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Sensitive detection of *Trypanosoma evansi* infection by polymerase chain reaction targeting invariable surface glycoprotein gene

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ABSTRACT

Trypanosoma evansi, an extracellular haemoprotozoan parasite, causes surra in a wide range of domestic and wild animals. In the present study, a diagnostic PCR assay was developed using primers targeting invariable surface glycoprotein (ISG) gene of *T. evansi,* which amplified a 196 bp product. The DNA was extracted from purified trypanosomes and *T. evansi* infected mice blood, and serially diluted ranging from 20 ng/µl to 0.002 pg/µl and from 90 ng/µl to 0.009 pg/µl, respectively. The diagnostic sensitivity of the PCR assay was 0.02 ng/µl (2×10^1 parasites) and 0.09 ng/µl (1×10^2 parasites) with purified parasite DNA and infected mice blood DNA, respectively. The PCR assay was also performed on extracted genomic DNA from 86 blood samples from the field out of which 3 animals were found positive by ISG-PCR assay. The developed ISG gene based PCR assay could be employed for sensitive detection of early infection, sub-clinical status of trypanosomosis and drug efficacy studies in animals.

Key words: Equines, Invariable surface glycoprotein gene, PCR, Surra*, Trypanosoma evansi*

Trypanosoma evansi, an extra-cellular haemoprotozoan parasite, multiplies in blood, body fluids and tissues resulting in a wasting disease commonly known as surra, is clinically characterized by fever, weight loss, hypoglycemia, severe anaemia, reduced reproductivity, abortion and may also leads to death in the acute phase of the disease. *Trypanosoma evansi* is transmitted mechanically by biting of blood sucking flies including tabanids (*Tabanus, Haematopota, Haematobia*). It affects a wide range of domestic livestock and wild animals (OIE 2012). The disease is having a world-wide distribution among all pathogenic parasitic spp., is 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). Diagnosis of surra is often difficult by using conventional parasitological techniques (Nantulya, 1990) due to the fluctuating nature of parasites. Limitations of various parasitological and serological techniques arise the need for more sensitive and specific DNA based assays like PCR. The PCR is a rapid, sensitive and specific DNA based assay detecting very low level of parasitaemia (Pruvot *et al*. 2010). The invariable surface glycoprotein (ISG) does not exhibit any antigenic variation (Tran *et al*. 2008) and are uniformly distributed over the entire surface of the trypanosomes (Nolan *et al*. 1997). is conserved among all the species and subspecies of the Trypanozoon subgenus including *T. evansi*, *T. brucei gambiense*, *T. brucei rhodesiense*, *T. equiperdum* and found as multiple copies (Pays and Nolan 1997, Tran *et al*. 2008). Hence, the present study was designed to explore the diagnostic sensitivity of the PCR assay targeting invariable surface glycoprotein gene of *T. evansi* in experimentally infected mice and naturally infected equines.

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MATERIALS AND METHODS

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

Propagation and purification of T.evansi: 1×10^5 trypanosomes were inoculated to Swiss albino mice 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

Table 1. Parameters used for optimization of ISG PCR

Test	Component	Quantity	PCR conditions
ISG PCR	Dream Taq PCR Master Mix	12.5µl	Initial denaturation: 95°C for 5min
	Primer forward (10µM/µl)	1.0µl	Denaturation: 95°C for 30sec
	Primer reverse (10µM/µl)	1.0µl	Annealing: 54–60°C for 30sec × 30
	Template DNA	1.0 µl (20 ng)	Extension: 72°C for 30sec
	Nuclease free water	up to 25µl	Final extension: 72°C for 5min

Table 2. Parasitic equivalence for serially diluted whole blood *T. evansi* DNA

DNA concentration	No. of parasites in successive dilutions
90 ng/µl	1×10^5 parasites
9.0 ng/µl	1×10^4 parasites
0.9 ng/µl	1×10^3 parasites
0.09 ng/µl	1×10^2 parasites
9.0 pg/µl	1×10^1 parasites
0.9 pg/µl	1×10^0parasites
0.09 pg/µl	1×10^{-1} parasites
0.009 pg/µl	1×10^{-2} parasites

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

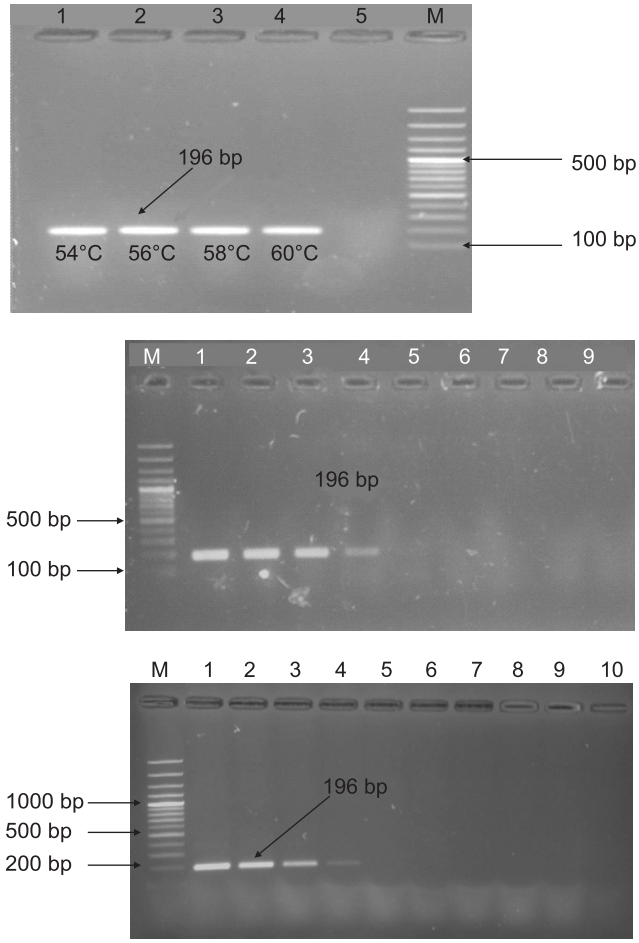
Estimated *T. evansi* DNA concentration	No. of parasites in successive dilutions from purified parasites
20 ng/µl	2×10^4
2 ng/µl	2×10^3
0.2ng/µl	2×10^2
0.02 ng/µl	2×10^1
2.0 pg/µl	2×10^0
0.2 pg/µl	2×10^{-1}
0.02 pg/µl	2×10^{-2}
0.002 pg/µl	2×10^{-3}

DEAE-cellulose anion exchange column chromatography as per Lanham and Godfray (1970) followed by centrifugation at 3,000 rpm for 5 min for obtaining the purified parasite pellet.

DNA extraction: The genomic DNA from purified parasites along with experimentally infected mice blood was extracted separately by the method of Sambrook and Russel (2001) with little modifications. Finally, the DNA pellet was dissolved in 100µl of nuclease free water and stored at –20°C till further use. The concentration and purity of isolated DNA was measured using nanodrop spectrophotometer.

Primer designing: A conserved region was selected from ISG gene sequences of *T. evansi* isolates. The primers were designed from the ISG gene sequence of *T. evansi* isolate of Indian horse origin available in the GenBank database (Accession No. KR858261) using conserved region of 1201 bp to 1397 bp of ISG gene sequence i.e., ISG196-F: AAAGCCACCGAAGATGCAGA and ISG196-R: TTGTCCCAATCCAGCCACTC.

Standardization of PCR: The PCR conditions were optimized with ISG primers at different annealing temperatures ranging from 54°- 60°C. PCR reactions were carried out in 25 µl mixture using DreamTaq Master Mixture containing 0.05 unit/µl Taq DNA polymerase, reaction



Figs 1–3. **1.** Lane 1–4, ISG primer amplified 196 bp product at different annealing temperatures; lane 5, negative control; lane M, 100 bp plus DNA marker. **2.** Sensitivity of PCR assay by dilution of purified *T. evansi* DNA. Lane M, 100bp plus DNA marker; lane 1–7, 20 ng/µl, 2 ng/µl, 0.2 ng/µl, 0.02 ng/µl, 2 pg/µl, 0.2 pg/µl and 0.02 pg/µl; lane 8, *Theileria equi* DNA; lane 9, negative control. **3.** Sensitivity of PCR assay by dilution of *T. evansi* infected mice blood DNA. Lane M, 100bp plus DNA marker; lane 1-8, 90 ng/µl, 9.0 ng/µl, 0.9 ng/µl, 0.09 ng/µl, 9.0 pg/µl, 0.9 pg/µl, 0.09 pg/µl, 0.009 pg/µl; lane 9, *Theileria equi* DNA; lane 10, negative control.

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 (20 ng/µl concentration). The PCR conditions were first standardized as given in Table 1. PCR amplified products were electrophoresed and visualized the PCR bands.

Serial dilutions of whole blood and purified T. evansi *DNA:* The trypanosomes numbers were calculated for DNA extraction in experimentally infected mice whole blood and purified parasites counted by Neubauer chamber. The DNA was isolated by phenol:chloroform:isoamyl alcohol method. The concentration of DNA from experimentally infected mice blood (5×10^7 trypanosomes/ml) and purified parasites (1×10^7 trypanosomes/ml) was estimated as 90ng/µl and 20 ng/µl, respectively. Serial dilutions of whole blood DNA and purified *T. evansi* genomic DNA were prepared to obtain a regular series of 10 fold dilutions ranging from 10^0 (90 ng/µl) to 10^{-7} (0.009 pg/µl) and 10^0 (20ng/µl) to 10^{-7} (0.002pg/µl), respectively (Tables 2, 3). PCR was performed using optimized conditions for ISG primer as described above. 1µl of serially diluted DNA was used in PCR.

Blood collection from experimentally infected mice and field equines: To know the detection sensitivity of the PCR assay in terms of duration post infection, 6 Swiss albino mice were experimentally infected with 1×10^4 trypanosomes intra-peritoneally. The blood samples were collected from each infected mouse at 24 h interval till peak parasitemia. The DNA was extracted and ISG-PCR assay was employed on these samples as per PCR condition described above. To know the applicability of this assay in field, 86 blood samples from equines were also collected from *T. evansi* endemic area adjoining to Hisar and Meerut and analyzed for detection of *T. evansi* and compared with OIE recommended gold standard TBR-PCR assay (Masiga *et al*. 1992).

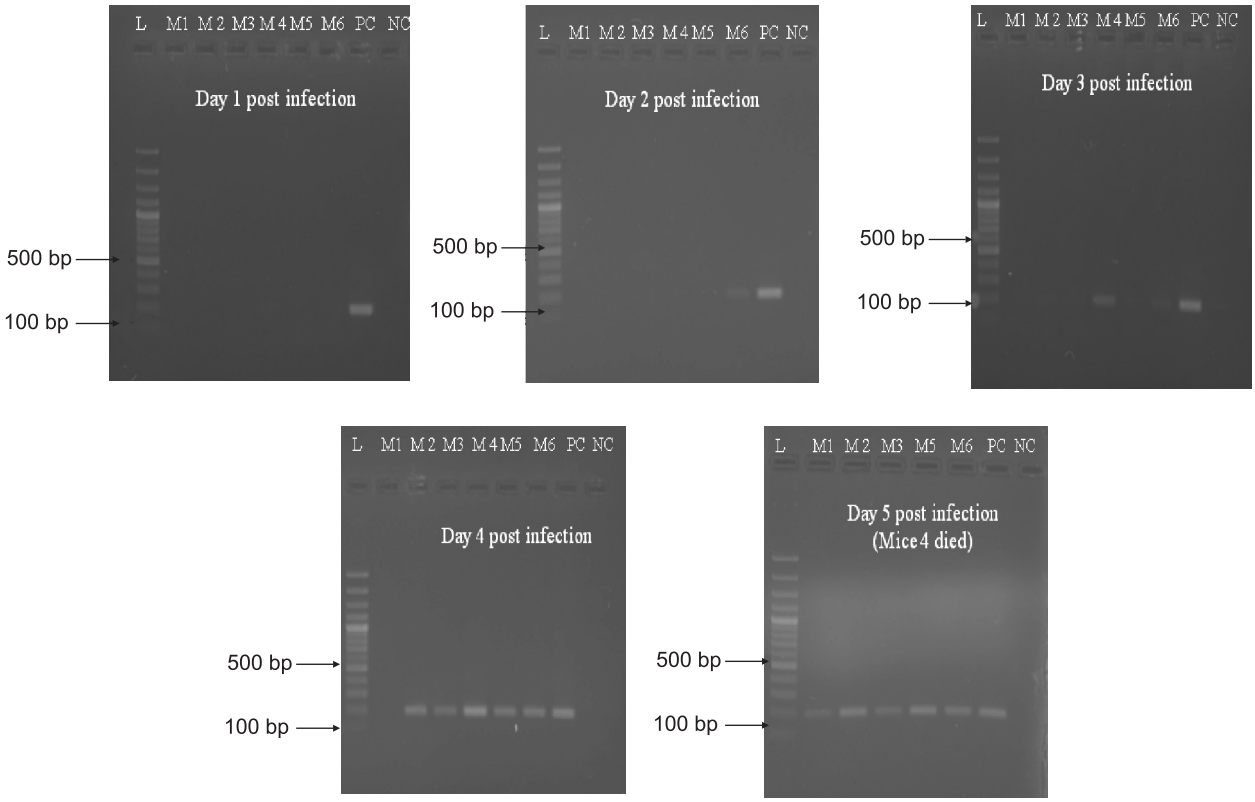


Fig. 4. Amplification of 196 bp amplicon using ISG primer from *T. evansi* experimentally infected mice blood. lane L, 100 bp plus DNA ladder; lane M1-M6, PCR products from 1–6 mice infected with *T. evansi;* lane PC, positive control; lane NC, negative control.

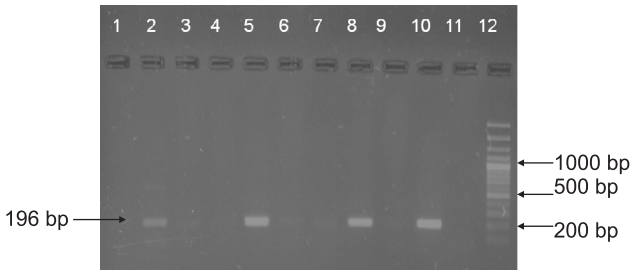


Fig. 5. Diagnostic ISG PCR on DNA samples extracted from blood of field equines for detection of *T. evansi* infection. Lane 12, 100bp plus DNA marker; lane 10, positive control; lane 11, negative control.

RESULTS AND DISCUSSION

For ISG-PCR assay optimization primers were designed using a conserved region sequence of ISG gene of *T. evansi* Indian isolate for amplification of 196 bp size product.

Based on better resolution and band strength we have selected 58°C annealing temperature (Fig.1). After optimization, the sensitivity of this assay was determined and found that ISG-PCR assay was able to detect up to 0.02 ng/µl of genomic DNA extracted from purified parasites (Fig. 2), and 0.09 ng/µl of DNA extracted from blood of *T. evansi* experimentally infected mice (Fig. 3). In our study, the diagnostic sensitivity of ISG based PCR assay for DNA of purified parasites was more sensitive than *T. evansi* infected whole blood DNA. This variation in sensitivity probably may be due to the presence of host cell DNA and other inhibitory factors for PCR in blood that lowers the sensitivity of the assay (Kabiri *et al*. 1999). Rudramurthy *et al*. (2013) reported detection sensitivity of 0.04 pg and 1.2 ng of DNA from purified trypanosomes and *T. evansi* infected rat blood samples, respectively in PCR based on ISG-75 sequence with amplification of 407 bp. This may be due to imprecision in DNA titration technique, reaction conditions, genetic variation of *T. evansi* strains, and greatly the DNA extraction method from infected blood etc. (Pruvot *et al*. 2010). In the present study, we designed the primers which amplified 196 bp size of gene fragment. A successful PCR assay requires efficient and specific amplification of the product, if the purpose is to develop a clinical assay to detect a specific DNA fragment, the product size of 120–300 bp after DNA amplification may be optimal (Dieffenbach *et al*. 1993). The short PCR products are typically amplified with higher efficiency than longer ones and longer PCR products will also require more extension times than short ones.

The standardized assay was also applied to extracted DNA samples from blood of 6 experimentally *T. evansi* infected mice to determine the sensitivity in terms of duration post infection. On day 1, none of mice was found positive, on day 2 post infection, 1 mouse was detected positive and on day 3 and 4 post infection, 2 and 5 mice detected positive, respectively. On fifth day post infection, all mice were found positive by PCR (Fig. 4). Further, 86 DNA samples extracted from the blood of field equines for detection of *T. evansi* infection. The DNA samples were tested with ISG-PCR (Fig. 5) and OIE recommended gold standard TBR-PCR assay. In both the assays, 3 blood samples were found positive for *T. evansi* infection. This study indicated that ISG-PCR assay could also be used for sensitive detection of surra in field equines.

The developed ISG gene based PCR assay in the present study could be employed for sensitive detection of early infection, sub-clinical status of trypanosomosis, localization of trypanosomes in different host tissues, for differentiation of *T. evansi* infected and treated animals and drug efficacy studies in animals.

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