever possible, recent BDV field isolates. Most of the limitations of virus isolation for the  
215 detection of BDV in serum or blood, tissues or semen can be overcome by the use of a proven, sensitive pan-

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<sup>1</sup> Please consult the WOAHA Web site: <https://www.woaha.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3>

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216 pestivirus reactive real-time RT-PCR. Some laboratories screen samples by real-time RT-PCR and undertake  
217 virus isolation on positive samples to collect BDV strains for future reference or research purposes.

218 For specific technical details of virus isolation procedures, including immunoperoxidase staining, refer to chapter  
219 3.4.7.

## 220 1.2. Nucleic acid detection methods

221 The complete genomic sequences of three BD viruses have been determined and compared with those of other  
222 pestiviruses (Becher *et al.*, 1998; Ridpath & Bolin, 1997). Phylogenetic analysis shows BD viruses to be more  
223 closely related to CSFV than to BVDV (Becher *et al.*, 2003; Van Rijn *et al.*, 1997; Vilcek & Nettleton, 2006; Vilcek  
224 *et al.*, 1997). Real-time RT-PCR for diagnosing pestivirus infection is now used widely and a number of formats  
225 have been described. Real-time RT-PCR assays have the advantages of being able to detect both infectious  
226 virus and residual nucleic acid, the latter being of value for investigating abortions and lamb deaths.  
227 Furthermore, the presence of virus-specific antibodies in a sample will have no adverse effect on the sensitivity  
228 of the real-time RT-PCR assay. These assays are also useful for screening semen and, when recommended  
229 nucleic acid extraction protocols are followed, are less affected by components of the semen compared with  
230 virus isolation. Because of the potential for small ruminants to be infected with genetically different strains of  
231 BDV or with strains of BVDV, a ~~proven~~ pan-pestivirus reactive real-time RT-PCR with proven high sensitivity  
232 should be used. To ensure that the genetic spectrum of BDV strains is sufficiently covered, it may be necessary  
233 to apply a broadly reactive BDV specific real time RT-PCR in parallel to maximise diagnostic sensitivity. Suitable  
234 protocols for both nucleic acid extraction as well as the real-time RT-PCR are described in chapter 3.4.7. All  
235 precautions to minimise laboratory contamination should be followed closely.

236 After testing samples in a pan-pestivirus reactive assay, samples giving ~~positive results~~ any level of reactivity  
237 should be investigated further by the application of a BDV-specific real-time RT-PCR (Willoughby *et al.*, 2006).  
238 It is important to note however that different genotypes of BDV may be circulating in some populations,  
239 especially wild ruminants such as chamois and deer, and may be transferred to sheep. An assay that is specific  
240 for the detection of BDV should be used with some caution as variants or previously unrecognised genotypes  
241 may not be detected, hence the value of initially screening samples with a pan-pestivirus reactive real-time RT-  
242 PCR. Nevertheless, there are also situations where a pan-pestivirus reactive real-time RT-PCR may have lower  
243 analytical sensitivity. Consequently, in any situation where BDV infection is suspected, the application of several  
244 diagnostic methods is recommended. Maternal serology can also play an important role as negative results  
245 should exclude the potential involvement of a pestivirus.

## 246 1.3. Enzyme-linked immunosorbent assay for antigen detection

247 ELISAs for the direct detection of pestivirus antigen in blood and tissues of infected animals have proven to be  
248 extremely useful for the detection of PI animals and the diagnosis of disease. The first ELISA for pestivirus  
249 antigen detection was described for detecting viraemic sheep and was later modified into a double MAbs capture  
250 ELISA for use in sheep and cattle (Entrican *et al.*, 1994). The test is most commonly employed to identify PI  
251 viraemic sheep using washed, detergent-lysed blood leukocytes. The sensitivity is close to that of virus isolation  
252 and it is a practical method for screening large numbers of blood samples. As with virus isolation, high levels of  
253 colostral antibody can mask persistent viraemia. The ELISA is more effective than virus isolation in the presence  
254 of antibody, but may give false-negative results in viraemic lambs younger than 2 months old. The ELISA is  
255 usually not sensitive enough to detect acute BDV infections on blood samples. As well as for testing leukocytes,  
256 the antigen ELISA can also be used on tissue suspensions, especially spleen, from suspected PI sheep and,  
257 as an alternative to immunofluorescence and immunoperoxidase methods, on cell cultures. Several pestivirus  
258 ELISA methods have been published but there are at present no commercially available kits that have been  
259 fully validated for detecting BDV. Prior to use for regulatory purposes, these kits should be validated in the region  
260 where they are to be used to ensure that a wide range of field strains of BDV can be detected and that they are  
261 suitable for the sample types to be tested.

## 262 1.4. Immunohistochemistry

263 Viral antigen demonstration is possible in most of the tissues of PI animals (Braun *et al.*, 2002; Thur *et al.*, 1997)  
264 although this is not a method that is routinely used for diagnostic purposes. This should be done on acetone-  
265 fixed frozen tissue sections (cryostat sections) or paraffin wax embedded samples using appropriate antibodies.  
266 Pan-pestivirus reactive antibodies with NS2-3 specificity are suitable. Tissues with a high amount of viral antigen  
267 are brain, thyroid gland, lung and oral mucosa. Skin biopsies have been shown to be useful for *in-vivo* diagnosis  
268 of persistent BDV infection.

## 269 2. Serological tests

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270 Antibody to BDV is usually detected in sheep sera using VN or an ELISA. The less sensitive agar gel immunodiffusion test  
271 is not recommended. Control positive and negative reference sera must be included in every test. These should give results  
272 within predetermined limits for the test to be considered valid. Single sera can be tested to determine the prevalence of  
273 BDV in a flock, region or country. For diagnosis, however, acute and convalescent sera are the best samples for confirming  
274 acute BDV infection. Repeat sera from one animal should always be tested alongside each other on the same plate to  
275 provide a reliable comparison of titres.

## 276 **2.1. Virus neutralisation test**

277 Due to antigenic diversity among pestiviruses the choice of test virus is difficult (Dekker *et al.*, 1995; Nettleton  
278 *et al.*, 1998). No single strain of BDV is ideal. A local strain that gives the highest antibody titre with a range of  
279 positive sheep sera should be used.

280 Because there are few cytopathogenic strains of BDV available, to achieve optimal analytical sensitivity, it is  
281 more usual to employ a representative local non-cytopathogenic strain and read the assay after  
282 immunoperoxidase staining of the cells. Proven highly sensitive, pestivirus-free sheep cells such as lamb testis  
283 or kidney cells are suitable and can be maintained as cryogenically frozen stocks for use over long periods of  
284 time. The precautions outlined for selection of pestivirus-free medium components are equally applicable to  
285 reagents to be used in VN tests. A recommended procedure follows.

### 286 **2.1.1. Test procedure**

- 287 i) The test sera are heat-inactivated for 30 minutes at 56°C.
- 288 ii) From a starting dilution of 1/4, serial twofold dilutions of the test sera are made in a cell-culture  
289 grade flat-bottomed 96-well microtitre plate, using cell culture medium as diluent. For each sample,  
290 three or four wells are used at each dilution depending on the degree of precision required. Also,  
291 for each sample and at each serum dilution, one well is left without virus to monitor for evidence of  
292 sample toxicity that could mimic viral cytopathology or interfere with virus replication. Control  
293 positive and negative sera should also be included in each batch of tests.
- 294 iii) An equal volume (e.g. 50 µl) of a stock of BDV containing 100 TCID<sub>50</sub> (50% tissue culture infective  
295 dose) is added to each well. A back titration of virus stock is also done in some spare wells to check  
296 the potency of the virus (acceptance limits ~~30-80~~ 300 TCID<sub>50</sub>).
- 297 iv) The plate is incubated for 1 hour at 37°C.
- 298 v) A flask of suitable cells (e.g. ovine testis or kidney cells) is trypsinised and the cell concentration is  
299 adjusted to  $2 \times 10^5$ /ml. 100 µl of the cell suspension is added to each well of the microtitre plate.
- 300 vi) The plate is incubated at 37°C for 4–5 days, either in a 5% CO<sub>2</sub> atmosphere or with the plate sealed.
- 301 vii) The wells are examined microscopically to ensure that there is no evidence of toxicity or cytopathic  
302 effect (CPE), then fixed and stained by immunoperoxidase staining using an appropriate MAb. The  
303 VN titre for each serum is the dilution at which the virus is neutralised in 50% of the wells. This can  
304 be calculated by the Spearman–Kärber or Reed Muench methods. A seronegative animal will show  
305 no neutralisation at the lowest dilution of serum (i.e. 1/4), equivalent to a final dilution of 1/8. For  
306 accurate comparison of antibody titres, and particularly to demonstrate significant (more than  
307 fourfold) changes in titre, samples should be tested in parallel in the same test.
- 308 viii) Occasionally there may be a need to determine whether antibody in a flock is against a virus  
309 belonging to a particular *Pestivirus* serogroup. A differential VN test can be used in which sera are  
310 titrated out against representative viruses from each of the four *Pestivirus* groups, i.e. BDV, BVDV  
311 types 1 and 2, and CSFV. Maximum titre will identify the infecting serotype and the spectrum of  
312 cross-reactivity with the other serotypes will also be revealed.

## 313 **2.2. Enzyme-linked immunosorbent assay**

314 An MAb-capture ELISA for measuring BDV antibodies has been described. Two pan-pestivirus MAbs that detect  
315 different epitopes on the immunodominant nonstructural protein NS 2/3 are used to capture detergent-lysed  
316 cell-culture grown antigen. The results correlate qualitatively with the VN test (Fenton *et al.*, 1991).

### 317 **2.2.1. Antigen preparation**

318 Use eight 225 cm<sup>2</sup> flasks of newly confluent FLM cells; four flasks will be controls and four will be infected.  
319 Wash the flasks and infect four with a 0.01–0.1 m.o.i. of Moredun cytopathic BDV. Allow the virus to  
320 adsorb for 2 hours at 37°C. Add maintenance media containing 2% FBS (free from BDV antibody), and  
321 incubate cultures for 4–5 days until CPE is obvious. Pool four control flask supernatants and separately

322 pool four infected flask supernatants. Centrifuge at 3000 **g** for 15 minutes to pellet cells. Discard the  
323 supernatants. Retain the cell pellets. Wash the flasks with 50 ml of PBS and repeat the centrifugation  
324 step as above. Pool all the control cell pellets in 8 ml PBS containing 1% Nonidet P40 and return 2 ml to  
325 each control flask to lyse the remaining attached cells. Repeat for infected cells. Keep the flasks at 4°C  
326 for at least 2 hours agitating the small volume of fluid on the cells vigorously every 30 minutes to ensure  
327 total cell detachment. Centrifuge the control and infected antigen at 12,000 **g** for 5 minutes to remove  
328 the cell debris. Supernatant antigens are stored at -70°C in small aliquots.

## 329 2.2.2. Test procedure

- 330 i) The two MAbs are diluted to a predetermined dilution in 0.05 M bicarbonate buffer, pH 9.6. All wells  
331 of a suitable ELISA-grade microtitre plate (e.g. Nunc maxisorb, Greiner 129b) are coated overnight  
332 at 4°C.
- 333 ii) After washing three times in PBST, a blocking solution of PBST containing 10% horse serum  
334 (PBSTH) is added to all wells, which are incubated at 37°C for 1 hour.
- 335 iii) The antigen is diluted to a predetermined dilution in PBSTH and alternate rows of wells are coated  
336 with virus and control antigens for 1 hour at 37°C. The plates are then washed three times in PBST  
337 before addition of test sera.
- 338 iv) Test sera are diluted 1/50 in PBSTH and added to duplicate virus and duplicate control wells for 1  
339 hour at 37°C. The plates are then washed three times in PBST.
- 340 v) Anti-ovine IgG peroxidase conjugate is diluted to a predetermined dilution in PBSTH and added to  
341 all wells for 1 hour at 37°C. The plates are washed three times in PBST.
- 342 vi) A suitable activated enzyme substrate/chromogen, such as ortho-phenylene diamine (OPD) or  
343 tetramethyl benzidine (TMB), is added). After colour development, the reaction is stopped with  
344 sulphuric acid and the absorbance read on an ELISA plate reader. The mean value of the two  
345 control wells is subtracted from the mean value of the two virus wells to give the corrected  
346 absorbance for each serum. Results are expressed as corrected absorbance with reference to the  
347 corrected absorbance of known positive and negative sera. Alternatively, ELISA titres can be  
348 extrapolated from a standard curve of a dilution series of a known positive reference serum.
- 349 If antigens of sufficient potency can be produced the MAb capture stage can be omitted. In this  
350 case alternate rows of wells are coated with virus and control antigen diluted to a predetermined  
351 dilution in 0.05 M bicarbonate buffer, pH 9.6, overnight at +4°C. The plates are washed and blocked  
352 as in step ii above. After washing, diluted test sera are added and the test proceeds from step iv as  
353 above.

## 354 C. REQUIREMENTS FOR VACCINES

### 355 1. Background

356 To be useful, a BDV vaccine should be effective when administered to female sheep before breeding to prevent  
357 transplacental infection. Experimental and commercial inactivated whole virus BDV vaccines have been produced in  
358 Europe (Brun *et al.*, 1993; Vantsis *et al.*, 1980). Unlike vaccines for BVDV, there is limited demand for vaccines against  
359 BDV and those produced have only been inactivated products. No live attenuated or recombinant subunit vaccines for  
360 BDV have been produced commercially.

361 Pestivirus contaminants of modified live virus vaccines have been found to be a cause of serious disease following their  
362 use in pigs, cattle, sheep and goats. Contaminated vaccines have included those used for the control of Aujeszky's disease,  
363 CSF, rotavirus, coronavirus, rinderpest, sheep pox and contagious pustular dermatitis. The insidious ability of pestiviruses  
364 to cross the placenta, and thus establish PI animals, gives them the potential to contaminate vaccines through cells, serum  
365 used as medium supplement, or seed stock virus. As nearly all isolates of pestiviruses are noncytopathic, they will remain  
366 undetected unless specific tests are carried out. Although such contamination should be less likely to be a problem with  
367 an inactivated vaccine, nevertheless steps should be taken to ensure that materials used in production are not  
368 contaminated.

#### 369 1.1. Characteristics of a target product profile

370 Traditionally, pestivirus vaccines fall into two classes: modified live or inactivated virus vaccines. The essential  
371 requirement for both types is to afford provide a high level of fetal infection. Only inactivated vaccines have been  
372 produced for BDV. Properly formulated inactivated vaccines are very safe to use but, to obtain satisfactory levels

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373 of immunity, they usually require booster vaccinations, which may be inconvenient. Because of the propensity  
374 for antigenic variability, the vaccine should contain strains of BDV that are closely matched to viruses found in  
375 the area in which they are used. This may present particular challenges with BDV in regions where several  
376 antigenic types have been found. Due to the need to customise vaccines for the most commonly encountered  
377 strains within a country or region, it is not feasible to produce a vaccine antigen bank that can be drawn upon  
378 globally.

379 Guidance for the production of veterinary vaccines is given in Chapter 1.1.8 *Principles of veterinary vaccine*  
380 *production*. The guidelines given here and in chapter 1.1.8 are intended to be general in nature and may be  
381 supplemented by national and regional requirements.

## 382 2. Outline of production and minimum requirements for vaccines

### 383 2.1. Characteristics of the seed

384 An ideal vaccine should contain a strain or strains of virus that give protection against all sheep pestiviruses.  
385 This may be challenging however, because of the range of pestiviruses with which sheep can be infected. There  
386 is considerable antigenic variation across these viruses – both between viruses that have been classified in the  
387 BDV genogroup as well as between viruses in the BVDV1 and BVDV2 genotypes (Becher *et al.*, 2003; Vilcek &  
388 Nettleton, 2006; Wensvoort *et al.*, 1989). Infection of sheep with the putative BVDV-3 genotype has also been  
389 described (Decaro *et al.*, 2012). It is likely that the antigenic composition of a vaccine will vary from region to  
390 region to provide an adequate antigenic match with dominant virus strains. Cross-neutralisation studies are  
391 required to establish optimal combinations. Nevertheless, it would appear that any BDV vaccine should contain  
392 at least a representative of the BDV and BVDV (type 1) groups. Characterisation of the biologically cloned  
393 vaccine viruses should include typing with MAbs and genotyping (Paton *et al.*, 1995).

#### 394 2.1.1. Quality criteria (sterility, purity, freedom from extraneous agents)

395 It is crucial to ensure that all materials used in the preparation of the bulk antigens have been extensively  
396 screened to ensure freedom from extraneous agents. This should include master and working seeds,  
397 the cell cultures and all medium supplements such as bovine serum. Some bovine viruses and  
398 particularly BVDV can readily infect small ruminants such as sheep. Therefore, it is particularly important  
399 to ensure that any serum used that is of bovine origin is free of both adventitious BVDV and antibodies  
400 against BVDV strains because low levels of either virus or antibody can mask the presence of the other.  
401 Materials and vaccine seeds should be tested for sterility and freedom from contamination with other  
402 agents, especially viruses as described in the chapter 1.1.8 and Chapter 1.1.9 *Tests for sterility and*  
403 *freedom from contamination of biological materials intended for veterinary use*.

404 If a vaccine passes basic tests, the efficacy of vaccination should ultimately be measured by the capacity  
405 to prevent transplacental transmission. Effective challenge of vaccinated pregnant ewes at 50–60 days  
406 gestation has been achieved by intranasal installation of virus or by mixing with PI sheep (Brun *et al.*,  
407 1993). Usually this reliably produces persistently viraemic offspring in non-immune ewes. In regions  
408 where multiple genotypes of BDV viruses are commonly encountered, efficacy in protecting against  
409 multiple strains should be measured.

### 410 2.2. Method of manufacture

#### 411 2.2.1. Procedure

412 Inactivated vaccines have been prepared using conventional laboratory techniques with stationary or  
413 rolled cell cultures. Inactivants have included formalin and beta-propiolactone. Adjuvants have included  
414 aluminium hydroxide and oil (Brun *et al.*, 1993; Vantsis *et al.*, 1980). Optimal yields depend on the cell  
415 type and isolate used. A commercial BDV vaccine containing two strains of virus has been prepared on  
416 ovine cell lines (Brun *et al.*, 1993). Cells must be produced according to a seed-lot system from a master  
417 cell seed (MCS) that has been shown to be free from all contaminating microorganisms. Vaccine should  
418 only be produced in cells fewer than 20 passages from the MCS. Control cells from every passage should  
419 be checked for pestivirus contamination. Standard procedures may be used, with the expectation for  
420 harvesting noncytopathic virus on days 4–7 after inoculation of cultures. The optimal yield of infectious  
421 virus will depend on several factors, including the cell culture, isolate used and the initial seeding rate of  
422 virus. These factors should be taken into consideration and virus replication kinetics investigated to  
423 establish the optimal conditions for large-scale virus production. Whether a live or inactivated vaccine,  
424 the essential aim will be to produce a high-titred virus stock. This bulk antigen preparation can  
425 subsequently be prepared according to the type of vaccine being considered.



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### 2.2.2. Requirements for ingredients

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BDV vaccines have usually been grown in cell cultures of ovine origin that are frequently supplemented with medium components of animal origin. The material of greatest concern is bovine serum due to the potential for contamination with BVD viruses and antibodies to these viruses. These adventitious contaminants not only affect the efficiency of production but also may mask the presence of low levels of infectious BVDV that may have undesirable characteristics. In addition to the virus seeds, all materials should be tested for sterility and freedom from contamination with other agents, especially viruses as described in chapters 1.1.8 and 1.1.9. Furthermore, materials of bovine or ovine origin should originate from a country with negligible risk for transmissible spongiform encephalopathies (see chapter 1.1.9).

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### 2.2.3. In-process controls

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In-process controls are part of the manufacturing process. Cultures should be inspected regularly to ensure that they remain free from gross bacterial contamination, and to monitor the health of the cells and the development or absence of CPE, as appropriate. While the basic requirement for efficacy is the capacity to induce an acceptable neutralising antibody response, during production, target concentrations of antigen required to achieve an acceptable response may be monitored indirectly by assessment of the quantity of infectious virus or antigen mass that is produced. Rapid diagnostic assays such as the ELISA are useful for monitoring BVDV antigen production. Alternatively, the quality of a batch of antigen may be determined by titration of the quantity of infectious virus present, although this may underestimate the quantity of antigen. For inactivated vaccines, infectivity is evaluated before inactivation. For inactivated vaccines the inactivation kinetics should be established so that a suitable safety margin can be determined and incorporated into the routine production processes. At the end of production, *in-vitro* cell culture assays should be undertaken to confirm that inactivation has been complete. These innocuity tests should include a sufficient number of passages and volume of inoculum to ensure that very low levels of infectious virus would be detected if present.

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### 2.2.4. Final product batch tests

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#### i) Sterility

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Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9.

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#### ii) Identity

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Identity tests should demonstrate that no other strain of BDV is present when several strains are propagated in a facility producing multivalent vaccines.

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#### iii) Safety

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Samples from inactivated vaccines should be tested rigorously for viable virus. Samples of the product should be passaged for a minimum of three passages in sensitive cell cultures to ensure absence of live BDV. This *in-vitro* monitoring can be augmented by injecting two BDV-seronegative sheep with 20 doses of unformulated antigen as part of a standard safety test. Presence of live virus will result in the development of a more convincing serological response than will occur with inactivated virus alone. The sheep sera can also be examined for antibody to other prescribed agents.

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Safety tests shall also consist of detecting any abnormal local or systemic adverse reactions to the vaccine by all vaccination route(s). Batch-to-batch safety tests are required unless safety of the product is demonstrated and approved in the registration dossier and production is consistent with that described in chapter 1.1.8. Vaccines must either be demonstrated to be safe in pregnant sheep (i.e. no transmission to the fetus), or should be licensed with a warning not to use them in pregnant animals.

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#### iv) Batch potency

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Vaccine potency is best tested in seronegative sheep in which the development and level of antibody is measured. BVD vaccines must be demonstrated to produce adequate immune responses when used in their final formulation according to the manufacturer's published instructions. The minimum quantity of infectious virus or antigen required to produce an acceptable immune response should be determined. An indirect measure of potency is given by the level of virus infectivity prior to inactivation. *In-vitro* assays should be used to monitor individual batches during production. The antigen content following inactivation can be assayed by MAb-capture ELISA and related to the results of established *in-vivo* potency results. It should be demonstrated

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480 that the lowest recommended dose of vaccine can prevent transplacental transmission of BDV in  
481 pregnant sheep.

## 482 **2.3. Requirements for authorisation/registration/licensing**

### 483 **2.3.1. Manufacturing process**

484 For registration of a vaccine, all relevant details concerning manufacture of the vaccine and quality  
485 control testing should be submitted to the relevant authorities. Unless otherwise specified by the  
486 authorities, information should be provided from three consecutive vaccine batches with a volume not  
487 less than 1/3 of the typical industrial batch volume.

488 There is no standard method for the manufacture of a BDV vaccine, but conventional laboratory  
489 techniques with stationary, rolled or suspension (micro-carriers) cell cultures may be used. Inactivated  
490 vaccines can be prepared by conventional methods, such as binary ethylenimine, formalin or beta-  
491 propiolactone inactivation (Park & Bolin, 1987). A variety of adjuvants may be used.

### 492 **2.3.2. Safety requirements**

493 *In-vivo* tests should be undertaken using repeat doses (taking into account the maximum number of  
494 doses for primary vaccination and, if appropriate, the first revaccination/booster vaccination) and contain  
495 the maximum permitted antigen load and, depending on the formulation of the vaccine, the maximum  
496 number of vaccine strains.

#### 497 i) Target and non-target animal safety

498 The safety of the final product formulation of inactivated vaccines should be assessed in susceptible  
499 young sheep that are free of maternally derived antibodies and in pregnant ewes. They should be  
500 checked for any local reactions following administration, and, in pregnant ewes, for any effects on  
501 the unborn lamb.

#### 502 ii) Reversion-to-virulence for attenuated/live vaccines and environmental considerations

503 In the event that a live virus vaccine was developed for BDV, virus seeds that have been passaged  
504 at least up to and preferably beyond the passage limit specified for the seed should be inoculated  
505 into young lambs to confirm that there is no evidence of disease. If a live attenuated vaccine has  
506 been registered for use in pregnant animals, reversion to virulence tests should also include  
507 pregnant animals. Live attenuated vaccines should not be transmissible to unvaccinated 'in-contact'  
508 animals.

#### 509 iii) Precautions (hazards)

510 BDV is not considered to be a human health hazard. Standard good microbiological practice should  
511 be adequate for handling the virus in the laboratory. While the inactivated virus in a vaccine should  
512 be identified as harmless for people administering the product, adjuvants included in the vaccine  
513 may cause injury to people. Manufacturers should provide adequate warnings that medical advice  
514 should be sought in the case of self-injection (including for adjuvants, oil-emulsion vaccine,  
515 preservatives, etc.) with warnings included on the product label/leaflet so that the vaccinator is  
516 aware of any danger.

### 517 **2.3.3. Efficacy requirements**

518 The potency of the vaccine should be determined by inoculation into seronegative and virus negative  
519 lambs, followed by monitoring of the antibody response. Antigen content can be assayed by infectivity  
520 titration prior to inactivation and subsequently by ELISA and adjusted as required to a standard level for  
521 the particular vaccine. Standardised assay protocols applicable to all vaccines do not exist. Live vaccine  
522 batches may be assayed by infectivity titration. Each production batch of vaccine should undergo potency  
523 and safety testing as batch release criteria. BVD vaccines must be demonstrated to produce adequate  
524 immune responses, as outlined above, when used in their final formulation according to the  
525 manufacturer's published instructions.

### 526 **2.3.4. Vaccines permitting a DIVA strategy (detection of infection in vaccinated animals)**

527 To date, there are no commercially available vaccines for BDV that support use of a true DIVA strategy.

### 528 **2.3.5. Duration of immunity**

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529 Inactivated vaccines are unlikely to provide sustained levels of immunity and it is likely that after an initial course  
530 of two or three injections annual booster doses may be required. Insufficient information is available to determine  
531 any correlation between vaccinal antibody titres in the dam and fetal protection. As there are likely to be different  
532 commercial formulations and these involve a range of adjuvants, there are likely to be different periods of  
533 efficacy. Consequently, duration of immunity data must be generated separately for each commercially available  
534 product by undertaking challenge tests at the end of the period for which immunity has been claimed.

### 535 **2.3.6. Stability**

536 There are no accepted guidelines for the stability of BDV vaccines, but it can be assumed that an inactivated  
537 virus vaccine should remain potent for at least 1 year if kept at 4°C and probably longer. Lower temperatures  
538 could prolong shelf life but adjuvants in a killed vaccine may preclude this. Bulk antigens that have not been  
539 formulated into finished vaccine can be reliably stored frozen at low temperatures, but the antigen quality should  
540 be monitored with *in-vitro* assays prior to incorporation into a batch of vaccine.

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612 **NB:** At the time of publication (2017) there were no WOAHA Reference Laboratories  
613 for border disease (please consult the WOAHA Web site:  
614 <https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3http://www.oie.int/>).

615 **NB:** FIRST ADOPTED IN 1996. MOST RECENT UPDATES ADOPTED IN 2017.

REUNIÓN DE LA COMISIÓN DE NORMAS BIOLÓGICAS

París, 5–9 de febrero de 2024

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CHAPTER 3.8.12.

SHEEP POX AND GOAT POX

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SUMMARY

Sheep pox and goat pox are contagious, viral diseases of sheep and goats characterised by fever, generalised papules or nodules, vesicles (rarely), internal lesions (particularly in the lungs), and death. Both diseases are caused by strains of capripoxvirus, all of which can infect sheep and goats. Although most of the strains examined cause more severe clinical disease in either sheep or goats, some strains have been isolated that are equally pathogenic in both species.

Sheeppox virus (SPPV) and goatpox virus (GTPV) are the causative agents of sheep pox and goat pox, and with lumpy skin disease virus (LSDV) make up the genus Capripoxvirus in the family Poxviridae. Sheep pox and goat pox are endemic in Africa north of the Equator, the Middle East and Asia, while some parts of Europe have experienced outbreaks recently. See WAHIS (<https://wahis.woah.org/#/home>) for recent information on distribution at the country level. Countries that reported outbreaks of the disease between 2010 and 2015 include Bulgaria, Chinese Taipei, Israel, Kazakhstan, Kyrgyzstan, Mongolia, Morocco, Greece and Russia, with Greece, Israel and Russia having experienced recurring incidences.

**Identification of the agent:** Laboratory confirmation of capripoxvirus is most rapid using the polymerase chain reaction (PCR) method in combination with a clinical history consistent with generalised capripoxvirus infection. Isolation of the virus is possible as capripoxviruses will grow on tissue culture of ovine, caprine or bovine origin, although field isolates may require up to 14 days to grow or require one or more additional tissue culture passage(s). The virus causes intracytoplasmic inclusions that can be clearly seen using haematoxylin and eosin staining. The antigen can also be detected in tissue culture using specific sera and immunoperoxidase or immunofluorescence techniques. Capripoxvirus antigen and inclusion bodies may be seen in stained cryostat or paraffin sections of biopsy or post-mortem lesion material.

~~An antigen detection enzyme-linked immunosorbent assay (ELISA) using a polyclonal detection serum raised against a recombinant immunodominant antigen of capripoxvirus has been developed.~~

**Serological tests:** The virus neutralisation test is the most specific serological test. The indirect immunofluorescence test is less specific due to cross-reactions with antibody to other poxviruses. Western blotting using the reaction between the P32 antigen of capripoxvirus with test sera is both sensitive and specific, but is expensive and difficult to carry out. An enzyme-linked immunosorbent assay (ELISA) has been developed and validated to detect antibodies to capripoxviruses, however it cannot differentiate between SPPV, GTPV and LSDV.

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32 *The use of this antigen, or other appropriate antigens expressed by a suitable vector, in an ELISA offers the*  
33 *prospect of an acceptable and standardised serological test in the future.*

34 **Requirements for vaccines:** *Live and inactivated vaccines have been used for the control of*  
35 *capripoxviruses. All strains of capripoxvirus so far examined share a major neutralisation site and some will*  
36 *cross protect. Inactivated vaccines give, at best, only short-term immunity.*

## 37 A. INTRODUCTION

38 The *Capripoxvirus* genus, in the family *Poxviridae*, consists of three species – lumpy skin disease virus (LSDV), which  
39 causes disease in cattle only (see Chapter 3.4.12), and sheeppox virus (SPPV) and goatpox virus (GTPV), which cause  
40 sheep pox and goat pox, respectively. Sheep pox and goat pox are characterised by disseminated cutaneous nodules and  
41 up to 100% mortality in fully susceptible breeds-naïve of sheep and goats. In indigenous animals, generalised disease and  
42 mortality are less common, although they are seen where disease has been absent from an area or village for a period of  
43 time, when intensive husbandry methods are introduced, or in association with other disease agents, such as peste des  
44 petits ruminants virus or foot and mouth disease virus. Sheep pox and goat pox are major constraints to the introduction  
45 of exotic breeds of sheep and goats to endemic areas, and to the development of intensive livestock production.

46 Strains of SPPV and GTPV can pass between sheep and goats, although most cause more severe clinical disease in only  
47 one their homologous host species. SPPV and GTPV are transboundary diseases that regularly spread into adjacent, non-  
48 endemic areas. Sheep pox and goat pox are endemic in Africa north of the Equator and parts of the Middle East and Asia  
49 (see WAHIS for most up-to-date information on distribution: <https://wahis.woah.org/#/home>). Outbreaks have been  
50 reported in non-endemic countries of Asia, Europe and the Middle East.

51 The incubation period of sheep pox and goat pox is between 8 and 13 days following contact between infected and  
52 susceptible animals. It may be as short as 4 days following experimental infection by intradermal inoculation ~~or mechanical~~  
53 ~~transmission by insects~~. Some breeds of European sheep, such as Soay, may die of acute infection before the  
54 development of skin lesions. In other breeds there is an initial rise in rectal temperature to above 40°C, followed in 2–5  
55 days by the development of, at first, macules – small circumscribed areas of hyperaemia, which are most obvious on  
56 unpigmented skin – and then of papules – hard swellings of between 0.5 and 1 cm in diameter – which may cover the body  
57 or be restricted to the groin, axilla and perineum. Papules may be covered by fluid-filled vesicles, but this is rare. Some  
58 researchers have distinguished between a vesicular and nodular form of sheep pox and goat pox (Zro *et al.*, 2014b).

59 Within 24 hours of the appearance of generalised papules, affected animals develop rhinitis, conjunctivitis and enlargement  
60 of all the superficial lymph nodes, in particular the prescapular lymph nodes. Papules on the eyelids cause blepharitis of  
61 varying severity. As the papules on the mucous membranes of the eyes and nose ulcerate, so the discharge becomes  
62 mucopurulent, and the mucosae of the mouth, anus, and prepuce or vagina become necrotic. Breathing may become  
63 laboured and noisy due to pressure on the upper respiratory tract from the swollen retropharyngeal lymph nodes, due to  
64 the developing lung lesions.

65 If the affected animal does not die in this acute phase of the disease, the papules start to become necrotic from ischaemic  
66 necrosis following thrombi formation in the blood vessels at the base of the papule. In the following  
67 5–10 days the papules form scabs, which persist for up to 6 weeks, leaving small scars. The skin lesions are susceptible  
68 to fly strike, and secondary pneumonia is common. Anorexia is not usual unless the mouth lesions physically interfere with  
69 feeding. Abortion is rare.

70 On post-mortem examination of the acutely infected animal, the skin lesions are often less obvious than on the live animal.  
71 The mucous membranes appear necrotic and all the body lymph nodes are enlarged and oedematous. Papules, which  
72 may be ulcerated, can usually be found on the abomasal mucosa, and sometimes on the wall of the rumen and large  
73 intestine, on the tongue, hard and soft palate, trachea and oesophagus. Pale areas of approximately 2 cm in diameter may  
74 occasionally be seen on the surface of the kidney and liver, and have been reported to be present in the testicles. Numerous  
75 hard lesions of up to 5 cm in diameter are commonly observed throughout the lungs, but particularly in the diaphragmatic  
76 lobes.

77 The clinical signs and post-mortem lesions vary considerably with breed of host and strain of capripoxvirus. Indigenous  
78 breeds are less susceptible and frequently show only a few lesions, which could be confused with insect bites or contagious  
79 pustular dermatitis. However, lambs that have lost their maternally derived immunity, animals that have been kept isolated  
80 and animals brought into endemic areas from isolated villages, particularly if they have been subjected to the stress of  
81 moving long distances and mixing with other sheep and goats, and their pathogens, can often be seen with generalised  
82 and sometimes fatal capripoxvirus infections. Invariably there is high mortality in unprotected imported breeds of sheep  
83 and goats following capripoxvirus infection. Surviving animals clear the infection, as there is no evidence of persistently

84 infected animals. Capripoxvirus is not infectious to humans. Capripoxvirus is inactivated at 56°C for 2 hours or 65°C for 30  
 85 minutes. The virus survives between pH 6.6–8.6. It is susceptible to highly alkaline or acid pH. The virus is sensitive to  
 86 various chemicals: sodium dodecyl sulphate, ether 20%, chloroform, formalin 1%, sodium 2%, iodine compounds, Virkon  
 87 2%, quaternary ammonium (0.5%), and phenol 2% for 15 minutes.

## 88 B. DIAGNOSTIC TECHNIQUES

89 **Table 1. Test methods available for diagnosis of sheep pox and goat pox and their purpose**

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Identification of the agent <sup>(a)</sup>						
Virus isolation	+	++	+	+++	+	–
Antigen detection	++	++	++	++	++	–
<u>IFAT</u>	±	±	±	++	±	≡
<u>IHC</u>	±	±	±	++	±	≡
PCR	++	+++	++	+++	++	–
Detection of immune response						
<u>VNI</u>	++	++	++	++	++	++
IFAT	+	+	+	+	+	+
<u>ELISA</u>	++	++	++	++	++	++

90 Key: +++ = recommended for this purpose; ++ recommended but has limitations;

91 + = suitable in very limited circumstances; – = not appropriate for this purpose.

92 IFAT = indirect fluorescent antibody test; IHC = immunohistochemistry; PCR = polymerase chain reaction;

93 VNI = virus neutralisation; ELISA = enzyme-linked immunosorbent assay.

94 <sup>(a)</sup>A combination of agent identification methods applied on the same clinical sample is recommended.

### 95 1. Identification of the agent

#### 96 1.1. Specimen collection and submission

97 Material for virus isolation and antigen detection should be collected by biopsy or at post-mortem from skin  
 98 papules, lung lesions or lymph nodes. Samples for virus isolation and antigen detection ~~enzyme-linked~~  
 99 ~~immunosorbent assay (ELISA)~~ should be collected within the first week of the occurrence of clinical signs, before  
 100 the development of neutralising antibodies. Samples for genome detection by polymerase chain reaction (PCR)  
 101 may be collected before or after the development of neutralising antibody responses. In addition to epithelial  
 102 lesions, nasal and buccal swabs can be collected because the virus will be present in nasal and saliva  
 103 discharges. Buffy coat from blood collected into EDTA (ethylene diamine tetra-acetic acid) during the viraemic  
 104 stage of capripoxvirus infection (before generalisation of lesions or within 4 days of generalisation), can also be  
 105 used for virus isolation.

106 Samples for histology should include tissue from the surrounding area and should be placed immediately  
 107 following collection into ten times the sample volume of 10% formalin or neutral buffered 10% formal saline.  
 108 Tissues in formalin have no special transportation requirements.

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109 Blood samples, for virus isolation from the buffy coat, should be collected in tubes containing anticoagulant,  
110 placed immediately on ice and processed as soon as possible. In practice, the blood samples may be kept at  
111 4°C for up to 2 days prior to processing, but should not be frozen or kept at ambient temperatures. Tissues and  
112 dry scabs for virus isolation, **antigen detection** and genome detection should preferably be kept at 4°C, on ice  
113 or at -20°C. If it is necessary to transport samples over long distances without refrigeration, the medium should  
114 contain 10% glycerol; the samples should be of sufficient size that the transport medium does not penetrate the  
115 central part of the biopsy, which should be used for virus isolation/detection.

## 116 1.2. Virus isolation

117 Lesion material for virus isolation and **genome antigen** detection is homogenised. The following is an example  
118 of one technique for homogenisation: The tissue is minced using sterile scissors and forceps, and then  
119 macerated in a steel ball bearing mixer mill or ground with a sterile pestle in a mortar with sterile sand and an  
120 equal volume of sterile phosphate buffered saline (PBS) or serum-free Modified Eagle's Medium (MEM)  
121 containing sodium penicillin (1000 international units [IU]/ml), streptomycin sulphate (1 mg/ml), mycostatin  
122 (100 IU/ml) or fungizone (2.5 µg/ml) and neomycin (200 IU/ml). The homogenised suspension is freeze-thawed  
123 three times and then partially clarified by centrifugation using a bench centrifuge at 600 **g** for 10 minutes. In  
124 cases where bacterial contamination of the sample is expected (such as when virus is isolated from skin  
125 samples), the supernatant can be filtered through a 0.45 µm pore size filter after the centrifugation step,  
126 however, the amount of virus in the supernatant might be reduced. Buffy coats may be prepared from 5–8 ml  
127 unclotted blood by centrifugation at 600 **g** for 15 minutes; the buffy coat is carefully removed into 5 ml of cold  
128 double-distilled water using a sterile Pasteur pipette. After 30 seconds, 5 ml of cold double-strength growth  
129 medium is added and mixed. The mixture is centrifuged at 600 **g** for 15 minutes, the supernatant is discarded  
130 and the cell pellet is suspended in 5 ml of growth medium, such as Glasgow's modified Eagle's medium  
131 (GMEM). After centrifugation at 600 **g** for a further 15 minutes, the resulting pellet is suspended in 5 ml of fresh  
132 GMEM. Alternatively, the buffy coat may be separated from a heparinised sample using a density gradient.

133 Capripoxvirus will grow in tissue culture of bovine, ovine or caprine origin, although primary or secondary  
134 cultures of lamb testis (LT) or lamb kidney (LK) cells are considered to be the most susceptible. Care needs to  
135 be taken to ensure they are not contaminated with viruses such as bovine viral diarrhoea virus, particularly those  
136 derived from a wool sheep breed (see chapter 1.1.9). Madin-Darby bovine kidney (MDBK) cells have been  
137 shown to be suitable for capripoxvirus isolation (Fay et al., 2020). The following is an example of an isolation  
138 technique: either 1 ml of buffy coat cell suspension or 1 ml of clarified biopsy preparation supernatant is  
139 inoculated on to a 25 cm<sup>2</sup> tissue culture flask of appropriate cells at 90% confluent LT or LK cells confluence,  
140 and the supernatant is allowed to adsorb for 1 hour at 37°C. The culture is then washed with warm PBS and  
141 covered with 10 ml of a suitable medium, such as GMEM, containing antibiotics and 2% fetal calf serum. If  
142 available, tissue culture tubes containing LT or LK cells and a, flying cover-slips, or tissue culture microscope  
143 slides, are can also be infected.

144 The flasks should be examined daily for 7–14 days for evidence of cytopathic effect (CPE). Contaminated flasks  
145 should be discarded. Infected cells develop a characteristic CPE consisting of retraction of the cell membrane  
146 from surrounding cells, and eventually rounding of cells and margination of the nuclear chromatin. At first only  
147 small areas of CPE can be seen, sometimes as soon as 4 days after infection; over the following 4–6 days these  
148 expand to involve the whole cell sheet. If no CPE is apparent by day 7, the culture should be freeze-thawed  
149 three times, and clarified supernatant inoculated on to fresh LT or LK cell cultures. At the first sign of CPE in the  
150 flasks, or earlier if a number of infected cover-slips are being used, a cover-slip should be removed, fixed in  
151 acetone and stained using H&E. Eosinophilic intracytoplasmic inclusion bodies, which are variable in size but  
152 up to half the size of the nucleus and surrounded by a clear halo, are indicative of poxvirus infection. Syncytia  
153 formation is not a feature of capripoxvirus infection. If the CPE is due to capripoxvirus infection of the cell culture,  
154 it can be prevented or delayed by inclusion of specific anti-capripoxvirus serum in the medium; this provides a  
155 presumptive identification of the agent. Some strains of capripoxvirus have been adapted to grow on African  
156 green monkey kidney (Vero) cells, but these cells are not recommended for primary isolation.

## 157 1.3. Electron microscopy

158 The characteristic poxvirus virion can be visualised using a negative-staining preparation technique followed by  
159 examination with an electron microscope. There are many different negative-staining protocols, an example is  
160 given below:

161 Material from the original tissue suspension is prepared for transmission electron microscope examination, prior  
162 to centrifugation, by floating a 400-mesh hexagon electron microscope grid, with piliform-carbon substrate  
163 activated by glow discharge in pentylamine vapour, on to a drop of the suspension placed on parafilm or a wax  
164 plate. After 1 minute, the grid is transferred to a drop of Tris/EDTA buffer, pH 7.8, for 20 seconds and then to a  
165 drop of 1% phosphotungstic acid, pH 7.2, for 10 seconds. The grid is drained using filter paper, air-dried and



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166 placed in the electron microscope. The capripoxvirus virion is brick shaped, covered in short tubular elements  
167 and measures approximately 290 × 270 nm. A host-cell-derived membrane may surround some of the virions,  
168 and as many as possible should be examined to confirm their appearance (Kitching & Smale, 1986).

169 The virions of capripoxvirus are indistinguishable from those of orthopoxvirus, but, apart from *Vaccinia* virus, no  
170 orthopoxvirus causes lesions in sheep and goats. However, capripoxvirus is distinguishable from the virions of  
171 parapoxvirus, that cause contagious pustular dermatitis, as they are smaller, oval in shape, and each is covered  
172 in a single continuous tubular element, which appears as striations over the virion.

#### 173 1.4. Histopathology

174 Material for histopathology and immunohistochemistry should be prepared by standard techniques (Parvin *et*  
175 *al.*, 2022). Following preparation, and staining with haematoxylin and eosin (H&E), and mounting of the formalin-  
176 fixed biopsy material, a number of sections should be examined by light microscopy. On histological  
177 examination, the most striking aspects of acute-stage skin lesions are a massive cellular infiltrate, vasculitis and  
178 oedema. Early lesions are characterised by marked perivascular cuffing. Initially infiltration is by macrophages,  
179 neutrophils and occasionally eosinophils, and as the lesion progresses, by more macrophages, lymphocytes  
180 and plasma cells. A characteristic feature of all capripoxvirus infections is the presence of variable numbers of  
181 'sheep pox cells' in the dermis. These sheep pox cells can also occur in other organs where microscopic lesions  
182 of sheep pox and goat pox are present. These cells are large, stellate cells with eosinophilic, poorly defined  
183 intracytoplasmic inclusions and vacuolated nuclei. Vasculitis is accompanied by thrombosis and infarction,  
184 causing oedema and necrosis. Epidermal changes consist of acanthosis, parakeratosis and hyperkeratosis.  
185 Changes in other organs are similar, with a predominant cellular infiltration and vasculitis. Lesions in the upper  
186 respiratory tract are characterised by ulceration.

187 Immunohistochemistry will show capripox virus antigen infiltrated macrophages throughout the subcutis. The  
188 capripox virus antigen can occasionally be detected in hair follicle epithelial cells, the endothelium and smooth  
189 muscle cells of the blood vessels, and histiocytic cells (Parvin *et al.*, 2022).

#### 190 1.5. Immunological methods

##### 191 1.5.1. Fluorescent antibody tests

192 Capripoxvirus antigen can also be identified on infected cover-slips or tissue culture slides using  
193 fluorescent antibody tests. Cover-slips or slides should be washed and air-dried and fixed in cold acetone  
194 for 10 minutes. The indirect test using immune sheep or goat sera is subject to high background colour  
195 and nonspecific reactions. However, a direct conjugate can be prepared from sera from convalescent  
196 sheep or goats or from rabbits hyperimmunised with purified *Capripoxvirus*. Uninfected tissue culture  
197 should be included as a negative control because cross-reactions, due to antibodies to cell culture  
198 antigens, can cause problems. The fluorescent antibody tissue section technique has also been used on  
199 cryostat-prepared slides.

#### 200 1.6. Nucleic acid recognition methods

201 Amplification methods for detection of ~~the viral DNA genome are specific to the genus *Capripoxvirus*~~ DNA are  
202 ~~and both specific and sensitive for detection~~ throughout the course of disease, including before and after the  
203 emergence of antibody responses. These methods include conventional PCR, real-time PCR, and most recently  
204 loop-mediated isothermal amplification (LAMP). Nucleic acid recognition methods can be used to detect the  
205 *Capripoxvirus* genome in biopsy, swab, blood, semen or tissue culture samples. It is important that nucleic acid  
206 extraction and PCR amplification methods are validated for the sample matrix being tested.

##### 207 1.6.1. Conventional PCR methods

208 Several conventional PCR methods have been reported with varying specificity for capripoxviruses in  
209 general, SPPV, or GTPV (Heine *et al.*, 1999; Ireland & Binopal, 1998; Zro *et al.*, 2014a). A conventional  
210 PCR assay that differentiates GTPV and LSDV from SPPV has been described (Lamien *et al.*, 2011a).  
211 Conventional PCR methods are particularly useful for obtaining sufficient genetic material necessary for  
212 species identification by subsequent sequence and phylogenetic analysis (Le Goff *et al.*, 2009).

213 The conventional gel-based PCR method described below is a simple, fast and sensitive method for the  
214 detection of capripoxvirus genome in EDTA blood, semen or tissue culture samples (Tuppurainen *et al.*,  
215 2005).

- 216 Test procedure
- 217 The extraction method described below can be replaced using commercially available DNA extraction
- 218 kits.
- 219 i) Freeze and thaw 200 µl of blood in EDTA, semen or tissue culture supernatant and suspend in
- 220 100 µl of lysis buffer containing 5 M guanidine thiocyanate, 50 mM potassium chloride, 10 mM
- 221 Tris/HCl (pH 8); and 0.5 ml Tween 20.
- 222 ii) Cut skin and other tissue samples into fine pieces using a sterile scalpel blade and forceps. Grind
- 223 with a pestle in a mortar. Suspend the tissue samples in 800 µl of the above mentioned lysis buffer.
- 224 iii) Add 2 µl of proteinase K (20 mg/ml) to blood samples and 10 µl of proteinase K (20 mg/ml) to tissue
- 225 samples. Incubate at 56°C for 2 hours or overnight, followed by heating at 100°C for 10 minutes.
- 226 Add phenol:chloroform:isoamylalcohol (25:24:1 [v/v]) to the samples in a 1:1 ratio. Vortex and
- 227 incubate at room temperature for 10 minutes. Centrifuge the samples at 16,060 g for 15 minutes at
- 228 4°C. Carefully collect the upper, aqueous phase (up to 200 µl) and transfer into a clean 2.0 ml tube.
- 229 Add two volumes of ice cold ethanol (100%) and 1/10 volume of 3 M sodium acetate (pH 5.3). Place
- 230 the samples at -20°C for 1 hour. Centrifuge again at 16,060 g for 15 minutes at 4°C and discard
- 231 the supernatant. Wash the pellets with ice cold 70% ethanol (100 µl) and centrifuge at 16,060 g for
- 232 1 minute at 4°C. Discard the supernatant and dry the pellets thoroughly. Suspend the pellets in
- 233 30 µl of nuclease-free water and store immediately at -20°C (Tuppurainen *et al.*, 2005).
- 234 Alternatively a column-based extraction kit may be used.
- 235 iv) The primers for this PCR assay were developed from the gene encoding the viral attachment
- 236 protein. The size of the expected amplicon is 192 bp (Ireland & Binopal, 1998). The primers have
- 237 the following gene sequences:
- 238 Forward primer 5'-TCC-GAG-CTC-TTT-CCT-GAT-TTT-TCT-TAC-TAT-3'
- 239 Reverse primer 5'-TAT-GGT-ACC-TAA-ATT-ATA-TAC-GTA-AAT-AAC-3'.
- 240 v) DNA amplification is carried out in a final volume of 50 µl containing: 5 µl of 10 × PCR buffer, 1.5 µl
- 241 of MgCl<sub>2</sub> (50 mM), 1 µl of dNTP (10 mM), 1 µl of forward primer, 1 µl of reverse primer, 1 µl of DNA
- 242 template (~10 ng), 0.5 µl of Taq DNA polymerase and 39 µl of nuclease-free water. The volume of
- 243 DNA template required may vary and the volume of nuclease-free water must be adjusted to the
- 244 final volume of 50 µl.
- 245 vi) Run the samples in a thermal cycler as follows: 2 minutes at 95°C; then 45 seconds at 95°C,
- 246 50 seconds at 50°C and 1 minute at 72°C (34 cycles); 2 minutes at 72°C and hold at 4°C until
- 247 analysis.
- 248 vii) Mix 10 µl of each sample with loading dye and load onto a 1.5% agarose gel in TAE buffer
- 249 (Tris/acetate buffer containing EDTA). Load a parallel lane with a 100 bp DNA-marker ladder.
- 250 Electrophoretically separate the products using approximately 8–10 V/cm for 40–60 minutes and
- 251 visualise with a suitable DNA stain and transilluminator.

## 252 1.6.2. Real-time PCR methods

253 Several highly sensitive and specific fluorescent detection-based real-time PCR methods have been

254 developed and validated (Balinsky *et al.*, 2008; Bowden *et al.*, 2008; Das *et al.*, 2012; Stubbs *et al.*,

255 2012). Each test detects a small conserved genetic locus within the capripoxvirus genome, but these

256 methods do not discriminate between SPPV, GTPV or LSDV. Real-time PCR methods for direct

257 capripoxvirus genotyping species differentiation without the need for gene sequencing have been

258 described (Haegeman *et al.*, 2013; Gelaye *et al.*, 2013; Lamien *et al.*, 2011b; Wolff *et al.*, 2021).

259 The real-time PCR method described below is a rapid, sensitive and specific method for the detection of

260 the genomic DNA from SPPV, GTPV or LSDV. This assay will is not designed to differentiate between

261 the capripoxvirus species.

262 DNA extraction from blood, and tissue and semen

263 A number of DNA extraction kits are commercially available for the isolation extraction of template DNA

264 for real-time PCR. Manufacturer's instructions should always be consulted for guidance on the

265 appropriate method for the sample type being extracted followed while using commercial extraction kits.

266 WOAHP Reference Laboratories can be contacted for advice on suitable commercial kits.

- 267 Real-time PCR
- 268 i) The real-time PCR method outlined below uses the primers and probe described by Bowden *et al.*  
269 (2008), and further validated by Stubbs *et al.* (2012). Cycling conditions and reagent concentrations  
270 can be altered to ensure optimal performance in individual laboratories.
- 271 ii) Forward and reverse primers should be prepared at concentrations of 20  $\mu$ M. A minor groove binder  
272 (MGB) TaqMan hydrolysis probe should be prepared at a concentration of 10  $\mu$ M.
- 273 Forward primer: 5'-AAA-ACG-GTA-TAT-GGA-ATA-GAG-TTG-GAA-3'
- 274 Reverse primer: 5'-AAA-TGA-AAC-CAA-TGG-ATG-GGA-TA-3'
- 275 Probe: 5'-FAM-TGG-CTC-ATA-GAT-TTC-CT-MGB-3'
- 276 iii) Mastermix is prepared by combining 10  $\mu$ l of 2  $\times$  real-time PCR mastermix with 0.4  $\mu$ l of forward  
277 primer, 0.4  $\mu$ l of reverse primer, 0.5  $\mu$ l of probe and 6.7  $\mu$ l of RNase free water per reaction.
- 278 iv) Add 2  $\mu$ l of extracted DNA to 18  $\mu$ l of mastermix in a 96-well PCR plate or PCR strip and perform  
279 real-time PCR according to the example given below or similar method:
- 280 v) 95°C for 10 minutes followed by 45 cycles of 95°C for 15 seconds, 60°C for 60 seconds.  
281 Fluorescence detection should be performed at the end of each cycle.
- 282 vi) Following completion of the real-time PCR, a cycle threshold ( $C_T$ ) should be set. Samples with  $C_T$   
283 values less than 35 are considered positive. Samples with a  $C_T$  value greater than 35 but less than  
284 45 are considered inconclusive and require further investigation. Samples which do not yield a  $C_T$   
285 value, i.e. the amplification curve does not cross the threshold, are considered negative.

### 286 1.6.3. Isothermal genome amplification

287 Molecular tests using loop-mediated isothermal amplification (LAMP) to detect capripoxvirus genomes  
288 are reported to provide sensitivity and specificity similar to real-time PCR with a simpler method and at  
289 lower cost (Das *et al.*, 2012; Murray *et al.*, 2013). Field validation of the Das *et al.* (2012) LAMP method  
290 assay has been further reported by (Omoga *et al.*, 2016) and a combination of this universal  
291 capripoxvirus test with two additional LAMP assays was reported to show utility in discriminating between  
292 to differentiate GTPV and from SPPV (Zhao *et al.*, 2014).

## 293 2. Serological tests

294 Detectable levels of antibodies develop 1 week after the animal shows clinical signs. The highest antibody levels are  
295 detected within 1–2 months after infection is detected.

### 296 2.1. Virus neutralisation

297 A test serum can either be titrated against a constant titre of capripoxvirus (100 TCID<sub>50</sub> [50% tissue culture  
298 infective dose]) or a standard capripoxvirus strain can be titrated against a constant dilution of test serum in  
299 order to calculate a neutralisation index. Because of the variable sensitivity of tissue culture to capripoxvirus,  
300 and the consequent difficulty of ensuring the use of 100 TCID<sub>50</sub>, the neutralisation index is the preferred method,  
301 although it does require a larger volume of test sera. The test is described using 96-well flat-bottomed tissue  
302 culture grade microtitre plates, but it can be performed equally well in tissue culture tubes with the appropriate  
303 changes to the volumes used, although it is more difficult to read an end-point in tubes. The use of Vero cells in  
304 the virus neutralisation test has been reported to give more consistent results (Kitching & Taylor, 1985).

#### 305 2.1.1. Test procedure

- 306 i) Test sera including a negative and a positive control are diluted 1/5 in Eagle's/HEPES (N-2-  
307 hydroxyethylpiperazine, N-2-ethanesulphonic acid) and inactivated at 56°C for 30 minutes.
- 308 ii) Next, 50  $\mu$ l of the first inactivated serum is added to columns 1 and 2, rows A to H of the microtitre  
309 plate. The second serum is placed in columns 3 and 4, the third in columns 5 and 6, the positive  
310 control serum is placed in columns 7 and 8, the negative control serum is placed in columns 9 and  
311 10, and 50  $\mu$ l of Eagle's/HEPES without serum is placed in columns 11 and 12 and to all wells of  
312 row H.
- 313 iii) A reference strain of capripoxvirus, usually a vaccine strain known to grow well in tissue culture,  
314 with a titre of over log<sub>10</sub> 6 TCID<sub>50</sub> per ml is diluted in Eagle's/HEPES in bijoux bottles to give a log  
315 dilution series of log<sub>10</sub> 5.0; 4.0; 3.5; 3.0; 2.5; 2.0; 1.5 TCID<sub>50</sub> per ml (equivalent to log<sub>10</sub> 3.7; 2.7; 2.2;  
316 1.7; 1.2; 0.7; 0.2 TCID<sub>50</sub> per 50  $\mu$ l).

- 317 iv) Starting with row G and the most diluted virus preparation, 50 µl of virus is added to each well in  
318 that row. This is repeated with each virus dilution, the highest titre virus dilution being placed in row  
319 A.
- 320 v) The plates are covered and incubated for 1 hour at 37°C.
- 321 vi) ~~LT cells are~~ An appropriate cell suspension (such as MDBK cells) is prepared from pregrown  
322 monolayers as a suspension of 10<sup>5</sup> cells/ml in Eagle's medium containing antibiotics and 2% fetal  
323 calf serum. Following incubation of the microtitre plates, 100 µl of cell suspension is added to all  
324 the wells, except wells H11 and H12, which serve as control wells for the medium. The remaining  
325 wells of row H are cell and serum toxicity controls.
- 326 vii) The microtitre plates are covered and incubated at 37°C for 9 days.
- 327 viii) Using an inverted microscope, the monolayers are examined daily starting at day 4 for evidence of  
328 CPE. There should be no CPE in the cells of row H. Using the 0240 KSGP vaccine strain of  
329 capripoxvirus, the final reading is taken on day 9, and the titre of virus in each duplicate titration is  
330 calculated according to the Kärber method. If left longer, there is invariably a 'breakthrough' of virus  
331 in which virus that was at first neutralised appears to disassociate from the antibody.
- 332 ix) *Interpretation of the results:* The neutralisation index is the log titre difference between the titre of  
333 the virus in the negative serum and in the test serum. An index of ≥1.5 is positive. The test can be  
334 made more sensitive if serum from the same animal is examined before and after infection.  
335 Because immunity to capripoxvirus is predominantly cell mediated, a negative result, particularly  
336 following vaccination in which the response is necessarily mild, does not imply that the animal from  
337 which the serum was taken is not protected.
- 338 ~~A constant virus/varying serum method has been described using serum dilutions in the range 1/5~~  
339 ~~to 1/500 and fetal calf muscle cells. Because these cells have a lower sensitivity to capripoxvirus~~  
340 ~~than LT cells, the problem of virus 'breakthrough' is overcome.~~

## 341 2.2. Indirect fluorescent antibody test

342 Capripoxvirus-infected tissue culture grown on flying cover-slips or tissue culture microscope slides can be used  
343 for the indirect fluorescent antibody test. Uninfected tissue culture control, and positive and negative control  
344 sera, should be included in the test. The infected and control cultures are fixed in acetone at -20°C for 10  
345 minutes and stored at 4°C. Dilutions of test sera are made in PBS, starting at 1/5, and positives are identified  
346 using an anti-sheep gamma-globulin conjugated with fluorescein isothiocyanate (Davies & Otema, 1978). Cross-  
347 reactions can occur with orf, bovine papular stomatitis virus and perhaps other poxviruses.

## 348 2.3. Western blot analysis

349 Western blotting of test sera against capripoxvirus-infected cell lysate provides a sensitive and specific system  
350 for the detection of antibody to capripoxvirus structural proteins, although the test is expensive and difficult to  
351 carry out (Chand *et al.*, 1994).

## 352 2.4. Enzyme-linked immunosorbent assay

353 ~~No validated ELISA is available for the serological diagnosis of SPP or GTP.~~

354 Both in-house and commercial enzyme-linked immunosorbent assay (ELISAs) are available, but these tests  
355 cannot discriminate between antibodies to different capripoxviruses (LSDV or SPPV/GTPV).

# 356 C. REQUIREMENTS FOR VACCINES

357 **[THIS SECTION IS UNDER REVIEW IN THE 2024/2025 REVIEW CYCLE]**

## 358 1. Background

### 359 1.1. Rationale and intended use of the product

360 A variety of attenuated live and inactivated capripoxvirus vaccines has been used to provide protection against  
361 sheeppox and goatpox. All strains of capripoxvirus of ovine, caprine or bovine origin examined so far share a  
362 major neutralising site, so that animals recovered from infection with one strain are resistant to infection with

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363 any other strain (Capstick, 1961). Consequently, it is possible to use a single strain of capripoxvirus to protect  
364 both sheep and goats against all field strains of virus, regardless of whether their origin was in Asia or Africa  
365 (Kitching *et al.*, 1986; Kitching & Taylor, 1985). However, field evidence suggests some strains are quite host-  
366 specific and are used only in sheep against SPPV and only in goat against GTPV.

367 A number of strains of capripoxvirus have had widespread use as live vaccines (Davies & Mbugwa, 1985), for  
368 example the Romanian and RM-65 strains used mainly in sheep and the Mysore and Gorgan strains used in  
369 goats. The real identity of the commonly used Kenyan sheep and goat pox vaccine virus (KSGP) 0240 was  
370 recently shown to be actually LSDV (Tuppurainen *et al.*, 2014). Virus strain identity and attenuation properties  
371 must be ascertained and taken into consideration when selecting vaccine strains for use in cattle, sheep and  
372 goats. The protective dose depends on the vaccine strain used. Immunity in sheep and goats against  
373 capripoxvirus following vaccination with the 0240 strain lasts over a year and the Romanian strain gave  
374 protection for at least 30 months.

375 Killed vaccines produced from tissue culture contain only the intracellular mature virion form of the virus, and  
376 lack the less robust but biologically crucial extracellular enveloped virion form. As a result, the vaccine does not  
377 stimulate immunity against the extracellular enveloped virion, resulting in poor protection. Killed capripoxvirus  
378 vaccines provide, at best, only temporary protection.

## 379 **2. Outline of production and minimum requirements for conventional vaccines**

380 General requirements set for the facilities used for the production of vaccines and for the documentation and record keeping  
381 throughout the whole manufacturing process are described in Chapter 1.1.8 *Principles of veterinary vaccine production*.  
382 The documentation should include the standard operating procedures (SOP) for the method of manufacture and each step  
383 for the testing of cells and reagents used in the process, each batches and the final product.

### 384 **2.1. Characteristics of the seed**

#### 385 **2.1.1. Biological characteristics**

386 A strain of capripoxvirus used for vaccine production must be accompanied by a history describing its  
387 origin and tissue culture or animal passage. It must be safe to use in all breeds of sheep and goats for  
388 which it is intended, including pregnant and young animals. It must be non-transmissible, remain  
389 attenuated after further tissue culture passage, and provide complete protection against challenge with  
390 virulent field strains for a minimum of 1 year. A quantity of master seed vaccine virus should be prepared  
391 and stored in order to provide a consistent working seed for regular vaccine production.

#### 392 **2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)**

393 Each master seed must be tested to ensure its identity and shown to be free from adventitious viruses,  
394 in particular pestiviruses, such as border disease and bovine viral diarrhoea virus, and free from  
395 contamination with bacteria, fungi and/or mycoplasmas. The general procedures for sterility or purity  
396 tests are described in chapter 1.1.9. The master seed must also be safe and produce no clinical reaction  
397 in all breeds of sheep or goats when given by the recommended route and stimulate complete immunity  
398 to capripoxvirus in all breeds of sheep and goats for at least 1 year. The necessary safety and potency  
399 tests are described in Section C.2.2.4 *Final product batch tests*.

### 400 **2.2. Method of manufacture**

401 The method of manufacture should be documented as the Outline of Production.

#### 402 **2.2.1. Procedure**

403 Vaccine seed should be lyophilised and stored in 2 ml vials at  $-20^{\circ}\text{C}$ . It may be stored wet at  
404  $-20^{\circ}\text{C}$ , but when wet, is more stable at  $-70^{\circ}\text{C}$  or lower. The virus should be cultured in primary or  
405 secondary LT or LK cells of wool sheep origin for maximum yield. Vero cells may also be used with  
406 suitably adapted strains.

407 Vaccine batches are produced on fresh monolayers of secondary LT or primary LK cells. A vial of seed  
408 virus is reconstituted with GMEM or another appropriate medium and inoculated on to an LT or LK  
409 monolayer that has been previously washed with warm PBS, and allowed to adsorb for 15 minutes at  
410  $37^{\circ}\text{C}$  before being overlaid with additional GMEM. After 4–6 days, there will be extensive (80–90%) CPE.  
411 The culture should be examined for any evidence of nonspecific CPE, medium cloudiness or change in  
412 medium pH. The culture is freeze–thawed three times, the suspension removed and centrifuged at 600

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413 **g** for 20 minutes. A second passage may be required to produce sufficient virus for a production batch.  
414 Live vaccine may be produced on roller bottles.

415 The procedure is repeated and the harvests from individually numbered flasks are each mixed separately  
416 with an equal volume of sterile and chilled 5% lactalbumin hydrolysate and 10% sucrose, and transferred  
417 to individually numbered bottles for storage at  $-20^{\circ}\text{C}$ . Prior to storage, 0.2 ml is removed from each bottle  
418 for sterility control. An additional 0.2 ml is removed for virus titration; 2 ml pools composed of 0.2 ml  
419 samples taken from ten bottles are used. A written record of all the procedures must be kept for all  
420 vaccine batches.

421 Inactivated vaccines are produced, usually from unattenuated field strains of capripoxvirus, grown in  
422 tissue culture as described above, inactivated with 0.03% formaldehyde, and mixed with an equal volume  
423 of alhydrogel as adjuvant. Formaldehyde is no longer considered to be a suitable inactivant for certain  
424 viral vaccines because its mode of action cannot be guaranteed to be totally effective in inactivating all  
425 the live virus. This has not been fully investigated for capripoxvirus.

### 426 **2.2.2. Requirements for substrate and media**

427 The specification and source of all ingredients used in the manufacturing procedure should be  
428 documented and the freedom from extraneous agents: bacteria, fungi, mycoplasma and any other  
429 viruses should be tested. The detailed testing procedure is described in the chapter 1.1.9. The use of  
430 antibiotics must meet the requirements of the licensing authority.

### 431 **2.2.3. In-process controls**

#### 432 i) Cells

433 Cells should be obtained from the testis or kidney of a healthy young lamb from a scrapie-free flock  
434 of a wool sheep breed. During cultivation, cells must be observed for any evidence of CPE, and for  
435 normal morphology (predominantly fibroblastic). They can usually be passaged successfully up to  
436 ten times. When used for vaccine production, uninfected control cultures should be grown in parallel  
437 and maintained for at least three additional passages for further observation. They should be  
438 checked for the presence of noncytopathic strains of bovine virus diarrhoea or border disease  
439 viruses by immunofluorescence or immunoperoxidase techniques. If possible, cells should be  
440 prepared and screened prior to vaccine production and stocked in 1–2 ml aliquots containing  $2 \times$   
441  $10^7$  cells/ml in sterile 10% DMSO (dimethyl sulphoxide) and 90% FBS (fetal bovine serum) solution  
442 stored in liquid nitrogen.

#### 443 ii) Serum

444 Bovine serum used in the growth or maintenance medium must be free from transmissible  
445 spongiform encephalopathies (TSEs) and antibody to capripoxvirus, and tested for contamination  
446 with pestivirus or any other viruses, extraneous bacteria, mycoplasma or fungi.

#### 447 iii) Medium

448 Medium must be tested free from contamination with pestivirus or any other viruses, extraneous  
449 bacteria, mycoplasma or fungi.

#### 450 iv) Virus

451 Seed virus and final vaccine must be titrated in tissue culture tubes or microtitre plates. Vaccine  
452 samples must be examined for the presence of adventitious viruses including cytopathic and  
453 noncytopathic strains of pestivirus, and should be mixed with a high-titre capripoxvirus-immune  
454 serum that has tested negative for antibody to pestivirus to prevent the vaccine virus itself interfering  
455 with the test. The vaccine bulk can be held at  $-20^{\circ}\text{C}$  or below until all sterility tests and titrations  
456 have been completed, at which time it should be freeze-dried in 1 ml aliquots in vials sufficient for  
457 100 doses. The vaccine harvest diluted with lactalbumin hydrolysate and sucrose should have a  
458 minimum titre  $\log_{10}$  4.5 TCID<sub>50</sub> per ml after freeze-drying, equivalent to a field dose of  $\log_{10}$  2.5  
459 TCID<sub>50</sub>. A further titration is carried out on five randomly chosen vials of the freeze-dried preparation  
460 to confirm the titre.

### 461 **2.2.4. Final product batch tests**

#### 462 i) Sterility/purity

463 Tests for sterility and freedom from contamination of biological materials intended for veterinary use  
464 may be found in chapter 1.1.9.

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465 ii) Safety  
466 The safety studies should be demonstrated by statistically valid vaccination studies using  
467 seronegative young sheep and goats of known susceptibility to capripox virus. The procedure  
468 described is suitable for vaccine strains such as 0240 that are equally immunogenic in both sheep  
469 and goats. The choice of target animal should be adapted for strains with a more restricted host  
470 preference.

471 iii) Potency  
472 Potency tests must be undertaken if the minimum immunising dose of the virus strain is not known.  
473 This is usually carried out by comparing the titre of a virulent challenge virus on the flanks of  
474 vaccinated and control animals. Following vaccination, the flanks of at least three animals and three  
475 controls are shaved of wool or hair. Log<sub>10</sub> dilutions of the challenge virus are prepared in sterile  
476 PBS and six dilutions are inoculated intradermally (0.1 ml per inoculum) along the length of the  
477 flank; four replicates of each dilution are inoculated down the flank. An oedematous swelling will  
478 develop at possibly all 24 inoculation sites on the control animals, although preferably there will be  
479 little or no reaction at the four sites of the most dilute inocula. The vaccinated animals should  
480 develop an initial hypersensitivity reaction at sites of inoculation within 24 hours, which should  
481 quickly subside. Small areas of necrosis may develop at the inoculation site of the most  
482 concentrated challenge virus. The macule/papule is measured at between 8 and 10 days post-  
483 challenge. The titre of the challenge virus is calculated for the vaccinated and control animals; a  
484 difference of log<sub>10</sub> titre > 2.5 is taken as evidence of protection.

## 485 2.3. Requirements for authorisation

### 486 2.3.1. Safety requirements

487 i) Target and non-target animal safety  
488 The vaccine must be safe to use in all breeds of sheep and goats for which it is intended, including  
489 young and pregnant animals. It must also be non-transmissible, remain attenuated after further  
490 tissue culture passage.

491 Safety tests should be carried out on the final product of each batch as described in Section C.2.2.4.

492 The safety of the vaccine in non-target animals must have been demonstrated using mice and  
493 guinea-pigs as described in Section C.2.2.4. There should be no evidence of pathology caused by  
494 the vaccine.

495 ii) Reversion-to-virulence for attenuated/live vaccines  
496 The selected final vaccine should not revert to virulence during a further passages in target animals.

497 iii) Environmental consideration  
498 Attenuated vaccine should not be able to perpetuate autonomously in cattle, sheep or goat  
499 populations. Vaccines using the 0240 strain should not be used in *Bos taurus* breeds. Strains of  
500 capripoxvirus are not a hazard to human health. There are no precautions other than those  
501 described above for sterility and freedom from adventitious agents.

### 502 2.3.2. Efficacy requirements

503 i) For animal production  
504 The efficacy of the vaccine must be demonstrated in vaccination challenge experiment under  
505 laboratory conditions. As described in Section C.2.2.4.

506 Once the potency of the particular strain being used for vaccine production has been determined  
507 in terms of minimum dose required to provide immunity, it is not necessary to repeat this on the  
508 final product of each batch, provided the titre of virus present has been ascertained.

509 ii) For control and eradication  
510 Vaccination is the only effective way to control the sheep pox and goat pox outbreaks in endemic  
511 countries. Unfortunately, currently no marker vaccines allowing the differentiation of infected from  
512 vaccinated animals are available.

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513 Immunity to virulent field virus following vaccination of sheep or goats with the 0240 strain lasts  
514 over 1 year, and protection against generalised infection following intradermal challenge lasts at  
515 least 3 years and is effective lifelong. The duration of immunity produced by other vaccine strains  
516 should be ascertained in both sheep and goats by undertaking controlled trials in an environment  
517 in which there is no possibility of field strains of capripoxvirus confusing the results. The inactivated  
518 vaccines provide immunity for less than 1 year, and for the reasons given at the beginning of this  
519 section, may not give immunity to the form of capripoxvirus usually associated with natural  
520 transmission.

### 521 2.3.3. Stability

522 All vaccines are initially given a shelf-life of 24 months before expiry. Real-time stability studies are then  
523 conducted to confirm the appropriateness of the expiry date. Multiple batches of the vaccine should be  
524 re-titrated periodically throughout the shelf-life to determine the vaccine variability.

525 Properly freeze-dried preparations of capripox vaccine, particularly those that include a protectant, such  
526 as sucrose and lactalbumin hydrolysate, are stable for over 25 years when stored at  $-20^{\circ}\text{C}$  and for 2–4  
527 years when stored at  $4^{\circ}\text{C}$ . There is evidence that they are stable at higher temperatures, but no long-  
528 term controlled experiments have been reported. The inactivated vaccines must be stored at  $4^{\circ}\text{C}$ , and  
529 their shelf- life is usually given as 1 year.

530 No preservatives other than a protectant, such as sucrose and lactalbumin hydrolysate, are required for  
531 the freeze-dried preparation.

## 532 3. Vaccines based on biotechnology

### 533 3.1. Vaccines available and their advantages

534 Currently, no recombinant vaccines for capripoxviruses are commercially available. However, a new generation  
535 of capripox vaccines is being developed that uses the capripoxvirus genome as a vector for the genes of other  
536 ruminant pathogens such as peste des petits ruminants (PPR) virus (Berhe *et al.*, 2003; Tuppurainen *et al.*,  
537 2014).

### 538 3.2. Special requirements for biotechnological vaccines, if any

539 Not applicable.

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609 \*  
610 \* \*

611 **NB:** There are WOA Reference Laboratories for sheep pox and goat pox (please consult the WOA Web site:  
612 <https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3http://www.oie.int/>).

613 Please contact the WOA Reference Laboratories for any further information on  
614 diagnostic tests, reagents and vaccines for sheep pox and goat pox

615 **NB:** FIRST ADOPTED IN 1989. MOST RECENT UPDATES ADOPTED IN 2017.

REUNIÓN DE LA COMISIÓN DE NORMAS BIOLÓGICAS

París, 5–9 de febrero de 2024

SECTION 3.9.

SUIDAE

CHAPTER 3.9.1.

AFRICAN SWINE FEVER  
(INFECTION WITH AFRICAN SWINE FEVER VIRUS)

SUMMARY

*African swine fever (ASF) is an infectious disease of domestic and wild pigs of all breeds and ages, caused by ASF virus (ASFV). The clinical syndromes vary from peracute, acute, subacute to chronic, depending on the virulence of the virus. Acute disease is characterised by high fever, haemorrhages in the reticuloendothelial system, and a high mortality rate. Soft ticks of the Ornithodoros genus, especially O. moubata and O. erraticus, have been shown to be both reservoirs and transmission vectors of ASFV. The virus is present in tick salivary glands and passed to new hosts (domestic or wild suids) when feeding. It can be transmitted sexually between ticks, transovarially to the eggs, or transtadially throughout the tick's life.*

*ASFV is the only member of the Asfarviridae family, genus Asfivirus.*

*Laboratory diagnostic procedures for ASF fall into two groups: detection of the virus and serology. The selection of the tests to be carried out depends on the disease situation and laboratory diagnostic capacity in the area or country.*

**Identification of the agent:** *Laboratory diagnosis must be directed towards isolation of the virus by inoculation of pig leukocyte or bone marrow cultures, the detection of antigen in smears or cryostat sections of tissues by fluorescent antibody test and/or the detection of genomic DNA by the polymerase chain reaction (PCR) or real-time PCR. The PCRs are excellent, highly sensitive, specific and rapid techniques for ASFV detection and are very useful under a wide range of circumstances. They are especially useful if the tissues are unsuitable for virus isolation and antigen detection. In doubtful cases, the material is passaged in leukocyte cell cultures and the procedures described above are repeated.*

**Serological tests:** *Pigs that survive natural infection usually develop antibodies against ASFV from 7–10 days post-infection and these antibodies persist for long periods of time. Where the disease is endemic, or where a primary outbreak is caused by a strain of low or moderate virulence, the investigation of new outbreaks should include the detection of specific antibodies in serum or extracts of the tissues submitted. A variety of methods such as the enzyme-linked immunosorbent assay (ELISA), the indirect fluorescent*

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30 antibody test (IFAT), the indirect immunoperoxidase test (IPT), and the immunoblotting test (IBT) is available  
31 for antibody detection.

32 **Requirements for vaccines:** At present, there is no vaccine for ASF. Commercially produced modified live  
33 virus vaccines are available and licenced under field evaluation in some countries.

## 34 A. INTRODUCTION

35 The current distribution of African swine fever (ASF) extends across more than 50 countries in three continents (Africa,  
36 Asia and Europe). Several incursions of ASF out of Africa were reported between the 1960s and 1970s. In 2007, ASF was  
37 introduced into Georgia, from where it spread to neighbouring countries including the Russian Federation. From there ASF  
38 spread to eastern European countries extending westwards and reaching the European Union in 2014. Further westward  
39 and southern spread in Europe has occurred since that time. In all these countries, both hosts – domestic pig and wild  
40 boar – were affected by the disease. In August 2018, the People’s Republic of China reported its first outbreak of ASF and  
41 further spread in Asia has occurred. ASF was identified on the island of Hispaniola (Haiti and the Dominican Republic) in  
42 2021. See WAHIS (<https://wahis.woah.org/#/home>) for recent information on distribution at the country level.

43 ASF virus (ASFV) is a complex large, enveloped DNA virus with icosahedral morphology. It is currently classified as the  
44 only member of the *Asfviridae* family, genus *Asfivirus* (Dixon *et al.*, 2005). More than 60 structural proteins have been  
45 identified in intracellular virus particles (200 nm) (Alejo *et al.*, 2018). More than a hundred infection-associated proteins  
46 have been identified in infected porcine macrophages, and at least 50 of them react with sera from infected or recovered  
47 pigs (Sánchez-Vizcaíno & Arias, 2012). The ASFV double-stranded linear DNA genome comprises between 170 and  
48 193 kilobases (kb) and contains between 150 and 167 open reading frames with a conserved central region of about 125  
49 kb and variable ends. These variable regions encode five multigene families that contribute to the variability of the virus  
50 genome. The complete genomes of several ASFV strains have been sequenced (Bishop *et al.*, 2015; Chapman *et al.*,  
51 2011; de Villiers *et al.*, 2010; Portugal *et al.*, 2015). Different strains of ASFV vary in their ability to cause disease, but at  
52 present there is only one recognised serotype of the virus detectable by antibody tests.

53 The molecular epidemiology of the disease is investigated by sequencing of the 3’ terminal end of the B646L open reading  
54 frame encoding the p72 protein major capsid protein, which differentiates up to 24 distinct genotypes (Achenbach *et al.*,  
55 2017; Boshoff *et al.*, 2007; Quembo *et al.* 2018). To distinguish subgroups among closely related ASFV, sequence analysis  
56 of the tandem repeat sequences (TRS), located in the central variable region (CVR) within the B602L gene (Gallardo *et al.*,  
57 2009; Lubisi *et al.*, 2005; Nix *et al.*, 2006) and in the intergenic region between the I73R and I329L genes, at the right  
58 end of the genome (Gallardo *et al.*, 2014), is undertaken. Several other gene regions such as the E183L encoding p54  
59 protein, the CP204L encoding p30 protein, and the protein encoded by the EP402R gene (CD2v), have been proved as  
60 useful tools to analyse ASFVs from different locations and hence track virus spread.

61 ASF viruses produce a range of syndromes varying from peracute, acute to chronic disease and subclinical infections.  
62 Pigs are the only domestic animal species that is naturally infected by ASFV. European wild boar and feral pigs are also  
63 susceptible to the disease, exhibiting clinical signs and mortality rates similar to those observed in domestic pigs. In contrast  
64 African wild pigs such as warthogs (*Phacochoerus aethiopicus*), bush pigs (*Potamochoerus porcus*) and giant forest hogs  
65 (*Hylochoerus meinertzhageni*) are resistant to the disease and show few or no clinical signs. These species of wild pig act  
66 as reservoir hosts of ASFV in Africa (Costard *et al.*, 2013; Sánchez-Vizcaíno *et al.*, 2015).

67 The incubation period is usually 4–19 days. The more virulent strains produce peracute or acute haemorrhagic disease  
68 characterised by high fever, loss of appetite, haemorrhages in the skin and internal organs, and death in 4–10 days,  
69 sometimes even before the first clinical signs are observed. Case fatality rates may be as high as 100%. Less virulent  
70 strains produce mild clinical signs – slight fever, reduced appetite and depression – which can be readily confused with  
71 many other conditions in pigs and may not lead to suspicion of ASF. Moderately virulent strains are recognised that induce  
72 variable disease forms, ranging from acute to subacute. Low virulence, non-haemadsorbing strains can produce subclinical  
73 non-haemorrhagic infection and seroconversion, but some animals may develop discrete lesions in the lungs or on the  
74 skin in areas over bony protrusions and other areas subject to trauma. Animals that have recovered from either acute,  
75 subacute or chronic infections may potentially become persistently infected, acting as virus carriers. The biological basis  
76 for the persistence of ASFV is still not well understood, nor it is clear what role persistence plays in the epidemiology of  
77 the disease.

78 ASF cannot be differentiated from classical swine fever (CSF) by either clinical or post-mortem examination, and both  
79 diseases should be considered in the differential diagnosis of any acute febrile haemorrhagic syndrome of pigs. Bacterial  
80 septicaemias may also be confused with ASF and CSF. Laboratory tests are essential to distinguish between these  
81 diseases.

82 In countries free from ASF but suspecting its presence, the laboratory diagnosis must be directed towards isolation of the  
83 virus by the inoculation of pig leukocyte or bone marrow cultures, the detection of genomic DNA by polymerase chain  
84 reaction (PCR) or the detection of antigen in smears or cryostat sections of tissues by direct fluorescent antibody test  
85 (FAT). Currently the PCR is the most sensitive technique and can detect ASFV DNA from a very early stage of infection in  
86 tissues, ethylene diamine tetra-acetic acid (EDTA)-blood and serum samples. The PCR is particularly useful if samples  
87 submitted are unsuitable for virus isolation and antigen detection because they have undergone putrefaction. Pigs that  
88 have recovered from acute, subacute or chronic infections usually exhibit a viraemia for several weeks making the PCR  
89 test a very useful tool for the detection of ASFV DNA in pigs infected with low or moderately virulent strains. Virus isolation  
90 by the inoculation of pig leukocyte or bone marrow cultures and identification by haemadsorption tests (HAD) are  
91 recommended as a confirmatory test when ASF is positive by other methods, particularly in the event of a primary outbreak  
92 or a case of ASF.

93 ~~As no vaccine is available, the presence of ASFV antibodies is indicative of previous infection and, as antibodies are~~  
94 ~~produced from the first week of infection and persist for long periods, they are a good marker for the diagnosis of the~~  
95 ~~disease, particularly in subacute and chronic forms.~~

96 Vaccines should be prepared in accordance with Chapter 1.1.8 *Principles of veterinary vaccine production*. Current ASF  
97 modified live virus (MLVs) vaccines are based on the live virus that have been naturally attenuated or attenuated by  
98 targeted genetic recombination through cell cultures (Gladue & Borca, 2022). MLV production is based on a seed-lot  
99 system consistent with the *European Pharmacopoeia* (11th edition) and that has been validated with respect to virus  
100 identity, sterility, purity, potency, stability, safety and immunogenicity (including spread), non-transmissibility, stability and  
101 immunogenicity. ASF MLV first generation vaccines – defined as those for which peer-reviewed publications are in the  
102 public domain – should meet or exceed the minimum standards as described below. Paramount–Demonstration of  
103 acceptable safety and efficacy against the epidemiologically relevant ASFV field strain(s) circulating in areas where the  
104 vaccine is intended for use are is required. At the present time, a variety of mutants (Forth *et al.*, 2023) and recombinants  
105 (Zhao *et al.*, 2023) have emerged globally, and the prevalence of these strains is increasing. In addition, there is a risk that  
106 vaccine strains will recombine with circulating strains. These conditions should be taken into account in vaccine  
107 development. acceptable efficacy should be shown against the B646L (p72) genotype II pandemic virus lineage currently  
108 circulating widely in domestic pigs and wild boar.

109 ASF MLV first generation vaccines allowing the differentiation of infected animals from vaccinated animals (DIVA) by  
110 suitable methods (e.g. serology-based tests) are preferred. Demonstration of MLV safety and efficacy in pigs at different  
111 growth stages (suckling piglets, nursery pigs, fattening pigs), the safety in breeding-age boars, gilts and pregnant sows,  
112 and onset and duration of protective immunity, are also preferred-but are not required to meet the minimum standard.  
113 Additional data will likely be required by Regulatory Authorities if these categories are included in the indications for the  
114 vaccine. Details of the onset of immunity (the interval of time elapsed between vaccination and challenge if protection is  
115 confirmed) and the duration of immunity (the time point at which vaccine-induced immunity begins to decline and provides  
116 less protection) are also required to meet minimum standards.

117 ASF epidemiology is complex with different epidemiological patterns of infection occurring in Africa and Europe. ASF  
118 occurs through transmission cycles involving domestic pigs, wild boar, wild African suids, and soft ticks (Sánchez-Vizcaíno  
119 *et al.*, 2015). In regions where *Ornithodoros* soft bodied ticks are present, the detection of ASFV in these reservoirs of  
120 infection contributes to a better understanding of the epidemiology of the disease. This is of major importance in  
121 establishing effective control and eradication programmes (Costard *et al.*, 2013).

122 ASF is not a zoonotic disease and does not affect public health (Sánchez-Vizcaíno *et al.*, 2009).

123 ASFV should be handled with an appropriate level of bio-containment, determined by risk analysis in accordance with  
124 Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal*  
125 *facilities*.

126 . . .

## 127 **C. REQUIREMENTS FOR VACCINES-UNDER REVIEW**

128 ~~At present there is no commercially available vaccine for ASF.~~ Commercially produced modified live virus vaccines are  
129 being evaluated and licensed for field use.

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## 130 1. Background

131 The ASF p72 genotype II strains (ASFV Georgia 2007/1 lineage) (NCBI, 2020) are recognised to be the current highest  
132 global threat for domestic pig production worldwide (Penrith *et al.*, 2022). However, genotype I attenuated strains and  
133 genotype I/II recombinant strains have been reported to be circulating. In Africa, multiple genotypes are circulating.

134 Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of Veterinary Vaccine Production*.  
135 Varying additional requirements relating to quality (including purity and potency), safety, and efficacy will apply in particular  
136 countries or regions for manufacturers to comply with local regulatory requirements.

137 Wherever live, virulent ASFV or ASF MLVs are stored, handled and disposed, the appropriate biosecurity level, procedures  
138 and practices should be used. The ASF MLV vaccine production facility should meet the requirements for containment  
139 outlined in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and*  
140 *animal facilities*.

141 An optimal ASF MLV first generation vaccine for the target host should have the following general characteristics (minimum  
142 standards):

- 143 • Safe: demonstrate absence of persistent fever as defined below (see Section 2.3.2) and clinical signs of acute or  
144 chronic ASF in vaccinated and in-contact animals, minimal and ideally no vaccine virus transmission, and absence  
145 of an increase in virulence (genetic and phenotypic stability);
- 146 • Efficacious: protects against mortality, reduces acute disease (fever accompanied by the appearance of clinical signs  
147 caused by ASF) and reduces vertical (boar semen and placental) and horizontal disease transmission;
- 148 • Quality – purity: free from wild-type ASFV and extraneous microorganisms that could adversely affect the safety,  
149 potency or efficacy of the product;
- 150 • Quality – potent stability: the log<sub>10</sub> virus titre maintained throughout the vaccine shelf life that guarantees the efficacy  
151 demonstrated by the established minimum immunising (protective) dose.
- 152 • Identity-Vaccine matching: based on the capacity to protect against the ASFV B646L (p72) genotype II pandemic  
153 strain or other p72 genotypes of recognised epidemiologic importance.

154 Vaccine production should be carried out using a validated, controlled and consistent manufacturing process.

155 ASF MLV first generation vaccines must be safe (i.e. an acceptable safety profile) for non-target species and the  
156 environment in general.

157 Ideally, ASF MLV first generation vaccines that meet the minimum standards should also fulfil the following additional  
158 general characteristics: i) prevents acute and persistent (carrier state) disease; ii) prevents horizontal and vertical disease  
159 transmission; iii) induces rapid protective immunity (e.g. < 2 weeks); and iv) confers stable, life-long immunity.

160 Furthermore, ASF MLV second and future generation vaccines should meet the minimum safety and efficacy standards  
161 as ASF MLV first generation vaccines, and ideally provide additional product profile benefits, including but not limited to: i)  
162 contain a negative marker allowing the differentiation of infected from vaccinated animals (DIVA) by reliable discriminatory  
163 tests such as serology-based tests; and ii) confer broad range of protection against other p72 genotype field strains of  
164 varying virulence (low, moderate, and high).

165 The majority of ASF global vaccine research groups and companies are currently focused on ASF MLV first generation  
166 vaccine candidates that are safe and efficacious against ASF viruses belonging to the ASFV p72 genotype II pandemic  
167 strain (ASFV Georgia 2007/1 lineage) (NCBI, 2020). More research is needed to determine whether these genotype II-  
168 specific MLVs can effectively protect against newly circulating variants of genotype II and recombinant strains.

169 Currently, two recombinant gene deleted MLV recombinant-vaccines (ASFV-G-ΔI177L and ASFV-G-ΔMGF) have been  
170 licensed for field use in Vietnam for use in domestic pigs following supervised field testing to evaluate the safety and  
171 effectiveness of several vaccine batches.

172 There are numerous, promising ASF MLV vaccine candidates targeting the p72 genotype II pandemic strain under  
173 development, including:

- 174 • A naturally attenuated field strain (Lv17/WB/Rei1) (Barasona *et al.*, 2019) being developed as an oral bait vaccine for  
175 wild boars;
  - 176 • A laboratory thermo-attenuated field strain (ASFV-989) (Bourry *et al.*, 2022);
-

177 • Single gene-deleted, recombinant viruses (e.g. SY18ΔI226R, ASFV-G-ΔA137R) (Gladue *et al.*, 2021; Zhang *et al.*,  
178 2021);

179 • Double gene-deleted, recombinant viruses (e.g. ASFV-G-Δ9GL/ΔUK; ASFV-SY18-ΔCD2v/UK; Arm-ΔCD2v-ΔA238L)  
180 (O'Donnell *et al.*, 2016; Pérez-Núñez *et al.*, 2022; Teklue *et al.*, 2020);

181 • Multiple gene-deleted, recombinant viruses (ASFV-G-ΔI177L/ΔLVR; ASFV-G-ΔMGF; BA74ΔCD2; HLJ/18-7GD;  
182 ASFVGZΔI177LΔCD2vΔMGF) (Borca *et al.*, 2021; Chen *et al.*, 2020; Kitamura *et al.*, 2023; Liu *et al.*, 2023;  
183 Monteagudo *et al.*, 2017; O'Donnell *et al.*, 2015).

184 Information regarding many of these MLV vaccine candidates can be found in a recent review publication (Brake, 2022).

185 Different DIVA strategies using serological methods (e.g. ELISA) or genome detection methods (e.g. differential real-time  
186 PCR) are not widely available for these ASF MLV first generation vaccine candidates. Therefore, there is still room for  
187 improvement with respect to marker vaccines and their companion diagnostic tests.

188 Inactivated (non-replicating) whole virus vaccines are not presently available and may be difficult to develop to meet  
189 minimum efficacy standards. Recombinant vectored, subunit vaccine candidates that can be produced in scalable vaccine  
190 platform expression systems and mRNA-based ASF vaccines are being evaluated in ongoing laboratory research, testing  
191 and evaluation in experimental challenge models. The publicly available *Center of Excellence for African Swine Fever*  
192 *Genomics* (ASFV Genomics, 2022<sup>1</sup>) that provides the structural protein predictions for all 193 ASFV proteins may help  
193 accelerate ASF first and second generation vaccine research and development.

194 Any future use of vaccine candidates should be based on a thorough risk–benefit assessment considering all safety and  
195 efficacy features, as well as the potential vaccination scenario. Fit-for-purpose vaccine use scenarios matched to the  
196 intended use in a domestic pig-specific type of production system may require different vaccine product profiles or may  
197 influence the focus of essential versus ideal vaccine requirements. Prudent use of ASF MLVs as part of strict, controlled  
198 vaccination programmes, especially in the areas where ASF is not prevalent, should be implemented.

199 It is important to know what genotypes of ASFV are circulating in a population before vaccination is introduced. Due to the  
200 potential risk of recombination events between circulating low and high virulent field strains with future licensed vaccine  
201 strains, and the possibility of reversion to virulence of vaccine strains, strict pharmacovigilance post-vaccination is  
202 essential. Field pharmacovigilance data should be collected and analysed during vaccination campaigns using ASF MLV  
203 first generation vaccines post-licensing. Active post-vaccination surveillance programmes for the detection of new ASF  
204 viruses that may arise from MLV vaccine strains and naturally circulating wild-type virus recombination, as well as revertant  
205 vaccine strains, should be implemented. It is also recommended that vaccine manufacturers carry out laboratory  
206 experiments to further evaluate the risk of vaccine virus recombination with field and vaccine strains.

207 As with any MLV vaccine, all ASF MLV vaccines should be used according to the label instructions, under the strict control  
208 of the country's Regulatory Authority.

209 The minimum standards given here and in chapter 1.1.8 are intended to be general in nature and may be supplemented  
210 by national, regional, and veterinary international medicinal product harmonised requirements. Minimum data requirements  
211 for an authorisation in exceptional circumstances (e.g. unexpected introduction of the virus, sudden outbreaks of the  
212 disease) should be considered where applicable.

## 213 **2. Outline of production and minimum requirements for vaccines**

### 214 **2.1. Characteristics of the seed virus**

#### 215 **2.1.1. Biological characteristics of the master seed virus**

216 ASF MLVs are generally produced from ASFV field strains derived from naturally attenuated field isolates  
217 or using DNA homologous (genetically targeted) recombination techniques in cell cultures to delete one  
218 or more ASFV genes or gene families. These molecular techniques typically involve replacement of the  
219 targeted ASFV gene(s) with one or more positive, marker fluorescent (e.g. BFP, eGFP, mCherry) or  
220 enzyme-based (e.g. β-glucuronidase) ASFV promoter-reporter gene systems that allow the use of  
221 imaging microscopy or flow cytometry to visualise, select, and clone gene-deleted, recombinant, ASF  
222 MLVs. MLV production is carried out in cell cultures based on a seed-lot system.

223 Master seed viruses (MSVs) for MLVs should be selected and produced based on their ease of growth  
224 in cell culture, virus yield (log<sub>10</sub> infectious titre) and genetic stability over multiple cell passages.

1 <http://asfvgenomics.com>, Accessed 4/4/2023.

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225 Preferably, a continuous well-characterised cell line (e.g. ZMAC-4; PIPEC; IPKM) (Borca *et al.*, 2021;  
226 Masujin *et al.*, 2021; Portugal *et al.*, 2020) is used to produce a master cell bank (MCB) on which the  
227 MSV and MSV-derived working seed virus (WSV) can be produced. The exact source of the underlying  
228 ASFV isolate, the whole genome sequence, and the passage history must be recorded.

### 229 **2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)**

230 Only MSVs that have been established as sterile, pure (free of wild-type parental virus and free of  
231 extraneous agents as described in Chapter 1.1.9 *Tests for sterility and freedom from contamination of*  
232 *biological materials intended for veterinary use*, and those listed by the appropriate licensing authorities)  
233 and immunogenic, should be used as the vaccine virus (WSV and vaccine batch production). Live  
234 vaccines must be shown not to cause disease or other adverse effects in target animals in accordance  
235 with chapter 1.1.8, Section 7.1 *Safety tests* (for live attenuated MSVs), that includes target animal safety  
236 tests, increase in virulence tests, assessing the risk to the environment) and if possible, no transmission  
237 to other animals.

238 Identity of the MSV must be confirmed using appropriate methods (e.g. through the use of vaccine strain-  
239 specific whole genome detection methods such as next generation sequencing).

240 Demonstration of MSV stability over several cell passages is necessary, typically through at least five  
241 passages (e.g. MSV+5). For those MLV vaccines for which attenuation is linked to specific characteristics  
242 (gene deletion, gene mutations, etc.), genetic stability of attenuation throughout the production process  
243 should be confirmed by full genome sequencing and confirmation of the vaccine phenotype, for example,  
244 by confirming the virus titre obtained by growth in the cell line used for production using suitable methods.  
245 Suitable techniques to demonstrate genetic stability may include but are not limited to: genome  
246 sequencing, biochemical, proteomic, genotypic (e.g. detection of genetic markers) and phenotypic strain  
247 characterisation. If final product yields (infectious titres) are relatively low, as is typically the case with  
248 ASFV, demonstration of stability is required for the maximum passage for use in the final product  
249 manufacturing as defined by the producer—genetic stability at a minimum of MSV+10 should be  
250 demonstrated to allow more flexibility in the outline of production. For example, if MSV+8 is the maximum  
251 passage for use in final product manufacturing, demonstration of genetic stability to at least MSV+10 is  
252 warranted.

### 253 **2.1.3. Validation as a vaccine strain**

254 The vaccine derived from the MSV must be shown to be satisfactory with respect to safety and efficacy.

255 Even if pigs are not known for susceptibility to transmissible spongiform encephalopathy (TSE) agents,  
256 consideration should also be given to minimising the risk of TSE transmission by ensuring that animal  
257 origin materials from TSE-relevant species, if no alternatives exist for vaccine virus propagation, comply  
258 with the measures on minimising the risk of transmission of TSE.

259 Ideally, the vaccine virus in the final product should generally not differ by more than five passages from  
260 the master seed lot.

261 ASF vaccines should be presented in a suitable pharmaceutical form (e.g. lyophilisate or liquid form).

## 262 **2.2. Method of manufacture**

### 263 **2.2.1. Procedure**

264 The MLV virus is used to infect swine primary cell cultures obtained from specific-pathogen free pigs, the  
265 requirements for which are defined in specific monographs (Chapter 2.3.3 *Minimum requirements for the*  
266 *organisation and management of a vaccine manufacturing facility*, Section 2.4.2). Compared with primary  
267 cell cultures, use of a continuous cell line generally allows for more consistency, higher serial volumes  
268 in manufacturing and aligns better with a seed lot system. Thus, preferably a master cell bank based on  
269 an established, continuous cell line shown to support genetically stable ASFV replication and acceptable  
270 titres over several passages should be used.

271 Cell cultures shall comply with the requirements for cell cultures for production of veterinary vaccines in  
272 chapter 1.1.8. Regardless of the production method, the substrate should be harvested under aseptic  
273 conditions and may be subjected to appropriate methods to release cell-associated virus (e.g. freeze-  
274 thaw cycles, detergent lysis). The harvest can be further processed by filtration and other purification  
275 methods. A stabiliser or other excipients may be added as appropriate. The vaccine is homogenised to  
276 ensure a uniform batch/serial.



277

## **2.2.2. Requirements for ingredients**

278

All ingredients used for vaccine production should be in line with requirements in chapter 1.1.8.

279

## **2.2.3. In-process controls**

280

In-process controls will depend on the protocol of production: they include virus titration of bulk antigen and sterility tests.

281

282

## **2.2.4. Final product batch tests**

283

### i) Sterility

284

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9.

285

286

### ii) Identity

287

Appropriate methods such as specific genome detection methods (e.g. specific differential real-time PCR) should be used for confirmation of the identity of the vaccine virus and differentiation from the parent strain of the virus as a potential contaminant.

288

289

290

### iii) Purity

291

Appropriate methods should be used to ensure that the final product batch does not contain any residual wild-type ASFV.

292

293

### iv) Safety

294

Batch safety testing is to be carried out unless consistent safety of the product is demonstrated and approved in the registration dossier and the production process is approved for consistency in accordance with the standard requirements referred to in chapter 1.1.8.

295

296

297

### v) Batch/serial potency

298

Virus titration is a reliable indicator of vaccine potency once a relationship has been established between the vaccine minimum immunising dose (MID) (minimum protective dose) and titre of the modified live vaccine in vitro. In the absence of a demonstrated correlation between the virus titre and protection, an efficacy test will be necessary (Section C.2.3.3 Efficacy requirements, below).

299

300

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302

### vi) Residual humidity/residual moisture

303

The test should be carried out consistent with VICH<sup>2</sup> GL26 (*Biologicals: Testing of Residual Moisture, 2003*<sup>3</sup>). Required for MLV vaccines presented as lyophilisates for suspension for injection.

304

305

306

## **2.3. Requirements for authorisation/registration/licensing**

307

### **2.3.1. Manufacturing process**

308

For regulatory approval of a vaccine, all relevant details concerning history of the pre-MSV, preparation of MSV, manufacture of the vaccine and quality control testing (Sections C.2.1 Characteristics of the seed and C.2.2 Method of manufacture) should be submitted to the authorities.

309

310

311

Information shall be provided from three preferably consecutive vaccine batches originating from the same MSV and representative of routine production, with a volume not less than 1/10, and more preferably with a volume not less than 1/3 of the typical industrial batch volume. The in-process controls are part of the manufacturing process.

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315

### **2.3.2. Safety requirements**

316

For the purpose of gaining regulatory approval, the following safety tests should be performed satisfactorily.

317

<sup>2</sup> VICH: International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medical Products

<sup>3</sup> [https://www.ema.europa.eu/en/documents/scientific-guideline/vich-gl26-biologicals-testing-residual-moisture-step-7\\_en.pdf](https://www.ema.europa.eu/en/documents/scientific-guideline/vich-gl26-biologicals-testing-residual-moisture-step-7_en.pdf)

318 As a minimum standard, vaccines should be tested for any pathogenic effects on healthy domestic pigs  
319 of the target age intended for use. Additional demonstration of MLV safety in breeding age gilts and  
320 pregnant sows is preferred but not required as a minimum standard. If in the future a vaccine intended  
321 for use in breeding animals is developed, an evaluation of the impact of the vaccine on reproductive  
322 performance will be a standard safety requirement.

323 i) Safety in young animals

324 Carry out the test by each recommended route of administration using, in each case, piglets a  
325 minimum of 6-4 weeks old and not older than 10-weeks old.

326 The test is conducted using no fewer than eight healthy piglets, and preferably no fewer than ten  
327 healthy piglets.

328 Use vaccine virus at the least attenuated passage level that will be present in a batch of the vaccine.

329 Administer to each piglet a quantity of the vaccine virus equivalent to not less than ten times the  
330 maximum virus titre (e.g. 50% haemadsorption dose [HAD<sub>50</sub>], 50% tissue culture infective dose  
331 [TCID<sub>50</sub>], quantitative PCR, etc.) (maximum release dose) likely to be contained in one dose of the  
332 vaccine.

333 To obtain individual and group mean baseline temperatures, the body temperature of each  
334 vaccinated piglet is measured on at least the 3 consecutive days preceding administration of the  
335 vaccine.

336 To confirm the presence or absence of fever accompanied by acute and chronic disease, observe  
337 the piglets 4 hours after vaccination and then at least once daily for at least 45 days, preferably 60  
338 days post-vaccination. Carry out the daily observations for signs of acute and chronic disease using  
339 a quantitative clinical scoring system adding the values for multiple clinical signs (e.g. Gallardo *et*  
340 *al.*, 2015a). These clinical signs should include fever, anorexia, recumbency, skin haemorrhage or  
341 cyanosis, joint swelling and necrotic lesions around the joints, respiratory distress and digestive  
342 findings).

343 At a minimum of 45 days post-vaccination, humanely euthanise all vaccinated piglets. Conduct  
344 gross pathology on spleen, lung, tonsil, and kidney tissue samples and at least three different lymph  
345 nodes (which should include lymph node closest to site of inoculation, gastrohepatic and  
346 submandibular nodes).

347 The vaccine complies with the test if:

348 • No piglet shows abnormal (local or systemic) reactions or notable signs of disease, or reaches  
349 the pre-determined humane endpoint defined in the clinical scoring system or dies from  
350 causes attributable to the vaccine;

351 • The average body temperature increase for all vaccinated piglets (group mean) for the  
352 observation period does not exceed 1.5°C above baseline; and no individual piglet shows a  
353 temperature rise above baseline greater than 2.5°C for a period exceeding 3 days.

354 • On each day during the observation period, the maximum increase in body temperature above  
355 the baseline observed for each pig will be used to calculate the daily group mean temperature  
356 rise. This mean value should not exceed 1.5°C and no individual pig should show a rise in  
357 temperature above baseline greater than 1.5°C for a period exceeding 3 consecutive days.

358 • No vaccinated pigs show notable signs of disease by gross pathology

359 ii) Safety test in pregnant sows and test for transplacental transmission

360 There is limited currently an absence of published information on ASFV pathogenesis in breeding-  
361 age gilts and in pregnant sows associated with ASFV transplacental infection and fetus  
362 abortion/stillbirth. If a label claim is pursued for use in breeding age gilts and sows, then a safety

363 study in line with VICH GL44 (*Guidelines on Target Animal Safety for Veterinary Live and*  
364 *Inactivated Vaccines, Section 2.2. Reproductive Safety Test, 2009<sup>4</sup>*) should be completed.

365 iii) Horizontal transmission

366 The test is conducted using no fewer than 12 healthy piglets, a minimum of 6 4-weeks old and not  
367 older than 10-weeks old and of the same origin, that do not have antibodies against ASFV, and  
368 blood samples are negative on real-time PCR. All piglets are housed together from day 0 and the  
369 number of vaccinated animals is the same as the number of naïve, contact animals. Co-mingle  
370 equal numbers of vaccinated and naïve, contact piglets from day 0 in the same pen or room.

371 Use vaccine virus at the least attenuated passage level that will be present between the master  
372 seed lot and a batch of the vaccine. Administer by each recommended route of administration to  
373 no fewer than six piglets a quantity of the vaccine virus equivalent to not less than the maximum  
374 virus titre (maximum release dose) likely to be contained in 1 dose of the vaccine.

375 To obtain individual and group mean baseline temperatures, the body temperature of each naïve,  
376 contact piglet is measured on at least the 3 consecutive days preceding co-mingling with vaccinated  
377 piglets. The body temperature of each naïve, contact piglet is then measured daily for at least 45  
378 days, preferably 60 days.

379 To confirm the presence or absence of fever accompanied by disease, observe the naïve, contact  
380 piglets daily for at least 45 days, preferably 60 days. On each day during the observation period the  
381 maximum increase in body temperature above the baseline observed for each pig will be used to  
382 calculate the daily group mean temperature rise. This mean value should not exceed 1.5°C and no  
383 individual pig should show a rise in temperature above baseline greater than 1.5°C for a period  
384 exceeding 3 consecutive days. Carry out the daily observations for signs of acute and chronic  
385 clinical disease using a quantitative clinical scoring system adding the values for multiple clinical  
386 signs (e.g. Gallardo *et al.*, 2015a). These clinical signs should include fever, anorexia, recumbency,  
387 skin haemorrhage or cyanosis, joint swelling and necrotic lesions around the joints, respiratory  
388 distress and digestive findings.

389 In addition, Blood should be taken from the naïve contact piglets at least twice a week for the first  
390 21 days post-vaccination and then on a weekly basis. From the blood samples, determine vaccine  
391 virus titres by quantitative virus isolation (HAD<sub>50</sub>/ml, TCID<sub>50</sub>/ml or other methods, e.g. titration using  
392 IPT or FAT detection). Quantitative PCR may be used to detect positive samples, but results should  
393 be confirmed by infectious virus titration as described above infectious virus titres by quantitative  
394 virus isolation (e.g. HAD<sub>50</sub>/ml or TCID<sub>50</sub>/ml) and using a real-time PCR test.

395 If the vaccine virus is non haemadsorbing or does not cause cytopathic effects, a real-time PCR  
396 test only may be used.

397 Collect blood (serum) samples from the naïve contact pigs at least at day 21 and day 28 days and  
398 carry out an appropriate test to detect vaccine virus antibodies. At a minimum of 45 days, humanely  
399 euthanise all naïve, contact piglets. Conduct gross pathology on spleen, lung, tonsil, and kidney  
400 tissue samples and at least three different lymph nodes. Determine virus titres in all collected  
401 samples by quantitative virus isolation (HAD<sub>50</sub>/mg or TCID<sub>50</sub>/mg) or other appropriate methods (e.g.  
402 IPT or FAT detection). Quantitative PCR may be used to detect positive samples, but results should  
403 be confirmed by infectious virus titration as described above and real-time(RT)-PCR (see Section  
404 B.1. Identification of the agent). If the vaccine virus is non-haemadsorbing or does not cause  
405 cytopathic effects, a real-time PCR test or other appropriate method (e.g. titration using IPT or FAT  
406 detection) may be used.

407 The vaccine complies with the test if:

- 408 • No vaccinated or naïve contact piglet shows abnormal (local or systemic) reactions or notable  
409 signs of disease, reaches the predetermined humane endpoint defined in the clinical scoring  
410 system or dies from causes attributable to the vaccine;
- 411 • On each day during the observation period the maximum increase in body temperature above  
412 the baseline observed for each pig will be used to calculate the daily group mean temperature  
413 rise. This mean value should not exceed 1.5°C and no individual pig should show a rise in

<sup>4</sup> [https://www.ema.europa.eu/en/documents/scientific-guideline/vich-gl44-target-animal-safety-veterinary-live-inactivated-vaccines-step-7\\_en.pdf](https://www.ema.europa.eu/en/documents/scientific-guideline/vich-gl44-target-animal-safety-veterinary-live-inactivated-vaccines-step-7_en.pdf)

414 temperature above baseline greater than 1.5°C for a period exceeding 3 consecutive days  
415 The average body temperature increase for all naïve, contact piglets (group mean) for the  
416 observation period does not exceed 1.5°C above baseline; and no individual piglet shows a  
417 temperature rise above baseline greater than 2.5°C for a period exceeding 3 days;

418 • No naïve, contact piglet shows notable signs of disease by gross pathology and no virus is  
419 detected in their blood or tissue samples;

420 • No or a low percentage of contact piglets test both real-time PCR positive and seropositive  
421 No naïve contact pigs test positive for antibodies to the vaccine virus.

422 iv) Post-vaccination kinetics of viral replication (MLV blood and tissue dissemination) study  
423 Prior to the reversion to virulence study (Section C2.3.2.v. below), a minimum of one study should  
424 be performed to determine the post-vaccination kinetics of virus replication in the blood (viremia),  
425 tissues and viral shedding.

426 The test consists of the administration of the vaccine virus from the master seed lot to no fewer  
427 than eight healthy piglets, and preferably ten healthy piglets, a minimum of 6-4-weeks old and not  
428 older than 10-weeks old and of the same origin, that do not have antibodies against ASFV, and  
429 blood samples are negative on real-time PCR.

430 Administer to each piglet, using the recommended route of administration most likely to result in  
431 spread (such as the intramuscular route or intranasal route), a quantity of the master seed vaccine  
432 virus equivalent to not less than the maximum virus titre (maximum release dose) likely to be  
433 contained in 1 dose of the final product of the vaccine.

434 Record daily body temperatures and observe inoculated animals daily for clinical disease for at  
435 least 45 days, preferably 60 days.

436 Carry out the daily observations for signs of acute and chronic clinical disease using a quantitative  
437 clinical scoring system adding the values for multiple clinical signs (e.g. Gallardo *et al.* (2015a).  
438 These clinical signs should include fever, anorexia, recumbency, skin haemorrhage or cyanosis,  
439 joint swelling and necrotic lesions around the joints, respiratory distress and digestive findings.

440 Collect blood samples from all the piglets at least two times per week from 3 days post-vaccination  
441 for the first 2 weeks, then weekly for the duration of the test. Determine vaccine virus titres by  
442 quantitative virus isolation (HAD<sub>50</sub>/ml or TCID<sub>50</sub>/ml) or other appropriate methods (e.g. titration using  
443 IPT or FAT detection). Quantitative PCR may be used to detect positive samples but results should  
444 be confirmed by infectious virus titration as described above and using a real-time PCR test. If the  
445 vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test only  
446 may be used.

447 Determine which blood timepoint(s) should be used in the design of the reversion to virulence study  
448 (Section C2.3.2.v. below), for example, specific blood sample(s) at specific timepoints that show  
449 the highest titres should be considered for selection and use in the reversion to virulence study.

450 Collect oral, nasal and faecal swab samples (preferably devoid of blood to minimise assay  
451 interference) at least two times per week from 3-days post-vaccination for the first 2 weeks, then  
452 weekly for the duration of the test. Test the swabs for the presence of vaccine virus. Determine  
453 virus titres in all collected samples by quantitative virus isolation (HAD<sub>50</sub>/ml or TCID<sub>50</sub>/ml) or other  
454 appropriate methods (e.g. titration using IPT or FAT detection). Quantitative PCR may be used to  
455 detect positive samples, but results should be confirmed by infectious virus titration as described  
456 above and using a real-time PCR test. If the vaccine virus is non-haemadsorbing or does not cause  
457 cytopathic effects, a real-time PCR test or other appropriate method (e.g. titration using IPT or FAT  
458 detection) may be used.

459 Euthanise at least two piglets on days 5, 7, 14, 21, and preferably on day 28 (±2 days at each  
460 timepoint) and collect spleen, lung, tonsil, kidney tissue samples and at least three different lymph  
461 nodes (which should include lymph node closest to site of inoculation, gastrohepatic and  
462 submandibular nodes). Determine virus titres in all collected samples by quantitative virus isolation  
463 (HAD<sub>50</sub>/mg or TCID<sub>50</sub>/mg) or other appropriate methods (e.g. titration using IPT or FAT detection).  
464 Quantitative PCR may be used to detect positive samples, but results should be confirmed by  
465 infectious virus titration as described above and using real-time PCR test. If the vaccine virus is

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non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test or other appropriate method (e.g. titration using IPT or FAT detection) may be used.

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Determine which tissue(s) and timepoint(s) should be used to aid in the design of the reversion to virulence study (Section C.2.3.2.v), for example, specific tissues at specific timepoints which show the highest titres should be considered for selection and use in the reversion to virulence study.

471 v) Reversion to virulence

472 The test carried out should be consistent with VICH GL41 (Examination of live veterinary vaccines  
473 in target animals for absence of reversion to virulence, 2008<sup>5</sup>).

474 The test for increase in virulence consists of the administration of the vaccine master seed virus to  
475 healthy piglets of an age (e.g. between 6-4 weeks and 10 weeks old) suitable for recovery of the  
476 strain and of the same origin, that do not have antibodies against ASFV, and blood samples that  
477 are negative on real-time PCR. This protocol is typically repeated five times.

478 First passage (p1)

479 Administer to no fewer than two piglets, and preferably no fewer than four piglets using the intended  
480 route of administration for the final product, a quantity of the master seed vaccine virus equivalent  
481 to not less than the maximum virus titre (maximum release dose) likely to be contained in 1 dose  
482 of the final product of the vaccine. Observe inoculated animals daily for the appearance of at least  
483 two and preferably at least three clinical signs and record daily body temperatures using a  
484 quantitative clinical scoring system adding the values for multiple clinical signs (e.g. Gallardo *et al.*,  
485 2015a) and record daily body temperatures.

486 Based on results from at least one completed post-vaccination kinetics of viral replication (MLV  
487 vaccine shed and spread (virus blood and tissue dissemination study (Section C.2.3.2.iv above),  
488 collect an appropriate quantity of blood from each piglet on the predetermined single timepoint(s)  
489 (day 5-3-13). Determine virus titres in individual blood samples by quantitative virus isolation  
490 (HAD<sub>50</sub>/ml or TCID<sub>50</sub>/ml) or other appropriate methods (e.g. titration using IPT or FAT detection).  
491 Quantitative PCR may be used to detect positive samples, but results should be confirmed by  
492 infectious virus titration as described above and by real-time PCR. If the vaccine virus is non-  
493 haemadsorbing or does not cause cytopathic effects, a real-time PCR test or other appropriate  
494 method (e.g. titration using IPT or FAT detection) may be used. Identify the individual blood  
495 sample(s) with the highest infectious titre and reserve for the subsequent *in-vivo* passage (second  
496 pass, p2).

497 Based on results from at least one completed vaccine virus-MLV blood and tissue distribution  
498 dissemination study (Section C.2.3.2.iv above), euthanise piglets on the predetermined timepoint  
499 (i.e. day 5, 7, 14, 21, or 28). Determine infectious virus titres in individual tissue samples by  
500 quantitative virus isolation (HAD<sub>50</sub>/ml or TCID<sub>50</sub>/ml) or other appropriate methods (e.g. titration using  
501 IPT or FAT detection). Quantitative PCR may be used to detect positive samples, but results should  
502 be confirmed by infectious virus titration as described above. If the vaccine virus is non-  
503 haemadsorbing or does not cause cytopathic effects, a real-time PCR test or other appropriate  
504 method (e.g. titration using IPT or FAT detection) may be used. Identify individual tissue sample  
505 type(s) with the highest infectious titre. Pool the tissues with the highest titres from different organs  
506 from all each animals with the highest titres and prepare at least a 40% virus suspension to obtain  
507 a virus titre within the range used for inoculation in PBS, pH 7.2 kept at 4°C or at -70°C for longer  
508 storage.

509 Test each blood and tissue sample pool used for inoculation by PCR to confirm the absence of  
510 potential viral agent contaminants (i.e. CSFV, FMDV, PRRS, PCV2). Blood and pooled tissue (p1)  
511 are used to inoculate 2 ml of positive material diluted to the maximum release dose likely to be  
512 contained in 1 dose of the vaccine using the intended route of administration for the final product  
513 to each of at least two and ideally at least four further pigs of the same age and origin.

514 Second pass (p2)

515 If no virus is found at passage 1 (p1), repeat the administration by the intended route ~~once~~ again  
516 with the same pooled material (blood and pooled tissue, p1) in another ten healthy piglets of the  
517 same age and origin. If no virus is found at this point during this second passage (p2) at this point,  
518 end the process here.

519 Second passage (p2)

520 If ~~however~~ virus is found in p1, carry out a second series of passages by administering 2 ml of  
521 positive material diluted to the maximum release dose likely to be contained in 1 dose of the vaccine

<sup>5</sup> [https://www.ema.europa.eu/en/documents/scientific-guideline/vich-gl41-target-animal-safety-examination-live-veterinary-vaccines-target-animals-absence-reversion\\_en.pdf](https://www.ema.europa.eu/en/documents/scientific-guideline/vich-gl41-target-animal-safety-examination-live-veterinary-vaccines-target-animals-absence-reversion_en.pdf)

522 using the intended route of administration for the final product to each of no fewer than two piglets,  
523 and preferably no fewer than four piglets of the same age and origin. Observe inoculated animals  
524 daily for the appearance of at least two and preferably at least three clinical signs using a  
525 quantitative clinical scoring system adding the values for multiple clinical signs (e.g. Gallardo *et al.*,  
526 2015a), and record daily body temperatures and determine infectious virus titres in individual blood  
527 and tissue samples as described for p1 above.

528 *Third and fourth pass (p3 and p4)*

529 If no virus is found at in (p2), repeat the intramuscular administration by the intended route once  
530 again with the same pooled material (blood and pooled tissue, p2) in another eight healthy piglets  
531 of the same age and origin. If no virus is found at this point, end the process here.

532 *Third and fourth passage (p3 and p4)*

533 If, however, virus is found on p2, carry out this passage operation no fewer than two additional  
534 times (p3 and p4) (to each of no fewer than two piglets, and preferably no fewer than four piglets  
535 of the same age and origin) and verifying the presence of the virus at each passage in blood and  
536 tissues. Observe inoculated animals daily for the appearance of at least two and preferably at least  
537 three clinical signs using a quantitative clinical scoring system adding the values for multiple clinical  
538 signs (e.g. Gallardo *et al.*, 2015a) and record daily body temperatures.

539 *Fifth passage (p5)*

540 Administer 2 ml of the blood and pooled tissue (p4) to each of at least eight healthy piglets of the  
541 same age and origin. Observe inoculated animals daily for at least 28 days post-inoculation for the  
542 appearance of at least two and preferably at least three clinical signs using a quantitative clinical  
543 scoring system adding the values for multiple clinical signs (e.g. Gallardo *et al.*, 2015a), and record  
544 daily body temperature and determine infectious virus titres in individual blood and tissue samples  
545 as described above.

546 The vaccine virus complies with the test if:

- 547 • No piglet shows abnormal (local or systemic) reactions, or notable signs of disease, or reaches  
548 the pre-determined humane endpoint defined in the clinical scoring system or dies from  
549 causes attributable to the vaccine; and
- 550 • There is no indication of increasing virulence (as monitored by daily body temperature  
551 accompanied by clinical sign observations) of the maximally passaged virus compared with  
552 the master seed virus.

553 At a minimum, a safe MLV vaccine shall demonstrate ALL the following features (minimal  
554 standards):

- 555 • Absence of fever (on each day during the observation period, the maximum increase in body  
556 temperature above the baseline observed for each pig will be used to calculate the daily group  
557 mean temperature rise. This mean value should not exceed 1.5°C and no individual pig should  
558 show a rise in temperature above baseline greater than 1.5°C (defined as average body  
559 temperature increase for all vaccinated piglets (group mean) for the observation period does  
560 not exceed 1.5°C above baseline; and no individual piglet shows a temperature rise above  
561 baseline greater than 2.5°C for a period exceeding 3 days);
- 562 • Absence of chronic and acute clinical signs and gross pathology over the entire test period or  
563 minimal chronic mild clinical signs (defined as e.g. mild swollen joints with a low clinical score  
564 that resolve within 1 week).
- 565 • Minimal (defined as no naïve, contact piglet shows notable signs of disease by clinical signs  
566 and gross pathology and no or a low percentage of contact piglets test both real-time PCR  
567 positive and seropositive) or no vaccine virus transmission (defined as no naïve, contact piglet  
568 shows notable signs of disease by clinical signs and gross pathology and no contact piglets  
569 test both real-time PCR positive and seropositive) over the entire test period;
- 570 • Absence of an increase in virulence (genetic and phenotypic stability) (complies with the  
571 reversion to virulence test).

572 In addition, for regulatory approval, ASF MLV the vaccines in their commercial presentation before  
573 being authorised for general use should be tested for safety in the under field conditions (see

574 chapter 1.1.8 Section 7.2.3). Additional Field safety studies generally evaluation studies may  
575 include measurement of body temperatures, observation of local or systemic reactions and, where  
576 appropriate, performance measurements but are not limited to: environmental persistence (e.g.  
577 determination of virus recovery from bedding or other surfaces), assessment of  
578 immunosuppression, and negative impacts on performance.

### 579 **2.3.3. Efficacy requirements**

#### 580 **i) Protective dose**

581 Vaccine efficacy is estimated in immunised animals directly, by evaluating their resistance to live  
582 virus challenge. The test consists of a vaccination/challenge trial in piglets a minimum of 6-4 weeks  
583 old and not more than 10-weeks old, free of antibodies to ASFV, and negative blood samples by  
584 real-time PCR. The test is conducted using no fewer than 15 and preferably no fewer than 24  
585 vaccinated pigs, and no fewer than five non-vaccinated control piglets.

586 The test is conducted to determine the minimal immunising dose (MID) (also referred to as the  
587 minimal protective dose [MPD] or protective fraction); using at least three groups of no fewer than  
588 five and preferably not fewer than eight vaccinated piglets per group, and one additional group of  
589 no fewer than five non-vaccinated piglets of the same age and origin as controls. Use vaccine  
590 containing virus at the highest passage level that will be present in a batch of vaccine.

591 Each group of piglets, except the control group, is immunised with a different vaccine virus content  
592 in the same vaccine volume. In at least one vaccinated group, piglets are immunised with a vaccine  
593 dose containing not more than the minimum virus titre (minimum release dose) likely to be  
594 contained in one dose of the vaccine as stated on the label.

595 Twenty-eight days ( $\pm 2$  days) after the single injection dose of vaccine (or if using two injections  
596 doses of the vaccine then 28 days [ $\pm 2$  days] following the second injection dose), challenge all the  
597 piglets by the intramuscular route. If previous studies have demonstrated acceptable efficacy using  
598 IM challenge, then a different challenge route (e.g. direct contact, oral or oronasal) may be used.  
599 Challenged, vaccinated piglets may be housed in one or more separate pens in the same room or  
600 in different rooms. Challenged, naïve controls can be housed in one or more rooms that are  
601 separate from challenged, vaccinated piglets.

602 Carry out the test using an ASFV representative strain of the epidemiologically relevant field  
603 strain(s) where the vaccine is intended for use (e.g. ASFV B646L [p72] genotype II pandemic strain  
604 and other p72 virulent genotype of recognised epidemiologic importance). For gene deleted,  
605 recombinant MLV viruses, if neither challenge virus type is available, then carry out the test with  
606 the parental, virulent virus used to generate the MLV recombinant virus. Use a  $10e3$ – $10e4$  HAD<sub>50</sub>  
607 (or TCID<sub>50</sub> for non-HAD viruses) challenge dose sufficient to cause death or meet the humane  
608 endpoint in 100% of the nonvaccinated piglets in less than 21 days. Higher or lower challenge  
609 doses can be considered if appropriately justified.

610 The rectal temperature of each vaccinated piglet is measured on at least the 3 days preceding  
611 administration of the challenge virus, at the time of challenge, 4 hours after challenge, and then  
612 daily for the observation period of at least 28-45 days, preferably 35-60 days. Observe the piglets  
613 at least daily for at least 28 days, preferably 35 days. Carry out the daily observations for signs of  
614 acute and chronic clinical disease using a quantitative clinical scoring system adding the values for  
615 multiple clinical signs (e.g. Gallardo *et al.*, 2015). These clinical signs should include fever,  
616 anorexia, recumbency, skin haemorrhage or cyanosis, joint swelling and necrotic lesions around  
617 the joints, respiratory distress and digestive findings.

618 Collect oral, nasal, anal and blood samples from the vaccinated challenged piglets at least two  
619 times once per week from 3 days post-challenge for at least 28-14 days, then weekly up to 35 days  
620 post-challenge and then every 14 days up to the end of the observation period, preferably 35 days.  
621 From the blood samples, determine infectious virus titres by quantitative virus isolation (HAD<sub>50</sub>/ml  
622 or TCID<sub>50</sub>/ml) or other appropriate methods (e.g. titration using IPT or FAT detection). Quantitative  
623 PCR may be used to detect positive samples, but results should be confirmed by infectious virus  
624 titration as described above and using a real-time PCR test. If the vaccine virus is non-  
625 haemadsorbing or does not cause cytopathic effects, a real-time PCR test only may be used.

626 At the end of the test period, humanely euthanise all vaccinated challenged piglets. Conduct gross  
627 pathology (and histopathology if considered necessary) on spleen, lung, tonsil, and kidney tissue  
628 samples and at least three different lymph nodes (which should include lymph node closest to site



629 of inoculation, gastrohepatic and submandibular nodes). Determine virus titres in all collected  
630 samples by quantitative virus isolation (HAD<sub>50</sub>/mg or TCID<sub>50</sub>/mg) or other appropriate methods (e.g.  
631 titration using IPT or FAT detection). Quantitative PCR may be used to detect positive samples, but  
632 results should be confirmed by infectious virus titration as described above and real-time PCR (see  
633 Section B.1. Identification of the agent). If the vaccine virus is non-haemadsorbing or does not  
634 cause cytopathic effects, a real-time PCR test or other appropriate method (e.g. titration using IPT  
635 or FAT detection) may be used.

636 The test is invalid if fewer than 100% the difference between in the number of unvaccinated control  
637 piglets infected with the live challenge virus and the number of vaccinated / challenged piglets  
638 vaccinated with the minimum release dose that die or reach a humane endpoint is not statistically  
639 significant.

640 The vaccine (or a specific vaccine virus dose if conducting a vaccine dose titration study) complies  
641 with the test if:

- 642 • No vaccinated challenged piglet dies or shows abnormal (local or systemic) reactions, reaches  
643 the humane endpoint or dies from causes attributable to ASF;
- 644 • On each day during the observation period the maximum increase in body temperature above  
645 the baseline observed for each pig will be used to calculate the daily group mean. This mean  
646 value should not exceed 1.5°C and no individual pig should show a rise in temperature above  
647 baseline greater than 2.0°C for a period exceeding 2 consecutive days. The average body  
648 temperature increase for all vaccinated challenged piglets (group mean) for the observation  
649 period does not exceed 2.0°C above baseline; and no individual piglet shows a temperature  
650 rise above baseline greater than 2.0°C;
- 651 • The vaccinated challenged piglets display a reduction or absence of typical acute clinical signs  
652 of disease and gross pathology and a reduction or absence of challenge virus levels in blood  
653 and tissues.

654 ii) Assessment for horizontal transmission (challenge virus shed and spread study)

655 The ASF basic reproduction number, R<sub>0</sub>, can be defined as the average number of secondary ASF  
656 disease cases caused by a single ASFV infectious pig during its entire infectious period in a fully  
657 susceptible population (Hayes *et al.*, 2021). In general, if the ASFV effective reproduction number  
658  $Re = R_0 \times (S/N)$  (S= susceptible pigs; N= total number of pigs in a given population) is greater than  
659 1.0, disease is predicted to spread. Ideally, ASF vaccination should reduce Re to less than 1.0 by  
660 reducing the number of susceptible, naïve, contact pigs exposed to vaccinated, infected pigs.

661 To evaluate ASF vaccine impact on ASF disease transmission, the test consists of a  
662 vaccination/challenge trial in piglets a minimum of 6-4 weeks old and not older than 10-weeks old,  
663 free of antibodies to ASFV, and negative blood samples by real-time PCR.

664 The test is conducted using no fewer than 15 healthy piglets at a ratio comprising twice the number  
665 of vaccinated piglets to naïve piglets (e.g. ten vaccinated and five naïve). Use vaccine containing  
666 virus at the highest passage level that will be present in a batch of the vaccine.

667 The quantity of vaccine virus administered to each pig is equivalent to be not less than the minimum  
668 virus titre (minimum dose) likely to be contained in one dose of the vaccine as stated on the label.  
669 Following immunisation, vaccinated and naïve piglets should continue to be co-mingled.

670 Twenty-eight days (±2 days) after the single injection dose of vaccine (or if using two injections  
671 doses of the vaccine then 28 days [±2 days] following the second injection dose), temporarily  
672 separate [into different pen(s) or room(s)] all vaccinated piglets from naïve piglets. Challenge all  
673 vaccinated piglets by the intramuscular or other previously verified route. Carry out the challenge  
674 using an ASFV representative strain of the epidemiologically relevant field strain(s) where the  
675 vaccine is intended for use (e.g. ASFV B646L [p72] genotype II pandemic strain and other p72  
676 virulent genotype of recognised epidemiological importance). For gene deleted, recombinant MLV  
677 viruses, if neither challenge virus type is available, then carry out the test with the parental, virulent  
678 virus used to generate the MLV recombinant virus. Use a 10e3–10e4 HAD<sub>50</sub> (or TCID<sub>50</sub> for non-  
679 HAD viruses challenge dose sufficient to cause death or met the humane endpoint in 100% of the  
680 nonvaccinated piglets in less than 21 days. Higher or lower challenge doses can be considered if  
681 appropriately justified.

682 Approximately 18–24 hours later, re-introduce naïve piglets to vaccinated, challenged piglets and  
683 allow for direct nose to nose contact exposure with vaccinated, challenged piglets. Allow for  
684 continuous contact exposure by co-mingling both groups through the end of the study. If more than  
685 one pen or room is used for co-housing, following reintroduction initially maintain a ratio of 2:1 of  
686 challenged, vaccinated piglets to contact exposed, naïve piglets.

687 The rectal temperature of each contact piglet is measured on at least the 3 days preceding  
688 administration of the challenge virus to vaccinated pigs, immediately prior to direct contact  
689 exposure, 4 hours post-contact exposure, and then daily for at least 28, preferably 35 days and  
690 twice a week for at least 60 days. Observe all contact exposed piglets at least daily for at least 28  
691 days, and then twice a week for at least 60 days preferably for at least 35 days.

692 Carry out the daily observations in each contact piglet for signs of acute and chronic clinical disease  
693 using a quantitative clinical scoring system adding the values for multiple clinical signs (e.g.  
694 Gallardo *et al.*, 2015a). These clinical signs should include fever, anorexia, recumbency, skin  
695 haemorrhage or cyanosis, joint swelling and necrotic lesions around the joints, respiratory distress  
696 and digestive findings.

697 In addition, blood should be taken from the naïve contact piglets at least twice a week from 3 days  
698 post-contact exposure for the duration collect blood samples from the contact piglets at least two  
699 times per week from 3 days post-contact for at least 14 days, then weekly up to 35 days post-  
700 contact exposure and then every 14 days up to the end of the test period. Determine virus titres in  
701 all collected samples by quantitative virus isolation (HAD<sub>50</sub>/mg or TCID<sub>50</sub>/mg) or other appropriate  
702 methods (e.g. titration using IPT or FAT detection). Quantitative PCR may be used to detect positive  
703 samples, but results should be confirmed by infectious virus titration as described above. From the  
704 blood samples, determine infectious challenge virus titres by quantitative virus isolation (HAD<sub>50</sub>/ml  
705 or TCID<sub>50</sub>/ml) and using a real-time PCR test. If the vaccine virus is non-haemadsorbing or does  
706 not cause cytopathic effects, a real-time PCR test only may be used.

707 Collect blood (serum) samples from the naïve contact pigs at least at day 21 and day 28 (±2 days),  
708 and at the end of the test period, and carry out an appropriate test to detect vaccine virus antibodies.

709 Collect oral, nasal and faecal swab samples (preferably devoid of blood to minimise assay  
710 interference) from all contact-exposed naïve piglets at least two times per week from 3-days post-  
711 contact exposure for the first 2 weeks, then weekly for the duration of the test and test swabs for  
712 the presence of challenge virus. Determine virus titres in all collected samples by quantitative virus  
713 isolation (HAD<sub>50</sub>/mg or TCID<sub>50</sub>/mg) or other appropriate methods (e.g. titration using IPT or FAT  
714 detection). Quantitative PCR may be used to detect positive samples, but results should be  
715 confirmed by infectious virus titration as described above. Determine virus titres in all collected  
716 samples by quantitative virus isolation (HAD<sub>50</sub>/ml or TCID<sub>50</sub>/ml) and using a real-time PCR test. If  
717 the vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test  
718 or other appropriate method (e.g. titration using IPT or FAT detection) may be used.

719 At the end of the test period, humanely euthanise all contact piglets. Conduct gross pathology on  
720 spleen, lung, tonsil, and kidney tissue samples and at least three different lymph nodes. (which  
721 should include lymph node closest to site of inoculation, gastrohepatic and submandibular nodes).  
722 Determine virus titres in all collected samples by quantitative virus isolation (HAD<sub>50</sub>/mg or  
723 TCID<sub>50</sub>/mg) or other appropriate methods (e.g. titration using IPT or FAT detection). Quantitative  
724 PCR may be used to detect positive samples, but results should be confirmed by infectious virus  
725 titration as described above. Determine virus titres in all collected samples by quantitative virus  
726 isolation (HAD<sub>50</sub>/mg or TCID<sub>50</sub>/mg) and real-time PCR (see Section B.1. Identification of the agent).  
727 If the vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-time PCR  
728 test or other appropriate method (e.g. titration using IPT or FAT detection) may be used.

729 The test is invalid if the vaccine fails to comply with the compliance criteria described for the  
730 protected dose test in vaccinated pigs (Section C.2.3.3.i above).

731 If the manufacturer claims that the vaccine induces sterilising immunity, the vaccine complies with  
732 the test for a reduction in horizontal disease transmission if all the following conditions are satisfied:

- 733 • No naïve, contact exposed piglet shows abnormal (local or systemic) reactions, reaches the  
734 defined humane endpoint or dies from causes attributable to ASF;
- 735 • No naïve, contact exposed piglet displays fever accompanied by typical signs of disease,  
736 including gross pathology.

- 737 • Naïve contact pigs show an absence of challenge virus in blood and tissues.
- 738 • No naïve contact pigs test positive for antibodies to the challenge virus.
- 739 Otherwise, the vaccine complies with the test for a reduction in horizontal disease transmission if:
- 740 • Naïve contact pigs show a reduction or absence of challenge virus levels in blood and tissues.
- 741 • None of or a reduced number of naïve contact exposed pigs test positive for antibodies to the
- 742 challenge virus.
- 743 At a minimum, an efficacious MLV vaccine shall demonstrate ALL the following features (minimal
- 744 standards):
- 745 • Protects against mortality;
- 746 • Reduces acute disease (fever accompanied by a reduction of typical clinical and pathological
- 747 signs of acute disease)
- 748 • Reduces levels of viral shedding and viraemia.
- 749 • Reduces horizontal disease transmission (no none of or a reduced number of naïve, contact
- 750 exposed piglets shows abnormal [local or systemic] reactions, reaches the humane endpoint
- 751 or dies from causes attributable to ASF, and displays fever accompanied by typical acute
- 752 disease signs caused by ASF) and test positive for antibodies to the challenge virus.
- 753 • Reduces levels of viral shedding and viraemia.
- 754 In general, for regulatory approval, ASF MLV addition, the vaccines in their commercial presentation
- 755 before being authorised for general use should be tested for efficacy in the under field conditions (see
- 756 chapter 1.1.8 Section 7.2.3). Additional field efficacy evaluation studies may generally include but are
- 757 not limited to: onset of immunity, duration of immunity, and impact on disease transmission measurement
- 758 of relevant efficacy parameters including but limited to mortality, clinical signs, impact on disease
- 759 transmission, performance parameters.

#### 2.3.4. Duration of immunity

760 Although not included in the guidance for ASF MLV first generation vaccines, manufacturers are

761 encouraged required, as part of the authorisation procedure, to define and demonstrate the duration of

762 immunity of a given vaccine by evaluation of potency at the end of the claimed period of protection.

763

#### 2.3.5. Stability

764 Stability of the vaccine should be demonstrated over the shelf life recommended for the product. Although

765 not included in the standards for first generation MLV ASF vaccines, manufacturers are encouraged

766 required, as part of the authorisation procedure, to generate data supporting the retention of

767 immunogenicity over a defined period of validity time of a lyophilised or other pharmaceutical form of the

768 ASF vaccine as part of the authorisation procedure.

769

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937 \*  
938 \* \*

939 **NB:** There are WOA Reference Laboratories for African swine fever  
940 (please consult the WOA Web site:  
941 <https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3>).  
942 Please contact the WOA Reference Laboratories for any further information on  
943 diagnostic tests and reagents for African swine fever

944 **NB:** FIRST ADOPTED IN 1990. MOST RECENT UPDATES ADOPTED IN 2021.





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**Anexo 17. Plantilla para que los candidatos a designación como expertos en laboratorios de referencia presenten su *curriculum vitae***

**REUNIÓN DE LA COMISIÓN DE NORMAS BIOLÓGICAS**

**París, 5–9 de febrero de 2024**

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Apellido	<input type="text"/>	Nombre(s)	<input type="text"/>
Correo electrónico	<input type="text"/>	Teléfono	<input type="text"/>
Nombre del laboratorio	<input type="text"/>	Nombre de la enfermedad	<input type="text"/>
País del laboratorio	<input type="text"/>	Fecha de presentación	<input type="text"/>

1. Títulos y cualificaciones; por favor, aporte los datos y los años de obtención.

2. Experiencia relevante, incluidos los puestos ocupados, con fechas y responsabilidades (que demuestre experiencia en diagnósticos de laboratorio).

- 
3. Información que demuestre el reconocimiento internacional de su experiencia: nombramientos, premios y pertenencia a comités y grupos de trabajo (relacionados con la enfermedad para la que solicita la designación).

4. Publicaciones en revistas revisadas por expertos y trabajos en prensa relacionados con la enfermedad o el agente patógeno respecto a los cuales solicita la designación (*Por favor, facilite aquellas publicaciones que destaquen su experiencia en la enfermedad específica: ponga en negrita su nombre en el título de las publicaciones, y también el del agente patógeno en cuestión*).

*Número de publicaciones como primer autor:*  
*Número de publicaciones como último autor:*  
*Número de publicaciones en otras posiciones:*

*Por favor, aporte la lista completa de publicaciones por orden cronológico.*

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**Anexo 18. Procedimiento de la OMSA para el registro de kits de diagnóstico - Resumen de los estudios de validación (kit Genelix™ de detección del VPPA por PCR en tiempo real)**

**REUNIÓN DE LA COMISIÓN DE NORMAS BIOLÓGICAS**

**París, 5–9 de febrero de 2024**

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**Nombre del kit de diagnóstico:** kit Genelix™ de detección del VPPA por PCR en tiempo real

**Fabricante:** Sanigen Co., Ltd.

**Número de procedimiento/aprobación:**

**Fecha de registro:** 052131

**Enfermedad:** Peste porcina africana (PPA)

**Agente patógeno:** virus de la PPA (VPPA)

**Tipo de prueba:** PCR en tiempo real

**Finalidad de la prueba**

El kit Genelix™ de detección del VPPA por PCR en tiempo real es un producto que detecta cualitativamente el VPPA y que confirma el diagnóstico de infección por el VPPA mediante un sistema de detección por PCR en tiempo real en sangre total, suero y tejidos porcinos en los que se sospecha de infección por el VPPA.

**Especies y muestras**

La especie diana es el cerdo doméstico y la prueba puede hacerse en sangre total, suero y tejidos porcinos. Para realizar pruebas se puede utilizar sangre entera almacenada con anticoagulantes. Se recomienda que las muestras se utilicen de inmediato tras su obtención. No obstante, si no es posible usarlas de inmediato, se pueden conservar unos días a 4 °C en una nevera, o bien pueden conservarse ultracongeladas a menos de -70 °C si se va a tardar más de 7 días en analizarlas. Las muestras deben dividirse en las cantidades que requieran las pruebas y conservarse a -20±5 °C en un congelador para evitar una descongelación reiterada. Si el procesado o el transporte implican una espera de más de 24 horas, deben conservarse a -20±5 °C. Debe evitarse la congelación y descongelación reiterada de las muestras.

**1. Información sobre el kit**

Consulte el prospecto del kit en el apartado sobre registros de la página web de la OMSA o bien contacte con el fabricante, Sanigen Co., Ltd., en:  
Telf.: +82-1833-8010  
Fax: +82-2-573-3134

**2. Resumen de los estudios de validación**

**Especificidad analítica**

**Conclusión:** Las pruebas de interferencia realizadas con las muestras positivas y negativas con cinco tipos de sustancias interferentes indicaron que no hay interferencia con los resultados. Se evaluó la prueba de reactividad cruzada para distinguir entre los analitos diana y no diana. Se confirmó la exclusividad con agentes patógenos relacionados con enfermedades porcinas o reactivos infecciosos (41 materiales, incluidas 16 bacterias, siete virus relacionados con enfermedades porcinas, y otros 18 virus). No se encontraron reactividades cruzadas significativas. Se sintetizaron nueve genotipos del gen p72 del VPPA, el analito diana del kit, y se comprobó su inclusividad. Todos los tipos del gen dieron positivo.

**Sensibilidad analítica**

**Conclusión:** Se realizó la prueba del límite de detección (LD) del kit Genelix™ de detección del VPPA por PCR en tiempo real para medir su sensibilidad analítica. Las concentraciones positivas bajas significativas se repitieron 24 veces, y los datos se volvieron a analizar mediante un análisis probit con un intervalo de confianza del 95 %; como resultado, la estimación máxima de 16,9 (1,7 x 10<sup>1</sup>) copias/μℓ se consideró el LD.

## Repetibilidad

**Conclusión:** La repetibilidad fue comprobada por una única persona, con un lote, durante 20 días, ejecutando la prueba dos veces al día, con duplicación de cada ejecución, y a tres concentraciones distintas. Como resultado de los experimentos realizados con dilución del ADN plasmídico del VPPA a los tres niveles de concentración de la muestra, se detectó el 100 % de las muestras, y el control negativo no presentó amplificación en ninguna muestras. El valor del coeficiente de variación (CV) fue inferior al 5 % en todos los casos

## Características diagnósticas:

Determinación del ciclo umbral y estimaciones de la sensibilidad (DSe) y la especificidad (DSp) diagnósticas:

### Conclusión:

El ciclo umbral o umbral de ciclos (Ct, por las siglas en inglés de *cycle threshold*) del kit Genelix™ de detección del VPPA por PCR en tiempo real es de 38,1 Ct. En el caso del LD, determinado mediante un análisis probit, el ciclo umbral se define como el valor Ct medio de la siguiente dilución más concentrada que se haya hallado hasta el LD establecido mediante un análisis probit. En la evaluación de determinación del ciclo umbral, el valor Ct medio fue de 38,1 a  $2,8 \times 10^1$  copias/μl, que es la concentración más cercana por encima del valor determinado mediante una prueba probit.

## Interpretación del resultado

- Los criterios de fijación del valor umbral y del valor basal según el equipo son los siguientes:

Equipo	Ciclo umbral	Valor basal inicial	Valor basal final
AB 7500	0,1	3	15
AB 7500 Fast	0,1	3	15
QuantStudio™ 5	0,4	3	15
Bio-rad CFX96™	100	3	15

- Si los resultados de los controles positivo y negativo coinciden con los criterios de la siguiente tabla, interprete los resultados de la muestra o muestras en estudio. Si los resultados de los controles no coinciden con los de la tabla, repita el experimento.

Tipo de control	Valor Ct
Control positivo	Ct ≤ 38,1
Control negativo	No detectado

- Compruebe el valor Ct de la muestra(s) empleando el software específico de cada equipo. Los datos relativos a la muestra se consideran positivos cuando Ct ≤ 38,1, y negativos cuando Ct > 38,1.

## Estimaciones de la sensibilidad (DSe) y la especificidad (DSp) diagnósticas e intervalos de confianza del 95%

- Para determinar la sensibilidad y la especificidad diagnósticas, se llevó a cabo una prueba comparativa empleando el método de referencia (validado y certificado por la OMSA), y los resultados se muestran a continuación.

Kit Genelix™ de detección del VPPA mediante PCR en tiempo real		VPPA/Sangre total y suero porcinos
Sensibilidad diagnóstica	n	187
	DSe	99,47 %
	IC	97,07 a 99,99%
Especificidad diagnóstica	n	553
	DSp	100 %
	IC	99,33 a 100,0%

Kit Genelix™ de detección del VPPA mediante PCR en tiempo real		VPPA/Tejido porcino
Sensibilidad diagnóstica	n DSe CI	22 100 % 84,56 a 100,0%
Especificidad diagnóstica	n DSp CI	450 100 % 99,18 a 100,0%

## Reproducibilidad

**Conclusión:** Los tres laboratorios de referencia de la OMSA para la PPA llevaron a cabo un estudio comparativo de la reproducibilidad. Para determinar la reproducibilidad de la prueba, se compararon los resultados obtenidos por tres laboratorios, en tres días distintos y llevando a cabo dos ejecuciones por día. Todos los resultados cualitativos coincidieron al 100% y cumplieron los criterios de aceptación, con un CV inferior al 5%. Los resultados de la prueba se muestran en la tabla siguiente:

Muestra nº	Coeficientes de variación (%)			
	Sanigen	Lab A	Lab B	Lab C
SNG-01	1,09	0,46	0,90	1,36
SNG-02	0,68	2,81	0,43	1,19
SNG-03	0,40	0,40	0,40	2,42
SNG-04	0,68	0,92	2,56	1,66
SNG-05	2,20	2,86	1,86	2,28
SNG-06	Negativo	Negativo	Negativo	Negativo
SNG-07	Negativo	Negativo	Negativo	Negativo
SNG-08	0,87	2,36	1,64	0,92
SNG-09	0,21	4,98	2,07	0,45
SNG-10	0,63	1,66	1,29	0,64
SNG-11	0,60	0,57	0,62	1,29
SNG-12	0,90	1,55	0,95	0,33
SNG-13	0,19	2,12	0,47	0,69
SNG-14	0,36	0,91	0,92	1,41
SNG-15	Negativo	Negativo	Negativo	Negativo
SNG-16	0,60	5,18	1,07	0,78
SNG-17	1,04	0,42	0,43	1,00
SNG-18	1,03	2,07	1,02	1,08
SNG-19	1,02	6,13	1,54	1,71
SNG-20	Negativo	Negativo	Negativo	Negativo

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- 
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**Anexo 19. Procedimiento de la OMSA para el registro de kits de diagnóstico - Resumen de los estudios de validación (Kit de prueba rápida Sentinel® de detección de anticuerpos contra el VPPA)**

**REUNIÓN DE LA COMISIÓN DE NORMAS BIOLÓGICAS**

**París, 5–9 de febrero de 2024**

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**Nombre del kit de diagnóstico:** Kit de prueba rápida Sentinel® de detección de anticuerpos contra el VPPA

**Fabricante:** Excelsior Bio-System Incorporation

**Número de procedimiento/aprobación:** 062233

**Fecha de registro:**

**Enfermedad:** Peste porcina africana

**Agente patógeno:** Virus de la peste porcina africana

**Tipo de prueba:** Ensayo inmunocromatográfico de flujo lateral (prueba rápida)

**Finalidad de la prueba:** Detección de anticuerpos asociados a una infección actual o una reacción inmunitaria a una exposición previa en un animal, un grupo de animales o una población definida. Para uso junto con otras pruebas o procedimientos de diagnóstico, como ayuda en el diagnóstico o en otras evaluaciones clínicas o epidemiológicas.

**Especies y muestras:** Suero porcino.

**1. Información sobre el kit**

Consulte el prospecto del kit en el apartado sobre registros de la página web de la OMSA o bien contacte con el fabricante en:

Página web: [ebs.com.tw/en/products/asfvrt](http://ebs.com.tw/en/products/asfvrt)

Correo electrónico: [sales@ebs.com.tw](mailto:sales@ebs.com.tw)

**2. Resumen de los estudios de validación**

**Especificidad analítica**

**Conclusión:**

- a) El Kit de prueba rápida Sentinel® de detección de anticuerpos contra el VPPA puede utilizarse para detectar en muestras de suero infección por diferentes genotipos (I, II, IX, X) del virus de la peste porcina africana.
- b) El Kit de prueba rápida Sentinel® de detección de anticuerpos contra el VPPA proporcionó resultados con una especificidad alta (93/95 = 97,89%; IC del 95% = 92,6% a 99,74%) y una reactividad cruzada muy baja al usarse con 95 muestras individuales para la detección de 19 agentes patógenos porcinos habituales (distintos del VPPA) de los cerdos domésticos.
- c) Los posibles factores de interferencia, como los anticoagulantes, la hemólisis (hemoglobina) y la lipemia (intralípidos), no afectaron a los resultados de la prueba.

**Sensibilidad analítica**

**Conclusión:**

Hubo una concordancia superior al 80% entre la prueba EURL-IPT (prueba de la inmunoperoxidasa) y la prueba Sentinel cuando los sueros tenían títulos de anticuerpos superiores a 1:5120.

**Repetibilidad**

**Conclusión:**

Para determinar la repetibilidad intraensayo, un técnico evaluó 4 sueros de referencia (fuerte, medio, débil y negativo) en pruebas en las que se analizaron por cuadruplicado. La repetibilidad entre ensayos se evaluó utilizando los mismos 4

sueros de referencia en 20 ejecuciones realizadas por tres técnicos en días distintos y con diferentes lotes de kits. Todas las ejecuciones intraensayo e interensayo de los cuatro sueros de referencia dieron resultados idénticos. La prueba rápida Sentinel® de detección de anticuerpos contra el VPPA presentó una repetibilidad del 100%. Según los informes de repetibilidad intraensayo e interensayo del Laboratorio Europeo de Referencia (EURL), se analizaron 10 sueros de referencia en una ronda/día durante 2 días, y cada ronda se analizó por duplicado. La prueba rápida Sentinel® de detección de anticuerpos contra el VPPA presentó una repetibilidad del 100%.

### Características diagnósticas:

#### Determinación del ciclo umbral:

La prueba rápida Sentinel® de detección de anticuerpos contra el VPPA es una prueba cualitativa. La muestra problema es positiva si aparecen dos líneas (tanto la línea C como la línea T), y negativa cuando sólo aparece la línea C. El ciclo umbral del título de anticuerpos es  $> 1:640$  ( $> 50\%$  de concordancia con la prueba EURL-IPT).

#### Estimaciones de la sensibilidad (DSe) y la especificidad (Dsp) diagnósticas:

Se analizaron 788 muestras de suero. A continuación, se indican los resultados obtenidos con la prueba EURL y el informe de evaluación de Excelsior Bio-System.

	EURL-IPT		Sin VPPA
	Positivo	Negativo	Negativo
Categoría 1: EURL-ASF-Ref1	8	2	-
Categoría 2: Suero de referencia experimental	122	23	-
Categoría 3: Muestras experimentales de cerdos infectados por el genotipo II del VPPA	148	96	-
Muestras de suero negativas de la <i>National Pingtung University of Science and Technology</i> (NPUST), Taiwán.	-	-	389
Total	278	121	389

Prueba rápida Sentinel® de detección de anticuerpos contra el VPPA		Muestras
Sensibilidad diagnóstica (DSe)	<b>81,65%</b> (IC del 95% = 76,60% a 86,02%)	Positivas según la prueba EURL-IPT: 278
Especificidad diagnóstica (Dsp)	<b>96,27%</b> (IC del 95% = 94,24% a 97,74%)	Negativas según la prueba EURL-IPT: 121 Ausencia de VPPA según la NPUST: 389

### Reproducibilidad

#### Conclusión:

El estudio de reproducibilidad fue realizado por el Instituto Pirbright y evaluado en tres laboratorios. Se analizaron 22 muestras positivas y 20 negativas, clasificadas según el resultado obtenido con ELISA (que se consideró la prueba estándar de referencia). Los resultados indican que la prueba rápida Sentinel® de detección de anticuerpos contra el VPPA puede dar un grado razonable de reproducibilidad entre los resultados de muestras replicadas obtenidos en distintos laboratorios. Los valores kappa de comparación entre laboratorios son los siguientes:



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<b>Interlaboratorio</b>	<b>Valor kappa</b>	<b>Resultado</b>
Lab 1 y Lab 2	<b>0,781</b> (IC del 95% = 0,582 a 0,981)	concordancia significativa
Lab 1 y Lab 3	<b>0,850</b> (IC del 95% = 0,695 a 1,000)	concordancia muy alta
Lab 2 y Lab 3	<b>0,791</b> (IC del 95% = 0,603 a 0,979)	concordancia significativa

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