Immunodiagnosis of and immunoprophylaxis against the haemoparasites *Babesia* sp. and *Anaplasma* sp. in domestic animals

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**Summary:** The haemoparasites *Babesia* bovis and *Anaplasma* marginale are extremely important pathogens of cattle, affecting more than 300 million animals worldwide. Species of less importance affect cattle, dogs, horses and sheep. No vaccine is available for widespread usage. This review summarises existing immunodiagnostic and immunoprophylactic methods currently available for these diseases and reports on future immunoprophylactic methods.

**KEYWORDS:** Anaplasma - Babesia - Immunodiagnosis - Immunoprophylaxis.

**INTRODUCTION**

The tick-borne haemoparasites *Babesia* sp. and *Anaplasma* sp. are major pathogens of domestic animals. The most important of these is the intra-erythrocytic protozoan *B. bovis*, which together with the less pathogenic species *B. bigemina* is widespread in most tropical and subtropical regions. Approximately 300 million cattle are at risk to these two species. Another species which affects cattle, *B. divergens* is restricted to Europe. Babesiosis is also important in sheep in north Africa, the Middle East and Asia. Less pathogenic species affect pigs in some regions of the Mediterranean and Africa, while pathogenic species of *Babesia* affect horses and dogs in Europe and Africa. Because of the importance of *B. bovis*, the review will largely concentrate on this parasite which is the most intensively studied of all *Babesia* sp.

Anaplasmosis is an intra-erythrocytic Rickettsia and usually occurs in tandem with *B. bovis* infections due to their sharing a common tick vector *Boophilus microplus*. This is especially so in Latin America and Oceania. However, *A. marginale* also occurs in some regions in isolation, especially in the USA and east Africa. Whilst infections caused by *A. marginale* are less pathogenic than those caused by *Babesia*, severe anaemia and production losses occur.

Sheep are also infected by *A. ovis* in the Middle East and Asia though this species is not regarded as being very important. As a consequence, this review will focus on *A. marginale*.

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Numerous studies have demonstrated the role of humoral immunity in the protective response of cattle to *B. bovis* infections. It was shown by Mahoney and co-workers (17) that the passive transfer of immune serum to susceptible calves conferred partial protection against *B. bovis* infection. The antibody subclass inducing immunity was IgG. It was later shown that IgG1 was largely responsible for induction of the immune response (12). Colostral antibody confers protection to calves which may persist for up to 6 months for *B. bovis* and 3-4 months for *B. bigemina* (25). In addition to humoral immunity, cell-mediated responses are also thought to be important in the protective immune response to *Babesia*. It is especially relevant that splenectomised animals are less severely affected than non-splenectomised animals (30) and it is thought that this is related to the role of cell-mediated responses causing lipid peroxidation which is observed during acute *B. bovis* infections (10). Another mechanism was recently observed with *B. bovis* infections demonstrating that some antibodies will induce opsonisation in vitro (14). Phagocytosis has been observed in most other babesial infections.

Studies on the induction of immunity with defined immunogens have led to the discovery that the major parasite antigens are non-protective but induce large antibody responses, possibly as a decoy mechanism. In addition, "spurious" antigens exist which are host-derived and, as a result of non-specific inflammation, induce strong antibody responses which are generally presumed to be of parasite, instead of host origin. Studies at CSIRO have shown that all protective antigens isolated to date are very minor components of the parasite and all induce only weak antibody responses. When native antigens are used in serodiagnostic tests, only very poor responses are detected if antibodies from animals immunised with these protective antigens are used. The major non-protective antigens account for most of the antibody response after natural infections and it is these that serodiagnostic procedures largely measure.

Upon infection, animals are immune to *B. bovis* for at least 4 years and to *B. bigemina* for 2-3 years (18, 19). In addition, immunity to one strain confers a high level of protection to other strains. In the short term at least, immunity to *B. bigemina* confers protection against subsequent *B. bovis* infections indicating that common protective antigens exist between these two species (31). *B. bovis* antigens also cross-protect against *B. ovis* infections of sheep (1).

**IMMUNODIAGNOSIS**

A large number of immunodiagnostic tests have been developed for the detection of antibodies to *B. bovis*. These include complement fixation (CF), indirect haemagglutination (IHA), latex agglutination, indirect fluorescent antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA) and a derivation of this (slide ELISA), and radio immunoassay (RIA). Only two of these tests are used routinely, namely IFAT and ELISA. Serodiagnosis of *B. bigemina* infections is less specific and only IFAT is regularly used. Serological assays for other species of *Babesia* are much less defined but generally IFAT procedures are used. The principles of the *B. bovis* ELISA and IFAT can be applied to serodiagnosis of the other species.
Indirect fluorescent antibody test (IFAT)

This test is the most widely used for diagnosis of all Babesia species. However, it has a number of limitations, especially those of operator fatigue, and in the case of B. bigemina at least, non-specificity.

The test is performed using thin films of infected blood with \( \approx 5\% \) parasitaemia. The blood is collected in an anticoagulant, washed three times in five volumes phosphate buffered saline (PBS) to remove immunoglobulins and then resuspended in two volumes PBS to which 1% bovine serum albumin (BSA) is added to cause adherence of the infected blood to the glass slide. Films are then fixed for 5 min in acetone before storage at \(-70^\circ\text{C}\).

Sera are serially diluted in two or three-fold steps starting with an initial dilution of 1/80-1/100. Sera are regarded as positive above this point. Sera may be used with or without heat inactivation at 56°C for 30 min. The slides are marked into 8-10 divisions each one cm square with an oil pen. To each test square is added 5-10 \( \mu \text{l} \) of every serum dilution using a fine pipette. The preparations are then incubated at 26°C for 1-2 h or at 4°C overnight, in a moist chamber. For controls, appropriate dilutions of positive and negative sera and a conjugate control are used with each test. After incubation, the slides are gently rinsed twice with PBS and given two 10 min washes with PBS. An appropriate dilution of anti-bovine IgG antibody labelled with fluorescein isothiocyanate which is commercially available is then added to each test square. Every new batch of conjugate must be titrated, the working range usually being between 1/1000-1/3000. After a further incubation of the slides with the conjugate at 4°C overnight, the slides are again given three 5 min washes in PBS. The wet slides are mounted in 10% glycerol in PBS and then examined by standard ultra-violet microscopy. A competent operator can examine about 150 samples per day.

Enzyme-linked immunosorbent assay (ELISA)

This test is, as yet, available only for the diagnosis of B. bovis infections.

Antigen is prepared by the following procedure as described by Waltisbuhl et al. (27). Infected blood (usually 5-10% parasitaemia) is collected from splenectomised calves into disodium ethylenediamine tetra-acetic acid (EDTA). The blood is washed three times in five volumes PBS and is then subjected to concentration of infected cells 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. To determine the optimal concentration of saline solution a series of hypotonic saline solutions are prepared, ranging from 0.35-0.50% NaCl, in 0.025% increments. Five volumes of each saline solution are then added to one volume of packed red cells, which are gently mixed and allowed to stand for 5 min. The mixtures are then centrifuged and the supernatant aspirated. An equal volume of plasma (retained from the original blood) is added to each tube containing packed red cells and mixed. Thin blood films, made from each of these resuspended blood cell mixtures, are fixed in methanol and stained with Giemsa. These films are examined by microscopy to determine which saline solution produced >95% intact infected erythrocytes by differentially lysing the uninfected red cells. Generally, the optimal differential lysis step is induced by 0.40-0.425% saline solutions. The bulk of the infected blood is then differentially lysed with the optimal saline solution and centrifuged. The sediment (>95% infected red cells) is resuspended in 1-2 volumes PBS and sonicated in appropriate volumes using medium power for 60-90 seconds. The sonicated material
is ultracentrifuged (105,000 g, 5°C, 60 min) and the supernatant retained. Haemoglobin is removed from the supernatant by mixing with CM sephadex in 0.05M PBS, pH 5.5. The relatively colourless supernatant is mixed with equal volumes of glycerol and stored in 2-5 ml aliquots at −20°C.

The ELISA test is performed as follows:

Antigen is diluted 10:1 in carbonate/bicarbonate buffer pH 9.6 and 200 µl aliquots are applied to 96 well polystyrene microtitre ELISA plates. The plates are covered and incubated either at room temperature (26°C) for 3 h or at 4°C overnight. The antigen solution is then aspirated and the plates blocked with 250 µl with 0.5% gelatin in carbonate buffer pH 9.6 for 1 h at room temperature. After five washes with PBS + 0.05% Tween 20, 200 µl of each test sera diluted in PBS are added. The plates are then incubated for 3 h at 20°C and again washed five times in PBS/Tween. Finally 200 µl of enzyme conjugated antiovine IgG diluted in PBS are added to each well. The plates are again covered and incubated at 4°C overnight. The plates are then washed five times with PBS/Tween and an appropriate substrate added and the plates sealed. During substrate incubation the plates are agitated with a microtitre plate agitator. At the end of substrate incubation time, the reaction is stopped with 50 µl of IM NaOH and the plates read on a microtitre plate reader. To control for inter-plate variation, positive and negative serum controls are included in each plate.

Each new batch of antigen and conjugate should be re-titrated using a checkerboard layout. In general terms, a working dilution of antigen would be between 1/400-1/1600 and of commercially available conjugate 1/1000-1/3000. A strong positive serum is included and the substrate reaction allowed to continue until its absorbency value is 1.0. Sera are then ranked from 1-10 relative to the positive control (Value 10) using absorbency values of test samples. Negative sera should rank 2 or less. The most suitable enzyme-labelled conjugate is horseradish peroxidase (HRP), whilst the safest and most stable substrate is 5-amino salicylic acid.

With this test it is possible to detect antibodies at least four years after a single infection. There should be 100% positive reactions with B. bovis-immune animals, 1-2% false positive reactions with negative sera and 1-2% false positive reactions with B. bigemina-immune animals.

Recently a modification of the standard ELISA was developed by Böse et al. (8). This method is dependent on extremely pure antigens produced by recombinant DNA and utilises a computer-based kinetics-linked immunosorbent assay programme (KELA) based on the technique of Barlough et al. (6). This technique measures the rate of substrate reaction over 2 min periods and calculates the regression coefficient or KELA slope value as the linear relationship between rate of substrate conversion by the enzyme and time. The advantage of this technique over the standard ELISA is that it avoids batch-to-batch variation of antigen and eliminates non-specificity due to red cell antigen contamination of native antigen.

Another modification of the standard ELISA was made recently (15). This technique, called Slide Enzyme-Linked Immunosorbent Assay (SELISA), utilises acetone-fixed smears of B. bovis-infected blood as the source of antigen. These are reacted in the standard way with primary antibody and HRP-conjugated second antibody with 4-chloro-1 napthol + H2O2 as a substrate. The slides are then viewed with a conventional light microscope. Infected cells stain bluish-purple. This test
correlates very well with ELISA and IFAT and has the advantage that expensive readers and/or fluorescent microscopes are not required for data collection. This test is very inexpensive to perform and would be ideal for laboratories in developing countries.

**Diagnosis of other species**

*a) B. divergens*

ELISA, IFAT and CF have all been used to diagnose infections with this parasite (7).

*b) Canine babesiosis*

IFAT and ELISA have been used to detect infections with either *B. canis* or *B. gibsoni* (28). These tests are largely experimental and are not in widespread usage.

*c) Equine babesiosis*

The CF test is the assay of choice and is widely used to detect carrier animals (13). The antigen source is derived from the lysis of infected red cells. Some use of IFAT is also made.

*d) Ovine babesiosis*

Although the literature pays scant attention to the diagnosis of *B. ovis* infections, work by the author, in collaboration with colleagues in Turkey, has shown that native *B. bovis* antigen is very effective in the detection of *B. ovis* antibodies by ELISA (1). IFAT is also used in a few laboratories, to good effect.

**IMMUNOPROPHYLAXIS OF BABESIA**

**Living, attenuated vaccines**

Vaccines utilising replicating *B. bovis* have been available in various forms since about 1900, but it was not until 1966 when a standardised, relatively avirulent strain of *B. bovis* was introduced in Australia that a reliable vaccine became commercially available (9). Currently, it is only in Australia and South Africa that this type of vaccine is readily obtained.

Living vaccines have a number of disadvantages. These include short shelf life, reversion to virulence when passaged in the host, reversion to transmissibility by the tick vector and the inadvertent co-transmission of other pathogens such as viruses (32). In spite of these shortcomings, the live attenuated vaccine used in Australia has proven to be extremely reliable and has been used extensively in the *Babesia* endemic zone. Smaller amounts of *B. bigemina* vaccine are used, whilst *A. centrale* is also used in conjunction with 80-90% of all *B. bovis* vaccine doses.

*B. bovis*-attenuated vaccines are produced by the rapid passage of virulent organisms through a minimum of 20 to a maximum of 30 passages in splenectomised calves. After 30 passages the strains lose immunogenicity and vaccine breakdowns occur. The vaccine is produced in a standard dose of $1 \times 10^7$ parasites and must be stored at 5°C and used within seven days. Some animals undergo severe reactions and require chemotherapy, but the majority show only mild symptoms. Once an animal has been immunised by this method it generally does not require further vaccinations and is immune for life. Natural infection results in only a very mild, transient parasitaemia in vaccinated animals.
B. divergens vaccines have also been produced in Sweden, the UK and Ireland but all proved to be virulent and the subsequent infections required chemotherapy to control them. Experimental vaccines using irradiation or passage through abnormal hosts have also been developed for this parasite but to date these have not yet been used commercially.

Non-living vaccines

a) Culture derived

Soluble exoantigens derived from B. bovis and B. bigemina infected blood cultures have been used in extensive field trials in Venezuela (20). Similarly, long-term cultures of B. canis have also been used to produce exoantigens in France (21). About 400,000 doses of this product, Pirodog, are used annually in France. It is claimed that protective immunity to the B. bovis and/or B. bigemina exoantigen vaccines lasts at least thirteen months, although another study (26) using B. bovis exoantigen prepared by the same method failed to induce any immunity. The other problem with this type of vaccine is cost. The production of non-viable vaccines using tissue-culture procedures is extremely labour-intensive and it is unlikely that this type of vaccine could be economically viable. However, in vitro cultured replicating vaccines, whilst relatively expensive to produce, could well fill a niche in those countries where only a small demand for the vaccine exists.

b) Genetically engineered

The new generation of vaccines will result from the synthesis of protective antigens using recombinant DNA techniques. Extensive studies by scientists at CSIRO, Brisbane, Australia, have reached the stage where a number of protective B. bovis antigens have been identified, cloned and expressed. These have been tested in vaccination/challenge experiments and shown to suppress parasitaemias by over 99%. Only minute amounts of these synthetic antigens are required to induce high levels of protection and fortuitously they are common to all strains of the parasite so far probed. It is also of interest that these antigens share conserved regions with other species of Babesia and it is likely that this property can be utilised to develop multivalent vaccines effective against other Babesia species. A synthetic Babesia vaccine will be extremely cost-effective.

ANAPLASMA MARGINALE INFECTIONS

IMMUNOLOGY

After recovery from infection with A. marginale, animals are partially or wholly immune to subsequent field challenge. Some virulent heterologous strains may cause mild parasitaemias, whilst only inapparent transient parasitaemias are seen with others. Sterile immunity has also been shown to last for at least eight months (16). Although the exact mechanism of immunity is not known, a number of mechanisms have been proposed. These are:

- antibody blockade of the erythrocyte binding site on the A. marginale initial body surface
- direct lysis of the initial bodies by antibody and/or complement, and
- phagocytosis and intra-erythrocytic killing of initial bodies and/or infected erythrocytes (22). Some evidence suggests that macrophages and/or T helper cells are also involved in the immune process.

The less pathogenic species *A. centrale* shares many common antigens with *A. marginale*. Upon infection it produces a mild clinical disease and, significantly, confers a good level of cross-protection to subsequent infection with virulent *A. marginale* organisms. This cross-protection has been exploited and is the basis for one of the two commercial vaccines currently available.

**IMMUNODIAGNOSIS**

A number of tests exist for the detection of *Anaplasma* antibodies. Because of the high degree of cross-reactivity which exists between *A. marginale* and *A. centrale*, and because of the crude nature of the test antigen, none of these tests can distinguish between the two species.

**Complement fixation test (CF)**

This test is undertaken using standard CF test procedures. Antigen consists of *Anaplasma* bodies which have been separated from erythrocytes by lysis (e.g. in a French pressure cell) and then washed free of most haemoglobin and cellular debris. A microtitre technique is now most commonly used as it requires very small quantities of reagents. It is essential that all reagents are correctly standardised and that the test is properly controlled. Detailed manuals for the conduct of both the standard (4) and the microtitre technique (5) for the CF test for anaplasmosis have been produced.

**Card agglutination test (CT)**

The advantages of this test are that it may be undertaken either in the laboratory or in the field and a result is available within a few minutes. The test is conducted on Brewer diagnostic cards using *Anaplasma* organisms stained with fast green dye as antigen. After mixing standard quantities of antigen and serum or plasma on a test area of the card, the card is tilted to and fro and the degree of agglutination visibly assessed after a set time (2). The sensitivity of the test is improved if a factor present in normal serum is included in the test system (3). Card test kits and associated equipment are available commercially in some countries.

**Capillary tube agglutination test (CA)**

This test is based on an assessment of the degree of agglutination occurring when *Anaplasma* antigen (consisting of partially purified *Anaplasma* bodies) is mixed with test serum in a capillary tube (23). The test was developed as an alternative to the much more involved complement fixation test but has largely been superseded by the more rapid CT.

**Enzyme-linked immunosorbent assay (ELISA)**

A sensitive ELISA for detecting *Anaplasma marginale* antibodies was described by Duzgun *et al.* (11). This test, which gave improved results in comparison to
previously reported ELISA's, utilised a "negative" antigen consisting of a preparation of red blood cells obtained prior to infection and a "positive" antigen derived from *A. marginale*-infected red blood cells taken from the same calf after infection. A net absorbance value was obtained by subtracting values obtained with test sera reacted with the positive and negative antigens. This technique markedly reduced false cross-reactions which were apparently the result of isoantigens. The procedure is similar to that described for the diagnosis of *B. bovis* infections.

**Radio-immunoassay (RIA)**

A radioimmunoassay utilising a similar technique to that described for the ELISA has been reported (33). Because this assay requires more sophisticated laboratory equipment than ELISA (which appears to have about the same sensitivity and specificity), the latter test is preferred under most circumstances.

**Indirect fluorescent antibody test (IFAT)**

Because of limitations on the number of IFA tests that can be performed by an operator in a day, other serological tests are generally preferred to IFAT. The test is performed as for babesiosis except that *A. marginale*-infected blood is used for preparation of antigen smears.

Non-specific fluorescence is the main problem encountered with the test. Antigen made from blood collected as soon as an adequate parasitaemia (5-10%) occurs is most likely to be suitable.

**IMMUNOPROPHYLAXIS OF *A. MARGINALE* INFECTIONS**

**Living vaccines**

Two types of living vaccine have been developed, one being *A. centrale*, the other involving the use of *A. marginale* attenuated by passage in non-bovine hosts such as deer or sheep. However, of the two, only the *A. centrale* vaccine has had widespread acceptance and this discussion will focus on it.

As stated earlier, this vaccine depends on the use of the mildly pathogenic *A. centrale* strain and upon its ability to induce cross-protection against subsequent *A. marginale* infection. The degree of reaction of the *A. centrale*-immune host to subsequent virulent *A. marginale* challenge infection is dependent on the strain. In some circumstances, only very mild infections ensue whereas in others quite severe reactions result which require chemotherapeutic control.

Animals immune to *A. marginale* remain protected against most strains at least, for life. This is also likely to occur with animals immunised with *A. centrale*. As with live *Babesia* vaccines, this vaccine suffers from a number of shortcomings including the need to be refrigerated prior to use and the likely co-transmission of other infectious agents. These two constraints limit its use in most tropical countries. The *A. centrale* vaccine is widely used in Australia, and to a lesser extent in Israel, South Africa and some South American countries.

**Non-living vaccines**

Like non-living *Babesia* vaccines, this type of vaccine has several theoretical advantages over live vaccines, namely:
- stability
- elimination of other infectious agents
- the non-transmission of the infection itself.

a) Killed whole A. marginale organisms

In the USA a commercial vaccine, Anaplas, which is composed of whole A. marginale organisms in an oil adjuvant is widely used. This vaccine induces partial protection against virulent heterologous strains but as it contains red cell contaminants, it also induces anti-erythrocytic isoantibodies which may result in isoerythrolysis of the new-born (29). Cattle that have been vaccinated and, subsequently, naturally infected remain carriers of the disease and may themselves suffer production losses as a result of relapses (24). The vaccine is thus only partially effective and has not been utilised outside the USA.

b) Genetically engineered

This new generation of highly defined synthetic antigens promises to overcome the shortcomings of existing A. marginale vaccines. Like synthetic Babesia vaccines, these should contain epitopes that induce cross-protection to a wide variety of A. marginale strains, preferably universal, and should be stable and cost-effective. Hopefully they should also induce long-lasting immunity after one or at most two doses.

Encouraging results have been reported by American workers with two native antigens, designated AM105 and AM106. Both contain epitopes to a number of strains of A. marginale and A. centrale. Animals immunised with them are protected against subsequent challenge with two defined strains (22). Whilst these workers have expressed these antigens in E. coli, no vaccination data has as yet been reported.

Workers at CSIRO and the Queensland Department of Primary Industries (QDPI), Brisbane, Australia, have also been jointly researching synthetic A. marginale vaccines and have recently had success with two distinct antigens, both of which have epitopes in common with two virulent strains of A. marginale as well as with A. centrale.

Whilst synthetic A. marginale vaccines are likely to be developed, from data published to date these are unlikely to be commercially available for a few years. As Babesia and Anaplasma generally occur in tandem it is desirable that synthetic vaccines to these two parasites should be used together, possibly as a multivalent vaccine.

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DIAGNOSTIC ET PROPHYLAXIE DE LA BABÉSIOSE ET DE L’ANAPLASMOSE CHEZ LES ANIMAUX DOMESTIQUES. — I.G. Wright.

Résumé : Les hémoparasites Babesia bovis et Anaplasma marginale sont des agents pathogènes responsables de maladies extrêmement graves, qui affectent plus de 300 millions de bovins dans le monde. D’autres espèces, qui provoquent des affections moins graves, touchent non seulement les bovins, mais aussi les chiens, les équidés et les ovins. Il n’existe pas de vaccin se prêtant à une large utilisation. Dans cet article, l’auteur présente les méthodes de diagnostic et de prophylaxie immunologiques disponibles actuellement, ainsi que les nouvelles méthodes d’immunoprophylaxie à l’étude.

MOTS-CLÉS : Anaplasma - Babesia - Diagnostic immunologique - Immunoprophylaxie.

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DIAGNÓSTICO Y PROFILAXIS DE LOS HEMOPARÁSITOS BABESIA Y ANAPLASMA EN LOS ANIMALES DOMÉSTICOS. — I.G. Wright.

Resumen: Los hemoparásitos Babesia bovis y Anaplasma marginale son los agentes patógenos responsables de afecciones extremadamente graves, que afectan a más de 300 millones de bovinos en todo el mundo. Otras especies provocan enfermedades menos graves, que afectan, además de a los bovinos, a los perros, los équidos y los ovines. No existe ninguna vacuna que se preste a una amplia utilización. En este artículo, el autor presenta los métodos de diagnóstico y de profilaxis inmunológicos actualmente disponibles, así como los nuevos métodos de inmunoprofilaxis que todavía están en estudio.

PALABRAS CLAVE: Anaplasma - Babesia - Diagnostico inmunologico - Inmunoprofilaxis.

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