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PCR assay for the detection of partial variable surface glycoprotein gene in *Trypanosoma evansi* in canines

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Abstract
The Polymerase Chain Reaction assay using the species-specific diagnostic RoTat 1.2 VSG primers targeting the partial VSG gene of *T. evansi* was standardized, which amplified a product of 205 bp length. The standardized PCR assay showed an analytical sensitivity of detection up to 1 pg DNA of *T. evansi* in our lab. A total of 314 blood samples were collected from dogs and 7.64% (24/314) were found positive by standardized PCR assay in and around Hyderabad, Telangana. A non-significant difference existed in the prevalence in male (7.58%) and female (7.69%) dogs. Age wise comparison of prevalence indicated a significantly higher rate of infection in 1-8 years (11.46%), > 8 years (5.88%), and < 1 year (1.38%) age dogs in descending order. The highest prevalence was noticed among Mongrels (15.68%) and then crossbred dogs (13.04%), Labradors (3.92%), German shepherd dogs (3.22%), Doberman (2.94%), Pomeranian (2.56%) in descending order and no prevalence was recorded in Saint Bernard, Pug and Golden Retriever.

Keywords: *Trypanosoma evansi*, variable surface glycoprotein, dog

Introduction
Trypanosoma evansi, a haemoprotozoan causing a significant disease called 'Surra'. In 1880, it was described by Griffith Evans for the first time in the blood of Indian equines and dromedaries (Hoare C A 1972) [1]. It is widely distributed and is mechanically transmitted by *Stomoxys*, *Tabanus*, and *Haematobia* spp. (Desquesnes *et al.*, 2013) [2]. Orally transmitted by feeding fresh infected meat in carnivores (Bhatia *et al.*, 2006) [3]. Dogs are more prone to *T. evansi* infection and clinically exhibit signs of anorexia, intermittent fever, anaemia, corneal opacity, pharyngeal oedema, cachexia, hoarse voice, staggering gait, conjunctivitis, lacrimation (Varshney *et al.*, 1998) [4] and death occurs within a month in acute cases.
Understanding the limitations of microscopy and immunological techniques in diagnosing *T. evansi* infection, a more sensitive and specific molecular technique like PCR is used to detect *T. evansi*. Hence, an attempt was made to standardize a PCR assay that amplifies a partial VSG gene of *T. evansi.*

Materials and Methods
Collection of *T. evansi* positive blood from dog and its propagation
Trypanosoma evansi, a dog strain was obtained from a male mongrel of 7 years old showing clinical signs of pyrexia (104.5 °F), corneal opacity, anaemia, anorexia, loss of body weight, generalized weakness (figure 1), hypersalivation, staggering gait and found positive by wet blood film examination under high power objective of the light microscope.
About 0.25 ml of blood was collected from the ear vein of the dog aseptically and inoculated intraperitoneally into a Swiss albino mouse for the propagation of *T. evansi* in the laboratory (Prior permission was taken from the Institutional IAEC approval No. for the project - 30/24/C.V.Sc., Hyd. IAEC-Mice, dated 12.06.2021).

Fig 1: Dog emaciated due to *Trypanosoma evansi* infection

Isolation of host cell-free *T. evansi* by Diethyl aminoethyl-cellulose chromatography

T. evansi infected mouse blood was collected in a sodium heparin vacutainer and immediately diluted with chilled PSG buffer (pH 8.0) in a 1:3 ratio. The diluted blood was charged drop by drop carefully along the sides on to DEAE cellulose column followed by a small quantity of PSG buffer without disturbing the column. The elute was collected as drops into Eppendorf tubes and centrifuged at 2,400 g for 20 min. The pellet of pure trypanosomes without host cells was washed twice and resuspended in PBS. Finally stored at -20 ℃ until further DNA extraction.

Genomic DNA extraction from *T. evansi*

DNA was extracted from host cell-free trypanosomes as described by Sambrook *et al*. (1989) [5] with slight modifications. The purified trypanosomes stored at -20 ℃ were thawed to room temperature, and centrifuged at 3,000 rpm for 10 min. Cell lysis buffer and Proteinase K were added to the sediment and incubated at 56 ℃ for 6 hr. It was then layered by 1ml PCI in 25:24:1, homogenized, and then centrifuged at 13,000 rpm for 10 min at 4 °C. Equal volume of Chloroform: Isoamyl alcohol (24:1) is added to an aqueous layer, and centrifuged. The final aqueous phase was aspirated and 10 M Ammonium acetate, absolute ethanol were added and mixed gently. Later the precipitated DNA was collected into a fresh Eppendorf tube, and 70% ethanol was added and centrifuged. The supernatant was discarded carefully retaining the DNA pellet and it was washed two times by 70% ethanol. Finally, the supernatant was decanted completely and airdried the pellet, and dissolved in TE buffer.

Agarose gel electrophoresis for checking the presence of gDNA of *T. evansi*

One µl of extracted gDNA of *T. evansi*, 4 µl of nuclease-free water and one µl of 6x gel loading dye were mixed thoroughly and loaded into wells along with the ethidium bromide stained agarose gel (0.7%). Electrophoresis was carried out at 50 V (1-1.5 hr), and the gel was documented in a gel documentation system.

Standardization of PCR assay

PCR reaction for the amplification of partial VSG gene of *T. evansi* using RoTat 1.2 VSG Forward 5' GCGGGGTGTTTAAAGCAATA 3' and Reverse primers 5' ATTAGTGCTGCGTGTGTTCG 3', was standardized as suggested previously (Claes *et al*., 2004) [6]. 25 µl of reaction volume containing 10 pmol of each primer, 2 µl of template DNA, 8.5 µl of nuclease free water, and 12.5 µl Master mix, was run under PCR conditions of 4 mins initial denaturation at 94 ℃, followed by 40 amplification cycles (94 ℃ for 30 sec, 59 ℃ for 30 sec and 72 ℃ for 30 sec) and final extension at 72 ℃ for 5 min. In every reaction, a negative control was run using nuclease free water. The amplified PCR products were electrophoresed on an ethidium bromide-stained 2% agarose gel (1-1.5 hr. at 75V) and visualized by Gel Doc.

Analytical sensitivity

Trails were performed in the laboratory to estimate the lowest quantity of template DNA that could diagnose *T. evansi* by producing a visible band. Appropriate descending 10-fold serial dilutions of template DNA were made in autoclaved triple distilled water, starting from a 100 ng concentration, and were subjected to standardized PCR conditions.

Whole blood samples of 314 dogs of both males and females of all age groups and breeds suspected of *Trypanosoma evansi* infection collected from in and around Hyderabad, were screened using a standardized PCR assay.

Results

DEAE cellulose purified *T. evansi* yielded DNA with a concentration of 517 ng/µl of TE buffer, with a purity of 1.83 (A260/A280 ratio). Electrophoresis of *T. evansi* DNA in 0.7% agarose gel showed a clear band (Figure 2). Standardized PCR assay for amplification of partial VSG gene of *T. evansi* with specific diagnostic primers has produced a single product of 205 bp length without any amplicon in the negative control (Figure 3) and it has also detected as low as 1 pg of gDNA of *T. evansi* (Figure 4).

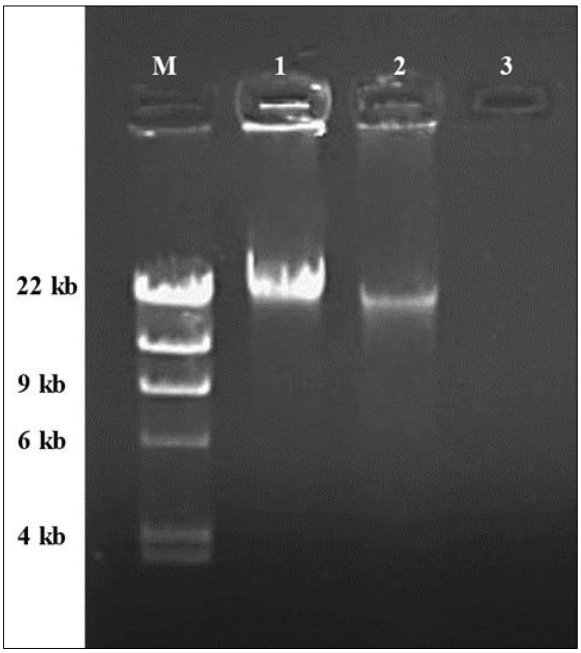

Fig 2: Genomic DNA of Dog Leucocyte and *Trypanosoma evansi* (0.7% agarose gel) M: Molecular marker (Lambda *Hind* III digest), 1: Dog Leucocyte DNA, 2: *Trypanosoma evansi* DNA

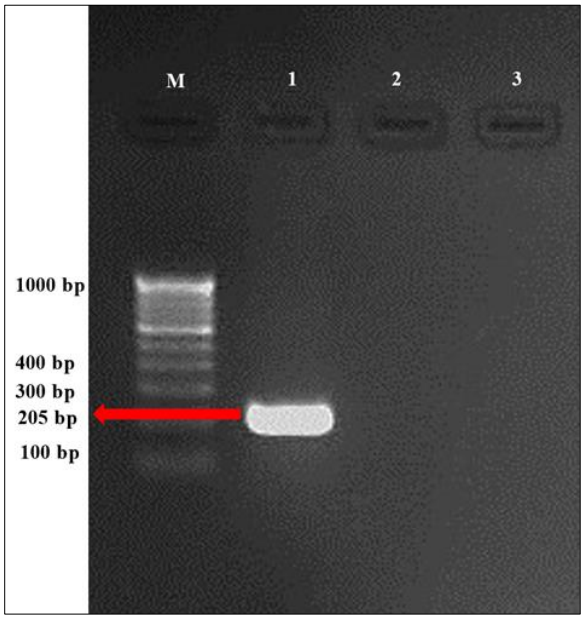

Fig 3: Standardization of PCR assay for the diagnosis of *Trypanosoma evansi* in canines M: 100 bp marker, 1: *Trypanosoma evansi* DNA, 2: Dog Leucocyte DNA, 3: Negative control

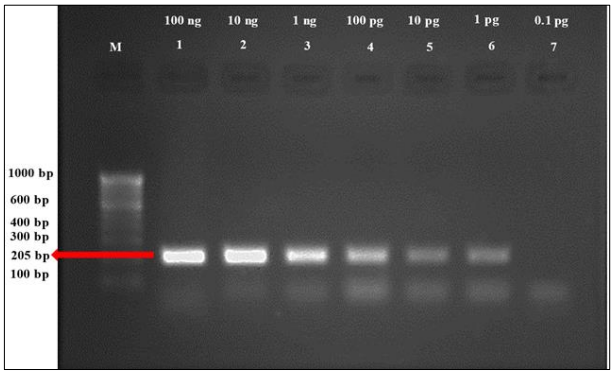

Fig 4: PCR assay with ten-fold serial dilutions of genomic DNA of *T. evansi* M: 100 bp marker, 1: Genomic DNA of *T. evansi* -100 ng, 2: 10 ng, 3: 1 ng, 4: 100 pg, 5: 10 pg, 6: 1 pg, 7: 0.1 pg.

314 dog samples collected from the field were tested under standardized PCR conditions and an amplicon of 205 bp length was found in 24 (7.64%) dogs (Figure 5). Non-significantly higher prevalence was reported in female (7.69% *i.e.*, 13/163) dogs than in male (7.58% *i.e.*, 11/145) dogs. Age-wise prevalence has revealed a significantly higher prevalence in adult dogs (11.46% *i.e.*, 18/157) of 1-8 years age than the dogs of > 8 years (5.88% *i.e.*, 5/85) and puppies < 1 year age. Breed-wise prevalence was recorded as 15.68% (16/102) in mongrel dogs followed by 13.04% (3/23) in crossbreed dogs, 3.92% (2/51) in Labradors, 3.22% (1/31) in German shepherd dogs, 2.94% (1/34) in Doberman, 2.56% (1/39) in Pomeranian and 0% (0/9) in St. Bernard, (0/11) Pug and (0/14) Golden Retriever in descending order.

Fig 5: Field diagnosis of canine Trypanosomosis by standardized PCR assay M: Gene Ruler of 100 bp DNA ladder, Lane 1 - Positive control, 3,5,6,8 - Positive dog blood samples, 2,4,7,9,10,11 - Negative dog blood samples, 12 - Negative control

Discussion

Apart from mechanical transmission, oral transmission is common in canines because of feeding infected fresh meat (Raina *et al.*, 1985) [7]. Thus, care must be taken regarding the feeding habits of dogs. Although there are many techniques, microscopic examination remains the golden standard test for the detection of *T. evansi* in the blood of an infected animal in the field. But it cannot detect subacute or chronic infection (Fernandez *et al.*, 2009) [8]. The PCR is very sensitive as it can multiply even traces of *T. evansi* DNA to a detectable quantity under appropriate cycling conditions (Uilenberg, 1998) [9].

The sensitivity of PCR assay in detecting as low as 1 pg of pure *T. evansi* DNA in our study is in accordance with the studies of Claes *et al.* (2004) [6] who diagnosed as low as 10 trypanosomes using the same primer sets, which is equivalent to 1 pg of *T. evansi* DNA (Borst *et al.*, 1982) [10]. PCR assays with different sensitivities using various sets of primers in the detection of *T. evansi* DNA were documented by various workers [11-14].

Sex-wise prevalence of canine Trypanosomosis in our study is in accordance with Prasad *et al.* (2015) [15], Chowdhury *et al.* (2005) [16], Alli *et al.* 2021 [17] indicating equal susceptibility of dogs of either sex to *T. evansi*. Very less prevalence in dogs of less than 1 year of age might be due to more attention paid by owners in providing proper housing, feeding habits, and less exposure to biting flies. Also, the low incidence of infection in dogs > 8 years might be attributable to the enhanced immune response of the dogs. The higher incidence in the age 1-8 years might be due to more risk of attacks of biting flies like *Tabanus*, living near carrier hosts like bovines, dogs which are used for guarding at dairy farms, fish ponds where there is more fly activity, ingestion of fresh infected meat or carcasses.

Significantly higher prevalence of Trypanosomosis in mongrels compared to other breed dogs might be due to the easy exposure to the attacks of biting flies, surroundings which are favourable for the propagation of vectors, living in the vicinity of slaughterhouses where there is a chance of feeding infected fresh carcasses, close relationship with carrier animals like buffaloes, cattle, etc., which transmits Trypanosomosis to susceptible hosts easily. The non-significant difference in the prevalence was found among different breeds indicating no susceptibility of breeds to *T. evansi*, also reported by previous researchers [15-17].

Conclusion

The VSG gene present in the genome of *T. evansi* is expressed in the host during all the developmental stages of *T. evansi.* Considering the analytical sensitivity and specificity, it was concluded that the PCR assay that targets the partial VSG gene can be used as a quick, reliable, and more sensitive method for the diagnosis of canine Trypanosomosis at all stages of infection.

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