CHAPTER 3.4.2.

BOVINE BABESIOSIS

SUMMARY

Bovine babesiosis is a tick-borne disease of cattle caused by protozoan parasites including Babesia bovis, B. bigemina and B. divergens. Rhipicephalus (Boophilus) microplus*, the principal vector of* B. bovis *and* B. bigemina*, is widespread in tropical and subtropical countries. The major vector of* B. divergens *is* Ixodes ricinus*. There are other important vectors that can transmit these pathogens, including* Haemaphysalis *and other* Rhipicephalus *spp.*

Detection of the agent: *In the case of live animals, thick and thin films of blood should be taken from a peripheral blood vessel or small capillary bed, for example, the tip of the tail. Demonstration of parasites in dead animals is possible by microscopic examination of smears of peripheral blood, brain, kidney, heart muscle, spleen and liver, provided decomposition is not advanced. The smears are fixed with methanol, stained with 10% Giemsa for 15–30 minutes, and examined at ×800–1000 magnification under oil immersion. Sensitive polymerase chain reaction assays are available that can detect and differentiate* Babesia *species in cattle.*

Serological tests: *Enzyme-linked immunosorbent assays (ELISAs) and competitive ELISA (C-ELISA) using recombinant* B. bovis *and* B. bigemina *merozoite proteins have largely replaced the indirect fluorescent antibody test (IFAT) for the detection of antibodies to* Babesia spp.*, because of processing efficiency and objectivity in interpretation of results. The IFA test has been widely used in the past, but serological cross-reactions make species diagnosis difficult in* B. bigemina*. Immunochromatographic tests (ICT) using recombinant* B. bovis *and* B. bigemina *merozoite proteins have also been developed and used for epidemiological surveys of those infections.*

Requirements for vaccines: *Vaccines consisting of live, attenuated strains of* B. bovis, B. bigemina *or* B. divergens *are produced in several countries either from the blood of infected donor animals or by* in-vitro *culture. The vaccines are provided in frozen or chilled forms. Frozen vaccine has the advantage of allowing post-production control of each batch, but has a much reduced post-thaw shelf life compared with chilled vaccine. The risk of contamination of the blood-derived vaccines makes thorough quality control essential, but this may be prohibitively expensive.*

Whilst in-vitro *production methods offer obvious advantages in terms of animal welfare, vaccine can also be successfully produced using* in-vivo *production systems under strict animal welfare guidelines. With either* in-vivo *or* in-vitro *systems, strict adherence to production protocols is essential to ensure consistency of vaccine and to avoid potential changes in virulence, immunogenicity and consequent protectiveness associated with continued passage of* Babesia *spp. organisms in either culture or splenectomised calves.*

With live Babesia *vaccines, it is prudent, for safety reasons, to limit use to calves less than one year of age, as these animals likely have nonspecific immunity. Older vaccinated animals should be held under surveillance and treated with a babesiacide if adverse events occur.*

Protective immunity develops in 3–4 weeks. A single vaccination usually provides lifelong immunity.

A. INTRODUCTION

Bovine babesiosis is caused by protozoan parasites of the genus *Babesia*, order Piroplasmida, phylum Apicomplexa. Of the species affecting cattle, two – *Babesia bovis* and *B. bigemina* – are widely distributed and of major importance in Africa, Asia, Australia, and the American continent, from the south of the United States to the north of Argentina. *Babesia divergens* is economically important in some parts of Europe. A recent study reported

severe clinical babesiosis caused by *Babesia* sp. Mymensingh in a cow (Sivakumar *et al.,* 2018). However, further studies are essential to confirm experimentally the virulence of this novel *Babesia* species and to determine its geographical distribution.

Tick species are the vectors of *Babesia* (Bock *et al.,* 2008). *Rhipicephalus* (*Boophilus*) *microplus* is the principal vector of *B. bigemina* and *B. bovis* and is widespread in the tropics and subtropics. The vector of *B. divergens* is *Ixodes ricinus*. Other important vectors include *Haemaphysalis* and other species of *Rhipicephalus*.

Babesia bigemina has the widest distribution but *B. bovis* is generally more pathogenic than *B. bigemina* or *B. divergens*. *Babesia bovis* infections are characterised by high fever, ataxia, anorexia, general circulatory shock, and sometimes also nervous signs as a result of sequestration of infected erythrocytes in cerebral capillaries. Anaemia and haemoglobinuria may appear later in the course of the disease. In acute cases, the maximum parasitaemia (percentage of infected erythrocytes) in circulating blood is less than 1%. This is in contrast to *B. bigemina* infections where the parasitaemia often exceeds 10% and may be as high as 30%. In *B. bigemina* infections, the major signs include fever, haemoglobinuria and anaemia. Intravascular sequestration of infected erythrocytes does not occur with *B. bigemina* infections. The parasitaemia and clinical appearance of *B. divergens* infections are somewhat similar to *B. bigemina* infections (Zintl *et al.,* 2003).

Infected animals develop a life-long immunity against reinfection with the same species. There is also evidence of a degree of cross-protection in *B. bigemina*-immune animals against subsequent *B. bovis* infections. Calves rarely show clinical signs of disease after infection regardless of the *Babesia* spp. involved or the immune status of the dams (Bock *et al.,* 2008; Zintl *et al.,* 2003).

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of babesiosis in cattle 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)]						
Microscopic examination	–	–	–	+++	+	–
PCR	+++	+++	+++	+++	+++	–
Detection of immune response						
ELISA	+++	+++	+++	–	+++	+++
IFAT	++	++	++	–	++	+++
ICT	–	–	–	–	++	+

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay;
IFAT = indirect fluorescent antibody test, ICT = immunochromatographic test.
[(a)]A combination of agent identification methods applied on the same clinical sample is recommended.

1. Detection of the agent

1.1. Direct microscopic examination

The traditional method of identifying the agent in infected animals is by microscopic examination of thick and thin blood films stained with Giemsa, a Romanowsky type stain (10% Giemsa in phosphate buffered saline (PBS) or Sörenson's buffer at pH 7.4). The sensitivity of thick films is such that it can detect

parasitaemias as low as 1 parasite in 10^6 red blood cells (RBCs). Species differentiation is good in thin films but poor in the more sensitive thick films. This technique is usually adequate for detection of acute infections, but not for detection of carriers where the parasitaemias are mostly very low. Parasite identification and differentiation can be improved by using a fluorescent dye, such as acridine orange, instead of Giemsa.

Samples from live animals should preferably be films made from fresh blood taken from capillaries, such as those in the tip of the ear or tip of the tail, as *B. bovis* is more common in capillary blood. *Babesia bigemina* and *B. divergens* parasites are uniformly distributed through the vasculature. If it is not possible to make fresh films from capillary blood, sterile jugular blood should be collected into an anticoagulant such as lithium heparin or ethylene diamine tetra-acetic acid (EDTA). The sample should be kept cool, preferably at 5°C, until delivery to the laboratory. Thin blood films are air-dried, fixed in absolute methanol for 10–60 seconds and then stained with 10% Giemsa for 15–30 minutes. It is preferable to stain blood films as soon as possible after preparation to ensure proper stain definition. Thick films are made by placing a small droplet of blood (approximately 50 µl) on to a clean glass slide and spreading this over a small area using a circular motion with the corner of another slide. This droplet is not fixed in methanol, but simply air-dried, heat-fixed at 80°C for 5 minutes, and stained in 10% Giemsa. This is a more sensitive technique for the detection of *Babesia* spp., as RBCs are lysed and parasites concentrated, but species differentiation is more difficult. Unstained blood films should not be stored with formalin solutions as formalin fumes affect staining quality. Moisture also affects staining quality.

Samples from dead animals should consist of thin blood films, as well as smears from cerebral cortex, kidney (freshly dead), spleen (when decomposition is evident), heart muscle, lung, and liver (Bock *et al.,* 2006). Organ smears are made by pressing a clean slide on to a freshly cut surface of the organ or by crushing a small sample of the tissue (particularly cerebral cortex) between two clean microscope slides drawn lengthwise to leave a film of tissue on each slide. The smear is then air-dried (assisted by gentle warming in humid climates), fixed for 10–60 seconds in absolute methanol, and stained for 15–30 minutes in 10% Giemsa. This method is especially suitable for the diagnosis of *B. bovis* infections using smears of cerebral cortex, but is unreliable if samples are taken 24 hours or longer after death has occurred, especially in warmer weather. Parasites can sometimes be detected in capillary blood taken from the lower limb region one or more days after death.

All stained films are examined under oil immersion using (as a minimum) a ×10 eyepiece and a ×100 objective lens. *Babesia bovis* is a small parasite, usually centrally located in the erythrocyte. It measures approximately 1–1.5 µm long and 0.5–1.0 µm wide, and is often found as pairs that are at an obtuse angle to each other. *Babesia divergens* is also a small parasite and is very similar morphologically to *B. bovis*. However, obtuse-angled pairs are often located at the rim of the erythrocyte. *Babesia bigemina* is typically pear-shaped, but many diverse single forms are found. It is usually a much bigger parasite (3–3.5 µm long and 1–1.5 µm wide), and is often found as pairs at an acute angle to each other or almost parallel. In acute cases, the parasitaemia of *B. bovis* seldom reaches 1% (measured in general circulation, rather than capillary blood), but with *B. bigemina* and *B. divergens* much higher parasitaemias are usual.

1.2. Nucleic acid-based diagnostic assays

Nucleic acid-based diagnostic assays are very sensitive particularly in detecting *B. bovis* and *B. bigemina* in carrier cattle (Buling *et al.,* 2007; Criado-Fornelio, 2007). Polymerase chain reaction (PCR-based techniques are reported to be as much as 1000 times more sensitive than microscopy for detection of *Babesia* spp., with detection at parasitaemia levels ranging from 0.001% to 0.0000001% (1 parasite in 10^9 RBCs) (Criado-Fornelio, 2007). A number of PCR techniques have been described that can detect and differentiate species of *Babesia* in carrier infections (Buling *et al.,* 2007; Criado-Fornelio, 2007). PCR assays to differentiate isolates of *B. bovis* have also been described. The application of the reverse line blot procedure, in which PCR products are hybridised to membrane-bound, species-specific oligonucleotide probes, to *Babesia* and, more recently, two quantitative PCR methods (Criado-Fornelio *et al.,* 2009) have enabled the simultaneous detection of multiple species, even in carrier state infections. However, current PCR assays generally do not lend themselves well to large-scale testing and at this time are unlikely to supplant serological tests as the method of choice for epidemiological studies. PCR assays are useful as confirmatory tests and in some cases for regulatory testing. Recently, loop-mediated isothermal amplification (LAMP) and multiplex LAMP methods have been developed (Iseki *et al*., 2007; Liu *et al.,* 2012) with higher sensitivity than that of PCR, although optimisation for detection of strains from different parts of the world needs to be addressed. This latter method requires expensive or

sophisticated equipment, while the LAMP method only requires an ordinary water bath and the results can be read with the naked eye. The LAMP method is, therefore, cost-effective, simple, and a rapid DNA amplification method that is suitable for pen-side diagnostics. However, care should be taken to avoid cross-contamination as sensitivity of the LAMP is usually very high.

1.2.1. Nested polymerase chain reaction for the simultaneous detection of *B. bovis* and *B. bigemina*

As the sensitivity of nested PCR is higher than that of PCR, nested PCR is a suitable assay for international trade, especially for *B. bovis,* which usually shows low parasitaemia or carrier status. Although there are several genes used for nested PCR, RAP-1 and AMA-1 have been widely used for *B. bovis* and *B. bigemina*, respectively.

i) DNA extraction

a) Whole blood sample is collected into a vacuum blood collection tube containing EDTA.

b) 200 µl bovine blood is placed in a 1.5 ml microfuge tube

c) 1 ml cold PBS is added and the mixture is centrifuged at 1000 ***g*** for 5 minutes at 4°C. This step is repeated three times.

d) The supernatant is discarded and the pellet is resuspended in 200 µl PBS.

e) After measuring DNA concentrations using a spectrophotometer, the samples can be stored at –20°C.

Note that the DNA samples can also be prepared from infected blood samples using commercial DNA extraction kits.

ii) Multiplex nested PCR

The following test procedure is adapted from Figueroa *et al.* (1993) with modifications for the multiplex nested PCR detection of *B. bovis* and *B. bigemina* based on *RAP-1* and *Spe*I-*Ava*I, respectively.

a) For the first round of PCR, 9 µl reaction mixture that includes 1 µl of 10 × reaction buffer, 200 µM of each dNTPs, 0.5 µM of outer forward (BoF and BilA) and reverse (BoR and BilB) primers, 0.5 units of Taq DNA polymerase (Applied Biosystems), and double distilled water is prepared.

b) 1 µl of the extracted DNA sample is added to the reaction mixture and then subjected to the following PCR cycling conditions. The initial enzyme activation at 95°C for 5 minutes is followed by 35 cycles, each of them consisting of a denaturing step at 95°C for 30 seconds, an annealing step at 55°C for 1 minute, and an extension step at 72°C for 1 minute. The final elongation step is at 72°C for 10 minutes.

c) For the nested round of PCR, 1 µl of the first PCR product is transferred to a new PCR tube that contains a reaction mixture with the same composition as that of the first PCR except for the outer primers, which were replaced with the inner forward (BoFN and BilAN) and reverse (BoRN and BilBN) primers.

iii) List of PCR primers

Parasite	PCR	Primer	Sequence (5'–3')
B. bovis	Primary	BoF	CAC-GAG-GAA-GGA-ACT-ACC-GAT-GTT-GA
		BoR	CCA-AGG-AGC-TTC-AAC-GTA-CGA-GGT-CA
	Nested	BoFN	TCA-ACA-AGG-TAC-TCT-ATA-TGG-CTA-CC
		BoRN	CTA-CCG-AGC-AGA-ACC-TTC-TTC-ACC-AT

Parasite	PCR	Primer	Sequence (5'–3')
B. bigemina	Primary	BilA	CAT-CTA-ATT-TCT-CTC-CAT-ACC-CCT-CC
		BilB	CCT-CGG-CTT-CAA-CTC-TGA-TGC-CAA-AG
	Nested	BilAN	CGC-AAG-CCC-AGC-ACG-CCC-CGG-TGC
		BilBN	CCG-ACC-TGG-ATA-GGC-TGT-GTG-ATG

a) PCR products are separated by electrophoresis (100 V) in 1.5 % agarose gels and 0.5 × Tris/borate/EDTA (TBE) buffer. Gels are then stained with a fluorescent DNA stain, visualised under ultraviolet light, and photographed.

iv) Interpretation of the results

a) Positive samples should have PCR products of the expected size (170 bp for *B. bigemina* and 298 bp *B. bovis*) similar to those of positive controls.

b) The assay must be repeated if the positive control remained negative, or if the negative controls are positive.

Note: Although the above described procedure is for the multiplex PCR assay, simplex nested PCR can also be performed to detect either *B. bovis* or *B. bigemina* using only the respective PCR primers.

Sivakumar *et al.* (2012) noted that the primers that target the *B. bigemina Spe*I-*Ava*I restriction fragment can also amplify *B. ovata* DNA. Therefore, care should be taken when testing DNA samples that were sourced from *B. ovata*-endemic regions.

Recently, a new, improved method for diagnostics based on apocytochrome b genes (CYTb) of *B. bovis* and *B. bigemina* was developed (Romero-Salas *et al.,* 2016), allowing the detection of as little as 0.1 fg DNA of each *Babesia* pathogen, and showing 100 and 1000 times higher sensitivity for *B. bovis* and *B. bigemina*, respectively, as compared with those described by Figueroa *et al.* (1993).

1.3. *In-vitro* culture

In-vitro culture methods have been used to demonstrate the presence of carrier infections of *Babesia* spp. (Holman *et al.,* 1993), and *B. bovis* has also been cloned in culture. The minimum parasitaemia detectable by this method will depend, to a large extent, on the facilities available and the skills of the operator, but could be as low as 10^{-10} (Friedhoff & Bose, 1994), making it a very sensitive method, with 100% specificity, for the demonstration of infection.

2. Serological tests

The indirect fluorescent antibody test (IFAT) was widely used in the past to detect antibodies to *Babesia* spp., but the *B. bigemina* test has poor specificity. Cross-reactions with antibodies to *B. bovis* in the *B. bigemina* IFAT were a particular problem in areas where the two parasites coexist. The IFA test also has the disadvantages of low sample throughput and subjectivity. The complement fixation test is no longer used for diagnosis of these infections.

Enzyme-linked immunosorbent assays (ELISA) have largely replaced the IFAT as the diagnostic test of choice for *Babesia* spp. because of the objectivity in interpretation of results and capacity to process high numbers of samples daily. An ELISA for the diagnosis of *B. bovis* infection that uses a whole merozoite antigen has undergone extensive evaluation (Molloy *et al.,* 1998). High sensitivity and specificity of this test was demonstrated in both Australia and Zimbabwe, although threshold values varied between laboratories (Molloy *et al.,* 1998). Indirect (Bono *et al.,* 2008; Boonchit *et al*., 2006) and competitive ELISAs (Goff *et al.,* 2003) using recombinant merozoite surface and rhoptry-associated antigens of *B. bovis* have also been developed. The competitive ELISA has been more widely validated in different laboratories, with the antigen recognised by antibody from diverse regions around the world (Goff *et al.,* 2006). Reduction in specificity of the indirect *B. bovis* ELISA using recombinant antigens has been noted in some situations (Bono *et al.,* 2008).

There is still no well validated ELISA available for *B. bigemina* despite the efforts of several investigators in different laboratories. ELISAs for antibodies to *B. bigemina* crude antigen typically have poor specificity. Competitive ELISAs developed and validated in Australia and USA (Goff *et al.,* 2008) are apparently the only ELISAs in routine use. Unlike *B. bovis* where animals are thought to remain carriers for life after infection, *B. bigemina* may clear infection and antibody levels may decline below the negative threshold within months after infection (Goff *et al.,* 2008). Inconclusive results may occur around the negative threshold values; and this phenomenon can provide a diagnostic challenge in animals where titres are declining if the animal clears infection.

ELISAs have also been developed for *B. divergens* using antigen derived from culture, *Meriones* or cattle, but there does not appear to be one that has been validated internationally (Zintl *et al.,* 2003).

The immunochromatographic test (ICT) is a rapid diagnostic method that detects antibodies against a specific antigen by means of specific antibodies and antigens impregnated on a nitrocellulose membrane-based test strip. ICT is a rapid, easy-to-read assay, very much needed under field situations, particularly in developing countries where equipment and electricity are limited. An ICT for simultaneous rapid serodiagnosis of bovine babesiosis caused by *B. bovis* and *B. bigemina* has been developed (Kim *et al.,* 2008).

2.1. *Babesia bovis* indirect enzyme-linked immunosorbent assay

Antigen preparation is based on a technique described by Waltisbuhl *et al.* (1987). Infected blood (usually 5–10% parasitaemia) is collected from a splenectomised calf into EDTA. The blood is first centrifuged and the plasma removed and stored for later use. Infected RBCs can also be obtained from *in-vitro* cultures. The RBCs are then washed three times in five volumes of phosphate-buffered saline (PBS), and infected cells are concentrated by differential lysis of uninfected cells in hypotonic saline solution. Infected cells are more resistant to lysis in hypotonic saline solutions than are uninfected cells.

To find the best concentration for particular infected blood, a series of hypotonic saline solutions are prepared, ranging from 0.35% to 0.50% NaCl in 0.025% increments. Five volumes of each saline solution are then added to one volume of packed RBCs, gently mixed and allowed to stand for 5 minutes. The mixtures are then centrifuged and the supernatants aspirated. An equal volume of plasma (retained from the original blood) is added to each tube containing packed RBCs, and the contents of the tubes are mixed. Thin blood films are prepared from each of these resuspended blood cell mixtures, fixed in methanol, and stained with Giemsa. These films are examined under a microscope to determine which saline solution lyses most uninfected RBCs but leaves infected RBCs intact. It should be possible to achieve >95% infection in the remaining intact RBCs.

The bulk of the packed RBCs are then differentially lysed with the optimal saline solution, centrifuged and the supernatant removed. The sediment (>95% infected RBCs) is lysed in distilled water at 4°C, and parasites are pelleted at 12,000 ***g*** for 30 minutes. The pellet is washed at least three times in PBS by resuspension and centrifugation at 4°C until minimal haemoglobin is in the supernatant. It is then resuspended in one to two volumes of PBS at 4°C, and sonicated in appropriate volumes using medium power for 60–90 seconds. The sonicated material is ultra-centrifuged (105,000 ***g*** for 60 minutes at 4°C) and the supernatant containing the solubilised merozoite antigen is retained. The supernatant is mixed with an equal volume of glycerol and stored in 2–5 ml aliquots at –70°C. Short-term storage at –20°C is acceptable for the working aliquot.

2.1.1. Test procedure

The following test procedure is based on that described by Molloy *et al.* (1998) with some modification.

i) 100 µl of antigen solution (with the antigen typically diluted in the range from 1/400 to 1/1600 in 0.1 M carbonate buffer (pH 9.6) is added to each well of a polystyrene 96-well microtitre plate. The plate is covered and incubated overnight at 4°C.

ii) The solution containing any unbound antigen is removed and the wells are then blocked for 1 hour at 20–25°C by the addition of 200 µl of a 2% solution of sodium caseinate in carbonate buffer (pH 9.6).

iii) After blocking, the wells are rinsed three times with PBS containing 0.1% Tween 20 (PBST); then 100 µl of diluted test and control bovine serum (diluted 1/100 in PBST containing 2%

skim milk powder) is added into each well, and the plates are incubated for 30 minutes at 20–25°C with shaking.

iv) The washing step consists of five rinses with PBST. During the last rinse, the plate is shaken for 5 minutes.

v) Next, 100 µl of peroxidase-labelled anti-bovine IgG diluted in PBST containing 2% skim milk powder is added and the plates are shaken for a further 30 minutes at 20–25°C. (NB: some batches of skim milk powder may contain immunoglobulins that can interfere with anti-bovine IgG conjugates and must be tested for suitability prior to use).

vi) Wells are washed as described in step iv above, and to each well is added 100 µl of peroxidase substrate/chromagen (ABTS [2,2'-Azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid)]) 0.3 g/litre in a glycerine/citric acid buffer; with H_2O_2 concentration of 0.01%). The substrate reaction is allowed to continue until the absorbance of a strong positive control serum included on each plate approaches 1.

vii) At this point, the reaction is stopped using an equal volume (100 µl) of ABTS Peroxidase Stop Solution (working concentration of 1% sodium dodecyl sulphate). Absorbance at 414 nm is read on a microtitre plate reader within 30 minutes. 3,3',5,5'-Tetramethyl benzidine (TMB) is also a suitable chromagen, but is stopped with equal volumes of 1 M phosphoric acid (H_3PO_4) and is read at wavelength 450 nm.

To control for inter-plate variation, known positive and negative sera are included in each plate. Test sera are then ranked relative to the positive control. ELISA absorbance results are expressed as a percentage of this positive control (per cent positivity). Positive and negative threshold values should be determined in each laboratory by testing as many known positive and negative sera as possible.

Each batch of antigen and conjugate should be titrated using a checkerboard layout. With this test, it is possible to detect antibodies at least 4 years after a single infection. There should be 95–100% positive reactions with *B. bovis*-immune animals, 1–2% false-positive reactions with negative sera, and <2% false-positive reactions with *B. bigemina*-immune animals.

2.2. *Babesia bovis* and *Babesia bigemina* competitive enzyme-linked immunosorbent assays

The format of these tests is based on that described by Goff *et al.* (2003). The tests are described together because of the similarity of the processes. They have also been developed with the intention of validation as international standard tests and include purified recombinant antigen dried onto microtitre wells for ease of standardisation, handling and distribution, and inter-laboratory use and comparison under varying conditions (Goff *et al.,* 2006; 2008). The assays are based on a species-specific, broadly conserved, and tandemly repeated B-cell epitope within the C terminus of the rhoptry-associated protein 1 (RAP 1), expressed as a histidine-tagged thioredoxin fusion peptide. The expressed purified antigen is coated and then dried onto microtitre wells; optimal concentration of antigen and monoclonal antibody (MAb) is determined by block titration. In the case of *B. bovis*, positive sera inhibit the binding of the epitope-specific MAb BABB'75A4; in the case of *B. bigemina*, positive sera inhibit the binding of MAb 64/04.10.3.

The specificity, sensitivity and predictive values of these competitive ELISAs have been calculated, and the test reliability compared across laboratories. For *B. bovis* (Goff *et al.,* 2006), based on random operator receiver (ROC) analysis, 21% inhibition was chosen as the threshold value to define positive and negative samples. Using this value, specificity was 100%, sensitivity 91.1%, and positive predictive value 100%; negative predictive value varied with prevalence, ranging from 99% at 10% prevalence to 55.6% at a prevalence of 90%. A competitive ELISA using recombinant merozoite surface antigen 2c (rMSA-2c) was evaluated in *Babesia bovis* in Argentina and showed 98% specificity and 96.2% sensitivity (Dominguez *et al.,* 2012). For *B. bigemina* (Goff *et al.,* 2008), using a hypothetical prevalence rate of 25% and threshold inhibition for a negative value at 16%, the assay had a specificity of 98.3% and sensitivity of 94.7%. When threshold inhibition was increased to 21%, specificity was 100% but sensitivity reduced to 87.2%; negative predictive value at 25% prevalence reduced from 98.2% to 95.9%; and positive predictive value increased to 100% from 94.9%. At 21% inhibition, negative predictive value varied from 97.0% at 10% prevalence to 48.2% with a prevalence of 95%; positive predictive values were 90.7% (10% prevalence),

95.7% (15% prevalence) and 100% at all higher prevalence rates. The attributes of both tests appear to meet standards required for international application.

2.3. Indirect fluorescent antibody test

2.3.1. Antigen preparation

Antigen slides are made from jugular blood or *in-vitro* culture, ideally when the parasitaemia is between 2% and 5%.

Blood is collected into a suitable anticoagulant (sodium citrate or EDTA), and is then washed at least three times in five to ten volumes of PBS to remove contaminating plasma proteins and, in particular, host immunoglobulins. After washing, the infected RBCs are resuspended in two volumes of PBS to which 1% bovine serum albumin (BSA) has been added. The BSA is used to enable RBCs to adhere to the glass slide. By preference, single-layered blood films are made by placing a drop of blood on to a clean glass slide, which is then spun in a cytocentrifuge. This produces very uniform smears. Alternatively, thin blood films may be made by the conventional manual technique (dragging with the end of another slide). Commercially available microscope slides containing wells can also be conveniently used. The films are air-dried and fixed for 5 minutes in an oven at 80°C or cold dry acetone for 1 minute at –20°C. Fixed blood films are then covered (e.g. with aluminium foil or brown paper sticking tape) so as to be airtight, and stored at –70°C until required (maximum 5 years).

2.3.2. Test procedure

Test sera are diluted 1/80 in PBS. Sera may be used with or without heat inactivation at 56°C for 30 minutes. The slides are marked into 8–10 divisions with an oil pen to produce hydrophobic divisions. In each test square, 5–10 µl of each serum dilution is added to a filter paper disc using a fine pipette. The preparations are then incubated at 37°C for 30 minutes, in a humid chamber. For controls, negative and weak positive sera (at the same /80 dilution) are used on each test slide.

After incubation, the slides are rinsed with a gentle stream of PBS to remove the filter paper discs. The slides are soaked for 10 minutes in racks in PBS followed by 10 minutes in water. The PBS and water are circulated using a magnetic stirrer. Diluted anti-bovine IgG antibody labelled with fluorescein isothiocyanate (FITC) is then added to each test square. The appropriate dilution is based on titration of every new batch of conjugate, with the working range usually being between 1/400 and 1/1200. Conjugated rabbit, donkey and chicken antibodies are usually more suitable for this purpose than goat antibodies. Currently, newer fluorochromes offer better options due to their improved photostability and brightness, and a wider pH range (for more information contact the WOAH Reference Laboratories). The slides with the conjugate are incubated again at 20–25°C for 30 minutes, and washed as above. The wet slides are mounted with cover-slips in a solution containing 1 part glycerol and 1 part PBS, and examined by standard fluorescence microscopy. A competent operator can examine approximately 150 samples per day.

2.4. Other tests

Other serological tests have been described in recent years, and include a dot ELISA, a slide ELISA, latex and card agglutination tests and an immunochromatographic test (Kim *et al.,* 2008). These tests show acceptable levels of sensitivity and specificity for *B. bovis* and, in the case of the dot ELISA and ICT, also for *B. bigemina*. However, none of these tests appears to have been adopted for routine diagnostic use in laboratories other than those in which the original development and validation took place. Adaptability of these tests to routine diagnostic laboratories should be therefore evaluated in the future.

C. REQUIREMENTS FOR VACCINES

1. Background

Cattle develop a durable, long-lasting immunity after a single infection with *B. bovis, B. divergens* or *B. bigemina*. This feature has been exploited in some countries to immunise cattle against babesiosis (Bock *et al.,* 2008; Mangold

et al., 1996; Pipano, 1997). Most of these live vaccines contain specially selected strains of *Babesia*, mainly *B. bovis* and *B. bigemina*, and are produced in government-supported production facilities as a service to the livestock industries, in particular in Australia, Argentina, South Africa and Israel. Some other countries possess the ability to produce vaccine on a smaller scale. An experimental *B. divergens* vaccine prepared from the blood of infected *Meriones unguiculatus* (mongolian gerbil) has also been used successfully in Ireland (Zintl *et al.,* 2003).

A killed *B. divergens* vaccine has also been prepared from the blood of infected calves (Zintl *et al.,* 2003), but little information is available on the level and duration of the conferred immunity. Other experimental vaccines containing *Babesia* spp. antigens produced *in vitro* have also been developed (Montenegro-James *et al.,* 1992), but the level and duration of protection against heterologous challenge are unclear. Despite the characterisation of various parasite proteins and the *B. bovis* genome (Brayton *et al.,* 2007) and considerable effort worldwide directed towards identification of candidate vaccine antigens, the prospects for recombinant vaccines against *Babesia* spp. remain challenging (Brown *et al.,* 2006). To date no effective subunit vaccine is available commercially.

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

This section will deal with the production of live babesiosis vaccines, mainly those against *B. bovis* and *B. bigemina* infections in cattle. Production involves infection of calves with selected strains, and use of the infected RBCs as vaccine (Bock *et al.,* 2008); or *in-vitro* culture methods to produce parasites for vaccine (Mangold *et al.,* 1996). Calves used for infection with these strains or, in the case of *in vitro* methods, as a source of serum and RBCs for culture, must be free of infectious agents that can be transmitted by products derived from their blood. In the case of *B. divergens*, blood of infected gerbils (*Meriones unguiculatus*) can be used instead of bovine blood. Evidence that changes in immunogenicity occur with repeat passages in calves, and that possible antigenic drift occurs during long-term maintenance of *B. bovis* in culture, must be managed by limiting the number of repeat passages or subcultures made before returning to the vaccine working seed stabilate. Whilst *in-vitro* production methods offer obvious advantages in terms of animal welfare, vaccine can also be successfully produced using *in-vivo* production systems under strict animal welfare guidelines. Close to 400,000 doses of vaccine per year have been successfully produced in Argentina under authority of SENASA (Servicio Nacional de Sanidad y Calidad Agroalimentaria, National Service for Agrifood Health and Quality) using *in-vitro* culture, and up to 850,000 doses per year have been successfully produced in Australia under authority of APVMA (Australian Pesticides and Veterinary Medicines Authority) using *in-vivo* techniques.

Babesia bovis and *B. bigemina* vaccines can be prepared in either frozen or chilled form depending on demand, transport networks and the availability of liquid nitrogen or dry ice supplies. Preparation of frozen vaccine (Bock *et al.,* 2008; Mangold *et al.,* 1996; Pipano, 1997) allows for thorough post-production testing of each batch. However, it has a much reduced shelf-life once thawed, is more costly to produce and more difficult to transport than chilled vaccine. The potential risk of contamination of this blood-derived vaccine makes pre- and post-production quality control essential, but may put production beyond the financial means of some countries in endemic regions.

2. Outline of vaccine production

2.1. Characteristics of the seed

2.1.1. Internationally available strains

Attenuated Australian strains of *B. bovis* and *B. bigemina* have been used effectively to immunise cattle in Africa, South America and South-East Asia (Bock *et al.,* 2008). Tick-transmissible and non-transmissible strains are available. A strain of *B. divergens* with reduced virulence for *Meriones* has also been developed (Zintl *et al.,* 2003).

2.1.2. Isolation and purification of local strains

Strains of *B. bovis*, *B. divergens* and *B. bigemina* that are free of extraneous agents, such as *Anaplasma*, *Eperythrozoon, Theileria, Trypanosoma* and various viral and bacterial agents are most readily isolated by feeding infected ticks on susceptible splenectomised cattle. The vectors and modes of transmission of the species differ, and these features can be used to separate the species (Friedhoff & Bose, 1994).

Babesia spp. can also be isolated from infected cattle by subinoculation of blood into susceptible splenectomised calves. A major disadvantage of this method is the difficulty of separating the *Babesia* spp. from contaminants such as *Anaplasma* and *Eperythrozoon*. Isolation of *B. divergens* is a relatively simple process because of the susceptibility of *Meriones* (Zintl *et al.,* 2003). Maintenance of isolated strains *in vitro* (Jorgensen & Waldron, 1994) can be used to eliminate most contaminants, but not to separate *Babesia* spp. Selective chemotherapy (for example, 1% trypan blue to eliminate *B. bigemina*) can be used to obtain pure *B. bovis* from a mixed *Babesia* infection, while rapid passage in susceptible calves will allow isolation of *B. bigemina*.

2.1.3. Attenuation of strains

Various ways of attenuating *Babesia* spp. have been reported. The most reliable method of reducing the virulence of *B. bovis* involves rapid passage of the strain through susceptible splenectomised calves. Attenuation is not guaranteed, but usually follows after 8 to 20 calf passages (Bock *et al.,* 2008). The virulence of *B. bigemina* decreases during prolonged residence of the parasite in latently infected animals. This feature has been used to obtain avirulent strains by infecting calves, splenectomising them 6–12 weeks after inoculation and then using the ensuing relapse parasites to repeat the procedure (Bock *et al.,* 2008). Attenuation of *B. divergens* in *M. unguiculatus* followed long-term maintenance *in vitro* has been achieved (Zintl *et al.,* 2003).

Attenuation of *Babesia* spp. with irradiation has been attempted, but the results were variable. Similarly, maintenance in vitro in modified media has been used experimentally.

Avirulent strains should be stored as stabilate for safety testing and for future use as master seed in the production of vaccine.

2.1.4. Preparation and storage of master seed

Avirulent strains are readily stored as frozen infected blood in liquid nitrogen or dry ice. Dimethyl sulphoxide (DMSO) and polyvinylpyrrolidone (PVP) MW 40,000 (Bock *et al.,* 2008) are the recommended cryopreservatives, as they allow for intravenous administration after thawing of the master seed.

For the DMSO procedure, infected blood is collected and chilled to 4°C. Cold cryoprotectant (4 M DMSO in PBS) is then added, while stirring slowly, to a final blood: cryoprotectant ratio of 1:1 (final concentration of DMSO is 2 M). This dilution procedure is carried out in an ice bath, and the diluted blood is dispensed into suitable containers (e.g. 5 ml cryovials), and frozen, as soon as possible, in the vapour phase of a liquid nitrogen container. The vials are stored in the liquid phase in a designated tank to prevent loss of viability and contamination. Stored in this way, master seed lots of *Babesia* spp. have been known to remain viable for 20 years.

Unlike DMSO, it has not been found necessary to work with stabilates containing PVP in an ice-bath (Standfast & Jorgensen, 1997). Pre- and post-thaw storage at 20–25°C have not affected infectivity. PVP is a complex polymer that does not permeate intact cell membranes. It has low toxicity for vertebrates and parasites and stabilate-containing PVP is infective when inoculated intravenously. PVP with a molecular weight of 40,000 is made up to a 20% solution with PBS and autoclaved to sterilise. Blood from an infected calf is slowly mixed with an equal volume of the 20% PVP in PBS solution to produce a final concentration of 10% PVP. The mixture is then dispensed into 5 ml cryovials, frozen in the vapour phase of liquid nitrogen by cooling at a rate of about 10°C per minute for 15 minutes and then stored in liquid nitrogen (Standfast & Jorgensen, 1997).

In-vitro cultures are based on the microaerophilous stationary phase method (Levy & Ristic, 1980). Blood infected with avirulent strains of *B. bovis* or *B. bigemina* is harvested from splenectomised calves and washed with VYM phosphate-buffered saline solution (Vega *et al.,* 1985) to remove the plasma and buffy coat. The VYM solution is composed of $CaCl_2.2H_2O$ (16.0 mg), KCl (400.0 mg), KH_2PO_4 (1415.4 mg), $MgSO_4.7H_2O$ (154.0 mg), $Na_2HPO_4.7H_2O$ (1450 mg), NaCl (7077.0 mg) and dextrose (20.5 g) in 1 litre of double-distilled deionised water containing 0.25 mM adenine and 0.50 mM guanosine. Individual 5% suspensions of infected and uninfected RBCs are then prepared in basic culture medium consisting of commercial M199 medium and normal bovine serum (60/40). The basic medium is supplemented with 18 mM HEPES (4-[2-Hydroxyethyl] piperazine-1-ethanesolfonic acid), 10 mM $NaHCO_2$, 100 µg/ml streptomycin sulphate and

100 U/ml penicillin G. The parasitised and normal RBC suspensions are mixed (1/1), dispensed into culture flasks and incubated under an atmosphere of 90% N_2, 5% O_2 and 5% CO_2 at 37°C. After 8 to 10 subcultures in different size culture flasks, the final complete cultures are spun at 1200 ***g*** for 10 minutes at 4°C and the supernatant removed. Packed parasitised RBCs are gently mixed with an equal volume (1/1) of 20% PVP in VYM solution, and dispensed in 2 ml cryovials. The parasitised RBCs are frozen in the vapour phase of liquid nitrogen by cooling at a rate of about 10°C per minute for 15 minutes and then stored in liquid nitrogen (Standfast & Jorgensen, 1997). Normal blood from donor cattle, which is used as a source of serum and uninfected RBCs for culture medium, is defibrinated with glass balls. The RBCs are washed and stored for up to 3 weeks in VYM solution at 4°C and normal serum is stored frozen at –20°C until use.

2.1.5. Preparation and storage of working seed

Working seed is prepared in the same way as master seed (Section C.2.1) using master seed as starting material.

2.1.6. Validation of safety and efficacy of working seed

The suitability of a working seed is determined by repeatability of infectivity in splenectomised calves, or in initiating cultures, and its safety and efficacy in non-splenectomised cattle. Repeatability is determined by inoculating several susceptible splenectomised calves and monitoring parasite progression by stained blood smears. The prepatent period and parasite progression should be relatively consistent between calves to allow inoculations to be scheduled with a degree of certainty.

In-vitro prepared working seed vials are thawed by immersing in water preheated to 40°C and directly dispensed in culture media. The *in-vitro* multiplication process starts with a 5% RBC suspension, which is progressively increased up to 10%. Working seed is considered acceptable when continuous cultures derived from it achieve 8–12% of RBCs parasitised by morphologically normal merozoites/trophozoites after the third subculture and growth in an atmosphere of 5% CO_2 in air at 37°C.

The safety and efficacy of the vaccine strain is determined by inoculating suitable numbers of susceptible adult cattle with vaccine prepared from RBCs from a calf that has been inoculated with the strain, or from an *in-vitro* culture process. Safety can be judged by monitoring body temperature, parasitaemia in stained blood films, and PCV depression following vaccination. Efficacy is judged by monitoring the same parameters following the inoculation of the vaccinated cattle with a heterologous strain. The purity of the working seed is tested by monitoring the cattle used in the safety test for evidence of possible extraneous agents or by thorough testing of the calf from which the stabilate was produced (see Section C.2.2.3). Bovine donors of uninfected blood used for *in-vitro* cultures are maintained in isolated pens and their health status monitored.

2.2. Method of manufacture

2.2.1. Production of frozen vaccine concentrate

First, 5–10 ml quantities of working seed are rapidly thawed by immersing the vials in water preheated to 37°C. The thawed material is used as soon as possible to infect a susceptible, splenectomised calf (free of potential vaccine extraneous agents) by intravenous inoculation. If DMSO is used as the cryopreservative, the thawed working seed must be kept on ice and inoculated within 30 minutes of thawing.

Blood suitable for vaccine is obtained by monitoring films of jugular blood and collecting the required volume of blood when a suitable parasitaemia is reached. A parasitaemia of 3.5×10^8/ml for *B. bovis* in jugular blood, or 3×10^7/ml for *B. bigemina*, is usually adequate for production of frozen vaccine. If a suitable *B. bovis* parasitaemia is not obtained, passage of the strain by subinoculation of 100–800 ml of blood into a second splenectomised calf may be necessary. Passage of *B. bigemina* through splenectomised calves is not recommended because of the potential for the attenuated strain to increase in virulence.

Blood from the infected donor calf is collected by jugular cannulation using preservative-free heparin as anticoagulant (5 IU heparin/ml blood).

In the laboratory, the parasitised blood is held at 20–25°C and mixed in equal volumes with 3 M glycerol in PBS supplemented with 5 mM glucose (final concentration of glycerol in blood mixture is 1.5 M) held at 37°C. The mixture is then equilibrated at 37°C for 30 minutes, and dispensed into suitable containers (e.g. 5 ml cryovials). The vials are cooled at approximately 10°C/minute in the vapour phase of liquid nitrogen and, when frozen, stored in the liquid phase (Bock *et al.,* 2008).

DMSO can be used as cryoprotectant in the place of glycerol. This is carried out in the same way as outlined for the preparation of master seed (Pipano, 1997).

When glycerolised frozen vaccine is diluted for use as vaccine, the diluent should be iso-osmotic and consist of PBS containing 1.5 M glycerol and 5 mM glucose. Similarly, the diluent used in vaccine cryopreserved with DMSO should be iso-osmotic, and should contain the same concentration of DMSO in PBS as the concentration of DMSO in the vaccine concentrate.

Frozen vaccine containing both *B. bovis* and *B. bigemina* can be prepared by mixing equal volumes of blood containing each of the parasites obtained from different donors (Mangold *et al.,* 1996). A trivalent vaccine containing RBCs infected with *B. bovis*, *B. bigemina* and *Anaplasma centrale* is also made in Australia. RBCs from three donors (one for each parasite) are concentrated by centrifugation and mixed with glycerol solution to produce the trivalent concentrate, which is thawed and mixed with a diluent before use (Bock *et al.,* 2008).

The recommended dose volume of vaccine after reconstitution and dilution ranges from 1 to 2 ml depending on local practices and requirements, but aims to deliver a minimum infective dose of parasites, based on the parasitaemia prior to freezing.

2.2.2. Production of chilled vaccine

Infective material used in the production of chilled vaccine is obtained in the same way as for frozen vaccine, but should be issued and used as soon as possible after collection. If it is necessary to obtain the maximum number of doses per calf, the infective material can be diluted to provide the required number of parasites per dose (usually from 2.5 to 10 $\times 10^6$). A suitable diluent is 10% sterile bovine serum in a balanced salt solution containing the following ingredients per litre: NaCl (7.00 g), $MgCl_2.6H_2O$ (0.34 g), glucose (1.00 g), Na_2HPO_4 (2.52 g), KH_2PO_4 (0.90 g), and $NaHCO_3$ (0.52 g).

In-vitro multiplication is carried out in 225 cm^2 tissue culture flasks, where 115 ml of complete culture medium is dispensed to achieve a depth of 5.0–5.2 mm. Ninety ml of supernatant is replaced daily by fresh medium and 50–75% of parasitised RBCs are replaced every 48 hours by uninfected RBCs (subculture). Parasitised RBCs containing *Babesia* spp. are harvested when the parasites show typical morphology and achieve the maximum parasitaemia still inside the RBCs. Ninety per cent of the basic medium from each flask is removed without disturbing the settled RBCs. The *Babesia*-parasitised RBCs, still suspended in the remaining medium, are then mixed 1/1 with balanced salt solution, dispensed into one bottle and refrigerated to 5°C until use. The suspensions of each *Babesia* species, both with a high concentration of parasites, are finally diluted with the same balanced salt solution enriched with 10% bovine serum to achieve a concentration of 10^7 *B. bovis and* 10^7 *B. bigemina* parasitised RBCs per 2 ml dose.

Where anaplasmosis is of concern, *Anaplasma centrale* may also be incorporated into the vaccine to make a trivalent vaccine effective against *B. bovis*, *B. bigemina* and *Anaplasma marginale*.

2.2.3. In-process control

i) Sources and maintenance of vaccine donors

A source of donors free of natural infections with *Babesia*, other tick-borne diseases, and other infectious agents transmissible with blood, should be identified. If a suitable source is not available, it may be necessary to breed donor calves under tick-free conditions specifically for the purpose.

Donor calves should be maintained under conditions that will prevent exposure to infectious diseases and to ticks and biting insects. In the absence of suitable facilities, the risk of contamination with the agents of infectious diseases present in the country involved should be estimated, and the benefits of local production of vaccine (as opposed to importation of a suitable product) should be weighed against the possible adverse consequences of spreading disease (Bock *et al.,* 2008).

ii) Surgery

Calves to be used as vaccine donors should be splenectomised to allow maximum yield of parasites for production of vaccine. This is easier in calves less than 3 months of age and must be performed under general anaesthesia.

iii) Screening of vaccine donors before inoculation

Donor calves should be examined for agents of all blood-borne infections prevalent in the country, including *Babesia, Anaplasma, Theileria*, *Eperythrozoon* and *Trypanosoma*. This can be done by routine examination of stained blood films after splenectomy, and preferably also by serological testing pre- and post-quarantine. Calves showing evidence of natural infections with any of these agents should be rejected or have infections chemically sterilised. The absence of other infective agents endemic in the country should also be confirmed; this may include the agents of enzootic bovine leucosis, bovine immunodeficiency virus, bovine pestivirus, bovine syncitial virus, infectious bovine rhinotracheitis, Akabane disease, Aino virus, ephemeral fever, bluetongue, foot and mouth disease, bluetongue, *Brucella abortus*, *Leptospira* spp., heartwater, Jembrana disease, Rift Valley fever, rabies, lumpy skin disease, contagious bovine pleuropneumonia and rinderpest. The test procedures will depend on the diseases prevalent in the country and the availability of tests, but should involve serology of paired sera and, in some cases, virus isolation or antigen or DNA detection (Bock *et al.,* 2008; Pipano, 1997).

iv) Monitoring of parasitaemias following inoculation

It is necessary to determine the concentration of parasites in blood collected for vaccine or in RBCs harvested from culture. There are accurate techniques for determining the parasite count, but the parasite concentration can be adequately estimated from the RBC count and the parasitaemia (% infected RBCs).

v) Collection of blood for vaccine

All equipment should be sterilised before use (e.g. by autoclaving). The blood is collected in heparin using strict aseptic techniques in a closed-circuit collection system, when the required parasitaemia is reached. The calf should be sedated (for example, with xylazine).

Up to 3 litres of heavily infected blood can be collected from a 6-month-old calf. If the calf is to live, the transfusion of a similar amount of blood from a suitable donor (or blood previously collected from the donor itself) is indicated. Alternatively, the calf should be killed immediately after collection of the blood.

Using *in-vitro* culture, a 225 cm^2 flask can routinely provide 1800 doses. Starting from one 225 cm^2 flask containing 11 ml of 8–10% parasitised RBCs, it is possible to harvest about 45,000 doses after 6 days of continuous growth.

vi) Dispensing of vaccine

All procedures are performed in a suitable environment, such as a laminar flow cabinet, using standard sterile techniques. Use of a mechanical or magnetic stirrer will ensure thorough mixing of infected RBCs and diluent throughout the dispensing process.

2.2.4. Batch control

The potency, safety and sterility of vaccine batches cannot be determined in the case of chilled vaccine, except by thorough testing of vaccine donors and adherence to the principles of good

manufacturing practice. Specifications of frozen vaccine depend on the code of practice of the country involved. The following are the specifications for frozen vaccine produced in Australia.

i) Sterility and freedom from contaminants

Standard tests for sterility are employed for each batch of vaccine and diluent. The absence of contaminants is determined by doing appropriate serological and molecular diagnostic testing of donor cattle for evidence of viral and bacterial infection. Potential contaminants include those agents listed in Section C.2.2.3.

ii) Safety

Adverse reactions of the cattle inoculated in the test for potency may be monitored by measuring parasitaemia, temperature and packed cell volume; or based on regular observation of the health and demeanour of vaccinated animals. Detailed monitoring is more usually associated with the development and testing of parasite strains as potential candidates for vaccine production. Only batches with pathogenicity levels equal to or lower than a predetermined standard are released for use. Vaccine is preferably used in calves less than 1 year of age.

Non-target species are of no concern. Some attenuated vaccine strains of *B. bovis* are tick transmissible and evidence suggests that they may return to virulence after transmission by ticks. This is of little consequence in endemic situations.

No withholding periods for milk or meat are necessary following use of the vaccine, unless required by local legislation.

iii) Potency

Frozen, glycerolised vaccine concentrate is thawed and diluted 1/10 with isotonic diluent (Bock *et al.,* 2008; Pipano, 1997).The prepared vaccine is then stored for 8 hours at 4°C, and 5 to 25 susceptible cattle (held in cattle tick-free areas) are each inoculated subcutaneously with a 2 ml dose of that vaccine batch. The inoculated cattle are then monitored for the presence of viable *Babesia* spp. infections by examination of stained blood smears, by PCR techniques or by evidence of seroconversion. Only batches with acceptable infectivity are released for use at a working dilution of 1/10. Greater than 95% of vaccinated cattle would be expected to develop immunity to *Babesia* spp. after a single inoculation with an adequate dose (1×10^7 parasites) in a chilled or frozen vaccine prepared, stored and transported according to appropriate protocols.

iv) Duration of immunity

Long-lasting immunity usually results from one inoculation. Protective immunity develops in 3–4 weeks, and lasts at least 4 years in most cases (Bock & de Vos, 2001). Evidence of *B. bovis* vaccine failures have been reported and are related to the choice of vaccine strain, the presence of heterologous field strains and host factors (Bock *et al.,* 2008). There is little evidence of time-related waning of immunity (Bock & de Vos, 2001).

v) Stability

When stored in liquid nitrogen, the frozen vaccine can be kept for 5 years. Sterile diluent can be kept for 2 years in a refrigerator. Thawed vaccine rapidly loses potency and cannot be refrozen.

vi) Preservatives

Benzylpenicillin (500,000 IU/litre) and streptomycin (370,000 µg/litre) are added to the vaccine concentrate prior to dispensing into cryotubes. No preservative is used.

vii) Use of vaccine

In the case of frozen vaccine, vials should be thawed by immersion in water preheated to 37°C. Glycerolised vaccine should be kept cool and used within 8 hours (Bock *et al.,* 2008),

while vaccine with DMSO as cryoprotectant should be kept on ice and used within 15–30 minutes of thawing (Pipano, 1997).

Chilled vaccine should be kept refrigerated and used within 4–7 days of preparation, depending on the viability of the parasites and the recommendation of the vaccine production facility.

The strains of *B. bovis, B. divergens* and *B. bigemina* used in the vaccine may be of reduced virulence, but may not be entirely safe. A practical recommendation is therefore to limit the use of vaccine to calves under the age of 1 year, when nonspecific immunity will minimise the risk of adverse reactions. If older animals are to be vaccinated, there is a greater risk of vaccine reactions. These reactions occur infrequently, but valuable breeding stock or pregnant animals warrant due attention and should be observed daily for 3 weeks after vaccination. Ideally, rectal temperatures of vaccinated cattle should be taken and the animals should be treated if significant fever develops. Reactions to *B. bigemina* and *B. divergens* are usually seen by day 6–8 and those to *B. bovis* by day14–18 (Bock *et al.,* 2008).

Babesiosis and anaplasmosis vaccines are often used concurrently, but it is preferable not to use any other vaccines at the same time (Bock *et al.,* 2008).

viii) Precautions

Babesia bovis and *B. bigemina* vaccines are not infective for humans. However, cases of *B. divergens* have been reported in splenectomised individuals. When the vaccine is stored in liquid nitrogen, the usual precautions pertaining to the storage, transportation and handling of liquid nitrogen and deep-frozen material applies.

2.3. Requirements for regulatory approval

Issues of safety, potency, stability of vaccine strains, non-target species and reversion to virulence are dealt with in preceding sections. The vaccine is only used to control babesiosis. Eradication of babesiosis is only undertaken through eradication of the tick vector and/or intensive chemotherapeutic regimes.

3. Vaccines based on biotechnology

No biotechnology-based vaccines are currently available.

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NB: There are WOAH Reference Laboratories for babesiosis (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for babesiosis

NB: FIRST ADOPTED IN 1991. MOST RECENT UPDATES ADOPTED IN 2021.