COMPARISON OF PARASITOLOGICAL, SEROLOGICAL AND MOLECULAR DIAGNOSTIC TESTS FOR DETECTION OF SUBCLINICAL TRYPANOSOMOSIS IN TWO ORGANISED DAIRY HERDS

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ABSTRACT

Trypanosomosis also known as 'Surra' caused by *Trypanosoma evansi* is of significant economic importance in livestock production. Diagnosis of subclinical infection and carrier state of disease is far difficult. Therefore, the present study was conducted to compare parasitological, serological and molecular diagnostic tests for detecting subclinical trypanosomosis in two dairy herds of Murrah buffalo (n=27) and crossbred cattle (n=22) in Hisar district of Haryana. Peripheral blood samples collected from all the animals were subjected to blood smear examination and DNA isolation for molecular detection by PCR, and like-wise serum samples were collected and subjected to latex agglutination test (TE-LAT) for *T. evansi* antigen detection. PCR products from positive samples were purified and sequenced for confirmation of *T. evansi* DNA. None of the animals in the two herds was found positive for *T. evansi* parasites by microscopic examination of thick blood smears. In buffalo farm, 29.62% samples each were found moderately and weak positive by TE-LAT, whereas PCR assay test yielded 40.74% samples positive for *T. evansi* DNA. In cattle farm, TE-LAT showed 18.18%, 31.89% and 9.09% samples to be strong, moderate and weak positive, whereas 50% samples were found PCR-positive for *T. evansi* DNA. Agreement among TE-LAT and PCR assay, as expressed by the kappa value, was found to be poor. To conclude, each test has its own merits and limitations. While TE-LAT is suitable for screening purpose, PCR assay confirms parasite in blood. For clinical diagnosis, results of laboratory diagnostic tests must, however, be correlated clinically before initiating the therapy.

Keywords: *Bubalus bubalis*, buffaloes, Trypanosomosis, diagnosis, subclinical infection, TE-LAT, PCR assay

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INTRODUCTION

Trypanosomosis commonly known as 'Surra' caused by *Trypanosoma evansi* is one of the most important haemoprotozoan diseases of domestic animals in Indian subcontinent through widespread morbidity and mortality (Pathak and Chhabra, 2011). Recent study conducted by Narladkar (2018) reported 2.69% prevalence of disease in subclinical form in country with an annual estimated loss of Rs 713.5 crore in cross-bred and Rs. 311.21 crores in buffaloes.

Clinical signs of Surra are not pathognomonic for making more than a tentative diagnosis. In field condition, diagnosis is done by examining blood smear for typical trypanosoma flagellate which is not consistent due to low and irregular parasitemia. Identification of parasite in smear form the basis of definitive diagnosis and is considered as gold standard test for clinical cases in field. However, in cases of subclinical infection, where low and irregular parasitemia occurs, it is difficult by direct microscopy (Singla *et al*., 2015).

In past, various serological tests for detecting antibodies against trypanosomal antigen or circulating trypanosomal antigens have been developed. For detecting circulating antibodies, card agglutination test (CATT), immunofluorescent antibody test (IFAT) and complement fixation test have been employed in various studies. Similarly, Ag-ELISA, dipstick colloidal immunoassay (DIA) and monoclonal antibody based latex agglutination test have been used for detecting circulating antigens. The serological tests have limitation due to their inability to distinguish between current infection and past infection because of persistent antibody titres (Singh *et al*., 2014). In antigen detecting assay results depends on multiple factors *viz.* sampling time, infection and disease status, treatment history, etc. (Shyma *et al*., 2012).

Because of limitation of conventional parasitological and serological techniques, disease diagnosis cannot be relied upon, therefore, it is imperative to use a test which is more sensitive and reliable. DNA based molecular assay, such as polymerase chain reaction (PCR) assay, offers identification of haemoprotozoa at levels far below the detection limit of the commonly used parasitological techniques. PCR is reported to be able to detect one trypanosome/ml of blood or as low as 1.0 pg of *Trypanosoma* DNA in the presence of host DNA (Penchenier *et al*., 1996). This technique has its own limitations of requirement of laboratory equipment and advantage of screening a large number of samples at a time. Therefore, PCR can be used as a diagnostic tool for epidemiological studies.

Keeping the above facts under consideration, the current study was conducted to compare parasitological, serological and molecular diagnostics for detecting subclinical trypanosomosis by screening two different organized dairy herds.

MATERIALS AND METHODS

Sample collection

Two organized dairy herds situated in Hisar District of Haryana, one having cross bred cattle (n=22) and other having Murrah buffalo (n=27) were selected for the present study. Animals selected for the study had not been treated with any chemotherapeutic agent for surra during past one month. This was to minimise the possibility of false positive reactions in TE-LAT. Slide was prepared on the spot and fixed with methanol.

Microscopic examination

Paired fixed thick blood smears for each sample were stained with Giemsa stain for 30 minutes. Blood smears were examined for the typical flagellate under oil emersion (100X). Slides were screened visualizing 50 fields for characteristics morphology of the parasite. Even a single parasite found in the slide was considered as positive case.

***Trypanosoma evansi* Latex agglutination test (TE-LAT)**

Standardized latex agglutination test based on monoclonal antibodies generated in Mice was used to determine the seropositivity of samples as suggested by Shyma *et al.* (2012). Twenty microliters of the latex reagent were taken in the cavity of the slide and an equal volume of serum added to it. The reagent and the serum sample were mixed by gentle swirling motion of the slide for five to ten minutes. Result were graded as strong positive, moderate positive, weak positive and negative based on the duration of test showing positive results. Clumping or granular aggregates formation is considered as positive as shown in Figure 1.

Molecular diagnosis

DNA from all the samples was extracted using a commercial kit (Qiagen). PCR assay, using primers based on repetitive nucleotide sequence (Table 1), was used to amplify the 257 bp product. PCR assay was carried out in 200 µl PCR tubes using Applied Biosystems thermocycler. Each 12.5 µl reaction mixture comprised of 2 µl of template DNA, 6.25 µl Dream Taq™ master mix, 0.5 µl of each forward and reverse primer, and 3.25 µl nuclease free water (NFW). The PCR conditions include initial denaturation at 95°C for 2 minutes; followed by 30 cycles of 95°C for 30 seconds (denaturation), 55°C for 30 seconds (annealing) and 72°C for 1 minute (extension); with a final extension step of 72°C for 10 minutes. Sequencing was done to confirm the isolates.

Agarose gel electrophoresis (AGE):

Five microlitres of amplified PCR product was directly loaded to electrophoresis in 1.5% agarose gel along with 100 bp DNA ladder mix with 6X loading dye. The images were captured and documented using gel documentation system (Bio-Rad, USA).

Statistical analysis

Kappa value was estimated using SPSS 16.0. Agreement among the TE-LAT and PCR assay as expressed by the kappa value was determined.

RESULTS AND DISCUSSION

None of the animals in the two herds was found positive for *T. evansi* parasites by microscopic examination of thick blood smears. Agglutination was evident in approximately 60% of the sample from both the herds using latex agglutination test. Table 2 shows the result of TE-LAT applied on the two herds. Results revealed 18.18% cross-bred cattle to be strong positive whereas not even a single buffalo was found to be strong positive for TE-LAT.

PCR assay declared 50% and 40.74% samples positive, with a characteristic band of 257 bp in AGE as shown in Table 3 and Figure 2. However, the amplified band exhibited variable intensity when visualized in agarose gel electrophoresis. Result of PCR assay revealed cattle to be more prone for infection as compared

to buffaloes. The results of both TE-LAT and PCR revealed that both the herds were having subclinical infection of *T. evansi*, with 50% to 60% herd prevalence. Similar results have been reported by Gonzales *et al*., 2003; Herrera *et al*., 2004; Singh *et al*., 2004 stating PCR assay to be more sensitive as compare to microscopic examination of stained smear.

Out of three test compared in present study, TE-LAT was the fastest and had higher sensitivity in terms of estimating the prevalence. Similar to our study, higher percentage of detection has been described using serological test in comparison with the PCR by Holland *et al*. (2001); Gonzales *et al*. (2003). Higher detection by serological assay can probably be caused due to maintenance of the titer of anti-*T. evansi* IgG antibodies in field animals, even after treatment with trypanocidal drugs. This is the reason it cannot be used to discriminate between active or inactive infections (Desquesnes, 2004; Desquesnes*et al*., 2009). Higher sensitivity of serological test, when compared to parasitological and molecular test, suggests their use in epidemiological studies, in which overall exposure to trypanosome infection is being investigated. However, antibody detecting serological tests do not indicate if the infection is active or if the animal is responded to therapy (Junior *et al*., 2019). In case of antigen detecting TE-LAT, the problems of 'false positive' serum samples have been encounteredinprevious studies. One of the possible reasons recorded in previous studies could be the antigen in circulation for a few weeks post-treatment of clinical cases in the field situation (Rayulu *et al*., 2009; Shyma *et al*., 2012). However, the false positive reactions due to this reason had been precluded in the present study.

Zero percent prevalence recorded using parasitological method clearly suggests present of very low parasitemia or might be due to subclinical nature of disease.Similar to our findings, Ramírez-Iglesias *et al*. (2011) on conducting the comparative study on four different diagnostic approaches using rabbit as experimental model revealed that the parasite can be detected by parasitological techniques in acute phase of infection, but neither in subclinical nor chronic phase of infection. They stated false negative results using direct microscopy during acute phase, which they related with fluctuations in the parasitemia. They also pointed out delay in detection by direct microscopy could be due to initial low parasitemia. They showed that PCR and ELISA could be used in chronic infection for 100% detection of the parasite, whereas PCR had an added advantage of detecting the parasite in pre-latency phase of infection. In the chronic phase, the parasitological techniques yielded a negative diagnosis, indicating the limitations of these methods for that phase, which was characterised by low parasitemia.

Reema *et al.* (2012) compared PCR with LAT (the same as TE-LAT), testing 55 crossbred cows and showed PCR to be specific with no cross- reactivity, whereas somecross- reactivity was seen using LAT with positive samples for babesiosis and theileriosis. They concluded that cross-reactivity observed in LAT could be either due to mixed infections or due to actual presence of circulating antigen because of previous exposure or treatment with trypanocidal drug(s) prior to sample collection. In accordance with the present study, Rayulu *et al.* (2012) while comparing parasitological examination with Ag-LAT (the same as TE-LAT)revealed low number of positive samples by wet blood film examination and microhematocrit centrifugal technique and reasoned that this could be due to the inherent low sensitivity of these tests or could be indiscriminate

Table 1. Primers based on repetitive nucleotide sequence used in PCR to detect *T. evansi* DNA from peripheral blood samples.

Parasite	Gene targeted	Primer	Sequence 5' -3'	Reference
Trypanosoma evansi	Repetitive nucleotide sequences	Forward	GCG CGG ATT CTT TGC AGA CGA	Ananyutthawongese *et al. (*1999)
		Reverse	TGC AGA CAC TGG AAT GTT ACT	

Table 2. Screening of two bovine herds using antigen detecting latex agglutination test based on mAbs (TE-LAT).

Grading of TE-LAT result	Cross-bred cattle farm (n=22)	Buffalo farm (n=27)
Strong positive	18.18%	Nil
Moderate positive	31.89%	29.62%
Weak positive	9.09%	29.62%
Negative	40.84	40.76

Table 3. Molecular detection of trypanosomosis in two bovine herds.

Results of PCR	Cross-bred cattle farm (n=22)	Buffalo farm (n=27)
Positive	50%	40.74%
Negative	50%	59.26%

Table 4. Showing results of kappa test applied on the results to determine the agreement between molecular and serological diagnostic assay.

Dairy herds		Kappa value	Asymp. Std. errora	Approx. Tb	Approx. Sig.
Murrah buffalo herd	Measure of agreement Kappa	0.152	0.170	0.871	0.384
	N of valid cases	27			
Cross bred cattle herd	Measure of agreement Kappa	-0.091	0.209	-0.434	0.665
	N of valid cases	22			

Not assuming the null hypothesis. Using the asymptotic standard error assuming the null hypothesis.

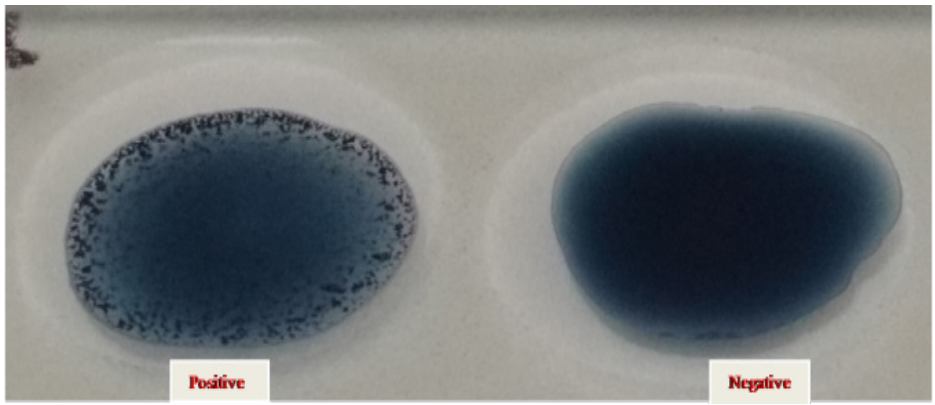

Figure 1. Positive and negative results of TE-LAT performed on a cavity slide.

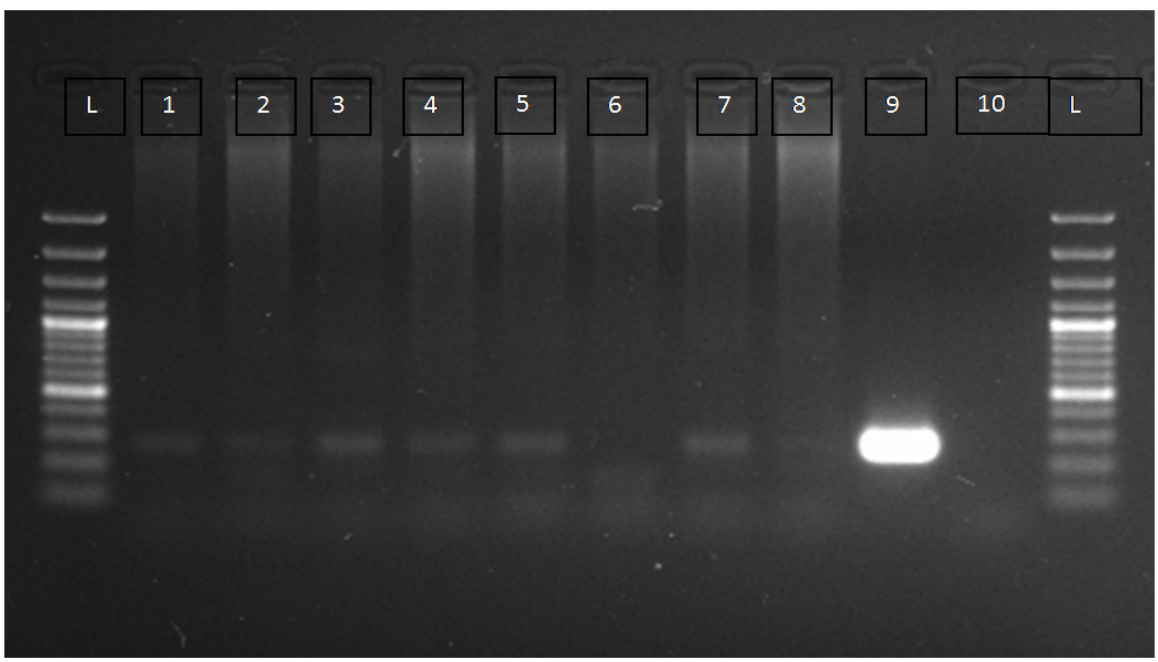

Figure 2. PCR amplification of *T. evansi* DNA extracted from peripheral blood samples.
Lane L: 100 bp DNA
Ladder; 1-8: test samples, all positive, except Lane 6 and 8
Samples; 9: Control positive showing 257 bp band; 11: Control negative.

treatment of suspected animals for trypanosomosis based on clinical signs. They showed positive results by Ag-LAT even after treatment as killed parasite antigens may remain in blood circulation for about four weeks after treatment. Post-treatment antigenaemia persisting for a few days to weeks has previously been reported (Thammasart *et al*., 2001; Singh and Chaudhri, 2002). Therefore, it is imperative to consider reliable history of the animals receiving anti-trypanosome treatment during past few weeks.

On statistical analysis, poor agreement was found between TE-LAT and PCR assay as expressed by Kappa Value determined using statistical software as shown in Table 4. These results revealing poor agreement between the tests is in agreement with the findings of Alves *et al.* (2017).

Different tests have their own limitations and advantages. Antibody detecting serological tests could be misleading as they cannot distinguish between present and past infection. Despite limitations discussed above, antigen detecting tests can be effectively used in correlation with the clinical signs and symptoms of the disease in animals, when detectable parasitaemia is not present, which otherwise could be confused with the symptoms of other haemoprotozoan infections (Sumbria *et al*., 2014). The early diagnosis of subclinical/carrier stage of infection by PCR assay technique and subsequent treatment helps in prevention of any serious outbreak at the farm especially during months having abundance of vector population for spread of disease. In present times, use of combination of several techniques can avoid the generation of false- negative results, restricting the transmission of disease and reducing the economic losses.To conclude, each test has its own merits and limitations, and the choice of tests to be used would depend on the situation, availability of resources and accessibility to laboratory. While TE-LAT is suitable for screening purpose, PCR assay confirms the parasites in blood of infected animals. For clinical diagnosis, the results of laboratory diagnostic tests must, however, be correlated clinically before initiating the therapy.

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