CHAPTER 3.3.15.

TURKEY RHINOTRACHEITIS (AVIAN METAPNEUMOVIRUS INFECTIONS)

SUMMARY

Avian metapneumovirus (*aMPV*) *principally causes an acute highly contagious upper respiratory tract infection sometimes combined with reproductive disorders, primarily of turkeys, chickens and ducks. The disease produced by aMPV was originally referred to as avian pneumovirus infection and avian rhinotracheitis; it also has been referred to as turkey rhinotracheitis* (*TRT*) *in turkeys and as the triggering pathogen in swollen head syndrome* (*SHS*) *in chickens. aMPV is a single-stranded non-segmented negative-sense RNA virus belonging to the family* Pneumoviridae, *genus* Metapneumovirus*. The disease can cause significant economic losses for the poultry industry, particularly when exacerbated by secondary pathogens. Other avian species known to support the replication of aMPVs, other than turkeys, chickens, Muscovy and Peking ducks, are pheasants, and guinea fowl. The disease has global distribution in poultry-producing regions, with only Oceania and Canada reported to be free of aMPV infection. Four antigenically distinct subgroups, A, B, C and D, of aMPV have been identified by neutralisation with monoclonal antibodies, possible limited cross reactivity in enzyme-linked immunosorbent assay* (*ELISA*), *and sequence analysis of the attachment glyco protein, G. Recent sequence data suggest that additional subgroups may exist in gulls and parakeets.*

Public health significance: *aMPV has not been reported to cause human infections. A human MPV has been identified worldwide as a pathogen causing bronchiolitis in infants, the elderly or the immunocompromised but the two viruses are clearly different.*

Detection of the agent: *Virus isolation in cell cultures, embryonated chicken eggs, and tracheal organ cultures, as well as molecular methods for identification of the nucleic acid, have all been used successfully to detect aMPV. The degree of success depends on the strain of virus, type and timeliness of sample collection, as well as storage and handling of specimens. Electron microscopy, virus neutralisation and molecular techniques can be used to identify the virus. Infectious virus can only usually be isolated for approximately ten days after infection.*

Monoclonal antibodies to the spike glycoprotein, G, have been used in virus neutralisation tests to differentiate subgroups A and B, while neutralisation tests using polyclonal antiserum have shown that subgroups A and B belong to a single serotype. Subgroup C is neutralised poorly by subgroup A or B monospecific antiserum, and not by monoclonal antibodies that differentiate subgroups A and B. These data suggest that subgroup C represents a second serotype of aMPV. Monospecific antiserum and monoclonal antibodies can be used for agent identification by virus neutralisation and immunofluorescence staining of infected cell cultures; however antigenic characteristics need to be considered. The immunodiffusion test has also been used to confirm aMPV isolates.

Molecular procedures based on the nucleoprotein (*N*), *matrix* (*M*), *fusion* (*F*), *small hydrophobic* (*SH*), *G and polymerase L genes of aMPV have been used for the detection and or genomic subgrouping of aMPV. Conventional reverse-transcription polymerase chain reaction* (*RT-PCR*) *procedures can be used for aMPV genomic subgrouping. Different sets of either subgroup-specific or broadly reactive PCR primers have been defined, however, a single set of RT-PCR primers directed to the N gene have been shown to detect subgroups A, B, C and D and could possibly be used as universal primers for the detection of aMPV. A pan-MPV real-time RT-PCR, that detects both aMPV and human MPV* (*see below*) *has also been developed.*

Serological tests: *The most commonly employed method is the ELISA. Other methods that have been used are virus neutralisation* (*VN*), *immunofluorescence and immunodiffusion tests. The VN test*

can be performed in primary tracheal organ cultures, chicken embryo fibroblast (CEF) and chicken embryo liver (CEL); several cell lines such as Vero, MA104 or QT35 have also been used successfully. Numerous commercial ELISA kits, as well as in-house assays, have been developed. For optimal sensitivity, homologous strain of aMPV should be used as antigen because of inter-subgroup variations in antigenicity. In many countries where the disease is endemic, vaccination is also practised, complicating interpretation of the results. Ideally, serum samples from birds in the acute phase of disease and also from convalescent birds should be obtained for testing.

Requirements for vaccines: *Two types of vaccine are commercially available for the control of TRT and SHS: live attenuated vaccines, and inactivated oil-emulsion adjuvanted vaccines.*

A. INTRODUCTION

Avian metapneumovirus (aMPV), previously referred to as avian pneumovirus (APV) and avian rhinotracheitis (ART) virus, causes an acute, highly contagious upper respiratory tract infection of turkeys and chickens and ducks. The onset of clinical signs and spread of infection through a flock can be rapid occurring as quickly as 2–4 hours. In turkeys, the virus causes a disease known as turkey rhinotracheitis (TRT). The aetiological agent is an enveloped virus with an unsegmented single-stranded negative-sense RNA virus of approximately 14 kilo bases contained in a nucleocapsid with a helical symmetry. The virus exhibits some characteristics of a pneumovirus, but differs from mammalian pneumoviruses at the molecular level. aMPV is the type strain of the genus, *Metapneumovirus,* in the family *Pneumoviridae* (Kuhn *et al.,* 2020). Metapneumoviruses have been detected in humans and are associated with respiratory tract infection in children (Van Den Hoogen *et al*., 2001). Avian and human metapneumoviruses have no non-structural NS1 and NS2 proteins and their gene order (3'-N-P-M-F-M2-SH-G-L-5') is different from that of mammalian pneumoviruses (3'-NS1-NS2-N-P-M-SH-G-F-M2-L-5') (Tanaka *et al*., 1995). aMPV has been further classified into four subgroups: A, B, C and D based on reactivity against monoclonal antibodies, cross reactivity in the enzyme-linked immunosorbent assay (ELISA) and neutralisation tests, and nucleotide sequence analysis (Cook *et al*., 1993a). However recent reports from North America suggest the existence of two new isolates that are distinct from A, B, C and D subgroups. Phylogenetic analysis based on L gene sequences show that they are closer to the subgroup C viruses than to A, B and D (Canuti *et al.,* 2019; Retallack *et al.,* 2019). A recent book chapter has been dedicated to these aspects and can be sourced for further reading (Brown & Eterradossi 2019).

Infection with aMPV can occur from a very young age in turkeys and is characterised by snicking, rales, sneezing, nasal discharge, foaming conjunctivitis, swelling of the infraorbital sinuses and submandibular oedema (Pringle, 1998). Secondary adventitious agents can dramatically exacerbate the clinical signs. In an uncomplicated infection, recovery is rapid and the birds appear normal in approximately 14 days. When husbandry is poor or secondary bacterial infection occurs, airsacculitis, pericarditis, pneumonia, and perihepatitis may prolong the disease and there may be an increase in morbidity and mortality (Mekkes & De Wit, 1999). Among secondary agents that have been shown to exacerbate and prolong clinical disease are *Bordetella avium*, *Pasteurella*-like organisms, *Mycoplasma gallisepticum*, *Chlamydophila* and *Ornithobacterium rhinotracheale* (Alkhalaf *et al*., 2002; Jirjis *et al*., 2004; Senne *et al*., 1997; Van Loock *et al*., 2006). In addition, other co-infections with viruses such as infectious laryngotracheitis, infectious bronchitis, paramyxovirus-1 (avian orthoavulavirus-1) or fungi such as *Aspergillus fumigatus* have been reported (Croville *et al.,* 2018). Morbidity can be as high as 100%, with mortality ranging from 0.5% in adult turkeys to 80% in young poults (Van De Zande *et al*., 1999). Clinical signs of infection in chickens include nasal discharge and depression, but they are less characteristic than those in turkeys. Severe respiratory distress may occur in broiler chickens particularly when exacerbated by secondary pathogens such as infectious bronchitis virus, mycoplasmas, and *Escherichia coli* (O'Brien, 1985; Pattison *et al*., 1989). Unlike subgroup A and B, the United States of America (USA) strain – Colorado, or subgroup C – has not been shown to naturally induce disease in chickens, although experimentally infected chickens were shown to be susceptible to a subgroup C turkey isolate of aMPV. Different strains of aMPV have been shown to have a specific tropism for chickens or turkeys (Cook *et al*., 1993b). Other species of birds have been reported to have been infected with aMPV, however clinical signs have rarely been reported. Viruses characterised as subgroup C aMPV and shown to have 75–83% nucleotide identity to the US Colorado subgroup C aMPV have been associated with respiratory signs and decreased egg production in ducks in France (Toquin *et al*., 2006). Retrospective molecular analysis of viruses isolated in the 1980s from turkeys in France indicates the presence of a fourth subgroup of aMPV designated subgroup D.

Most recently a series of experimental studies was published in which the host range of aMPV A, B, C and D was assessed in turkeys, chickens and Muscovy ducks (Brown *et al.,* 2019). Overall these trials showed that aMPV-A, B, Turkey C and D were viruses well adapted to Galliformes, especially turkeys. An aMPV-C duck isolate was well adapted to ducks, however chickens and turkeys seroconverted and were positive by virus isolation. Likewise, the

turkey aMPV-C virus was well adapted to turkeys yet was also isolated from chickens. Other experimental studies suggest that direct contact is necessary for bird-to-bird spread of the disease (Alkhalaf *et al*., 2002). In commercial conditions aerogenous infection following airborne transmission is also likely as the disease is restricted to the respiratory tract. Following experimental infection of 2-week-old turkeys with aMPV alone, the virus was detected in the respiratory tract for only a few days (Bayon-Auboyer *et al*., 1999). However, in birds inoculated with aMPV and *B. avium,* virus was detected for up to 7 days post-inoculation (dpi) (Collins & Gough, 1988; Cook *et al*., 1993b). There is no evidence that aMPV can result in a latent infection and no carrier state is known to exist. Although neonatal turkeys are occasionally infected (Shin *et al*., 2002a), there have been no reports of vertical transmission of aMPV.

In growing turkeys, virus replication is limited to the upper respiratory tract with a short viraemia. Replication of both attenuated and virulent strains of aMPV persists for approximately 10 dpi (Van De Zande *et al*., 1999). Limited replication occurs in the trachea, and lung, but virus has not been shown to replicate in other tissues following natural infection (Cook, 2000). Sequential histopathological and immunocytochemical studies have shown viral replication in the turbinates causing a serous rhinitis with increased glandular activity, epithelial exfoliation, focal loss of cilia, hyperaemia and mild mononuclear infiltration in the submucosa and eosinophilic intracytoplasmic inclusions in the ciliated cells of the turbinates at 2 dpi. A catarrhal rhinitis with mucopurulent exudate, damage to the epithelial layer and a copious mononuclear inflammatory infiltration in the submucosa was seen 3–4 dpi. Transient lesions were seen in the trachea, with little or no lesions present in the conjunctiva and other tissues (Giraud *et al*., 1988; Majo *et al*., 1995). Respiratory infection is less severe in laying turkeys; however, there may be a drop in egg production of up to 70% (Stuart, 1989) and the quality of eggs during the recovery period, up to 3 weeks, may be poor. In experimentally infected laying turkeys, viral replication has been demonstrated in both the respiratory and genital tracts up to 9 dpi.

In chickens, there is strong evidence to suggest aMPV is one of the aetiological agents of swollen head syndrome (SHS). The syndrome is characterised by respiratory disease, apathy, swelling of infraorbital sinuses and unilateral or bilateral periorbital and facial swelling, extending over the head. These signs are frequently followed by cerebral disorientation, torticollis and opisthotonos. Although mortality does not usually exceed 1–2%, morbidity may reach 10%, and egg production is frequently affected (Gough *et al*., 1994; Morley &Thomson, 1984; O'Brien, 1985; Pattison *et al*., 1989; Picault *et al*., 1987; Tanaka *et al*., 1995).

Serological evidence suggests aMPV is widespread throughout the world and of considerable economic importance, particularly in turkeys. Oceania and Canada are the only regions that have not reported aMPV in poultry (Cook, 2000). There is serological and molecular evidence that aMPV occurs in a variety of other avian species, including pheasants, guinea fowl, ostriches, passerines and various waterfowl (Shin *et al*., 2002b), but there is no evidence of disease except in pheasants.

aMPV has not been reported to cause human infections. A human MPV has been identified worldwide as a pathogen causing bronchiolitis in infants, the elderly or the immunocompromised. hMPV is genetically most related to aMPV subgroup C (amino acid identity can be 88% or higher for the most conserved proteins). However hMPV and aMPV-C exhibit striking differences in their SH and G proteins (approximately 30% amino acid identity) demonstrating the two viruses are clearly different.

A more detailed account of the disease and its causal virus may be found in Swayne *et al.* (2020).

B. DIAGNOSTIC TECHNIQUES

Isolation and identification of the agent provide the most certain diagnosis of aMPV, but are not usually attempted for routine diagnostic purposes as the virus may prove difficult to isolate and these tests are labour intensive. Thus indirect tests are more frequent: i) immunological demonstration of specific antibodies to the virus in serum or ii) molecular demonstration of viral RNA in tissues or tissue secretions. Available methods for diagnosis are shown in Table 1 and will have different degrees of usage depending on the objectives.

Table 1. Test methods available 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[a]						
Agent isolation	+[b]	–	–	+[e]	–	–
Virus detection by RT-PCR	+[b]	++[d]	–[b]	+++	–	+[c]
Virus characterisation (nucleotide sequencing)	+	–	–	+++	–	–
Antigen detection in respiratory tissues	+[b]	–	–	+++	–	–
Detection of immune response						
ELISA	+++[b]	+++	+++	+[g]	+++	+++
VN	+[f]	+[f]	–	–	+	++[f]

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
RT-PCR = reverse-transcription polymerase chain reaction;
ELISA = enzyme-linked immunosorbent assay; VN = virus neutralisation.
[a]A combination of agent identification methods applied on the same clinical specimen is recommended.
[b]If performed on a large scale and always negative in an area where no live vaccination is performed, or to check for subclinical aMPV.
[c]Could be used post-vaccination to check replication of live vaccine in the respiratory tract.
[d]Could be negative if infection occurred several weeks before testing.
[e]Labour intensive and needs to be complemented with genotyping using full length genome sequences.
[f]Labour intensive however, critical to correlate the presence of detected antibody with protection.
[g]Could be used for case confirmation under certain circumstances, for example if two series of serological samples 3 weeks apart are available.

1. Detection of the agent

To maximise the chances of successfully isolating the virus, a multiple approach to diagnosis is recommended. This is particularly relevant when dealing with different subgroups or genotypes that may require varied *in-vitro* virus isolation methods. This was illustrated in the USA with the failure of the first attempts to isolate subgroup C aMPV. The USA subgroup C has not been associated with ciliostasis, in tracheal organ cultures (Senne *et al*., 1997), and the agent was only cultured following multiple embryo and cell culture passages. This was in contrast to the experience in Europe and elsewhere in which tracheal organ cultures and/or Vero cells were shown to be the most reliable method for the primary isolation of subgroup A, B, C and D of aMPV (Giraud *et al*., 1988).

1.1. Collection and selection of diagnostic specimens

It is very important to take samples for attempted virus isolation in the early stages of infection as the virus may be present only in the sinuses and turbinates for a short period. Ideally, the upper respiratory tract of live birds in the acute phase of the disease should be sampled using sterile swabs (Stuart, 1989). The most successful samples have been nasal exudates, choanal cleft swabs and scrapings of sinus and turbinate tissue. The virus has also been isolated from trachea and lungs, and occasionally viscera of affected turkey poults. Isolation of virus is rarely successful from birds showing severe chronic signs as

the extreme clinical signs are usually due to secondary adventitious agents. This certainly applies to SHS of chickens in which the characteristic signs appear to be due to secondary (e.g. *Escherichia coli*) bacterial infection. Furthermore, for reasons that are unclear, virus isolation from chickens may be more difficult than from turkeys.

It is essential that samples should be sent immediately on ice to the diagnostic laboratory. When delays of more than 3 days are expected, the samples should be frozen prior to dispatch. Swabs for attempted virus isolation should be sent on ice fully immersed in viral transport medium. Swabs for polymerase chain reaction (PCR) analysis can be sent dry, but on ice or frozen.

For virus isolation, a 20% (v/v) suspension of the nasal exudate or homogenised tissue is made in phosphate-buffered saline (PBS) or brain–heart infusion (BHI) broth containing antibiotics, at pH 7.0–7.4. This is then clarified by centrifugation at 1000 ***g*** for 10 minutes and the supernatant is passed through a 450 nm membrane filter.

1.2. Culture and Identification of avian metapneumovirus (aMPV)

The best method for primary virus isolation from infected birds is in tracheal organ cultures or embryonated turkey or chicken eggs with subsequent cultivation in cell cultures; serial passage on Vero cells has also been found to be a sensitive method for the isolation of aMPV (Giraud *et al*., 1988). The original isolation of aMPV in South Africa in the late 1970s and the more recent Colorado aMPV were carried out in embryonated eggs, however subgroup A and B aMPV isolations have routinely been made in tracheal organ cultures. Subgroup C aMPV, do not cause ciliostasis in organ cultures; for this reason: embryonating chicken eggs and subsequent passage on to cell culture are the preferred method for virus isolation (Senne *et al*., 1997). All four aMPV subgroups can be isolated using Vero cells.

Tracheal organ cultures are prepared from turkey embryos or very young turkeys obtained from flocks free of specific antibodies to aMPV. Tracheas from chicken embryo or 1- to 2-day-old chicks may also be used. Transverse sections of trachea are rinsed in PBS (pH 7.2), placed one section per tube in Eagles medium with antibiotics, and held at 37°C. After incubation, the media is removed from the cultures and 0.1 ml of bacteria-free inoculum is added. After incubation for 1 hour at 37°C, growth medium is added and the cultures are incubated at 37°C on a roller apparatus, rotating at 30 revolutions per hour. Cultures are examined daily after agitation on a laboratory mixer to remove debris from the lumen. Ciliostasis may occur within 7 days of inoculation on primary passage, but usually is produced rapidly and consistently only after several blind passages.

For isolation in eggs, 6- to 8-day-old embryonated chicken or turkey eggs from flocks known to be free of aMPV antibodies are inoculated by the yolk-sac route with 0.2–0.3 ml of bacteria-free material from infected birds and incubated at 37°C. If there is no evidence of infection (embryo stunting or mortality) after the first passage, yolk sac material should be processed for a second blind embryo passage. Within 7–10 days, there is usually evidence of stunting of the embryos with few deaths. Serial passage is often required before the agent causes consistent embryo mortality. Isolation in embryonating eggs is a slow, expensive, labour intensive process and requires multiple subsequent cell culture passages for identification.

Various cell cultures have been used for the primary isolation of aMPV, including chicken embryo cells, Vero cells and more recently the QT-35 cells, with varying degrees of success. Primary isolation of the USA subgroup C has been made after multiple (5–6 serial passages) in Vero cell cultures. However, once the virus has been adapted to growth in embryonating eggs or tracheal organ cultures, in which it grows only to low titres, the virus will readily replicate to moderate titres following multiple passages in a variety of primary chicken or turkey embryo cells, Vero cells, and QT-35 cells (Cook, 2000). The primary isolation of all four subgroups of aMPV has proven successful following serial passage on Vero cells. The virus produces a characteristic cytopathic effect (CPE) with syncytial formation within 7 days. Identification of virus-infected cell cultures can be by immunofluorescence staining of infected cells or molecular methods.

Paramyxovirus-like morphology of the virus can be observed by negative-contrast electron microscopy. Pleomorphic fringed particles, roughly spherical and 80–200 nm in diameter are commonly seen. Occasionally much larger filamentous forms are present, which may be up to 1000 nm in length. The surface projections are 13–14 nm in length and the helical nucleocapsid that can sometimes be seen

emerging from disrupted particles, is 14 nm in diameter with an estimated pitch of 7 nm per turn (Collins & Gough, 1988; Giraud *et al*., 1988).

1.3. Molecular identification

Reverse-transcription PCR (RT-PCR) is a significantly more sensitive and rapid method for the detection of aMPV than standard virus isolation methods because of the fastidious nature of aMPV . RT-PCR procedures targeted to the F, M, N and G genes are used for the detection of aMPV; however, because of molecular heterogeneity between aMPV strains, most RT-PCR procedures are subgroup specific or do not detect all subgroups (Bayon-Auboyer *et al*., 1999; Pedersen *et al*., 2000; 2001). Subgroup specific assays are successfully used for the detection and diagnosis of endemic strains (Mase *et al*., 2003; Naylor *et al*., 1997; Pedersen *et al*., 2001). However, limitations of subgroup -specific assays need to be recognised when conducting diagnostic testing for respiratory disease. Primers directed to conserved regions of the N gene have been shown to have broader specificity, detecting representative isolates from A, B, C, and D subgroups (Bayon-Auboyer *et al*., 1999). RT-PCR assays directed to the G gene have also been successfully used for genotype or subgroup identification (Lwamba *et al*., 2005; Mase *et al*., 1996). A variety of RT-PCR techniques have been developed and evaluated and these have been extensively reviewed elsewhere (Njenga *et al*., 2003).

Nasal exudates, choanal cleft swabs, and turbinate specimens collected 2–7 days post-exposure are the preferred specimen (Cook *et al*., 1993b; Pedersen *et al*., 2001; Stuart, 1989). It is imperative to collect specimens when clinical signs are first exhibited as recent studies have shown that the maximum amount of virus is present in the trachea and nasal turbinates at 3 days post-inoculation and viral RNA persists for 9 days in the trachea and up to 14 days in the nasal turbinates (Velayudhan *et al*., 2005). It has been shown that aMPV can be detected from specimens collected 7–10 days post-exposure, however the viral concentration is considerably less thus reducing success of detection (Alkhalaf *et al*., 2002; Pedersen *et al*., 2001). Swabs from a single flock can be pooled in groups of not more than five to allow the processing of samples from a larger number of birds and therefore increasing the potential recovery rate.

Template RNA for RT-PCR can be extracted from homogenised tissue, dry swabs or wet swab pools with silica column or magnetic bead commercial RNA extraction reagents according to the manufacturer's protocol.

Table 2. Example of primers that can be used for the detection of a region of the N gene of subgroups A, B, C and D of aMPV (Bayon-Auboyer et al., 1999; Lemaitre et al., 2018)

Target	Primer ID	Sequence 5' → 3'	Position	Product size	Type of test
N Gene	Nc	5'–TTC-TTT-GAA-TTG-TTT-GAG-AAG-A–3'	632–653	RT primer	End point RT-PCR
	Nx	5'–CAT-GGC-CCA-ACA-TTA-TGT-T–3'	830–812	115	
	Nd	5'–AGC-AGG-ATG-GAG-AGC-CTC-TTT-G–3'	716–737	115	
N Gene	PanMPV /N1fwdA	5'-CTG-TTT-GTG-AAC-ATT-TTY-ATG-CA-3'	718–740 (aMPV A/B/D)		SYBR green real time RT-PCR
	PanMPV /N1AMPVDfwdA	5'-CTG-GTT-GTG-AAC-ATA-TTC-ATG-CA-3'	727–749 (aMPV C)		
	PanMPV /N1RevB	5'-ACA-GAG-ACA-TGG-CCT-AAC-ATD-AT-3'	824–802 (aMPV A/B/D) 833–811 (aMPV C)		

1.3.1. Example protocol (end-point RT-PCR)

i) Synthesis of the cDNA can be carried out in 20 µl volume with the Nc RT primer (or any convenient primer, such as an oligodT or the reverse primer of the primer pair that will be

used in the PCR) and a suitable reverse transcriptase enzyme. Heat 1 µl RT primer (2 pmol), 1 µl dNTP mix (10 mM each), with extracted RNA and sterile distilled water (QS to 20 µl) to 65°C for 5 minutes.

ii) Chill quickly and pulse centrifuge.

iii) Add 4 µl 5× First-Strand buffer, 2 µl 0.1 M DTT, and 1 µl of a suitable RNase.

iv) Heat contents to 42°C for 2 minutes and add 1 µl (200 units) of reverse transcriptase enzyme, mix gently.

v) RT is conducted at 42°C for 50 minutes followed by 70°C for 15 minutes for inactivation of RT enzyme.

vi) PCR amplification can be conducted with a suitable DNA polymerase according to manufacturer's instructions. Amplification conditions are as follows: 94°C for 15 minutes and 30 cycles of 94°C for 20 seconds, 51.0°C for 45 seconds (for the Nd/Nx primer pair, if another pair is used, the annealing temperature should be adapted), 72°C for 45 seconds with a final extension of 72°C for 10 minutes.

Several RT-PCR assays directed to the F, G and M genes have been successfully used for subgroup identification and detection or diagnosis of endemic aMPV (Goyzm *et al*., 2000; Jirjis *et al*., 2004; Majo *et al*., 1995). Nucleotide sequence and phylogenetic analysis of the G gene has been used to genotype subgroup A, B, C and D aMPV and is the recommended procedure for subgroup identification of an unidentified virus. Recommended RT-PCR procedures for sequence analysis of the G gene have been described (Lwamba *et al*., 2005; Toquin *et al*., 2006). A real-time RT-PCR has been demonstrated recently to allow the specific detection, identification and quantification of aMPV subgroups A, B, C and D (Guionie *et al*., 2007) and another protocol was developed for the broad spectrum detection of all MPVs (aMPV and hMPV) (Lemaitre *et al.,* 2018) and could therefore serve for the detection of emerging aMPV of yet-to-be-defined subgroups.

Procedures for the identification of subgroup A and B RNA in diagnostic specimens have also been described (Naylor *et al*., 1997), as have procedures for the detection of subgroup A and C viruses (Pedersen *et al*., 2001). Isolation of aMPV from chickens is difficult and has succeeded only in a limited number of cases; for this reason, molecular tests have been used primarily for the detection of aMPV in chickens (Mase *et al*., 1996). It is important to remember that RT-PCR detects viral RNA, not live virus, so the significance of a positive PCR result in terms of detecting an active infection has to be established.

2. Serological tests

Serology is the most common method of diagnosis of aMPV infections, particularly in unvaccinated flocks, because of difficulties in isolating and identifying aMPV. The most commonly employed method is the ELISA; however, virus neutralisation, microimmunofluorescence and immunodiffusion tests have been used. A number of commercial and in-house ELISA kits are available that are suitable for testing both turkey and chicken serum; however, differences in sensitivity and specificity between commercial kits have been reported (Eterradossi *et al*., 1995; McFarlane-Toms & Jackson, 1998; Mekkes & De Wit, 1999). Competitive or blocking ELISA kits incorporating an aMPV-specific monoclonal antibody have been developed. These kits claim to have a broad spectrum of sensitivity and specificity for all subgroups of aMPV and can be used for testing sera from a variety of avian species. In-house ELISA antigens, as described below, have been prepared in a variety of substrates including various cell cultures and tracheal organ cultures (Chiang *et al*., 2000). Generally, aMPV antibodies are less well detected when a heterologous strain of aMPV is used as antigen, even though the strains appear closely related by virus neutralisation test (Eterradossi *et al*., 1995). The situation is further complicated by discrepancies in the ability of different ELISAs to detect vaccinal antibody when different aMPV strains are used as coating antigens (Eterradossi *et al*., 1995). In-house assays using a homologous antigen have been used extensively for the surveillance of endemic aMPV strains. Ideally, both acute and convalescent serum samples should be obtained for testing. In chickens, the serological response to aMPV infection is weak when compared to the response in turkeys.

2.1. Enzyme-linked immunosorbent assay

The following protocol (Chiang *et al*., 2000), or alternative methods with well documented results (Giraud *et al*., 1987; Grant *et al*., 1987; Gulati *et al*., 2000; 2001; Luo *et al*., 2004), may be used.

Virus is propagated in chicken embryo fibroblast (CEF) or Vero cell cultures until 70–100% of the monolayer is simultaneously infected (3–4 days). The cell culture fluid is decanted and the monolayer washed with PBS (pH 7.2). The monolayer is lysed with 0.5 ml (per 75 cm^2 flask) of a 0.5% non-ionic detergent solution (IGEPAL CA-630 or Nonidet P-40) on a rocking platform for 1 hour at 4°C. Following physical disruption of lysed cells, the whole virus antigen lysate is clarified at 3000 ***g*** for 15 minutes. Uninfected cell cultures are treated in the same manner for a negative control antigen. Serial dilutions of antigen are tested against serial dilutions of anti-species IgG horseradish peroxidase conjugate in a checker-board fashion to determine the optimal antigen/conjugate dilution. A working dilution of the aMPV antigen and normal antigen (100 µl) are coated onto flat-bottom microtitre plates with a carbonate/bicarbonate coating buffer (Chiang *et al*., 2000). Each serum is tested against aMPV and normal antigen for determination of the S/P ratio. Coated plates are incubated at 4°C overnight and washed a total of five times with a Tween 20 wash solution (Chiang *et al*., 2000) prior to use or three times prior to long-term storage at –70°C. Residual wash solution remains on the plate when the plates are frozen. Following storage and equilibration to room temperature, the plates are washed twice and blotted dry prior to use.

2.1.1. Test method

i) Dilute test sera 1/40 in dilution/blocking buffer (Chiang *et al*., 2000).

ii) Apply 50 µl test sera and working dilutions of positive and negative sera to aMPV antigen and normal antigen-coated wells.

iii) Incubate at room temperature for 1 hour.

iv) Wash plates five times with Tween 20 wash solution

v) Apply 50 µl of the working dilution of anti-species IgG horseradish peroxidase conjugate to each well and incubated for 1 hour at room temperature.

vi) Wash plates five times with Tween 20 wash solution

vii) Apply 100 µl of the prepared ortho-phenylenediamine (OPD) chromogen/substrate solution to each well and incubate for 10 minutes in the dark. Combine the following reagents for preparation of OPD in a suitable substrate.

viii) Stop the reaction with 25 µl/well of 2.5 M sulphuric acid.

ix) Read the OD at 490/450 nm.

The results are expressed as the OD difference between the virus antigen-coated and negative control antigen-coated wells. Determine the mean OD_{490} reading for each duplicate set of wells with the positive and negative antigen for each serum. The antigens are usually calibrated so that a sample with an OD_{490} difference between the antigen-coated and negative control antigen-coated wells of more than 0.2 is considered positive (upon development of the method in a laboratory, this threshold may need to be re-evaluated under local conditions, by assessing a panel of negative sera with the newly prepared antigens). Sporadic nonspecific positive reactions are inherent with the ELISA, especially with chicken or duck sera, and immunofluorescence may be used for confirmation testing.

C. REQUIREMENTS FOR VACCINES

1. Background

Two types of vaccine are commercially available for the control of TRT: live attenuated vaccines, or inactivated oil-emulsion adjuvanted vaccines. The possibility exists of developing live recombinant vaccines based on a fowlpox vector expressing the F protein of aMPV (Stuart, 1989), DNA vaccines encoding various aMPV proteins (Tanaka *et al*., 1995) and, more recently, genetically attenuated aMPV produced by reverse genetics.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

1.1. Live vaccines: methods of use

Live TRT vaccines are produced from virus strains that have been attenuated by serial passage in embryonating eggs, tracheal organ cultures or cell culture (various cell lines or chicken embryo fibroblasts), or by alternate passages using a combination of these methods. Commercially available live attenuated TRT vaccines have been derived from subgroup A or subgroup B aMPV isolates in Europe, and from a subgroup C aMPV isolate in the USA. The aMPV subgroup to which the vaccine belongs should be mentioned in the vaccine label, as this information is relevant to the development of efficient post-vaccination serological monitoring. Live TRT vaccines are intended for use in young birds to induce an active immune response that will help to prevent the respiratory disease caused by aMPV. Additionally, live TRT vaccines are also used in parent turkeys to produce a primary response prior to vaccination near to point-of-lay using inactivated vaccine (see below).

Live TRT vaccines are usually applied several times by coarse spray, in the drinking water, or by oculonasal administration. There is a published report on the use of a single *in-ovo* injection (Shin *et al*., 2002b), but, more often, the first TRT live vaccination is administered to turkeys at day-old or up to 7 days of age. The second TRT live vaccine is either applied around 6 weeks of age (when only two vaccinations are performed), or around 3 weeks of age (when there is a third application) or after 6 weeks of age. The rationale for these repeated vaccinations is linked first to the difficulties of inducing a prolonged antibody response lasting for the whole life of the meat turkeys, and second to the need to avoid TRT vaccination in young turkeys when they have recently been vaccinated against haemorrhagic enteritis (vaccines against haemorrhagic enteritis virus [HEV], are usually administered at around 28 days of age to avoid interference with maternally derived antibodies [MDA] to HEV). Although it has been published that MDA to TRTV do not prevent infection of day-old turkeys by virulent aMPV strains (Toquin *et al*., 2003), it has been observed that some interference between MDA and some live TRT vaccines may occur and result in lower vaccine take in young turkeys with higher MDA levels. Clinical cross-protection between live vaccine and challenge virus belonging to subgroups A or B (and *vice versa*) has been reported (Cook *et al*., 1993b; Velayudhan *et al*., 2005). Protective immunity was also observed when birds immunised against aMPV subgroups A or B were subsequently challenged with a subgroup C virulent virus, but not in the converse experiment .

Avian metapneumoviruses are very easily neutralised in the environment by physical and chemical agents and thus ensuring good live vaccination against these viruses may be demanding. If the vaccine is given in the drinking water, clean water with a neutral pH must be used and it must be free from smell or taste of chlorine or metals. Skimmed milk powder may be added at a rate of 2 g per litre. Care must be taken to ensure that all birds receive their dose of vaccine. To this end, all water should be removed (cut off) for 2–3 hours before the medicated water is made available and care must be taken that no residual water remains in the water adduction pipes or in the drinkers. If the vaccine is given by spray, high quality water with a neutral pH and with no disinfectant residues should be used. A specific nebuliser should be used that will be used for no other purpose but vaccination. This apparatus should ideally allow for constant pressure throughout the vaccination process (and thus for a constant size of the vaccine droplets). The turkeys to be vaccinated should be grouped together prior to vaccination and several passes with the nebuliser should be performed to ensure that all birds are indeed exposed to the spray. The ventilation and heating of the poultry house should be turned as low as practical, so that the nebulised vaccine is neither eliminated by ventilation, nor inactivated by overheating (heating moreover favours evaporation, which decreases the size of the nebulised vaccine droplets and cause an increase proportion of the vaccine to reach the lower respiratory tract, a phenomenon that has been suspected to contribute to adverse reactions to live vaccination). It is important that the birds are allowed to calm down immediately after spraying as a non-negligible amount of the vaccine may be absorbed when the birds preen their feathers after being exposed to the vaccine spray.

aMPV vaccines have been reported not to interfere with Newcastle disease vaccines in chickens (Van De Zande *et al*., 1999; Yu *et al*., 1992); however the compatibility of TRT vaccines is not documented in turkeys. As with other vaccines, only healthy birds should be vaccinated. Vials of lyophilised vaccine should be kept at temperatures between 2°C and 8°C up to the time of use.

1.2. Inactivated vaccines: method of use

Inactivated aMPV vaccines are mostly used to produce high, long-lasting and uniform levels of antibodies in breeder turkeys that have previously been primed by live vaccine or by natural exposure to

field virus during rearing. As the rationale to use inactivated vaccines in breeders is to improve their protection not only against the respiratory signs of TRT, but also against the reproductive signs (egg-drops) associated with aMPV infection, it is not uncommon that the inactivated aMPV vaccines also associate this virus with several other viruses also involved in respiratory and/or reproductive disorders. The usual programme is to administer the inactivated vaccine at least 4–6 weeks after the last live vaccination, up to 28 weeks of age in turkeys, avoiding the 4 last weeks before lay. The inactivated vaccine is manufactured as a water-in-oil emulsion, and has to be injected into each bird. The preferred routes are intramuscular in the leg muscle, avoiding proximity to joints, tendons or major blood vessels or the subcutaneous route. A multidose syringe may be used, subject to the apparatus being in full working order and in accordance with manufacturers' instructions and recommended hygiene practices. All equipment should be cleaned and sterilised between flocks, and vaccination teams should exercise strict hygiene when going from one flock to another. The vaccine should not be frozen; it should be stored at between 4°C and 8°C instead (but should be allowed to reach room temperature before injection). Inactivated vaccines should not be exposed to bright light or high temperatures.

Only healthy birds, known to be sensitised by previous exposure to aMPV, should be vaccinated. Used in this way the inactivated vaccine should produce a good antibody response that will protect the breeders against respiratory and reproductive signs during the period of lay (Van De Zande *et al*., 2000). The precise level and duration of immunity conferred by inactivated vaccines will depend mainly on the concentration of antigen present per dose. The manufacturing objective should be to obtain a high antigen concentration and hence a highly potent vaccine.

2. Outline of production and minimum requirements for vaccines

2.1. Characteristics of the seed

See also Chapter 1.1.8 Principles of veterinary vaccine production and Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use*.

2.1.1. Biological characteristics of the master seed

i) Live vaccines

The identity of live aMPV vaccines of subgroups A, B or C that are kept as master seeds for vaccine production should ideally be confirmed by deep sequencing so that any subsequent contamination by adventitious aMPV strains can be detected in the purity checks.

ii) Inactivated vaccines

For inactivated vaccines, the most important characteristics are high yield and good antigenicity.

2.1.2. Quality criteria

i) Purity

The seed live vaccine virus or inactivated vaccine must be shown to be free from extraneous viruses, bacteria, mycoplasma and fungi, particularly from avian pathogens. This includes freedom from contamination with other strains of aMPV.

Seed virus must be shown to be stable, with no tendency to revert to virulence. This can be confirmed by carrying out at least five consecutive turkey-to-turkey passages at 2- to 6-day intervals. Use turkeys not older than 3 weeks and free of MDAs against aMPV. Passage may be achieved by natural spreading or by inoculating a suspension prepared from the mucosa of the turbinates and upper trachea of the previously inoculated birds, or from tracheal swabs. Care must be taken to avoid contamination by viruses from previous passages. It must be shown that the virus was transmitted. The stability should be evaluated by demonstrating that there is no indication of an increased severity in the clinical signs when comparing the maximally passaged virus with the unpassaged vaccine. A scoring system may be used to quantify the severity of the signs.

2.1.3. Validation as a vaccine strain

Data on efficacy should be obtained before bulk manufacture of vaccine begins. The vaccine should be administered to birds in the way in which it will be used in the field. Live vaccine can be given to young birds and the response measured serologically and by resistance to experimental challenge. In the case of killed vaccines, a test must be carried out in older birds that go on to lay, using the recommended vaccination schedule, so that the prolonged seroconversion can be demonstrated. A scoring system may be used to quantify the severity of the signs

i) Live vaccine

Safety: Ten field doses of the vaccine candidate are administered by the oculonasal route to each of 10 turkeys of the minimum age recommended for vaccination and free from antibodies to aMPV. Observe the turkeys at least daily for 21 days. The vaccine fails the test if any turkey dies or shows signs of disease attributable to the vaccine. If more than two turkeys show abnormal clinical signs or die due to causes not related to the vaccine, the test must be repeated. This test is performed on each batch of final vaccine.

Efficacy test: efficacy should be tested for each of the recommended routes of vaccination. Use turkeys that are not older than the minimum age recommended for vaccination and are free of antibodies against aMPV. Administer one field dose of vaccine of the minimum recommended titre by one of the recommended routes to each of 20 turkeys, keeping 10 turkeys as non-vaccinated controls. After 21 days, challenge all turkeys by oculonasal administration of a suitable dose of a virulent strain of aMPV (suitable challenge viruses can be provided by the WOAH Reference Laboratory for TRT[1]). Observe the turkeys daily for 10 days and register their clinical signs individually. The vaccine fails the test unless at least 90% of the vaccinated turkeys survive without showing either clinical signs or lesions evocative of aMPV infection. A scoring system may be used to quantify the severity of the signs. If less than 80% of the non-vaccinated turkeys exhibit clinical signs following challenge, or more than 10% of the control or inoculated birds die from causes not attributable to the test, the test is invalid. Providing results are satisfactory, this test needs to be carried out on only one batch of all those batches prepared from the same seed lot.

ii) Inactivated vaccine

Safety of the inactivated vaccine should be tested for all recommended administration routes and with a batch of vaccine whose activity is at least the maximal activity of future commercial batches. One dose, or a double dose to ensure maximal activity, of vaccine is administered to specific antibody negative (SAN) or specific pathogen free (SPF) turkeys. Clinical signs in vaccinated turkeys are checked daily and for 14 days. The vaccine passes the test if no signs are observed and no death can be attributed to the vaccine. The test is invalid if nonspecific death occurs.

Efficacy test: as drops in egg production are not easily reproduced experimentally, vaccine-induced protection against egg drop following virulent aMPV challenge may be difficult to document and thus protocols aimed at demonstrating the reduction in excretion levels are also acceptable. Alternatively, the induction of a long-lasting immune response following injection of the inactivated vaccine may also be used. For the latter experiment, at least 20 unprimed turkeys are given one dose of vaccine at the recommended age (near to point-of-lay) by one of the recommended routes, and the antibody response is measured between 4 and 6 weeks after vaccination by ELISA or serum neutralisation. If a primary vaccination with a live vaccine is recommended, an additional group of turkeys is given only the primary vaccination so that the actual effect of the inactivated vaccine can be indeed assessed individually.

1 https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3

2.2. Methods of manufacture

2.2.1. Procedure

Seed virus may be propagated in various cell culture systems. The bulk is distributed in aliquots and freeze-dried in sealed containers.

The vaccine must be manufactured in suitable clean and secure accommodation, well separated from diagnostic facilities or commercial poultry.

Production of the vaccine should be on a seed-lot system using a suitable strain of virus of known origin and passage history. Specific pathogen free eggs must be used for all materials employed in propagation and testing of the vaccine. Live vaccines can be produced in eggs or cell cultures. Inactivated vaccines may be made using virulent virus grown in cell culture or embryonating eggs. A high virus concentration is required. These vaccines are made as water-in-oil emulsions. A typical formulation is to use 80% mineral oil to 20% virus suspension, with suitable emulsifying and preservative agents.

2.2.2. Requirements for ingredients

i) Ingredients of animal origin

All ingredients of animal origin, including serum and cells, must be checked for the presence of viable bacteria, viruses, fungi or mycoplasma. Ingredients of animal origin should be sourced from a country with negligible risk for transmissible spongiform encephalopathies (TSEs).

SPF eggs must be used for all materials employed in propagation and testing of the vaccine.

ii) Preservatives

A preservative may be required for vaccine in multidose containers. The concentration of the preservative in the final vaccine and its efficacy until the end of the shelf life should be checked. A suitable preservative already established for such purposes should be used.

2.2.3. In-process control

i) Antigen content

Having grown the virus to a high concentration, its titre should be assayed by use of tracheal organ culture or cell cultures, as appropriate, to the strain of virus being used. The antigen content or infectious titre required to produce satisfactory batches of vaccine should be based on determinations made on test vaccine that has been shown to be effective in laboratory and field trials.

ii) Inactivation of inactivated vaccines

Inactivation is often done with either ß-propiolactone or formalin. The inactivating agent and the inactivation procedure must be shown under the conditions of vaccine manufacture to inactivate the vaccine virus and any potential contaminants, e.g. bacteria that may arise from the starting materials.

Prior to inactivation, care should be taken to ensure a homogeneous suspension free from particles that may not be penetrated by the inactivating agent. A test for inactivation of the vaccine should be carried out on each batch of both the bulk harvest after inactivation and the final product. The test selected should be appropriate to the vaccine virus being used and should consist of at least two passages in susceptible cell cultures, embryos or turkeys, with ten replicates per passage. No evidence of the presence of any live virus or microorganism should be observed.

iii) Sterility of inactivated vaccines

Oil used in the vaccine must be sterilised by heating at 160°C for 1 hour, or by filtration, and the procedure must be shown to be effective. Tests appropriate to oil-emulsion vaccines are

carried out on each batch of final vaccine as described, for example, in the European Pharmacopoeia.

2.2.4. Final product batch test

i) Identity

The identity of a live aMPV vaccine can be confirmed at the batch level by incubating an appropriate dilution of the vaccine with a monospecific anti-aMPV antiserum, then inoculating the mix to susceptible SAN or SPF eggs, or susceptible tracheal organ or cell cultures. The neutralised vaccine should not exhibit any infectivity.

The identity of inactivated aMPV vaccine can be confirmed at the batch level by administrating the vaccine to SAN or SPF chickens, and demonstrating that the vaccine does induce aMPV-specific antibodies. In some instances, this test can be combined with the potency test in order to reduce the number of animals used in the experiments.

ii) Sterility and absence of extraneous agents

Tests for sterility and freedom from contamination of biological materials by bacteria, fungi, mycoplasma and extraneous agents are described in Chapter 2.3.4 *Minimum requirements for the production and quality control of vaccines*.

iii) Safety

a) Live vaccine safety test

As described under Section C.2.1.3.i *Live vaccine* (safety), ten field doses of vaccine are administered by the oculonasal route to each of 10 turkeys of the minimum age recommended for vaccination and free from antibodies to aMPV. Observe the turkeys at least daily for 21 days. The vaccine fails the test if any turkey dies or shows signs of disease attributable to the vaccine. If more than two turkeys show abnormal clinical signs or die due to causes not related to the vaccine, the test must be repeated. This test is performed on each batch of final vaccine.

b) Extraneous agents in inactivated vaccines

Ten SPF turkeys, free of maternal antibodies to aMPV and 14–28 days of age, are inoculated by the recommended routes with twice the field dose. The birds are observed for 3 weeks. No abnormal local or systemic reaction should develop. The test is performed on each batch of final vaccine, unless controls at earlier production stages complemented by implementation of good manufacturing practices advocate for the safety of the overall process.

iv) Residual live vaccine in inactivated vaccines

The process described in Section C.2.2.3.ii *In process controls* may be performed on each batch of final product.

v) Potency

a) Live vaccine potency test

A potency test (virus titration) in embryonating eggs, tracheal organ cultures or suitable cell cultures, as appropriate to the vaccine virus, must be carried out on each serial (batch) of vaccine produced. The vaccine titre at the time of issue must be high enough to guarantee that the minimum virus titre per dose will be maintained at least until the expiry date. In addition, the method described in Section C.2.1.3.i *Live vaccine* (efficacy test) must be used and yield satisfactory results on one batch representative of all the batches prepared from the same seed lot.

b) Inactivated vaccine potency test

The potency test for inactivated vaccines is developed from the results of the efficacy test on a representative batch of vaccine the master seed virus, by measuring antibody production.

As explained in Section C.2.1.3.ii *Live vaccine*, the following protocol may be followed: at least 20 unprimed turkeys are given one dose of vaccine at the recommended age (near to point-of-lay) by one of the recommended routes, and the antibody response is measured between 4 and 6 weeks after vaccination by ELISA or serum neutralisation. If a primary vaccination with a live vaccine is recommended, an additional group of turkeys is given only the primary vaccination so that the actual effect of the inactivated vaccine can be indeed assessed individually.

2.3. Requirements for regulatory approval

2.3.1. Manufacturing process

For registration of vaccine, all relevant details concerning the manufacture of the vaccine and quality control testing (see Section C.2.1 *Characteristics of the seed* and C.2.2 *Methods of manufacture*) should be submitted to the authorities. This information shall be provided from three consecutive vaccine batches with a volume not less than 1/3 of the typical industrial batch volume.

2.3.2. Safety requirements

i) Target and non-target animal safety

Live attenuated aMPV vaccines of subgroup A, B and C (galliforms origin) will infect both turkeys and chickens, but ducks are not susceptible to these viruses (see Section A. *Introduction* and Brown *et al.,* 2019).

No interaction of live aMPV vaccines with non-target avian species has been documented so far. Any information regarding a negative effect in a non-target animal species should be provided in the vaccine instructions for use.

ii) Reversion-to-virulence for attenuated/live vaccines and environmental considerations

It is critical that the potential of live attenuated aMPV vaccines to revert to virulence is assessed prior to regulatory approval (See Section C.2.1.2.i *Quality criteria* [purity] above).

Environmental considerations to be taken into account in the regulatory approval process include the knowledge of the aMPV strains that circulate in the area where the licensed vaccine will be used, as this knowledge may help i) in selecting the vaccines suitable for controlling these strains and ii) in deciding whether it is justified or not to introduce a live attenuated aMPV vaccine strain possibly significantly different from the local aMPV strains.

iii) Precautions (hazards)

aMPV is not recognised as a zoonotic agent, however precaution should be implemented in the manufacturing steps or during vaccination to minimise the exposure of staff to vaccine aerosols. Oil-emulsion vaccines cause serious injury to the vaccinator if accidentally injected into the hand or other tissues. In the event of such an accident the person should go at once to a hospital, taking the vaccine package and manufacturer's datasheet with them. Each vaccine bottle and package should be clearly marked with a warning of the serious consequences of accidental self-injury.

2.3.3. Efficacy requirements

The tests, challenge models and criteria used to assess the efficacy of aMPV vaccines are described in Sections C.2.1.3.i *Live vaccine* and C.2.1.3.ii *Inactivated vaccine*. When assessing efficacy in an aMPV challenge model, it is advisable that the selected challenge virus be representative of contemporary aMPV strains that circulate in the area where the licensed vaccine will be used.

2.3.4. Vaccines permitting a DIVA strategy

No DIVA (detection of infection in vaccinated animals) vaccines are commercially available for aMPV.

2.3.5. Duration of immunity

The cell-mediated response to aMPV infection has been reported to be the main line of defence and protection has been reported to last as long as 22 weeks under experimental conditions following vaccination (Bao *et al.*, 2020; Williams *et al.,* 1991). However, in the field repeat vaccinations (2 – 3 times) are practiced in order to stimulate the cell mediated response in the respiratory tract (Rautenschlein, 2020)

2.3.6. Stability

Evidence should be provided on at least one representative batch of vaccine to show that the vaccine passes the batch potency test at 3 months beyond the requested shelf life.

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NB: There is a WOAH Reference Laboratory for turkey rhinotracheitis
(please consult the WOAH Web site for the most up-to-date list:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact WOAH Reference Laboratories for any further information on
diagnostic tests, reagents and vaccines for turkey rhinotracheitis

NB: FIRST ADOPTED IN 2008. MOST RECENT UPDATES ADOPTED IN 2022.