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Canine Monocytic Ehrlichiosis among working dogs of organised kennels in India: A comprehensive analyses of clinico-pathology, serological and molecular epidemiological approach

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ABSTRACT

Canine Monocytic Ehrlichiosis (CME) is a serious tick-borne rickettsial disease affecting canine populations globally. Besides few reports from stray and pet dogs from localised geographical regions (cities/towns/small states), a comprehensive study on prevalence of *Ehrlichia canis (E. canis)* among working dogs from different geo-climatic zones of India was pertinently lacking. Study of CME among these dog populations was thus carried out, encompassing clinical aspects and different diagnostic methodologies viz., microscopy, serology and molecular biology. During the two-year study period, clinical specimens from 225 cases suspected of canine ehrlichiosis were examined for clinical pathology and presence of the haemoparasites. Overall prevalence of ehrlichiosis by microscopic examination, commercial dot-ELISA kit and nested PCR assay was estimated to be 1.3%, 19.1% and 5.8%, respectively, which were found to be statistically significant by McNemar Chi squared test ($p < 0.05$). It was also observed that possibly due to widespread use of doxycycline therapy in field, CME presently does not remain a potential threat which it uses to pose earlier. However, concurrent infections of *E. canis* and *Babesia gibsoni* were found to be mostly fatal. Keeping in view of high number of apparently healthy dogs (24) out of total positive cases (46) observed during the study, it is recommended that prevalence studies on CME should also involve screening of apparently healthy dogs. Phylogenetic analysis carried on partial sequencing of 16S rRNA of *E. canis* strains revealed that all of the Indian strains clustered in a single clade with other *E. canis* species from India and rest of the world. Molecular divergence was observed among the sequences of Brazilian and American isolates which were also included in the present study. These findings have thus opened a new paradigm for planning of pragmatic control strategies against CME.

1. Introduction

Ehrlichia canis (*E. canis*) is an obligate, intra-cytoplasmic, pleomorphic, Gram negative rickettsial member of the family *Anaplasmataceae*. This pathogen is responsible for causing Canine Monocytic Ehrlichiosis (CME) in canines and probably in felines, globally. It is transmitted exclusively by the brown dog tick, *Rhipicephalus sanguineus* sensu lato (Dumler et al., 2001). *E. canis* has a tissue tropism for reticulo-endothelial cells of the liver, spleen and lymph nodes and replicates primarily in the mononuclear macrophages (Swango et al., 1989). The disease is often referred to as 'tropical canine pancytopaenia', because of its high prevalence rates in tropics and subtropical zones with associated decrease in leucocytes and platelets in clinical cases. In absence of timely therapeutic interventions, the disease pathophysiology continues and the mononuclear cell invasions results into various severe clinical and fatal outcomes (Gal et al., 2008).

The most common laboratory diagnosis of the CME involves conventional microscopy for detection of morulae within the mononuclear cells (mostly monocytes) in thin blood stained smears. Serological based laboratory assays viz. indirect immunofluorescence antibody (IFA) test (Bartsch and Greene, 1996) and enzyme linked immunosorbent assay (ELISA; Harrus et al., 2002) are also used for

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detection of anti-*E. canis* IgG antibodies. Besides these serological assays, two of the pen-side serological kits viz. Immunocomb dot-ELISA test (Biogal Galed Laboratories, Israel) and SNAP3Dx assay (IDEXX Laboratories, USA) utilizing synthetic peptides derived from major immuno-dominant *E. canis* proteins are also commercially available for the field use (Harrus et al., 2002). Presently, molecular tools like polymerase chain reaction (PCR) and sequencing are considered to be more sensitive methods for detection and characterization of *E. canis* (Harrus et al., 1998a, 2004).

Till date, studies on CME from India have been carried out in small geographical locations (cities/towns/small states) and that too, they have been carried out either on pet or on stray dogs and somehow, majority of these studies fail to address the clinical and diagnostic challenges being presently posed by the disease (Juyal et al., 1994; Samaradni et al., 2003; Lakshmanan et al., 2006; Wise and Tarlinton, 2012; Abd Rani et al., 2011; Singla et al., 2011; Milanjeet et al., 2014; Bhadesiya and Raval, 2015; Singla et al., 2016; Kottadamane et al., 2016, 2017). Besides these lacunae, these prevalence studies were also solely based on either commercial serology kit or on molecular based techniques and thus failed to analyse the practical relevance of both the methodologies in existing field conditions. These points are quite pertinent from the fact that nowadays there has been lot of awareness on control measures viz. anti-tick/acaricide applications and recommended doxycycline therapy against the disease. At present, working dogs being housed in organised kennels also form a key corpus of dog population in this country and due to intensive way of housing; these dogs are unrelentingly on the high risk of acquiring CME. With this background, this study was envisaged to correlate the haematological parameters, serum biochemistry profiles and histopathology findings of canine ehrlichiosis cases along with their diagnostic assays results. These lab findings were then thoroughly analysed so as to bridge the existing knowledge gap on the actual scenario of CME in the organised kennels of this country.

2. Materials and methods

2.1. Study area and sampling design

Blood and serum samples were collected from working dogs (guard, tracker and sniffer dogs of the security agencies), clinically suspected for canine ehrlichiosis and also from apparently healthy dogs but in-contact dogs that were co-housed with previously confirmed cases of CME. These kennels were located in different geo-climatic zones encompassing the north-eastern states (Assam and Nagaland), eastern state (West Bengal), northern states (Jammu & Kashmir, Uttar Pradesh and Delhi) and south-western state (Maharashtra) of this country. These organised kennels exclusively maintained pedigreed German Shepherd and Labrador Retriever breeds of dogs. This is akin to many other countries as these two breeds' forms the main corpus of working dog population, globally. Moreover, the kennels under study followed recommended vaccination, acaricide and deworming schedules and had standard management practices in place. A total of 225 serum and blood samples were collected from 90 German Shepherds, 132 Labrador Retrievers and three Cocker Spaniels over a two year study period from March 2012 to April 2014. Three animals (two Labradors and one German Shepherd) died during the study and their necropsy tissue samples were subjected to histopathology examination.

Canines affected with concurrent infections of viral or bacterial or other parasitic diseases were comprehensively ruled out by battery of assays which included molecular assays (PCR or RT-PCR) and pen-side kits for differential diagnosis of canine ehrlichiosis with infections of Canine Adeno Virus-1 (CAV-1), Canine Adeno Virus-2 (CAV-2), Canine Parvo Virus (CPV), Canine Distemper Virus (CDV), Canine Corona Virus (CCV), Rabies, *Leptospira* spp, *Babesia* spp., Mycoplasma, *Trypanosoma evansi*, Canine Heart Worm, *Anaplasma phagocytophilum* and *Borrelia burgdorferi*. Thus it was meticulously ensured that canines affected with concurrent infections of viral or bacterial pathogen were ruled out and those solely affected with *E. canis* (barring six dogs having dual infection of *E. canis* and *B. gibsoni*) were only incorporated in this study.

2.2. Microscopy

Giemsa stained thin blood smears were prepared from ear punctures (capillary blood) and examined under 1000× magnification of the microscope for presence of *E. canis* morulae.

2.3. Haematology

Whole blood collected from cephalic vein in BD® vacutainers (containing EDTA as anticoagulant) was used for haematological analysis. Different haematological parameters viz., haemoglobin (Hb), packed cell volume (PCV), platelet counts, total leukocyte count (TLC), total RBC count (TRBC) and differential leukocyte count (DLC) were carried out in MS4s vet haematology analyser (MELET SCHLOESING Laboratories, France). Erythrocyte sedimentation rate (ESR) was studied using automated LEXUS ESR 2010 analyser (Lab One®, India) along with the controls as per manufacturer instructions.

2.4. Biochemistry

Serum harvested from blood collected in BD® vacutainers containing clot activator was used for biochemical profiling. Estimation of biochemical parameters such as total bilirubin, total protein, albumin, globulin, albumin: globulin ratio (A/G), aspartate amino transferase (AST/SGOT), alanine amino transferase (ALT/SGPT), urea (BUN) and creatinine (CRE) were carried out in an automated clinical chemistry analyser (EM 360™, ERBA diagnostics Mannheim GmbH, Germany) as per manufacturer's recommendations.

2.5. Histopathology

Processing and staining of tissue sections for histopathology examination with hematoxylin and eosin staining was performed as per standard procedure (Fischer et al., 2008).

2.6. Serology

Serum samples were screened for presence of antibodies to *E. canis* by a commercial dot-ELISA Immunocomb® Canine Ehrlichia Antibody test kit (Biogal Galed Laboratories, Israel). The assay was used for detection of anti-*E. canis* antibody titre. The intensity of the test spot corresponds to the antibody titre in the clinical sample and was measured on a scale of S0, S1, S2, S3, S4, S5 and S6. As per Waner et al. (2000), S3 of the ibid kit has been calibrated to a cut-off titre of 1:80 of IFAT, which is considered the gold standard for the detection of antibodies against *Ehrlichia.* Antibody titres corresponding to S3 and above (S4 and S5) have been considered as positive while samples scoring S0, S1 and S2 in the study were considered as negative for the test.

2.7. Nucleic acid amplification of the 16S ribosomal RNA gene segment

2.7.1. Nucleic acid extraction

DNA was extracted from whole blood using DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) as per manufacturer's protocol. The extracted DNA was estimated for quality and quantity using Nano Drop Spectrophotometer (ND-1000, Thermo Fisher Scientific, USA) and stored at −20 °C until further use.

2.7.2. Nucleic acid amplification for Ehrlichia canis

Nested PCR for amplification of *E. canis* DNA was performed according to the cycling conditions and primers described by Wen et al. (1997). Positive control (Oklahoma strain of *E. canis* received from Prof.

Table 1
Details of the samples found to be positive for *E. canis* by any of the diagnostic methodology (microscopy/Immunocomb dot-ELISA kit/nested-PCR) along with signalment (Yrs-Years; F-Female dogs; F*-Neutered female dogs; M-Male dogs; Lab-Labrador Retriever; GSD-German Shepherd; CS- Cocker Spaniel; MODS-Multiple Organ Dysfunction Syndrome; DIC-Disseminated Intravascular Coagulopathy; URT-Upper Respiratory Tract; P-Positive; N-Negative).

	Breed	Age (yrs)	Sex	Anamnesis	Microscopy for *E. canis*	Microscopy for Babesia	Immunocomb assay titer	PCR for *E. canis*	PCR for Babesia	Clinical Outcome
1	GSD	8.0	M	Pyrexia and Anorexia	**N**	**N**	**1:160 (P)**	**N**	**N**	Animals responded to treatment and
2	Lab	7.0	F*	Sprain Muscle thigh left/ hind leg	**N**	**N**	**1:80 (P)**	**N**	**N**	were discharged as fit
3	Lab	10.0	M	URT Infection	**N**	**N**	**1:80 (P)**	**N**	**N**	
4	Lab	5.0	M	Renal Failure; Epilepsy; Anemia; MODS	**N**	**P**	**1:20(N)**	**P**	**P**	**Died**; histopathological finding were indicative of interstitial pneumonic changes in lungs; perivascular lymphocytic infiltration in kidneys
5	Lab	5.0	M	Epilepsy and Dermatitis paw	**N**	**P**	**1:160 (P)**	**P**	**P**	**Died**; histopathological findings were indicative of toxemic shock
6	GSD	2.5	F*	Jaundice	**N**	**P**	**1:80 (P)**	**P**	**P**	Animal responded to treatment and was discharged as fit
7	GSD	2.5	M	Jaundice; Renal Failure	**N**	**P**	**1:40 (N)**	**P**	**P**	**Died**; histopathological findings were indicative of hemorrhagic shock due to DIC
8	GSD	2.0	M	Bilateral Epistaxsis	**N**	**N**	**1:320 (P)**	**P**	**N**	Animals responded to treatment and
9	Lab	5.0	M	Apparently healthy	**N**	**N**	**1:80 (P)**	**N**	**N**	were discharged as fit
10	Lab	7.5	M	Apparently healthy	**N**	**N**	**1:80 (P)**	**N**	**N**	
11	Lab	8.5	M	Apparently healthy	**N**	**N**	**1:160 (P)**	**N**	**N**	
12	Lab	9.0	M	Apparently healthy	**N**	**N**	**1:160 (P)**	**N**	**N**	
13	Lab	5.0	M	Apathy; Anorexia; Pyrexia	**N**	**N**	**1:80 (P)**	**N**	**N**	
14	Lab	8.5	M	Apparently healthy	**N**	**N**	**1:80 (P)**	**N**	**N**	
15	Lab	4.0	M	Apparently healthy	**N**	**N**	**1:80 (P)**	**N**	**N**	
16	Lab	10.0	M	Apathy; Pyrexia; Anorexia	**N**	**N**	**1:80 (P)**	**N**	**N**	
17	GSD	4.0	M	Apparently healthy	**N**	**N**	**1:80 (P)**	**N**	**N**	
18	GSD	4.0	M	Apparently healthy	**N**	**N**	**1:80 (P)**	**N**	**N**	
19	GSD	4.0	M	Apparently healthy	**N**	**N**	**1:80 (P)**	**N**	**N**	
20.	CS	4.5	M	Pyrexia; Anorexia	**N**	**N**	**1:80 (P)**	**N**	**N**	
21	Lab	8.5	F	Apparently healthy	**N**	**N**	**1:160 (P)**	**N**	**N**	
22	Lab	1.0	F	Apathy; Anorexia	**N**	**N**	**1:80 (P)**	**N**	**N**	
23	Lab	0.5	M	Apparently healthy	**N**	**N**	**1:80 (P)**	**N**	**N**	
24	Lab	0.5	M	Apparently healthy	**N**	**N**	**1:80 (P)**	**N**	**N**	
25	Lab	7.5	M	Apathy; Anorexia; Pyrexia	**N**	**N**	**1:160 (P)**	**N**	**N**	
26	Lab	2.5	F*	Apparently healthy	**N**	**N**	**1:80 (P)**	**N**	**N**	
27	Lab	5.0	M	Apathy; Pyrexia	**N**	**N**	**1:160 (P)**	**N**	**N**	
28	Lab	0.5	F	Apparently healthy	**N**	**N**	**1:80 (P)**	**N**	**N**	
29	GSD	0.5	M	Apparently healthy	**N**	**N**	**1:160 (P)**	**N**	**N**	
30	CS	1.5	M	Pyrexia (104.2 °F/40.1 °C)	**P**	**N**	**1:80 (P)**	**P**	**N**	
31	Lab	7.0	F*	Apparently healthy	**N**	**N**	**1:80 (P)**	**N**	**N**	
32	GSD	2.0	F*	Apparently healthy	**N**	**N**	**1:80 (P)**	**N**	**N**	
33	Lab	4.5	F*	Jaundice & Pyrexia (104.8 °F/40.4 °C)	**P**	**N**	**1:160 (P)**	**P**	**N**	Animals responded to treatment and were discharged as fit
34	GSD	1.0	M	Pyrexia (104.4 °F/40.2 °C)	**N**	**N**	**1:80 (P)**	**P**	**N**	
35	Lab	2.5	M	Anemia	**N**	**N**	**1:320 (P)**	**P**	**N**	
36	CS	5.0	M	Pyrexia (105 °F/40.6 °C); Anorexia; Vomition	**N**	**P**	**1:320 (P)**	**P**	**P**	
37	GSD	2.0	F*	Apparently healthy	**N**	**N**	**1:320 (P)**	**P**	**N**	
38	Lab	8.0	M	Weak; lethargic; apathic	**N**	**N**	**1:160 (P)**	**N**	**N**	
39	Lab	4.5	M	Apparently healthy	**N**	**P**	**1:320 (P)**	**P**	**P**	
40	Lab	4.0	F*	Apparently healthy	**N**	**N**	**1:160 (P)**	**N**	**N**	
41	GSD	6.0	M	Apparently healthy	**N**	**N**	**1:80 (P)**	**N**	**N**	
42	GSD	1.0	F	Anaemia and Pyrexia	**P**	**N**	**1:20 (N)**	**P**	**N**	
43	Lab	3.5	M	Apparently healthy	**N**	**N**	**1:160 (P)**	**N**	**N**	
44	Lab	1.5	M	Apparently healthy	**N**	**N**	**1:80 (P)**	**N**	**N**	
45	GSD	0.5	M	Apathy; Anorexia	**N**	**N**	**1:160 (P)**	**N**	**N**	
46	Lab	3.5	F*	Apparently healthy	**N**	**N**	**1:320 (P)**	**N**	**N**	

Jere W. McBride, University of Texas Medical Branch, Galveston, Texas, USA), negative control (genomic DNA from healthy dog) and a non-template control were run in all the PCR experiments to rule out the possibility of contamination related false positive results. The PCR amplified products were resolved on ethidium bromide stained 1.5% agarose gel in Tris acetate-EDTA (TAE) buffer and visualized under UV light in a gel documentation system (Alpha Imager® EP, Alpha Innotech, San Leandro, CA, USA). Detection of DNA from other haemoprotozoan parasites (*Trypanosoma evansi* and *Babesia* species) in the clinical samples were screened by the primers and thermal conditions as described previously (Wuyts et al., 1994; Ikadai et al., 2004). Primers and their sequences used for screening blood samples in this study are illustrated in Suppl. 1.

2.8. Sequencing of amplified products and bioinformatics analysis

The PCR products after electrophoretic separation were excised from the gel and extracted using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) following manufacturer's instructions. The purified PCR products were assessed for quality and quantity. The PCR products

were custom sequenced at BioServe Biotechnologies (India) Pvt Ltd, Hyderabad, India. The sequence chromatogram was visualized in BioEdit Sequence Alignment Editor software version 7.0.5 (Isis Therapeutics, Carlsbad, CA, USA). Mega Blast was performed with the deduced sequence within the non-redundant nucleotide database (http://www.ncbi.nlm.nih.gov/Blast) to confirm the presence of *Ehrlichia canis*.

Nucleotide dataset used for phylogentic reconstruction consisted of 11 sequences generated in this study and 28 partial 16S ribosomal RNA gene segment that were retrieved from NCBI GenBank (including two from India JX861392 and LC053451 generated from different studies). Multiple sequence alignment was performed using inbuilt ClustalW algorithm in MEGA6 software (Tamura et al., 2013). The best fit nucleotide substitution model calculated using online jModelTest 2 generated Kimura2 parameter with gamma distributed rate variation among sites with 5 rate categories [5 discrete categories of gamma (+G, parameter = 0.7247)] (Darriba et al., 2012). The phylogenetic tree and evolutionary distances was calculated using K2 + G nucleotide substitution model in MEGA 6.0 software. The branch lengths were measured in terms of the number of substitutions per site. Robustness of different nodes was assessed with bootstrap analysis using 1000 iterations of the nucleotide data set.

2.9. Statistical tools

Chi-square test was used for analysing significant difference in presence of *E. canis* among German Shepherd and Labrador Retriever breeds (http://www.socscistatistics.com/tests/chisquare/Default2.aspx). Paired results of different diagnostic methods (dot- ELISA, PCR and microscopy) were analysed by using McNemar's Chi squared test (http://www.graphpad.com/quickcalcs/mcNemar) to predict significant difference between the test results.

3. Results

3.1. Microscopy, serology and nested PCR

Overall prevalence of CME based on 225 samples by three different detection techniques, microscopic examination, commercial dot-ELISA and by nested PCR was 1.33% (03/225), 19.11% (43/225) and 5.78% (13/225), respectively (Table 2). No significant difference at $p < 0.05$ was observed among German Shepherd and Labrador Retriever breeds in relation to prevalence of *E. canis* by any of the mentioned diagnostic methods. Cases of *E. canis* among Cocker Spaniels were not included for breed wise estimation because of far too less sample numbers (three only). Chi-square statistic and p values for microscopy, dot-ELISA and nested PCR were 0.0749/0.784, 1.41/0.235 and 0.94/0.33, respectively. Most importantly, difference between the prevalence observed by the three diagnostic methods were found to be statistically significant by McNemar Chi-squared test ($p < 0.05$).

Microscopic examination on the 225 peripheral blood smears revealed that only three samples i.e. one sample each from Cocker Spaniel, Labrador Retriever and German Shepherd breeds to be positive for *E. canis* morulae (Table 1). Among these three microscopic positive samples, only two samples were found to be positive in dot-ELISA while all the three samples were confirmed to be nested PCR positive.

Serological screening by dot-ELISA kit on the 225 serum samples revealed that 43 samples were positive for *E. canis* antibodies (Table 1). Out of these 43 serologically positive samples, two (4.87%) were positive by microscopy and 10 (23.25%) were positive by nested PCR. Three dot-ELISA negative samples were although positive by PCR and one among these was also positive by microscopy as well. Significant difference was observed between serology and microscopy/nested PCR results ($p < 0.05$).

Nested PCR targeting partial 16S rRNA gene of *E. canis* DNA in overall (225) blood samples, amplified a single 389 bp fragment in only 13 blood samples (Table 1). Out of these 13 nested PCR positive cases, 10 were positive and rest three were found to be negative in dot-ELISA assay. The clinical samples which were positive for *E. canis* by any of the test (PCR/serology/microscopy) were also screened for *Babesia* spp. by generic 18S rRNA PCR as reported by Ikadai et al. (2004). Importantly, six out of 13 *E. canis* PCR positive samples were also found positive for *Babesia* spp. by generic PCR and were later confirmed by sequencing for *B. gibsoni* (Table 1).

Table 2
Prevalence of *E. canis* in dogs as observed by three diagnostic methods dot-ELISA (commercial kit), PCR and microscopic examination of Giemsa stained thin smears. The prevalence is shown as percentage and the number of cases in parentheses.

		Prevalence			Overall Prevalence
		Microscopy	Dot-ELISA	PCR	
Sample size studied = 225		1.33% (3/225)	19.11% (43/225)	5.78% (13/225)	20.44% (46/225)
Breed	Labrador	0.76% (1/132)	21.21% (28/132)	3.78% (5/132)	21.96% (29/132)
	GSD	1.11% (1/90)	11.63% (12/90)	6.60% (6/90)	15.56% (14/90)
	Cocker Spaniel	33.3% (1/3)	100% (3/3)	66.7% (2/3)	100% (3/3)

3.2. Haematological & biochemical analysis

Haematology and biochemical parameters of dogs positive for *E. canis* have been illustrated in Tables 3 and 4, respectively. From the study, it was generally observed that haematological and biochemical profiles were not very aberrant in most of the clinical cases. Importantly, lowered values of erythrocyte sedimentation rate (ESR) and absence of hypoglobulinemia was observed in all the clinical cases under study.

3.3. Histopathology examination

Life threatening cases solely because of *E. canis* pathogen have not been reported in this study and most of the sero-positive dogs were apparently healthy. On the contrary, mortality rate was observed in

Table 3
Haematological parameters obtained from the blood samples from 40 dogs positive for *E. canis*. Parameters of 6 dogs that were found positive for dual infection of *E. canis* and *Babesia gibsoni* have not been incorporated in this table. Changes indicate increase, decrease or no change in the haematological parameters (Normal values* are as per the laboratory reference range standardised by Central Military Veterinary Laboratory for dogs).

Haematology Parameters	Normal values*	Number of dogs		
		Increased	Decreased	Unchanged
1. Haemoglobin (g/dl)	12–18	0	8	32
2. Packed Cell Volume (% PCV)	37–55	0	8	32
3. Erythrocyte Sedimentation Rate (ESR; mm/h fall)	5–25	0	40	0
4. Total Erythrocyte Count (TEC, N × 10^6 µl)	5.5–8.5	0	6	34
5. Platelet Counts (N × 10^5 µl)	2–9	0	12	28
6. Total Leucocyte Count (TLC, N × 10^3 µl)	6–17	4	4	32
7. Differential Leucocyte Count (%)				
a) Neutrophils	60–73	8	6	26
b) Lymphocytes	12–30	12	2	26
c) Monocytes	3–10	0	2	38
d) Eosinophils	2–10	0	4	36
e) Basophils	0–0 (rare)	2	0	38

Table 4
Biochemical parameters obtained from 40 dogs positive for *E. canis*. Parameters of 6 dogs that were found positive for dual infection (*E. canis* and *B. gibsoni*) had not been incorporated in this table. Changes indicate increase, decrease or no change in the biochemical parameters (Normal values* are as per the laboratory reference range standardised by Central Military Veterinary Laboratory for dogs).

Biochemical Parameters	Normal values*	Number of dogs		
		Increased	Decreased	Unchanged
1. Total bilirubin (mg%)	0.1–0.6	2	2	36
2. Total protein (mg%)	5.5–7.5	6	2	32
3. Albumin (mg%)	2.6–4.0	0	16	24
4. Globulin (mg%)	2.1–3.7	8	0	32
5. A/G ratio (Albumin/ Globulin)	0.7–1.9	0	14	26
6. AST (IU/L) (Aspartate amino transferase)	8–48	10	0	30
7. ALT (IU/L) (Alanine amino transferase)	8–58	8	0	32
8. BUN (mg%) (Blood urea nitrogen)	8.8–26	8	0	32
9. Creatinine (mg%)	0.5–1.6	6	0	34

animals concurrently infected with *E. canis* and *B. gibsoni*. Three dogs out of the six animals having dual infection of *E. canis* and *B. gibsoni* succumbed to death (Table 1). Post-mortem and histopathology examinations from these dogs were indicative of chronic renal failure, toxaemic shock and haemorrhagic shock due to disseminated intravascular coagulopathy (Suppl. 2).

3.4. Sequencing of 16S rRNA fragment and phylogenetic analysis

Amplicons from 13 *E. canis* DNA samples, collected during the study were sequenced and out of these 11 sequences were submitted to GenBank, NCBI. The details of sequence accession numbers have been presented in Suppl. 3. Phylogenetic analysis was carried out using 39 sequences i.e. 11 *E. canis* sequences generated from the present study, 20 *E. canis* sequences retrieved from NCBI along with sequences of eight other Rickettsial organisms (*Ehrlichia muris, Ehrlichia chaffeensis, Ehrlichia ewingii, Ehrlichia ruminantium, Anaplasma platys, Anaplasma marginale, Anaplasma phagocytophilum* and *Neorickettsia helminthoeca*). All the sequences reported in this study formed a single clade with rest of the *E. canis* isolates reported from India and other countries (Fig. 1). Minor differences were observed in *E. canis* sequences of puma (JQ260848) and jaguarundi (JQ260852) isolated from Brazil and in Oklahoma strain (NR118741) isolated from USA. Hence, these sequences branched into separate individual clade. Interestingly, isolates from lion from Brazil (JQ260853) and human isolate from Venezuela (AF363712) clustered in the same clade as most of the *E. canis* sequences (Fig. 1).

4. Discussion

India, due to varied agro-climatic zones is endemic to many vector borne parasitic diseases including Canine Monocytic Ehrlichiosis as has been evident by previous studies from this country. However, these studies mainly focused on pet dogs bought to the clinics or on stray dogs of some selected cities/parts of this country. Prevalence studies of CME from Northern India were mainly concentrated from Ludhiana city in Punjab over the last few years in pet dogs and non-descript local dogs that were bought to the clinic. Singla et al. (2011) reported a high seroprevalence of 61% in pet dogs when tested by commercial serological kit. The same group in their molecular studies through nested PCR in 2016 in the same city detected meagre 0.39% prevalence of *E. canis* DNA in pet dogs. Milanjeet et al. (2014) from the same Ludhiana city reveals prevalence of 2.34% by microscopy and 41.59% by PCR. In a recent study from the same location by Kottadamane et al. (2017), prevalence of 14.28% and 86.90% were reported by microscopy and serology. A multi-centric study by Abd Rani et al. (2011) reported 20.6% of molecular prevalence study among the stray dogs from the cities of Delhi, Mumbai, Ladakh region of Jammu and Kashmir and from the small state of Sikkim in Eastern India. A study conducted in Western India state of Goa by Wise and Tarlinton (2012) revealed the presence of anti-*E. canis* antibodies among 19% of the dogs out of 40 dogs were screened. Samaradni et al. (2003) from Nagpur, Maharashtra (Western India) reported 18.9% prevalence by microscopy. A single study conducted in Chennai city from South India by Lakshmanan et al. (2006) reported 50% prevalence by PCR.

Moreover, all these prevalence studies have been either based on microscopy or serology or PCR assay. No attempt whatsoever had been made to compare these various diagnostic approaches. Present study has been attempted to gauge the pattern of *E. canis* infection among working dogs in India. A sizeable population of working dogs in India principally comprise of Labrador Retriever and German Shepherd breeds. Deployment of these working dogs in difficult terrains like grassland and forests posses a potential exposure to the infected wild canids; while carrying out duties in semi-urban or rural areas these animals get exposed to the stray dogs. Thus, these working dogs are also at the continuous risk of exposure to ticks and other tick borne diseases. With this background, present study was undertaken simultaneously using, microscopy, dot-ELISA and nested PCR. These diagnostic results were then retrospectively correlated with clinical manifestations, haematology parameters, biochemical profiles and clinico-pathological findings of the clinical cases so as to have an actual view of CME existing in the organised kennels of this country.

The conventional microscopy of stained thin blood smears in the present study detected *E. canis* morulae in only three cases, while six smears also displayed small form of *Babesia* i.e. *B. gibsoni* (Table 1). The limitations of microscopy in detecting *E. canis* morulae has also been reported by prior studies (Mylonakis et al., 2004; Singla et al., 2011; Abd Rani et al., 2011) and therefore make it a less sensitive technique for confirmatory diagnosis of canine ehrlichiosis. Moreover, sensitivity of *E. canis* detection by microscopy is low due to the fact that in chronic cases number of infected cells in peripheral circulation is very less to be detected by microscopic examination. It is considered that sensitivity may be augmented to some extent by examining buffy coat smears rather than examining blood smears; nevertheless in our study we didn't find any difference between the two approaches.

Present study also revealed a greater number (43) of sero-positive samples *vis-à-vis* 13 nested PCR positive cases. Three PCR positive dogs were sero-negative by dot ELISA. Thus, 23.25% sero-positive canines were nested PCR positive, while only 4.65% sero-positives were positive by microscopy. Parana da Silva Souza et al. (2010) had also observed that 34.5% IFAT positive dogs were PCR positive for *E.* canis. Wen et al. (1997) reported 16S rRNA nested PCR as highly specific and sensitive method for detection of *E. canis* DNA from blood samples. Nested PCR protocol has been claimed to be comparable 0.2 pg to of *E. canis* DNA by Southern blotting. Harrus et al. (1998b) had reported that serology based diagnostic assays like indirect immunofluorescence antibody test are not reliable methods to determine persistence of infection or success of treatment as titres have been shown to remain high for long periods even after elimination of the parasite. In contrast, very recent acute condition may not generate enough antibodies sufficient enough to be detected by serology. This phenomenon was observed with two sera samples found to be negative by dot-ELISA (titre 1:20) but judged positive for *E. canis* by microscopy and nested PCR. Alternately, chronic infections may result in negative results with serology. This may be due to pancytopenia and bone marrow failure (Saito and Walker, 2016). *E. canis* DNA in infected dog blood can be detected earlier than serum antibodies, while the latter continue to persist even after doxycycline therapy (Moroff et al., 2014). Moreover, cross reactivity between different *Ehrlichia* spp. makes the definitive sero-diagnosis of *E. canis* difficult. Furthermore, cases with sero-positive and

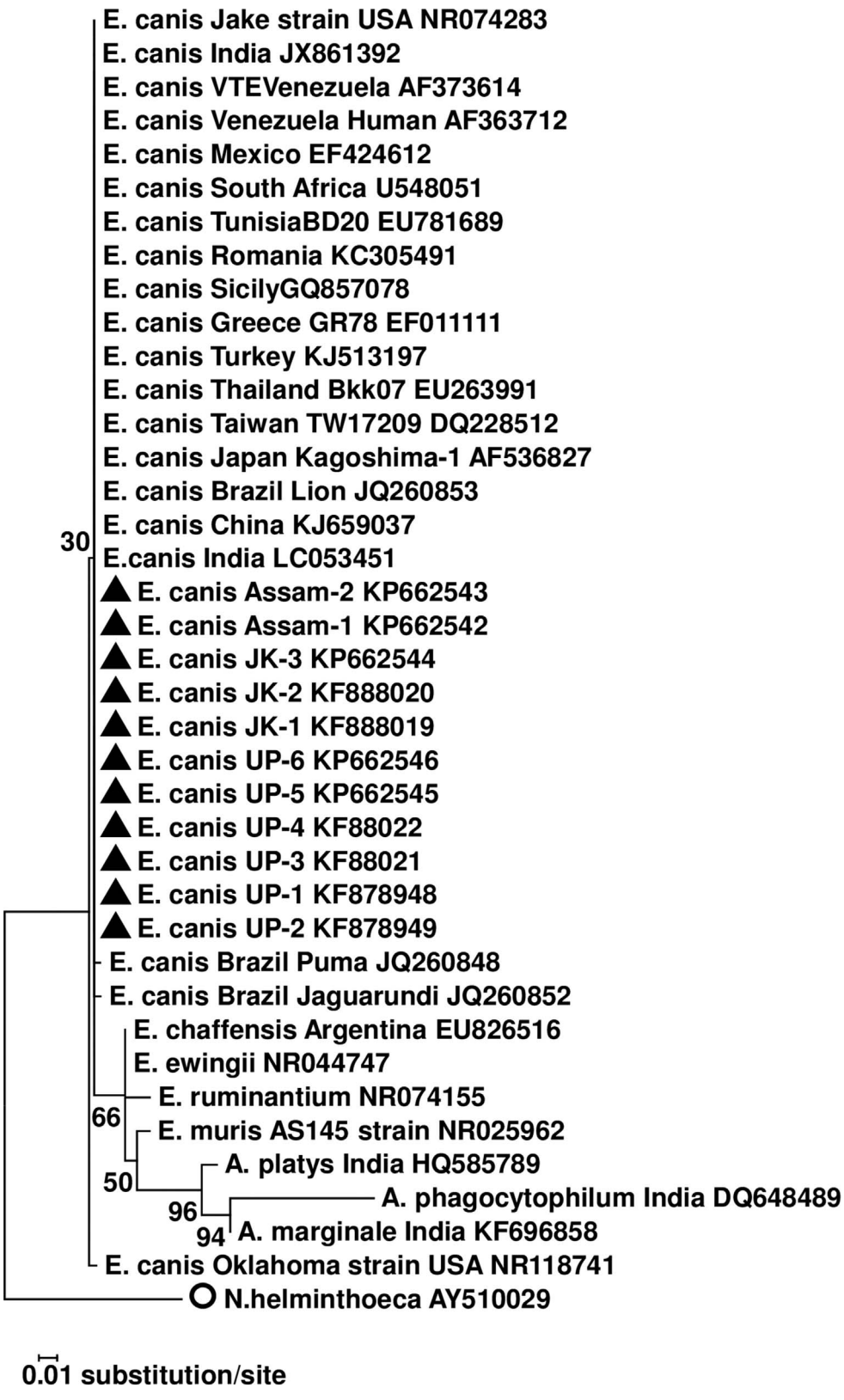

Fig. 1. Molecular phylogenetic tree based on 16S ribosomal RNA sequences of *Ehrlichia canis* and other closely related rickettsia species (39 nucleotide sequences). The evolutionary tree was inferred based on maximum likelihood method by Kimura2 parameter model with gamma distributed rate variation among sites (MEGA6.0). Sequences UP-1, UP-2, UP-3, UP-4, UP-5, UP-6, Assam-1, Assam-2, JK-1, JK-2 and JK-3 were generated during the study are marked with solid triangles. The phylogenetic tree is drawn to scale, with branch lengths measured in the number of substitutions per site. There were a total of 313 positions in final dataset. Bootstrap support values are shown next to the branches. *N. helminthoeca* sequence was used to root the tree (marked with blank circle).

negative nested PCR may also be an indication of the carrier state or subclinical infection due to sequestration of the parasite in the splenic macrophages (Harrus et al., 1998a,b; Waner, 2008). Nakaghi et al. (2008) did not observe any significant difference between the results IFAT kit (VMRD Inc., USA) and dot-ELISA kit (Biogal Galed Laboratories, Israel), though the latter detected more sero-positives (70% by ELISA versus 63.3% by IFAT). In light of observed facts, it is emphasized that serological assays (dot-ELISA) without confirmation by molecular assay (nested PCR) may lead to biased results, as cross reactivity among *Ehrlichia* species and persistence of antibodies for long periods in dogs even after their successful treatment may result in reporting of false positive cases. This possibility leads to the overestimation of CME

in a region/country under study. The same was also observed in our study, where a greater number (43) of sero-positive samples (19.1% of sero-prevalence) vis-à-vis 13 nested PCR positive cases (5.8% of molecular prevalence) was recorded in a fixed set of a dog population. Thus, the serological results should be interpreted with caution and should be complemented by nested PCR results so as to avoid misdiagnosis.

Although, clinical signs and pathology of *E. canis* infection in dogs may vary within or between geographical locations but none the less this pathogen is considered to be most pathogenic amongst other causative agents of canine ehrlichiosis *viz. Ehrlichia chaffensis*, *Anaplasma platys* and *Anaplasma phagocytophilum* (Nair et al., 2016). It is thus beyond doubt that CME due to *E. canis* results in marked anaemia due to significant decrease in PCV, TRBC and haemoglobin (gm/dl). But over the last decade, there has been lot of awareness on canine ehrlichiosis in organised kennels and stringent management practices have helped to reduce the incidence and prevalence of the disease. As per our observations, most veterinarians in India prescribe doxycycline at recommended dose along with supportive B-vitamins and iron supplementations for treatment of minor ailments like pyrexia or as chemoprophylaxis, especially during the tick season. It is expected that due to widespread application of doxycycline, CME is no more a real potent threat to dogs in organised kennels of this country as it used to be, in spite of its high prevalence. This may be one of the reasons that no severe chronic case of ehrlichiosis was encountered during the entire study period. From the interpretation derived from clinical presentation of clinical CME cases with their haematological and biochemical profile pictures it was inferred in this study that severe form of chronic ehrlichiosis is not very common in the organised kennels of this country. Thus keeping in view of endemic status of CME in this country and subclinical state of infection it is recommended prevalence studies should also involve screening of apparently healthy dogs.

It was also observed in present study that most of the *E. canis* infected dogs seem to recover after appropriate therapy, unless otherwise complicated with *B. gibsoni* co-infection or other secondary infections. These findings are consistent with the study by Villaescusa et al. (2012) in Spain. As CME progresses into a chronic form, the clinical outcome may be often grave (Harrus et al., 1997). Though authors did not observe such serious cases of CME alone and the cases with grave prognosis were observed in only cases affected with mixed infections. Three dogs out of the six animals having dual infection of *E. canis* and *B. gibsoni* succumbed to death while no casualty solely because *E. canis* could be incriminated during the entire course of this study. Changes in various organs like spleen, liver, kidney and lungs were similar to earlier studies carried out by de Castro et al. (2004) and Welzl et al. (2001). Nevertheless, continuous exposure to tick vector and due to lack of timely treatment chronic ehrlichiosis may be encountered in stray dogs as reported by other researchers from India (Wise and Tarlinton, 2010; Abd Rani et al., 2011; Bhadesiya and Raval, 2015).

Phylogenetic analysis of 16S ribosomal RNA gene revealed that barring few minor variations, the gene is largely conserved and hence populations of *E. canis* are largely homogenous. However, for developing diagnostics and vaccines for *E. canis* multi-locus sequence typing of the pathogens along with other associated pathogens like *A. platys* which have been reported from this country must be carried out. *E. canis* is considered to have a limited host range among canines and to some extent among felids. Nucleic acid of *E. canis* has been detected from human beings in Venezuela (Unver et al., 2001) as well from ruminants (ovines) in Turkey and South Africa (Parzy et al., 2009). However pathogen has not been isolated from any human subject till date but raises concerns over 'One-Health' issues. Wider host range and the similarity among the geographically distinct isolates indicate a global spread of the pathogen and its vectors (Parzy et al., 2009). These hint at zoonotic potential of *E. canis* and its potential ability to cross species barrier of both hosts and vectors, though human infections are considered to be rare and accidental (McQuiston et al., 2003).

5. Conclusion

CME is endemic in India and still remains a disease of importance in pet dogs, stray dogs and working dogs of this country. The disease manifests in various forms and its diagnosis by conventional microscopy is prone to errors and insufficiency. Regrettably, due to prerequisites of sophisticated technical resources and higher cost elements for PCR and serological assays, practising veterinarians mostly carry out an empirical treatment leading to indiscriminate use of doxycycline and raising concerns on possibilities of positive selection of drug resistant strain of *E. canis*. It is suggested that doxycycline should be used with caution only when there is active infection in the affected dogs. Thus, practising vets need to be supported strongly by sound diagnostic services to ascertain the status of CME in clinical cases. The present study concludes with the recommendation that researchers/clinicians should not solely rely on clinical signs and smear microscopy/serological methods (antibody based detection methods); but should correlate with detection of active infection (antigen or nucleic acid based detection methodologies). These findings will definitely assists in planning of pragmatic control strategies against CME especially in countries where its endemic scenario exists.

Conflict of interest statement

We declare that the authors have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.prevetmed.2017.08.012.

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