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Research Article

IDENTIFICATION OF EHRLICHIA CANIS BY PCR WITH PHYLOGENETIC ANALYSIS IN DOGS FROM SOUTH INDIA

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ABSTRACT

Canine monocytic ehrlichiosis (CME) or canine hemorrhagic fever is a potentially fatal tick-borne disease in tropical countries associated with its deleterious effect on the health of dogs. Clinically affected (n=130) and apparently healthy dogs (n=20) for the purpose of deworming or vaccination brought to Teaching Veterinary Clinical Complex (TVCC), Veterinary College and Research Institute (VC &RI), Namakkal were screened for *E. canis* by microscopic examination and polymerase chain reaction assay. The PCR assay revealed a positivity of 8.0 percent samples for *E. canis* targeting Vir-B9 gene and generating an amplicon of 380 bp. However, conventional microscopic blood smear examination failed to detect *E. canis* owing to the low parasitaemia. The sequence of *E. canis* had a 100.0 per cent homology with the *E. canis* of dogs in India and USA. The tick vector from infected dogs was identified as *Rhipicephalus sanguineus*. Dogs below1 year age group, females and pure breeds were found to be more affected.

Keywords: *E. canis*, Epidemiological factors, PCR, Phylogenetic analysis

India has a wide range of climatic zones from montane and semi-arid regions to the wet tropic which is conducive for a diverse range of vectors. In India,17.0 per cent of households were reported to own a pet, however, the information on the prevalence, epidemiology, diagnosis and management of canine vector-borne diseases (CVBD) is scarce (Abd Rani *et al.*, 2011). Amongst haemoparasitic infections, ehrlichiosis in canines gained significance in the recent years in India (Bhattacharjee and Sarmah, 2013). *E. canis* is the pathogenic and most commonly encountered Ehrlichia species in dogs, transmitted by the bite of *R. sanguineus* (Shaw *et al.*, 2001) with non-regenerative anaemia and often a fatal haemorrhagic syndrome. Moreover, clinical cases of ehrlichiosis which are treated inappropriately may recover clinically entering the subclinical phase and may remain persistent healthy carriers of *E. canis* for long period. The recombinations in the outer membrane major antigen protein genes of Ehrlichiae lead to evasion of host defense mechanism by organisms, resulting in persistent infection (Greene *et al.*, 2012).

Definitive diagnosis can also be made on the basis of demonstration of morulae in leukocytes from blood smears or tissue aspirates of lung, spleen and lymph nodes; however, microscopic examination of stained blood smears is not sensitive and time consuming too. Nevertheless, PCR is considered to be the sensitive tool for detection of Ehrlichia in healthy carriers (Greene *et al.*, 2012). Serological tests also fail to distinguish a current infection from previous infection and cross-reactivity is of a serious concern in the differentiation of Ehrlichia species (Warner *et al.*, 2001). Hence, this study reports the occurrence of ehrlichiosis in clinically cases and apparently healthy dogs by molecular approach, phylogenetic characterization of *E. canis* and its epidemiology in Namakkal, Tamil Nadu of South India.

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MATERIALS AND METHODS

Blood samples (n=150) were collected from clinical cases (n=130) suggestive of ehrlichiosis viz. anorexia, pyrexia, lymphadenopathy, pale mucosa, vomiting, diarrhea, melena, epistaxis and splenomegaly and apparently healthy dogs (n=20) along with relevant epidemiological data at TVCC, VC & RI, Namakkal of Tamil Nadu. 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 were prepared from all the dogs and subjected to Giemsa staining for demonstration of *E. canis*. The blood samples were collected in EDTA coated vacutainers and DNA was extracted by using DNeasy blood and tissue kit as per the recommended protocol. Known positive DNA procured from external source (Department of Veterinary Parasitology, Madras veterinary College, Chennai) was used as positive control.

The primers for PCR as recommended by Kledmanee *et al.* (2009) and Corales *et al.* (2014) were got synthesized (Eurofins and Sigma, India) with the following nucleotide sequences (5'-3') targeting Vir-B9 protein gene of *E. canis*.

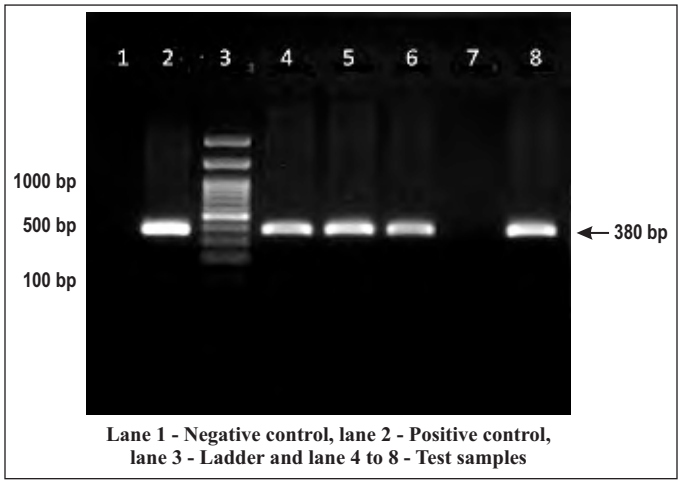
Forward– CCATAAGCATAGCTGATAACCCTGTTA CAA and reverse –TGGATAATAAAACCGTACTAT GTATGCTAG for the generation of amplicon size of 380

bp in positive cases. The extracted DNA was amplified using the selected primers with following reaction mixture and cycling conditions. Reaction mixture: Total volume: 25 µl; DNA template –3 µl, master mix-13 µl, forward and reverse primer (10 pico moles, each) - 1 µl each and molecular grade water -7 ml. Cycling conditions were: initial denaturation 94 °C/4 min, denaturation 94 °C/30 sec, annealing 50 °C/30 sec and extension 72 °C/30 sec with 35 cycles; and final extension 72 °C/2 min. The gel was visualized under UV transilluminator and the amplicons of appropriate size (380 bp) were identified by comparison with the 100 bp ladder. The images were documented using the gel documentation system (Vilber Lourmat, France). Two positive amplicons were purified, sequenced and subjected to phylogenetic analysis (Eurofins, India) for confirmation.

The ticks collected from the study population were identified. Ticks were collected from inside the ears and in-between the digits of dogs and stored in sterile storage vials with 70.0 per cent ethanol as preservative. The male ticks were processed as per standard parasitological procedure and the morphological characters were identified based on the keys cited by Dantas-Torres (2008) and predisposition to ehrlichios is by age, breed, sex and season was statistically analysed by Chi-square test. All statistical analyses were conducted using SPSS software (Version 16).

RESULTS AND DISCUSSION

The molecular prevalence by PCR was found to be 8.0 percent detecting the Vir-B9 protein gene of *E. canis* with the amplicon size of 380 bp (Fig. 1). The infection could be detected in 8.46 per cent of the suspected clinical cases, and 5.0 per cent of the dogs which were apparently healthy. In this study, conventional microscopic examination failed to detect *E. canis* owing to the low parasitaemia and difficulty in demonstration of morulae in blood cells. This is in agreement with that of Abd Rani *et al.* (2011) who recorded no prevalence for *E. canis* by microscopic examination in Northern and Southern parts of India. However, Senthil Kumar *et al.* (2009) and Bhattacharjee and Sarmah (2013) recorded a prevalence of 6.0 per cent and 2.12 per cent in Chennai and North east India and Assam, respectively for *E. canis* by peripheral blood smear examination. In this study, the prevalence of *E. canis* (Vir-B9 gene) by PCR was found to be low, whereas previously, Lakshmanan *et al.* (2007) and Abd Rani *et al.* (2011) recorded a high prevalence of 50.0 and 20.6 per cent, respectively for *E. canis* targeting 16S rRNA and 18S in rRNA genes, respectively by PCR in India. The sensitivity and specificity of PCR when compared to blood smear examination is 100 per cent.



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

Fig. 1. PCR amplified products of Vir-B9 protein gene of *E. canis* in 1.5 % agarose gel showing bands at 380 bp

The variation in the results could be due to the treatment of the clinically suspected cases, prevalence of transmitting ticks in local regions and timely control of ticks. The positivity in apparently healthy dogs in this study could be attributed to the sensitivity of PCR which is the most efficient tool in the detection of subclinical and inapparent infections with a very low level of parasitaemia (Abd Rani *et al.*, 2011).

The sequence of *E. canis* positive sample was found to possess a 100.0 per cent homology with the *E. canis* of dogs in India (Uttar Pradesh) and USA. It also had a 83.5 per cent homology with *E. chaffenesis* of humans in USA and 82.9 per cent homology with the *Ehrlichia* sp. of human in USA (Table 1, Fig. 2). The phylogenetic analysis of *E. canis* in this study is in accordance with that of De Visser (2012) who also found a strong relationship between North and South American strain of Vir-B9 gene.

Table 1

Comparison of homology of the *E. canis* from positive case with published sequencing results

Haemoparasites	GenBank accession number	Haemoparasites with target gene	Place of isolation
E. canis	JF706287.1	*E. canis* strain Caper VirB9-like gene	Uttar Pradesh
	AY205339.1	*E. canis* strain Arizona VirB9 gene	USA
	CP000107.1	*E. canis* strain Jake gene	USA
	AF546158.1	*E. canis* type IV secretion component (virB9) gene	USA
	CP007474.1	*Ehrlichia* sp. HF	USA
	AF392617.1	*E. chaffeensis* GTP cyclohydrolase II (ribA) gene	USA

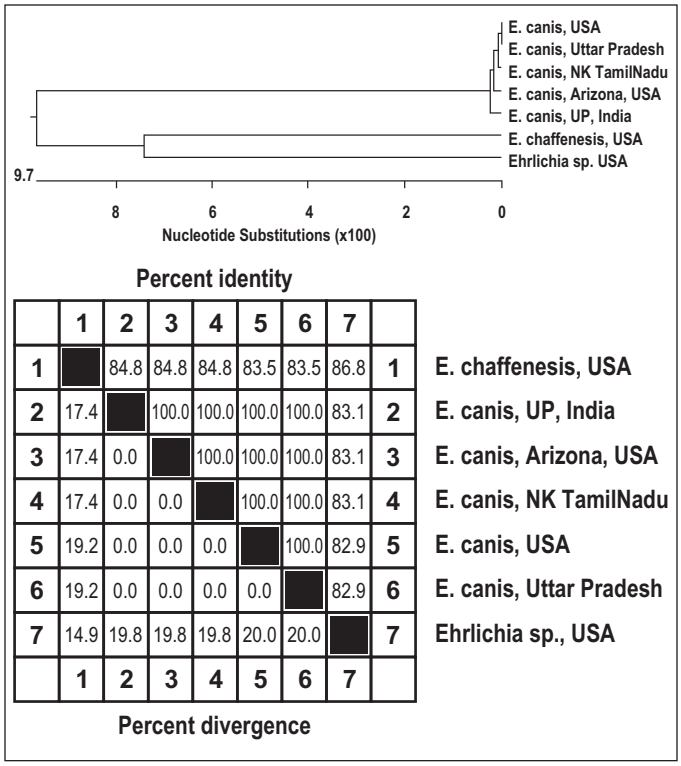


Fig. 2. Phylogenetic relationship of Vir-B9 protein gene of *E. canis* with other published isolates (GenBank) and its homology and divergence

The complete homology of the sequence with the sequence recorded in other geographical areas could be attributed to the highly conserved genetic profile of these parasites (Kamani *et al.*, 2013).

The ticks collected from the study population were identified as *R. sanguineus*. The tick vector, *R. sanguineus* might be the transmitting agent of *E. canis* in this study and this finding is in agreement with that of previous reports (Abd Rani *et al.*, 2011 and Bhattacharjee and Sarmah, 2013) in India. Among the positive dogs, a high prevalence was noticed in below1 year age group (11.76%) followed by 1-2 years (10.0%), 2-4 years (9.37%) and >4 years age group (2.27%); in pure breeds (9.43%) followed by non–descript dogs (5.88%); and in winter (12.19%), followed by southwest monsoon (8.33%) and northeast monsoon (7.31%). Females (19.04%) were found to be affected more than males (3.7%) with a statistically, highly significant difference ($p<0.01$). However, no statistical difference ($p>0.05$) could be detected for the prevalence of *E. canis* between the age groups, breeds and seasons (Table 2). A high prevalence of *E. canis* recorded in young female dogs in this study is in contrast to that of Trapp *et al.* (2006), Avizeh *et al.* (2010) and Dhankar *et al.* (2011) who recorded a high prevalence in adults and males. However, Greene *et al.* (2012) concluded that no predilection could exist to age and sex in ehrlichiosis. Statistically, significant

Table 2

Age, sex, breed and season-specific prevalence of *E. canis* infection

Epidemiological determinants		Total	No. of (n=150)	Per cent positives (n=12)	P value positives
	<1years	34	4	11.76	
	1-2 years	40	4	10.0	
	2-4 years	32	3	9.37	0.404^{NS}
	>4years	44	1	2.27	
Sex	Male	108	4	3.7	
	Female	42	8	19.04	0.0018**
Breed	Pure	106	10	9.43	
	Cross	10	-	-	
	ND	34	2	5.88	0.503^{NS}
Season	Southwest monsoon	12	1	8.33	
	Northeast monsoon	82	6	7.31	
	Winter	41	5	12.19	0.667^{NS}
	Summer	15	-	-	

^{NS}Non-significant, *Significant at 95.0 per cent level, **Significant at 99.0 per cent level

difference was found with respect to sex only in the prevalence of *E. canis*. The high prevalence of *E. canis* in pure breeds (GSD, Labrodar retriever, Dobermann) is in concurrence with that of Kelly (2000) and Dhankar *et al.* (2011). The high susceptibility of breeds like GSD could be associated with the poor ability of these breeds to mount an adequate humoral and cell-mediated immune response (Greene *et al.*, 2012) and also the long hair coat which prevents the easy removal of the infesting vectors (Senthil Kumar *et al.*, 2009). The high prevalence observed in winter (12.19%) might be due to the prolonged subclinical period in chronic cases or recrudescence when exposed to stress or corticosteroid therapy, as Greene *et al.* (2012) also supported that there could be an even seasonal distribution of *E. canis* in dogs. Previously, Milanjeet *et al.* (2014) observed a high prevalence of *E. canis* in summer.

In the present study, PCR assay was found to be more sensitive than conventional microscopic examination in detecting *E. canis*. Since, the clinically healthy dogs may act as carriers and the dogs in the chronic phase of the disease may not improve on treatment. Hence, diagnosis at an early stage is of paramount importance to ensure successful treatment and PCR assay is ideally suited to epidemiological investigations and to arrive at correct and unequivocal diagnosis.

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