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PCR-based Identification of *Trypanosoma evansi* targeting Internal-transcribed Spacer 1 of rDNA in experimentally infected Mice

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ABSTRACT

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Trypanosoma evansi (*T. evansi*) is a causative agent of disease called surra, affecting wide range of domestic and wild animals. In this study, a PCR assay was developed using primers targeting ITS-1 region flaking between 5.8 S and 18 S subunits of rRNA. The test was employed using serially diluted DNA extracted from purified trypanosomes ranging from 200 ng/µl to 0.00002 pg/µl and *T. evansi* infected mice blood ranging from 350 ng/µl to 0.00035 ng/µl. The diagnostic sensitivity of PCR assay was found to be 0.2 pg/µl and 0.035 ng/µl with purified parasite DNA and infected mice blood DNA, respectively.

Keywords: *Trypanosma evansi*, Surra, ITS-1, PCR, rDNA, Equine

INTRODUCTION

Trypanosoma evansi is an extra-cellular haemoprotozoan parasite, multiplies in blood, body fluids and tissues and resulting in a wasting disease commonly known as Surra. It affects a wide range of domestic livestock and wild animals (OIE, 2012). Parasite is transmitted mechanically by biting flies such as *Tabanus, Stomoxys* and *Lyperosia* species and transmission has also been observed in the carnivores after feeding on meat infected with *T. evansi* (Herrera *et al*., 2004) and during vaccination campaigns (Davila and Silva, 2000). The disease is having world-wide distribution among all pathogenic parasitic spp. and prevalent in Africa, Central and South America, Middle East and Asia regions (Reid, 2002). In India, *T. evansi* infections are widely prevalent in equines in different geographical parts including Gujarat, Haryana, Himachal Pradesh, Jammu and Kashmir, Punjab, Rajasthan, Uttarakhand and Uttar Pradesh (Kumar *et al*., 2013).

The diagnosis of surra is often difficult because of the chronic and fluctuating nature of parasitaemia and the cryptic nature of the parasite into less accessible tissues such as the nervous system making diagnosis difficult (Nantulya, 1990). The PCR is a very much rapid, sensitive and specific DNA-based assay detecting very low level of parasitaemia (Pruvot *et al*., 2010). Various level of sensitivity has been achieved by using different primers targeting different sequence; favoured targets are those which are present in a high copy number in the genome because high copies of the target leads to greater amplification by PCR. The PCR assay has been reported to be more sensitive than conventional parasitological techniques in experimentally infected cattle (Wuyts *et al*., 1995), water buffaloes (Holland *et al*., 2001), mice (Ijaz *et al*., 1998; Fernandez *et al*., 2009) and naturally infected domestic and wild mammals species (Herrera *et al*., 2005). The TBR primers targeting satellite DNA are (Masiga *et al.,*

1992) are considered as gold standard for PCR assay (OIE, 2012); allows the detection of a single parasite (0.1 pg of DNA) and on enzymatic digestion of DNA can lead to the detection of 0.01 trypanosome by hybridization (Masiga *et al.,* 1992; Fernandez *et al*., 2009).

The present study was performed to study diagnostic sensitivity of PCR targeting internal transcribed spacer 1 of rDNA of *T. evansi*. The PCR-based diagnostic sensitivity was performed with purified parasite DNA and *T. evansi*-infected whole blood DNA.

MATERIALS AND METHODS

Source of isolate used: *T. evansi* isolate of pony origin (*T. ev*-India-NRCE-Horse1/Hisar/Haryana) was used for standardization of ITS1 PCR. This isolate is being maintained and cryopreserved in liquid nitrogen at National Research Centre on Equines, Hisar.

Propagation of *T. evansi* isolates in mice and DNA isolation: Initial inoculation to Swiss albino mice was done with 1×10^5 trypanosomes by intra-peritoneal route. The parasitaemia was examined daily and at peak parasitaemia mice were sacrificed and blood was collected by cardiac puncture.

Trypanosomes were purified from whole blood by DEAE-cellulose anion exchange column chromatography as per the methods of Lanham and Godfray (1970) followed by centrifugation at 3000 rpm for 5 minutes for obtaining the purified parasite pellet.

The genomic DNA from purified parasite was extracted by method of Sambrook and Russel (2001) with little modifications. Briefly, 200 µl of purified parasite was treated with 200 µl of solution I (10 mM Tris, pH-7.6 ; 10 mM KCl; 10 mM $MgCl_2$) and 48 µl of 1/10x NonidetP$_{40}$ was added to lyse the cells, mixed well by vortexing for 10–15 seconds, spinned at 2000 rpm for 10 minutes. Supernatant was poured off and pellet resuspended in 60 µl of solution II (10 mM Tris, pH-7.6; 10 mM KCl; 10 mM $MgCl_2$; 0.5M NaCl; 0.5% SDS; 2 mM EDTA). 10 µl of proteinaseK was added followed by mixing and kept at 55°C for 2 hours. 30 µl Tris saturated phenol (pH-8.0) was added and vortexing for 2–5 seconds, spinned at 10,000 rpm for 1 minute, upper phase was transferred to a clean micro-centrifuge tube. This aqueous phase was treated with 40 µl of phenol: chloroform: isoamyl alcohol (PCI) in 25:24:1 ratio and centrifuged at 10,000 rpm for 1 minute at room temperature. Upper aqueous phase was collected in a clean centrifuged tube again and treated with 60 µl volume of chloroform: isoamyl alcohol (24:1) and finally collected after centrifugation at 10,000 rpm for 10 minutes at RT. To the aqueous phase, double volume of ice cold absolute ethanol was added and spinned at 12,000 rpm for 8 minutes. Ethanol was poured off gently and 400 µl of 70% ethanol was added to wash the DNA with spinning at 12,000 rpm for 8 minutes. The DNA pellet settled at the bottom of the tube and ethanol was discarded and pellet was air dried. Finally, DNA pellet was resuspended in 100 µl of nuclease free water and stored at -20°C or less temperature. The concentration and purity of isolated DNA was measured using Nanodrop spectrophotometer (Bio Photometer plus, Eppendorf).

Standardization of PCR using ITS-1 Primer: The conserved sequence data of ITS 1 gene of above said *T.* evansi isolate (recently sequenced at NRCE) was used for designing of primer which amplified 151 bp product of ITS-1 region between 18 S and 5.8 S subunit of rRNA. The PCR conditions were standardized using ITS1 NRCE primer (Table 1) with purified *T. evansi* DNA. Reactions were carried out in 25 µl mixture using DreamTaq Master Mixture containing 0.05 unit/µl Taq DNA polymerase, reaction buffer, 4 mM $MgCl_2$, 0.4 mM of each dNTP (dATP, dCTP, dGTP and dTTP), 0.4 µM of each primer and 1.0 µl templates DNA (15 ng/µl concentration). The PCR conditions were first standardized as given in Table 2.

PCR-amplified products were electrophoresed in 2% agarose in 1 × TAE buffer and visualized under UV light with the help of staining dye EtBr and optimum condition of amplification were selected which giving a visual size band without any non-specific amplification.

Serial dilutions of whole blood and purified *T. evansi* DNA: Whole blood and purified parasites genomic DNA obtained by phenol: chloroform: isoamyl alcohol method as described above, concentration was obtained by spectrophotometric technique. Estimated concentration of DNA from

Table 1: Details of primers targeting ITS-1 region of *T.evansi* for PCR study

Oligo name	Sequence 5'-3'	Length	Tm (°C)	GC (%)	Product Size
ITS1-NRCE-F	CAGTAGTAATAACACAGAGAAT	22	49.4	31.8	151 bp
ITS1-NRCE-R	AATAGGAAGCCAAGTCAT	18	53.5	38.8	

Table 2: Parameters used for optimization of ITS-1 PCR

Test	Component	Quantity	PCR Conditions
ITS1-PCR NRCE	Dream Taq PCR Master Mix	12.5 µl	Initial denaturation at 94°C for 5 min; Denaturation at 94°C for 30 sec, annealing at 48–55°C for 30 sec & extension at 72°C for 30 sec for 35 cycles; Final extension: 72°C for 2 min
	Primer Forward (10µM/µl)	1.0 µl	
	Primer Reverse (10µM/µl)	1.0 µl	
	Template DNA	1.0 µl (20 ng)	
	Nuclease free water	upto 25 µl	

Table 3: Parasitic equivalence for serially diluted purified *T. evansi* DNA

S.No.	Estimated *T. evansi* DNA Concentration	Equivalence of number of *T. evansi* genome (/µl)
1.	200 ng/µl	2.0×10^{6} genomic equivalence
2.	20 ng/µl	2.0×10^{5} genomic equivalence
3.	2 ng/µl	2.0×10^{4} genomic equivalence
4.	0.2 ng/µl	2.0×10^{3} genomic equivalence
5.	0.02 ng/µl	2.0×10^{2} genomic equivalence
6.	2 pg/µl	2.0×10^{1} genomic equivalence
7.	0.2 pg/µl	2.0×10^{0} genomic equivalence
8.	0.02 pg/µl	2.0×10^{-1} genomic equivalence
9.	0.002 pg/µl	2.0×10^{-2} genomic equivalence
10.	0.0002 pg/µl	2.0×10^{-3} genomic equivalence

purified parasite and whole blood was 200 ng/µl and 350 ng/µl, respectively. Serial dilutions of purified *T. evansi* genomic DNA and whole blood DNA prepared to obtain a regular series of 10 fold dilutions ranging from 10^{0} (200 ng/µl) to 10^{-10} (0.00002 pg/µl) and from 10^{0} (350ng/µl) to 10^{-7} (0.00035 ng/µl), respectively.

Sensitivity PCR: The PCR were performed with optimized conditions for ITS-1 NRCE primer as described in Table 2. 2 µl of serially diluted DNA from whole blood and purified *T. evansi* DNA used in PCR reaction. Parasite equivalent/genomic equivalent used in each PCR reaction calculated from estimated DNA quantity with the consideration that a single parasite genomic weight is 0.1 pg (Borst *et al*., 1982). Calculated parasite equivalence for estimated DNA concentration (purified parasite) for each dilution was given in Table 3. Whole blood DNA was extracted from known no of parasites (Table 4).

RESULTS AND DISCUSSION

Development and improvement of methods to control surra are of prime importance and the presently available diagnostic tests for detection of *T. evansi*, involve direct demonstration of parasites by stained thin blood film examination, only reliable in acute stage of disease able to detect parasitaemia ≈ 10^{5}/ml of blood (Paris *et al*., 1982). Serological techniques are unable to differentiate present and past form of infection (Njiru *et al.,* 2004). To increase the sensitivity molecular methods has been developed. In this regard, PCR-based techniques have shown very high sensitivity (Masiga *et al.*, 1992) for detection of *T. evansi*, varying according to primer used. The primer targeting highly repetitive satellite DNA considered as gold standard primer for diagnosis of *T. evansi* infection (Masiga *et al*., 1992; OIE, 2012).

Table 4: Parasitic equivalence for serially diluted whole blood *T. evansi* DNA

S.No.	Concentration of DNA	No. of parasites in\ successive dilutions
1.	350 ng/µl	4×10^{5} parasite/µl of blood
2.	35 ng/µl	4×10^{4} parasite/µl of blood
3.	3.5 ng/µl	4×10^{3} parasite/µl of blood
4.	0.35 ng/µl	4×10^{2} parasite/µl of blood
5.	0.035 ng/µl	4×10^{1} parasite/µl of blood
6.	0.0035 ng/µl	4×10^{0} parasite/µl of blood
7.	0.00035 ng/µl	4×10^{-1} parasite/µl of blood

In this study, the ITS1 NRCE primers were designed to amplify ITS-1 region between 18 S and 5.8 S subunit of rRNA. ITS1 gene fragments were amplified using genomic DNA extracted from purified trypanosomes. Desired amplicons of approximately 151 bp size for ITS1 gene was obtained as resolved by 2% agarose gel electrophoresis and revealed by EtBr staining (Fig. 1).

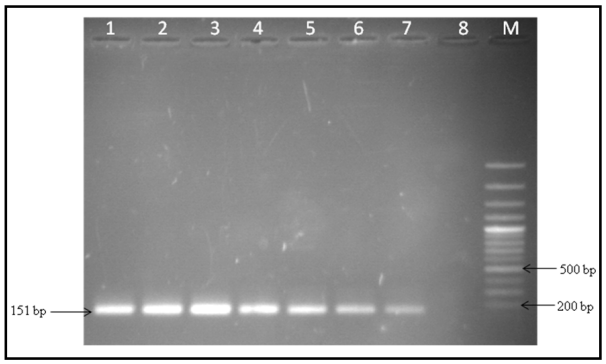

Fig. 1: Lane 1-7: ITS1 NRCE primer amplified 151 bp product at different temperatures 52°C- 58°C; Lane 8: Negative control; Lame M: 100 bp plus DNA marker

The optimum amplification was at 54°C. Gold standard TBR primer set (Masiga *et al*., 1992) was used by different researchers and revealed different level of sensitivity ranging from 1 parasite in infected tsetse flies (Masiga *et al*., 1992), 0.01 pg of purified DNA, about 0.1 parasite (Pruvot *et al*., 2010) to detected up to 0.001 pg about 0.01 parasites (Fernandez *et al*., 2009), with ISG-75 primer sensitivity PCR detection limit was 0.04 pg/µl (Rudramurthy *et al*., 2013) While our ITS1 NRCE primer was able to detect up to 7th dilution (0.2 pg/2 µl) or 2 parasitic equivalence from purified parasite DNA (Fig. 2), which was less as compared to TBR PCR and ISG-75 diagnostic sensitivity. From whole blood DNA dilutions detection sensitivity of ITS1 NRCE primer was 0.035 ng/2µl of blood (Fig. 3), which was comparatively higher than 1.2 ng/µl with ISG-75 primer (Rudramurthy *et al*., 2013).

Routine parasitological and serological methods have the limitation to detect active infection in latent or carrier stage of disease. The PCR assay developed in this study was able to efficiently detect *T. evansi* DNA at very low level, 2.0 pg of purified parasite DNA and 0.035 ng of infected whole blood DNA. The present study findings can be used as a rapid, reliable molecular tool for sensitive detection of *T. evansi in* carrier and treated animals.

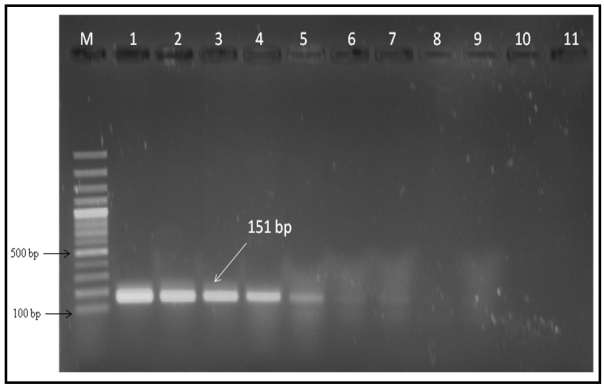

Fig. 2: Sensitivity of PCR assay by dilution of purified *T. evansi* DNA in water; Lane M: 100 bp plus DNA marker; Lane 1,2,3,4,5,6,7,8,9 and 10 = 200 ng/µl, 20 ng/µl, 2 ng/µl, 0.2 ng/µl, 0.02 ng/µl, 2 pg/µl, 0.2 pg/µl, 0.02 pg/µl, 0.002 pg/µl and 0.0002 pg/µl, respectively; Lane 11: Negative control

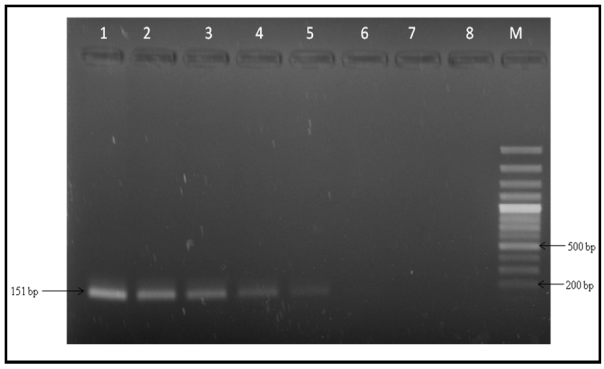

Fig. 3: Sensitivity of PCR assay by dilution of *T. evansi* infected mice blood DNA in water; Lane M: 100 bp plus DNA marker; Lane 1,2,3,4,5,6,7 and 8 = 350 ng/µl, 35 ng/µl, 3.5 ng/µl, 0.35 ng/µl, 0.035 ng/µl, 0.0035 ng/µl and 0.00035 ng/µl, respectively; Lane 8: Negative control

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