

Tick-borne diseases of Cattle

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Part 1. Diagnostic Overview

Summary

Babesiosis and anaplasmosis are tick-borne diseases of cattle caused by the organisms *Babesia bovis*, *Babesia bigemina* and *Anaplasma marginale*. A vector, *Boophilus microplus*, is widespread in the warm, humid northern parts of Australia.

Identification of the causal organism: The organisms can be seen in thin smears of blood, brain, kidney, liver and spleen from recently dead cattle, and in thick and thin smears of blood from the peripheral capillary circulation such as the tip of the tail of live cattle. The smears are fixed with methanol, stained with Giemsa and examined at x1000 magnification under oil immersion. Polymerase chain reaction assays and other procedures can detect and differentiate among the organisms in cattle but are not useful in assessing the clinical significance of infection.

Serological tests: The indirect fluorescent antibody test has been widely used for detecting antibodies to *B bovis* and *B bigemina* but enzyme-linked immunosorbent assays (ELISA) are rapidly gaining popularity. Serological tests for *B bovis* are generally both sensitive and specific but most tests for *B bigemina* suffer from cross-reactions making species differentiation difficult.

Commercial ELISA kits are preferred to the card agglutination test (CAT) for the detection of antibodies to *A marginale* because the CAT, although sensitive, specific and relatively inexpensive, is subjective and results are very sensitive to temperature and humidity fluctuations.

Aetiology

Babesiosis (piroplasmiasis) and anaplasmosis are economically important tick-borne diseases of cattle collectively known as “tick fever” that were introduced to Australia in the nineteenth century.¹ There are three organisms: *Babesia bovis* (syn. *B argentina* and *B berbera*), *Anaplasma marginale* and *B bigemina*, in that order of importance. These three organisms have a world-wide distribution in tropical and sub-tropical regions.

In Australia, these organisms are transmitted by the cattle tick *Boophilus microplus* in warm, humid, northern regions of the country. *Theileria buffeli* (*T sergenti/T orientalis* group),² another tick-borne parasite transmitted by *Haemaphysalis* spp, is widespread but of minor economic significance although it can cause death on rare occasions.³

Clinical Signs

Babesiosis

The clinical signs that develop during infections with *B bovis* or *B bigemina* are similar but the courses of the diseases differ markedly. Babesiosis due to *B bovis* is characterised by fever up to 42°C, anorexia, depression, increased respiratory rate particularly on exertion, muscle tremor, reluctance to move, anaemia and jaundice. These signs are often seen before parasites can be detected in blood smears.

Clinical signs in babesiosis due to *B bigemina* develop late in infection when patent parasitaemias are usually advanced. Cattle do not appear to be as sick as those with *B bovis*, but haemoglobinuria occurs more consistently, anaemia and jaundice occur more rapidly and death can occur with little warning.

The fever during infections may cause pregnant cattle to abort⁴ and bulls to show reduced fertility lasting six to eight weeks.⁵

Cattle infected with *B bigemina* may exhibit irritability and aggression but signs of cerebral derangement such as circling, head pressing, mania and convulsions have only been reported in cases of *B bovis* infection. Severe cases of cerebral babesiosis are refractory to treatment.

Cattle with advanced babesiosis are very susceptible to stress and sometimes collapse and die while being driven to a yard for treatment. Babesiosis is more severe in older cattle and is unusual in cattle less than 9 months old.

Anaplasmosis

Anaplasmosis, like babesiosis, increases in severity with the age of the animal. It is usually more protracted than babesiosis, temperatures of affected cattle steadily increasing to a maximum of 41°C over a 10-21 day period. This is accompanied by anaemia, weakness and respiratory distress particularly after exercise. Depression and anorexia are not marked in the early stages, but become more obvious as the disease progresses. There may be jaundice and frequently a marked loss of condition. Haemoglobinuria is not seen in uncomplicated *A marginale* infections although the urine is often brown due to the presence of bile pigments. Severe outbreaks are accompanied by abortions in pregnant cattle.

Epidemiology

Babesiosis

Both *B bovis* and *B bigemina* are transmitted transovarially (from one generation to the next via the egg) by *B microplus*, a one-host tick, with transmission after final development in the salivary glands of the larval stage (*B bovis*) or the nymphal and adult stages of the tick (*B bigemina*). *B bovis* infection does not persist in *B microplus* beyond the larval stage while *B bigemina* can pass from one generation to the next even when the ticks feed on non-susceptible hosts.⁶

The prepatent period after *B bovis* infection is generally 6-12 days with peak parasitaemias reached about 3-5 days later. In the case of *B bigemina*, the prepatent period is 12-18 days from the time ticks first attach but this can be shortened by transfer of ticks amongst cattle in close proximity.⁶

Cattle between 3 and 9 months of age have higher innate resistance to most tick-borne diseases and consequently disease incidence and corresponding mortality are typically lower for this stock class.⁷ If a sufficiently high proportion of a herd are consistently exposed to *Babesia* spp as calves a state of endemic stability may develop in which clinical tick fever is rarely seen.⁸

B bovis infection rates in *B microplus* are often very low. As a result, infection does not always follow exposure to ticks if infestation levels are low. Droughts, dipping practices or use of tick-resistant breeds of cattle may delay transmission of *B bovis* for months or years resulting in an unstable disease situation.⁸

B bigemina transmission rates are higher than *B bovis* because more ticks carry it and therefore endemic stability is more likely to develop to *B bigemina* than to *B bovis* in regions where both are present.⁸

Bos indicus breeds almost invariably experience milder signs of babesiosis than *Bos taurus* breeds. This phenomenon is thought to be a result of the evolutionary relationship between *Bos indicus* cattle, *Boophilus* spp. and *Babesia* spp.⁸

B bovis infection lasts for at least four years in *Bos taurus* cattle and *B bigemina* usually less than 6 months. Immunity to both parasites remains for at least four years regardless of the status of the infection.⁶ Most cattle with a significant *B indicus* content lose *B bovis* infection within 2 years, but immunity to *B bovis* lasts at least 3 years.⁹

Because the *B bovis* infection does not persist in ticks beyond the larval stage, infected cattle are the main reservoir of infection. However, in the case of *B bigemina* where the infection can persist from one generation of ticks to the next without reinfection and the carrier state in cattle is often short-lived, ticks play an important role not only as vectors, but also as reservoirs of infection.

A positive result of a serological test for *B bovis* is a clear indication of prior infection. Antibodies usually persist for at least four years after a single infection,

presumably due to the long duration of the carrier state, particularly in *Bos taurus* cattle. However, the proportion of false negative results to serological tests increases as the animals begin to eliminate the infection.

Serodiagnosis of *B bigemina* is considerably more difficult. One-way cross-reaction of antibodies to *B bovis* with *B bigemina* antigens can cause false positive results in some tests and the relatively short duration of the carrier state leads to false negative results because of declining antibody titres.

Anaplasmosis

A marginale in Australia is usually transmitted biologically by the vector *B microplus* although mechanical and *in utero* transmission may also occur. Transmission of *A marginale* by *B microplus* is by trans-stadial and intra-stadial means, not transovarial means. The transfer of larval and adult male *B microplus* from tick infested to uninfested cattle under field conditions does occur. Intra-stadial transmission by interhost transfer of male *B microplus* from infected to susceptible cattle may be the most important method because males can survive for longer than two months. For ticks to transmit the infection, it is therefore necessary to have *A marginale*-infected cattle in close contact with susceptible cattle.¹⁰

Despite being more resistant to *A marginale* infection than *Bos taurus* cattle, *Bos indicus* cattle can suffer from anaplasmosis¹¹ and *Bos taurus* and *Bos indicus* cross-bred cattle ($\frac{1}{2}$ to $\frac{3}{4}$ *Bos indicus*) have been reported as equally susceptible to anaplasmosis.^{12, 13}

Antibody titres to *A marginale* fall over time but there is little field evidence of immunity waning to *A marginale*.¹⁴ Animals inoculated with *A centrale* remain infected for a long time, probably for life¹⁵; there is no indication that *A marginale* would be different. A positive result to a serological test indicates prior exposure but the proportion of false negative results increases with time.

Occurrence and Distribution

Babesiosis and anaplasmosis are listed in the OIE Terrestrial Animal Health Code and present in parts of Australia where the vector, *B microplus*, is present. Distribution of the cattle tick and the disease status in different States are outlined in *Animal Health in Australia Report*.¹⁶

B microplus requires high humidity and ambient temperatures of at least 15-20°C for egg laying and hatching, and so persists only in northern and north-eastern, coastal and sub-coastal regions of Australia.⁴ Tick populations in these areas fluctuate widely and influence the epidemiology of babesiosis and anaplasmosis in the region.

Babesiosis and anaplasmosis are not notifiable within the prescribed tick-infested parts of Queensland, Northern Territory and Western Australia. No regulatory control measures are in place in these parts other than those aimed at preventing the spread of ticks into tick-free areas or states. It is the responsibility of property owners to implement the necessary control or risk management strategies to minimise losses from tick fever, including use of vaccine.

In the other States and in the tick-free parts of Queensland, Northern Territory and Western Australia, babesiosis and anaplasmosis are notifiable. Outbreaks are managed under state legislation, primarily through quarantine of infected and adjoining properties, and eradication of the tick. Cattle in tick-free areas can, subject to government approval, be vaccinated against tick fever if they are to be moved to endemic regions at some future time.

While cattle moving into the endemic area are at high risk, most outbreaks occur in cattle born in the endemic area for reasons explained under epidemiology.

More than 8 million cattle in tick-infested parts are potentially at risk to tick fever and, in Queensland alone, 150 to 300 outbreaks are reported annually with many more going unreported. At least 80% of these outbreaks are due to *B bovis*, 10-15% to *A marginale* and the balance to *B bigemina*.

Costs due to tick fever are incurred not only from mortality, ill-thrift, abortions, loss of milk/meat production and from control measures (such as acaricide treatments, purchase of vaccines and medications), but also because of its effect on international cattle trade. Losses and control of babesiosis and anaplasmosis were calculated in 1999 to cost the Australian cattle industry US\$16.9m per annum with a further US\$6.4m attributed directly to the impact of the vector.¹⁷

Cattle develop a long-lasting immunity after a single infection with *B bovis*, *B bigemina* or *A marginale*, which is the basis for the development and use of live attenuated blood based vaccines.⁸

Gross Pathology

Babesiosis

The main pathological change after *B bovis* infection is the development of a hypotensive shock syndrome with intravascular stasis and sequestration of parasitised red blood cells in the peripheral circulation. Anaemia is progressive. Dead animals are usually anaemic, with jaundice, haemoglobinuria, excess thick granular bile, ecchymotic haemorrhages of the epicardium and endocardium, and congestion of the brain and visceral organs. A cherry pink discolouration of the cerebral cortex is characteristic of acute *B bovis* infections. The spleen is markedly swollen with bulging of the contents from the cut surface. When the disease is more protracted the carcass may be pale with slight jaundice, the kidneys and brain moderately congested or even pale, the spleen and liver only slightly enlarged, and the heart may present a more haemorrhagic appearance than in acute cases. Thick, granular bile is a constant finding.

The pathogenesis of *B bigemina* infection is almost entirely related to parasite-induced intravascular haemolysis. Anaemia, jaundice, haemoglobinuria and excess thick granular bile are commonly seen in fatal cases but not congestion of the brain and visceral organs. Cardiac haemorrhages and splenic enlargement are not as marked as after *B bovis* infection, but pulmonary oedema is a more regular feature.⁶

Anaplasmosis

In anaplasmosis, anaemia develops due to extensive erythrophagocytosis initiated by parasite-induced damage of red blood cells and anti-erythrocytic antibodies.

Intravascular haemolysis is not a feature. At necropsy, there is marked anaemia and frequently emaciation. Jaundice may be present. The liver is enlarged and orange in colour, the bile may be thickened, and the spleen often enlarged and softened. Cardiac haemorrhages are sometimes seen. The urine is darker than normal but free of haemoglobin.

Diagnostic Tests (General)

Demonstration of parasites

The specimens required vary according to the degree of sickness of the animals, whether they are alive or dead and whether a dead animal has decomposed to any extent. Specimens from treated cattle are frequently unsuitable for examination. Jugular blood smears are of less value than capillary smears in the diagnosis of tick fever.

It is not possible to differentiate between infections with *B bovis*, *B bigemina* and *A marginale* on the basis of clinical signs and gross pathological changes alone; therefore, investigators should always take and store samples of tissues from cases of suspected tick fever. Beyond their use for diagnostic purposes, these samples can be a valuable source for epidemiological and economic evaluations of the role of each tick fever parasite.

Diagnostic procedures are used to identify parasites causing acute disease and to detect infection in healthy carriers for the purposes of disease and regulatory control.

During primary infections, parasites are relatively abundant for up to 10 days and 20 days in the blood of cattle infected with *Babesia* and *Anaplasma* respectively. Thereafter, parasitism persists at low levels for variable periods, being more likely to be of long duration for *B bovis* and *A marginale* than for *B bigemina*.

A diagnosis of the clinical disease or cause of death is usually made by demonstrating the parasites microscopically in blood and/or organ smears and by ascribing a level of significance to the infection based on the number of organisms present and other factors. Sensitive PCR assays can demonstrate the presence of organisms, but do not lend themselves well to large-scale testing, pose significant quality control and cost problems and are not very useful in determining the significance of the infection. Serological tests to detect specific antibodies are not of value in the clinical stage of the disease, and are used only for research, epidemiological studies or detection of carriers for movement control.

Detection in blood smears

Thin peripheral blood smears stained with a Romanowsky type stain (for example Giemsa) are required for detecting all three tick fever organisms and for differentiating between species.

B bovis is a small parasite, usually centrally located in the erythrocyte. The single form is small and round measuring 1 to 1.5 μm . The paired form of *B bovis* is typically pear-shaped, usually separated at an obtuse angle, with a rounded distal end

and each measuring approximately 1.5 µm by 2.5 µm. *B bigemina* singles are irregular in shape and large sometimes equalling the diameter of the erythrocyte. The paired form of *B bigemina* is joined at an acute angle within the mature erythrocyte, pear-shaped, but with a pointed distal end. Each pair is approximately 3 to 5 µm long and 1 to 1.5 µm wide, but usually with two discrete red-staining dots of nuclear material in each parasite (*B bovis* always has only one). Pairs of *B bovis* that are larger than any *B bigemina* are occasionally seen.

A marginale appear as dense, roundish, dark blue or purple intraerythrocytic bodies approximately 0.3 to 1.0 µm in diameter with most situated on or near the margin of the erythrocyte. The larger organisms appear slightly irregular with each being an aggregate of 8 subunits. Anaplasms are neither morphologically striking nor distinctively stained by the Romanowsky method and they are difficult to distinguish from specks of foreign matter and distortions of the erythrocytes in imperfectly made smears.

The temptation to submit only the blood film made during the collection of blood from the jugular vein or tail vessels should be resisted. Whereas such a specimen is usually less contaminated than a specimen of capillary blood, it may contain 20-fold fewer *B bovis* organisms than the latter, because this parasite has a predilection for the capillary circulation.

Thick blood films are 10 times more sensitive than thin blood films and are therefore very useful for the detection of low level *Babesia* spp infections. Blood is not spread over a large area and is not fixed before staining, thus allowing lysis of the red blood cells and concentration of the parasites. The sensitivity of this technique is such that it can detect parasitaemias in the range of 1 parasite in 10^6 - 10^7 erythrocytes.¹⁸

Species differentiation is good in thin films but poor in thick films. Thin films are usually adequate for detection of acute infections, but not for detection of carriers where the parasitaemias are mostly very low. Parasite identification and differentiation can be improved by using a fluorescent dye, such as acridine orange instead of Giemsa.¹⁹

Diagnoses are sometimes not confirmed at the laboratory because badly prepared or unsuitable specimens are sent. At other times, the specimens are good, but confirmation is not possible because of factors associated with the disease itself. *Babesia* may be difficult to detect during the recovery stage when parasitaemia falls rapidly, and when specimens are collected a day or so after specific treatment. *A marginale* is sometimes difficult to recognise in the recovering animal because of the presence of an intense anaemic response as punctate basophilic granules may resemble *A marginale* and confuse the diagnosis.

Table 1. Specimens required from suspected cases of tick fever

Animals Available	Appropriate Specimens
Acutely sick	Thin and thick capillary blood smears from as many cattle as possible
Dead animals	Thin blood smears from an extremity Organs Smears (in decreasing order of importance): brain, kidney, heart muscle, spleen, liver
Subacutely sick animals and recovering animals	Thin and thick blood smears from as many cattle as possible Blood in EDTA for haematology Blood serum

Detection in brain tissue

Postmortem deterioration occurs more slowly in the brain than in any other tissue, and in infected animals the brain is a richer source of *B bovis* than other organs. After the parasitaemia crisis and when organisms are rare in other organs and peripheral blood, brain smears are useful in establishing a diagnosis in dead animals. The predilection of *B bovis*, but not *B bigemina*, for brain capillaries helps to identify the causative organism when parasites have degenerated to be morphologically indistinguishable. *B bovis* is frequently present in detectable numbers in the brains of clinically normal carriers, and some judgement may be required in deciding whether it is pathologically significant based on parasite numbers, gross pathology and case history.²⁰

Detection in visceral organs and decomposing tissue

All three parasites can be demonstrated in smears of visceral organs, but their morphology changes with post mortem degeneration. The availability of a freshly dead animal ensures that the clinical diagnosis of babesiosis or anaplasmosis will be confirmed or negated - providing correct specimens, including smears of peripheral blood, are taken. It is sometimes difficult to decide whether *A marginale* is present among the tissue debris of an organ smear, and also *B bigemina* may round up and contract to resemble *B bovis* in internal organs. Anaplasmas are more easily recognised in thin blood films, and the morphology of *Babesia* spp in the peripheral circulation deteriorates less rapidly after death. Therefore, an accompanying thin peripheral blood smear is needed for confirmation of anaplasmosis and may also be needed to differentiate *Babesia* spp.

Parasites are destroyed by autolysis in the host. The time taken depends on the ambient temperature and the degree of post mortem bacterial invasion. Parasites may disappear in less than 24 hours after death in hot conditions but may be recognisable for up to 3 days in cool weather. Organs deteriorate at different rates, and some organs are more heavily parasitised than others. Thus, kidney smears are preferred to spleen smears from the recently dead animal because parasites are usually more readily found in kidney than in spleen. However, the spleen appears less susceptible than other visceral organs to bacterial invasion and therefore should always be sampled when post mortem decomposition is evident.

Direct immunofluorescence is useful for the identification of the causative organism in decomposing tissue.²¹ Bovine antisera against *B bovis* and *B bigemina*, respectively, conjugated with fluorescein isothiocyanate (FITC), enable these parasites to be identified when they are morphologically indistinguishable in blood films and organ smears. However, because this procedure cannot be done on fixed blood smears, duplicate smears should be collected. The sensitivity of this test makes it unreliable when the parasitaemia is low.

Interpretation of results

When the laboratory reports that no organisms were seen, the possibility of the disease condition being tick fever cannot be entirely excluded. Conversely, a positive finding does not invariably mean that tick fever is the primary cause of what is observed.

B bovis is by far the most common cause of tick fever in Australia but there is little correlation between clinical severity and the parasitaemia in circulating blood. Even in very sick animals it is often present in small numbers only and, consequently, a positive finding in blood smears is usually reported as being significant, regardless of the parasitaemia.

With *B bigemina*, interpretation of the results is often difficult because this parasite is sometimes found in clinically healthy animals. A report of a significant *B bigemina* infection is based on 1% or more of red blood cells being infected. A parasitaemia of less than 1% may be regarded as significant if there is evidence of anaemic changes and the history is strongly suggestive of tick fever.

Results of examination of samples for *A marginale* are also difficult to interpret. A positive result is usually reported as being significant if more than 5% of red blood cells are infected, or where the infection is accompanied by marked anaemic changes of the cells and the history is suggestive of tick fever.

Differentiation of *B bovis* and *B bigemina* is difficult when gross morphological changes have occurred in the parasites. Also, degenerate parasites of both species can resemble *A marginale*. These problems are likely when animals are sampled after treatment with tick fever drugs, or some time after death. In such cases, duplicate smears of blood and of organs sampled at necropsy are examined with the Direct Fluorescent Test.

Theileria buffeli is a very common infection in Australia, and organisms are often present in readily detectable numbers in blood films of clinically healthy cattle. It is a cause of disease only on rare occasions and detection in a blood smear will be considered to be significant only if the parasitaemia is very high (that is, more than 20% of erythrocytes infected) and is associated with clinical or haematological evidence of a haemolytic condition. Some morphological forms of *T buffeli* can be confused with *B bovis* and degenerating *T buffeli* can resemble *A marginale*.

Histopathology

Babesiosis

The most striking microscopic change seen in acute *B bovis* infections is sequestration of parasitised erythrocytes and resultant congestion in capillaries and visceral organs, especially in the brain and kidneys.⁶ Sequestration is not a feature of *B bigemina* infections with parasitised cells evenly distributed throughout the circulatory system and little or no congestion.⁶

Variable degrees of centrilobular necrosis of the liver are seen in *B bovis* and *B bigemina* infections. In both infections, but particularly with *B bovis*, there are marked accumulations of macrophages, neutrophils, lymphocytes and plasma cells within the sinusoids and central veins of the liver. Biliary retention with distension of canaliculi occurs in some cases. Mild to moderate necrosis of tubule epithelium with cast formation is seen in the kidneys of most *B bovis* infected animals. Excess haemosiderin is present in macrophages in the liver, lymph nodes, lungs and to a lesser extent in the spleen and kidneys. Phagocytosis of erythrocytes and cell debris is most marked in the hepatic and renal lymph nodes, but can also be detected in the liver and spleen. In the spleen there is congestion and a marked reduction in the ratio of white to red pulp. Germinal centres generally contain few cells, and the surrounding layer of small lymphocytes may be greatly reduced. There is generalised hyperplasia of the reticuloendothelial system.

Pulmonary oedema is seen more frequently in *B bigemina* than in *B bovis* cases as is extensive necrosis of the red pulp of the spleen.⁶

Anaplasmosis

Hepatic necrosis is also a feature of this disease and is generally more severe than in babesiosis. The distribution of necrosis is centrilobular. Fatty infiltration of hepatic parenchymal cells is a constant feature, which is not found in babesiosis probably because of the more protracted course of anaplasmosis. Biliary retention is marked in most cases of anaplasmosis as are accumulations of haemosiderin in cells of the reticuloendothelial system, particularly in the lung and renal and hepatic lymph nodes. Splenic changes are similar to those occurring in babesiosis except that there are generally more extensive accumulations of plasma cells in the red pulp in anaplasmosis. Lymphocytes, plasma cells and neutrophils accumulate in lymph node sinuses and hepatic sinusoids and into areas of hepatic necrosis. Changes indicative of renal degeneration are generally mild.¹⁰

Confirmation of the carrier state

It may become necessary to confirm a carrier state in an animal following a positive tick fever serological test associated with livestock movements particularly for export or in the validation of a serological test. Various methods have been used for this with the newer polymerase chain reaction (PCR) assays currently being the most economic and practical option, but others can be used depending on the circumstances.

PCR assays have proven to be very sensitive particularly in detecting *B bovis* and *B bigemina* in carrier cattle. Detection levels as low as three parasitised erythrocytes in 20 µL of packed cells (or 1 per 10⁹ cells) have been claimed.²² A number of PCR techniques have been described that can detect and differentiate species of *Babesia* in carrier infections.^{23, 24, 25, 26} PCR assays to differentiate isolates of *B bovis* have also been described.²⁷ The application of the reverse line blot procedure, in which PCR products are hybridized to membrane-bound, species-specific oligonucleotide probes, to *Babesia* has enabled the simultaneous detection of multiple species even in carrier state infections.²⁸

PCR-based tests to detect *A marginale* infection in carrier cattle have also been developed.²⁹ A sensitive and potentially specific PCR based test capable of identifying as few as 30 infected erythrocytes per mL of blood, or about 1 per 10⁸ erythrocytes has been developed.³⁰

In-vitro culture methods have been used to demonstrate the presence of carrier infections of *Babesia* spp,³¹ and *B bovis* has also been cloned in culture. The minimum parasitaemia detectable by this method will depend, to a large extent, on the facilities available and the skills of the operator,¹⁸ but could be as low one organism in 10¹⁰ red cells,¹⁹ making it a very sensitive method for the demonstration of infection. An added benefit is that it is 100% specific.

Confirmation of infection in a suspected carrier animal can also be made by transfusing approximately 500 mL of jugular blood intravenously into a splenectomised calf known to be *Babesia/Anaplasma*-free, and monitoring the calf for the presence of infection. This method is cumbersome and expensive, and obviously not suitable for routine diagnostic use.

The administration of a corticosteroid releases *B bovis* from immune control in a high proportion of carriers, enabling diagnosis in thick smears or even thin films.³² Betamethasone given daily at the rate of 0.05 mg/kg for 5 days produced relapses during the course of treatment or within a week of its completion. Corticosteroids are not as useful in invoking relapses in *B bigemina* infections, but are reported to in anaplasmosis.³³

Splenectomy also appears to have differing effects in causing relapse parasitaemias in carrier animals in the three infections. It is followed regularly by patent parasitaemia with *B bigemina* and *A marginale*, but less frequently with *B bovis*.

Detection of immune response

Serological tests for babesiosis

If serological reagents or kits containing biologicals are imported, an Australian Quarantine Inspection Service (AQIS) permit may be required and their use may be restricted to Quarantine Approved Premises. Details are available on the AQIS website.

The indirect fluorescent antibody (IFAT) test has been widely used to detect antibodies to *Babesia* spp, but the *B bigemina* test has poor specificity and both have the disadvantages of low sample throughput and subjectivity.³⁴ *B bovis* antisera react with *B bovis* and *B bigemina* parasites in an IFAT but *B bigemina* antisera react only with *B bigemina* parasites and not with *B bovis* parasites.³⁵ This antibody cross-reactivity as measured by the *B bigemina* IFAT was restricted to the period during, and shortly after, recovery.³⁶

Cross-reactions with antibodies to *B bovis* in the *B bigemina* IFAT are a particular problem in areas where the two parasites coexist. Drug-sterilisation of infected animals is followed by a rapid loss of reactivity in the test.^{37, 38}

An enzyme-linked immunosorbent assay (ELISA) for the diagnosis of *B bovis* infection that uses a whole merozoite antigen has a reported sensitivity and specificity of 100% and 99.4%, respectively.³⁹ When 647 field sera were tested, 96% agreement with the IFAT was reported. More recent competitive ELISAs using recombinant merozoite surface and rhoptry associated antigens of *B bovis* may perform similarly, but have not been widely validated.^{40, 41, 42} Initial evaluation in Australia suggests that the test developed by Goff *et al*⁴⁰ performs similarly to the whole merozoite antigen ELISA (Molloy, unpublished).

Despite the efforts of several investigators in different laboratories, there is still not a good well-validated ELISA available for *B bigemina*. ELISAs for antibodies to *B bigemina* typically have poor specificity. In one study, *B bigemina* immune serum reacted similarly with antigens prepared from parasitised and non-parasitised erythrocytes and this was attributed to cross-reactions with bovine fibrinogen.⁴³

A *B bigemina* indirect ELISA kit using recombinant immunodominant antigen developed at the International Livestock Research Institute (ILRI)⁴⁴ is marketed by SVANOVA Biotech AB. This test has undergone only preliminary validation for use in Australia (Molloy, unpublished). Initial results suggest that the test may be useful but specificity would be a problem if the positive threshold prescribed by the manufacturer was applied.

A competitive ELISA developed and validated in Australia and shown to have sensitivity and specificity of 95.7% and 97%, respectively, is the only test in routine use.⁴⁵ There was no evidence of cross-reaction of antibodies to *B bovis*. In the absence of any other workable test for *B bigemina*, the procedure for this assay has been included in the test methods.

Serological tests for anaplasmosis

Complement fixation (CF) tests for anaplasmosis have been used extensively for many years. However, the CF test is ineffective for identifying cattle persistently infected with *A marginale* and can no longer be recommended as a reliable assay to detect infected animals.⁴⁶

The card agglutination test (CAT) is most often used in Australia for the regulatory certification of export cattle, because of its greater sensitivity.^{47, 48} However, some countries of destination insist on the CF Test.

A competitive ELISA using recombinant major surface protein 5 (rMSP5) of *A marginale* is available in kit form from VMRD.^{29, 30} In one study, the sensitivity and specificity of the rMSP5 ELISA for detection of antibodies to *A marginale* or *A centrale* in sera were 96% and 95%, respectively.³⁰ In an Australian study, the sensitivity and specificity were 100% and 98%, respectively, which was very similar to these characteristics for the CAT. The agreement between this ELISA and the CAT depended on the positive threshold selected for the ELISA but the maximum achievable agreement was 91.5%.⁴⁸ It was concluded that the competitive inhibition ELISA is a useful alternative to the CAT for detection of *A marginale* and *A centrale* infection in cattle.

An indirect ELISA also based on a rMSP5 antigen of *A marginale* is available in kit form from SVANOVA Biotech AB.⁴⁹, but Australian evaluation is incomplete.

Part 2. Test Methods

Microscopic detection of *Babesia* and *Anaplasma* organisms

Principle of the test

Babesia and *Anaplasma* are intra-erythrocytic organisms, which are detectable by light microscopic examination of stained smears of blood and organs prepared during the acute phase of the infection.

Reagents and materials

Sorensen's Phosphate Buffer

Prepare solution A by dissolving 9.465 g Na₂HPO₄ in 1 litre of distilled water and solution B by similarly dissolving 9.07 g KH₂PO₄, giving a concentration of 1/15 M for each salt. Mix solution A and solution B in ratio of 4:1 to give pH 7.4. Check with pH meter.

Giemsa's Stain

Some commercial preparations give suboptimal staining of *Babesia*. The reasons lie in the variable composition of the component dyes,⁵⁰ in particular the quantities and proportions of the thiazine compounds (methylene blue, azure A, azure B, azure C and methylene violet). It is good practice to obtain a minimal quantity of stain, test it, and if it proves satisfactory to purchase a large consignment from the batch. Most commercially available liquid stains give poor results. Make up fresh supplies as required from the powder as follows: Add 4 g Giemsa powder to 250 mL of AR glycerol in a dry and clean 500 mL flask. Stopper the flask and place in a 56°C oven for 24 hours. Add 250 mL AR methanol and stir on a magnetic stirrer for 24 h. Bottle in an oven-dried 500 mL bottle.

Test procedure: Microscopic detection of *Babesia* and *Anaplasma* organisms

Preparation of thin blood smears

- Greasy slides (from handling with the fingers) should be cleaned thoroughly before use.
- When handling the slide, hold by the edges to avoid contaminating the area where the blood smear will be, or has been made.
- Clip or fold the hair from the tail tip or from a site on the ear; brush off any dirt and scurf.
- Prick the tail tip or ear with a sharp needle, scalpel or razor blade to obtain capillary bleeding.
- Allow the blood to well up before touching the corner of the 'pusher' slide onto the top of the drop of blood.
- Transfer the blood from the corner of the 'pusher' slide to one end of the slide on which the smear is to be made.
- Wipe the end of the 'pusher' slide clean and dry.
- Then touch the 'pusher' slide down on the specimen slide, allowing the blood to spread along its back edge.

- With the 'pusher' slide at a 30⁰ angle on the specimen slide, move it smoothly forward in one quick movement maintaining light pressure. This should spread an even, bullet shaped film of blood onto the specimen slide.
- Wave the slide to air dry it or *gently* heat over a flame (for example, a lighted match) if very cold or wet.
- Identify by writing with a lead pencil on the thick end of the blood smear itself, on frosted section of slide, or on the glass with a diamond stylus (diamond scribe, diamond pencil).
- Wrap in clean dry paper or tissues or place in slide mailer and at all times keep away from moisture (do not put in refrigerator), formalin fumes, flies and cockroaches.

Preparation of thick blood smears

- Place a drop of capillary blood on the middle of the slide.
- The amount is not critical but should not be greater than the volume of half a match-head.
- The blood can be conveniently spread using the corner of another slide.
- Use a circular motion to gradually increase the area of the drop of blood until it is 0.5-1.0 cm in diameter.
- The smear is the right thickness when it is just possible to see the hands of a watch or print through it.
- Keep the slide on a horizontal surface until the smear is dry. Identify and wrap in paper or tissue.
- The whole operation should be carried out in as clean a manner as possible.
- Avoid exposure of the smear to moisture, insects or formalin fumes.

Preparation of brain smears

- Using scissors or a scalpel blade, slice a piece of grey matter from the cerebrum, that is, the grey layer over most of the two large cerebral hemispheres.
- Place a piece about the size of a match-head about 2 cm from the end of the specimen slide.
- Take a second slide and lay it on top of the grey matter, but with the long axis at right angles to the first.
- Press down carefully and move the top slide sideways to the far end of the lower slide (this crushes the brain and spreads it thinly).
- Dry either by waving it in the air or by warming over a flame on a cold or wet day. Identify and wrap in clean paper or tissue.

Preparation of visceral organ smears

- Make a fresh cut in the organ (kidney, heart muscle, liver, spleen) and squeeze out a little blood and prepare a thin smear of the blood.
- Alternatively, make impression smears by lightly applying the freshly cut surface of a piece of the organ to the surface of the slide in several places.

Causes of unsuitable specimens

- Smears exposed in transit to formalin and formalin fumes: pack smears in airtight container.
- Wrong Specimens: Jugular blood smears are much less sensitive than capillary blood smears for examination for *B bovis* organisms. Organ smears are often

unsuitable for detecting *A marginale* and for distinguishing *B bigemina* from *B bovis* infections.

- Lysed cells: Severe postmortem autolysis; smears took too long to dry or were affected by condensation, for example, after being placed in a refrigerator.
- Smears contaminated: Blood smears prepared from a wet or dirty tail will be contaminated with bacteria and may therefore be unsuitable for examination.
- Smears too thick.
- Smears stuck together or cover-slipped when wet.
- Organs too decomposed.
- Dirty microscope slides.

Staining of thin films

1. Place slides on staining rack film-side up.
2. Fix with absolute methanol for 1 minute.
3. Drain off excess methanol.
4. Flood surface of slide with Giemsa freshly diluted to 10% and mixed thoroughly in buffer (use 3-5 mL per slide).
5. After 15-30 minutes wash off stain with either buffer solution or tap water. Wash vigorously but for no longer than 5 seconds, or the specimen will become destained.
6. Dry slides in air or in warm oven.

Staining of thick blood smears⁵¹

1. Ensure that the smear is thoroughly dry (if not it can be warmed at 50-60°C briefly) before placing on rack. Fixation in acetone is optional.
2. Gently apply 5% Giemsa in buffer.
3. Stain for 30 minutes (can use 10% Giemsa for 10-15 minutes).
4. Wash stain off gently but thoroughly, and dry as above.

Quality Control Aspects

The sensitivity and specificity of microscopic detection of tick fever organisms are strongly influenced by a number of factors such as:

- The quality and selection of specimens submitted.
- The quality of the staining process; some brands of Giemsa result in stain deposit on the slide, as does the use of poor quality water.
- The quality of the equipment; the microscope needs to operate at the limit of the resolution obtainable with a light microscope (proper alignment, quality optics, oil immersion, x1000 magnification) to allow detection of tick fever organisms, especially *Anaplasma*.
- The skills of the microscopist.

The subjectivity of the test can be reduced by ensuring:

- All microscopists are adequately trained.
- Any diagnosis is confirmed by a second skilled operator.
- Skills of operators are validated periodically within and between laboratories.
- Submitters are trained in preparation of suitable specimens.

To improve the sensitivity of the procedure, allowance has to be made for the quality of the specimens received. As a general guide, the following rules for the examination of field smears have been found useful in the authors' laboratories.

- Good (or poor) blood smears and serious outbreak with history suggesting tick fever: Examine until parasites are found with maximum examination time of 30 minutes (perhaps more for bad smears);
- Good blood smears, not serious outbreak and unpromising case history - 5 minutes;
- Poor blood smears, not serious outbreak and unpromising case history - 10 minutes;
- Good organ smears - 3 minutes;
- Poor organ smears - 10 minutes.

Interpretation of Results

Interpretation relies heavily on the number of organisms present ("parasitaemia") and the significance ascribed it based on the reported history. For more details see Table 2 and the discussion in the section on Diagnostic Tests (General).

Comments

This procedure is useful to confirm the cause of disease during the acute phase of tick fever but it is not suitable for subsequent confirmation of exposure.

Table 2 Characteristics of Giemsa stained *Babesia* spp and *Anaplasma* spp for differentiation purposes

Character	<i>B bovis</i>	<i>B bigemina</i>	<i>A marginale</i>	<i>A centrale</i>	<i>Theileria buffeli</i>
Parasitaemia in blood smears	Low, clinical severity less related to parasitaemia in circulating blood than with other spp	Can infect up to 50% of erythrocytes	Parasitaemia varies depending on stage of infection. High parasitaemias common in acute phase, may be lower later with marked anaemic response	As for <i>A marginale</i>	Most animals carriers, parasitaemia low but detectable
Parasitaemia in organ smears	Higher in peripheral circulation than in blood, very high in brain	Same as in blood, do not accumulate in peripheral circulation	Difficult to detect, do not accumulate in peripheral circulation	Difficult to detect	Difficult to detect, do not accumulate in peripheral circulation
Size and appearance	Single parasites round and smaller than erythrocyte radius at 1-1.5 μm . Paired form separated at an obtuse angle, pear-shaped bodies 1.5 by 2.5 μm in size with a rounded distal end (but rare forms larger).	Single parasites larger than erythrocyte radius and irregular in shape. Paired form separated at an acute angle, pear-shaped, with a pointed distal end, and can be equal to the erythrocyte diameter.	Solid densely staining chromatin dots; early stages dust-like, mature stages 0.3-1.0 μm , circular, larger mature forms squarish to raspberry-like	As for <i>A marginale</i>	Single parasites usually smaller than <i>B bovis</i>
Structure and confusing factors	Nuclei round and well-defined. Seen as a single red staining dot. Cytoplasm abundant and dense	Nucleus seen as two discrete red staining dots. Many diverse single forms are found (especially large triangular singles)	Howell-Jolly bodies common in anaemic conditions; slightly larger than anaplasms and smoothly rounded, central in erythrocyte. Stain or dirt particles, irregular shape, may be refractile on focusing up and down.	As for <i>A marginale</i>	Rod and bayonet-shaped forms distinctive. Round to oval single forms also seen which can be confused with <i>B bovis</i> but have a broad nucleus as an acorn-like cap at one end of the parasite
Division and location in erythrocyte	Dividing forms usually at obtuse angle (90° - 180°). Rarely at periphery of erythrocyte	Dividing forms usually at acute angle. Rarely at periphery of erythrocyte	Mature forms divide into smaller bodies. More than 50% peripherally in red cells	As for <i>A marginale</i> . Majority located more centrally in erythrocytes	"Maltese Cross" dividing forms with 4 daughter cells
Clinical significance of parasitaemia	Any positive smear is probably significant	1% or more infected erythrocytes but less if accompanied with anaemic response	5% or more infected erythrocytes but less if accompanied with anaemic response	Will be seen only in vaccinated cattle in Australia.	Normally non-pathogenic

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

Reagents and materials

Antigen preparation is based on a technique described by Waltisbuhl *et al.*⁵² Infected blood (usually 5–10% parasitaemia) is collected from a splenectomised calf into EDTA. The erythrocytes are recovered by centrifugation and washed three times in five volumes of phosphate buffered saline (PBS), and then infected cells are concentrated by differential lysis of uninfected cells in hypotonic saline solution. Infected cells are more resistant to lysis in hypotonic saline solutions than are uninfected cells. A series of hypotonic saline solutions are prepared, ranging from 0.35% to 0.50% NaCl, in 0.025% increments. To find the optimum concentration for differential lysis, five volumes of each saline solution are then added to one volume of packed erythrocytes, gently mixed and allowed to stand for 5 minutes.

The mixtures are then centrifuged and the supernatants are aspirated. An equal volume of plasma (retained from the original blood) is added to each tube containing packed erythrocytes, and the contents of the tubes are mixed. Thin blood films are prepared from each of these resuspended blood cell mixtures, fixed in methanol, and stained with Giemsa. These films are examined under a microscope to determine which saline solution lyses most uninfected erythrocytes but leaves infected erythrocytes intact. It should be possible to achieve greater than 95% infection in the remaining intact erythrocytes. The bulk of the packed erythrocytes is then differentially lysed with the optimal saline solution and centrifuged. The sediment (greater than 95% infected erythrocytes) is lysed in distilled water at 4°C and then the parasites are recovered by centrifugation at 12,000 g for 30 minutes. The pellet is washed three times in PBS by resuspension and centrifugation at 4°C. It is then resuspended in one to two volumes of PBS at 4°C, and sonicated using medium power for 60–90 seconds. The sonicated material is ultracentrifuged, (105,000 g at 4°C for 60 minutes) and the supernatant retained. The supernatant is mixed with an equal volume of glycerol and stored in 2 or 5 mL aliquots at minus 70°C. Short-term storage at minus 20°C is acceptable for the working aliquot.

Test procedure

- i) 100 µL of antigen, diluted appropriately (typically from 1/400 to 1/1600) in 0.1 M carbonate buffer, pH 9.6, is added to each well of a polystyrene 96-well microtitre plate. The plates are covered and incubated at 4°C overnight.
- ii) Antigen is removed and the wells blocked at room temperature for 2 hours by the addition of 200 µl of a 2% solution of sodium caseinate in carbonate buffer.
- iii) After blocking, the wells are rinsed briefly with PBS containing 0.1% Tween 20 (PBST) and 100 µl of bovine serum diluted 1/100 in PBST containing 2% skim milk powder is added, and the plates are incubated at room temperature for 2 hours.
- iv) The washing step consists of a brief rinse with PBST, followed by three 5-minute washes with the same buffer (during which the plates are shaken vigorously), followed by a further brief rinse.

- v) Next, 100 μ L of peroxidase-labelled anti-bovine IgG diluted appropriately in PBST containing skim milk powder is added and the plates are shaken at room temperature for a further 30 minutes. (NB: some batches of skim milk powder may contain immunoglobulins that can interfere with anti-bovine IgG conjugates),
- vi) Wells are washed as described in step (iv) above, and 100 μ L of peroxidase substrate TMB is added to each well. The substrate reaction is allowed to continue until the absorbance of a strong positive control serum included on each plate approaches 1. At this point the reaction is stopped by addition of 50 μ L of 2M phosphoric acid and the absorbance at 450 nm is read on a microtitre plate reader.

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

Quality Control Aspects

The test is not commercially available; therefore criteria for validity will depend on the control sera and conditions used in individual laboratories. In DPI&F laboratories, using local control sera, the positive threshold is 25% of the positive control with the negative control consistently less than 10% of the positive control.

Sera should be tested in duplicate wherever possible.

If the test is performed correctly, there should be 95–100% positive reactions with *B. bovis*-immune animals, 1–2% false-positive reactions with negative sera, and less than 2% false-positive reactions with *B. bigemina* immune animals. It is possible to detect antibodies at least 4 years after a single infection.

***Babesia bigemina* enzyme-linked immunosorbent assay**

Reagents and materials

A competitive inhibition ELISA uses a monoclonal antibody (MAb) (D6) directed against an immunodominant 58 kDa antigen (rhoptry-associated antigen 1 (RAP-1)) identified by a number of groups in *B. bigemina* isolates.⁴⁵ The antigen used in the ELISA is a 26 kDa peptide, encoded by a 360 bp fragment of the p58 gene, expressed in *Escherichia coli* and affinity purified. This antigen can also be used in an indirect ELISA format, but some cross-reactivity of antibodies to *B. bovis* should be expected.

Test Procedure

- i) The recombinant 26 kDa antigen is diluted in 0.1 M carbonate buffer, pH 9.6, to a concentration of approximately 2 µg/mL and 100 µL is added to each well of a 96-well microtitre plate. The plates are incubated at 4°C overnight.
- ii) Excess antigen is removed and the wells are then blocked at room temperature for 1 hour by addition of 200 µL per well of a 2% solution of sodium caseinate in carbonate buffer.
- iii) Following a brief rinse (3 x 200 µL) with PBS containing 0.1% Tween 20 (PBST), 100 µL of undiluted serum is added and the plates are incubated at room temperature for 30 minutes with gentle shaking.
- iv) The plates are then washed with PBST (5 x 200 µL rinse, 5-minute soak with shaking), and 100 µL of peroxidase-labelled MAb D6 diluted to a concentration of 0.03 µg/mL in PBST containing 2% skim milk powder is added to each well. The plates are then incubated at room temperature for 30 minutes with gentle shaking.
- v) Plates are washed again, 100 µL TMB peroxidase substrate is added to each well, and the plates are incubated in the dark until the absorbance of the conjugate control wells (no serum) approaches 1. At this point the reaction is stopped by the addition of 50 µL of 2 M phosphoric acid and the absorbance is read at 450 nm. Positive and negative control sera should be included on each test plate.

Quality Control Aspects

Percent inhibition (PI) is calculated relative to a control well to which monoclonal antibody and conjugate is added but no serum. Because the test is not commercially available criteria for validity will depend on the control sera and conditions used in individual laboratories. In DPI&F laboratories, using local control sera the positive control is expected to exhibit more than 90% inhibition and the negative control less than 10% inhibition. A positive threshold of 30% or more is used.

Interpretation of results

The PI for test sera is calculated relative to the conjugate control ($PI = 100 - [100 \times \text{test absorbance} / \text{conjugate control absorbance}]$). Positive and negative threshold values should be determined in each laboratory by testing as many known positive and negative sera as possible.

The specificity of the ELISA has been estimated at 97.0% and the sensitivity for detection of antibodies in experimentally infected cattle is 95.7%.⁴⁵

Card agglutination test for *Anaplasma*

The advantages of the CAT are that it is sensitive, relatively inexpensive and requires few facilities to carry out, but it can be affected by temperature and humidity. Antigen only or a diagnostic kit including freeze dried Bovine Serum Factors (BSF) can be purchased from the USDA (Appendix 1). The following instructions differ slightly from those supplied with the CAT kit, but have been found to improve specificity and sensitivity under Australian conditions.

Reagents and materials

- (i) Control Sera: Negative serum plus a weak positive control are essential. Medium and strong positive control sera can also be included.
- (ii) Antigen: Must not be frozen and is stored at 4°C.
- (iii) BSF: This is serum selected from an animal with a high conglutinin level. If the conglutinin level is unknown, fresh serum from a healthy animal known to be free from *Anaplasma* can be used. The Jersey breed is often suitable. The BSF must be stored at minus 70°C in small aliquots, a fresh aliquot being used each time tests are performed (not reused after initial freeze - thaw). The inclusion of BSF improves the sensitivity of the test.
- (iv) Test Plates: clear Perspex/plastic or glass plate, marked with 18 mm diameter circles.
- (v) Glass stirring rod: to mix serum, BSF and antigen on the plate.
- (vi) 5-20 µL pipettor and tips.
- (vii) Platform rocker: to mix reagents during test development.
- (viii) Humidity chamber (lunch box): to prevent mixed test reagents from desiccating while developing on platform rocker.
- (ix) Electronic temperature probe: insert probe into humidity chamber via a hole punched in the lunchbox.
- (x) Wash bottles containing H₂O and 70% EtOH/H₂O
- (xi) Light box and timer

Test procedure

- (i) Ensure all samples for the CAT have had uninterrupted incubation at room temperature (22 to 24°C) for 48 hours to reduce natural conglutinin levels, which may cause false positives.
- (ii) Ensure all test components are at room temperature (22 to 24°C) before use (this constant temperature is critical for the test).
- (iii) Firstly validate the reagents and the test conditions by running a validation plate using only the control serum. The incubation time may have to be decreased if the room temperature is too high, but do not allow the time to drop below 4 minutes.

- (iv) On each circle of the test plate, place next to, but not touching each other:
 - 10 μ L of BSF
 - 10 μ L of sample serum or control serum
 - 5 μ L of CAT antigen
- (v) Ensure a low positive and a negative control serum are present on every test plate and that the first and last positions on the plate have a negative control. These are used as an indicator of the correct conditions.
- (vi) Mix well with glass stirrer beginning with the first negative control and ending with the positive control. After mixing each test, wipe glass stirrer with a clean tissue to prevent cross-contamination.
- (vii) Place the test plate in a humid chamber and rock at 100-110 RPM for 6 minutes (or the adjusted time determined by the validation plate).
- (viii) Read immediately against the back light.
- (ix) Read results of the reference sera first. If the positive and negative reference results are satisfactory, proceed to next step. If reference results are unsatisfactory, repeat the test. Responses to further test failures are determined by detailed examination of the various control elements in the procedure.
- (x) Positive Result: characteristic granular sediment and clumping of sediment in a ring around the edges of each test.
- (xi) Negative Result: no characteristic clumping or granular sediment.
- (xii) Suspect Result: fine sediment is visible but no ring has formed.
- (xiii) Gently rinse the plate samples off the plate with a wash bottle of H₂O and into approved AQIS waste container. When all traces of colour have gone, rinse the plate with 70% EtOH/H₂O and wipe it with clean tissue.

Quality Control Aspects

As mentioned above a low positive and a negative control serum are present on every test plate and the first and last positions on the plate have a negative control. Criteria for validity will depend on the control sera and conditions used in individual laboratories. In the authors' laboratories using local control sera, the low positive control is expected to exhibit 1+ clumping while the negative control should have no characteristic clumping

Interpretation of results

Ensure control samples are testing correctly. Results are recorded as Negative, Suspect or Positive according to the following criteria:

- Negative result: No characteristic clumping.
- Suspect result: Trace of characteristic clumping.
- Positive result: characteristic clumping 1+ to 3+.

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Appendix 1 Serological test reagent supplies

Diagnostic kits for the Anaplasma Card Agglutination test are available from: USDA, APHIS, VS, National Veterinary Services Laboratories (NVSL), P.O. Box 844, Ames, IA 50010, (515) 663-7200. It is however more cost effective to buy the antigen only and produce the bovine serum factor locally.

A competitive ELISA kit using recombinant major surface protein 5 (rMSP5) of *A marginale* is available from VMRD, Inc, 4641 Pulman-Albion Rd., PO Box 502, Pullman, Washington 99163, USA; Tel 1 509 3345815; Fax 1 509 3325356; www.vmr.com.

A *B bigemina* indirect ELISA kit using recombinant immunodominant antigen and an indirect ELISA for *A marginale* based on a rMSP5 antigen developed at the International Livestock Research Institute (ILRI) is marketed by SVANOVA Biotech AB. Kits are available from SVANOVA Biotech AB, Uppsala Science Park, Dag Hammarskjölds Väg 32, SE-751 83 Uppsala, Sweden, Tel 46 18 654900, Fax 46 18 654999, info@svanova.com, www.svanova.com.

An AQIS permit may be required to import the test kits or antigen and the tests may have to be performed in Quarantine Approved Premises.