CHAPTER 2.4.2.

BOVINE BABESIOSIS

SUMMARY

Babesiosis is a tick-borne disease of cattle caused by the protozoan parasites Babesia bovis, B. bigemina, B. divergens and others. Boophilus spp., the principal vectors of B. bovis and B. bigemina, are widespread in tropical and subtropical countries. The major vector of B. divergens is Ixodes ricinus. Other important vectors include Haemaphysalis and Rhipicephalus spp.

Identification of the agent: Demonstration of parasites in dead animals is possible by microscopic examination of smears of blood, brain, kidney, liver and spleen, provided decomposition is not advanced. The smears are fixed with methanol, stained with 10% Giemsa for 20–30 minutes, and examined at ×800–1000 magnification under oil immersion. In the case of live animals, thick and thin films of capillary blood should be taken from, for example, the tip of the tail. Sensitive polymerase chain reaction assays are available that can detect and differentiate Babesia species in cattle.

Serological tests: The indirect fluorescent antibody (IFA) test is the most widely used test for the detection of antibodies to B. bovis and B. divergens, but enzyme-linked immunosorbent assays are gaining popularity. The IFA test has been used for detection of antibodies to B. bigemina, but serological cross-reactions make species diagnosis difficult. The complement fixation test has also been used to detect antibodies against B. bovis and B. bigemina.

Requirements for vaccines and diagnostic biologicals: Vaccines consisting of live, attenuated strains of B. bovis, B. bigemina or B. divergens are produced in several countries from the blood of infected donor animals. The vaccines are provided in frozen or chilled forms. Production of frozen vaccine is usually recommended as it allows thorough post-production control of each batch. The risk of contamination of this blood-derived vaccine makes thorough quality control essential, but it may be prohibitively expensive.

Live Babesia vaccines are not entirely safe. A practical recommendation is to limit their use to calves, preferable less than 1 year old, when nonspecific immunity will minimise the risk of vaccine reactions. When older animals are to be vaccinated, the risk of reaction warrants close surveillance and treatment with a babesiacide if reactions occur.

Protective immunity develops in 3–4 weeks and lasts for several years after a single vaccination.

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 Central and South America. Babesia divergens is economically important in some parts of Europe.

Tick species are the vectors of Babesia (18). 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, Rhipicephalus and other Boophilus spp.

Babesia bigemina has the widest distribution but generally, B. bovis is more pathogenic than B. bigemina or B. divergens. 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. 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 (41).

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 (4, 10).

## B. DIAGNOSTIC TECHNIQUES

### 1. Identification of the agent

The traditional method of identifying the agent in infected animals is by microscopic examination of thick and thin blood films stained with, for example, Giemsa. The sensitivity of this technique is such that it can detect parasitaemias as low as 1 parasite in 10⁰ red blood cells (RBCs) (8). 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 (8). A quantitative buffy coat method using acridine orange to stain parasites in capillary tubes was developed to demonstrate *Plasmodium* in human blood and could potentially also detect low *Babesia* parasitaemias, but differentiation is likely to be poor (8).

Samples from live animals should preferably be 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 smears from capillary blood, sterile jugular blood should be collected into an anticoagulant such as ethylene diamine tetra-acetic acid (EDTA) (e.g. 1 mg/ml). Heparin may affect the colour characteristics of the staining and is not recommended. The sample should be kept cool, preferably at 5°C, until delivery to the laboratory, again preferably within hours of collection. Thin blood films are air-dried, fixed in absolute methanol for 1 minute, and stained in 10% Giemsa stain for 20–30 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 drop (approximately 50 µl) of blood on to a clean glass slide. This droplet is then air-dried, heat-fixed at 80°C for 5 minutes, and stained in 10% Giemsa for 15–20 minutes. Unstained blood smears should not be stored with formalin solutions as it may affect staining quality.

Samples from dead animals should consist of thin blood films, as well as smears from (in order of preference), cerebral cortex, kidney, liver, spleen and bone marrow. 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 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 5 minutes in absolute methanol, and stained for 20–30 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 drop (approximately 50 µl) of blood on to a clean glass slide. This droplet is then air-dried, heat-fixed at 80°C for 5 minutes, and stained in 10% Giemsa for 15–20 minutes. Unstained blood smears should not be stored with formalin solutions as it may affect staining quality.

All stained smears are examined under oil immersion using (as a minimum) a ×8 eyepiece and a ×60 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 a much longer parasite, and is often found as pairs at an acute angle to each other. *Babesia bigemina* is typically pear-shaped, but many diverse single forms are found. It is 3–3.5 µm long and 1–1.5 µm wide, and paired forms often have two discrete red-staining dots in each parasite (*B. bovis* and *B. divergens* always have only one). In acute cases, the parasitaemia of *B. bovis* seldom reaches 1%, but with *B. bigemina* and *B. divergens* much higher parasitaemias are the norm. Thick blood films are especially useful for the diagnosis of low level *B. bovis* infections, as are organ smears (2).

Polymerase chain reaction (PCR) assays have proven to be very sensitive particularly in detecting *B. bovis* and *B. bigemina* incarrier cattle (9, 11, 17, 34, 36, 38). Thammasirirak et al. found their PCR-enzyme-linked immunosorbent assay (ELISA) to be at least 1000 times more sensitive than thin blood smears for detection of *B. bovis* (38), and detection levels as low as three parasitised erythrocytes in 20 µl of packed cells have been claimed (37). A number of PCR techniques have been described that can detect and differentiate species of *Babesia* in carrier infections (9, 11, 17, 36). PCR assays to differentiate isolates of *B. bovis* have also been described (6). The application of the reverse line blot procedure, in which PCR products are hybridised to membrane-bound, species-specific oligonucleotide probes, to *Babesia* (21) has 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.

*In-vitro* culture methods have been used to demonstrate the presence of carrier infections of *Babesia* spp. (22), 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 (8), but could be as low as $10^{-10}$ (19), making it a very sensitive method for the demonstration of infection. An added benefit is that it is 100% specific.

Confirmation of infection in a suspected carrier animal can also be made by transfusing approximately 500 ml of jugular blood intravenously into a splenectomised calf known to be *Babesia*-free, and monitoring the calf for the presence of infection. This method is cumbersome and expensive, and obviously not suitable for routine diagnostic use. Mongolian gerbils (*Meriones unguiculatus*) can, however, be used to demonstrate the presence of *B. divergens* (41).

2. **Serological tests**

The indirect fluorescent antibody (IFA) test is widely used 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* IFA test are a particular problem in areas where the two parasites coexist. The IFA test has the disadvantages of low sample throughput and subjectivity. The complement fixation (CF) test has been described as a method to detect antibodies against *B. bovis* and *B. bigemina* (1). This test has been used to qualify animals for importation into some countries. The test is based on the procedure previously described and validated for the detection of antibody against *Babesia caballi* and *Theileria equi* (see Chapter 2.5.8 Equine piroplasmosis). An ELISA for the diagnosis of *B. bovis* infection that uses a whole merozoite antigen has undergone extensive evaluation (12, 29, 39). Competitive ELISAs using recombinant merozoite surface and rhoptry associated antigens of *B. bovis* have recently been developed (7, 14, 20) but have not yet been widely validated. Despite the efforts of several investigators in different laboratories, there is still no well-validated ELISA available for *B. bigemina*. ELISAs for antibodies to *B. bigemina* typically have poor specificity. In one study (16), *B. bigemina* antiserum appeared to react non-specifically with fibrinogen. To the best of our knowledge a competitive ELISA developed and validated in Australia (30) is the only ELISA in routine use. In the absence of any other workable test for *B. bigemina*, the procedure for that assay has been included here. ELISAs have also been developed for *B. divergens* (9) using antigen derived from culture, *Meriones* or cattle, but there does not appear to be one that has been validated internationally.

a) **Babesia bovis** enzyme-linked immunosorbent assay

Antigen preparation is based on a technique described by Waltisbuhl et al. (39). Infected blood (usually 5–10% parasitaemia) is collected from a splenectomised calf into EDTA. The blood is washed three times in five volumes of phosphate buffered saline (PBS), and then 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. A series of hypotonic saline solutions are prepared, ranging from 0.35% to 0.50% NaCl, in 0.025% increments. To find the best concentration, five volumes of each saline solution is then added to one volume of packed RBC, which are gently mixed and allowed to stand for 5 minutes.

The mixtures are then centrifuged and the supernatants are aspirated. An equal volume of plasma (retained from the original blood) is added to each tube containing packed RBC, 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 RBC but leaves infected RBC intact. It should be possible to achieve >95% infection in the remaining intact RBC. The bulk of the packed RBC is then differentially lysed with the optimal saline solution and centrifuged. The sediment (>95% infected RBC) is lysed in distilled water at 4°C, and parasites are pelleted at 12,000 g for 30 minutes. The pellet is washed three times in PBS by resuspension and centrifugation at 4°C. 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 ultracentrifuged, (105,000 g for 60 minutes at 4°C) and the supernatant 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.

- **Test procedure**
  
  i) 100 µl of this antigen, diluted 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) Antigen is removed and the wells are then blocked for 2 hours at room temperature by the addition of 200 µl of a 2% solution of sodium caseinate in carbonate buffer.
  
  iii) After blocking, the wells are rinsed briefly with PBS containing 0.1% Tween 20 (PBST) and 100 µl of bovine serum diluted 1/100 in PBST containing either 5% normal horse serum or 5% skim milk powder is added, and the plates are incubated for 2 hours at room temperature.
iv) The washing step consists of a brief rinse with PBST, followed by three 5-minute washes with the same buffer (during which the plate is shaken vigorously), and finally the plates are given a further brief rinse.

v) Next, 100 µl of peroxidase-labelled anti-bovine IgG diluted appropriately in PBST containing horse serum or skim milk is added and the plates are shaken for a further 30 minutes at room temperature. (NB: some batches of skim milk powder may contain immunoglobulins that can interfere with anti-bovine IgG conjugates).

vi) Wells are washed as described in step iv above, and 100 µl of peroxidase substrate (ABTS [2,2'-Azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid]) is added to each well. The substrate reaction is allowed to continue until the absorbance of a strong positive control serum included on each plate approaches 1. At this point the absorbance at 414 nm is read on a microtitre plate reader.

To control for inter-plate variation, known positive and negative sera are included in each plate (29). Test sera are then ranked relative to the positive control. ELISA results are expressed as a percentage of this positive control (percentage 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. The most suitable enzyme label for the conjugate is horseradish peroxidase. ABTS or tetramethyl benzidine (TMB) are suitable substrates. 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.

b) Babesia bigemina enzyme-linked immunosorbent assay

This ELISA is based on an immunodominant 58 kDa antigen identified by a number of groups in B. bigemina isolates from Australia, Central America and Texas, United States of America, Egypt and Kenya (30). A monoclonal antibody (MAb) (D6) (Tick Fever Centre, Qld, Australia) directed against this antigen has been used to develop a competitive inhibition ELISA (30). The antigen used in the ELISA is a 26 kDa peptide (Tick Fever Centre, Qld, Australia), encoded by a 360 bp fragment of the p58 gene, expressed in Escherichia coli and affinity purified. This antigen can also be used in an indirect ELISA format, but some cross-reactivity of antibodies to B. bovis should be expected.

• Test procedure

i) The recombinant 26 kDa antigen is diluted in 0.1 M carbonate buffer, pH 9.6, to a concentration of approximately 2 µg/ml and 100 µl is added to each well of a 96-well microtitre plate. The plates are incubated overnight at 4°C.

ii) Excess antigen is removed and the wells are then blocked for 1 hour at room temperature by addition of 200 µl per well of a 2% solution of sodium caseinate in carbonate buffer.

iii) Following a brief rinse (3 × 200 µl) with PBS containing 0.1% Tween 20 (PBST), 100 µl of undiluted serum is added and the plates are incubated for 30 minutes at room temperature with gentle shaking.

iv) The plates are then washed with PBST (5 × 200 µl rinse, 5-minute soak with shaking), and 100 µl of peroxidase-labelled MAb D6 diluted to a concentration of 0.03 µg/ml in PBST containing 2% skim milk powder is added to each well. The plates are then incubated at room temperature for 30 minutes with gentle shaking.

v) Plates are washed again, 100 µl TMB peroxidase substrate is added to each well, and the plates are incubated in the dark until the absorbance of the conjugate control wells (no serum) approaches 1. At this point the reaction is stopped by the addition of 50 µl of 1 M sulphuric acid and the absorbance is read at 450 nm. Positive and negative control sera should be included on each test plate.

The per cent inhibition (PI) for test sera is calculated relative to the conjugate control (PI = 100 – [100 × test absorbance/conjugate control absorbance]). Positive and negative threshold values should be determined in each laboratory by testing as many known positive and negative sera as possible.

The specificity of the ELISA has been estimated at 97.0% and the sensitivity for detection of antibodies in experimentally infected cattle is 95.7% (30).

c) Indirect fluorescent antibody test

• Antigen preparation

Antigen slides are made from jugular blood, 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 from 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 adhere RBCs 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 technique (dragging with the end of another slide). The films are air-dried and fixed for 5 minutes in an oven at 80°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).

- **Test procedure**

Test and control sera are diluted 1/30 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. To each test square 5–10 µl of each serum dilution is added using a fine pipette. The preparations are then incubated at 37°C for 30 minutes, in a humid chamber. For controls, dilutions of weak positive and negative sera are used on each test slide.

After incubation, the slides are gently rinsed once with PBS, and given two 10-minute washes with PBS followed by water. An appropriate dilution of anti-bovine IgG antibody labelled with fluorescein isothiocyanate (which is available commercially) is then added to each test square. Every new batch of conjugate must be titrated, the working range usually being between 1/400 and 1/1200. Conjugated rabbit and chicken antibodies are usually more suitable for this purpose than goat antibodies. The slides with the conjugate are incubated again at room temperature for 30 minutes, and washed as above. The wet slides are mounted with cover-slips in 1/1 glycerol and PBS, and examined by standard fluorescence microscopy. A competent operator can examine approximately 150 samples per day.

- **Complement fixation**

The CF test is used by some countries for general diagnosis and to qualify cattle for importation. A brief description is provided here of antigen production and test protocols used by the United States Department of Agriculture (1). The methods are essentially the same as those described in this Terrestrial Manual for the microtitration CF test for equine piroplasmosis.

- **Solutions**

*Alsever’s solution:* prepare 1 litre of Alsever’s solution by dissolving 20.5 g glucose; 8.0 g sodium citrate; 4.2 g sodium chloride in sufficient distilled water. Adjust to pH 6.1 using citric acid, and make up the volume to 1 litre with distilled water. Sterilise by filtration.

*Stock veronal buffer (5×):* dissolve the following in 1 litre of distilled water: 85.0 g sodium chloride; 3.75 g sodium 5,5 diethyl barbituric; 1.68 g magnesium chloride (MgCl2.6H2O); 0.28 g calcium chloride. Dissolve 5.75 g of 5,5 diethyl barbituric acid in 0.5 litre hot (near boiling) distilled water. Cool this acid solution and add to the salt solution. Make up to 2 litre with distilled water and store at 4°C. To prepare a working dilution, add one part stock solution to four parts distilled water. The final pH should be from 7.4 to 7.6.

- **Antigen production**

Blood is obtained from cattle with a high parasitaemia (e.g. 30% parasitaemia for *B. bovis* and 60% for *B. bigemina*), and mixed with equal volumes of Alsever’s solution as an anticoagulant. The plasma/Alsever’s supernatant and buffy coat are removed when the RBCs have settled to the bottom of the flask. The RBCs are washed several times with cold veronal buffer and then disrupted. The antigen is recovered from the lysate by centrifugation at 30,900 g for 30 minutes.

The recovered antigen is washed several times in cold veronal buffer by centrifugation at 20,000 g for 15 minutes. Polyvinyl pyrrolidone (5% w/v) is added as a stabiliser and the preparation is mixed on a magnetic stirrer for 30 minutes, strained through two thicknesses of sterile gauze, dispensed into 2 ml volumes and freeze-dried. The antigen can then be stored at below −50°C for several years.

- **Test procedure – Microtitration method**

i) The specificity and potency of each batch of antigen should be checked against standard antisera of known specificity and potency. Optimal antigen dilutions are also determined in a preliminary checkerboard titration.

ii) Test sera are inactivated for 30 minutes at 58°C and tested in dilutions of 1/5 to 1/320. Veronal buffer is used for all dilutions.
Chapter 2.4.2. – Bovine babesiosis

iii) Complement is prepared and titrated spectrophotometrically to determine the 50% haemolytic dose (C’H50) and used in the test at five times C’H50. The haemolytic system (sensitised RBCs) consists of equal parts of a 2% sheep RBC suspension and veronal buffer with optimally diluted haemolysin.

iv) The total volume of the test is 0.125 ml, made up of equal portions (0.025 ml) of antigen, complement (five times C’H50) and diluted serum. Incubation is performed for 1 hour at 37°C.

v) A double portion (0.05 ml) of the haemolytic system (sensitised sheep RBCs) is added and the plates are incubated for a further 45 minutes at 37°C with shaking after 20 minutes.

vi) The plates are centrifuged for 5 minutes at 300 g before being read over a mirror.

vii) The reaction in each well is recorded as follows: 100% lysis = 0 or negative, 75% lysis = 1+, 50% lysis = 2+, 25% lysis = 3+, 0% lysis = 4+. A 2+ reaction (50% lysis) or stronger at the 1/5 dilution is recorded as positive, with titre results reported as the reaction, if any, at the next dilution higher than the greatest serum dilution with a 4+ reaction (e.g. 1+ at 1:10, for a sample with a 4+ reaction at 1/5 and a 1+ reaction at 1/10). A full set of controls must be included in each test, including positive and negative sera, as well as control antigen prepared from normal (uninfected) horse RBCs.

Anticomplementary samples are examined by the IFA test.

e) Other tests

Other serological tests have been described in recent years, and include a dot ELISA (31), a slide ELISA (25), and latex and card agglutination tests (3, 26). These tests show acceptable levels of sensitivity and specificity for B. bovis and, in the case of the dot ELISA, 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 is therefore unknown.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

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 (4, 27, 35). 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, Israel and Uruguay. An experimental B. divergens vaccine prepared from the blood of infected Meriones has also been used successfully in Ireland (41).

A killed B. divergens vaccine is prepared in Austria from the blood of infected calves (15), but little information is available on the level and duration of the conferred immunity. Experimental vaccines containing antigens produced in vitro have also been developed (3, 32), but the level and duration of protection against heterologous challenge are unclear. Parasite proteins have been characterised and there has been some progress towards the development of subunit vaccines (11, 33). 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 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 blood as vaccine (4). Calves used for infection with these strains 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. In-vitro culture methods have also been used to produce parasites for vaccine (24, 27). However, the relatively high cost of production from culture and evidence of possible antigenic drift during long-term maintenance in culture, make mass culture of Babesia impractical in most laboratories at present.

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 is preferred (4, 27, 35), as it allows for thorough post-production control of each batch. However, it is more costly to produce and more difficult to transport than chilled vaccine. The potential risk of contamination of this blood-derived vaccine makes post-production control essential, but may put production beyond the financial means of some countries in endemic regions (13). A production facility supplying an annual market of fewer than 50,000 doses is unlikely to operate without financial support.
1. Seed management

a) Characteristics of the seed

• 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 (4). Tick-transmissible and nontransmissible strains are available. A strain of B. divergens with reduced virulence for Meriones has also been developed (40).

• Isolation and purification of local strains

Strains of B. bovis, B. divergens and B. bigemina that are free of contaminants, 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 (19).

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 (41). Maintenance of isolated strains in vitro (23) 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 passaging in susceptible calves will allow isolation of B. bigemina (2).

• 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 (4).

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 after 3 months and then using the ensuing relapse parasites to repeat the procedure (4).

Attenuation of B. divergens for Meriones followed long-term maintenance in vitro (40).

b) 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 MW 40,000 (4) are the recommended cryopreservatives, as they allow for intravenous administration after thawing of the master seed. A detailed account of the freezing technique using DMSO is reported elsewhere (28). Briefly, it involves the following:

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:protectant ratio of 1:1 with the final concentration of DMSO being 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 have been known to remain viable for 20 years.

c) Preparation and storage of working seed

Working seed is prepared in the same way as master seed (Section C.1.b) using master seed as starting material.

d) Validation of safety and efficacy of working seed

The suitability of a working seed is determined by inoculating suitable numbers of susceptible cattle with vaccine prepared from it and then challenging them and susceptible controls with a virulent, heterologous strain. Both safety and efficacy can be judged by monitoring fever, parasitaemias in stained blood films, and
depression of packed cell volumes. The purity of the working seed is tested by monitoring the cattle used in the safety test for evidence of possible contaminants as mentioned in Section C.4.b.

2. Method of manufacture

a) Production of frozen vaccine concentrate

First, 5–10 ml quantities of working seed are rapidly thawed by immersing the vials in water preheated to 40°C. The thawed material is kept on ice and used as soon as possible (within 30 minutes if DMSO is used) to infect a susceptible, splenectomised calf (free of potential vaccine contaminants) by intravenous inoculation.

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 $1 \times 10^8$/ml (approximately 2% parasitaemia in jugular blood) is usually adequate for production of 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* is not recommended.

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

In the laboratory, the parasitised blood is mixed in equal volumes with 3 M glycerol in PBS supplemented with 5 mM glucose (final concentration of glycerol 1.5 M) 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 (4).

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 (35).

If glycerolised frozen vaccine is to be diluted, 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.

Frozen vaccine containing both *B. bovis* and *B. bigemina* can be prepared (27) by mixing equal numbers of the parasites obtained from different donors. A 3 in 1 vaccine containing packed red cells infected with *B. bovis*, *B. bigemina* and *Anaplasma Centrale* is also made in Australia. Packed cells from 3 donors are concentrated and mixed to produce the trivalent concentrate which on thawing is mixed with a diluent before use (4).

The recommended dose of vaccine after reconstitution and dilution ranges from 1 to 2 ml depending on local practices and requirements.

b) 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 $10^7$). 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$_2$O (0.34 g), glucose (1.00 g), Na$_2$HPO$_4$ (2.52 g), KH$_2$PO$_4$ (0.90 g), and NaHCO$_3$ (0.52 g).

Blood containing *B. divergens* may be diluted in Hanks’ solution. If diluent is not required, sterile acid citrate dextrose or citrate phosphate dextrose should be used as the anticoagulant, at a rate of one part to four parts blood, to provide the glucose necessary for parasite survival.

3. In-process control

a) 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 (4).

b) Surgery
Donor calves 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 best done under general anaesthesia.

c) 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, 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. The absence of other infective agents endemic in the country should also be confirmed; these may include the agents of enzootic bovine leukosis, bovine immunodeficiency virus, bovine pestivirus, infectious bovine rhinotracheitis, Akabane disease, ephemeral fever, bluetongue, foot and mouth disease, 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, antigen or DNA detection (4, 35).

d) Monitoring of parasitaemias following inoculation
It is necessary to determine the concentration of parasites in blood collected for vaccine. There are accurate techniques for determining the parasite count (2), but the parasite concentration can be estimated from the RBC count and the parasitaemia (% infected RBCs).

e) 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 when the required parasitaemia is reached. This is best done if the calf is sedated with, for example, xylazine and with the use of a closed-circuit collection system.

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 is indicated. Alternatively, the calf should be killed immediately after collection of the blood.

f) 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 blood and diluent throughout the dispensing process.

4. Batch control
The potency, safety and sterility of vaccine batches cannot be determined in the case of chilled vaccine, and 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.

a) 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 testing of donor cattle and by inoculating donor lymphocytes into sheep and then monitoring them for evidence of viral infection. Potential contaminants include the agents of enzootic bovine leukosis, infectious bovine rhinotracheitis, bovine pestivirus, ephemeral fever, Akabane disease, Aino virus, bluetongue, Brucella abortus and Leptospira, foot and mouth disease, lumpy skin disease, rabies, Rift Valley fever, rinderpest, contagious bovine pleuropneumonia, heartwater, Jembrana disease, and pathogenic Theileria and Trypanosoma spp. (4, 35).

b) Safety
Vaccine reactions of the cattle inoculated in the test for potency (see Section C.4.c) are monitored by measuring parasitaemia, fever and depression of packed cell volume. Only batches with pathogenicity levels equal to or lower than a predetermined standard are released for use.

c) Potency
Frozen, glycerolised vaccine concentrate is thawed and diluted 1/10 with isotonic diluent (4, 35). The prepared vaccine is then incubated for 8 hours at 4°C, and five cattle are inoculated subcutaneously with
2 ml doses each. The inoculated cattle are then monitored for the presence of infections by examination of stained blood smears. Only fully infective batches are released for use at a working dilution of 1/10.

d) Duration of immunity

Long-lasting immunity usually results from one inoculation. 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 (4). There is little evidence of time-related waning of immunity (5).

e) Stability

When stored in liquid nitrogen, the 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.

f) Preservatives

Penicillin (500,000 IU/litre) and streptomycin (370,000 µg/litre) are added to the vaccine at the time of dispensing.

g) Use of vaccine

In the case of frozen vaccine, vials should be thawed by immersion in water preheated to 37–40°C. Glycerolised vaccine should be kept cool and used within 8 hours (4), while vaccine with DMSO as cryoprotectant should be kept on ice and used within 15–30 minutes of thawing (35).

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

The strains of *B. bovis*, *B. divergens* and *B. bigemina* used in the vaccine may be of reduced virulence, but will not be entirely safe. A practical recommendation is, therefore, to limit the use of vaccine to calves, when nonspecific immunity will minimise the risk of vaccine reactions. If older animals are to be vaccinated, there is a risk of severe 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 day 10–16 (4).

Protective immunity develops in 3–4 weeks, and lasts at least 4 years in most cases (4).

Babesiosis and anaplasmosis vaccines are often used concurrently, but it is not advisable to use any other vaccines at the same time (4).

h) 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 deep-frozen material applies.

5. Tests on the final product

a) Safety

See Section C.4.b.

b) Potency

See Section C.4.c.

REFERENCES


Chapter 2.4.2. — Bovine babesiosis


* * *

**NB:** There is an OIE Reference Laboratories for Bovine babesiosis (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: [www.oie.int](http://www.oie.int)).