**Annex 10. Item 5.1. – Chapter 3.4.1. Bovine anaplasmosis**

2 MEETING OF THE WOAH BIOLOGICAL STANDARDS COMMISSION

3 **Paris, 4–8 September 2023**

4

## 5 SEC T I O N 3. 4.

6 **BO V I NA E**

# 7 C H A P T E R 3 . 4 . 1 .

8 **B OV I N E A N AP L A S M OS IS**

9 **SUMMARY**

1. ***Definition of the disease:*** *Bovine anaplasmosis results from infection with* Anaplasma marginale*. A second*
2. *species,* A. centrale*, has long been recognised and usually causes benign infections.* Anaplasma marginale
3. *is responsible for almost all outbreaks of clinical disease.* Anaplasma phagocytophilum *and* A. bovis, *which*
4. *infect cattle, ~~have been recently~~ are also included within the genus ~~but they are not reported to~~.* Anaplasma
5. phagocytophilum *can cause ~~clinical~~ self-limiting disease in cattle. There are no reports of disease associated*
6. *with* A. bovis *infection. The organism is classified in the genus* Anaplasma *belonging to the family*
7. Anaplasmataceae *of the order* Rickettsiales*.*
8. ***Description of the disease:*** *Anaemia, jaundice in acute, severe cases and ~~sudden~~ unexpected death are*
9. *characteristic signs of bovine anaplasmosis. Other signs include rapid loss of milk production and weight,*
10. *but the clinical disease can only be confirmed by identifying the organism. Once infected, cattle may remain*
11. *carriers for life, and identification of these animals depends on the detection of specific antibodies using*
12. *serological tests, or of rickettsial DNA using molecular amplification techniques. The disease is typically*
13. *transmitted by tick vectors, but mechanical transmission by biting insects or by needle can occur.*
14. ***Detection ~~Identification~~ of the agent:*** *Microscopic examination of blood or organ smears stained with*
15. *Giemsa stain is the most common method of identifying* Anaplasma *in clinically affected animals. In these*
16. *smears,* A. marginale *organisms appear as dense, rounded, intraerythrocytic bodies approximately 0.3–*
17. *1.0 µm in diameter situated on or near the margin of the erythrocyte.* Anaplasma centrale *is similar in*
18. *appearance, but most of the organisms are situated toward the centre of the erythrocyte. It can be difficult*
19. *to differentiate* A. marginale *from* A. centrale *in a stained smear, particularly with low levels of rickettsaemia.*
20. *Commercial stains that give very rapid staining of* Anaplasma spp. *are available in some countries.*
21. Anaplasma phagocytophilum *can only be observed in infected granulocytes, mainly neutrophils and* A. bovis
22. *can only be observed in infected monocytes ~~infecting granulocytes, mainly neutrophils~~.*
23. *It is important that smears be well prepared and free from foreign matter. Smears from live cattle should*
24. *preferably be prepared from blood drawn from the jugular vein or another large vessel. For post-mortem*
25. *diagnosis, smears should be prepared from internal organs* (*including liver, kidney, heart and lungs*) *and*
26. *from blood retained in peripheral vessels. The latter are particularly ~~desirable~~ useful if post-mortem*
27. *decomposition is advanced.*
28. ***Serological tests:*** *A competitive enzyme-linked immunosorbent assay* (*C-ELISA*) *has ~~been demonstrated~~*
29. *~~to have~~ good sensitivity in detecting carrier animals. Card agglutination is the next most frequently used*
30. *assay. The complement fixation test* (*CFT*) *is no longer considered a reliable test ~~for disease certification of~~*
31. *individual animals due to variable sensitivity. Cross reactivity between* Anaplasma *spp. can complicate*
32. *interpretation of serological tests. In general, the C-ELISA has the best specificity, with cross-reactivity*
33. *described between* A. marginale, A. centrale, A. phagocytophilum *and* Ehrlichia *spp. Alternatively, an*
34. *indirect ELISA ~~using the CFT with modifications~~* (*I-ELISA*) *is a reliable test used in many laboratories and*
35. *can be prepared in-house for routine diagnosis of anaplasmosis. Finally, a displacement double-antigen*
36. *sandwich ELISA has been developed to differentiate between* A. marginale *and* A. centrale *antibodies.*
37. ***Nucleic-acid-based tests*** *~~have been used~~ are often used in diagnostic laboratories and experimentally,*
38. *and are capable of detecting the presence of low-level infection in carrier cattle and tick vectors. A nested*
39. *conventional polymerase chain reaction (PCR*) *~~reaction is necessary~~ has been used to identify low-level*
40. *carriers ~~using conventional polymerase chain reaction~~* ~~(~~*~~PCR~~*~~)~~, *~~and~~ although nonspecific amplification can*
41. *occur. ~~Recently,~~ Real-time PCR assays ~~with~~ have analytical sensitivity equivalent to nested conventional*
42. *PCR ~~have been described~~ and are preferable in a diagnostic setting to reduce the risk of amplicon*
43. *contamination.*
44. ***Requirements for vaccines:*** *Live vaccines are used in several countries to protect cattle against*
45. ~~A. marginale~~ *~~infection~~ bovine anaplasmosis. A vaccine consisting of live* A. centrale *is most widely used and*
46. *gives partial protection against challenge with virulent* A. marginale*. Vaccination with* A. centrale *leads to*
47. *infection and long-term persistence in many cattle. Vaccinated cattle are typically protected from disease*
48. *caused by* A. marginale*, but not infection.*
49. Anaplasma centrale *vaccine is provided in chilled or frozen forms. Quality control is very important as other*
50. *blood-borne agents that may be present in donor cattle can contaminate vaccines and be disseminated*
51. *broadly. For this reason, frozen vaccine is recommended as it allows thorough post-production quality control,*
52. *which limits the risk of contamination with other pathogens.*
53. Anaplasma centrale *vaccine is not entirely safe. A practical recommendation is to restrict its use, as far as*
54. *possible, to calves, as nonspecific immunity will minimise the risk of some vaccine reactions that may require*
55. *treatment with tetracycline or imidocarb. Partial immunity develops in 6–8 weeks and lasts for several years*
56. *after a single vaccination. In countries where* A. centrale *is exotic, it cannot be used as a vaccine against* A.
57. marginale.

## 67 A. INTRODUCTION

1. Outbreaks of bovine anaplasmosis are due to infection with *Anaplasma marginale. Anaplasma centrale* is capable of
2. ~~producing~~ can produce a moderate degree of anaemia, but clinical outbreaks in the field are extremely rare. ~~New species~~
3. ~~of~~ *~~Anaplasma~~*~~,~~ Other members of the family Anaplasmataceae that infect cattle include *A. phagocytophilum* and *A. bovis*
4. (Dumler *et al*., 2001)*~~,~~* ~~with a primary reservoir~~. *Anaplasma phagocytophilum* has a broad host range and causes the
5. diseases human granulocytic anaplasmosis (HGE), equine granulocytic anaplasmosis (EGA), and canine granulocytic
6. anaplasmosis (CGA), in humans, horses, and dogs, respectively (Matei *et al.,* 2019). In northern Europe ~~in rodents~~, *A.*
7. *phagocytophilum* causes tick-borne fever, primarily affecting lambs. In cattle, *A. phagocytophilum* infections have been
8. reported ~~to infect cattle, but do not cause~~ from many geographical regions, however the association with disease is less
9. commonly reported. Naturally occurring clinical disease as reported in Germany was characterised by fever (39.5–41.7°
10. C), sudden reduction in milk production, lower limb oedema, and stiffness with leukopenia, erythropenia, neutropenia,
11. lymphocytopenia and monocytopenia. The affected animals recovered without antibiotic treatment (~~Dreher~~ *~~et al.,~~* ~~2005;~~
12. ~~Hofmann-Lehmann~~ *~~et al.~~*~~, 2004~~ Silaghi *et al*., 2018).
13. The most marked clinical signs of bovine anaplasmosis are anaemia and jaundice, the latter occurring in acute severe,
14. cases or late in the disease. Haemoglobinaemia and haemoglobinuria are not present, and this may assist in the differential
15. diagnosis of bovine anaplasmosis from babesiosis, which is often endemic in the same regions. The disease can only be
16. confirmed, however, by identification of the organism in erythrocytes from the affected animal. Caution must be exercised
17. if using nucleic acid techniques alone to diagnose *A. marginale* in anaemic cattle. Persistent, low-level infection can be
18. detected by these techniques and may lead to a misdiagnosis of bovine anaplasmosis. Visualisation of *A. marginale* bodies
19. in erythrocytes is therefore required for confirmation.
20. *Anaplasma marginale* occurs in most tropical and subtropical countries and is widely distributed in ~~some more~~ temperate
21. regions. *Anaplasma centrale* was first described from South Africa. The organism has since been imported by other
22. countries – including Australia and some countries in South America, South-East Asia and the Middle East – for use as a
23. vaccine against *A. marginale.*
24. *Anaplasma* species ~~were~~, though originally ~~regarded~~ described as protozoan parasites, ~~but further research showed they~~
25. ~~had no significant attributes to justify this description. Since the last major accepted revision of the~~ are obligate intracellular
26. Gram-negative bacteria. Based on taxonomy established in 2001 (Dumler *et al.*, 2001), the Family *Anaplasmataceae*
27. (Order *Rickettsiales*) is ~~now~~ composed of ~~four~~ five genera, *Anaplasma*, *Ehrlichia, Neorickettsia,* ~~and~~ *Wolbachia*~~. The genus~~
28. and *Aegyptianella* ~~is retained within the Family~~ *~~Anaplasmataceae~~* ~~as genus~~ *~~incertae sedis~~*~~. The revised genus~~. The genus
29. *Anaplasma* ~~now~~ contains *Anaplasma marginale* as the type species, *A. phagocytophilum* the agent of human granulocytic
30. ehrlichiosis (formerly *Ehrlichia phagocytophila* and *E. equi*), *A. platys,* and *A. bovis* (formerly *E. bovis*)*. ~~Haemobartonella~~*
31. ~~and~~ *~~Eperythrozoon~~* ~~are now considered most closely related to the mycoplasmas.~~
32. *Anaplasma* species are transmitted either mechanically or biologically by arthropod vectors. ~~Reviews based on careful~~
33. ~~study~~ Detection of ~~reported transmission experiments list up~~ pathogen DNA within a tick is insufficient to ~~19 different ticks~~
34. ~~as capable of~~ determine the ability of a particular tick species to transmit a pathogen. Studies demonstrating transmission
35. of the pathogen are critical in determining the potential role of a particular tick species in pathogen transmission ~~transmitting~~
36. *~~A. marginale~~* ~~(Kocan~~ *~~et al.~~*~~, 2004). These are:~~ *~~Argas persicus, Ornithodoros lahorensis,~~*. Many studies have demonstrated
37. the transmission ability of *Dermcentor ~~albipictus, D.~~ andersoni*, *~~D. hunteri, D. occidentalis,~~ D. variabilis*,
38. *~~Hyalomma excavatum, H. rufipes, Ixodes ricinus, I. scapularis,~~* and *D. albipictus*. Additionally, transmission by multiple
39. *Rhipicephalus* species is well recognised including *R. annulatus* ~~(formerly~~ *~~Boophilus annulatus),~~ R. bursa*, *R. calcaratus,*
40. *R. decoloratus, R. evertsi, R. microplus~~, R. sanguineus~~* ~~and~~ *~~R. simus.~~* ~~However, the classification of several ticks in these~~
41. ~~reports has been questioned.~~ and *R. sanguineous*. Other species of *Rhipicephalus* also likely serve as biological vectors
42. of *A. marginale*. *Anaplasma marginale* DNA has been widely reported in *Hyalomma* species, and transmission has been
43. demonstrated with *H. excavatum*. It is likely that multiple *Hyalomma* species also serve as vectors of *A. marginale* (Shkap
44. *et al*., 2009).
45. Intrastadial or transstadial transmission ~~is the usual mode~~ can occur, even in the one-host, *Rhipicephalus* species. Male
46. ticks may be particularly important as vectors, as they ~~can become persistently infected and serve as a reservoir~~ are most
47. likely to move between cattle searching for ~~infection~~ female ticks. Experimental demonstration of vector competence does
48. not necessarily imply a role in transmission in the field. However, *Rhipicephalus* species are clearly important vectors of
49. anaplasmosis in ~~countries such as~~ Australia ~~and countries in~~, many regions of Africa, and Latin America~~, and some species~~
50. ~~of~~. *Dermacentor* spp. are efficient vectors in the United States of America (USA).
51. Various other biting arthropods have been implicated as mechanical vectors, particularly in the USA. Experimental
52. transmission has been demonstrated with a number of species of *Tabanus* (horseflies), and with mosquitoes of the genus
53. *Psorophora* ~~(Kocan~~ *~~et al.~~*~~, 2004)~~. The importance of biting insects in the natural transmission of anaplasmosis appears to
54. vary greatly from region to region. *Anaplasma marginale* also can be readily transmitted during vaccination against other
55. diseases unless a fresh or sterilised needle is used for injecting each animal. Similar transmission by means of unsterilised
56. surgical instruments has been described (Reinbold *et al.*, 2010a).
57. The ~~main~~ only known biological vector~~s~~ of *A. centrale* ~~appear to be multihost ticks~~ is *R. simus*, endemic in Africa~~, including~~
58. *~~R. simus.~~* ~~The~~*.* Though multiple transmission studies have been done, there is no evidence that the common cattle tick
59. (*R. microplus*) ~~has not been shown to be~~ can serve as a vector for *A. centrale*. This is ~~of relevance~~ relevant where
60. *A. centrale* is used as a vaccine in *R. microplus*-infested regions.
61. *Anaplasma marginale* infection has not been reported in humans. ~~Thus,~~ There is ~~no~~ minimal risk of field or laboratory
62. transmission to workers ~~and~~ from laboratories working with *A. marginale* ~~may operate at the lowest biosafety level,~~
63. ~~equivalent to BSL1~~. Nevertheless the agent should be handled with appropriate biosafety and containment procedures as
64. determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the*
65. *veterinary laboratory and animal facilities*).

## 133 B. DIAGNOSTIC TECHNIQUES

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

|  |  |
| --- | --- |
| **Method** | **Purpose** |
| [Population](https://oieoffice365.sharepoint.com/sites/ScienceDepartment/Documents%20partages/Admin/G_SCIENCE/TERRESTRIAL%20MANUAL%20ENGLISH/Manual%202024/Chapters%20after%20BSC1/Annex10_3.04.01_Table_1a_pop_freedom_from_infection.pdf) [freedom from](https://oieoffice365.sharepoint.com/sites/ScienceDepartment/Documents%20partages/Admin/G_SCIENCE/TERRESTRIAL%20MANUAL%20ENGLISH/Manual%202024/Chapters%20after%20BSC1/Annex10_3.04.01_Table_1a_pop_freedom_from_infection.pdf) [infection](https://oieoffice365.sharepoint.com/sites/ScienceDepartment/Documents%20partages/Admin/G_SCIENCE/TERRESTRIAL%20MANUAL%20ENGLISH/Manual%202024/Chapters%20after%20BSC1/Annex10_3.04.01_Table_1a_pop_freedom_from_infection.pdf) | [Individual animal](https://oieoffice365.sharepoint.com/sites/ScienceDepartment/Documents%20partages/Admin/G_SCIENCE/TERRESTRIAL%20MANUAL%20ENGLISH/Manual%202024/Chapters%20after%20BSC1/Annex10_3.04.01_Table_1b_individual_freedom_to_move.pdf) [freedom from](https://oieoffice365.sharepoint.com/sites/ScienceDepartment/Documents%20partages/Admin/G_SCIENCE/TERRESTRIAL%20MANUAL%20ENGLISH/Manual%202024/Chapters%20after%20BSC1/Annex10_3.04.01_Table_1b_individual_freedom_to_move.pdf) [infection prior to](https://oieoffice365.sharepoint.com/sites/ScienceDepartment/Documents%20partages/Admin/G_SCIENCE/TERRESTRIAL%20MANUAL%20ENGLISH/Manual%202024/Chapters%20after%20BSC1/Annex10_3.04.01_Table_1b_individual_freedom_to_move.pdf) [movement](https://oieoffice365.sharepoint.com/sites/ScienceDepartment/Documents%20partages/Admin/G_SCIENCE/TERRESTRIAL%20MANUAL%20ENGLISH/Manual%202024/Chapters%20after%20BSC1/Annex10_3.04.01_Table_1b_individual_freedom_to_move.pdf) | [Contribute to](https://oieoffice365.sharepoint.com/sites/ScienceDepartment/Documents%20partages/Admin/G_SCIENCE/TERRESTRIAL%20MANUAL%20ENGLISH/Manual%202024/Chapters%20after%20BSC1/Annex10_3.04.01_Table_1c_bov_anaplas_eradication_policy.pdf) [eradication](https://oieoffice365.sharepoint.com/sites/ScienceDepartment/Documents%20partages/Admin/G_SCIENCE/TERRESTRIAL%20MANUAL%20ENGLISH/Manual%202024/Chapters%20after%20BSC1/Annex10_3.04.01_Table_1c_bov_anaplas_eradication_policy.pdf) [policies](https://oieoffice365.sharepoint.com/sites/ScienceDepartment/Documents%20partages/Admin/G_SCIENCE/TERRESTRIAL%20MANUAL%20ENGLISH/Manual%202024/Chapters%20after%20BSC1/Annex10_3.04.01_Table_1c_bov_anaplas_eradication_policy.pdf) | [Confirmation](https://oieoffice365.sharepoint.com/sites/ScienceDepartment/Documents%20partages/Admin/G_SCIENCE/TERRESTRIAL%20MANUAL%20ENGLISH/Manual%202024/Chapters%20after%20BSC1/Annex10_3.04.01_Table_1d_bov_anaplas_confirm_clinical_case.pdf) [of clinical](https://oieoffice365.sharepoint.com/sites/ScienceDepartment/Documents%20partages/Admin/G_SCIENCE/TERRESTRIAL%20MANUAL%20ENGLISH/Manual%202024/Chapters%20after%20BSC1/Annex10_3.04.01_Table_1d_bov_anaplas_confirm_clinical_case.pdf) [cases](https://oieoffice365.sharepoint.com/sites/ScienceDepartment/Documents%20partages/Admin/G_SCIENCE/TERRESTRIAL%20MANUAL%20ENGLISH/Manual%202024/Chapters%20after%20BSC1/Annex10_3.04.01_Table_1d_bov_anaplas_confirm_clinical_case.pdf) | [Prevalence](https://oieoffice365.sharepoint.com/sites/ScienceDepartment/Documents%20partages/Admin/G_SCIENCE/TERRESTRIAL%20MANUAL%20ENGLISH/Manual%202024/Chapters%20after%20BSC1/Annex10_3.04.01_Table_1e_prevalence_for_surveillance.pdf) [of infection –](https://oieoffice365.sharepoint.com/sites/ScienceDepartment/Documents%20partages/Admin/G_SCIENCE/TERRESTRIAL%20MANUAL%20ENGLISH/Manual%202024/Chapters%20after%20BSC1/Annex10_3.04.01_Table_1e_prevalence_for_surveillance.pdf) [surveillance](https://oieoffice365.sharepoint.com/sites/ScienceDepartment/Documents%20partages/Admin/G_SCIENCE/TERRESTRIAL%20MANUAL%20ENGLISH/Manual%202024/Chapters%20after%20BSC1/Annex10_3.04.01_Table_1e_prevalence_for_surveillance.pdf) | [Immune status in](https://oieoffice365.sharepoint.com/sites/ScienceDepartment/Documents%20partages/Admin/G_SCIENCE/TERRESTRIAL%20MANUAL%20ENGLISH/Manual%202024/Chapters%20after%20BSC1/Annex10_3.04.01_Table_1f_immune_status_individuals.pdf) [individual animals or](https://oieoffice365.sharepoint.com/sites/ScienceDepartment/Documents%20partages/Admin/G_SCIENCE/TERRESTRIAL%20MANUAL%20ENGLISH/Manual%202024/Chapters%20after%20BSC1/Annex10_3.04.01_Table_1f_immune_status_individuals.pdf) [populations (post-](https://oieoffice365.sharepoint.com/sites/ScienceDepartment/Documents%20partages/Admin/G_SCIENCE/TERRESTRIAL%20MANUAL%20ENGLISH/Manual%202024/Chapters%20after%20BSC1/Annex10_3.04.01_Table_1f_immune_status_individuals.pdf) [vaccination)](https://oieoffice365.sharepoint.com/sites/ScienceDepartment/Documents%20partages/Admin/G_SCIENCE/TERRESTRIAL%20MANUAL%20ENGLISH/Manual%202024/Chapters%20after%20BSC1/Annex10_3.04.01_Table_1f_immune_status_individuals.pdf) |
| Microscopic examination | – | ~~+~~ – | – | +++ | – | – |
| Detection of the agent(a) |
| PCR | – | ++ ~~+~~ | – | +++ | – | – |
| Detection of immune response |
| CAT(b) | – | – | – | – | + | + |
| C-ELISA(b) | +++ | +++ | +++ | – | +++ | +++ |
| IFAT(b) | + | – | – | – | ++ | ++ |
| ~~CFT~~ | ~~–~~ | ~~–~~ | ~~–~~ | ~~–~~ | ~~+~~ | ~~–~~ |
| ddasELISA | ~~–~~ | ~~–~~ | ~~–~~ | ~~–~~ | ~~–~~ | ~~++~~ |

1. Key: +++ = recommended for this purpose; ++ recommended but has limitations;

136 + = suitable in very limited circumstances; – = not appropriate for this purpose.

1. Agent id. = agent identification; CAT = card agglutination test; ~~CFT = complement fixation test;~~
2. C-ELISA = competitive enzyme-linked immunosorbent assay; ddasELISA = displacement double-antigen, sandwich ELISA;
3. IFAT = indirect fluorescent antibody test; PCR = polymerase chain reaction.
4. (a)A combination of agent identification methods applied on the same clinical sample is recommended.
5. (b)These tests do not distinguish infected from vaccinated animals.

#### 1. Detection of the agent

##### 1.1. Microscopic examination

1. Samples from live cattle should include thin blood smears and blood collected into an anticoagulant. Air-dried thin
2. blood smears can be kept satisfactorily at room temperature for at least 1 week. The blood sample in anticoagulant
3. should be held and transferred at 4°C, unless it can reach the laboratory within a few hours. This sample is useful for
4. preparing fresh smears if those submitted are not satisfactory. In addition, a low packed cell volume ~~and/~~or erythrocyte
5. count can help to substantiate the involvement of *A. marginale* when only small numbers of the parasites are detected
6. in smears, ~~for example~~ particularly during the recovery stage of the disease.
7. In contrast to *Babesia bovis, A. marginale-*~~does~~ infected erythrocytes do not accumulate in capillaries, so blood drawn
8. from the jugular or other large vessel is satisfactory. *Anaplasma marginale* replicate in the erythrocytes to form small
9. membrane-bound colonies, also termed inclusion bodies or initial bodies. ~~Because of the rather indistinctive~~
10. ~~morphology of~~ *~~Anaplasma~~* These initial bodies can be visualised on a blood smear, but are small and easily confused
11. with debris or stain precipitate (see Figure 1)*.* Thus it is essential that smears are well prepared ~~and~~ , including
12. ensuring slides are free ~~from foreign matter, as specks~~ of debris ~~can confuse diagnosis~~ and stain is recently filtered
13. (Watman #1 filter paper). Thick blood films as are used sometimes for the diagnosis of babesiosis are not appropriate
14. for the diagnosis of anaplasmosis, as *~~Anaplasma~~ A. marginale* are difficult to identify once they become dissociated
15. from erythrocytes.

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1. ***Figure. 1.*** *Anaplasma marginale initial bodies. A Diff-Quick stained blood smear from a bovine experimentally infected*
2. *with A. marginale. Arrows point to the A. marginale initial bodies. Photo from S. Noh.*
3. Samples from dead animals should include air-dried thin smears from the liver, kidney, heart and lungs and from a
4. peripheral blood vessel. The latter is particularly recommended should there be a significant delay before post-
5. mortem examination because, under these circumstances, bacterial contamination of organ smears often makes
6. identification of *~~Anaplasma~~ A. marginale* equivocal. Brain smears, which are useful for the diagnosis of some forms
7. of babesiosis, are of no direct value for diagnosing anaplasmosis, but should be included for differential diagnosis
8. where appropriate.
9. Blood from organs, rather than organ tissues *per se*, is required for smear preparation, as the aim is to be able to
10. examine microscopically intact erythrocytes for the presence of *~~Anaplasma~~ A. marginale colonies.* Organ-derived
11. blood smears can be stored satisfactorily at room temperature for several days.
12. Both blood and organ smears can be stained in 10% Giemsa stain for approximately 30 minutes after fixation in
13. absolute methanol for 1 minute. After staining, the smears are rinsed three or four times with tap water to remove
14. excess stain and are then air-dried. Conditions for Giemsa staining vary from laboratory to laboratory, but distilled
15. water is not recommended for dilution of Giemsa stock. Water should be pH 7.2–7.4 to attain best resolution with
16. Giemsa stain. Commercial stains that give very rapid staining of *~~Anaplasma~~ A. marginale* are available in some
17. countries. Smears ~~are~~ must be examined under oil immersion at a magnification of ×700–1000.
18. *Anaplasma marginale* ~~appear as dense,~~ initial bodies are round~~ed and~~ deeply stained ~~intraerythrocytic bodies,~~ and
19. approximately 0.3–1.0 µm in diameter. Most of these bodies are located on or near the margin of the erythrocyte.
20. This feature distinguishes *A. marginale* from *A. centrale,* as in the latter most of the organisms have a more central
21. location in the erythrocyte. However, particularly at low levels of rickettsaemia, differentiation of these two species in
22. smears can be difficult. Appendages associated with the *Anaplasma* ~~body~~ initial body have been described in some
23. isolates of *A. marginale* ~~(Kreier & Ristic, 1963; Stich~~ *~~et al~~*~~., 2004)~~.
24. The percentage of infected erythrocytes varies with the stage and severity of the disease. Maximum rickettsaemias
25. in excess of 50% may occur with *A. marginale.* Multiple infections of individual erythrocytes are common during
26. periods of high rickettsaemias.
27. The infection becomes visible microscopically 2–6 weeks following transmission. During the course of clinical
28. disease, the rickettsaemia approximately doubles each day for up to about 10 days, and then decreases at a similar
29. rate. Severe anaemia may persist for some weeks after the parasites have become virtually undetectable in blood
30. smears. Following recovery from initial infection, cattle remain latently infected for life.

##### 1.2. Polymerase chain reaction

1. Nucleic-acid-based tests to detect *A. marginale* ~~infection~~ in ~~carrier~~ infected cattle have been developed although not
2. ~~yet~~ fully validated. The analytical sensitivity of polymerase chain reaction (PCR)-based methods has been estimated
3. at 0.0001% infected erythrocytes, but at this level, only a proportion of carrier cattle would be detected. A nested PCR
4. has been used to identify *A. marginale* carrier cattle with a capability of identifying as few as 30 infected erythrocytes
5. per ml of blood, well below the lowest levels in carriers. However, nested PCR is time consuming as it requires two
6. full PCR reactions, and poses significant quality control ~~and specificity~~ problems for routine use (Torioni De Echaide
7. *et al.*, 1998). Real-time PCR assays are reported to achieve a level of analytical sensitivity equivalent to nested PCR
8. ~~has also been described for identification of~~ *~~A. marginale~~* and should be considered instead of the nested PCR (Carelli
9. *et al.*, 2007; Decaro *et al.*, 2008~~; Reinbold~~ *~~et al.~~*~~, 2010b~~). ~~Two~~ Advantages of ~~this technique~~ the real-time PCR, which
10. uses a single closed tube for amplification and analysis, are reduced opportunity for amplicon contamination and a
11. semi-quantitative assay result. Equipment and reagents needed for real-time PCR ~~is~~ are expensive~~, requires~~
12. ~~preventive maintenance,~~ and may be beyond the capabilities of some laboratories. ~~Real-time PCR assays may target~~
13. ~~one of several genes (Carelli~~ *~~et al.~~*~~, 2007; Decaro~~ *~~et al.~~*~~, 2008), or 16S rRNA (Reinbold~~ *~~et al.~~*~~, 2010b), and are reported~~
14. ~~to achieve a level of analytical sensitivity equivalent to nested conventional PCR (Carelli~~ *~~et al.~~*~~, 2007; Decaro~~ *~~et al.~~*~~,~~
15. ~~2008; Reinbold~~ *~~et al.~~*~~, 2010b).~~
16. The most widely cited assays for the detection *A. marginale* in individual animals use a probe for increased specificity
17. and are designed to detect *msp1b* (Carelli *et al.*, 2007) or *msp5* (Futse *et al.,* 2003) in genomic DNA extracted from
18. whole blood. The assay based on detection of *msp1b* has been partially validated to detect the pathogen in individual
19. animals and was used to define samples for the validation of a C-ELISA (Carelli *et al.*, 2007; Chung *et al.,* 2014). The
20. analytical test performance of this assay is robust, and exclusivity testing confirmed other bacterial and protozoal tick-
21. borne pathogens of cattle were not detected. The assay, evaluated using 51 blood samples from 18 cattle herds in
22. three regions of southern Italy, had 100% concordance with nested PCR.
23. *Msp1b* is a multigene family. Based on the annotation of the St. Maries strain of *A. marginale*, the designed primers
24. and probe will amplify multiple members of this gene family, including *msp1b-1*, *msp1b-2*, and *msp1-pg3*). This may
25. help increase diagnostic sensitivity, but may pose challenges if quantification of the pathogen is desired. Additionally,
26. some *A. marginale* strains have single nucleotide polymorphisms in *msp1b* within the primer and probe binding
27. regions. Thus, if *msp1b* is used as a diagnostic target, primer and probe design should consider local *A. marginale*
28. strains. *Msp1b* has the advantage as a target in that orthologs of this gene family are absent in the related *A.*
29. *phagocytophilum* and *Ehrlichia* spp., including *E. ruminantium*, thus helping ensure specificity of the test.
30. *Msp5* has also been used as a target to detect *A. marginale* in cattle in field samples and more frequently in
31. experimental samples (Futse *et al.,* 2003). *Msp5* is highly conserved among *A. marginale* strains and is a single copy
32. gene, thus providing some advantages as a target for ensuring detection of widely variant strains of *A. marginale*.
33. However, the related *Anaplasma* spp. and *Ehrlichia* spp. all have *msp5* orthologs with 50% identity to an *E.*
34. *ruminantium* gene (NCBI accession: L07385.1), thus specificity must be determined in laboratory and field samples.
35. Additionally, little work has been done to validate an *msp5*-based real-time PCR test for diagnostic purposes.
36. A third primer–probe set is designed to detect *A. marginale* using real-time, reverse transcriptase PCR. The primers
37. amplify a 16sRNA gene segment from *A. marginale* and *A. phagocytophilum*, while the probe differentiates between
38. the two species (Reinbold *et al.*, 2010b). The analytical performance of this assay is robust. However, the diagnostic
39. sensitivity, specificity, and of particular importance with 16sRNA sequence-based tests, exclusivity for other tick-
40. borne pathogens of cattle have not been evaluated. Additionally, this assay is designed for use following RNA
41. extraction and reverse transcription, which is more laborious and expensive than DNA extraction. Bacterial RNA is
42. rapidly degraded, and this may ultimately reduce diagnostic sensitivity of this assay.
43. In regions that use *A. centrale* as a vaccine, it may be useful to differentiate between *A. marginale* and *A. centrale*
44. infected/vaccinated animals. PCR is best suited for this task. The real-time PCR assay developed by Carelli *et al.*
45. can also be used in a duplex reaction to detect and differentiate between *A. centrale* and *A. marginale* (Decaro *et al.*,
46. 2008). Primers and probe have been designed to specifically amplify a region of *A. centrale groEL*, but not *A.*
47. *marginale* groEL, despite 97% sequence identity between the two genes. The *A. marginale-*specific primers and
48. probes perform similarly in the single and duplex PCR (Carelli *et al.*, 2007). Using the same 51 field samples from
49. cattle in Italy, the *A. centrale* assay had less analytical sensitivity compared with nested PCR and discordance in 4
50. of 51 samples between an *A. centrale* reverse line blot test and the duplex PCR assay.
51. ***Table 1.*** *Oligonucleotides used in PCR assays to detect* A. marginale *and* A. centrale

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Assay** | **Reference** | **Oligonucleotides(a)** | **Sequence 5’–3’(b)** | **Amplicon size (bp)** | **NCBI****accession number** |
| Real-time PCR | Carelli *et al.,*2007 | *Am\_msp1b*\_F | TTG-GCA-AGG-CAG-CAG-CTT | 95 | M59845 |
| *Am\_msp1b*\_R | TTC-CGC-GAG-CAT-GTG-CAT |
| *Am\_msp1b*\_PB | TCG-GTC-TAA-CAT-CTC-CAG-GCT-TTC- AT |
| Real-time PCR | Futse *et al.,*2003 | *Am\_msp5*\_F | GCC-AAG-TGA-TGG-TGA-TAT-CGA | 151 | M93392 |
| *Am\_msp5*\_R | AGA-ATT-AAG-CAT-GTG-ACC-GCT-G |
| *Am\_msp5*\_PB | AAC-GTT-CAT-GTA-CCT-CAT-CAA |
| Reverse- transcription real-time PCR | Reinbold *et al.,* 2010 | *16S rRNA*\_F(c) | CTC-AGA-ACG-AAC-GCT-GG | 142 | M60313 |
| *16S rRNA* \_R(c) | CAT-TTC-TAG-TGG-CTA-TCC-C |
| *Am\_16S rRNA*\_PB(d) | CGC-AGC-TTG-CTG-CGT-GTA-TGG-T |
| Real-time PCR(d) | Decaro *et al.,* 2008 | *Ac\_groEL*\_F(e, f) | CTA-TAC-ACG-CTT-GCA-TCT-C | 77 | CP001759.1 |
| *Ac\_groEL*\_R(e, f) | CGC-TTT-ATG-ATG-TTG-ATG-C |
| *Ac\_groEL*\_PB(e, f) | TCA-TCA-TTC-TTC-CCC-TTT-ACC-TCG-T |

1. (a)*Am* denotes *A. marginale*, *Ac* denotes *A. centrale*, Pb denotes probe sequence.
2. (b)Fluorophores and quenchers not included in probe sequences.
3. (c)Amplifies *A. phagocytophilum* and *A. marginale* 16S rRNA gene.
4. (d)Probe is specific for *A. marginale* 16S rRNA gene.
5. (e)Can be used as a duplex PCR with *msp1b* primers and probe based on Carelli *et al.,* 2007.
6. (f)Primers and probe amplify *A. centrale groEL*.

#### 2. Serological tests

1. In general, unless animals have been treated or are at a very early stage of infection (<14 days), serology using the
2. competitive enzyme-linked immunosorbent assay (C-ELISA), indirect ELISA (I-ELISA) or card agglutination test (CAT)
3. (see below) may be the preferred methods of identifying infected animals in most laboratories. *Anaplasma marginale*
4. infections usually persist for the life of the animal. However, except for occasional small recrudescences, *~~Anaplasma~~*
5. *A. marginale initial bodies* cannot readily be detected in blood smears after acute rickettsaemia and, ~~even~~ end-point PCR
6. may not detect the presence of *~~Anaplasma~~* the pathogen in blood samples from asymptomatic carriers. Thus, a number of
7. serological tests have been developed with the aim of detecting persistently infected animals.
8. A feature of the serological diagnosis of anaplasmosis is the highly variable results with regard to both sensitivity and
9. specificity reported for many of the tests from different laboratories. This is due at least in part to inadequate ~~evaluation~~
10. validation of the tests using significant numbers of known positive and negative animals. ~~Importantly, the capacity of severa~~l
11. ~~assays to detect known infections of long-standing duration has been inadequately addressed.~~ An exception is a C-ELISA
12. (see below), which ~~has been~~ was initially validated using true positive and negative animals defined by nested PCR (Torioni
13. De Echaide *et al.*, 1998)~~, and the card agglutination assay, for which relative sensitivity and specificity in comparison with~~
14. ~~the C-ELISA has been evaluated (Molloy~~ *~~et al.~~*~~, 1999).~~ And updated in 2014 (Chung *et al*., 2014). Therefore, while most of
15. the tests described in this section are useful for obtaining broad-based epidemiological data, caution is advised on their
16. use for disease certification. The C-ELISA, I-ELISA and CAT are described in detail below.
17. It should be noted that there is a high degree of cross-reactivity between *A. marginale* and *A. centrale*, as well as cross-
18. reactivity with both *A. phagocytophilum* and *Ehrlichia* spp. in serological tests (Al-Adhami *et al.*, 2011; Dreher *et al*., 2005).
19. While the infecting species can sometimes be identified using antigens from homologous and heterologous species,
20. equivocal results are obtained on many occasions. Efforts have been made to develop tests that differentiate between
21. naturally acquired immunity to *A. marginale* and vaccine acquired immunity due to immunisation with *A. centrale* (Bellezze
22. *et al*., 2023; Sarli *et al*., 2020).

##### 2.1. Competitive enzyme-linked immunosorbent assay

1. ~~A C-ELISA using a recombinant antigen termed~~ Major surface protein 5 (MSP5) is an immunodominant protein
2. expressed by A. *marginale*, *A. ovis*, and *A. centrale*. In *A. marginale* the gene is highly conserved making it a useful
3. target across broad geographical regions with high *A. marginale* strain diversity (Knowles *et al.*, 1996; Torioni De
4. Echaide *et al.*, 1998). Thus, a C-ELISA based on recombinantly expressed (rMSP5 ~~and MSP5-~~) in combination with
5. an MSP5-specific monoclonal antibody (mAb) has proven very sensitive and specific for detection of *Anaplasma-*
6. infected animals (~~Hofmann-Lehmann~~ *~~et al.~~*~~, 2004~~ Molloy *et al*., 1999; Reinbold *et al.*, 2010b; Strik *et al.*, 2007). ~~Al~~l
7. *~~A. marginale~~* ~~strains tested, along with~~ Additionally, A. *ovis* and *A. centrale*, express ~~the~~ MSP5 ~~antigen~~ and ~~induce~~
8. infected animals produce antibodies against the immunodominant epitope recognised by the MSP5-specific ~~mAb. A~~
9. ~~recent report~~ mAb used in the C-ELISA. This C-ELISA was updated in 2014 to improve performance by using
10. glutathione S-transferase (GST) instead of maltose binding protein (MBP) as the tag on the rMSP5 (Chung *et al*.,
11. 2014). This assay no longer requires adsorption to remove the antibodies directed against MBP, thus it is faster and
12. easier than the previous version of the C-ELISA. The diagnostic sensitivity is 100% and the diagnostic specificity is
13. 99.7% using a cut-off of 30% inhibition as determined by receiver operating characteristic (ROC) plot (Chung *et al*.,
14. 2014). For this validation, 385 sera defined as negative were from dairy cattle maintained in tick-free facilities from
15. farms with no clinical history of bovine anaplasmosis. The 135 positive sera were from cattle positive for *A. marginale*
16. using nested PCR and serology.
17. One study suggested that antibodies from cattle experimentally infected with *A. phagocytophilum* will test positive in
18. the C-ELISA (Dreher *et al.*, 2005). However, in another study no cross-reactivity could be demonstrated, and the mAb
19. used in the assay did not react with *A. phagocytophilum* MSP5 in direct binding assays (Strik *et al.*, 2007). Cross
20. reactivity has been demonstrated between *~~A. marginale~~* ~~and~~ *~~Ehrlichia~~* ~~spp, in naturally and experimentally infected~~
21. ~~cattle (Al-Adhami~~ *~~et al~~*~~, 2011). Earlier studies had shown that the C-ELISA was 100% specific using 261 known~~
22. ~~negative sera from a non-endemic region, detecting acutely infected cattle as early as 16 days after experimental tick~~
23. ~~or blood inoculation, and was demonstrated to detect cattle that have been experimentally infected as long as 6 years~~
24. ~~previously (Knowles~~ *~~et al.~~*~~, 1996). In detecting persistently infected cattle from an anaplasmosis-endemic region that~~
25. ~~were defined as true positive or negative using a nested PCR procedure, the rMSP5 C-ELISA had a sensitivity of~~
26. ~~96% and a specificity of 95% (Torioni De Echaide~~ *~~et al.~~*~~, 1998)~~ *A. marginale* and *Ehrlichia* sp. BOV2010 isolated in
27. Canada, in naturally and experimentally infected cattle (Al-Adhami *et al*, 2011).
28. Test results using the rMSP5 C-ELISA are available in less than 2~~.5~~ hours. A test kit is available commercially that
29. contains specific instructions. Users should follow the manufacturer’s instructions. I~~n general, however, it is conducted~~
30. ~~as follows.~~
31. ~~2.1.1. Kit reagents~~
32. ~~A 96-well microtitre plate coated with rMSP5 antigen,~~
33. ~~A 96-well coated adsorption/transfer plate for serum adsorption to reduce background binding,~~
34. ~~100×Mab-peroxidase conjugate,~~
35. ~~10× wash solution and ready-to-use conjugate-diluting buffer,~~
36. ~~Ready-to-use substrate and stop solutions,~~
37. ~~Positive and negative controls~~
38. ~~2.1.2. Test procedure~~
39. i) Add 70 µl of undiluted serum sample to the coated adsorption/transfer plate and incubate at
40. ~~room temperature for 30 minutes.~~
41. ~~ii) Transfer 50 µl per well of the adsorbed undiluted serum to the rMSP5-coated plate and incubate~~
42. ~~at room temperature for 60 minutes.~~
43. ~~iii) Discard the serum and wash the plate twice using diluted wash solution.~~
44. iv) Add 50 µl per well of the 1× diluted MAb-peroxidase conjugate to the rMSP5-coated plate wells,
45. ~~and incubate at room temperature for 20 minutes.~~
46. ~~v) Discard the 1×diluted MAb-peroxidase conjugate and wash the plate four times using diluted~~
47. ~~wash solution.~~
48. ~~vi) Add 50 µl per well of the substrate solution, cover the plate with foil, and incubate for 20 minutes~~
49. ~~at room temperature.~~
50. ~~vii) Add 50 µl per well of stop solution to the substrate solution already in the wells and gently tap~~
51. ~~the sides of the plate to mix the wells.~~
52. ~~viii) Immediately read the plate in the plate reader at 620, 630 or 650 nm.~~
53. ~~2.1.3. Test validation~~
54. ~~The mean average optical density (OD) of the negative control must range from 0.40 to 2.10. The average per~~
55. ~~cent inhibition of the positive control must be ≥30%.~~
56. ~~2.1.4. Interpretation of the results~~
57. ~~The % inhibition is calculated as follows:~~

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  |  | ~~Sample OD × 100~~ |  |  |
| ~~100~~ | ~~–~~ | ~~Mean negative control OD~~ | ~~=~~ | ~~Per cent inhibition~~ |

1. ~~% inhibition = 100[1 – (Sample OD ÷ Negative Control OD)]~~
2. ~~Samples with <30% inhibition are negative. Samples with ≥30% inhibition are positive.~~
3. ~~Specificity of the MSP5 C-ELISA may be increased by using a higher percentage inhibition cut-off value~~
4. ~~(Bradway~~ *~~et al~~*~~., 2001); however the effect of this change on sensitivity has not been thoroughly evaluated.~~
5. ~~Recently, an improved MSP5 C-ELISA was developed by replacing rMBP-MSP5 with rGST-MSP5 in addition~~
6. ~~to an improvement in the antigen-coating method by using a specific catcher system. The new rMSP5-GST~~
7. ~~C-ELISA was faster, simpler, had a higher specificity and an improved resolution compared with the rMSP5-~~
8. ~~MBP C-ELISA with MBP adsorption (Chung~~ *~~et al~~*~~., 2014).~~

##### 2.2. Indirect enzyme-linked immunosorbent assay

1. An I-ELISA was first developed using the CAT antigen, which is a crude *A. marginale* lysate (see below). ~~and it~~ The
2. test can be implemented where the commercial C-ELISA is not available. Unlike the C-ELISA, most reagents, such
3. as buffers and ready-to dissolve substrates, are available commercially in many countries. Any laboratory can prepare
4. the antigen using local strains of *A. marginale*, though standardised methods have not been developed. I-ELISA uses
5. small amounts of serum and antigen that ~~and the sensitivity and specificity of the test standardised with true positive~~
6. ~~and negative sera is as good as for the C-ELISA. As it~~ can be prepared in each laboratory. ~~Only the general procedure~~
7. is described here (Barry *et al.*, 1986). For commercial kits, the manufacturer’s instructions should be followed. In the
8. ~~case of in-house I-ELISA~~ The sensitivity and specificity of the test was 87.3% and 98.4–99.6% respectively, though
9. this varied by laboratory (Nielsen *et al*., 1996). For general methods, refer to Barry *et al.* (1986). ~~Initial bodies and~~
10. ~~membranes are obtained as for the complement fixation test (Rogers~~ *~~et al.,~~* ~~1964). This antigen is treated with 0.1%~~
11. ~~sodium dodecyl sulphate for 30 minutes prior to fixing the antigen to the microtitre plate.~~ For each laboratory, the
12. specific amount of antigen ~~has to~~ must be ~~adjusted~~ optimised to obtain the best reading and the least expenditure.
13. Alternatively, rMSP5 can be used as the antigen in this test. This eliminates the need for preparation and
14. standardisation of antigen derived from splenectomised, *A. marginale* infected animals (Silva *et al*., 2006). In a
15. comparison between I-ELISA using the CAT antigen and rMSP with a histidine tag (rMSP5-HIS), these two I-ELISAs
16. performed identically. In this comparison, IFAT was used as the gold standard test (Silva *et al*., 2006).
17. Test results using the I-ELISA are available in about 4 to 5 hours. It is generally conducted as follows:

|  |  |  |
| --- | --- | --- |
| 355 | **2.2.1.** | **Test reagents** |
| 356 |  | A 96-well microtitre plate coated with ~~crude~~ *A. marginale* antigen, |
| 357 |  | PBS/Tween buffer, (PBS 0.1 M, pH 7.2, Tween 20 0.05%), |
| 358 |  | Blocking reagent (e.g. commercial dried skim milk) |
| 359 |  | Tris buffer 0.1 M, MgCl2, 0.1 M, NaCl, 005 M, pH 9.8 |
| 360 |  | Substrate *p*-Nitrophenyl phosphate disodium hexahydrate |
| 361 |  | Positive and negative controls. |

1. 2.2.2. Test procedure (this test is run in triplicate)
2. i) Plates can be prepared ahead of time and kept under airtight conditions at –20°C.
3. ii) Carefully remove the plastic packaging before using plates, being careful not to touch the bottom
4. of them as this can distort the optical density reading.
5. iii) Remove the lid and deposit 200 l PBST20 solution in each well and incubate for 5 minutes at
6. room temperature (RT).
7. iv) For one plate, dissolve 1.1 g of skim milk (blocking agent) in 22 ml of PBST20.
8. v) Remove the plate contents and deposit in each well 200 µl of blocking solution, put the lid on
9. and incubate for 60 minutes at 37°C.
10. vi) Wash the plate three times for 5 minutes with PBST20.
11. vii) Dilute all serum samples including controls 1/100 in PBST20 solution.
12. viii) Remove the contents of the plate and deposit 200 µl of diluted serum in each of the three wells
13. for each dilution, starting with the positive and negative and blank controls.
14. ix) Incubate plate at 37°C covered for 60 minutes.
15. x) Wash three times as described in subsection vi.
16. xi) Dilute 1/1000 anti-IgG bovine alkaline phosphatase conjugate in PBST20 solution. Add 200 µl
17. of the diluted conjugate per well. Incubate the covered plate at 37°C for 60 minutes.
18. xii) Remove the lid and wash three times as described in point vi above ~~make three washes with~~
19. ~~PBST20~~.
20. xiii) Remove the contents of the plate and deposit 195 µl of 0.075% *p*-Nitrophenyl phosphate
21. disodium hexahydrate in Tris buffer in each well and incubate for 60 minutes at 37°C.
22. xiv) The reaction is quantified by a microplate reader spectrophotometer, adjusted to 405 nm
23. wavelength. The data are expressed in optical density (OD).
24. 2.2.3. Data analysis
25. Analysis of results should take into account the following parameters.
26. i) The mean value of the blank wells.
27. ii) The mean value of the positive wells with their respective standard deviations.
28. iii) The mean value of negative wells with their respective standard deviations.
29. iv) The mean value of the blank wells is subtracted from the mean of all the other samples if not
30. automatically subtracted by the ELISA reader.
31. v) Control sera are titrated to give optical density values ranging from 0.90 to 1.50 for the positive
32. and, 0.15 to 0.30 for the negative control.
33. Positive values are those above the cut-off calculated value which is the sum of the average of the
34. negative and two times the standard deviation.
35. ~~For purposes of assessing the consistency of the test operator, the error “E” must alsoo be estimated;~~
36. ~~this is calculated by determining the percentage represented by the standard deviation of any against~~
37. ~~their mean serum.~~
38. As with all diagnostic tests, it is important to measure reproducibility. For more details see Chapter
39. 2.2.4 *Measurement uncertainty*.

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

1. ***A. centrale* antibodies**
2. In regions where vaccination with *A. centrale* is used to control bovine anaplasmosis, differentiation between
3. *A. centrale*-vaccinated and *A. marginale*-infected animals may be useful. Because there is often high amino acid
4. identity between *A. marginale* and *A. centrale* surface proteins, identifying unique targets for serological assays for
5. this purpose is difficult. Epitopes from MSP5 (aa28-210, without the transmembrane region) that are not shared
6. between *A. marginale* and *A. centrale* were used to develop a displacement double-antigen sandwich ELISA
7. (ddasELISA) (Bellezze *et al*., 2023; Sarli *et al*., 2020). The recombinant MSP5 epitopes from *A. marginale* or *A.*
8. *centrale* are expressed in *E. coli* with a histidine tag and purified. The ELISA plates are then coated with either the
9. recombinant *A. marginale* MSP5 epitope, or the *A. centrale* MSP5 epitope and blocked. Serum is added to the wells
10. and allowed to incubate. Following washing, a combination of biotinylated and non-biotinylated recombinant proteins
11. are added to improve specificity of the reaction (see below for specifics). The protein–biotin binding to the serum
12. antibody is detected with a peroxidase-streptavidin based detection system. The optical density for the *A. marginale*
13. MSP5-coated well (ODAm) and the OD for the *A. centrale* MSP5 (ODAc) coated well for each animal is measured. If
14. the OD for either target is <0.2, the sample is excluded from the analysis. For the remaining samples, the ratio
15. between the OD values (ODAm/ODAc) is calculated. If the ratio is >0.38 the sample is considered positive for anti-
16. *A. marginale* antibodies, and a ratio ≤ 0.38 is classified as vaccinated with *A. centrale*.
17. For the detection of *A. marginale* the test has a diagnostic specificity of 98% and a diagnostic sensitivity of 98.9%.
18. For 702 field samples evaluated, 131 (19%) had an OD <0.2 in the ddasELISA and thus were excluded from the
19. analysis. Of those animals, 52% were nested PCR positive for *A. marginale*, 23% were nested PCR positive for *A.*
20. *centrale*, 4.6% were nested PCR positive for *A. marginale* and *A. centrale*, 20% were nested PCR negative for both,
21. suggesting the ddasELISA may lack sensitivity.
22. Of the 571 ddasELISA positive field samples, the agreement between the ddasELISA and nested PCR was 84% and
23. the kappa coefficient was 0.70 (95% CI: 0.635–0.754), indicating substantial agreement between tests. There was
24. agreement between the ddasELISA and nested PCR for 93% of the *A. marginale* ddasELISA positive samples and
25. 86% of the *A. centrale* ddasELISA positive samples. Additionally, 36 nested PCR negative samples tested positive
26. for antibodies against *A. marginale* (*n*=28) or *A. centrale* (*n*=8) by ddasELISA. This test could not identify animals
27. with co-infections, meaning animals vaccinated with *A. centrale* that are then infected with *A. marginale*, which is not
28. uncommon.
29. Test results using the ddasELISA are available in 5–6 hours. It is conducted as outlined below, see Bellezze *et al.*,
30. 2023 for more details.
31. 2.3.1. Test reagents
32. i) A 96-well microtitre plate coated with either *A. marginale* or A. centrale recombinant protein
33. ii) PBS/Tween buffer (PBS (50mM sodium phosphate, 150 mM NaCL, pH 7.2) with 0.05% Tween-

435 20)

1. iii) Blocking reagent (PBS with 10% commercial dried skim milk)
2. iv) Purified recombinant *A. marginale* MSP5 epitopes and *A. centrale* epitopes
3. v) Biotinylated recombinant *A. marginale* MSP5 epitopes and *A. centrale* epitopes
4. vi) Streptavidin-horse radish peroxidase (HRP) detection system
5. vii) Chromogenic substrate (1 mM 2,2’-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-
6. diammonium salt in0.05 M sodium citrate, pH 4.5, 0.0025% V/V H2O2 (100 μl/well).
7. viii) ELISA plate reader (405 nm reading)
8. ix) Positive and negative control sera for *A. marginale* and *A. centrale*
9. 2.3.2. Test procedure
10. i) Plates are coated overnight.
11. ii) Block with blocking buffer for 1 hour at room temperature and wash three times with PBS/Tween
12. buffer.
13. iii) Add undiluted serum 100 ul/well and incubate for 1 hour at 25°C at 100 rpm.
14. iv) Wash three times with PBS/Tween buffer.
15. v) Add 100 μl of *A. marginale* MSP5-biotin (1 μg/ml) plus *A. centrale* MSP5 (10 μg/ml) to
16. *A. marginale* test wells. Add *A. centrale* MSP5-biotin (1 μg/ml) plus *A. marginale* MSP5
17. (10 μg/ml) in PBS/Tween buffer + 10% fat-free dried milk to *A. centrale* test wells.
18. vi) Incubate 1 hour at 25°C, 100 rpm and wash the plate five times with PBS/Tween buffer.
19. vii) To detect the bound protein–biotin complex, add streptavidin-HRP diluted in 1/500 in
20. PBS/Tween buffer with 10% dried milk for 1 hour at 25°C, 100 rpm.
21. viii) Wash five times with PBS/Tween buffer.
22. ix) Add chromogenic substrate based on manufacturer’s instructions.
23. x) The reaction is measured by microplate reader spectrophotometer at 405 nm wavelength. The
24. data are expressed in optical density (OD).
25. xi) OD405nm <0.2 is considered negative.
26. xii) Results are expressed as the ratio between antibodies specific for *A. marginale* MSP5 and for
27. *A. centrale* MSP5 (ODAm/ODAc). If the ratio is >0.38 the sample is considered positive for anti-
28. *A. marginale* antibodies, and a ratio ≤ 0.38 is classified as vaccinated with *A. centrale*.

##### 2.4. Card agglutination test

1. ~~The advantages of the CAT are that it is sensitive~~ The sensitivity of the CAT is from 84% to 98% (Gonzalez *et al*.,
2. 1978; Molloy *et al*., 1999) and the specificity is 98.6% (Molloy *et al*., 1999). Though sometimes giving variable results,
3. the CAT can be useful under certain circumstances, as it may be undertaken either in the laboratory or in the field,
4. and it gives a result within a few minutes. Nonspecific reactions may be a problem, and subjectivity in interpreting
5. assay reactions can result in variability in test interpretation. In addition, the CAT antigen, which is a ~~suspension~~
6. lysate of *A. marginale* ~~particles~~ isolated from erythrocytes, can be difficult to prepare and can vary from batch to batch
7. and laboratory to laboratory. To obtain the antigen, splenectomised calves are infected by intravenous inoculation
8. with blood containing *~~Anaplasma~~ A. marginale*-infected erythrocytes. When the rickettsaemia exceeds 50%, the
9. animal is exsanguinated, the infected erythrocytes are washed, lysed, and the erythrocyte ghosts and *~~Anaplasma~~*
10. ~~particles~~ *A. marginale* are pelleted. The pellets are sonicated, washed, and then resuspended in a stain solution to
11. produce the antigen suspension.
12. A test procedure that has been slightly modified from that originally described (Amerault & Roby, 1968; Amerault *et*
13. *al.*, 1972) is as follows, and is based on controlled conditions in a laboratory setting:
14. 2.4.1. Test procedure
15. i) Ensure all test components are at a temperature of 25–26°C before use (this constant
16. temperature is critical for the test).
17. ii) On each circle of the test card (a clear perspex/plastic or glass plate marked with circles that
18. are 18 mm in diameter), place next to each other, but not touching, 10 µl of bovine serum factor
19. (BSF), 10 µl of test serum, and 5 µl of CAT antigen [39](#_bookmark118). Negative and low positive control sera
20. must be tested on each card.
21. iii) BSF is serum from a selected animal with high known conglutinin level. If the conglutinin level
22. is unknown, fresh serum from a healthy animal known to be free from *Anaplasma* can be used.
23. The BSF must be stored at –70°C in small aliquots, a fresh aliquot being used each time the
24. tests are performed. The inclusion of BSF improves the sensitivity of the test.
25. iv) Mix well with a glass stirrer. After mixing each test, wipe the stirrer with clean tissue to prevent
26. cross-contamination.
27. v) Place the test card in a humid chamber and rock at 100–110 rpm for 7 minutes.
28. vi) Read immediately against a backlight. Characteristic clumping of the antigen (graded from +1
29. to +3) is considered to be a positive result. The test is considered to give a negative result when
30. there is no characteristic clumping.
31. A latex card agglutination test, a relatively simple and rapid test platform, has been partially validated.
32. This test uses rMSP5-HIS rather than *A. marginale* lysate and does not require BSF. The
33. performance of this test was compared with that of the I-ELISA using rMSP5-HIS as the antigen. The
34. relative sensitivity was 95.2% and relative specificity was 91.86% (Ramos *et al.,* 2014).
35. 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).

##### ~~2.4. Complement fixation test~~

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

##### 2.5. Indirect fluorescent antibody test

1. Because of the limitations on the number of indirect fluorescent antibody (IFA) tests that can be performed daily by
2. one operator, other serological tests are generally preferred to the IFA test. The IFA test is performed as described
3. for bovine babesiosis in chapter 3.4.2, except that *A. marginaIe* infected blood is used for the preparation of antigen
4. smears. A serious problem encountered with the test is nonspecific fluorescence. The reported sensitivity is 97.6%
5. and specificity 89.6% (Gonzalez *et al*., 1978). Antigen made from blood collected as soon as adequate rickettsaemia
6. (5–10%) occurs is most likely to be suitable. Nonspecific fluorescence due to antibodies adhering to infected
7. erythrocytes can be reduced by washing the erythrocytes in an acidic glycine buffer before antigen smears are
8. prepared. Infected erythrocytes are washed twice in 0.1 M glycine buffer (pH 3.0, centrifuged at 1000 ***g*** for 15 minutes
9. 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
10. with other members of the *Anaplasmataceae* family, and specifically an *Ehrlichia* spp. identified as BOV2010 (Al-
11. Adhami *et al.,* 2011).

##### 2.6. Complement fixation test

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

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#### 1. Background

## C. REQUIREMENTS FOR VACCINES

1. Several immunisation methods have been used to protect cattle against anaplasmosis in countries where the disease is
2. endemic, but none is ideal to date ~~(McHardy, 1984)~~. A review of *A. marginale* vaccines and antigens has been published
3. (Kocan *et al.*, ~~2003~~ 2010; Noh *et al.,* 2012). Use of the less pathogenic *A. centrale,* which gives partial cross-protection
4. against *A. marginale,* is the most widely accepted method, although not used in many countries ~~where the disease is~~
5. ~~exotic~~, including north America.
6. In this section, the production of live *A. centrale* vaccine is described. It involves infection of a susceptible, splenectomised
7. calf and the use of its blood as a vaccine. Detailed accounts of the production procedure are available and reference should
8. be made to these publications for details of the procedures outlined here (Bock *et al.*, 2004; de Vos & Jorgensen, 1992;
9. Pipano, 1995).
10. Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*.
11. The guidelines given here and in Chapter 1.1.8 are intended to be general in nature and may be supplemented by national
12. and regional requirements.
13. *Anaplasma centrale* vaccine can be provided in either frozen or chilled ~~form~~ depending on demand, transport networks,
14. and the availability of liquid nitrogen or dry ice supplies. Frozen vaccine is recommended in most instances, as it allows
15. for thorough post-production quality control of each batch. It is, however, more costly to produce and more difficult to
16. transport than chilled vaccine. The risk of contamination makes post-production control essential, but may be prohibitively
17. expensive.

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

##### 2.1. Characteristics of the seed

1. 2.1.1. Biological characteristics
2. *Anaplasma centrale* was isolated in 1911 in South Africa and has been used as a vaccine in South
3. America, Australia, Africa, the Middle East, and South-East Asia. It affords only partial, but adequate,
4. protection in regions where the ~~challenging~~ circulating strains are of moderate virulence (e.g.
5. Australia) (Bock & de Vos, 2001). In the humid tropics where *A. marginale* ~~appears to~~ may be ~~a very~~
6. more virulent ~~rickettsia~~, the protection afforded by *A. centrale* may be inadequate to prevent disease
7. in some animals.
8. *Anaplasma centrale* usually causes benign infections, especially if used in calves under 9 months of
9. age. Severe reactions following vaccination have been reported when adult cattle are inoculated. The
10. suitability of an isolate of *A. centrale* as a vaccine can be determined by inoculating susceptible cattle,
11. monitoring the subsequent reactions, and then challenging the animals and susceptible controls with
12. a virulent local strain of *A. marginale.* Both safety and efficacy can be judged by monitoring
13. rickettsaemias in stained blood films and the depression of packed cell volumes of inoculated cattle
14. during the vaccination and challenge reaction periods.
15. Infective material for preparing the vaccine is readily stored as frozen stabilates of infected blood in
16. liquid nitrogen or dry ice. Dimethyl sulphoxide (DMSO) ~~and~~ or polyvinylpyrrolidone M.W. 40,000
17. (Bock *et al.*, 2004) are the recommended cryopreservatives, as they allow for intravenous
18. administration after thawing of the stabilate. A detailed account of the freezing technique using DMSO
19. is reported elsewhere (Mellors *et al.,* 1982), but briefly involves the following: infected blood is
20. collected, chilled to 4°C, and cold cryoprotectant (4 M DMSO in PBS) is added slowly with stirring to
21. a final blood:protectant ratio of 1:1, to give a final concentration of 2 M DMSO. The entire dilution
22. procedure is carried out in an ice bath and the diluted blood is dispensed into suitable containers
23. (e.g. 5 mI cryovials), and frozen, as soon as possible, in the vapour phase of a liquid nitrogen
24. container.
25. 2.1.2. Quality criteria
26. Evidence of purity of the *A. centrale* isolate can be determined by serological testing of paired sera
27. from the cattle used in the safety test for possible ~~contaminants~~ pathogens that may be present (Bock
28. *et al.*, 2004; Pipano, 1997). Donor calves used to expand the seed for vaccine production should be
29. examined for all blood-borne infections prevalent in the vaccine-producing country, including
30. *Babesia*, *Anaplasma*, *Ehrlichia*, *Theileria* and *Trypanosoma*. This can be done by routine examination
31. of stained blood films after splenectomy, PCR, and preferably also by serology. Any calves showing
32. evidence of natural infections of any of these agents should be rejected. The absence of other
33. infective agents should also be confirmed. These may include the agents of enzootic bovine leukosis,
34. mucosal disease, infectious bovine rhinotracheitis, ephemeral fever, Akabane disease, bluetongue,
35. and foot and mouth disease~~, and rinderpest~~. The testing procedures will depend on the diseases
36. prevalent in the country and the availability of tests but should involve serology of paired sera at the
37. very least and, in some cases, virus isolation, antigen, or DNA/RNA detection (Bock *et al.*, 2004;
38. Pipano, 1981; 1997).

##### 2.2. Method of manufacture

1. 2.2.1. Procedure
2. i) Production of frozen vaccine
3. Quantities of the frozen stabilate (5–10 ml) are thawed by immersing the vials in water preheated to
4. 40°C. The thawed material is kept on ice and used as soon as possible (within 30 minutes) to infect
5. a susceptible, splenectomised calf by intravenous inoculation.
6. The rickettsaemia of ~~the~~ this donor calf is monitored daily by examining stained films of jugular blood,
7. and the blood is collected for vaccine production when suitable rickettsaemias are reached. A
8. rickettsaemia of 1 × 108/ml (approximately 2% rickettsaemia in jugular blood) is the minimum required
9. for production of vaccine as this is the dose to vaccinate a bovine. If a suitable rickettsaemia is not
10. obtained, passage of the strain by subinoculation of 100–200 ml of blood to a second splenectomised
11. calf may be necessary.
12. Blood from the donor is collected by aseptic jugular or carotid cannulation using heparin as an
13. anticoagulant (5 International Units [IU] heparin/ml blood). The use of blood collection units for human
14. use are also suitable and guarantee sterility and obviate the need to prepare glass flasks that make
15. the procedure more cumbersome.
16. In the laboratory, the infective blood is mixed in equal volumes with 3 M glycerol in PBS supplemented
17. with 5 mM glucose at 37°C (final concentration of glycerol 1.5 M). The mixture is then equilibrated at
18. 37°C for 30 minutes and dispensed into suitable containers (e.g. 5 ml cryovials). The vials are cooled
19. at approximately 10°C/minute in the vapour phase of liquid nitrogen and, when frozen, stored in the
20. liquid phase (Bock *et al.,* 2004).
21. DMSO can be used as a cryoprotectant in the place of glycerol. This is done in the same way as
22. outlined for the preparation of seed stabilate (Mellors *et al.,* 1982; Pipano, 1981).
23. If glycerolised vaccine is to be diluted, the diluent should consist of PBS with 1.5 M glycerol and 5 mM
24. glucose (Jorgensen *et al.,* 1989). Vaccine cryopreserved with DMSO should be diluted with diluent
25. containing the same concentration of DMSO as in the original cryopreserved blood (Pipano *et al.,*
26. 1986).
27. ii) Production of chilled vaccine
28. Infective material for chilled vaccine is prepared in the same way as for frozen vaccine, but it must
29. be issued and used as soon as possible after collection. The infective blood can be diluted to provide
30. 1 × 107 parasites per dose of vaccine. A suitable diluent is 10% sterile bovine serum in a
31. glucose/balanced salt solution containing the following quantities per litre: NaCI (7.00 g), MgCI2.6H2O
32. (0.34 g), glucose (1.00 g), Na2HPO4(2.52 g), KH2PO4(0.90 g), and NaHCO3(0.52 g).
33. If diluent is not available, acid citrate dextrose (20% [v/v]) or citrate phosphate dextrose (20% [v/v])
34. should be used as anticoagulant to provide the glucose necessary for survival of the organisms.
35. iii) Use of vaccine
36. In the case of frozen vaccine, vials should be thawed by immersion in water, preheated to 37°C to
37. 40°C, and the contents mixed with suitable diluent to the required dilution. If glycerolised vaccine is
38. prepared, it should be kept cool and used within 8 hours (Bock *et al.,* 2004). If DMSO is used as a
39. cryoprotectant, the prepared vaccine should be kept on ice and used within 15–30 minutes (Pipano,
40. 1981). The vaccine is most commonly administered subcutaneously.
41. iv) Chilled vaccine should be kept refrigerated and used within 4–7 days of preparation.
42. The strain of *A. centrale* used in the vaccine is of reduced virulence, but is not entirely safe. A practical
43. recommendation is, therefore, to limit the use of vaccine to calves, where nonspecific immunity will
44. minimise the risk of vaccine reactions. When older animals have to be vaccinated, there is a risk of
45. severe reactions. These reactions occur infrequently, but valuable breeding stock or pregnant
46. animals obviously warrant close attention, and should be observed daily for 3 weeks post-
47. vaccination. Clinically sick animals should be treated with oxytetracycline or imidocarb at dosages
48. recommended by the manufacturers. Protective immunity develops in 6–8 weeks and usually lasts
49. for several years.
50. Anaplasmosis and babesiosis vaccines are often used concurrently, but it is not advisable to use any
51. other vaccines at the same time (Bock *et al.,* 2004).
52. 2.2.2. Requirements for substrates and media
53. *Anaplasma centrale* ~~cannot~~ can be cultured in ~~vitro~~ *Rhipicephalus appendiculatus* and *Dermacentor variabilis*
54. cells lines, though antigen expression and immunogenicity of the cultured *A. centrale* need to be tested (Bell-
55. Sakyi *et al*., 2015). No substrates or media other than buffers and diluents are used in vaccine production.
56. DMSO or glycerol should be purchased from reputable companies.
57. 2.2.3. In-process controls
58. i) Source and maintenance of vaccine donors
59. A source of calves free from natural infections of *~~Anaplasma~~ A. marginale* and other tick-borne
60. diseases should be identified. If a suitable source is not available, it may be necessary to breed the
61. calves under tick-free conditions specifically for the purpose of vaccine production.
62. The calves should be maintained under conditions that will prevent exposure to infectious diseases
63. and to ticks and biting insects. In the absence of suitable facilities, the risk of contamination with the
64. agents of infectious diseases present in the country involved should be estimated, and the benefits
65. of local production of vaccine weighed against the possible adverse consequences of spreading
66. disease (Bock *et al.*, 2004).
67. ii) Surgery
68. Donor calves should be splenectomised to allow maximum yield of organisms for production of
69. vaccine. This is best carried out in young calves and under general anaesthesia.
70. iii) Screening of vaccine donors before inoculation
71. As for preparation of seed stabilate, donor calves for vaccine production should be examined for all
72. blood-borne infections prevalent in the vaccine-producing country, including *Babesia*, *Anaplasma*,
73. *Ehrlichia*, *Theileria* and *Trypanosoma*. This can be done by routine examination of stained blood films
74. after splenectomy, and preferably also by serology. Any calves showing evidence of natural infections
75. of any of these agents should be rejected. The absence of other infective agents should also be
76. confirmed. These may include the agents of enzootic bovine leukosis, bovine viral diarrhoea,
77. infectious bovine rhinotracheitis, ephemeral fever, Akabane disease, bluetongue, and foot and mouth
78. disease. The testing procedures will depend on the diseases prevalent in the country and the
79. availability of tests, but should involve serology of paired sera at the very least and, in some cases,
80. virus isolation, antigen, or DNA/RNA detection (Bock *et al.*, 2004; Pipano, 1981; 1997).
81. iv) Monitoring of rickettsaemias following inoculation
82. It is necessary to determine the concentration of rickettsia in blood being collected for vaccine. The
83. rickettsial concentration can be estimated from the erythrocyte count and the rickettsaemia
84. (percentage of infected erythrocytes).
85. v) Collection of blood for vaccine
86. All equipment should be sterilised before use (e.g. by autoclaving). Once the required rickettsaemia
87. is reached, the blood is collected in heparin using strict aseptic techniques. This is best done if the
88. calf is sedated and with the use of a closed-circuit collection system.
89. Up to 3 litres of heavily infected blood can be collected from a 6-month-old calf. If the calf is to live,
90. the transfusion of a similar amount of blood from a suitable donor is indicated. Alternatively, the calf
91. should be killed immediately after collection of the blood.
92. vi) Dispensing of vaccine
93. All procedures are performed in a suitable environment, such as a laminar flow cabinet, using
94. standard sterile techniques. Use of a mechanical or magnetic stirrer will ensure thorough mixing of
95. blood and diluent throughout the dispensing process. Penicillin (500,000 lU/litre) and streptomycin
96. (370,000 µg/litre) are added to the vaccine at the time of dispensing.
97. 2.2.4. Final product batch tests
98. The potency, safety and sterility of vaccine batches cannot be determined in the case of chilled vaccine, and
99. specifications for frozen vaccine depend on the country involved. The following are the specifications for frozen
100. vaccine produced in Australia.
101. i) Sterility and purity
102. Standard tests for sterility are employed for each batch of vaccine and diluent (see Chapter 1.1.9
103. *Tests for sterility and freedom from contamination of biological materials intended for veterinary use*).
104. The absence of contaminants is determined by doing appropriate serological testing of donor cattle,
105. by inoculating donor lymphocytes into sheep and then monitoring them for evidence of viral infection,
106. and by inoculating cattle and monitoring them serologically for infectious agents that could potentially
107. contaminate the vaccine. Cattle inoculated during the test for potency (see Section C.2.2.4.iii) are
108. suitable for the purpose. Depending on the country of origin of the vaccine, these agents include the
109. causative organisms of enzootic bovine leukosis, infectious bovine rhinotracheitis, bovine viral
110. diarrhoea, ephemeral fever, Akabane disease, Aino virus, bluetongue, parainfluenza, foot and mouth
111. disease, lumpy skin disease, rabies, Rift Valley fever, contagious bovine pleuropneumonia,
112. Jembrana disease, heartwater, pathogenic *Theileria* and *Trypanosoma spp*., *Brucella abortus*,
113. *Coxiella*, and *Leptospira* (Bock *et al.*, 2004; Pipano, 1981; 1997). Other pathogens to consider include
114. the causal agents of bovine tuberculosis and brucellosis as they may spread through contaminated
115. blood used for vaccine production. Most of these agents can be tested by means of specific PCR and
116. there are many publications describing primers, and assay conditions for any particular disease.
117. ii) Safety
118. Vaccine reactions of the cattle inoculated in the test for potency (see Chapter 1.1.8 *Principles of*
119. *veterinary vaccine production*) are monitored by measuring rickettsaemia and depression of packed
120. cell volume. Only batches with pathogenicity levels equal to or lower than a predetermined standard
121. are released for use.
122. iii) Potency
123. Vaccine is thawed and diluted 1/5 with a suitable diluent (Bock *et al.*, 2004). The diluted vaccine is
124. then incubated for 8 hours at 4°C, and five cattle are inoculated subcutaneously with 2 ml doses. The
125. inoculated cattle are monitored for the presence of infections by examination of stained blood smears.
126. All should become infected for a batch to be accepted. A batch proving to be infective is
127. recommended for use at a dilution of 1/5 with isotonic diluent.

##### 2.3. Requirements for authorisation

1. 2.3.1. Safety
2. The strain of *A. centrale* used in vaccine is of reduced virulence but is not entirely safe. A practical
3. recommendation is, therefore, to limit the use of vaccine to calves, where nonspecific immunity will
4. minimise the risk of vaccine reactions. When older animals have to be vaccinated, there is a risk of
5. severe reactions. These reactions occur infrequently, but valuable breeding stock or pregnant
6. animals obviously warrant close attention, and should be observed daily for 3 weeks post-
7. vaccination. Clinically sick animals should be treated with oxytetracycline or imidocarb at dosages
8. recommended by the manufacturers.
9. *Anaplasma centrale* is not infective to other species, and the vaccine is not considered to have other
10. adverse environmental effects. The vaccine is not infective for humans. When the product is stored
11. in liquid nitrogen, the usual precautions pertaining to the storage, transportation and handling of deep-
12. frozen material applies.
13. 2.3.2. Efficacy requirements
14. ~~Partial but long-lasting immunity results from one inoculation. There is no evidence that repeated~~
15. ~~vaccination will have a boosting effect.~~ Immunisation with live *A. centrale* results in long-term infection
16. of the vaccinee, thus repeated vaccination is unnecessary. Infection with *A. centrale* does not prevent
17. subsequent infection with *A. marginale*, but does at least result in protection from disease (Shkap *et*
18. *al*., 2009). The vaccine is used for control of clinical anaplasmosis in endemic areas. It will not provide
19. sterile immunity, and should not be used for eradication of *A. marginale*.
20. 2.3.3. Stability
21. The vaccine can be kept for 5 years when stored in liquid nitrogen. Once thawed, it rapidly loses its
22. potency. Thawed vaccine cannot be refrozen.

#### 3. Vaccines based on biotechnology

1. There are no vaccines based on biotechnology available for anaplasmosis.

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1. **NB:** There is a WOAH Reference Laboratory for anaplasmosis (please consult the WOAH Web site:
2. [https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3)](https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3)
3. Please contact the WOAH Reference Laboratory for any further information on
4. diagnostic tests, reagents and vaccines for bovine anaplasmosis
5. **NB:** First adopted in 1991. Most recent updates adopted in 2015.