

SECTION 2.4.

BOVIDAE

CHAPTER 2.4.1.

BOVINE ANAPLASMOSIS

SUMMARY

Definition of the disease: Bovine anaplasmosis results from infection with *Anaplasma marginale*. A second species, *A. centrale*, has long been recognised. Whether it truly represents a separate species is unclear. *Anaplasma marginale* is responsible for almost all outbreaks of clinical disease. A third species, *A. phagocytophilum*, has recently been reported to infect cattle. However, natural infection appears to be rare and *A. phagocytophilum* does not cause clinical disease. The organism is classified in the genus *Anaplasma* belonging to the family Anaplasmataceae of the order Rickettsiales.

Description of the disease: Anaemia and jaundice are characteristic signs of anaplasmosis, but the clinical disease can only be confirmed by identifying the organism. Once infected, cattle may remain carriers for life, and identification of these animals depends on the detection of specific antibodies using serological tests, or of rickettsial DNA using amplification techniques.

Identification of the agent: Microscopic examination of blood or organ smears stained with Giemsa stain is the most common method of identifying *Anaplasma* in clinically affected animals. In these smears, *A. marginale* appear as dense, rounded, intraerythrocytic bodies approximately 0.3–1.0 µm in diameter with most situated on or near the margin of the erythrocyte. *Anaplasma centrale* is similar in appearance, but most of the organisms are situated away from the margin of the erythrocyte. It can be difficult to differentiate *A. marginale* from *A. centrale* in a stained smear, particularly with low levels of rickettsmia. Commercial stains that give very rapid staining of *Anaplasma* are available in some countries.

It is important that smears be well prepared and free from foreign matter. Smears from live cattle should preferably be prepared from blood drawn from the jugular vein or another large vessel. For post-mortem diagnosis, smears should be prepared from internal organs (including liver, kidney, heart and lungs) and from blood retained in peripheral vessels. The latter are particularly desirable if post-mortem decomposition is advanced.

Serological tests: A competitive enzyme-linked immunosorbent assay (C-ELISA) has been demonstrated to have good sensitivity in detecting carrier animals. Card agglutination, indirect ELISA, dot ELISA and indirect fluorescent antibody tests also can be used. The complement fixation (CF) test is no longer considered a reliable test for disease certification of individual animals due to variable sensitivity. Cross reactivity between *Anaplasma* spp. can complicate interpretation of serological tests. In general, the C-ELISA has the best specificity, with well characterized cross-reactivity only between *A. marginale* and *A. centrale*.

Nucleic-acid-based tests have been used experimentally, and are capable of detecting the presence of low-level infection in carrier cattle and tick vectors. Caution is warranted with polymerase chain reaction-based assays when used diagnostically, as a nested reaction is necessary to identify low-level carriers and nonspecific amplification can occur.

Requirements for vaccines and diagnostic biologicals: Live vaccines are used in several countries to protect cattle against *A. marginale* infection. A vaccine consisting of live *A. centrale* is most widely used and gives partial protection against challenge with virulent *A. marginale*.

Anaplasma centrale vaccine is provided in chilled or frozen forms. Quality control is very important as other blood-borne agents that may be present in donor cattle can contaminate vaccines and be disseminated broadly. For this reason, frozen vaccine is recommended as it allows thorough post-production quality control, which limits the risk of contamination with other pathogens.

Anaplasma centrale vaccine is not entirely safe. A practical recommendation is to restrict its use, as far as possible, to calves, as nonspecific immunity will minimise the risk of some vaccine reactions that may require treatment with tetracycline or imidocarb. Partial immunity develops in 6–8 weeks and lasts for several years after a single vaccination.

A. INTRODUCTION

Outbreaks of bovine anaplasmosis are usually due to infection with *Anaplasma marginale*. *Anaplasma centrale* is capable of producing a moderate degree of anaemia, but clinical outbreaks in the field are extremely rare. Appendages associated with the *Anaplasma* body have been observed in certain isolates of *A. marginale* (19); although this parasite has been termed *A. caudatum*, it is not considered to be a separate species. A third species, *A. phagocytophilum*, has recently been reported to infect cattle. However, natural infection appears to be rare and *A. phagocytophilum* does not cause clinical disease (8, 20).

Anaplasma marginale occurs in most tropical and subtropical countries, and in some more temperate regions. *Anaplasma centrale* was first described from South Africa. The organism has since been imported by other countries – including Australia and some countries in South America, South-East Asia and the Middle East – for use as a vaccine against *A. marginale*.

Anaplasma species were originally regarded as protozoan parasites, but later research showed they had no significant attributes to justify this description. Since the last major accepted revision of the taxonomy in 2001 (9), the Family *Anaplasmataceae* (Order *Rickettsiales*) is now composed of four genera, *Anaplasma*, *Ehrlichia*, *Neorickettsia*, and *Wolbachia*. The genus *Aegyptianella* is retained within the Family *Anaplasmataceae* as *genus incertae sedis*. The revised genus *Anaplasma* now contains *Anaplasma marginale* as the type species, *A. phagocytophilum* (formerly *Ehrlichia phagocytophila*, *E. equi* and the unclassified agent of human granulocytic ehrlichiosis), *A. platys*, and *A. bovis*. *Haemobartonella* and *Eperythrozoon* are now considered most closely related to the mycoplasmas.

Anaplasma species are transmitted either mechanically or biologically by arthropod vectors. Reviews based on careful study of reported transmission experiments list up to 19 different ticks as capable of transmitting *A. marginale* experimentally (15, 21). These are: *Argas persicus*, *Ornithodoros lahorensis*, *Boophilus annulatus*, *B. calcaratus*, *B. decoloratus*, *B. microplus*, *Dermacentor albipictus*, *D. andersoni*, *D. hunteri*, *D. occidentalis*, *D. variabilis*, *Hyalomma excavatum*, *H. rufipes*, *Ixodes ricinus*, *I. scapularis*, *Rhipicephalus bursa*, *R. evertsi*, *R. sanguineus* and *R. simus*. The authors concluded that some of these reports, including those of *R. bursa*, *H. excavatum* and *O. lahorensis*, were not entirely convincing, and that the ticks identified as *A. persicus* were probably either *A. sanchezi* or *A. radiatus*. Intrastadial or transstadial transmission is the usual mode, even in the one-host *Boophilus* species. Male ticks may be particularly important as vectors; they can become persistently infected and serve as a reservoir for infection (17). Experimental demonstration of vector competence does not necessarily imply a role in transmission in the field. However, *Boophilus* species are clearly important vectors of anaplasmosis in countries such as Australia and countries in Africa, and some species of *Dermacentor* are efficient vectors in the United States of America (USA).

Various other biting arthropods have been implicated as mechanical vectors, particularly in the USA. Experimental transmission has been demonstrated with a number of species of *Tabanus* (horseflies), and with mosquitoes of the genus *Psorophora* (15, 33). The importance of biting insects in the natural transmission of anaplasmosis appears to vary greatly from region to region. *Anaplasma marginale* also can be readily transmitted during vaccination against other diseases unless a fresh or sterilised needle is used for injecting each animal. Similar transmission by means of unsterilised surgical instruments has been described.

The main biological vectors of *A. centrale* appear to be multihost ticks peculiar to Africa, including *R. simus*. The common cattle tick (*B. microplus*) has not been shown to be a vector. This is of relevance where *A. centrale* is used as a vaccine in *B. microplus*-infested regions.

B. DIAGNOSTIC TECHNIQUES

The most marked clinical signs of anaplasmosis are anaemia and jaundice, the latter occurring late in the disease. Haemoglobinaemia and haemoglobinuria are not present, and this may assist in the differential diagnosis of anaplasmosis from babesiosis, which is often endemic in the same regions. The disease can only be confirmed, however, by identification of the organism.

1. Identification of the agent

Samples from live cattle should include thin blood smears and blood collected into an anticoagulant. Air-dried thin blood smears will keep satisfactorily at room temperature for at least 1 week. The blood sample in anticoagulant should be held and transferred at 4°C, unless it can reach the laboratory within a few hours. This sample is useful for preparing fresh smears if those submitted are not satisfactory. In addition, a low packed cell volume and/or erythrocyte count can help to substantiate the involvement of *Anaplasma* when only small numbers of the parasites are detected in smears, such as may occur in the recovery stage of the disease.

In contrast to *Babesia bovis*, *Anaplasma* do not accumulate in capillaries, so blood drawn from the jugular or other large vessel is satisfactory. Because of the rather indistinctive morphology of *Anaplasma*, it is essential that smears be well prepared and free from foreign matter, as specks of debris can confuse diagnosis. Thick blood films as used for the diagnosis of babesiosis are not appropriate for the diagnosis of anaplasmosis, as *Anaplasma* are difficult to identify once they become dissociated from erythrocytes.

Samples from dead animals should include air-dried thin smears from the liver, kidney, heart and lungs and from a peripheral blood vessel. The latter is particularly recommended should there be a significant delay before post-mortem examination because, under these circumstances, bacterial contamination of organ smears often makes identification of *Anaplasma* equivocal. Brain smears, which are useful for the diagnosis of some forms of babesiosis, are of no direct value for diagnosing anaplasmosis, but should be included for differential diagnosis where appropriate.

Blood from organs, rather than organ tissues *per se*, is required for smear preparation, as the aim is to be able to examine microscopically intact erythrocytes for the presence of *Anaplasma*. Organ-derived blood smears will store satisfactorily at room temperature for several days.

Both blood and organ smears can be stained in 10% Giemsa stain for approximately 30 minutes after fixation in absolute methanol for 1 minute. After staining, the smears are rinsed three or four times with tap water to remove adhering stain, and are then air-dried. Conditions for Giemsa staining vary from laboratory to laboratory. Commercial stains that give very rapid staining of *Anaplasma* are available in some countries¹. Smears are examined under oil immersion at a magnification of ×700–1000.

Anaplasma marginale appear as dense, rounded and deeply stained intraerythrocytic bodies, approximately 0.3–1.0 µm in diameter. Most of these bodies are located on or near the margin of the erythrocyte. This feature distinguishes *A. marginale* from *A. centrale*, as in the latter most of the organisms have a more central location in the erythrocyte. However, particularly at low levels of rickettsemia, differentiation of these two species in smears can be difficult.

The percentage of infected erythrocytes varies with the stage and severity of the disease. Maximum parasitaemias in excess of 50% may occur with *A. marginale*. Multiple infections of individual erythrocytes are common during periods of high parasitaemias.

The infection becomes visible microscopically 2–6 weeks following transmission. During the course of clinical disease, the parasitaemia approximately doubles each day for up to about 10 days, and then decreases at a similar rate. Quite severe anaemia may persist for some weeks after the parasites have become virtually undetectable in blood smears. Following recovery from initial infection, most cattle remain latently infected for life.

An expensive procedure, but one that may occasionally be justified to confirm infection, particularly in latently infected cattle, is the inoculation of blood from the suspect animal into a splenectomised calf. A quantity (up to 500 ml) of the donor's blood in anticoagulant is inoculated intravenously into the splenectomised calf, which is then tested by blood smear examination at least every 2–3 days. If the donor is infected, *Anaplasma* will be observed in smears from the splenectomised calf generally within 4 weeks, but this period may extend up to 8 weeks.

Nucleic-acid-based tests to detect *A. marginale* infection in carrier cattle have been developed (11–13, 36). The analytical sensitivity of polymerase chain reaction (PCR)-based methods has been estimated at 0.0001% infected erythrocytes, but at this level only a proportion of carrier cattle would be detected (11, 12). A sensitive and potentially specific nested PCR has been used to identify *A. marginale* carrier cattle (36). This technique is capable of identifying as few as 30 infected erythrocytes per ml of blood, equivalent to a parasitaemia of approximately 0.00001%, well below the lowest levels in carriers. However, nested PCR poses significant quality control problems for routine use (36). Laboratories running this assay should recognise problems in specificity due

1 Commercial stains include Camco-Quik and Diff-Quik, Baxter Scientific Products, McGaw Park, Illinois, USA, and Hema 3 and Hema-Quik, Curtin-Matheson, Houston, Texas, USA.

to nonspecific amplification. An additional step such as restriction enzyme analysis, Southern hybridisation, or sequencing can confirm the specificity of the fragment amplified in nested PCR.

In general, unless animals have been treated or are at a very early stage of infection (<14 days), serology using the competitive enzyme-linked immunosorbent assay (C-ELISA) or card agglutination test (CAT) (see below) is the preferred method of identifying infected animals.

2. Serological tests

Anaplasma infections usually persist for the life of the animal. However, except for occasional small recrudescences, *Anaplasma* cannot readily be detected in blood smears after an acute parasitaemic episode. Thus, a number of serological tests have been developed with the aim of detecting persistently infected animals.

A feature of the serological diagnosis of anaplasmosis is the highly variable results with regard to both sensitivity and specificity reported for many of the tests from different laboratories. This is due at least in part to inadequate evaluation of the tests using significant numbers of known positive and negative animals. Importantly, the capacity of several assays to detect known infections of long-standing duration has rarely been adequately addressed. An exception is C-ELISA (see below), which has been validated using true positive and negative animals defined by nested PCR (36), and the card agglutination assay, for which relative sensitivity and specificity in comparison with the C-ELISA has been evaluated (24). Therefore, while most of the tests described in this section are useful for obtaining broad-based epidemiological data, caution is advised on their use for disease certification. Both the C-ELISA and card agglutination test are described in detail below.

It should be noted that there is a high degree of cross-reactivity between *A. marginale* and *A. centrale* in serological tests. While the infecting species can sometimes be identified using antigens from homologous and heterologous species, equivocal results are obtained on many occasions.

a) Competitive enzyme-linked immunosorbent assay

A C-ELISA using a recombinant antigen termed rMSP5 and MSP5-specific monoclonal antibody (MAb) has proven very sensitive and specific for detection of *Anaplasma*-infected animals (18, 27, 35, 37). All *A. marginale* strains tested, *A. ovis* and *A. centrale*, express the MSP5 antigen and induce antibodies against the immunodominant epitope recognised by the MSP5-specific MAb. A recent report suggests that antibodies from cattle experimentally infected with *A. phagocytophilum* will test positive in the C-ELISA (8). However, in another study no cross-reactivity could be demonstrated, and the MAb used in the assay did not react with *A. phagocytophilum* MSP5 in direct binding assays (35). Thus, additional work is necessary to clarify these conflicting results. The test was 100% specific using 261 known negative sera from a non-endemic region, detecting acutely infected cattle as early as 16 days after experimental tick or blood inoculation, and was demonstrated to detect cattle that have been experimentally infected as long as 6 years previously (18). In detecting persistently infected cattle from an anaplasmosis-endemic region that were defined as true positive or negative using a nested PCR procedure, the rMSP5 C-ELISA had a sensitivity of 96% and a specificity of 95% (36). An independent study using an indirect ELISA (I-ELISA) validates the use of rMSP5 as a diagnostic antigen (32). However, initial studies suggest that in its current format the indirect rMSP5 ELISA is less sensitive than the C-ELISA (32).

Test results using the rMSP5 C-ELISA are available in less than 2.5 hours. A test kit available commercially contains specific instructions. In general, however, it is conducted as follows.

- **Kit reagents**

- A 96-well microtitre plate coated with rMSP5 antigen,

- A 96-well coated adsorption/transfer plate for serum adsorption to reduce background binding,

- 100 × MAb/peroxidase conjugate,

- 10 × wash solution and ready-to-use conjugate-diluting buffer,

- Ready-to-use substrate and stop solutions,

- Positive and negative controls

- **Test procedure**

- i) Add 70 µl of undiluted serum sample to the coated adsorption/transfer plate and incubate at room temperature for 30 minutes.

- ii) Transfer 50 µl per well of the adsorbed serum to the rMSP5-coated plate and incubate at room temperature for 60 minutes.

- iii) Discard the serum and wash the plate twice using diluted wash solution.
- iv) Add 50 µl per well of the 1 × diluted MAb/peroxidase conjugate to the rMSP5-coated plate, and incubate at room temperature for 20 minutes.
- v) Discard the 1 × diluted MAb/peroxidase conjugate and wash the plate four times using diluted wash solution.
- vi) Add 50 µl per well of the substrate solution, cover the plate with foil, and incubate for 20 minutes at room temperature.
- vii) Add 50 µl per well of stop solution to the substrate solution already in the wells and gently tap the sides of the plate to mix the wells.
- viii) Read the plate in the plate reader at 620 nm.

- **Test validation**

The mean optical density (OD) of the negative control must range from 0.40 to 2.10. The per cent inhibition of the positive control must be ≥30%.

- **Interpretation of the results**

The % inhibition is calculated as follows:

$$100 - \frac{\text{Sample OD} \times 100}{\text{Mean negative control OD}} = \text{Per cent inhibition}$$

Samples with <30% inhibition are negative. Samples with ≥30% inhibition are positive.

Specificity of the MSP5 C-ELISA may be increased by using a higher percentage inhibition cut-off value (5); however the effect of this change on sensitivity has not been thoroughly evaluated.

b) Card agglutination test

The advantages of the CAT are that it is sensitive, may be undertaken either in the laboratory or in the field, and gives a result within a few minutes. Nonspecific reactions may be a problem, and subjectivity in interpreting assay reactions can result in variability in test interpretation. In addition, the CAT antigen, which is a suspension of *A. marginale* particles, can be difficult to prepare and can vary from batch to batch and laboratory to laboratory. Splenectomised calves are infected by intravenous inoculation with blood containing *Anaplasma*-infected erythrocytes. When the parasitaemia exceeds 50%, the animal is exsanguinated, the infected erythrocytes are washed, lysed, and the erythrocyte ghosts and *Anaplasma* particles are pelleted. The pellets are sonicated, washed, and then resuspended in a stain solution to produce the antigen suspension.

A test procedure that has been slightly modified from that originally described (1, 2) is as follows:

- i) Ensure all test components are at a temperature of 25–26°C before use (this constant temperature is critical for the test).
- ii) On each circle of the test card (a clear perspex/plastic or glass plate marked with circles that are 18 mm in diameter), place next to each other, but not touching, 10 µl of bovine serum factor (BSF), 10 µl of test serum, and 5 µl of CAT antigen². Negative and low positive control sera must be tested on each card.

BSF is serum from a selected animal with high known conglutinin level. If the conglutinin level is unknown, fresh serum from a healthy animal known to be free from *Anaplasma* can be used. The Jersey breed is often suitable. The BSF must be stored at –70°C in small aliquots, a fresh aliquot being used each time the tests are performed. The inclusion of BSF improves the sensitivity of the test.
- iii) Mix well with a glass stirrer. After mixing each test, wipe the stirrer with clean tissue to prevent cross-contamination.
- iv) Place the test card in a humid chamber and rock at 100–110 rpm for 7 minutes.
- v) Read immediately against a backlight. Characteristic clumping of the antigen (graded from +1 to +3) is considered to be a positive result. The test is considered to give a negative result when there is no characteristic clumping.

² The test as conducted in the USA and Mexico uses larger volumes of reagents: antigen (15 µl), serum (30 µl), and bovine serum factor (30 µl), and a 4-minute reaction time (see step iv).

c) Complement fixation test

The complement fixation (CF) test has been used extensively for many years; however, it shows variable sensitivity (ranging from 20 to 60%), possibly reflecting differences in techniques for antigen production, and poor reproducibility. In addition, it has been demonstrated that the CF assay fails to detect a significant proportion of carrier cattle (5). It is also uncertain as to whether or not the CF test can identify antibodies in acutely infected animals prior to other assays (6, 24). Therefore, the CF test is no longer recommended as a reliable assay for detecting infected animals.

d) Additional ELISAs

Indirect enzyme-linked immunosorbent assay – an I-ELISA based on the use of a normal red blood cell antigen (negative antigen) and an *A. marginale*-infected red blood cell antigen (positive antigen) has been found to be reliable for the detection of *A. marginale*-positive sera (10). Although more cumbersome than tests using only one antigen, this test eliminates those sera that have high levels of nonspecific activity due to iso-antibodies to normal red blood cell components. The test correctly identified all 100 known positive sera taken from cattle up to 3 years after infection, while 3% of negative sera, 2% of *Babesia bovis* and 4% of *B. bigemina* sera gave false-positive results.

Dot enzyme-linked immunosorbent assay – a dot ELISA has also been described. Compared with the I-ELISA, the dot ELISA has the potential advantages of being rapid, inexpensive and simple to perform. The dot ELISA has been reported to have a sensitivity of 93% and a specificity of 96% (25).

e) Indirect fluorescent antibody test

Because of the limitations on the number of indirect fluorescent antibody (IFA) tests that can be performed daily by one operator, other serological tests are generally preferred to the IFA test. The IFA test is performed as described for bovine babesiosis in Chapter 2.4.2, except that *A. marginale*-infected blood is used for the preparation of antigen smears. A serious problem encountered with the test is nonspecific fluorescence. Antigen made from blood collected as soon as adequate parasitaemia (5–10%) occurs is most likely to be suitable. Nonspecific fluorescence due to antibodies adhering to infected erythrocytes can be reduced by washing the erythrocytes in an acidic glycine buffer before antigen smears are prepared (26). Infected erythrocytes are washed twice in 0.1 M glycine buffer (pH 3.0, centrifuged at 1000 g for 15 minutes at 4°C) and then once in PBS, pH 7.4.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Several immunisation methods have been used to protect cattle against anaplasmosis in countries where the disease is endemic, but none is ideal (22). Recently a review of *A. marginale* vaccines and antigens has been published (16) Use of the less pathogenic *A. centrale*, which gives partial cross-protection against *A. marginale*, is the most widely accepted method, although not used in North America. Another method involves the use of a strain of *A. marginale* attenuated by passage in nonbovine hosts, such as deer or sheep (34).

In this section, the production of live *A. centrale* vaccine is described. It involves infection of a susceptible, splenectomised calf and the use of its blood as a vaccine. Detailed accounts of the production procedure are available and reference should be made to these publications for details of the procedures outlined here (3, 7, 29).

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.

Anaplasma centrale vaccine can be provided in either frozen or chilled form depending on demand, transport networks, and the availability of liquid nitrogen or dry ice supplies. Frozen vaccine is recommended in most instances, as it allows for thorough post-production quality control of each batch. It is, however, more costly to produce and more difficult to transport than chilled vaccine. The risk of contamination makes post-production control essential, but may be prohibitively expensive.

1. Seed management**a) Characteristics of the seed**

Anaplasma centrale was isolated in 1911 in South Africa, and has been used as a vaccine in South America, Australia, Africa, the Middle East, and South-East Asia. It affords only partial, but adequate, protection in regions where the challenging strains are of moderate virulence (e.g. Australia) (4). In the humid tropics where *A. marginale* appears to be a very virulent parasite, the protection afforded by *A. centrale* may be inadequate to prevent disease in some animals.

Anaplasma centrale usually causes benign infections, especially if used in calves under 9 months of age. Severe reactions following vaccination have been reported when adult cattle are inoculated.

b) Preparation and storage of stabilate

Infective material is readily stored as frozen stabilates of infected blood in liquid nitrogen or dry ice. Dimethyl sulphoxide (DMSO) and polyvinylpyrrolidone M.W. 40,000 (3) are the recommended cryopreservatives, as they allow for intravenous administration after thawing of the stabilate. A detailed account of the freezing technique using DMSO is reported elsewhere (23), but briefly involves the following: infected blood is collected, chilled to 4°C, and cold cryoprotectant (4 M DMSO in PBS) is added slowly with stirring to a final blood:protectant ratio of 1:1, to give a final concentration of 2 M DMSO. The entire 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.

c) Validation as a vaccine

The suitability of an isolate of *A. centrale* as a vaccine can be determined by inoculating susceptible cattle, monitoring the subsequent reactions, and then challenging the animals and susceptible controls with a virulent local strain of *A. marginale*. Both safety and efficacy can be judged by monitoring parasitaemias in stained blood films and the depression of packed cell volumes of inoculated cattle during the vaccination and challenge reaction periods. Evidence of purity of the isolate can be determined by serological testing of paired sera from the cattle used in the safety test for possible contaminants that may be present (3, 30).

2. Method of manufacture

a) Production of frozen vaccine

Quantities of the frozen stabilate (5–10 ml) are 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) to infect a susceptible, splenectomised calf by intravenous inoculation.

The parasitaemia of the donor calf is monitored daily by examining stained films of jugular blood, and the blood is collected for vaccine production when suitable parasitaemias are reached. A parasitaemia of 1×10^8 /ml (approximately 2% parasitaemia in jugular blood) is the minimum required for production of vaccine. If a suitable parasitaemia is not obtained, passage of the strain by subinoculation of 100–200 ml of blood to a second splenectomised calf may be necessary.

Blood from the donor is collected by aseptic jugular or carotid cannulation using heparin as an 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 at 37°C (final concentration of glycerol 1.5 M). 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 (3).

DMSO can be used as a cryoprotectant in the place of glycerol. This is done in the same way as outlined for the preparation of stabilate (23, 28).

If glycerolised vaccine is to be diluted, the diluent should consist of PBS with 1.5 M glycerol and 5 mM glucose (14). Vaccine cryopreserved with DMSO should be diluted with diluent containing the same concentration of DMSO as in the original cryopreserved blood (31).

b) Production of chilled vaccine

Infective material for chilled vaccine is prepared in the same way as for frozen vaccine, but it must be issued and used as soon as possible after collection. The infective blood can be diluted to provide 1×10^7 parasites per dose of vaccine. A suitable diluent is 10% sterile bovine serum in a glucose/balanced salt solution containing the following quantities per litre: NaCl (7.00 g), $MgCl_2 \cdot 6H_2O$ (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).

If diluent is not available, acid citrate dextrose (20% [v/v]) or citrate phosphate dextrose (20% [v/v]) should be used as anticoagulant to provide the glucose necessary for survival of the organisms.

c) Use of vaccine

In the case of frozen vaccine, vials should be thawed by immersion in water, preheated to 37°C to 40°C, and the contents mixed with suitable diluent to the required dilution. If glycerolised vaccine is prepared, it should

be kept cool and used within 8 hours (3). If DMSO is used as a cryoprotectant, the prepared vaccine should be kept on ice and used within 15–30 minutes (28). The vaccine is most commonly administered subcutaneously.

Chilled vaccine should be kept refrigerated and used within 4–7 days of preparation.

The strain of *A. centrale* used in vaccine is of reduced virulence, but is not entirely safe. A practical recommendation is, therefore, to limit the use of vaccine to calves, where nonspecific immunity will minimise the risk of vaccine reactions. When older animals have to be vaccinated, there is a risk of severe reactions. These reactions occur infrequently, but valuable breeding stock or pregnant animals obviously warrant close attention, and should be observed daily for 3 weeks post-vaccination. Clinically sick animals should be treated with oxytetracycline or imidocarb at dosages recommended by the manufacturers.

Protective immunity develops in 6–8 weeks and usually lasts for several years.

Anaplasmosis and babesiosis vaccines are often used concurrently, but it is not advisable to use any other vaccines at the same time (3).

3. In-process control

a) Source and maintenance of vaccine donors

A source of calves free from natural infections of *Anaplasma* and other tick-borne diseases should be identified. If a suitable source is not available, it may be necessary to breed the calves under tick-free conditions specifically for the purpose of vaccine production.

The 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 weighed against the possible adverse consequences of spreading disease (3).

b) Surgery

Donor calves should be splenectomised to allow maximum yield of organisms for production of vaccine. This is best carried out in young calves and under general anaesthesia.

c) Screening of vaccine donors before inoculation

Donor calves should be examined for all blood-borne infections prevalent in the vaccine-producing country, including *Babesia*, *Anaplasma*, *Cowdria*, *Theileria* and *Trypanosoma*. This can be done by routine examination of stained blood films after splenectomy, and preferably also by serology. Any calves showing evidence of natural infections of any of these agents should be rejected. The absence of other infective agents should also be confirmed. These may include the agents of enzootic bovine leucosis, mucosal disease, infectious bovine rhinotracheitis, ephemeral fever, Akabane disease, bluetongue, foot and mouth disease, and rinderpest. The testing procedures will depend on the diseases prevalent in the country and the availability of tests, but should involve serology of paired sera at the very least and, in some cases, virus isolation, antigen, or DNA/RNA detection (3, 28, 30).

d) Monitoring of parasitaemias following inoculation

It is necessary to determine the concentration of parasites in blood being collected for vaccine. The parasite concentration can be estimated from the erythrocyte count and the parasitaemia (percentage of infected erythrocytes).

e) Collection of blood for vaccine

All equipment should be sterilised before use (e.g. by autoclaving). Once the required parasitaemia is reached, the blood is collected in heparin using strict aseptic techniques. This is best done if the calf is sedated 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 for frozen vaccine depend on 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 (see Chapter 1.1.9 Tests for sterility and freedom from contamination of biological materials).

The absence of contaminants is determined by doing appropriate serological testing of donor cattle, by inoculating donor lymphocytes into sheep and then monitoring them for evidence of viral infection, and by inoculating cattle and monitoring them serologically for infectious agents that could potentially contaminate the vaccine. Cattle inoculated during the test for potency (see Section C.4.c) are suitable for the purpose. Depending on the country of origin of the vaccine, these agents include the causative organisms of enzootic bovine leukosis, infectious bovine rhinotracheitis, mucosal disease, ephemeral fever, Akabane disease, Aino virus, bluetongue, parainfluenza, foot and mouth disease, lumpy skin disease, rabies, Rift Valley fever, rinderpest, contagious bovine pleuropneumonia, Jembrana disease, heartwater, pathogenic *Theileria* and *Trypanosoma spp.*, *Brucella abortus*, *Coxiella*, and *Leptospira* (3, 28, 30).

b) Safety

Vaccine reactions of the cattle inoculated in the test for potency (see Section C.4.c) are monitored by measuring parasitaemia 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

Vaccine is thawed and diluted 1/5 with a suitable diluent (3). The diluted vaccine is then incubated for 8 hours at 4°C, and five cattle are inoculated subcutaneously with 2 ml doses. The inoculated cattle are monitored for the presence of infections by examination of stained blood smears. All should become infected for a batch to be accepted. A batch proving to be infective is recommended for use at a dilution of 1/5 with isotonic diluent.

d) Duration of immunity

Partial but long-lasting immunity results from one inoculation. There is no evidence that repeated vaccination will have a boosting effect.

e) Stability

The vaccine can be kept for 5 years when stored in liquid nitrogen. Once thawed, it rapidly loses its potency. Thawed vaccine cannot be refrozen.

f) Preservatives

No preservatives are added. Penicillin (500,000 IU/litre) and streptomycin (370,000 µg/litre) are added to the vaccine at the time of dispensing.

g) Precautions (hazards)

The vaccine is not infective for humans. When the product is stored in liquid nitrogen, the usual precautions pertaining to the storage, transportation and handling of deep-frozen material applies.

5. Tests on final product

a) Safety

See Section C.4.b.

b) Potency

See Section C.4.c.

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