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Comparative evaluation of agglutination assay with microscopy and polymerase chain reaction for detection of *Trypanosoma evansi* in bovines of Punjab

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ABSTRACT

Present investigation was aimed to evaluate the diagnostic efficacy of CATT/*T.evansi* in comparison to Giemsa stained thin blood smear examination and PCR for detection of bovine trypanosomosis in Punjab. Analysis of 264 blood and sera samples revealed 1.89% (5/264), 34.47% (91/264) and 51.89% (137/264) positivity by GSTBS, CATT/*T. evansi* and PCR, respectively, for *Trypanosoma evansi*. Sensitivity of CATT/*T. evansi* was 100% and 30.65% as compared to GSTBS and PCR, while its specificity was 66.79% and 61.42%, respectively. PCR is more sensitive and specific, and is able to detect the latent infections, however, it is time and cost ineffective technique. CATT/*T. evansi* is a quick and easy pen-site test suitable for mass screening of *T. evansi* endemic in developing countries.

Key words: Bovines, CATT, PCR, Punjab, *Trypanosoma evansi*

Trypanosoma evansi, the causative organism of surra, is a salivarian trypanosome, originating from Africa and spreading towards the Middle East, Turkey, India, Russia and South-East Asia (Desquesnes *et al.* 2013). It causes fever, anaemia, loss of appetite and weight, emaciation, nervous signs and/or abortion, cachexia and death, with or without more peculiar signs related to the host species (Gardiner and Mahmoud 1990).

The diagnosis of subclinical infection is far difficult as this disease is characterized by fluctuating parasitaemia with periods of paroxysms and intermission (Singla *et al.* 1996). In bovines, the level of parasitaemia is frequently low and fluctuating, specifically during the chronic stage, leaving the presence of the trypanosomes undetected by classical parasitological techniques (Nantulya 1990). Although many studies have been published to evaluate and compare the sensitivities of different serological and molecular diagnostic techniques for *T. evansi* in cattle and buffaloes (Masake *et al*. 2002), the discrepancies are predominating. Moreover, in Punjab, comparative study of microscopy, serology and molecular diagnosis for *T. evansi* in cattle has not been conducted yet; hence, this study was designed.

MATERIALS AND METHODS

Sample collection: Blood samples (264) were collected from animals having history of recurrent fever, emaciation, and decrease in milk yield from different places of Ludhiana and Jalandhar districts of Punjab state. About 5 ml blood was collected from the jugular vein of each animal aseptically in EDTA coated vials and glass vial, respectively for DNA isolation and serum separation. The extracted DNA and collected sera were stored at –20°C for further analysis by PCR and CATT/*T. evansi*.

Blood film: Two thin blood films were prepared and stained with Giemsa stain for 30–45 min, washed with distilled water to remove excess of stain after that the slides were left to dry and then examined under oil immersion lens (Coles 1986).

CATT/T. evansi test: The CATT/*T. evansi* kit was used (Institute of Tropical Medicine, Antwerp, Belgium) and the test was performed as per Bajyana and Hamers (1988). Agglutination patterns were scored as " – (negative), ± or + (weakly positive), and ++ or +++ (strongly positive).

PCR assay: Genomic DNA was extracted from the blood samples using the protocol of blood genomic DNA kit. The PCR reactions were performed using forward primer TR3 5' GCG CGG ATT CTT TGC AGA CGA 3' and reverse primer TR4 5' TGC AGA CAC TGG AAT GTT ACT 3' (Wuyts *et al.* 1994) to amplify repetitive nucleotide sequences of *T. evansi.* The PCR reaction mixture (25 µl) was constituted by 12.5 µl of KAPA 2G fast hot start ready mix (2X containing KAPA2G fast hot start DNA polymerase, KAPA 2G fast hot start PCR buffer, 0.2 mM dNTP each, 1.5 mM $MgCl_2$), 1.25 µl of 10 pmol TR3/TR4 primer. The reaction was set in automated thermocycler with the following programme: initial denaturation at 95°C (5 min), 30 cycles of denaturation at 95°C (30 sec), annealing at 57°C (1min), and extension at 72°C (1.5 min) and final

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extension at 72°C for 10 min. The amplified PCR products were separated by electrophoresis on 1% agarose gel and visualized under UV transilluminator for detection of 257 bp product size.

Nucleotide sequence analysis: The individual 257 bp product obtained by the primers specific for *T. evansi* were custom sequenced. The nucleotide sequences were subjected to BLASTn analysis (Altschul *et al.* 1990) for determining the similarity with the sequences present in the nucleotide database. The final sequences were submitted to NCBI database and the accession numbers is AB979445.1.

RESULTS AND DISCUSSION

Trypanosomes were demonstrated in 5 animals in Giemsa stained thin blood smears (GSTBS). Of the 5 positive animals, 4 had history of anorexia, decrease milk yield, weight loss, while 1 cattle had corneal opacity. In the present investigation, percentage positivity of *T. evansi* by PCR was high (51.9%) as compared to CATT/*T. evansi* (34.5%) and parasitological methods (1.89%) as depicted in Table 1. On comparing the sensitivity of CATT/ *T. evansi* with conventional microscopy, 86 out of 259 samples negative by blood smear examination were found positive by CATT/*T. evansi* (32± and +; 54 ++ and +++), while no sample negative by CATT was positive by blood smear microscopy. Thus CATT/*T.evansi* displayed higher sensitivity than the microscopy for detection of trypanosomes.

In general, serological techniques are useful for detection of a past infection but not for detection of an active infection with *T. evansi*. The results of CATT/*T. evansi* obtained with parasitologically positive and negative animals are given in Table 2. When a ± score was taken as cut-off, test sensitivity and specificity were 100% and 66.79%, respectively. The serological tests identified the animals with sub-patent infections which could not be detected by parasitological methods. Moreover, the per cent positivity was high (34.5) in animals by CATT/ *T. evansi* as compared to 1.89 by GSTBS. This finding confirms the usage of CATT instead of parasitological methods for more reliable results, as CATT/*T.evansi* employs highly specific antigens such as VSG *RoTat* 1.2 *T. evansi* (Verloo *et al.* 2001).

On comparing the sensitivity of CATT/ *T. evansi* with PCR assay, 49 out of 127 samples negative by PCR were found positive by CATT/ *T. evansi*, while 95 of 173 samples negative by CATT/*T. evansi* were found positive by PCR (Table 3). Intensity of PCR bands was variable in different test samples depending upon the level of infection in the test samples (Fig. 1). When a score ± was taken as the cut off, the sensitivity and specificity of CATT in comparison to PCR were found to be 30.65% and 61.42% respectively (Table 3). Our study revealed that PCR is the most sensitive diagnostic technique as compared to serological based test because it can detect the parasite at early stage of

Table 1. Prevalence of trypanosomosis using different diagnostic tests

	Total number of samples examined	Total positive samples	Total negative samples	Percent positivity
GSTBS	264	5	259	1.89
CATT	264	91	173	34.5
PCR	264	137	127	51.9

Table 2. Results obtained with CATT/*T. evansi* in relation to parasitological findings

GSTBS	Scores CATT/*T. evansi*			Total
	-	± and +	++ and +++	
Negative	173	32	54	259
Positive	0	3	2	05
Total	173	35	56	264

Sensitivity of CATT w.r.t. GSTBS

$$= \frac{Number\ of\ True\ positive}{Number\ of\ True\ positive + Number\ of\ False\ negative} \times 100$$

$$= \frac{5}{5+0} \times 100 = 100\%$$

Sensitivity of CATT w.r.t. PCR

$$= \frac{Number\ of\ True\ negative}{Number\ of\ True\ negative + Number\ of\ False\ positive} \times 100$$

$$= \frac{173}{173+86} \times 100 = 66.79\%$$

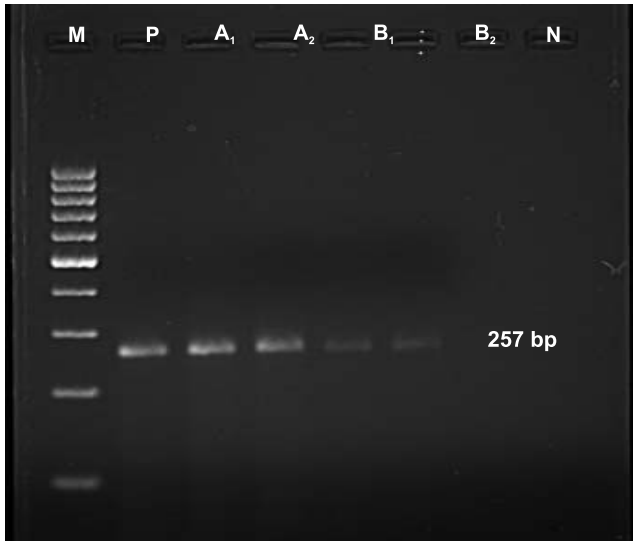


Fig. 1. Agarose gel (1.5%) electrophoresis showing amplified DNA from *T. evansi* (257 bp) targeting repetitive nucleotide sequence. Lane M, Molecular size marker 100 bp plus; lane P, positive control; lane A1 and A2, strongly positive cases; lane B1 and B2, weak positive cases; lane N, negative control.

Table 3. Results obtained with PCR in relation to CATT/*T. evansi*

PCR assay	Scores CATT/*T. evansi*			Total
	-	± and +	++ and +++	
Negative	78	19	30	127
Weak positive	63	7	14	84
Strong positive	32	9	12	53
Total	173	35	56	264

Sensitivity of CATT w.r.t. PCR

$$= \frac{Number\ of\ True\ positive}{Number\ of\ True\ positive + Number\ of\ False\ negative} \times 100$$

$$= \frac{42}{42+95} X\ 100 = 30.65\%$$

Specificity of CATT w.r.t. PCR

$$= \frac{Number\ of\ True\ negative}{Number\ of\ True\ negative + Number\ of\ False\ positive} \times 100$$

$$= \frac{78}{78+49} \times 100 = 61.42\%$$

development. PCR is known to possess high sensitivity in terms of parasite detection (Masake *et al.* 2002). However, PCR is a time consuming and cost ineffective test as a single PCR reaction costs about $4 as compared to CATT/ *T. evansi* that costs around $ 0.8. Moreover CATT/*T. evansi* can be easily employed in field conditions. Being pan site test it has the advantage over the conventional parasitological techniques and is cost effective, simple and rapid as compared to molecular diagnosis based PCR assay which requires sophisticated laboratories and presently it is not feasible to use PCR in field. CATT/ *T. evansi* can be used for sero-epidemiology of surra and is helpful in early diagnosis and treatment of the chronic cases of surra.

Although CATT/ *T. evansi* can be misleading as it cannot distinguish between present and past infection, yet it can be effectively used in correlation with the clinical signs and symptoms of the disease in animals, when detectable parasitaemia is not available, which otherwise can be confused with the symptoms of other pathognomic haemoprotozoan infections. (Sumbria *et al.* 2014).

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