Molecular prevalence and risk factors for the occurrence of canine monocytic ehrlichiosis

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ABSTRACT: Evaluation of blood samples collected from 214 dogs from Ludhiana, Punjab (India) was performed for the presence of *Ehrlichia canis* using PCR-based assays targeting a portion of the 16S rRNA gene. Of the total samples subjected to routine blood smear examination, the morulae of *E. canis* were detected in 2.34% samples. Nested PCR assay produced amplicons of expected size (389 bp) specific for *E. canis* in 41.59% (89/214) of samples. The results of multivariate analysis showed that the prevalence of *E. canis* was higher in the summer as compared to the winter season ($P = 0.031$) and in dogs younger than six-month-old as compared to older dogs ($P < 0.001$), while breed and sex of the host were not significantly associated with the occurrence of the disease.

Keywords: canine monocytic ehrlichiosis; *Ehrlichia canis*; haematological abnormalities; nested PCR; risk factors

Canine monocytic ehrlichiosis (CME) caused by the obligatory intracellular pleomorphic rickettsia, *Ehrlichia canis*, is considered to be of global importance in canines. The organism is transmitted by the bite of the brown dog tick, *Rhipicephalus sanguineus*, and causes severe clinical manifestations in affected animals (Groves et al. 1975). *E. canis* is characterised by the presence of intracytoplasmic inclusion bodies (morulae) in circulating monocytes and lymphocytes. CME is generally suspected when the animal presents with a compatible history of living in or travel to an endemic region and previous tick exposure, and when typical clinical signs and characteristic haematological findings (both in terms of parasitological and pathological interpretations) are present. However, it is difficult to reach a definitive diagnosis based only on clinical and haematological abnormalities as natural infections may be present with a variety of clinical signs that vary between different geographical regions (Asgarali et al. 2012).

The detection of morulae of *E. canis* in stained blood smears is a valuable diagnostic tool in the acute disease (Hildebrandt et al. 1973; Mylonakis et al. 2003). However, apart from being time-consuming, it lacks sensitivity for subclinical and chronic disease (Woody and Hoskins 1991). Serology (ELISA and IFA) is the most commonly used diagnostic method in CME; however, cross-reactivity and an inability to differentiate between current and past infections are disadvantages of these methods (Waner et al. 2001). Therefore, molecular techniques like polymerase chain reaction (PCR) using parasite-specific primers provides a better diagnostic tool in terms of both sensitivity as well as specificity and have been widely used in the laboratory diagnosis of canine ehrlichiosis (Cadman et al. 1994; Iqbal et al. 1994; Hegarty et al. 1997; Wen et al. 1997; Carlos et al. 2007). Further, correlation of various risk factors viz. age, sex, breed of host and season with the prevalence of the disease has been documented in past (Harrus et al. 1997; Lakshmanan et al. 2006; Singh et al. 2011; Silva et al. 2012) but similar reports from this part of the country using molecular assays are lacking. Therefore, the current study was undertaken to investigate the PCR-based prevalence of infection and its correlation with various risk factors.

MATERIAL AND METHODS

Geographical area. The study was conducted in the Ludhiana district of the Punjab state, in the north-western region of India. This region lies be-

tween the northern latitudes 30°34' and 31°01' and the eastern longitudes 75°18' and 76°20'. Summers are very hot and winters very cold with annual temperatures in the range from 1 °C to 46 °C (min/max), and an average annual rainfall of 565.9 mm. These environmental conditions provide favourable and conducive conditions for the survival and propagation of ticks and *Rhipicephalus sanguineus* is the major tick infesting canines (Gill and Gill 1977).

Samples. A total of 214 blood samples were collected aseptically from the cephalic vein of selected dogs presented to the Small Animal Clinics, Teaching Veterinary Clinical Complex, GADVASU, Ludhiana (n = 114) as well as local private veterinary clinics (n = 100) over a period of one year (April 2012 to March 2013). Blood was collected into EDTA-coated vials. Dogs were selected on the basis of the presence of naturally acquired tick infestation at the time of presentation and/or on the basis of clinical signs consistent with haemoprotozoan infection viz. fever, epistaxis, corneal opacity, anaemia etc. The collected blood samples were utilised immediately for the preparation of thin blood smears and estimation of haematological parameters (haemoglobin, TLC, DLC and total platelet count) and were then kept at –20 °C until DNA extraction. Microscopic examination of blood samples was done after staining the prepared thin blood smears with Giemsa as per a standard protocol (Coles 1986) and examined under the oil immersion objective of the microscope to detect the morula of *E. canis*. The obtained results were compared to that of the PCR assay.

Genomic DNA isolation. For conducting the PCR assays, genomic DNA was isolated from whole blood using QIAamp® DNA blood mini kit (QIAGEN, GmbH, Germany) following the manufacturer's recommendations. In brief, 200 µl of the blood samples were mixed with 20 µl of proteinase K and 200 µl of lysis buffer and incubated at 56 °C for 10 min. Then, 200 µl of ethanol were added to the sample, and the mixture was applied to a QIAamp Mini spin column and centrifuged at 8000 rpm for 1 min. Thereafter, two washing steps were performed with 500 µl each of wash buffers 1 and 2. Finally, 150 µl of elution buffer were added to the column, and DNA was eluted in 1.5 ml Eppendorf tubes after centrifugation and stored at –20 °C until use. Genomic DNA of *E. canis* was isolated and utilised as a positive control from an infected blood sample showing rickettsiaemia in blood smear examination. Genomic DNA was also isolated from the whole blood of an infection-free puppy (confirmed by blood smear and buffy coat examination) and used as a negative control along with nuclease-free water.

PCR protocols. The PCR assays (primary as well as nested) were designed to target a portion of the 16S rRNA gene so as to amplify all *Ehrlichia* spp. (primary) and *E. canis* (nested PCR) as described by Murphy et al. (1998). The sequences of the primers were as follows:

Primary PCR assay:

ECC: 5' AGAACGAACGCTGGCGGCAAGC 3'

ECB: 5' CGTATTACCGCGGCTGCTGGCA 3'

Nested PCR assay:

ECAN5: 5' CAATTATTTATAGCCTCTGGCTC-TGGCTATAGGA 3'

HE3: 5' TATAGGTACCGTCATTATCTTCCCTAT 3'

Two rounds of PCR in a final volume of 25 µl were carried out in a PCR thermal cycler (Applied Biosystems, USA). In the primary PCR assay for amplification of all canine ehrlichial species, the master mix consisted of 2.5 µl of 10× PCR buffer (MBI Fermentas), 0.5 µl of 10mM dNTP mix (MBI Fermentas), 1.5 µl of 25mM $MgCI_2$ (MBI Fermentas), 1.0 U of recombinant *Taq* DNA polymerase (MBI Fermentas), 1 µl each (20 pmol) of the ECC and ECB primers and 5 µl of template DNA isolated from field samples. The volume was made up to 25 µl with nuclease-free water. The cycling conditions were as follows: initial denaturation at 94 °C for 3 min, 30 cycles of denaturation at 94 °C for 1 min, annealing at 65 °C for 1 min, and extension at 72 °C for 2 min, and the final extension was performed at 72 °C for 8 min. In order to carry out the nested PCR specific for *E. canis*, the master mix was the same as described above, but instead of template DNA, 1 µl of the primary PCR product was used and amplified with 20 pmol each of ECAN5 and HE3 primers. The PCR conditions were as follows: initial denaturation at 94 °C for 3 min, first step consisted of three cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 2 min and extension at 72 °C for 1.5 min while the second step consisted of 37 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 2 min and extension at 72 °C for 1.5 min and the final extension was performed at 72 °C for 8 min.

The PCR products (primary as well as nested) were checked for amplification by electrophoresis on a 1.5% agarose gel and visualised using a gel documentation system (Syngene, UK). In order to check the specificity of the assays, isolated genomic

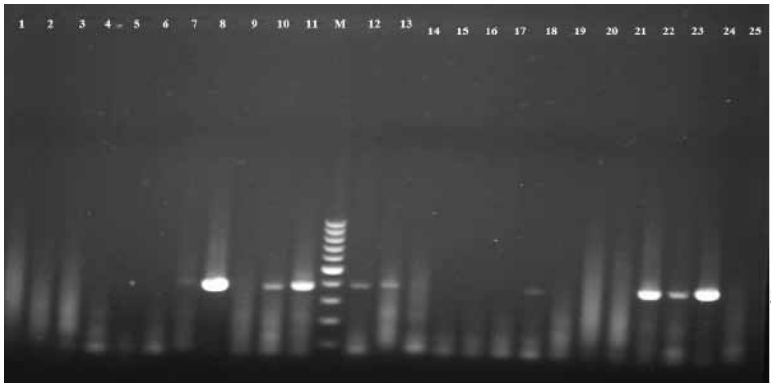

Figure 1. Field application of nested PCR assay. Lane M = Generuler^{TM} 100 bp ladder, lane 1–10 and 12–24 = field collected samples, lane 11 = positive control; lane 25 = negative control

DNA of *Babesia canis*, *B. gibsoni*, *Hepatozoon canis* and *Trypanosoma evansi* isolated from the microscopically positive cases was also tested in the PCR to determine amplification, if any.

Haematological evaluations. The whole blood samples of canines immediately after collection were used for determination of haemoglobin concentration – Hb (g/dl), total leukocyte count – TLC (× 10^3/µl) and total platelet count – PLT (× 10^3/µl) by utilising a fully automated blood analyser, the ADVIA 2120 Haematology System (Siemens Health Care Diagnostics Inc. IL, USA). Differential leukocyte count – DLC (%) was performed manually under oil immersion power (100×) of a bright field microscope in a Giemsa-stained thin blood smear (Jain 1986) and 100 leucocytes were counted per slide. The Tukey's multiple comparison test (GraphPad Prism 4) was used to compare the mean values of haemoglobin, leukocytes, neutrophils, lymphocytes and platelet counts betwen dogs with and without *E. canis* infection.

Statistical analysis. All data analyses were performed using a statistical software program (SPSS for Windows, Version 19.0, USA). Association between the prevalence of CME by nested PCR and various risk factors viz. sex, age and breed of the host; and season was carried out using the Chi squared (χ^2-test). Variables with significant association at $P < 0.05$ (two-sided) were subjected to the multivariate logistic regression model. The results were each expressed as *P*-values and odds ratios (OR) with a 95% confidence interval (CI 95%).

RESULTS

Blood smear examination. Examination of Giemsa-stained peripheral thin blood smears revealed 2.34% (5/214) positivity for the morulae of *E. canis*. The morulae were compact showing aggregations of initial corpuscles staining dark/navy blue in colour.

PCR assays. PCR products obtained from the primary PCR, when employed as templates in nested PCR produced amplicons of 389 bp in 41.59% (89/214) of the samples (Figure 1). Regarding the sensitivity of nested PCR, it was observed that amplification was achieved even from those primary PCR products (75 samples) that could not be visualised on agarose gel electrophoresis. Further, the PCR primers used in the present assay did not amplify any product when the genomic DNA of *B. canis*, *B. gibsoni*, *H. canis* and *T. evansi* was used as a template, revealing the specificity of these primers.

Haematological evaluations. Blood samples of dogs positive for *E. canis* by blood smear examination as well as nested PCR assay showed a significant drop ($P < 0.01$) in the haemoglobin level indicating that CME leads to anaemia in dogs (details in Table 1). Further, samples positive in the

Table 1. Comparison of various haematological parameters using Tukey's multiple comparison test

Parameter (units)	Normal range*	Blood smear	Nested PCR	Negative
Hb (g/dl)	12–18	8.57 ± 1.54[a]	8.23 ± 0.57[a]	12.53 ± 0.42
TLC (/µl)	6000–17 000	6672 ± 1274	5474 ± 1238[b]	12746 ± 1471
N (%)	60–70	87.2 ± 2.8	83.8 ± 3.4	76.3 ± 3.9
L (%)	12–30	11.5 ± 2.1	14.0 ± 3.2	18.5 ± 1.7
Platelets (× 10^3/µl)	200–900	34.5 ± 4.6[b]	52.6 ± 8.8	89.1 ± 10.2

[a]$P < 0.01$, [b]$P < 0.05$, *Kahn et al. (2005)

Table 2. Final logistic regression model for factors associated with the prevalence of canine monocytic ehrlichiosis by nested PCR on the animal level

Variable	Regression coefficient (β)	Standard error (SE)	*P*-value	Odds	Confidence interval (CI 95%)
Age	–1.540	0.260	0.000	1.333	0.628–2.833
Sex	0.339	0.181	0.062	0.956	0.551–1.658
Breed	0.241	0.233	0.300	1.340	0.632–2.845
Season	–0.486	0.225	0.031	2.600	1.277–5.296

nested PCR assay showed a significant decrease in TLC ($P < 0.05$) indicating leucopaenia. Also, samples positive in the blood smear examination recorded a significant ($P < 0.05$) decrease in platelet count indicating thrombocytopaenia.

Age-wise prevalence of CME. Animal age was found to be associated with the prevalence of CME ($P < 0.001$; OR: 1.33; CI 95%: 0.62–2.83) but had a negative correlation (β = –1.540) (Table 2). Thus, among the different age groups of dogs screened by nested PCR maximum infection was recorded in dogs < six months of age (47.05%), followed by the six months to one year age group (42.50%). Infection levels were lowest in the > one year age group (40.00%) (Table 3).

Sex-wise prevalence of CME. The association of the sex of the host and the prevalence of CME was non-significant ($P = 0.339$; OR: 0.956; CI 95%: 0.55–1.65) with a positive correlation (β = 0.339) and a higher prevalence was recorded in females (42.22%) in comparison to males (41.12%) (Table 3).

Breed-wise prevalence of CME. A non-significant association between the prevalence of CME and the breed of the dog was observed ($P = 0.300$; OR: 1.340; CI 95%: 0.63–2.84). A β-value of 0.241 was recorded with maximum prevalence in German Shepherds (58.82%) followed by Pugs (45.83%),

Table 3. Distribution of canine monocytic ehrlichiosis in accordance with various risk factors

Risk factor	Parameter	Number	Blood smear (%)	Nested PCR (%)
Age	0–6 months	34	1 (2.94)	16 (47.05)
	6 months–1 year	40	1 (2.50)	17 (42.50)
	1 year	140	3 (2.14)	56 (40.00)
	χ^2-value		6.141**	0.574
Sex	male	124	4 (3.22)	51 (41.12)
	female	90	1 (1.11)	38 (42.22)
	χ^2-value		7.361*	0.026
Breed	Labrador	71	1 (1.41)	29 (40.84)
	German Shepherd	34	1 (2.94)	20 (58.82)
	Pug	24	0	11 (45.83)
	others	35	0	12 (38.89)
	non-descript	50	3 (6)	17 (34)
	χ^2-value		9.731**	6.268
Season	summer	64	2 (3.12)	32 (50.00)
	rainy	78	2 (2.56)	37 (47.43)
	winter	72	1 (1.38)	20 (27.77)
	χ^2-value		0.228	8.852**
Total		214	5 (2.33)	89 (41.58)

*$P < 0.01$, **$P < 0.05$

Others include: Pomeranian (8), Saint Bernard (9), Dalmatian (3), Boxer (3), Great Dane (3), Cocker Spaniel (2), Rottweiler (4), Napoleon Mastiff (2)

while the lowest prevalence was recorded in non-descript breeds (34.0%) (Table 3).

Seasonal dynamics of CME. The current study indicates that season plays a very important role in the prevalence of CME. A significant association between the prevalence of CME and the season was observed (P = 0.031; OR: 2.60; CI 95%: 1.27–5.29). A β-value of –0.486 was recorded between the prevalence and the various seasons (summer followed by rainy and winter), thus indicating a decrease in prevalence with a decrease in ambient temperature (Table 3).

DISCUSSION

The laboratory diagnosis of CME is a challenging task, since the disease presents varied clinical, haematological, as well as biochemical abnormalities (Waner 2008). In this regard, apart from the conventional parasitological diagnostic techniques (cytology), serological and PCR based assays are now also increasingly utilised for the diagnosis of the disease, especially in epidemiological surveys, owing to their higher sensitivity and specificity.

In the past various studies have been carried out regarding the molecular prevalence of *E. canis* worldwide and the prevalence rate has been reported to range from 3.1% to 88.0% (Murphy et al. 1998; Dagnone et al. 2003; Bulla et al. 2004; Macieira et al. 2005; Diniz et al. 2007; Carvalho et al. 2008; Alexandre et al. 2009; Dagnone et al. 2009; Faria et al. 2010; Silva et al. 2012). As regards the Indian scenario, a small number of published reports are available: the prevalence of *E. canis* was reported to be 50% (49/98) in Chennai (Lakshmanan et al. 2007) and 20.6% from four different regions of India (Abd Rani et al. 2011). In the present study nested PCR assay targeting a portion of the 16S rRNA gene detected 41.59% (89/214) of samples to be positive for *E. canis*. The variation in the prevalence reported by various workers may be attributed to sample size, geographical area, climatic conditions which directly influence the tick population and time of sample collection. Further it may also be emphasised that the high prevalence rate obtained in the current study may be due to the fact that the study was carried out on dogs referred to the Veterinary Clinics presenting clinical manifestations consistent with CME. It has been shown that nested PCR is one hundred times more sensitive than one-step PCR for the diagnosis of CME (Bulla et al. 2004). Further, no false positive results have been found with this assay demonstrating that it is very specific. It has been used during the acute phase of the disease when antibodies are not detectable (Wen et al. 1997). Also, *E. canis* DNA has been shown to be detected using nested PCR in the buffy coats of experimentally infected dogs from the fourth day of infection (Wen et al. 1997).

In the present study peripheral blood smear examination revealed a very low prevalence (2.33%) of CME in comparison to the nested PCR assay-based detection (41.59%). The lower prevalence recorded by blood smear examination is due to the fact that it lacks sensitivity (Woody and Hoskins 1991), because it is time consuming (Mylonakis et al. 2003). Also, the chances of successfully finding the morulae in stained smears are very low, particularly in the subclinical stage of the disease (Harrus and Waner 2011) as it is assumed that during this phase, the pathogen is sequestered within the spleen and hence is not detected in blood (Waner 2008). Further, the inability of differentiating morulae from the inclusions present during severe bacterial infections (like Dohle bodies), inflammation, auto-immune diseases, viral infections (like canine distemper) and severe tissue destruction may result in false positive results (Schalm 2000). Therefore, molecular diagnosis, based on sensitive and specific PCR assays will be helpful especially in the early diagnosis of canine ehrlichiosis along with the identification of the infecting species, thereby helping with the taxonomic classification (Iqbal et al. 1994).

Profound haematological changes have been found to occur during the acute, subclinical and chronic phases of CME (Harrus et al. 1999; Waner 2008). Among the various haematological alterations frequently observed in affected dogs, anaemia, which is usually normocytic, normochromic and non-regenerative, suggesting a restricted or absent bone marrow response is a very important finding and has been reported by many workers (Dagnone et al. 2003; Bulla et al. 2004; Niwetpathomwat et al. 2006; Nakaghi et al. 2008; Borin et al. 2009; Gaunt et al. 2010; Silva et al. 2012). Similarly, in the present study a significant drop ($P < 0.01$) in haemoglobin levels was observed suggesting that CME is an important cause of anaemia in canines. Further, samples positive by nested PCR assay showed a significant decrease in TLC ($P < 0.05$) indicating leucopaenia. Similar findings indicating leucopaenia in dogs suffering from CME have been previously reported (Niwetpathomwat et al. 2006; Alexandre et al. 2009).

Also, samples positive by blood smear examination exhibited a significant ($P < 0.05$) decrease in platelet count indicating thrombocytopaenia in the present study though the data was not found to be significant when nested PCR was employed. Thrombocytopaenia has been considered to be the most common and consistent haematological finding in dogs affected with CME as well as experimentally infected dogs. Mild to severe thrombocytopaenia in *Ehrlichia*-infected dogs has been documented (Codner and Farris-Smith 1986; Waner et al. 1997; Bulla et al. 2004; Macieira et al. 2005; Niwetpathomwat et al. 2006; Silva et al. 2012). The thrombocytopaenia recorded can be explained by the immune-mediated destruction, increased consumption of platelets by sequestration or by decreased production, vasculitis and platelet function abnormalities (Lappin et al. 2010).

Further, dogs negative for CME in the present study also exhibited a lower platelet count when compared with the normal range but the count was higher than the samples positive for CME. It has been observed that not all thrombocytopaenic dogs are positive for CME, suggesting that it is not the only cause of thrombocytopaenia in dogs (Dagnone et al. 2003; Trapp et al. 2006; Gaunt et al. 2010). Numerous diseases can result in thrombocytopaenia including immune-mediated thrombocytopaenia, neoplastic processes, inflammatory diseases or other infectious agents (Grindem et al. 2002).

As far as the age- and sex-wise prevalence of CME is concerned several authors have observed the prevalence of haemoprotozoan infections to be highest in young dogs (Ezekoli et al. 1983; Abdullahi et al. 1990; Samaradni et al. 2005; Lakshmanan et al. 2006). In the current study it was also seen that age had a significant effect on the occurrence of CME which is consistent with earlier studies. In terms of sex-wise prevalence various workers have reported no significant difference in the prevalence of the disease in males and females using either conventional or serological and molecular methods (Lakshmanan et al. 2006; Kumar et al. 2009; Singh et al. 2011; Silva et al. 2012). In the current study it was also observed that sex had no significant effect on the occurrence of CME which is in agreement with earlier studies.

In terms of breed-wise variation, German shepherd dogs have been found to be the most susceptible dog breed for canine ehrlichiosis (Huxsoll et al. 1972; Heerden 1982; Lakshmanan et al. 2006). This may be due to the defective cell-mediated immune response seen in this breed. In the current study the disease prevalence was highest in this breed as compared to others although the data was statistically non-significant. Season-wise, the disease was found to be most prevalent in the summer season followed by rainy. It was least prevalent in the winter season. The probable reason behind this trend may be correlated to the seasonal activity of the brown dog tick, *Rhiphicephalus sanguineus* which is most abundant during hot and humid periods of the year (Soulsby 1982), thus resulting in a higher incidence of haemoprotozoan infections in warm months during warmer seasons (Harrus et al. 1997; Eljadar 2010; Dutta et al. 2013). Further, differences in climatic conditions have been determined to be important factors which influence the population dynamics of ticks in a particular region thereby resulting in a variable prevalence pattern of CME (Costa Jr et al. 2007).

In summary, it can be concluded that for the diagnosis of CME, apart from the routinely used conventional methods, PCR-based assays should also be employed in order to elucidate the true spectrum of the disease. As far as haematological alterations are concerned anaemia, leucopaenia and/or thrombocytopaenia must be included in the differential diagnosis when these are observed during routine laboratory evaluations.

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