

## SECTION 3.4.

# BOVINAE

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### CHAPTER 3.4.1.

## BOVINE ANAPLASMOSIS

### SUMMARY

**Definition of the disease:** Bovine anaplasmosis results from infection with *Anaplasma marginale*. A second species, *A. centrale*, has long been recognised and usually causes benign infections. *Anaplasma marginale* is responsible for almost all outbreaks of clinical disease. *Anaplasma phagocytophilum* and *A. bovis*, which infect cattle, are also included within the genus. *Anaplasma phagocytophilum* can cause self-limiting disease in cattle. There are no reports of disease associated with *A. bovis* infection. The organism is classified in the genus *Anaplasma* belonging to the family Anaplasmataceae of the order Rickettsiales.

**Description of the disease:** Anaemia, jaundice in acute, severe cases and unexpected death are characteristic signs of bovine anaplasmosis. Other signs include rapid loss of milk production and weight, but the clinical disease can only be confirmed by identifying the organism. Once infected, cattle may remain carriers for life, and identification of these animals depends on the detection of specific antibodies using serological tests, or of rickettsial DNA using molecular amplification techniques. The disease is typically transmitted by tick vectors, but mechanical transmission by biting insects or by needle can occur.

**Detection of the agent:** Microscopic examination of blood or organ smears stained with Giemsa stain is the most common method of identifying *Anaplasma* in clinically affected animals. In these smears, *A. marginale* organisms appear as dense, rounded, intraerythrocytic bodies approximately 0.3–1.0  $\mu\text{m}$  in diameter situated on or near the margin of the erythrocyte. *Anaplasma centrale* is similar in appearance, but most of the organisms are situated toward the centre of the erythrocyte. It can be difficult to differentiate *A. marginale* from *A. centrale* in a stained smear, particularly with low levels of rickettsaemia. Commercial stains that give very rapid staining of *Anaplasma* spp. are available in some countries. *Anaplasma phagocytophilum* can only be observed in infected granulocytes, mainly neutrophils and *A. bovis* can only be observed in infected monocytes.

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 useful if post-mortem decomposition is advanced.

**Serological tests:** A competitive enzyme-linked immunosorbent assay (C-ELISA) has good sensitivity in detecting carrier animals. Card agglutination is the next most frequently used assay. The complement fixation test (CFT) is no longer considered a reliable test 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 cross-reactivity described between *A. marginale*, *A. centrale*, *A. phagocytophilum* and *Ehrlichia* spp. Alternatively, an indirect ELISA (I-ELISA) is a reliable test used in many laboratories and can be prepared in-house for routine diagnosis of anaplasmosis. Finally, a displacement double-antigen sandwich ELISA has been developed to differentiate between *A. marginale* and *A. centrale* antibodies.

**Nucleic-acid-based tests** are often used in diagnostic laboratories and experimentally, and are capable of detecting the presence of low-level infection in carrier cattle and tick vectors. A nested conventional polymerase chain reaction (PCR) has been used to identify low-level carriers, although nonspecific amplification can occur. Real-time PCR assays have analytical sensitivity equivalent to nested conventional PCR and are preferable in a diagnostic setting to reduce the risk of amplicon contamination.

**Requirements for vaccines:** Live vaccines are used in several countries to protect cattle against bovine anaplasmosis. A vaccine consisting of live *A. centrale* is most widely used and gives partial protection against challenge with virulent *A. marginale*. Vaccination with *A. centrale* leads to infection and long-term persistence in many cattle. Vaccinated cattle are typically protected from disease caused by *A. marginale*, but not infection.

*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. In countries where *A. centrale* is exotic, it cannot be used as a vaccine against *A. marginale*.

## A. INTRODUCTION

Outbreaks of bovine anaplasmosis are due to infection with *Anaplasma marginale*. *Anaplasma centrale* can produce a moderate degree of anaemia, but clinical outbreaks in the field are extremely rare. Other members of the family Anaplasmataceae that infect cattle include *A. phagocytophilum* and *A. bovis* (Dumler *et al.*, 2001). *Anaplasma phagocytophilum* has a broad host range and causes the diseases human granulocytic anaplasmosis (HGE), equine granulocytic anaplasmosis (EGA), and canine granulocytic anaplasmosis (CGA), in humans, horses, and dogs, respectively (Matei *et al.*, 2019). In northern Europe, *A. phagocytophilum* causes tick-borne fever, primarily affecting lambs. In cattle, *A. phagocytophilum* infections have been reported from many geographical regions, however the association with disease is less commonly reported. Naturally occurring clinical disease as reported in Germany was characterised by fever (39.5–41.7° C), sudden reduction in milk production, lower limb oedema, and stiffness with leukopenia, erythropenia, neutropenia, lymphocytopenia and monocytopenia. The affected animals recovered without antibiotic treatment (Silaghi *et al.*, 2018).

The most marked clinical signs of bovine anaplasmosis are anaemia and jaundice, the latter occurring in acute severe, cases or late in the disease. Haemoglobinaemia and haemoglobinuria are not present, and this may assist in the differential diagnosis of bovine anaplasmosis from babesiosis, which is often endemic in the same regions. The disease can only be confirmed, however, by identification of the organism in erythrocytes from the affected animal. Caution must be exercised if using nucleic acid techniques alone to diagnose *A. marginale* in anaemic cattle. Persistent, low-level infection can be detected by these techniques and may lead to a misdiagnosis of bovine anaplasmosis. Visualisation of *A. marginale* bodies in erythrocytes is therefore required for confirmation.

*Anaplasma marginale* occurs in most tropical and subtropical countries and is widely distributed in 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, though originally described as protozoan parasites, are obligate intracellular Gram-negative bacteria. Based on taxonomy established in 2001 (Dumler *et al.*, 2001), the Family Anaplasmataceae (Order Rickettsiales) is composed of five genera, *Anaplasma*, *Ehrlichia*, *Neorickettsia*, *Wolbachia* and *Aegyptianella*. The genus *Anaplasma* contains *Anaplasma marginale* as the type species, *A. centrale*, *A. phagocytophilum* the agent of human granulocytic ehrlichiosis (formerly *Ehrlichia phagocytophila* and *E. equi*), *A. platys*, and *A. bovis* (formerly *E. bovis*).

*Anaplasma* species are transmitted either mechanically or biologically by arthropod vectors. Detection of pathogen DNA within a tick is insufficient to determine the ability of a particular tick species to transmit a pathogen. Studies demonstrating transmission of the pathogen are critical in determining the potential role of a particular tick species

in pathogen transmission. Many studies have demonstrated the transmission ability of *Dermcentor andersoni*, *D. variabilis*, and *D. albipictus*. Additionally, transmission by multiple *Rhipicephalus* species is well recognised including *R. annulatus*, *R. bursa*, *R. calcaratus*, *R. decoloratus*, *R. evertsi*, *R. microplus* and *R. sanguineus*. Other species of *Rhipicephalus* also likely serve as biological vectors of *A. marginale*. *Anaplasma marginale* DNA has been widely reported in *Hyalomma* species, and transmission has been demonstrated with *H. excavatum*. It is likely that multiple *Hyalomma* species also serve as vectors of *A. marginale* (Shkap *et al.*, 2009).

Intrastadial or transstadial transmission can occur, even in the one-host, *Rhipicephalus* species. Male ticks may be particularly important as vectors, as they are most likely to move between cattle searching for female ticks. Experimental demonstration of vector competence does not necessarily imply a role in transmission in the field. However, *Rhipicephalus* species are clearly important vectors of anaplasmosis in Australia, many regions of Africa, and Latin America. *Dermacentor* spp. 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*. 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 (Reinbold *et al.*, 2010a).

The only known biological vector of *A. centrale* is *R. simus*, endemic in Africa. Though multiple transmission studies have been done, there is no evidence that the common cattle tick (*R. microplus*) can serve as a vector for *A. centrale*. This is relevant where *A. centrale* is used as a vaccine in *R. microplus*-infested regions.

*Anaplasma marginale* infection has not been reported in humans. There is minimal risk of field or laboratory transmission to workers from laboratories working with *A. marginale*. Nevertheless, the agent should be handled with appropriate biosafety and containment procedures as determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).

## B. DIAGNOSTIC TECHNIQUES

*Table 1. Test methods available for the diagnosis of bovine anaplasmosis and their purpose*

Method	Purpose					
	Population freedom from infection <sup>(a)</sup>	Individual animal freedom from infection prior to movement <sup>(b)</sup>	Contribute to eradication policies <sup>(c)</sup>	Confirmation of clinical cases <sup>(d)</sup>	Prevalence of infection – surveillance <sup>(e)</sup>	Immune status in individual animals or populations (post-vaccination) <sup>(f)</sup>
Microscopic examination	–	–	–	+++	–	–
Detection of the agent <sup>(g)</sup>						
PCR	–	++	–	+++	–	–
Detection of immune response						
CAT <sup>(h)</sup>	–	–	–	–	+	+
C-ELISA <sup>(h)</sup>	+++	+++	+++	–	+++	+++
IFAT <sup>(h)</sup>	+	–	–	–	++	++
ddasELISA	–	–	–	–	–	++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;  
+ = suitable in very limited circumstances; – = not appropriate for this purpose.

PCR = polymerase chain reaction; CAT = card agglutination test; C-ELISA = competitive enzyme-linked immunosorbent assay;

IFAT = indirect fluorescent antibody test; ddasELISA = displacement double-antigen, sandwich ELISA.

<sup>(a)</sup>See Appendix 1 of this chapter for justification table for the scores given to the tests for this purpose.

<sup>(b)</sup>See Appendix 2 of this chapter for justification table for the scores given to the tests for this purpose.

<sup>(c)</sup>See Appendix 3 of this chapter for justification table for the scores given to the tests for this purpose.

<sup>(d)</sup>See Appendix 4 of this chapter for justification table for the scores given to the tests for this purpose.

<sup>(e)</sup>See Appendix 5 of this chapter for justification table for the scores given to the tests for this purpose.

<sup>(f)</sup>See Appendix 6 of this chapter for justification table for the scores given to the tests for this purpose.

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

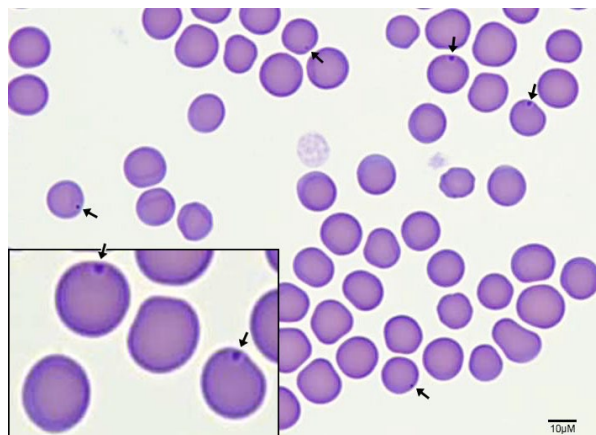
<sup>(h)</sup>These tests do not distinguish infected from vaccinated animals.

## 1. Detection of the agent

### 1.1. Microscopic examination

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

In contrast to *Babesia bovis*, *A. marginale*-infected erythrocytes do not accumulate in capillaries, so blood drawn from the jugular or other large vessel is satisfactory. *Anaplasma marginale* replicate in the erythrocytes to form small membrane-bound colonies termed inclusion bodies. These inclusion bodies can be visualised on a blood smear, but are small and easily confused with debris or stain precipitate (see Figure 1). Thus it is essential that smears are well prepared, including ensuring slides are free of debris and stain is recently filtered (Watman #1 filter paper). Thick blood films as are used sometimes for the diagnosis of babesiosis are not appropriate for the diagnosis of anaplasmosis, as *A. marginale* are difficult to identify once they become dissociated from erythrocytes.



**Fig. 1. *Anaplasma marginale* inclusion bodies.** A stained blood smear from a bovine experimentally infected with *A. marginale*. Arrows point to the *A. marginale* inclusion bodies.  
Photo from S. Noh.

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 *A. marginale* equivocal. Brain smears, which are useful for the diagnosis of some forms of babesiosis, are of no direct value for diagnosing anaplasmosis, but should be included for differential diagnosis where appropriate.

Blood from organs, rather than organ tissues *per se*, is required for smear preparation, as the aim is to microscopically examine intact erythrocytes for the presence of *A. marginale* colonies. Organ-derived blood smears can be stored 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 excess stain and are then air-dried. Conditions for Giemsa staining vary from laboratory to laboratory, but distilled water is not recommended for dilution of Giemsa stock. Water should be pH 7.2–7.4 to attain best resolution with Giemsa stain. Commercial stains that give very rapid staining of *A. marginale* are available in some countries. Smears must be examined under oil immersion at a magnification of  $\times 700$ – $1000$ .

*Anaplasma marginale* inclusion bodies are round deeply stained and approximately 0.3–1.0  $\mu\text{m}$  in diameter. Most of these bodies are located on or near the margin of the erythrocyte. This feature distinguishes *A. marginale* from *A. centrale*, as in the latter most of the organisms have a more central location in the erythrocyte. However, particularly at low levels of rickettsaemia, differentiation of these two species in smears can be difficult. Appendages associated with the *Anaplasma* inclusion body have been described in some isolates of *A. marginale*.

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

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

## 1.2. Polymerase chain reaction

Nucleic acid-based tests to detect *A. marginale* in infected cattle have been developed although not fully validated. 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. A nested PCR has been used to identify *A. marginale* carrier cattle with a capability of identifying as few as 30 infected erythrocytes per ml of blood, well below the lowest levels in carriers. However, nested PCR is time consuming as it requires two full PCR reactions, and poses significant quality control problems for routine use (Torioni De Echaide *et al.*, 1998). Real-time PCR assays are reported to achieve a level of analytical sensitivity equivalent to nested PCR and should be considered instead of the nested PCR (Carelli *et al.*, 2007; Decaro *et al.*, 2008). Advantages of the real-time PCR, which uses a single closed tube for amplification and analysis, are reduced risk of amplicon contamination and a semi-quantitative assay result. Equipment and reagents needed for real-time PCR are expensive and may be beyond the capabilities of some laboratories.

The most widely cited assays for the detection *A. marginale* in individual animals use a probe for increased specificity and are designed to detect *msp1b* (Carelli *et al.*, 2007) or *msp5* (Futse *et al.*, 2003) in genomic DNA extracted from whole blood. The assay based on detection of *msp1b* has been partially validated to detect the pathogen in individual animals and was used to define samples for the validation of a C-ELISA (Carelli *et al.*, 2007; Chung *et al.*, 2014). The analytical test performance of this assay is robust, and exclusivity testing confirmed other bacterial and protozoal tick-borne pathogens of cattle were not detected. The assay, evaluated using 51 blood samples from 18 cattle herds in three regions of southern Italy, had 100% concordance with nested PCR.

*Msp1b* is a multigene family. Based on the annotation of the St. Maries strain of *A. marginale*, the designed primers and probe will amplify multiple members of this gene family, including *msp1b-1*, *msp1b-2*, and *msp1-pg3*). This may help increase diagnostic sensitivity, but may pose challenges if quantification of the pathogen is desired. Additionally, some *A. marginale* strains have single nucleotide polymorphisms in *msp1b* within the primer and probe binding regions. Thus, if *msp1b* is used as a diagnostic target, primer and probe design should consider local *A. marginale* strains. *Msp1b* has the advantage as a target in that orthologs of this gene family are absent in the related *A. phagocytophilum* and *Ehrlichia* spp., including *E. ruminantium*, thus helping ensure specificity of the test.

*Msp5* has also been used as a target to detect *A. marginale* in cattle in field samples and more frequently in experimental samples (Futse *et al.*, 2003). *Msp5* is highly conserved among *A. marginale* strains and

is a single copy gene, thus providing some advantages as a target for ensuring detection of widely variant strains of *A. marginale*. However, the related *Anaplasma* spp. and *Ehrlichia* spp. all have *msp5* orthologs with 50% identity to an *E. ruminantium* gene (NCBI accession: L07385.1), thus specificity must be determined in laboratory and field samples. Additionally, little work has been done to validate an *msp5*-based real-time PCR test for diagnostic purposes.

A third primer–probe set is designed to detect *A. marginale* using real-time, reverse transcriptase PCR. The primers amplify a 16sRNA gene segment from *A. marginale* and *A. phagocytophilum*, while the probe differentiates between the two species (Reinbold *et al.*, 2010b). The analytical performance of this assay is robust. However, the diagnostic sensitivity, specificity, and of particular importance with 16sRNA sequence-based tests, exclusivity for other tick-borne pathogens of cattle have not been evaluated. Additionally, this assay is designed for use following RNA extraction and reverse transcription, which is more laborious and expensive than DNA extraction. Bacterial RNA is rapidly degraded, and this may ultimately reduce diagnostic sensitivity of this assay.

In regions that use *A. centrale* as a vaccine, it may be useful to differentiate between *A. marginale* and *A. centrale* infected/vaccinated animals. PCR is best suited for this task. The real-time PCR assay developed by Carelli *et al.* can also be used in a duplex reaction to detect and differentiate between *A. centrale* and *A. marginale* (Decaro *et al.*, 2008). Primers and probe have been designed to specifically amplify a region of *A. centrale groEL*, but not *A. marginale groEL*, despite 97% sequence identity between the two genes. The *A. marginale*-specific primers and probes perform similarly in the single and duplex PCR (Carelli *et al.*, 2007). Using the same 51 field samples from cattle in Italy, the *A. centrale* assay had less analytical sensitivity compared with nested PCR and discordance in 4 of 51 samples between an *A. centrale* reverse line blot test and the duplex PCR assay.

**Table 2. Oligonucleotides used in PCR assays to detect *A. marginale* and *A. centrale***

Assay	Reference	Oligonucleotides <sup>(a)</sup>	Sequence 5'–3' <sup>(b)</sup>	Amplicon size (bp)	NCBI accession number
Real-time PCR	Carelli <i>et al.</i> , 2007	<i>Am_msp1b_F</i>	TTG-GCA-AGG-CAG-CAG-CTT	95	M59845
		<i>Am_msp1b_R</i>	TTC-CGC-GAG-CAT-GTG-CAT		
		<i>Am_msp1b_PB</i>	TCG-GTC-TAA-CAT-CTC-CAG-GCT-TTC-AT		
Real-time PCR	Futse <i>et al.</i> , 2003	<i>Am_msp5_F</i>	GCC-AAG-TGA-TGG-TGA-TAT-CGA	151	M93392
		<i>Am_msp5_R</i>	AGA-ATT-AAG-CAT-GTG-ACC-GCT-G		
		<i>Am_msp5_PB</i>	AAC-GTT-CAT-GTA-CCT-CAT-CAA		
Reverse-transcription real-time PCR	Reinbold <i>et al.</i> , 2010	<i>16S rRNA_F</i> <sup>(c)</sup>	CTC-AGA-ACG-AAC-GCT-GG	142	M60313
		<i>16S rRNA_R</i> <sup>(c)</sup>	CAT-TTC-TAG-TGG-CTA-TCC-C		
		<i>Am_16S rRNA_PB</i> <sup>(d)</sup>	CGC-AGC-TTG-CTG-CGT-GTA-TGG-T		
Real-time PCR <sup>(d)</sup>	Decaro <i>et al.</i> , 2008	<i>Ac_groEL_F</i> <sup>(e, f)</sup>	CTA-TAC-ACG-CTT-GCA-TCT-C	77	CP001759.1
		<i>Ac_groEL_R</i> <sup>(e, f)</sup>	CGC-TTT-ATG-ATG-TTG-ATG-C		
		<i>Ac_groEL_PB</i> <sup>(e, f)</sup>	TCA-TCA-TTC-TTC-CCC-TTT-ACC-TCG-T		

<sup>(a)</sup>*Am* denotes *A. marginale*, *Ac* denotes *A. centrale*, *Pb* denotes probe sequence.

<sup>(b)</sup>Fluorophores and quenchers not included in probe sequences.

<sup>(c)</sup>Amplifies *A. phagocytophilum* and *A. marginale* 16S rRNA gene.

<sup>(d)</sup>Probe is specific for *A. marginale* 16S rRNA gene.

<sup>(e)</sup>Can be used as a duplex PCR with *msp1b* primers and probe based on Carelli *et al.*, 2007.

<sup>(f)</sup>Primers and probe amplify *A. centrale groEL*.

## 2. Serological tests

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), indirect ELISA (I-ELISA) or card agglutination test (CAT) (see below) may be the preferred methods of identifying infected animals in most laboratories. *Anaplasma*

*marginale* infections usually persist for the life of the animal. However, except for occasional small recrudescences, *A. marginale* inclusion bodies cannot readily be detected in blood smears after acute rickettsaemia and, end-point PCR may not detect the presence of the pathogen in blood samples from asymptomatic carriers. 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 validation of the tests using significant numbers of known positive and negative animals. An exception is a C-ELISA (see below), which was initially validated using true positive and negative animals defined by nested PCR (Torioni De Echaide *et al.*, 1998) and updated in 2014 (Chung *et al.*, 2014). 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. The C-ELISA, I-ELISA and CAT are described in detail below.

It should be noted that there is a high degree of cross-reactivity between *A. marginale* and *A. centrale*, as well as cross-reactivity with both *A. phagocytophilum* and *Ehrlichia* spp. in serological tests (Al-Adhami *et al.*, 2011; Dreher *et al.*, 2005). While the infecting species can sometimes be identified using antigens from homologous and heterologous species, equivocal results are obtained on many occasions. Efforts have been made to develop tests that differentiate between naturally acquired immunity to *A. marginale* and vaccine acquired immunity due to immunisation with *A. centrale* (Bellezze *et al.*, 2023; Sarli *et al.*, 2020).

## 2.1. Competitive enzyme-linked immunosorbent assay

Major surface protein 5 (MSP5) is an immunodominant protein expressed by *A. marginale*, *A. ovis*, and *A. centrale*. In *A. marginale* the gene is highly conserved making it a useful target across broad geographical regions with high *A. marginale* strain diversity (Knowles *et al.*, 1996; Torioni De Echaide *et al.*, 1998). Thus, a C-ELISA based on recombinantly expressed (rMSP5) in combination with an MSP5-specific monoclonal antibody (mAb) has proven very sensitive and specific for detection of *Anaplasma*-infected animals (Molloy *et al.*, 1999; Reinbold *et al.*, 2010b; Strik *et al.*, 2007). Additionally, *A. ovis* and *A. centrale*, express MSP5 and infected animals produce antibodies against the immunodominant epitope recognised by the MSP5-specific mAb used in the C-ELISA. This C-ELISA was updated in 2014 to improve performance by using glutathione S-transferase (GST) instead of maltose binding protein (MBP) as the tag on the rMSP5 (Chung *et al.*, 2014). This assay no longer requires adsorption to remove the antibodies directed against MBP, thus it is faster and easier than the previous version of the C-ELISA. The diagnostic sensitivity is 100% and the diagnostic specificity is 99.7% using a cut-off of 30% inhibition as determined by receiver operating characteristic (ROC) plot (Chung *et al.*, 2014). For this validation, 385 sera defined as negative were from dairy cattle maintained in tick-free facilities from farms with no clinical history of bovine anaplasmosis. The 135 positive sera were from cattle positive for *A. marginale* using nested PCR and serology.

One study suggested that antibodies from cattle experimentally infected with *A. phagocytophilum* will test positive in the C-ELISA (Dreher *et al.*, 2005). 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 (Strik *et al.*, 2007). Cross reactivity has been demonstrated between *A. marginale* and *Ehrlichia* sp. BOV2010 isolated in Canada, in naturally and experimentally infected cattle (Al-Adhami *et al.*, 2011).

Test results using the rMSP5 C-ELISA are available in less than 2 hours. A test kit is available commercially that contains specific instructions. Users should follow the manufacturer's instructions.

## 2.2. Indirect enzyme-linked immunosorbent assay

An I-ELISA was first developed using the CAT antigen, which is a crude *A. marginale* lysate (see below). The test can be implemented where the commercial C-ELISA is not available. Unlike the C-ELISA, most reagents, such as buffers and ready-to dissolve substrates, are available commercially in many countries. Any laboratory can prepare the antigen using local strains of *A. marginale*, though standardised methods have not been developed. I-ELISA uses small amounts of serum and antigen that can be prepared in each laboratory. The sensitivity and specificity of the test was 87.3% and 98.4–99.6% respectively, though this varied by laboratory (Nielsen *et al.*, 1996). For general methods, refer to Barry

*et al.* (1986). For each laboratory, the specific amount of antigen must be optimised to obtain the best reading and the least expenditure.

Alternatively, rMSP5 can be used as the antigen in this test. This eliminates the need for preparation and standardisation of antigen derived from splenectomised, *A. marginale* infected animals (Silva *et al.*, 2006). In a comparison between I-ELISA using the CAT antigen and rMSP with a histidine tag (rMSP5-HIS), these two I-ELISAs performed identically. In this comparison, IFAT was used as the gold standard test (Silva *et al.*, 2006).

Test results using the I-ELISA are available in about 4 to 5 hours. It is generally conducted as follows:

### 2.2.1. Test reagents

A 96-well microtitre plate coated with *A. marginale* antigen,

PBS/Tween buffer, (PBS 0.1 M, pH 7.2, Tween 20 0.05%),

Blocking reagent (e.g. commercial dried skim milk)

Tris buffer 0.1 M, MgCl<sub>2</sub>, 0.1 M, NaCl, 0.05 M, pH 9.8

Substrate *p*-Nitrophenyl phosphate disodium hexahydrate

Positive and negative controls.

### 2.2.2. Test procedure (this test is run in triplicate)

- i) Plates can be prepared ahead of time and kept under airtight conditions at –20°C.
- ii) Carefully remove the plastic packaging before using plates, being careful not to touch the bottom of them as this can distort the optical density reading.
- iii) Remove the lid and deposit 200 µl PBST20 solution in each well and incubate at room temperature (RT) for 5 minutes.
- iv) For one plate, dissolve 1.1 g of skim milk (blocking agent) in 22 ml of PBST20.
- v) Remove the plate contents and deposit in each well 200 µl of blocking solution, put the lid on and incubate at 37°C for 60 minutes.
- vi) Wash the plate three times for 5 minutes with PBST20.
- vii) Dilute all serum samples including controls 1/100 in PBST20 solution.
- viii) Remove the contents of the plate and deposit 200 µl of diluted serum in each of the three wells for each dilution, starting with the positive and negative and blank controls.
- ix) Incubate plate at 37°C covered for 60 minutes.
- x) Wash three times as described in point vi.
- xi) Dilute 1/1000 anti-IgG bovine alkaline phosphatase conjugate in PBST20 solution. Add 200 µl of the diluted conjugate per well. Incubate the covered plate at 37°C for 60 minutes.
- xii) Remove the lid and wash three times as described in point vi above.
- xiii) Remove the contents of the plate and deposit 195 µl of 0.075% *p*-Nitrophenyl phosphate disodium hexahydrate in Tris buffer in each well and incubate at 37°C for 60 minutes.
- xiv) The reaction is quantified by a microplate reader spectrophotometer, adjusted to 405 nm wavelength. The data are expressed in optical density (OD).

### 2.2.3. Data analysis

Analysis of results should take into account the following parameters.

- i) The mean value of the blank wells.
- ii) The mean value of the positive wells with their respective standard deviations.
- iii) The mean value of negative wells with their respective standard deviations.



- iv) The mean value of the blank wells is subtracted from the mean of all the other samples if not automatically subtracted by the ELISA reader.
- v) Control sera are titrated to give optical density values ranging from 0.90 to 1.50 for the positive and, 0.15 to 0.30 for the negative control.

Positive values are those above the cut-off calculated value which is the sum of the average of the negative and two times the standard deviation.

As with all diagnostic tests, it is important to measure repeatability. For more details see Chapter 2.2.4 *Measurement uncertainty*.

### 2.3. Displacement double-antigen sandwich ELISA to differentiate between *A. marginale* and *A. centrale* antibodies

In regions where vaccination with *A. centrale* is used to control bovine anaplasmosis, differentiation between *A. centrale*-vaccinated and *A. marginale*-infected animals may be useful. Because there is often high amino acid identity between *A. marginale* and *A. centrale* surface proteins, identifying unique targets for serological assays for this purpose is difficult. Epitopes from MSP5 (aa28-210, without the transmembrane region) that are not shared between *A. marginale* and *A. centrale* were used to develop a displacement double-antigen sandwich ELISA (ddasELISA) (Bellezze et al., 2023; Sarli et al., 2020). The recombinant MSP5 epitopes from *A. marginale* or *A. centrale* are expressed in *E. coli* with a histidine tag and purified. The ELISA plates are then coated with either the recombinant *A. marginale* MSP5 epitope, or the *A. centrale* MSP5 epitope and blocked. Serum is added to the wells and allowed to incubate. Following washing, a combination of biotinylated and non-biotinylated recombinant proteins are added to improve specificity of the reaction (see below for specifics). The protein-biotin binding to the serum antibody is detected with a peroxidase-streptavidin based detection system. The optical density for the *A. marginale* MSP5-coated well (ODAm) and the OD for the *A. centrale* MSP5 (ODAc) coated well for each animal is measured. If the OD for either target is <0.2, the sample is excluded from the analysis. For the remaining samples, the ratio between the OD values (ODAm/ODAc) is calculated. If the ratio is >0.38 the sample is considered positive for anti-*A. marginale* antibodies, and a ratio ≤ 0.38 is classified as vaccinated with *A. centrale*.

For the detection of *A. marginale* the test has a diagnostic specificity of 98% and a diagnostic sensitivity of 98.9%. For 702 field samples evaluated, 131 (19%) had an OD <0.2 in the ddasELISA and thus were excluded from the analysis. Of those animals, 52% were nested PCR positive for *A. marginale*, 23% were nested PCR positive for *A. centrale*, 4.6% were nested PCR positive for *A. marginale* and *A. centrale*, 20% were nested PCR negative for both, suggesting the ddasELISA may lack sensitivity.

Of the 571 ddasELISA positive field samples, the agreement between the ddasELISA and nested PCR was 84% and the kappa coefficient was 0.70 (95% CI: 0.635–0.754), indicating substantial agreement between tests. There was agreement between the ddasELISA and nested PCR for 93% of the *A. marginale* ddasELISA positive samples and 86% of the *A. centrale* ddasELISA positive samples. Additionally, 36 nested PCR negative samples tested positive for antibodies against *A. marginale* (n=28) or *A. centrale* (n=8) by ddasELISA. This test could not identify animals with co-infections, meaning animals vaccinated with *A. centrale* that are then infected with *A. marginale*, which is not uncommon.

Test results using the ddasELISA are available in 5–6 hours. It is conducted as outlined below, see Bellezze et al., 2023 for more details.

#### 2.3.1. Test reagents

- i) A 96-well microtitre plate coated with either *A. marginale* or *A. centrale* recombinant protein
- ii) PBS/Tween buffer (PBS (50mM sodium phosphate, 150 mM NaCl, pH 7.2) with 0.05% Tween-20)
- iii) Blocking reagent (PBS with 10% commercial dried skim milk)
- iv) Purified recombinant *A. marginale* MSP5 epitopes and *A. centrale* epitopes
- v) Biotinylated recombinant *A. marginale* MSP5 epitopes and *A. centrale* epitopes
- vi) Streptavidin-horse radish peroxidase (HRP) detection system

- vii) Chromogenic substrate (1 mM 2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt in 0.05 M sodium citrate, pH 4.5, 0.0025% V/V H<sub>2</sub>O<sub>2</sub> (100 µl/well).
- viii) ELISA plate reader (405 nm reading)
- ix) Positive and negative control sera for *A. marginale* and *A. centrale*

### 2.3.2. Test procedure

- i) Plates are coated overnight.
- ii) Block with blocking buffer for 1 hour at room temperature and wash three times with PBS/Tween buffer.
- iii) Add undiluted serum 100 µl/well and incubate at 25°C for 1 hour at 100 rpm.
- iv) Wash three times with PBS/Tween buffer.
- v) Add 100 µl of *A. marginale* MSP5-biotin (1 µg/ml) plus *A. centrale* MSP5 (10 µg/ml) to *A. marginale* test wells. Add *A. centrale* MSP5-biotin (1 µg/ml) plus *A. marginale* MSP5 (10 µg/ml) in PBS/Tween buffer + 10% fat-free dried milk to *A. centrale* test wells.
- vi) Incubate at 25°C for 1 hour at 100 rpm and wash the plate five times with PBS/Tween buffer.
- vii) To detect the bound protein-biotin complex, add streptavidin-HRP diluted in 1/500 in PBS/Tween buffer with 10% dried milk for 1 hour at 25°C, 100 rpm.
- viii) Wash five times with PBS/Tween buffer.
- ix) Add chromogenic substrate based on manufacturer's instructions.
- x) The reaction is measured by microplate reader spectrophotometer at 405 nm wavelength. The data are expressed in optical density (OD).
- xi) OD<sub>405nm</sub> < 0.2 is considered negative.
- xii) Results are expressed as the ratio between antibodies specific for *A. marginale* MSP5 and for *A. centrale* MSP5 (OD<sub>Am</sub>/OD<sub>Ac</sub>). If the ratio is >0.38 the sample is considered positive for anti-*A. marginale* antibodies, and a ratio ≤ 0.38 is classified as vaccinated with *A. centrale*.

## 2.4. Card agglutination test

The sensitivity of the CAT is from 84% to 98% (Gonzalez *et al.*, 1978; Molloy *et al.*, 1999) and the specificity is 98.6% (Molloy *et al.*, 1999). Though sometimes giving variable results, the CAT can be useful under certain circumstances, as it may be undertaken either in the laboratory or in the field, and it 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 lysate of *A. marginale* isolated from erythrocytes, can be difficult to prepare and can vary from batch to batch and laboratory to laboratory. To obtain the antigen, splenectomised calves are infected by intravenous inoculation with blood containing *A. marginale*-infected erythrocytes. When the rickettsaemia exceeds 50%, the animal is exsanguinated, the infected erythrocytes are washed, lysed, and the erythrocyte ghosts and *A. marginale* 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 (Amerault & Roby, 1968; Amerault *et al.*, 1972) is as follows, and is based on controlled conditions in a laboratory setting:

### 2.4.1. Test procedure

- 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 antigen<sup>1</sup>. 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 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.

A latex card agglutination test, a relatively simple and rapid test platform, has been partially validated. This test uses rMSP5-HIS rather than *A. marginale* lysate and does not require BSF. The performance of this test was compared with that of the I-ELISA using rMSP5-HIS as the antigen. The relative sensitivity was 95.2% and relative specificity was 91.86% (Ramos *et al.*, 2014).

## 2.5. 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 3.4.2, except that *A. marginale* infected blood is used for the preparation of antigen smears. A serious problem encountered with the test is nonspecific fluorescence. The reported sensitivity is 97.6% and specificity 89.6% (Gonzalez *et al.*, 1978). Antigen made from blood collected as soon as adequate rickettsaemia (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. 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. Recently published data show that the IFA, like the C-ELISA, can cross react with other members of the *Anaplasmataceae* family, and specifically an *Ehrlichia* spp. identified as BOV2010 (Al-Adhami *et al.*, 2011).

## 2.6. Complement fixation test

The complement fixation test (CFT) was used extensively for many years; however, it has variable sensitivity (ranging from 20 to 60%), possibly reflecting differences in techniques for antigen production, and poor reproducibility. In addition, the CF assay fails to detect a significant proportion of carrier cattle (Bradway *et al.*, 2001). It is also uncertain as to whether or not the CF test can identify antibodies in acutely infected animals prior to other assays (Coetzee *et al.*, 2007; Molloy *et al.*, 1999). Therefore, the CF test is no longer recommended as a reliable assay for detecting infected animals.

# C. REQUIREMENTS FOR VACCINES

## 1. Background

Several immunisation methods have been used to protect cattle against anaplasmosis in countries where the disease is endemic, but none is ideal to date. A review of *A. marginale* vaccines and antigens has been published (Kocan *et al.*, 2010; Noh *et al.*, 2012). 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 many countries, including north America.

<sup>1</sup> The test as conducted in the USA and Mexico uses larger volumes of reagents: antigen (15 µl), serum (30 µl), and bovine serum factor (30 µl), and a 4-minute reaction time (see step iv).

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 (Bock *et al.*, 2004; de Vos & Jorgensen, 1992; Pipano, 1995).

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 either frozen or chilled 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.

## 2. Outline of production and minimum requirements for conventional vaccines

### 2.1. Characteristics of the seed

#### 2.1.1. Biological characteristics

*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 circulating strains are of moderate virulence (e.g. Australia) (Bock & de Vos, 2001). In the humid tropics where *A. marginale* may be more virulent, 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. 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 rickettsaemias in stained blood films and the depression of packed cell volumes of inoculated cattle during the vaccination and challenge reaction periods.

Infective material for preparing the vaccine is readily stored as frozen stabilates of infected blood in liquid nitrogen or dry ice. Dimethyl sulphoxide (DMSO) or polyvinylpyrrolidone M.W. 40,000 (Bock *et al.*, 2004) 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 (Mellors *et al.*, 1982), 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.

#### 2.1.2. Quality criteria

Evidence of purity of the *A. centrale* isolate can be determined by serological testing of paired sera from the cattle used in the safety test for possible pathogens that may be present (Bock *et al.*, 2004; Pipano, 1997). Donor calves used to expand the seed for vaccine production should be examined for all blood-borne infections prevalent in the vaccine-producing country, including *Babesia*, *Anaplasma*, *Ehrlichia*, *Theileria* and *Trypanosoma*. This can be done by routine examination of stained blood films after splenectomy, PCR, 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 leukosis, mucosal disease, infectious bovine rhinotracheitis, ephemeral fever, Akabane disease, bluetongue, and foot and mouth disease. 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 (Bock *et al.*, 2004; Pipano, 1981; 1997).

## 2.2. Method of manufacture

### 2.2.1. Procedure

#### i) 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 rickettsaemia of this donor calf is monitored daily by examining stained films of jugular blood, and the blood is collected for vaccine production when suitable rickettsaemias are reached. A rickettsaemia of  $1 \times 10^8$ /ml (approximately 2% rickettsaemia in jugular blood) is the minimum required for production of vaccine as this is the dose to vaccinate a bovine. If a suitable rickettsaemia 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). The use of blood collection units for human use are also suitable and guarantee sterility and obviate the need to prepare glass flasks that make the procedure more cumbersome.

In the laboratory, the infective 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 (Bock *et al.*, 2004).

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 seed stabilate (Mellors *et al.*, 1982; Pipano, 1981).

If glycerolised vaccine is to be diluted, the diluent should consist of PBS with 1.5 M glycerol and 5 mM glucose (Jorgensen *et al.*, 1989). Vaccine cryopreserved with DMSO should be diluted with diluent containing the same concentration of DMSO as in the original cryopreserved blood (Pipano *et al.*, 1986).

#### ii) 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  $1 \times 10^7$  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<sub>2</sub>.6H<sub>2</sub>O (0.34 g), glucose (1.00 g), Na<sub>2</sub>HPO<sub>4</sub> (2.52 g), KH<sub>2</sub>PO<sub>4</sub> (0.90 g), and NaHCO<sub>3</sub> (0.52 g).

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.

#### iii) 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 (Bock *et al.*, 2004). If DMSO is used as a cryoprotectant, the prepared vaccine should be kept on ice and used within 15–30 minutes (Pipano, 1981). The vaccine is most commonly administered subcutaneously.

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

The strain of *A. centrale* used in the 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 (Bock *et al.*, 2004).

### 2.2.2. Requirements for substrates and media

*Anaplasma centrale* can be cultured in *Rhipicephalus appendiculatus* and *Dermacentor variabilis* cells lines, though antigen expression and immunogenicity of the cultured *A. centrale* need to be tested (Bell-Sakyi *et al.*, 2015). No substrates or media other than buffers and diluents are used in vaccine production. DMSO or glycerol should be purchased from reputable companies.

### 2.2.3. In-process controls

#### i) Source and maintenance of vaccine donors

A source of calves free from natural infections of *A. marginale* 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 (Bock *et al.*, 2004).

#### ii) 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.

#### iii) Screening of vaccine donors before inoculation

As for preparation of seed stabilate, donor calves for vaccine production should be examined for all blood-borne infections prevalent in the vaccine-producing country, including *Babesia*, *Anaplasma*, *Ehrlichia*, *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 leukosis, bovine viral diarrhoea, infectious bovine rhinotracheitis, ephemeral fever, Akabane disease, bluetongue, and foot and mouth disease. 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 (Bock *et al.*, 2004; Pipano, 1981; 1997).

#### iv) Monitoring of rickettsaemias following inoculation

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

#### v) Collection of blood for vaccine

All equipment should be sterilised before use (e.g. by autoclaving). Once the required rickettsaemia 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.

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

## 2.2.4. Final product batch tests

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.

i) Sterility and purity

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 intended for veterinary use*).

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.2.2.4.iii) 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, bovine viral diarrhoea, ephemeral fever, Akabane disease, Aino virus, bluetongue, parainfluenza, foot and mouth disease, lumpy skin disease, rabies, Rift Valley fever, contagious bovine pleuropneumonia, Jembrana disease, heartwater, pathogenic *Theileria* and *Trypanosoma spp.*, *Brucella abortus*, *Coxiella*, and *Leptospira* (Bock et al., 2004; Pipano, 1981; 1997). Other pathogens to consider include the causal agents of bovine tuberculosis and brucellosis as they may spread through contaminated blood used for vaccine production. Most of these agents can be tested by means of specific PCR and there are many publications describing primers, and assay conditions for any particular disease.

ii) Safety

Vaccine reactions of the cattle inoculated in the test for potency (see Chapter 1.1.8 *Principles of veterinary vaccine production*) are monitored by measuring rickettsaemia and depression of packed cell volume. Only batches with pathogenicity levels equal to or lower than a predetermined standard are released for use.

iii) Potency

Vaccine is thawed and diluted 1/5 with a suitable diluent (Bock et al., 2004). 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.

## 2.3. Requirements for authorisation

### 2.3.1. Safety

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.

*Anaplasma centrale* is not infective to other species, and the vaccine is not considered to have other adverse environmental effects. 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.

### 2.3.2. Efficacy requirements

Immunisation with live *A. centrale* results in long-term infection of the vaccinee, thus repeated vaccination is unnecessary. Infection with *A. centrale* does not prevent subsequent infection with *A. marginale*, but does at least result in protection from disease (Shkap *et al.*, 2009). The vaccine is used for control of clinical anaplasmosis in endemic areas. It will not provide sterile immunity, and should not be used for eradication of *A. marginale*.

### 2.3.3. 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.

## 3. Vaccines based on biotechnology

There are no vaccines based on biotechnology available for anaplasmosis.

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**NB:** There is a WOA Reference Laboratory for anaplasmosis (please consult the WOA Web site: <https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3http://www.oie.int/>)

Please contact the WOA Reference Laboratory for any further information on diagnostic tests, reagents and vaccines for bovine anaplasmosis

**NB:** FIRST ADOPTED IN 1991. MOST RECENT UPDATES ADOPTED IN 2024.

**Appendix 1: Bovine anaplasmosis**  
**Intended purpose of test: population freedom from infection**

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
C-ELISA +++ Bovine	Serum rMSP5-GST	Reference tests were nested PCR and IFAT. Dsp = 99.7% Dse = 100% 30% inhibition as determined by ROC analysis	1. 358 known non-infected cattle from dairy herds maintained in tick free barns and no clinical history of clinical anaplasmosis 2. 135 known positive sera as defined by nested PCR. 3. Intra-test comparison with 163 diagnostic samples with possible false positives based on rMSP5-GST C-ELISA. Test positive confirmation done with IFAT	See reference	1. Updated version with improved specificity 2. High sensitivity, detects persistently infected animals 3. Commercially available 4. Uses a standardised antigen 5. Target antigen is highly conserved among <i>A. marginale</i> strains, thus detects infection with all strains of <i>A. marginale</i> . 6. Rapid	1. Does not differentiate between infection with <i>A. marginale</i> and <i>A. centrale</i> 2. May cross react with anti- <i>Ehrlichia</i> antibodies. 3. May not be readily available in all countries. 4. Requires a microplate absorbance reader. 5. Low percent of false positive results.	Chung <i>et al.</i> , 2014
IFAT+ Bovine	Serum Glass slides with RBCs infected with <i>A. marginale</i>	Reference test was blood smear DSe 97.6% Dsp 89.6%	48 cattle raised in anaplasmosis free region 82 animals from endemic region	See reference	1. Antigen is relatively easy to produce and store 2. Does not require many reagents	1. Low specificity. 2. Time consuming and labour intensive so not suitable for high throughput. 3. Requires fluorescent microscope and blood smears with high rickettsemia	Gonzalez <i>et al.</i> , 1978

**Appendix 2: Bovine anaplasmosis**  
**Intended purpose of test: Individual animal freedom from infection prior to movement.**

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
PCR ++	Whole blood Various gene targets	Partial validation has been published	51 cattle from 18 herds in three regions of southern Italy were tested by RLB <sup>1</sup> for <i>A. marginale</i> , <i>A. centrale</i> , <i>A. bovis</i> , <i>T. buffeli</i> , <i>B. bovis</i> , <i>A. phagocytophilum</i> , and <i>B. bigemina</i> . All cattle except 4 were positive for at least one of these pathogens	See reference	Good reported concordance between nested PCR and real time PCR. High analytic sensitivity (10 <sup>1</sup> DNA copies).	Must be performed in a lab equipped to extract DNA and have thermocyclers for real time PCR. Though not determined empirically, it is likely that PCR has less sensitivity than C-ELISA in detection of persistently infected cattle	Carelli <i>et al.</i> , 2007.
C-ELISA +++ Bovine	Serum rMSP5-GST	Reference tests were nested PCR and IFAT. Dsp = 99.7% Dse = 100% 30% inhibition as determined by ROC analysis	1. 358 known non-infected cattle from dairy herds maintained in tick free barns and no clinical history of clinical anaplasmosis 2. 135 known positive sera as defined by nested PCR. 3. Intra-test comparison with 163 diagnostic samples with possible false positives based on rMSP5-GST C-ELISA. Test positive confirmation done with IFAT	See reference	1. Updated version with improved specificity 2. High sensitivity, detects persistently infected animals 3. Commercially available 4. Uses a standardised antigen 5. Target antigen is highly conserved among <i>A. marginale</i> strains, thus detects infection with all strains of <i>A. marginale</i> 6. Rapid	1. Does not differentiate between infection with <i>A. marginale</i> and <i>A. centrale</i> 2. May cross react with anti- <i>Ehrlichia</i> antibodies 3. May not be readily available in all countries 4. Requires a microplate absorbance reader	Chung <i>et al.</i> , 2014.

<sup>1</sup>RLB is the reverse line blot test.

**Appendix 3: Bovine anaplasmosis**  
**Intended purpose of test: contribute to eradication policies**

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
C-ELISA +++ Bovine	Serum rMSP5-GST	Reference tests were nested PCR and IFAT. Dsp = 99.7% Dse = 100% 30% inhibition as determined by ROC analysis	1. 358 known non-infected cattle from dairy herds maintained in tick free barns and no clinical history of clinical anaplasmosis 2. 135 known positive sera as defined by nested PCR 3. Intra-test comparison with 163 diagnostic samples with possible false positives based on rMSP5-GST C-ELISA. Test positive confirmation done with IFAT	See reference	1. Updated version with improved specificity 2. High sensitivity, detects persistently infected animals 3. Commercially available 4. Uses a standardised antigen 5. Target antigen is highly conserved among <i>A. marginale</i> strains, thus detects infection with all strains of <i>A. marginale</i> 6. Rapid	1. Does not differentiate between infection with <i>A. marginale</i> and <i>A. centrale</i> 2. May cross react with anti- <i>Ehrlichia</i> antibodies 3. May not be readily available in all countries 4. Requires a microplate absorbance reader 5. Low percent of false positive results	Chung <i>et al.</i> , 2014)

**Appendix 4: Bovine anaplasmosis**  
**Intended purpose of test: confirmation of clinical cases**

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
Microscopic examination +++	Whole blood	No robust validation has been published	N/A	N/A	<ol style="list-style-type: none"> <li>1. Most laboratories have the capacity to make and examine blood smears</li> <li>2. <i>A. marginale</i> infected erythrocytes readily visible in clinically affected animals</li> </ol>	<ol style="list-style-type: none"> <li>1. <i>A. marginale</i> colonies are small and can be difficult to differentiate from debris if animal has low rickettsemia</li> <li>2. Requires experience to identify <i>A. marginale</i> colonies</li> <li>3. Difficult to differentiate between <i>A. marginale</i> and <i>A. centrale</i></li> </ol>	
PCR +++	Whole blood Various gene targets	Partial validation has been published	51 cattle from 18 herds in three regions of southern Italy were tested by RLB <sup>1</sup> for <i>A. marginale</i> , <i>A. centrale</i> , <i>A. bovis</i> , <i>T. buffeli</i> , <i>B. bovis</i> , <i>A. phagocytophilum</i> , and <i>B. bigemina</i> . All cattle except 4 were positive for at least one of these pathogens	See reference	Good reported concordance between nested PCR and real time PCR. High analytic sensitivity (10 <sup>1</sup> DNA copies)	<ol style="list-style-type: none"> <li>1. Must be performed in a lab equipped to extract DNA and have thermocyclers for real-time PCR</li> <li>2. Important to use PCR in conjunction with diagnosis of anaemia and blood smear because PCR can detect low level rickettsemia leading to misdiagnosis</li> </ol>	Carelli et al., 2007

N/A: not available.

<sup>1</sup>RLB is the reverse line blot test.

**Appendix 5: Bovine anaplasmosis**  
**Intended purpose of test: prevalence of infection – surveillance**

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
CAT +	Serum Lysates of <i>A. marginale</i> isolated from red blood cells.	Reference test was blood smear. DSe 84.1 <sup>1</sup> -100 <sup>2%</sup> Dsp 97.9 <sup>1</sup> -98.6 <sup>2%</sup>	48 cattle raised in anaplasmosis free region. 82 animals from endemic region. <sup>1</sup> 86 sera from experimentally infected cattle and 183 sera from <i>A. marginale</i> free area <sup>2</sup>	See references	1. Can be done in field or in the laboratory	1. Antigen derived from infected cattle are difficult to produce and standardise 2. May have false negative and false positive results 3. Variation between tests depending on environmental conditions and the laboratory	<sup>1</sup> Gonzalez <i>et al.</i> , 1978. <sup>2</sup> Molloy <i>et al.</i> , 1999.
C-ELISA +++ Bovine	Serum rMSP5-GST	Reference tests were nested PCR and IFAT. Dsp = 99.7% Dse = 100% 30% inhibition as determined by ROC analysis	1. 358 known non-infected cattle from dairy herds maintained in tick free barns and no clinical history of clinical anaplasmosis 2. 135 known positive sera as defined by nested PCR 3. Intra-test comparison with 163 diagnostic samples with possible false positives based on rMSP5-GST C-ELISA. Test positive confirmation done with IFAT	See reference	1. Updated version with improved specificity 2. High sensitivity, detects persistently infected animals 3. Commercially available 4. Uses a standardised antigen 5. Target antigen is highly conserved among <i>A. marginale</i> strains, thus detects infection with all strains of <i>A. marginale</i> 6. Rapid	1. Does not differentiate between infection with <i>A. marginale</i> and <i>A. centrale</i> . 2. May cross react with anti- <i>Ehrlichia</i> antibodies. 3. May not be readily available in all countries. 4. Requires a microplate absorbance reader. 5. Low percent of false positive results	Chung <i>et al.</i> , 2014
IFAT++ Bovine	Serum Glass slides with RBCs infected with <i>A. marginale</i>	Reference test was blood DSe 97.6% Dsp 89.6%	1. 48 cattle raised in anaplasmosis free region 2. 82 animals from endemic region	See references	1. Antigen is relatively easy to produce and store 2. Does not require many reagents	1. Relatively high false positive rate 2. Time consuming and labour intensive so not suitable for high throughput 3. Requires fluorescent microscope and blood smears with high rickettsemia	Gonzalez <i>et al.</i> , 1978



**Appendix 6: Bovine anaplasmosis**  
**Intended purpose of test: immune status of individual animals**

Test with score and species	Sample type and target analytes	Accuracy	Test population used to measure accuracy	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
CAT +	Serum Lysates of <i>A. marginale</i> isolated from red blood cells.	Reference test was blood smears. DSe 84.1 <sup>1</sup> –100 <sup>2</sup> % Dsp 97.9 <sup>1</sup> –98.6 <sup>2</sup> %	48 cattle raised in anaplasmosis free region. 82 animals from endemic region. <sup>1</sup> 86 sera from experimentally infected cattle and 183 sera from <i>A. marginale</i> free area. <sup>2</sup>	See references	1. Can be done in field or in the laboratory 2. All materials and reagents are readily available	1. Antigen derived from infected cattle is difficult to produce and standardise 2. May have false negative and false positive results 3. Variation in test performance depending on the laboratory	<sup>1</sup> Gonzalez <i>et al.</i> , 1978 <sup>2</sup> Molloy <i>et al.</i> , 1999
C-ELISA +++ Bovine	Serum rMSP5-GST	Reference tests were nested PCR and IFAT Dsp = 99.7% Dse = 100% 30% inhibition as determined by ROC analysis	1. 358 known non-infected cattle from dairy herds maintained in tick free barns and no clinical history of clinical anaplasmosis 2. 135 known positive sera as defined by nested PCR 3. Intratest comparison with 163 diagnostic samples with possible false positives based on rMSP5-GST C-ELISA. Test positive confirmation done with IFAT	See reference	1. Updated version with improved specificity 2. High sensitivity, detects persistently infected animals 3. Commercially available 4. Uses a standardised antigen 5. Target antigen is highly conserved among <i>A. marginale</i> strains, thus detects infection with all strains of <i>A. marginale</i> 6. Rapid	1. Does not differentiate between infection with <i>A. marginale</i> and <i>A. centrale</i> 2. May cross react with anti- <i>Ehrlichia</i> antibodies 3. May not be readily available in all countries 4. Requires a microplate absorbance reader 5. Low percent of false positive results	Chung <i>et al.</i> , 2014
IFAT++ Bovine	Serum Glass slides with red blood cells infected with <i>A. marginale</i> .	Reference test was blood smear. DSe 97.6% Dsp 89.6%	1. 48 cattle raised in anaplasmosis free region 2. 82 animals from endemic region	See reference	1. Antigen is relatively easy to produce and store 2. Does not require many reagents	1. Relatively high false positive rate 2. Time consuming and labour intensive so not suitable for high throughput 3. Requires fluorescent microscope and blood smears with high rickettsemia	Gonzalez <i>et al.</i> , 1978