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Changing trends in diagnostics of trypanosomosis in animals

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ABSTRACT

Animal trypanosomosis presents special problems with regard to diagnosis since the clinical signs are not pathognomonic and standard parasitological techniques are not sufficiently sensitive. Formol-gel and mercuric chloride tests using serum of infected animals were adopted as a routine diagnostic tool for trypanosomosis, however, these chemical tests suffer from inherent problem of non specificity. To overcome this problem, alternative methods of diagnosis were developed, which detected antibody responses to antigens of infecting trypanosomes. Indirect immunofluorescent antibody test, enzyme immunoassay (ELISA) and the card agglutination test for trypanosomosis (CATT) were found to be useful tests for the diagnosis of *Trypanosoma evansi* infections in view of their sensitivity and specificity. However, antibody detection tests failed to distinguish between current and past infections because of persistent antibody titres. Recently, development of assays for the detection of circulating trypanosomal antigens in infected animals has circumvented this problem since antigen-positivity indicates existing infection. Latex agglutination test, being simple to perform**,** rapid, convenient, cost-effective could be quite suitable for field-level diagnosis and screening of trypanosomosis. Presently molecular diagnostic techniques like polymerase chain reaction (PCR) and DNA probes for detection of parasitic DNA have been used more efficiently as these indicate a sure sign of an active infection. These techniques eliminate the possibilities of cross-reaction and offer high specificity and sensitivity for detection of trypanosomosis in animals.

Key words: Diagnosis, Latex agglutination test, Monoclonal antibody, PCR, Trypanosomosis

India owns the largest livestock population in the world but recovery of produce from this sector is lower than its potential. The long term debilitating effects of parasitic infestations in livestock assume greater importance in terms of production losses (Rao 2006). *Trypanosoma evansi,* a blood protozoan parasite, causes a serious disease known as 'surra' in domestic and wild animals. Surra has been known in India since time immemorial, but its etiology was first brought to the notice of the rest of the world in 1880 by Griffith Evans. *Trypanosoma evansi* infection is widely prevalent in different parts of the country and is of significant economic importance in livestock production (Singh and Tiwari 2012, Sharma *et al.* 2013). The total net-benefit from effective surra control for a typical village in a moderate/high risk area was estimated to be US $158,000 per annum (Dobson *et al.* 2009). India is thought to be the major source from where surra has spread within livestock throughout the continent of Asia and Islands of the Indian Ocean (Singh 1989, Singh and Raisinghani 1990, Pathak 1999, Juyal *et al.* 2005). Though it has been proved experimentally that human serum has trypanocidal activity against *T. evansi* (Juyal *et al.* 1998), yet surra in human beings was reported in India (Joshi *et al.* 2005, Powar *et al.* 2006, Shah *et al.* 2011). This report assumes significance for it indicates possible zoonotic threat in future (Laha and Sasmal 2007, Singla *et al.* 2014). Though trypanosomes have been studied over the past several years, their definite diagnosis still suffers from low sensitivity and specificity. The true account of epidemiological picture of surra in India is lacking because the infections often do not manifest any real pathognomonic clinical signs (Singh and Chhabra 2008, Singh 1985). In India, treatment of animals for trypanosomosis on symptomatological basis is a quite common practice in the field leading to development of resistance in animals to currently available trypanocidal drugs. Accurate diagnosis of 'surra' is extremely important, to identify animals for treatment, to track the prevalence of the disease and to avoid misuse of the trypanocidal drugs. In the present review different diagnostic techniques available till date are discussed with special reference to field-oriented tests for large scale screening of animals suffering from surra.

Trypanosomosis is responsible for fluctuating nature of parasitaemia, which is often difficult to be detected by the commonly used parasitological methods. The situation becomes even worse in chronic infections, which are of common recurrence under stress conditions or due to overuse of corticosteroids under field conditions (Gupta *et al.* 2003, Kumar *et al.* 2012). The limitations in terms of

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low sensitivity of parasitological diagnostic techniques had been a driving force for research into alternate techniques such as serological and DNA based methods, which have got a great potential for unequivocal identification of the causative agent with higher sensitivity.

Parasitological methods

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 technique is suitable for screening large number of animals. This method, however, is insensitive as most of the times infected animals may be missed (Singh 1985). Examination of the buffy coat is more sensitive than examination of whole blood films as trypanosomes got concentrated just above the buffy coat. The method was initially used for the detection of avian trypanosomes and later gained wide application through various modifications (Kelly and Schillinger 1983). The micro-haematocrit centrifugation (MHCT) technique is particularly more useful as the status of anaemia in the test animals can be assessed at the same time. However, use of electricity limits its application in the field. This shortcoming overcame through the use of the battery-operated minicentrifuge described by Kelly and Schillinger (1983), but this has not yet received widespread appraisal.

Another method (Srivastava 1998), the miniature anion-exchange chromatography technique (MAECT), a technique used to diagnose *T. gambiense* infections in man was also established for diagnosis of animal trypanosomiosis (Reid *et al*. 2001). The application was not used in the field for routine diagnosis due to its more cumbersome procedure.

Laboratory animals, particularly rodents were found highly susceptible for multiplication of *T. evansi* when inoculated (Monzon 1990), hence these animals can be used for diagnosis of suspected animals due to high multiplication rate of *T. evansi* both in rat and mice (Singla *et al.* 2002). Singh and Chaudhri (1998) inoculated *T. evansi* suspected blood samples collected from different places and different hosts, intraperitoneally in the rats for diagnosis of surra and found mouse inoculation test (MIT) to be the gold standard technique, which is highly sensitive and specific than any other method. This, however, is not a practical technique due to ethical reasons regarding the use of live animals and diagnosis is not immediate. In addition, the cost of maintaining the animals makes the method prohibitively expensive and laborious for routine diagnosis, especially in the field. Despite several constraints, Laha and Sasmal (2007) recommended MIT as an alternative method, particularly where the facilities are not adequate for carrying out the serological and molecular diagnosis of *T. evansi*.

The parasitological examinations frequently fail to detect latent infections because parasitaemia is scanty in peripheral blood in the chronic forms (Killick-Kendrick 1968, Singh 1985). Despite improvement in parasitological techniques for detection of trypanosomes, a high proportion of infections are never detected (Luckins 1992) though they are used widely in the field even today. These drawbacks have necessitated the development of alternative methods of diagnosis.

Immunoassays for the detection of T. evansi *antibodies*

Several serological tests have been developed to detect circulating antibodies in the serum samples of *T. evansi* infected / suspected animals for diagnosis. The complement fixation test (CFT) was successfully applied to the diagnosis of *T. evansi* infections in several domestic animal species. Complement fixation tests, however, are prone to interference by anti-complementary activity in sera from several animal species and there can be difficulties in the preparation of satisfactory complement fixing antigens (Gill 1970, Sabanshiev 1973). Moreover, the test itself is difficult to perform as requires supplies of sheep red blood cells, complement, a centrifuge and a refrigerator. Thus, it is frequently unsuitable as a routine diagnostic tool.

Formol-gel and mercuric chloride test using serum of infected animal was adopted as a routine diagnostic tool for camel trypanosomosis. In experimental camel infections, Leach (1961) observed that the mercuric chloride test became positive 10 to 15 days post-infection and negative reactions occurred 2 to 3 months following treatment with a trypanocide. The tests were not specific for any one disease. Moreover, investigators failed to reproduce, let alone correlate, the results of this test with patent parasitaemia in subsequent studies (Killick-kendrick 1968). Gill (1964) observed that indirect haemagglutination test (IHAT) was more sensitive in the detection of anti-*T. evansi* antibodies but at the same time it failed to differentiate between homologous and heterologous antigens. Bansal and Pathak (1971) employed gel diffusion test for demonstrating *T. evansi* antigen and antibody in sera of infected camels, horses and buffaloes.

One of the most significant improvements in trypanosomosis serological diagnosis was the introduction of the indirect immunofluorescent antibody test (IFAT) (Williams *et al.* 1963). The IFAT is one of the most commonly applied serological diagnostic tests for this disease (Wery *et al.* 1970). The antigens used are usually prepared by fixing smears of parasitized blood using a variety of fixatives (Zwart *et al.* 1973), but the major problem of this method was the difficulty in preparation and storage of large numbers of blood smears, moreover the antigens so prepared showed substantial non-specific reactions. The IFAT, however, has major disadvantages since it requires sophisticated equipment and cannot be performed in the field.

Jon (1988) found that the micro-ELISA test was suitable for detecting trypanosome- specific antibodies of both Ig classes (IgG and IgM) in sera of goats experimentally

infected with *T. evansi* from 9 days to 17 weeks post inoculation. Singh *et al*. (1992 1994) detected *T. evansi* infection in camels by Ag-ELISA and Ab-ELISA, and found that ELISA is a superior alternative approach for diagnosis of latent surra in camels which signify active disease. Singh *et al*. (1995a) reported that the antigen ELISA is more sensitive and specific compared to antibody ELISA and wet blood examination for the diagnosis of latent trypanosomosis in buffaloes and horses. Rebeski *et al*. (2000) evaluated antigen-coating procedures of ELISA for detection of trypanosomal antibodies. Improved assay performance of the indirect ELISA method in detection of trypanosomal antibodies was noticed with the use of antigen-precoated and air-dried polystyrene 96-well plates, particularly in laboratories under tropical conditions. Dot-ELISA and a competitive inhibition ELISA (CI-ELISA) were also optimized for the detection of antibodies against *T. evansi* in dromedary camels (Shahardar *et al*. 2003). Sehrawat and Singh (2006) standardized a sensitive antibody-detection ELISA for assessing sero-prevalence of trypanosomosis in camel population of north-western regions of India by using high-titred rabbit anti-camel IgG_3 hyperimmune serum as secondary antibody.

Bajyana and Hamers (1988) developed card agglutination trypanosome test (CATT), based on the use of a widespread variable antigen type (VAT) RoTat 1.2 of *T. evansi* for the detection of trypanosome antibodies in the sera of pigs, cattle, buffaloes, horses and camels. Chaudhri *et al.* (1995) validated CATT in *T. evansi* infected crossbred cattle and buffaloes of India, pre- and post-treatment with quinapyramine prosalt. The test was found to be the most sensitive diagnostic method for detection of *T. evansi* antibodies in infected sera samples and did not reveal cross reactivity of *T. evansi* antigen in the test with *Theileria annulata* and *Babesia bigemina*. Card agglutination trypanosome test detected agglutination titres from day 14 post infection (PI) till death in experimentally infected calves (Chaudhri *et al.* 1996). Hilali *et al*. (2004) evaluated CATT/*T. evansi* for detection of antibodies against *T. evansi* in experimentally and naturally infected buffaloes. Anti-*T. evansi* antibodies were detected in buffalo samples by CATT/*T. evansi* which were found negative by parasitological examination. Singla *et al.* (2013) in their serodiagnostic studies on surra in cattle and buffaloes said CATT/*T.evansi* test as a pen-site test with higher field applicability. Card agglutination test was recommended by OIE for routine diagnosis of trypanosomosis in domestic animal. Shahardar *et al*. (2004) detected *T. evansi* antibodies by Ouchterlony's double immunodiffusion (DID) and counter immuno-electrophoresis (CIEP) tests, however, these tests are more of academic interest due to their low sensitivity.

Immunoassays for the detection of T. evansi *circulating antigens*

Serological diagnosis using antibody detection is hampered by its inability to distinguish between current and past infections because of persistent titres and occurrence of false positive results. The first attempt to detect circulating trypanosome antigens was made in Chagas' disease (Araujo 1982) but the sensitivity obtained was low. Further, counter immune-electrophoresis was employed efficiently to detect the circulating antigens of *T. evansi* experimentally (Singh and Chhabra 1993, Singh 1995b). Later, Rae and Luckins (1984) developed a *T. evansi* antigen detection system, using polyclonal anti-*T. evansi* antibodies for the detection of circulating *T. evansi* antigens in experimentally infected rabbits. 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. Monoclonal antibodies (mAbs) were produced against procyclic forms of *T. congolense*, *T. vivax, T. brucei brucei* and *T. b. rhodesiense* and these were used for the detection of species-specific antigens of trypanosomes by ELISA and IFAT. Monoclonal antibody based Ag-ELISA was not only of diagnostic importance, but also useful as tool for evaluating the efficacy of treatment (Nantulya *et al*. 1987). A mAb based antigen detection ELISA was also developed for the diagnosis of rhodesiense sleeping sickness in clinically suspected human cases (Nantulya 1989). In India, Swarnkar *et al*. (1993) used double antibody sandwich ELISA for the detection of *T. evansi* antigens in surra suspected cattle and buffaloes of Rajasthan and found it to be highly sensitive.

Nantulya (1994) developed monoclonal antibody-based simple and field-oriented latex agglutination test for the detection of circulating invariant trypanosomal antigens in *T. evansi* infections. Olaho-Mukani *et al*. (1996) screened camels by it and found *T. evansi* antigens in 46.3% camels, indicating that it is a sensitive, reliable and rapid field diagnostic test for the detection of *T. evansi* antigens in camels. A dipstick colloidal dye immunoassay (DIA) was developed using polyclonal antibodies and monoclonal antibody by Kashiwazaki *et al*. (2000). The performance of DIA in detecting *T. evansi* antigens was compared with Ag-ELISA. It was considered that DIA was an ideal field diagnostic test even though it had lower sensitivity than Ag-ELISA. Jeyabal *et al*. (2003) standardized an ELISA to detect circulating immune complexes (CIC) of *T. evansi* in cattle and buffaloes.

Rayulu *et al.* (2007) developed monoclonal antibody (mAb) based immunoassays like latex agglutination test (LAT) and Ag-ELISA using monoclonal antibody (IgA isotype) produced against surface membrane of *T. evansi* for the diagnosis of *T. evansi* antigens in domestic animals. The diagnostic sensitivity and diagnostic specificity were recorded as 95.38% and 59.74% for LAT and 83.08% and 67.14% for Ag-ELISA, respectively, using microhaematocrit technique (MHCT) as reference test, and 90.33% and 88.30% for LAT using Ag-ELISA as reference test. Later, monoclonal antibody based latex agglutination test was used for detection of *T. evansi* antigen in sera samples of cattle (Shyma *et al.* 2012a), buffaloes (Shyma

et al. 2012b), and equines (Shyma *et al.* 2011) and the results were compared with PCR of their corresponding blood samples, to determine the sensitivity of the test. The latex reagent was prepared by coating the latex microbeads with anti-*T. evansi* murine monoclonal antibody (IgA isotype) produced against cell membrane antigens of *T. evansi*. Twenty microlitres of the latex reagent were taken in the cavity of the slide and an equal volume of field serum sample was added, mixed by gentle swirling motion of the slide for five to ten minutes. The samples scored positive on occurrence of clumps or granular aggregates within five minutes. The results revealed a good correlation between LAT and PCR and found both the tests more sensitive than parasitological methods. It was concluded that latex agglutination test, being simple to perform**,** rapid, convenient, cost-effective could be quite suitable for field-level diagnosis and screening of trypanosomosis. However, PCR on the other hand has its role in monitoring the efficacy of trypanocidal treatment.

Molecular techniques for detection of T. evansi

A wide range of DNA based technologies have been used for detection of trypanosome infection using polymerase chain reaction (PCR), random amplification of polymorphic DNA (RAPD), kDNA minicircle analysis, hybridization using repetitive DNA sequences and DNA microarrays. Based on molecular DNA analysis, kDNA analysis and multi-locus isoenzymes, *T. evansi* stocks from various geographical regions showed a high degree of similarity to each other. A similar conclusion was drawn by serological analysis, which showed *T. evansi* to have a limited variant surface glycoprotein (VSG) antigenic repertoire (Lun *et al*. 1992).

A PCR-based *T. evansi* detection technique was developed and it had a sensitivity of detecting 0.5 pg of parasite DNA or one single parasite in 10µl of blood sample. The amplification of PCR in crude blood collected on microscopic glass slide was subsequently used for diagnosis of *T. evansi* in dairy cattle (Wuyts *et al*. 1994). Boid *et al*. (1996) described that detection of parasite DNA by PCR was a sure sign of an active infection with high specificity and sensitivity. The kDNA-PCR has shown that all *T. evansi* isolates fall within four sub-isotypes and there exists an identity with *T. equiperdum.* Reifenberg *et al.* (1997) described molecular characterization of trypanosome isolates from naturally infected domestic animals using PCR. Ijaz *et al.* (1998) used PCR in the identification of *T. evansi* in different stages of infection in mice and compared with standard microscopic examination method. During the acute phase of infection, parasites were detected by PCR 3 days earlier than by microscopy. The authors suggested that PCR could be used for the diagnosis of camel typanosomosis during both acute and chronic phases of infection and for use in the evaluation of treatment.

Watanapokasin *et al.* (1998) developed DNA fingerprints from isolates of *T. evansi* by PCR-based amplification using arbitrary primers (AP-PCR). The technique was applied in association with parasitological and serological examinations to investigate animal to animal transmission during an outbreak of surra in Thailand. Basagoudanavar *et al.* (2001) employed PCR in camel blood samples and found PCR sensitive to detect 0.5 ng of template DNA. Omanwar *et al*. (1999a) studied the specificity and sensitivity of PCR using oligonucleotide primers constructed from *T. evansi* repetitive DNA sequence. They amplified template DNA of *T. evansi* derived from buffaloes, camels and horses to a threshold sensitivity level of 0.5 pg and detected DNA from as few as five organisms in 10 µl crude blood samples following experimental infection of calves with 5×10^5 *T. evansi* parasites. Omanwar *et al*. (1999b) applied PCR for identification of *T. evansi* (derived from a buffalo) using two-oligonucleotide primers specific for kinetoplast minicircle and revealed the specific 994bp product. Dot-blot hybridization of total genomic DNA with the probe was found useful in detecting bubaline, cameline and equine strains of *T. evansi* down to 10 pg of parasite template DNA.

In Kenya, PCR and procyclic transformation test (PTT) were used to characterize trypanosomes from field infections of camels. The *in vitro* transformation was used to distinguish between *T. brucei* and *T. evansi. T. evansi* was isolated in 76% of the cases, confirming it to be the most important species causing trypanosomosis (Masiga and Nyang 2001). Ventura *et al*. (2001) developed PCR for the detection of *T. evansi* based on random amplified polymorphic DNA (RAPD) fragment. The taxon specificity of this PCR remained uncertain since it was tested on limited species/strains of trypanosomes. Omanwar *et al*. (2001) carried out AP-PCR using four random oligonucleotide primers (three 10mer and one 11mer) to study DNA polymorphism in *T. evansi* isolates from buffaloes, horses and camels. They reported that there is greater microheterogeneity between camel and buffalo isolates, followed by buffalo and horse, and horse and camel isolates, in descending order.

Kosum Chansiri and Khuchareontaworn (2002) developed a highly sensitive and specific PCR based assay for the detection of *T. evansi* in the blood of different animals and of the vector. The primer set was designed and synthesized to amplify a single band of 257bp PCR that was subsequently examined by ELISA. The sensitivity limit of PCR-ELISA was 0.01 pg corresponds to 1 parasite/ml of blood. No cross-reactivity of the assay was observed against *Babesia bovis, B. bigemina, Anaplasma marginale, Theileria annulata* and host DNA. They further suggested that technique of PCR-ELISA is not only beneficial for diagnosis of the parasite but useful for epidemiological study and designing rational trypanosomosis control programme. Ngaira *et al*. (2004) analysed *T. evansi* for the presence of RoTat 1.2 VSG gene by PCR and for the presence of expressed RoTat 1.2 VSG by Western blot analysis. However, results indicated that this gene was absent in some *T. evansi* strains infecting camels in Kenya. Transcript encoding a predominant *T. evansi* VSG RoTat

1.2 was cloned and expressed as a recombinant protein in *Spodoptera frugiperda* and *Trichoplusia* (insect) cells by Lejon *et al*. (2005). It was also reported that ELISA, CATT/*T. evansi* and LATEX/*T. evansi* tests using this recombinant VSG were more sensitive and specific for the diagnosis of *T. evansi* antibodies in camels. Singh *et al.* (2004) reported PCR as 100% efficient diagnostic test for diagnosis of *T. evansi* infection in camels as compared to parasitological and serological methods.

Aradaib and Majid (2006) developed and evaluated a nested polymerase chain reaction (nPCR), for rapid detection of *T. evansi* in experimentally infected mice and naturally infected camels (*Camelus dromedarius*). Four oligonucleotide primers selected from nuclear repetitive gene of *T. evansi*, were designed and used for PCR amplifications. The nPCR-based assay provides a valuable tool to study the epidemiology of *T. evansi* infection in camels and other susceptible animal populations. Njiru *et al.* (2007) investigated the use of inter-simple sequence repeats (ISSR) and microsatellites in revealing polymorphism among *T. evansi* isolates. They reported that both ISSR and microsatellites markers were useful in detecting genetic variability within *T. evansi.* Shahardar *et al.* (2007) employed PCR assay for detection of *T. evansi* in Indian dromedary camels using ribosomal DNA amplimers (20mer sense and 16mer antisense primer) based on structural 18S and 5.8S ribosomal sequence specific for kinetoplastida taxon. Li e*t al.* (2009) used complementary DNA microarray to analyze the gene expression profiles in the liver and spleen of mice infected with *T. evansi* (STIB 806) at the peak parasitemia (7 days after infection) and the results provided a comprehensive profile of changes in gene expression in these organs which was helpful in understanding the pathogenesis of Surra at a molecular level. Recently it has been seen that real time TaqMan assay was at least two fold more sensitive than conventional parasitological methods (Sharma *et al.* 2012). A cost effective duplex PCR for detection and management of concurrent latent infections of *Babesia bigemina* and *Trypanosoma evansi* have been employed in dairy animals from Punjab (Sharma *et al.* 2013). Ahmed *et al.* (2011) recommend that blood is transferred onto FTA cards whole followed by elution in Chelex®100 as the best approach for field level molecular diagnosis. Loop-mediated isothermal amplification (LAMP) was designed using the serum resistance-associated (SRA) gene of *Trypanosoma brucei rhodesiense* and found to have great potential for use in the HAT-endemic countries (Njiru *et al.* 2008)

The current diagnostic procedures of surra in domestic animals rely mostly on demonstration of organisms by light microscopy which misses about 80–90% of the infection. Though mouse inoculation test is observed as the most sensitive (100%), the test is impracticable for large scale epidemiology. Although, serology for antibody detection has an important place in defining the carrier status, antibodies to *T. evansi* infection persist after drug treatment complicating the correlation between patent infection and serological response. Development of sensitive and specific diagnostic methods to detect current infection is foremost important for early diagnosis of disease and control of disease. The authors have evaluated a new antigen detecting enzyme assay using monoclonal antibody produced against surface membrane of *T. evansi* coated on latex beads. The detection of circulating antigens of *T. evansi* gives a sure symbol of active infection and hence chemotherapeutic measures can be carried out appropriately. The test has been found to be rapid and more sensitive method than WBF. Moreover, the test is simple to perform neither requiring multiple and complex procedural steps, nor the use of sophisticated equipment for reading the results. Modern DNA based techniques such as PCR, duplex PCR, real time PCR, DNA microarrays although highly specific have limitations. However, these studies are limited only to well equipped laboratories and no commercial test kit of PCR is available so far for detection of *T. evansi* infection in animals.

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