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M Fibi Rani
Department of Veterinary Parasitology, College of Veterinary Science, Hyderabad, PVNR TVU, Telangana, India

GS Sreenivasamurthy
Department of Veterinary Parasitology, College of Veterinary Science, Korutla, PVNR TVU, Telangana, India

M Udaya Kumar
Department of Veterinary Parasitology, College of Veterinary Science, Hyderabad, PVNR TVU, Telangana, India

P Kalyani
Department of Veterinary Biotechnology, College of Veterinary Science, Hyderabad, PVNR TVU, Telangana, India

Corresponding Author:
M Fibi Rani
Department of Veterinary Parasitology, College of Veterinary Science, Hyderabad, PVNR TVU, Telangana, India

Comparison of molecular and parasitological techniques for the diagnosis of canine trypanosomosis

M Fibi Rani, GS Sreenivasamurthy, M Udaya Kumar and P Kalyani

Abstract
Trypanosoma evansi, a kinetoplastid haemoprotozoan is the most pathogenic parasite in animals, causing a significant disease called "Surra". The objective of the present study is to determine the prevalence rates of canine Trypanosomosis using the Polymerase Chain Reaction (PCR) assay and parasitological techniques like wet blood film examination and Giemsa staining techniques. Out of 314 canine blood samples examined, 5 (1.59%) dogs were found positive by Giemsa staining technique, only 3(0.95%) dogs were found positive by peripheral wet blood film examination, whereas Polymerase Chain Reaction assay targeting the partial Variable Surface Glycoprotein gene has amplified a 205 bp length product in 24 samples with an overall prevalence of 7.64% in and around Hyderabad, Telangana, India. These findings suggest that the Polymerase chain reaction assay is a more sensitive and specific diagnostic tool than parasitological techniques for the detection of *Trypanosoma evansi* in the infected dog's blood.

Keywords: Canine trypanosomosis, giemsa staining, polymerase chain reaction, parasitaemia.

1. Introduction

Trypanosomosis is an important and widely prevalent haemoprotozoan disease caused by *Trypanosoma evansi.* It has a worldwide distribution and the widest host range. Transmission occurs mechanically, and non-cyclically by biting flies like *Tabanus, Stomoxys,* and *Haematobia* (Radwanska *et al.,* 2018) [1]. Oral transmission occurs by ingestion of fresh infected meat or carcass in dogs and wild carnivores, and also iatrogenic transmission occurs through contaminated needles (Bhatia *et al.,* 2006; Singh *et al.,* 1993) [2, 3].

Trypanosomosis in dogs was first described by Bevan in 1908 (Bevan 1913) [4]. In dogs, death occurs within a month in case of acute infection and is characterized by anorexia, progressive anaemia, intermittent fever, edema of the head and throat, hoarse voice, generalized weakness, paresis of hindquarters, staggering gait, corneal opacity (Chaudhuri *et al.,* 2009) [5] and alterations in the haematological and biochemical profile during the course of infection (Sarvanan *et al.*, 2005) [6]. The clinical signs are non-specific and vary with the host range, hence various diagnostic methods from conventional parasitological techniques to molecular techniques are available for the diagnosis of canine Trypanosomosis (Reid, 2001) [7].

Detection of trypanosomes by microscopy has been the golden standard test. However, it can be used to demonstrate trypanosomes during acute infection only. Although serological tests are sensitive, they rely on antibody detection and fail to distinguish a current infection from past infections which may result in false positives. A molecular technique like Polymerase Chain Reaction based on the detection of *T. evansi* DNA has been used widely (Masiga *et al.,* 1992; Ijaz *et al.,* 1998; Omanwar *et al.,* 1999) [8-10]. Hence, our study was undertaken to determine the reliability of parasitological techniques as compared with the PCR assay targeting partial VSG gene for the diagnosis of *Trypanosoma evansi* infection in canines at the field level.

2. Materials and Methods

2.1 Collection of blood samples

A total of 314 dogs of either sex and all ages suspected for Trypanosomosis were selected from clinics and animal birth control centres in different parts of Hyderabad. The dogs were suspected based on a history of pyrexia, anorexia, generalized weakness, corneal opacity (figure 1), anaemia, edema of the head and throat, hoarse voice, and staggering gait.

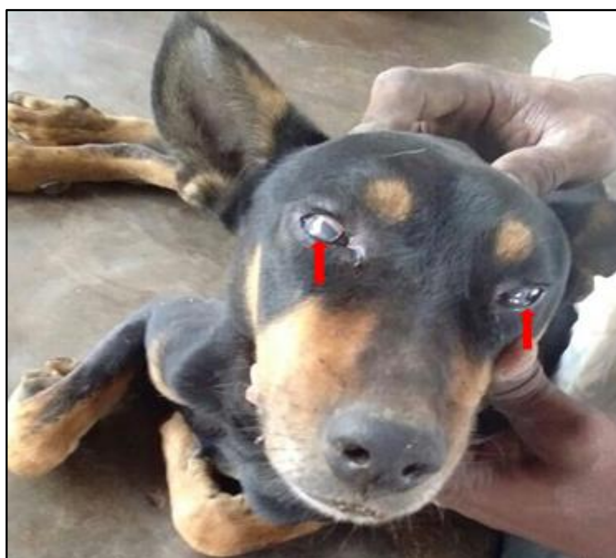


Fig 1: Bilateral corneal opacity in a dog due to Trypanosomosis

Under aseptic conditions, 2 ml of blood was collected by a sterile syringe from a cephalic vein of each dog into sterile sodium heparin-coated vacutainer and transported to the laboratory on the ice at 4 °C within 4 hr of collection and stored at -20 °C for extraction of DNA for further use in the PCR assay. A drop of blood from the marginal ear vein of the dog was immediately taken onto the slide for the wet blood film examination and smear preparation.

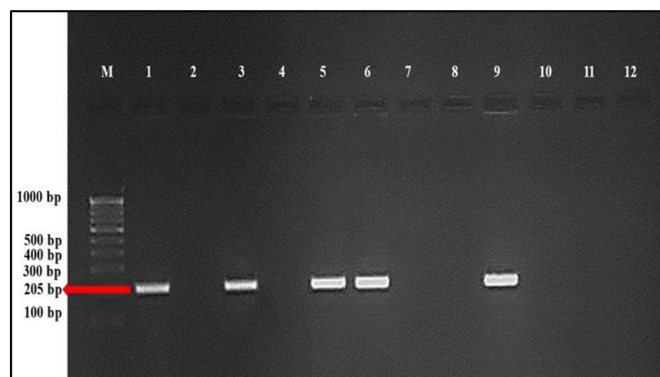
2.2 DNA extraction from canine blood samples and its amplification

DNA was isolated from the dog's blood by the phenol-chloroform method of DNA extraction (Muieed *et al.,* 2011) [11] with minor modifications. Nine parts of cell lysis buffer (0.2 M NaCl, 10 mM Tris HCl (pH 8.0), 10 mM EDTA (pH 8.0), 1% SDS) and 1/100 volume of proteinase K (100 µg/ml) were added to one part of blood sample in 2 ml Eppendorf tubes, followed by incubation in the dry bath at 56 °C for 6 hours until the solution was clear. After incubation, an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added and centrifuged at 12,000 rpm for 15 min at 4 °C.

The aqueous phase was carefully aspirated, mixed with 2.5 volumes of absolute ethanol, and incubated at -20 °C for the precipitation of genomic DNA. The DNA was concentrated by centrifugation at 12,000 rpm for 15 min at 4 °C. The supernatant was removed and adhering drops on the sides of the Eppendorf tube were blotted carefully without disturbing the DNA pellet. The DNA pellet thus obtained was air-dried and suspended in TE buffer (pH 8.0) and stored at -20 °C until used for amplification by PCR assay.

The PCR assay was carried out to evaluate its efficacy in the diagnosis of *T. evansi* and to compare it with parasitological techniques. The species-specific diagnostic primers, RoTat 1.2 VSG Forward 5'GCGGGGTGTTTAAAGCAATA3' and RoTat 1.2 VSG Reverse 5'ATTAGTGCTGCGTGTGTTC-G3' primers targeting partial VSG gene were used as described by class *et al.* 2004 [12]. The PCR reaction was performed in a 25 µl reaction mixture containing 10 pmol of each forward and reverse primers, 2 µl of sample DNA, 8.5 µl of Nuclease free water, 12.5 µl of Master mix, and run under the cycling conditions of initial denaturation at 94 °C for 4 min, followed by 40 amplification cycles of 1 min denaturation at 94 °C, 1 min annealing at 59 °C, and 1 min extension at 72 °C, followed by a final extension at 72 °C for 5 min.

The amplified PCR products were subjected to electrophoresis in ethidium bromide-stained (0.5 µg/ml) 2% (W/V) agarose gel, at 50 volts. After sufficient migration of dye (1-1.5 hr), the amplified PCR products on the gel were visualized by the gel documentation system and documented (figure 2).



M-Gene Ruler of 100bp DNA ladder, 1-Positive control of *T. evansi*, 3, 5, 6, 9-Positive dog blood samples, 2, 4, 7, 8, 10, 11-Negative dog blood samples, 12-Negative control

Fig 2: Field diagnosis of canine Trypanosomosis by PCR assay

2.3 Parasitological techniques

2.3.1 Wet blood film examination

A drop of blood was aseptically collected from the marginal ear vein of each suspected dog onto a clean grease-free microscopic slide, coverslip was placed on it and examined immediately for live and motile trypanosomes under a 45X objective of a bright field microscope (figure 3).

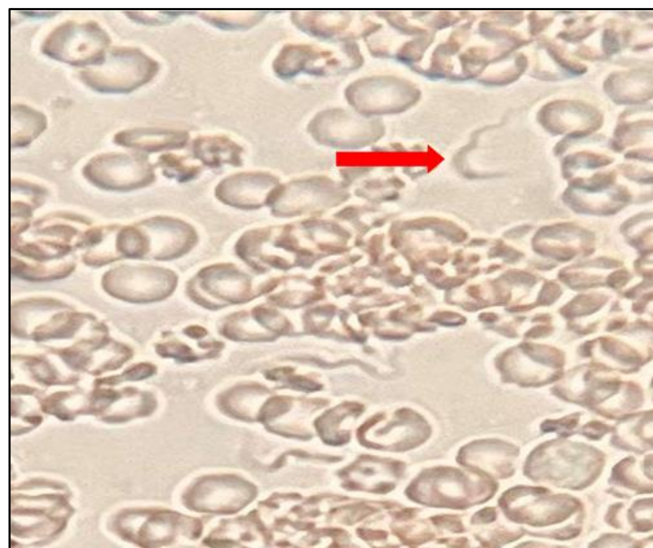


Fig 3: Wet blood film of dog showing live & motile *Trypanosoma evansi* (45X)

2.3.2 Giemsa staining technique

The blood smears prepared on glass slides were air-dried and fixed in methanol for 2 to 3 mins. Later they are stained with Giemsa stain of 1:10 dilution for 30 mins, rinsed in running tap water, and air-dried. A drop of cedar wood oil was placed on stained smears and were observed under the oil immersion lens (100X) of the microscope for the presence of *Trypanosoma evansi* (figure 4).

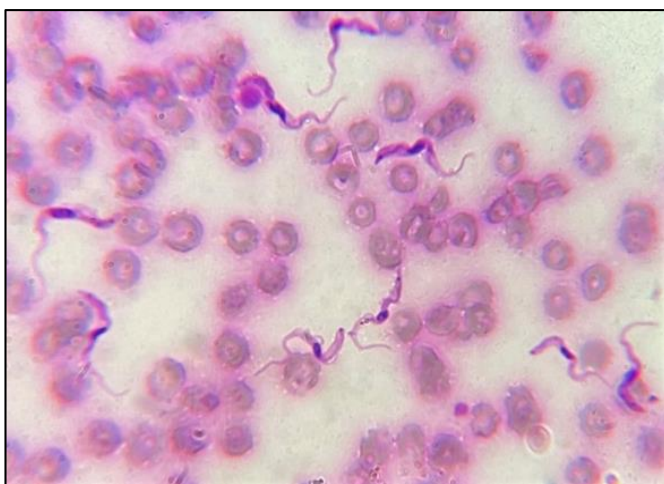


Fig 4: Giemsa stained blood smear of dog showing *Trypanosoma evansi* (100X)

3. Results

The PCR assay has amplified a PCR product of 205 bp length in 24 out of 314 blood samples of dogs tested for canine Trypanosomosis, indicating an overall prevalence of 7.64%. Whereas, microscopically examination of Giemsa stained smears revealed numerous *Trypanosoma* which are slender in shape with an undulating membrane, a free flagellum, a central nucleus, and a posterior kinetoplast in 5 out of 314 (1.59%) dog samples examined and only 3 out of 314 dogs (0.95%) revealed active and motile *T. evansi* on peripheral wet blood film examination. The results (table1) clearly indicate that the PCR assay is more sensitive and specific than microscopic examination methods in the diagnosis of canine Trypanosomosis.

Table 1: Comparison of PCR assay with the parasitological techniques in the field diagnosis of canine Trypanosomosis

Diagnostic method performed	No. of suspected dogs examined	No. of dogs found positive	Percent efficacy
Wet blood film examination	314	3	0.95%
Giemsa staining technique	314	5	1.59%
PCR assay	314	24	7.64%

4. Discussion and conclusion

Although different techniques have been developed for the demonstration of *T. evansi,* microscopy remains as the golden standard test at the field level. But the limitation is that the diagnostic sensitivity is low during chronic or latent infections (Fernandez *et al.,* 2009) [13]. The DNA amplification test i.e., PCR is extremely sensitive as it can amplify even minute quantities of *T. evansi* DNA to a detectable quantity if the number of cycles is sufficiently high as suggested by Uilenberg, 1998 [14].

In the present study, the PCR assay targeting partial VSG gene in the genome of *T. evansi* amplified a product of 205 bp length in 24/314 blood samples of dogs tested for canine Trypanosomosis, with an overall prevalence of 7.64% canine Trypanosomosis in and around Hyderabad, Telangana. Almost similar incidences but with different diagnostic techniques were reported by Alli *et al.* (2021) [15] who reported the prevalence of 7.28% in dogs in Telangana, Alanazi (2018) [16] who reported 4.3% in dogs in Saudi Arabia, Nadeem *et al.* (2011) [17] who reported a prevalence of 6% in equines in Gujranawala, Pakistan.

Whereas the Giemsa staining technique detected *T. evansi* in 5/314 (1.59%) dogs examined and peripheral WBF examination detected *T. evansi* in only 3/314 (0.95%) dogs screened, these are in agreement with Asif *et al.* (2020) [18] who reported a 2.5% prevalence in dogs, Alli *et al.* (2020) [19] who reported 0.66% by staining techniques and 0.33% prevalence by WBF, Prasad *et al.* (2015) [20] who reported prevalence of 1.60% in dogs by WBF, Chowdhury *et al.* (2005) [21] who reported 1.72% (5/290) dogs in Kolkata, Singh *et al.* (1993) [3] reported a higher prevalence of 3.12% (2/64) in dogs in Ludhiana by WBF examination. The lower incidences reported by microscopic examination techniques in the present study might be due to inherent low sensitivity, and collection of blood samples during stages of low or intermittent parasitaemia i.e., chronic or latent infection.

From the results, it is evident that the PCR assay is superior to the parasitological tests for the diagnosis of canine Trypanosomosis, as a higher percentage of positive cases (7.64%) was detected by PCR as compared to microscopic examination, which revealed a positive percentage of 1.59% (5/314) by Giemsa staining technique, and only 0.95% (3/314) by wet blood film examination. The highest prevalence recorded by the PCR assay might be due to the detection of flagellates during all stages of infection, even during the stages of low parasitemia which conventional tests fail to detect.

The present study confirmed that the PCR assay is a highly sensitive, specific, and reliable method over parasitological techniques in the diagnosis of canine Trypanosomosis, as it targets the partial VSG gene in the genome of *T. evansi* which could be used for the diagnosis of early, middle and late stages of infection in the field. Unlike microscopic examination techniques, PCR assay is a very sensitive and effective method of detection even during the stages of low parasitaemia, particularly during the chronic stage or prepatent period of the disease.

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