Trypanosomiasis in domestic animals: the problems of diagnosis

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Summary: Animal trypanosomiasis presents special problems with regard to diagnosis. The clinical signs are not pathognomonic and the standard techniques for the detection of trypanosomes are not sufficiently sensitive. Although significant improvements have been made in diagnosis, a high proportion of infections still remain undetected as the chronic, more common form of the disease, is often aparasitaemic. In the face of these constraints, alternative methods of diagnosis have been developed, most of which are for the detection of antibody responses to the antigens of the infecting trypanosomes. The most useful of these tests, in view of their sensitivity and specificity, are the indirect immunofluorescent antibody test, enzyme immunoassay (ELISA) and the card agglutination test for trypanosomiasis (CATT) which is used for the diagnosis of Trypanosoma evansi *infections. However, there are several shortcomings in antibody detection tests: the antigens used are ill-defined, thus making standardisation of the tests rather difficult with regard to sensitivity and specificity. Furthermore, some of the tests are not applicable to the field. Moreover, the presence of antibody in the serum does not necessarily reflect an existing infection, as antibodies may persist for several months following recovery. Recently, development of assays for the detection of circulating trypanosomal antigens in the blood of infected animals has circumvented this problem since antigen-positivity indicates existing infection. These new assays have not yet been fully evaluated in the field, but the data generated so far do indicate that the diagnostic strategy for the future is likely to be a combination of one of the more sensitive standard trypanosome detection techniques with antigen-trapping ELISA.*

KEYWORDS: Antigen detection - Diagnosis - Domestic animals - Parasitology - Sensitivity - Serology - Specificity - Trypanosomiasis.

INTRODUCTION

Trypanosomiasis is one of the major haemoparasitic diseases of domestic animals. The tsetse (*Glossina*)-transmitted form of the disease is endemic throughout the tropical regions of Africa where the vector is prevalent. The major pathogenic tsetse-transmitted trypanosome species are *Trypanosoma congolense*, *T. vivax* and *T. brucei* in cattle, sheep and goats and *T. simiae* in pigs. Animal trypanosomiasis is also encountered outside the tsetse fly belt, where the most important pathogenic

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trypanosome species, *T. vivax* and *T. evansi*, are transmitted mechanically by biting flies, while *T. equiperdum* is transmitted sexually. The principal domestic animals affected by *T. evansi* are camels, pigs, water buffaloes and cattle. *T. equiperdum* causes the disease in horses and donkeys.

The clinical manifestation of trypanosomiasis in animals is influenced by the host as well as the trypanosome species and "strain". In general, the disease is characterised by severe anaemia, weight loss, reduced productivity, infertility and abortion, with death occurring in some animals during the acute phase of the disease. Animals which survive often remain infected for several months or years, exhibiting a low level of fluctuating parasitaemia which serves as a reservoir for the disease. Occasionally, however, the infected animals may undergo spontaneous recovery (35, 37). Owing to these varied clinical manifestations, diagnosis of trypanosomiasis cannot be based on clinical signs alone. Laboratory confirmation of the diagnosis is an absolute necessity.

The standard laboratory method for diagnosis of trypanosomiasis is to demonstrate and identify trypanosomes in the blood of the infected animal. There are several techniques for parasite detection, which include direct microscopy, concentration techniques and animal inoculation. An alternative approach to laboratory diagnosis is to detect anti-trypanosomal antibodies in the serum of the infected animal. For this approach too there is a variety of techniques. Finally, techniques have also been developed for the detection of trypanosomal antigens in blood as a means of diagnosis. This article discusses the various approaches to laboratory diagnosis and the problems associated with them, with particular emphasis on the problems of immunodiagnosis.

PARASITOLOGICAL DIAGNOSIS

The easiest technique for detection of trypanosomes in peripheral blood is by direct microscopic examination of blood, either by the wet film method to detect motile trypanosomes or, as stained thick and thin smears, when parasites are identified on the basis of their morphology by light microscopy. Examination of wet blood films is quick and the method is suitable for screening large numbers of animals. This method, however, is insensitive as half of the infected animals may be missed (5).

The sensitivity of direct microscopic examination can be improved through concentration of the parasites by centrifugation. When unclotted blood is spun in a haematocrit centrifuge, the trypanosomes are concentrated at the buffy coat. Examination of the buffy coat is thus more sensitive than examination of blood films. Initially used for the detection of avian trypanosomes (6), this technique has gained wide application through various modifications (18, 26, 52). The microhaematocrit centrifugation technique is particularly useful in that the status of anaemia in the test animals can be assessed at the same time. This technique, however, requires the use of electricity which limits its application in the field. There are prospects for circumventing this shortcoming through the use of the battery-operated minicentrifuge described by Kelly and Schillinger (18), but this has not yet received widespread appraisal.

Improvement in the sensitivity of the concentration methods can be achieved if red blood cells are removed from the blood sample prior to centrifugation. The red

blood cells are removed by lysis either by hypotonic shock or through the use of a detergent (N. van Meirvenne, personal communication). Erythrocytes can also be removed from the test blood sample by diethyl amino-ethyl (DEAE) anion exchange chromatography (24). The miniature anion-exchange chromatography technique (MAECT) as it is known (24), is widely used to diagnose *T. gambiense* infections in man but is yet to be established as a routine test for diagnosis of animal trypanosomiasis due to its more cumbersome procedure. The technique likely to gain wider application due to its simplicity, is that employing sodium dodecyl sulphate for lysis of the red cells prior to centrifugation (N. van Meirvenne, personal communication).

Blood from suspect animals can also be inoculated into susceptible laboratory animals, usually mice or rats. This technique is more sensitive than direct microscopic examination of the blood sample (40). Animal inoculation has the added advantage that trypanosome isolates can be collected for other studies in the laboratory. This, however, is not a practical technique because diagnosis is not immediate. In addition, the cost of maintaining the animals makes the method prohibitively expensive for routine diagnosis, especially in the field. Furthermore, some trypanosome isolates, notably East African *T. vivax*, *T. simiae* and, to a lesser extent, *T. congolense*, do not infect laboratory rodents.

It can thus be seen that despite several improvements in the techniques for trypanosome detection, a high proportion of infections still go undetected as the majority of infections are chronic and often aparasitaemic. Also, the intermittent parasitaemia arising from the phenomenon of antigenic variation (9) may preclude detection of the parasites even in acute infections (26). Moreover, some of the techniques are not practical enough to be applied in the field. These drawbacks have necessitated the development of alternative methods of diagnosis.

ANTIBODY DETECTION TESTS

Several techniques have been developed for immunodiagnosis of trypanosomiasis. Most of these are based on the detection of immune responses of the animal to the infection. As early as 1899, it was shown that sera from trypanosome-infected animals caused lysis of trypanosomes (48). Trypanosome lysis was later shown to be both complement- and antibody-dependent. This was the basis for the development of the complement fixation (CF) test for the diagnosis of trypanosomiasis. The CF test was successfully applied to the diagnosis of *T. evansi* infections in several domestic animal species (43) with the aim of guarding against introduction of trypanosome-infected animals into the United States. The same test was central to the design of a successful eradication campaign against *T. equiperdum* infections in horses in Canada and South Africa. The main handicap of the CF test is the difficulty encountered in preparation and standardisation of the antigen for use in the test (12). Moreover, the test itself is difficult to perform; it is cumbersome and requires supplies of sheep red blood cells, complement, a centrifuge and a refrigerator. Thus, it is frequently unsuitable as a routine diagnostic tool.

The next generation of tools for immunodiagnosis of trypanosomiasis emanated from the observation that sera from trypanosome-infected animals were gelatinised

on treatment with various chemicals, such as formalin and mercuric chloride (14). This observation, first recorded with sera from syphilitic patients, was the basis for the development of the formol gel test and the mercuric chloride test for the diagnosis of animal trypanosomiasis. Both tests were later adopted as a routine diagnostic tool for camel trypanosomiasis (7, 20). In experimental camel infections, Leach (21) observed that the mercuric chloride test became positive ten to fifteen days post-infection and negative reactions occurred two to three months following treatment with a trypanocide. The basis for the reactions observed when formalin or mercuric chloride were added to serum was that these chemicals precipitate immunoglobulins (13), irrespective of the antigens responsible for the production of the immunoglobulins. Hence, the tests were not specific for any one disease (7). Moreover, later investigators failed to reproduce, let alone correlate, the results of these tests with patent parasitaemia in subsequent studies (19, 23).

Major improvements in the specificity and sensitivity of immunodiagnostic techniques were recorded through the introduction of the indirect haemagglutination test (10, 11). The test was applied to the diagnosis of trypanosomiasis in camels (16, 39) and other ruminants (43). The basic problems with this technique were that the antigens used were not well-defined and as a result, the test was difficult to standardise with regard to sensitivity and specificity (8). Moreover, coupling of the antigen onto the red blood cells can be irregular; the cells may be unstable on storage and non-specific agglutination can be encountered due to heterophile antibodies.

One of the most significant improvements in trypanosomiasis serodiagnosis was the introduction of the indirect immunofluorescent antibody test (IFAT) (50). The IFAT is one of the most commonly applied serodiagnostic tests for trypanosomiasis (49, 51). The antigens used are usually prepared by fixing smears of parasitised blood using a variety of fixatives (53), but there are two major problems associated with this method of antigen preparation. First, preparation of large numbers of blood smears to provide a standardised antigen preparation is cumbersome and the slides require ultra-low temperatures for storage and transportation. Second, the antigens so prepared provide substantial non-specific reactions. The technique for antigen preparation has recently been greatly improved (17) so that the test can give results which are specific enough to differentiate, to a limited extent, between infections with different trypanosome species in ruminants. The antigens are fixed in a mixture of acetone and formaldehyde in suspension (17) and can be stored and transported with greater ease. The IFAT, however, has major disadvantages since it requires sophisticated equipment and cannot be performed in the field.

The introduction of enzyme immunoassays was a major breakthrough in the field of immunodiagnosis. Enzyme immunoassays require simple equipment, the technique is straightforward and sensitive, and can be used for large-scale screening of samples. In trypanosomiasis, the test more commonly known as ELISA (enzyme-linked immunosorbent assay) was first used for diagnosis of *T. rhodesiense* infections in man (46). Luckins *et al.* (22, 23) later used it for diagnosis of *T. evansi* infections and obtained results comparable to those of the immunofluorescent technique with regard to sensitivity and specificity. A major limitation of ELISA in its application as a routine diagnostic test, is the nature of the antigens used in the assay. The antigen is usually a crude trypanosome lysate, the quality of which is ill-defined. This makes the test difficult to standardise with regard to specificity and sensitivity. A recent report, however, has demonstrated a much improved test which under laboratory conditions has been reported to be specific enough to distinguish between infections

with different trypanosome species through the use of purified antigens (15). With selection and purification of suitable antigens for use in the assay, ELISA is, therefore, likely to become the antibody-detection test of choice for trypanosomiasis diagnosis.

The persistent disadvantage of trypanosomiasis serodiagnosis has always been the lack of well-defined, standardised antigens. This problem, however, was solved for *T. b. gambiense* to some extent, by the introduction of the card agglutination test for trypanosomiasis (CATT) (25). This test predominantly detects antibodies to the surface coat antigens of a commonly occurring trypanosome antigenic variant (VAT). The antigens are fixed and stabilised on the parasite using formaldehyde (29) so that whole trypanosomes can be used in a direct agglutination test. The *T. b. gambiense* test has been applied to the diagnosis of camel trypanosomiasis with some success (54), though not in the field. This is because the VAT used in the test is found in the repertoire of VAT's expressed by *T. b. brucei, T. b. gambiense* and *T. evansi* (45). Applied to the diagnosis of *T. evansi* in water buffaloes, however, the test was found to give good results only after the inclusion of a specific anti-buffalo immunoglobulin (2, 3). Further improvement in the sensitivity of the test with regard to the diagnosis of *T. evansi* infections has recently been introduced by Bajyana Songa and Hamers (4) through substitution of the VAT used in the present test with a predominant VAT of *T. evansi*. The test is at present the simplest serological test for diagnosis of *T. evansi* infections. Its application to the diagnosis of the other species of pathogenic trypanosomes in livestock, however, is unlikely to succeed due to difficulties in stabilising their surface antigens by fixation.

All the serological tests described above measure host antibody responses to antigens of the infecting trypanosomes, specifically (i.e. using trypanosome antigens) or non-specifically (i.e. measurement of immunoglobulin rise). Antibody detection systems, however, can provide only a presumptive diagnosis as they do not differentiate between current and past infections. Therefore, a positive antibody detection test does not necessarily form a good basis for the decision to treat the animal. This is because anti-trypanosomal antibody levels may persist for several months following successful trypanocidal therapy (22, 23) or spontaneous self-cure (35, 37). They are thus more useful as epidemiological tools rather than as diagnostic procedures. For this reason, attention has continued to be focussed on the development of tests that can differentiate between exposure and a current infection.

ANTIGEN DETECTION TESTS

An alternative approach to antibody detection would involve the use of assays to detect trypanosome-specific antigen in the blood of infected animals as a means of diagnosis. Demonstration of trypanosomal antigens in the blood of the infected animal would be synonymous with parasitological diagnosis (47) and hence an indicator of current infection. The first attempt to detect circulating trypanosome antigens was made in Chagas' disease (1) but the sensitivity obtained was low. Later, Rae and Luckins (41) developed a *T. evansi* antigen detection system, using polyclonal anti-*T. evansi* antibodies. This polyclonal antibody system has, however, been found to have low specificity because cross-reactions occur with non-targeted trypanosome species and possibly with other parasitic diseases.

In this regard, the development of trypanosome species-specific monoclonal antibodies first reported by Nantulya and co-workers (27, 34, 37, 38), and later, by Richardson *et al.* (42), has been a major breakthrough in the diagnosis of African trypanosomiasis. The antibodies derived by Nantulya and colleagues were extensively cross-tested against several common haemoparasites of animals (28, 38) or man, and cross-reactivity was not observed. Using the *T. brucei* group-specific monoclonal antibody, a sandwich ELISA was developed for diagnosis of *T. brucei* infections in cattle and *T. rhodesiense* and *T. gambiense* infections in man (28, 36). Applying the same sandwich ELISA to the diagnosis of *T. evansi* infections in various animal species, circulating antigens were detected as early as six days following experimental infection and the "antigenaemia" persisted as long as the animals remained infected (30). The antigenaemia correlated well with parasitological findings. Applied to the diagnosis of *T. evansi* infections in camels from an endemic area (33), the test was able to detect circulating antigen in 92% of the animals with parasitologically confirmed diagnosis. Perhaps more importantly, the test also detected antigens in 55% of the animals in the same herd which had been missed by parasite detection techniques. Several camels from a trypanosomiasis-free area were all negative for antigens, indicating the high specificity of the assay. A tube-ELISA, as opposed to a microtitre plate-ELISA, has since been developed. This test gave similar results (33), thus providing, for the first time, a potentially suitable test for immunodiagnosis of individual animals in the field.

The principle of the assay is that the monoclonal antibody is used to coat a micro-ELISA plate or polystyrene tubes and excess coating antibody is discarded. Test serum is then added to the coated plates. The antigen in serum is captured by the coating antibody. Since the antigen trapped has several combining sites, a second antibody which is enzyme-labelled is introduced. The labelled antibody will bind to the free combining sites on the captured antigen. Any excess free labelled antibody is washed off and the reaction is then revealed by the addition of substrate and chromogen. From the protocol it can be seen that the same reagents can be used for diagnosis of the disease in several animal host species, since host species-specific anti-immunoglobulin reagents are not required.

The species-specific monoclonal antibodies against *T. congolense* and *T. vivax* have also been used to develop antigen-capture sandwich ELISA for the diagnosis of these infections in cattle (31, 34). Applied to the diagnosis of trypanosomiasis in cattle in an endemic area, the antigen-trapping ELISA gave positive results in 96% of the animals with parasitologically confirmed diagnosis (32). Sera from control cattle from a trypanosomiasis-free area were all negative for antigens. Interestingly, a high proportion of animals had mixed infections and a significant number (52.6%) of the animals from the same herd which had been missed by parasite detection techniques, were found to be infected as shown by the tests for antigen (32). Thus, whereas these tests may miss a small fraction of the early infections, this is more than compensated for by their ability to detect those cases which would be missed by parasitological diagnosis (5).

These antigen-trapping assays are easy to perform; the results can be read visually; large numbers of serum specimens can be analysed at a time; they can be applied to the diagnosis of individual animals; and, they are more sensitive than the techniques for parasitological diagnosis. Thus, these assays seem to provide an answer to some of the important questions regarding diagnosis of animal trypanosomiasis. The assays for diagnosis of bovine and camel trypanosomiasis are currently under field evaluation

in several African countries, as part of a collaborative project involving the International Atomic Energy Agency of the Food and Agriculture Organisation of the United Nations and ILRAD, funded by the Government of the Netherlands.

Although these tests are still under evaluation, certain conclusions can be drawn at this stage. First, there is a small proportion of animals in which antigens cannot be detected despite the patent parasitaemia. These results have also been observed under experimental conditions where very early in infection (31, 34), high parasitaemia may not be associated with the presence of antigen in serum. Since the basis for this test is that trypanosomes have to be destroyed to release the antigens in circulation, it is conceivable that the test will not be positive until after a critical number of trypanosomes have been destroyed by the immune response of the host. Second, there is a high proportion of animals without parasitaemia which test positive for antigens. Considering that the control animals from trypanosomiasis-free areas do not test positive for antigen, the cases detected by this test may represent true infections that cannot be diagnosed by parasitological techniques. The most effective diagnostic strategy, therefore, will be to combine antigen-trapping ELISA with one of the more sensitive standard trypanosome detection methods.

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LES TRYPANOSOMOSES DES ANIMAUX DOMESTIQUES : LES DIFFICULTÉS DU DIAGNOSTIC. – V.M. Nantulya.

Résumé: La trypanosomose animale pose des problèmes de diagnostic particuliers. Bien que des progrès considérables aient été réalisés, de nombreux cas passent encore inaperçus, car les signes cliniques de cette affection ne sont pas pathognomoniques, et les techniques habituelles de détection des trypanosomes ne sont pas suffisamment sensibles. En outre, dans la forme chronique de la maladie, qui est la plus courante, la parasitémie n'est pas fréquente. Etant donné ces difficultés, d'autres méthodes de diagnostic qui, pour la plupart, détectent la réponse immunitaire aux antigènes des formes infectantes, ont été mises au point. Parmi les épreuves les plus utiles pour le diagnostic, compte tenu de leur sensibilité et de leur spécificité, figurent une méthode de détection des anticorps par immunofluorescence indirecte, une méthode immuno-enzymatique (ELISA), et une technique d'agglutination directe (méthode CATT : Card Agglutination Test for Trypanosomiasis), utilisée pour déceler les infections à Trypanosoma evansi. Cependant, les insuffisances des méthodes de détection des anticorps sont nombreuses : les antigènes recherchés étant mal définis, il est difficile de normaliser ces techniques, en termes de sensibilité et de spécificité ; par ailleurs, certains tests ne sont pas utilisables sur le terrain. De plus, la présence d'anticorps dans le sérum ne signe pas nécessairement une infection, les anticorps pouvant persister plusieurs mois après traitement. La

récente mise au point de techniques de détection des antigènes des trypanosomes circulants (dans le sang des animaux infectés) a permis de résoudre ce problème, l'antigenémie indiquant l'existence de l'infection. L'évaluation de ces nouvelles méthodes sur le terrain n'est pas terminée, mais les résultats obtenus laissent à penser que, dans l'avenir, on associera probablement l'une des techniques actuelles de détection des trypanosomes les plus sensibles, à un test ELISA détectant les antigènes par immunocapture.

MOTS-CLÉS : Animaux domestiques - Détection des antigènes - Diagnostic - Parasitologie - Sensibilité - Sérologie - Spécificité - Trypanosomose.

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TRIPANOSOMIASIS EN ANIMALES DOMÉSTICOS: PROBLEMAS DE DIAGNÓSTICO. − V.M. Nantulya.

Resumen: La tripanosomiasis animal plantea problemas de diagnóstico específicos. Si bien se pueden señalar progresos considerables al respecto, numerosos casos pasan inadvertidos ya que los signos clínicos de la enfermedad no son patognómicos y las técnicas actuales de detección de tripanosomas carecen de la sensibilidad necesaria. Por otra parte, en la forma crónica de la enfermedad, que es la más corriente, no siempre los parásitos están presentes en la sangre. Para hacer frente a estas dificultades, se han elaborado otros métodos de diagnóstico, en su mayoría destinados a detectar las respuestas inmunitarias a los antígenos de los tripanosomas infectantes. Entre las pruebas que se consideran más útiles por su sensibilidad y su especificidad cabe destacar la de detección de los anticuerpos por inmunofluorescencia indirecta, la inmunoenzimática (ELISA) y la prueba de aglutinación directa (o CATT: Card Agglutination Test for Trypanosomiasis), empleada para detectar las infecciones por Trypanosoma evansi. *Sin embargo, presentan muchas insuficiencias: los antígenos buscados no están bien definidos y, por consiguiente, parece difícil conseguir normalizar estas técnicas cuyas sensibilidad y especificidad no se han demostrado y que no se pueden aplicar en su totalidad a la investigación de la tripanosomiasis. Además, la presencia de anticuerpos en el suero no significa necesariamente una infección, ya que éstos pueden subsistir varios meses después del tratamiento. La reciente puesta a punto de técnicas que permiten detectar los antígenos de los tripanosomas circulantes (en la sangre de los animales infectados) ha permitido resolver este problema; la positividad frente a los antígenos indica que existe infección. No se ha terminado de evaluar estas técnicas, pero los resultados ya obtenidos permiten pensar que las pruebas de diagnóstico futuras asociarán probablemente una de las técnicas actuales de detección de tripanosomas a la prueba ELISA.*

PALABRAS CLAVE: Animales domésticos - Detección de antígenos - Diagnóstico - Especificidad - Parasitología - Sensibilidad - Serología - Tripanosomiasis.

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