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Research Article

DEVELOPMENT OF PCR TO IDENTIFY THE PREVALENCE OF *TRYPANOSOMA EVANSI* IN DOGS FROM SOUTH INDIA

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ABSTRACT

Dogs are continuously exposed to a spectrum of haemoparasitic diseases and the information on the distribution and epidemiology of trypanosomosis in India is still under reported. Dogs brought to Teaching Veterinary Clinical Campus, Veterinary College and Research Institute, Namakkal of Tamil Nadu were screened for the haemoparasite, *T. evansi* by polymerase chain reaction. The PCR was developed for detection of *Trypanosoma evansi* in dogs targeting Beta tubulin mRNA gene at 997bp,which revealed a positivity of 1.33 per cent. The target gene of *T. evansi* identified was found to possess nearly a complete homology (99.6-99.8%) with that in China and India. The parasite could not be detected by microscopic examination of the Giemsa stained peripheral blood smears. High prevalence was noticed in dogs of less than 1 year age group (2.94%), males (1.85%) and non-descript dogs (2.94%).

Key words: Epidemiological factors, PCR, Phylogenetic analysis, Prevalence, *T. evansi*

Haemoparasitism is one factor which exerts deleterious effects and poses serious health problems in domestic dogs such as retarded growth, generalized ill health, lowered resistance to infectious diseases and reduced work efficiency (Bwalya, 2012). Canines are continuously exposed to a spectrum of vector-borne haemoprotozoan infections *viz.*, babesiosis, trypanosomosis and hepatozoonosis, due to an unchecked annoyance of arthropod vectors (Singh *et al.*, 2012). In Asia, the geographic distribution of *T. evansi* is steadily increasing and in India, the outbreaks of canine trypanosomosis are well documented (Ravindran *et al.*, 2008).

Trypanosomosis is caused by the protozoa *Trypanosoma evansi*, transmitted by *Stomoxys* and *Tabanus*, and characterized by intermittent fever, progressive emaciation, oedema of head, corneal opacity, anaemia and death (Tresamol *et al.*, 2013). It is a polymorphic disease where clinical form varies from host to host which makes the putative diagnosis difficult (Ravindran *et al.*, 2008). The disease can be diagnosed by a number of methods but their sensitivity and specificity in the detection of subclinical and carrier status is not reliable. The conventional microscopic examination may be inaccurate owing to difficulty in the demonstration of low and intermittent parasitaemia. Hence, PCR could be the useful method with improved sensitivity of detection, despite being relatively expensive and technical (Desquesnes *et al.*, 2013). Since the incidence of trypanosomosis in dogs has been reported rarely with a paucity in the prevalence data in this region, this paper reports the prevalence of trypanosomosis in dogs by molecular approach with its epidemiological significance.

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MATERIALS AND METHODS

Dogs (n=150) from a cross section of dog population irrespective of their age, sex, breed presented apparently healthy (n=20) and with the complaint of anorexia (n=130) to Teaching Veterinary Clinical Campus (TVCC), Veterinary College and Research Institute (VC&RI), Namakkal of Tamil Nadu, India from September, 2015 to April, 2016 were screened for trypanosomosis. Methodology of the sample collection in the clinics was performed as per the guidelines of the Institute and did not require animal ethical committee approval. Peripheral blood smears along with associated epidemiological data were collected from the dogs and subjected to Giemsa staining for demonstration of the trypanosomes. The blood samples were collected in EDTA coated vacutainers and the sample DNA was extracted by using Dneasy blood and tissue kit (Qiagen, Netherland) as per the protocol. Known positive DNA procured from external source (Department of Veterinary Parasitology, Madras veterinary College, Chennai) was used as positive control. A PCR master mix of 2x (Ampliqon, Denmark) concentration was used in the reaction mixture and 100 bp DNA ladder (Gene Direx, USA) was used in the electrophoresis. The primers were designed by using Fast PCR Software, custom synthesized (Eurofins and Sigma, India), targeting Beta tubulin mRNA gene, (DQ786574.1) with the following nucleotide sequence (5'-3'): Forward–AATGGACTCCGTACGTGC and reverse-GTCCATACCCTCGCCAGTGTAC with the amplicon size of 997 bp.

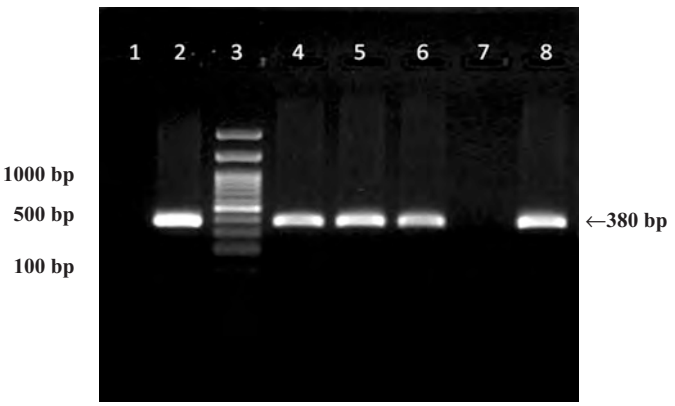
The extracted DNA was amplified using the selected primers with following reaction mixture and cycling conditions. Reaction mixture: DNA template–3µl, master mix-13 µl, forward and reverse primer (10 pico moles,

each) - 1 µl each and molecular grade water -7 µl. Cycling conditions: Initial denaturation-95°C/8min, denaturation -94°C/30sec, annealing -55°C/30 sec and extension -72°C/45 sec with 25 cycles; and final extension -72°C/10 min. The electrophoresed gel was visualized under UV transilluminator and the bands of appropriate size were identified by comparison with the100 bp ladder. Positive amplified products were sequenced and subjected to phylogenetic analysis (Eurofin) for confirmation.

RESULTS AND DISCUSSION

The microscopic examination of the Giemsa stained peripheral blood smears (n=150) revealed no trypanosomes. The absence of trypanosomes by conventional blood smear examination could be attributed to the poor sensitivity of the test associated with a low parasitaemia. Previously a low prevalence of 1.72, 0.4 and 0.21 per cent were found in dogs in Kolkata, Chennai and Ludhiana of India, respectively by microscopic method (Chowdury *et al.*, 2005; Senthil Kumar *et al.*, 2009; Singh *et al.*, 2012).

Out of the clinical cases (n=130) screened, a positivity of 1.33 per cent (2 cases) for *T. evansi* was observed by PCR (Fig. 1), whereas all the apparently healthy dogs (n=20) were negative by PCR. The sequence of *T. evansi* positive sample targeting beta tubulin mRNA gene revealed 99.6-99.8 per cent homology with the *T. evansi* of mice in China and camel in India (Uttar Pradesh and Rajasthan) and 99.8 per cent homology with *T. brucei* of humans in UK (Table 1 and Fig. 2). A low molecular prevalence of *T. evansi* was observed in this study, however, Ravindran *et al.* (2008) recorded a higher molecular prevalence of 7.7 per cent by PCR in Madhya Pradesh of India. Previously, Omanwar *et al.* (1999) developed a PCR targeting the 994 bp kinetoplast of *T. evansi* and Taylor *et al.* (2008) developed a Taq-Man PCR assay using ribosomal DNA of *T. evansi*. The phylogenetic



Lane 1- Negative Control, **Lane 2-** Positive control, **Lane 3-** Ladder and **Lane 4 to 8-** Test samples

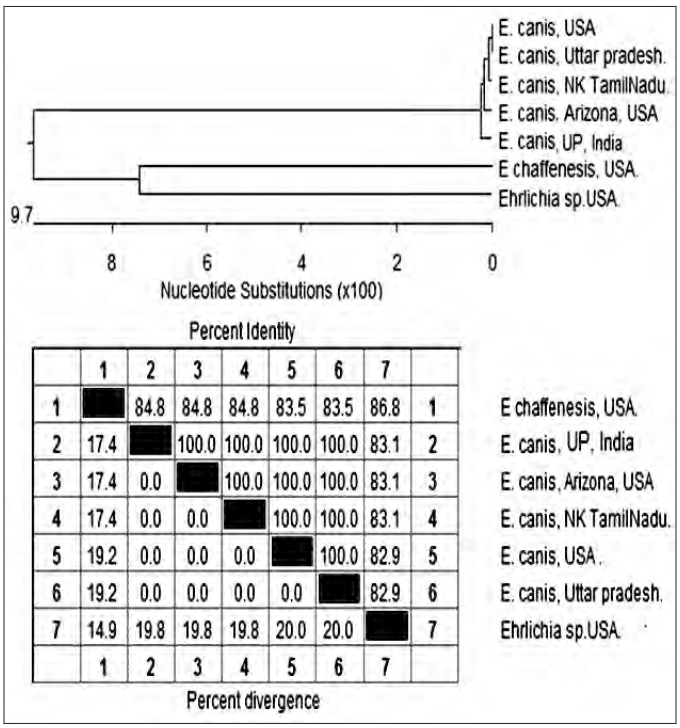
Fig. 1. PCR amplified products of Beta tublin mRNA gene of *T. evansi* in 1.5 per cent agarose gel showing bands at 997 bp

Table 1
Comparison of homology of the *T. evansi* from positive cases with published sequencing results

Haemo parasite	GenBank accession no.	Haemoparasites with target gene	Place of isolation
T. evansi	GQ483462.1	*T. evansi*, beta tubulin mRNA gene	Uttar Pradesh
	EU483116.1	*T. evansi*, beta tubulin mRNA gene	Rajasthan
	EU418847.1	*T. evansiclone*, beta tubulin mRNA gene	Uttar Pradesh
	DQ786574.1	*T. evansi*, beta tubulin mRNA gene	China
	XM1218934.1	*T. brucei. brucei*, beta tubulin partial mRNA gene	UK
	FN554964.1	*T. brucei* gambiense DAL972 chromosome 1	UK

analysis of *T. evansi* in this study is in agreement with that of Villareal *et al.* (2013) who earlier recorded a complete homology for the sequence of rRNA ITS region of *T. evansi* with that published in Phillipines and Gen Bank.

A high prevalence of *T. evansi* was noticed in under 1 year age group (2.94%) followed by 1-2 years age group (2.5%), in non–descript dogs (2.94%) followed by pure breeds (0.94%) and the prevalence was observed only in males (1.85%). The prevalence of *T. evansi* infection was found predominantly in south-west monsoon (8.33%). However, no statistical difference ($p>0.05$) could be detected for the prevalence of *T. evansi* between the age groups, breeds and seasons (Table 2).



	1	2	3	4	5	6	7	
1		84.8	84.8	84.8	83.5	83.5	86.8	1
2	17.4		100.0	100.0	100.0	100.0	83.1	2
3	17.4	0.0		100.0	100.0	100.0	83.1	3
4	17.4	0.0	0.0		100.0	100.0	83.1	4
5	19.2	0.0	0.0	0.0		100.0	82.9	5
6	19.2	0.0	0.0	0.0	0.0		82.9	6
7	14.9	19.8	19.8	19.8	20.0	20.0		7
	1	2	3	4	5	6	7	

E chaffenesis, USA.
E. canis, UP, India
E. canis, Arizona, USA
E. canis, NK TamilNadu.
E. canis, USA.
E. canis, Uttar pradesh.
Ehrlichia sp.USA.

Fig. 2. Phylogenetic relationship of beta tubulin mRNA gene of *T. evansi* with other published isolates (GenBank) and its homology and divergence

Table 2
Age, sex, breed and season-specific prevalence of *T. evansi* infection

Epidemiological determinants		Total (n=150)	No. of positives (n=2)	Per cent positives	P value
Age	<1 year	34	1	2.94	0.550NS
	1-2 years	40	1	2.5	
	2-4 years	32	-	-	
	>4years	44	-	-	
Sex	Male	108	2	1.85	0.374NS
	Female	42	-	-	
Breed	Pure	106	1	0.94	0.629NS
	Cross	10	-	-	
	ND	34	1	2.94	
Season	Southwest monsoon	12	1	8.33	0.104NS
	Northeast monsoon	82	1	1.21	
	Winter	41	-	-	
	Summer	15	-	-	

NS: Non-significant

A high prevalence of *T. evansi* recorded in under 1 year age group of dogs is in accordance with that of Bhatia and Shah (2001). A high prevalence of *T. evansi* recorded in males is in concurrence with that of Chowdury *et al.* (2005) and in contrast, Singh *et al.* (2012), Konto *et al.* (2014) and Lakshmi Prasad *et al.* (2015) recorded a high prevalence in females for this haemoparasitic infection. A higher prevalence of *T. evansi* was detected in non-descript dogs than in other breeds and this finding is in accordance with that of Senthil Kumar *et al.* (2009), whereas Chowdury *et al.* (2005) and Saravanan *et al.* (2005) reported the occurrence of *T. evansi* in pure breeds (Labrador Retriever and German Shepherd) of dogs. The high prevalence in non-descript dogs might be due to the poor management and health status associated with them. However, there was no statistically significant difference (P>0.05) found to exist among the different ages, sex and breeds of positive cases, and different seasons.

In this study, PCR was developed by targeting Beta tubulin mRNA gene of *T. evansi* to identify the distribution of the disease in dogs which did not show any apparent signs of trypanosomosis, as clinical trypanosomosis is rarely presented for treatment in this region. Though a low prevalence was detected, PCR was found to be highly useful in detecting the cases of mild or low parasitaemia, as microscopic examination often fails to detect these cases due to its poor sensitivity. There was no statistically significant difference in the susceptibility to *T. evansi* with respect to age, sex, breed and seasons in Namakkal region of Tamil Nadu.

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