

NEW METHODS FOR THE DIAGNOSIS OF *BABESIA BIGEMINA* INFECTION

S. MORZARIA; J. KATENDE; A. KAIRO; V. NENE & A. MUSOKE

International Laboratory for Research on Animal Diseases, P.O. Box 30709, Nairobi, Kenya

Accurate diagnosis of Babesia bigemina infection, an economically important tick-transmitted protozoan parasite of cattle, is essential in the management of disease control and in epidemiological studies. The currently used methods of diagnosis are blood smear examination and serological tests which include agglutination and immunofluorescence tests. These tests have been used in the field but because they lack sensitivity and specificity, newer and improved methods of diagnosis are being developed. The quantitative buffy coat (QBC) method, using microhaematocrit tubes and acridine orange staining allows rapid and quicker diagnosis of B. bigemina and other blood parasites compared to light microscopic examination of stained smears. Parasite specific monoclonal antibodies have been used in antigen/antibody capture enzymelinked immunosorbent assays with greater sensitivity and specificity than previously described serological tests. Similarly, DNA probes, derived from a repetitive sequence of the B. bigemina genome, offer a method of detecting very small numbers of parasites which are undetectable by conventional microscopy. An extrachromosomal DNA element, present in all the tick-borne protozoan parasites so far tested, provides an accurate means of differentiating mixed parasite populations in infected animals. These improved methods will greatly facilitate epidemiological studies.

Key words: *Babesia bigemina* - antigens - diagnosis - DNA probe - quantitative buffy coat test

Babesia bigemina is a tick-transmitted protozoan parasite of cattle. The parasite occurs mainly in the southern hemisphere and its distribution coincides with the distribution of its main vectors *Boophilus decoloratus*, *B. microplus* and *B. annulatus*. This main areas affected are Central and South America, Africa, India, South East Asia and Australia. The disease caused by the parasite is variously referred to as Texas fever, Redwater fever or Cattle Tick fever. The greatest economic impact of the disease is in areas where highly susceptible *Bos taurus* cattle are maintained. Losses usually occur when the endemic stability of the disease is disturbed, either by the introduction of naive cattle into endemic areas or by unreliable and sporadic tick control.

In many parts of the world cattle are often infected with several related haemoprotozoan parasites in addition to *B. bigemina*. For example, *B. bovis* occurs concurrently in Australia, South America and parts of Africa and Asia. In most parts of Africa, *Theileria* species also occur concurrently. Thus accurate identification of the parasite is very important in the management of the disease. Additionally, over-

all control strategies require sound epidemiological information on the prevalence and incidence of the disease in affected areas. This paper briefly reviews the methods available for the identification of *B. bigemina* and discusses the potential of new diagnostic tools, based on DNA and monoclonal antibody technology which are currently under development.

REQUIREMENTS FOR DIAGNOSIS

Four different phases of *B. bigemina* infection need to be considered in relation to the requirements for accurate diagnosis. The first is the low parasitaemic phase during the incubation of the disease, when less than one erythrocyte per thousand is infected. At this parasitaemia the infection is often undetectable under field conditions yet early diagnosis is often crucial for the successful treatment of disease. The second phase is the acute infection when parasites are easily detectable in stained blood smears by light microscopy. Accurate diagnosis is important, especially when mixed infections with the haemoprotozoan parasites are present in order to select the appropriate chemotherapy. The third phase is

during recovery when monitoring of the declining parasitaemia enables one to judge the effectiveness of chemotherapy. The fourth phase is a variable period of several months after recovery when the animal becomes a "carrier" and develops *B. bigemina* antibodies. In this phase the parasitaemia is extremely low and therefore the infection is difficult to detect. Recognition of the carrier phase and differentiation from early infection is important. This is also the phase when specific antibodies provide important information for epidemiological studies. The four phases described above can be identified by one or several tests. Some of the important methods used are described below.

DIRECT METHODS OF DIAGNOSIS

Light microscopy – Characteristic intra-erythrocytic parasites of between 2.5-3.5 μm can be observed in Giemsa-stained blood smears on examination by light microscopy. There is a range of morphological forms varying from single round vacuolate stages (trophozoites) to pyriform stages (merozoites). The size, shape and the characteristic staining of the parasite makes it relatively easy for an experienced diagnostician to identify at the species level. Low parasitaemias of up to one infected erythrocyte per 10,000 erythrocytes can be detected and identified by careful examination (100-200 fields or equivalent 0.5 μl to blood) of well stained thin and thick blood smears.

Fluorescence microscopy – Winters (1967) described an acridine orange staining method of blood smears for examination under a fluorescent microscope. He found that the sensitivity of detection of *B. bigemina* parasites using this method was much higher than detection by examination of Giemsa-stained blood smears. A recent improvement in this technique is the development of the quantitative buffy coat (QBC) method which stains and concentrates parasitised blood in one step (Levine et al., 1989). This method concentrates 50-60 μl of acridine orange stained blood in a small area in a capillary tube. Thus a significantly larger volume of blood than used on blood smears can be examined in a short time, increasing the sensitivity 100 fold. The QBC method is being widely used in the diagnosis of malaria (Rickman et al., 1989). The method also detects other cattle haemoparasites such as *Babesia*, *Theileria*, *Anaplasma*, *Borrelia* and *Eperythrozoon*. Commercially available kits

(Becton Dickinson) containing specialized pre-coated acridine orange capillary tubes, a centrifuge and fluorescence set up are adaptable for laboratory and field use. The method has great potential for large scale field surveys.

In our experience the QBC method is as sensitive as Giemsa-stained blood smear examination except in early infections when the QBC method provides a much more rapid diagnosis. The parasitised erythrocytes, unlike *Plasmodium* species, do not concentrate at the buffy-coat/erythrocyte interphase, instead the infected cells are scattered throughout the erythrocyte layer. We have recently found that *Borrelia* and *Eperythrozoon* parasites, causing pyrexia in cattle, can be easily detected using the QBC method. These organisms are extremely difficult to identify in Giemsa-stained smears.

INDIRECT METHODS OF DIAGNOSIS

Recent developments in recombinant DNA technology have led to the generation of probes which provide improved methods of diagnosis and characterization for a variety of parasites. The new methods enable identification of DNA sequences which are conserved and specific for a parasite species. DNA fragments from sequences can be amplified by inserting them into a plasmid or a bacteriophage and growing the in bacteria in a process called DNA cloning. These amplified DNA fragments can then be purified and used as parasite specific probes. Technically, the DNA probe is labelled with a radioisotope, denatured to two single stranded molecules and then applied to the denatured test DNA sample. The probe will hybridize only to complementary sequences present in the test sample thus demonstrating homology and relatedness. The hybridization signal can be visualized by autoradiography.

Typically, repetitive DNA sequences, which represent between 1-12% of the total parasite genome (Borst et al., 1980; Castro et al., 1981; Allsopp & Allsopp, 1988), or parasite species specific highly conserved ribosomal RNA (rRNA) sequences are used as probes. Such high copy number sequences in a parasite provide greater sensitivity than the use of single copy DNAs as probes. Repetitive sequences are also known to evolve rapidly within a species and these changes can be visualised by digestion with restriction enzymes to identify restriction fragment length polymorphism (RFLP).

Therefore such probes can also be used to characterize and distinguish among isolates, stocks and strains of parasites. DNA probes have been used in the diagnosis of a variety of parasitic infections (Franzen, et al., 1984; Barker, 1987; Greig & Ashall, 1987; Ole Moi-yoi, 1987; Waters & McCutchan, 1989). The advent of the polymerase chain reaction (PCR) technology, which enables amplification of target (test sample) DNA molecules by greater than a million-fold has greatly enhanced the sensitivity of DNA probes for use in the detection and identification of parasites from carrier animals.

Repetitive sequence — Buening and co-workers (1990) have cloned a 6.3 kb repetitive DNA sequence from a Mexican isolate of *B. bigemina* and have tested its suitability as a probe for diagnosis. They found the probe to be specific for *B. bigemina* and it did not cross-hybridise at high stringency with the major haemoparasites such as *B. bovis*, *A. marginale*, *T. parva* and *Trypanosoma brucei*. The probe has been shown to identify *B. bigemina* isolates from North and Central America, Africa and Australia. Thus the DNA sequence appears to be conserved among *B. bigemina* isolates from widely separated geographical regions and may have potential for use all over the world. The radio-labelled probe hybridized with as little as 10 pg of *B. bigemina* DNA and as few as 150 infected erythrocytes. Therefore the probe may be useful in detecting carrier animals. Figueroa et al. (1991) have also tested the sensitivity of the radio-labelled probe in experimentally infected cattle and have found that the detection of *B. bigemina* correlated well with the parasites observed by light microscopy. They found that the DNA probe was more sensitive than light microscopy, immunofluorescent tests or the complement fixation test for the detection of parasites in the infected cattle.

Figueroa & Buening (1989) have also tested the sensitivity of the 6.3 kb repetitive sequence probe using non-radioisotopic labelling. They showed that the probe, labelled by random-primed incorporation of digoxigenin deoxyuridine triphosphate, detected as little as 1 ng of DNA (equivalent of 0.001% parasitaemia) from seven different *B. bigemina* isolates. Thus the non-radioactive probe has a much higher sensitivity than light microscopy. This probe may have an important use in epidemiological studies of *B. bigemina*. The PCR technology will enhance its sensitivity and work is underway to

characterize the 6.3 kb repetitive sequence from different isolates in order to identify conserved sequences and design appropriate primers for detection of *B. bigemina* parasites (Figueroa, personal communication). The application of this technology will enable detection of carrier animals, on which the accuracy of information is lacking in most epidemiological studies. An additional benefit of such a sensitive DNA probe will be the identification of the parasite in infected ticks.

7.1 kb extrachromosomal element — Part of our studies on the biology of *T. parva* has focused on the characterization of a multicopy, extrachromosomal genetic element (A. Kairo & V. Nene, unpublished results). This genetic element is double stranded DNA, linear in structure and migrates with a mobility of about 7.1 kb in agarose gels. The gel purified 7.1 kb DNA crosshybridises, at high stringency, with a similar size DNA molecule in genomic DNA preparations of other species of *Theileria*; *T. annulata*, *T. mutans*, *T. taurotragi*, *T. buffeli* and *T. sergenti*. There are no RFPLs of the 7.1 kb DNA in different stocks of *T. parva*, however, there are simple and consistent RFPLs of this DNA molecule in other species of *Theileria*. This makes the 7.1 kb element a useful diagnostic probe. The 7.1 kb DNA also detected similar size DNA molecules in *B. bovis* and *B. bigemina* DNA. Simple RFLP between the two *Babesia* species was detected using the probe.

Approximately 6.0 kb of the *T. parva* DNA element has been cloned and sequenced and appears to contain genes which are normally encoded by mitochondrial DNA such as cytochrome b, polypeptide I and polypeptide III-like subunits of cytochrome c oxidase. The identification of polymorphic regions within the sequences of these genes amongst different *Babesia* species should enable generation of highly specific and sensitive oligonucleotide probes for use in PCR technology.

SEROLOGICAL TESTS

Complement fixation, passive haemagglutination, capillary tube agglutination, card agglutination and indirect fluorescent antibody (IFA) tests for the diagnosis of *B. bigemina* have been developed (Montenegro et al., 1981). The most widely used is the IFA test. The antigen commonly used in this test is derived from merozoites either grown in culture or obtained from an infected animal. The test has a higher

sensitivity and specificity compared to the other tests and is useful in experimental situations when infection with *B. bigemina* only is involved. However, it has a serious limitation for use as an epidemiological tool because of its one-way cross-reactivity with serum containing *B. bovis* antibodies (Goodger, 1971).

Relatively sophisticated enzyme-linked immunosorbent assays (ELISA) also suffer from the short-coming mentioned above because of the use of crude antigens which show cross-reactivity with various other blood parasites. With the advent of monoclonal antibody (MAb) and molecular biology technologies attention has focused on the utilization of purified, parasite species-specific antigens for enhanced sensitivity and specificity of ELISA. Such assays have been recently developed for the diagnosis of African trypanosomiasis (Nantulya, 1989; Nantulya & Lindqvist, 1989) and have been shown to be more sensitive than the parasitological techniques used in the diagnosis of chronic trypanosomiasis. Katende et al. (1990) have developed assays based on similar principles for *T. mutans* and have shown that these assays have much better sensitivity and specificity than the IFA test.

Antigen and antibody ELISAs – Recently at ILRAD antigen/antibody capture ELISAs for *B. bigemina*, based on MAbs reacting to epitopes on a 200 kD surface antigen of the merozoite stage, have been developed. These assays surpass the sensitivity and specificity of the IFA test in current use.

For the antigen capture ELISA, one MAb, immobilized onto a microtitre plate well is used to trap circulating antigen in test serum and the second horseradish peroxidase (HRP) conjugated MAb is used as an indicator antibody for detection of the trapped antigen. The reaction is visualised by adding a solution of substrate/chromogen (hydrogen peroxide/2,2-azinobis 3-ethyl-benzylthiazole 6-sulphonic acid, ammonium salt). In animals infected with *B. bigemina* sporozoite stabilate, the antigen capture ELISA detected the presence of circulating *B. bigemina* at the same time as the parasites were first detected in blood smears. The antigen presence correlated with the presence of parasites detected by blood smear examination.

For an antibody capture ELISA, a *B. bigemina* specific MAb, bound to a microtitre

plate well, is used to capture the relevant antigen from a parasite lysate. Following removal of the "uncaptured" antigen by extensive washing, the test bovine serum sample is applied. Specific antigen/antibody reaction is then visualized by an HRP-conjugated antbovine antibody. The test, using the 200 kD *B. bigemina* specific MAbs, shows no cross-reactions with sera containing antibodies to *B. bovis*, *T. parva*, *T. taurotragi* and *A. marginale*. Thus it has a higher specificity than the IFA test. In experimentally infected cattle, the presence of antibodies is detected on day 12 following infection with a sporozoite stabilate. This is three days earlier than the IFA test. High ELISA antibody titres have persisted for as long as 90 days following infection.

Comparison of antigen and antibody capture ELISA with the IFA test on field sera has shown that the combined results of the antigen and antibody capture ELISAs detect higher numbers of infected animals than the IFA test. Preliminary studies have shown that during the presence of high *B. bigemina* antibody titres circulating antigens are not detected and vice-versa. Further studies are in progress to validate the test using sera from experimentally and naturally infected cattle. This test shows great promise for use in epidemiological studies.

CONCLUSIONS

Several methods and tests are available for the diagnosis of *B. bigemina* infection in cattle. In general the direct methods of diagnosis using light microscopy and the QBC method are useful in detecting parasitaemia in the early and acute phases of the infection. The QBC method is more sensitive than the examination of Giemsa-stained blood smears by light microscopy. Thus it has potential for use in the early detection of infection.

Parasites can also be detected by using parasite specific DNA sequences as probes. A *B. bigemina* specific repetitive sequences has been cloned and characterized. The 6.3 kb repetitive sequence hybridises with several isolates of *B. bigemina* but does not detect the DNA from other bovine haemoparasites. The probe is highly represented in the total parasite genome and hence demonstrates high sensitivity. Thus it may have a role in the detection of carrier animals.

An extrachromosomal element from the *T. parva* genome has been used as a probe to differentiate between species of *Babesia*. The element has been found to be present in all the *Theileria* and *Babesia* species so far examined. The presence of simple RFLPs between species can be exploited to detect interspecies differences. The method is extremely useful in detecting mixed haemoprotozoan infections in cattle.

If appropriate oligonucleotide primers can be designed from both the repetitive DNA sequence and the extrachromosomal DNA element, PCR technology can be utilized for ultrasensitive detection and diagnosis of *B. bigemina*. At present the use of radio-labelled DNA probes for routine detection of parasites is expensive and until cheaper non-radioisotopic probes are developed the DNA probe technology will remain a research tool.

Of the many serological tests currently available, the recently developed antigen and antibody detection ELISA-based methods show the greatest promise for large-scale epidemiological surveys.

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