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Research Article

CLINICAL AND HAEMATO-BIOCHEMICAL OBSERVATIONS IN DOGS NATURALLY INFECTED WITH CANINE MONOCYTIC EHRLICHIOSIS

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ABSTRACT: The present study reported the clinical observations and haemato-biochemical abnormalities associated with *Ehrlichia canis* in dogs naturally infected with canine monocytic ehrlichiosis (CME) referred to a Teaching Veterinary Hospital, Ludhiana, Punjab. A total of 41 dogs infected with *E. canis* based on microscopic examination of Leishman-stained thin blood smears (n=17) and/or polymerase chain reaction assay (n=24) were included in the study. Clinical findings were recorded, and haemato-biochemical data of dogs with CME was compared with that of 44 apparently healthy dogs. Most common clinical manifestations associated with CME included tick infestation (90.2%), melena (75.6%), pale mucous membrane (61%), lymphadenopathy (48.8%) and epistaxis (41.5%). When compared with healthy dogs, the mean values of platelets, haemoglobin, haematocrit, total erythrocyte count, total protein and albumin were significantly ($p<0.001$) decreased. Conversely, the mean values of absolute neutrophil count, alkaline phosphatase, total bilirubin and blood urea nitrogen were significantly ($p<0.05$ to $p<0.0001$) increased in dogs with CME. The current study indicated that CME is very much common in dog population of Punjab, thus stressing the need to make dog owners aware regarding tick control measures. Major clinical and haemato-biochemical abnormalities in infected dogs are required to be addressed while undertaking therapeutic management in these dogs.

Key words: Clinical signs, Dog, Ehrlichiosis, Haemato-biochemical alterations, PCR.

INTRODUCTION

Canine monocytic ehrlichiosis (CME) is a major tick borne disease affecting dog population all over the world with a higher incidence rates reported in tropical countries (Mittal *et al*. 2017). The disease is mainly caused by intra-monocytic gram-negative, obligate intracellular bacterium, *Ehrlichia canis*, which is transmitted trans-stadially and intra-stadially by the brown dog tick, *Rhipicephalus sanguineous* (Bremer *et al*. 2005). The parasite results in formation of intra-cytoplasmic aggregates, commonly known as morulae, within the monocytes (Sainz *et al*. 2015). Bleeding tendencies manifested as mucosal and subcutaneous haemorrhages, haematuria, melena, epistaxis and prolonged bleeding from blood collection sites are the typical clinical findings observed in dogs with CME (Harrus and Waner 2011). The most common haematological alterations associated with the disease include anaemia and thrombocytopenia. In addition, infected dogs also have a tendency to develop multi-organ dysfunction including hepatic and renal abnormalities (Kottadamane *et al*. 2017).

Under hospital settings, the disease is mainly diagnosed on the basis of clinical and haemato-biochemical finding alongside demonstration of morulae within the monocytes on microscopy. Subclinical and chronic forms of the disease are usually asymptomatic. Furthermore, there could be low parasitaemia, making routine microscopy less sensitive for the detection of *E. canis* parasite in the suspected dogs. Moreover, serological tests like enzyme-linked immunosorbent assay and indirect fluorescence antibody test, fails to distinguish between current and earlier infections (Bélanger *et al*. 2002). Thus, advanced molecular techniques like

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polymerase chain reaction (PCR) assay are the diagnostic methods of choice for confirmatory diagnosis of CME (Harrus and Waner 2011).

A better understanding of the patho-physiology and alterations in the haemato-biochemical parameters associated with canine ehrlichiosis will surely help in management of the disease in a more effective way. Limited studies are available about clinical manifestations and haemato-biochemical alterations in dogs affected with ehrlichiosis from Northwest region of India (Kottadamane *et al.* 2017). So, the present study was carried out to investigate clinical and haemato-biochemical alterations in dogs naturally infected with ehrlichiosis.

MATERIALS AND METHODS

The present study was conducted at the Multispecialty Veterinary Hospital of Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana, Punjab. In routine, blood samples from dogs with clinical signs suggestive of haemo-parasites were subjected to thin blood smear examination by conventional microscopy. In the present study, 17 dogs found positive for *E. canis* morulae by microscopy during the period from January 2016 to July 2021 were included. In addition, 24 dogs found positive for CME by PCR assay during the period from October 2020 to July 2021 were also included in the present study making a total of 41 dogs with CME. For these dogs, presenting complaints, clinical examination findings and haemato-biochemical results, if any, were recorded from patient's clinical records by screening Teaching Veterinary Hospital Database Management System. The permission for sampling was duly approved by college's Institutional Animal Ethics Committee wide no. V-11011(13)/2/2021-CPCSEA-DADF.

Haemato-biochemical analysis

From each dog, about 6 mL of blood sample was obtained in a sterile disposable syringe by cephalic or lateral saphenous venipuncture. From this, about 3 mL of blood was transferred into an EDTA vial for performing haematology, blood smear examination and isolation of genomic DNA for performing PCR assay. The remaining 3 mL of the blood was placed in a vial without anticoagulant and used for serum harvesting and analyzing biochemical parameters.

Haematological parameters including haemoglobin (Hb; g/dL), total erythrocyte count (TEC; × 10^6/µL), haematocrit (%), platelet count (× 10^3/µL) and total leukocyte count (TLC; cells/µL) were measured by automatic blood analyzer (ADVIA 2120 Haematology System, Siemens Healthcare Diagnostic Inc., USA). Differential leucocyte count was performed manually as per the standard method. Biochemical parameters including total protein (g/dL), albumin (g/dL), total bilirubin (mg/dL), alkaline phosphatase (ALP; U/L), alanine aminotransferase (ALT; U/L), blood urea nitrogen (BUN; mg/dL) and creatinine (mg/dL) were analyzed by clinical chemistry analyzer (Vitros® 350 Chemistry System, Ortho-Clinical Diagnostics Inc., SA) using diagnostic kits according to manufacturers' recommendations.

Thin blood smears were stained with Leishman stain and examined microscopically under oil immersion lens (100X) in order to detect inclusion bodies or morulae stages of *Ehrlichia* species in monocytes (Fig. 1).

PCR assay

For PCR assay, the whole genomic DNA was isolated from blood samples by using Geneaid® DNA blood mini kit (GS300). The extracted DNA was stored at -20°C until further use. The PCR assay was standardized using the primers described by Kledmanee *et al.* (2009) targeting a portion of the VirB9 gene of *Ehrlichia* species. Subsequently, the forward and reverse primers Ehr1401F

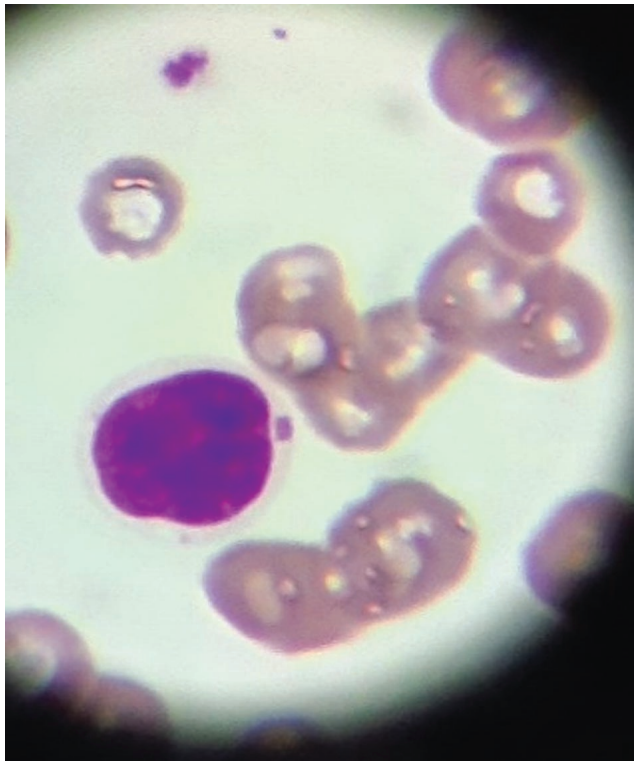

Fig. 1. Giemsa-stained peripheral blood smear of affected dog showing monocyte infected with morulae (→) of *E. canis* (100X).

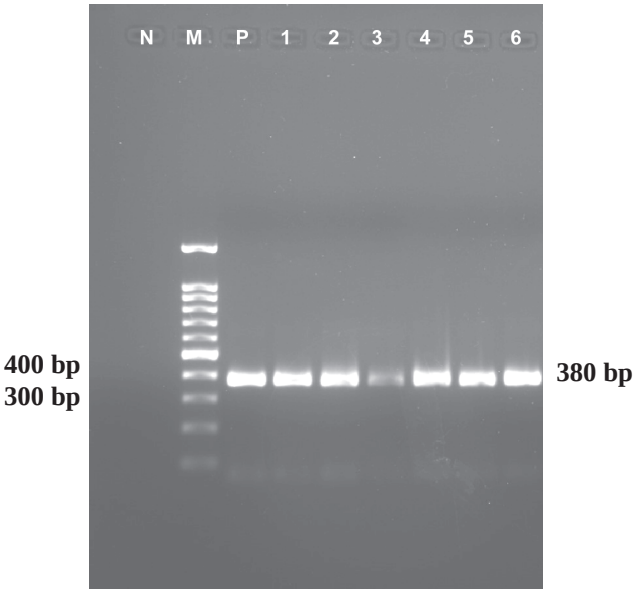

Fig. 2. Application of *E. canis* specific PCR assay on clinical samples.

(Lane M: 100 bp DNA Ladder, Lane P: Positive control, Lane N: Negative control, Lane 1-6 : Selected clinical samples).

and Ehr1780R, respectively, were used to identify *E. canis* species giving a product of 380 bp. The PCR products were resolved in 1.5% agarose gel containing Good view nucleic acid stain (Helix Biosciences) and visualized under Molecular Imager®ChemiDoc™ XRS (Bio-Rad, USA). The genomic DNA sample isolated from the blood of *E. canis*-infected dog was used as a positive control and genomic DNA from a healthy dog was used as a negative control (Fig. 2).

For comparison of the haemato-biochemical parameters of dogs with ehrlichiosis, apparently healthy dogs (n=44) presented for routine general check-ups or preventive care were included as a control group. The selection criteria included microscopic screening of dogs and those free from any vector borne haemo-parasitic infections were included. These dogs consisted of Labrador Retriever (26; 59%), German Shepherd (7; 15.9%), Pug (3; 6.8%), Mix breed (2; 4.5%), and Cocker Spaniel, Doberman, Lhasa Apso, American Pitbull, Pomeranian and Spitz (1 each; 2.3% each). There were 29 male dogs (65.9%) and 15 female dogs (34.9%) and the mean age of dogs were 4.7±0.4 years (range: 1 to 10 years).

Statistical analysis

Data were analyzed using Minitab statistical software (Version 14.2, State College, PA, USA). Frequencies, proportions, means, standard errors and ranges were calculated. The mean values of haemato-biochemical parameters were compared between healthy and diseased dogs using two sample *t*-test, and the differences were considered statistically significant at $p<0.05$.

RESULTS AND DISCUSSION

In this study, dogs were diagnosed for CME either based on microscopy (n=17) or by PCR assay (n=24). Dogs found positive for CME by microscopy were not further subjected to PCR assay for confirmation. The PCR assay was employed in only those dogs that were highly suspected for CME based on their clinico-haematological findings but were negative on microscopy. The conventional microscopy has a limitation in detecting *E. canis* morulae as reported in previous studies (Singla *et al.* 2011, Mittal *et al.* 2017). In the present study, during screening of the hospital canine patient data, it was observed that dogs with clinico-haematological findings characteristic of ehrlichiosis responded completely to the tetracycline antibiotics (like doxycycline), despite being negative on blood smear examinations. Although, molecular techniques, such as PCR assay, are more sensitive methods for detection of *E. canis* (Mittal *et al.* 2017), yet these being costlier and time consuming cannot be employed for routine examination of samples submitted to the clinical diagnostic laboratories of hospitals for detection of blood parasites.

Out of the 41 dogs, 12 (29.2%) were Germen Shepherd, 9 (22%) were Labrador Retriever, 4 each were Pug and Pomeranian (9.8% each), 3 were Gaddi (7.3%), 2 each were American Pitbull, Rottweiler and Golden Retriever (4.9% each) and 1 each were Beagle, Dachshund and Saint Bernard (2.4% each). There were 29 male dogs (70.7%) and 12 female dogs (29.3%). Majority of the affected dogs were >1 year of age (30; 73.2%) as compared with dogs ≤1 year of age (11; 26.8%). The mean age was 3.5±0.44 years (range: 0.5 to 11 years). Month-wise, majority of cases were reported during April to July (21; 51.2%) followed by November to March (15; 36.6%) and August to October (5; 12.2%). Higher susceptibility of Germen Shepherd dogs to CME might be due to its inherent inability to produce adequate blast cells causing inadequate cellular immune response against the causative agent, i.e., *E. canis* (Nyindo *et al*. 1980). In relation to gender predisposition, male dogs (70.7%) were more infected with CME as compared with the female dogs (29.3%) which could be attributed to more liking of male dogs by the pet owners which might resulted into over presentation of male dogs in the hospital. Young adult dogs >1 year of age were found to be more affected with CME which corroborates with the results of Trapp *et al.* (2006), Guedes *et al.* (2015) and Kottadamane *et*

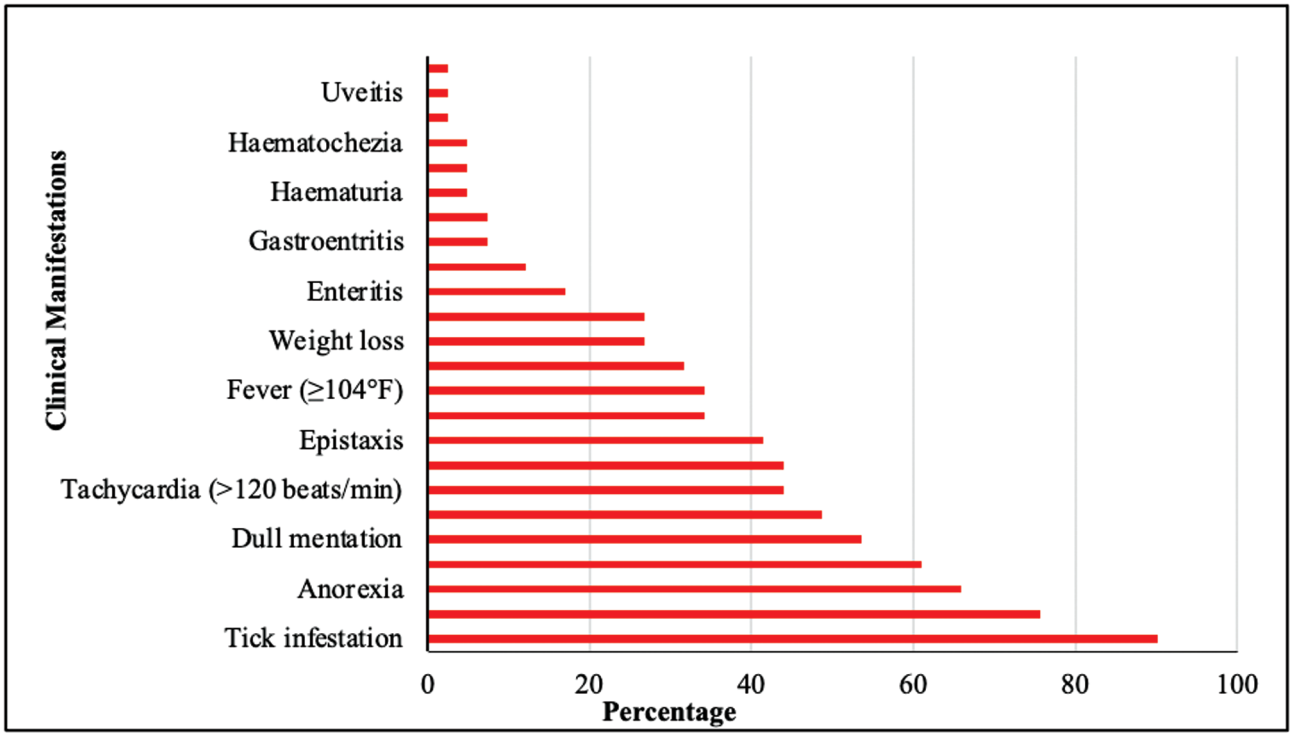

Fig. 3. Distribution of presenting and clinical examination findings in dogs with ehrlichiosis (N=41).

al. (2017). Young dogs may be protected from infection because of less exposure to the outside environment including ticks and other dogs. Majority of cases were presented during the summer season (*i.e.*, April to July) which coincides with the time period during which the vector population is high for the transmission of disease to the susceptible dogs (Kottadamane *et al.* 2017, Mittal *et al.* 2017).

Dogs with CME were presented with a wide variety of clinical manifestations, of which, the most frequently reported observations were tick infestation (37; 90.2%), melena (31; 75.6%), anorexia (27; 65.9%), pale mucosa (25; 61%), lethargy (22; 53.7%) and lymphadenopathy (20; 48.8%). Other clinical signs are depicted in Fig. 3. Three dogs were also presented with seizures which have been rarely observed in dogs with ehrlichiosis. Diverse type of clinical signs recorded in dogs with CME have been reported in the previous studies (Foglia Manzillo *et al.* 2006, Harrus and Waner 2011, Mylonakis *et al.* 2019), and these signs indicated multi-organ dysfunctions due to underlying pathological changes such as vasculitis in dogs with CME (Foglia Manzillo *et al.* 2006, Mylonakis *et al.* 2019).

The haemato-biochemical alterations in dogs infected with CME are presented in Table 1. As compared to the healthy dogs, the affected dogs had significantly lower mean levels of Hb, haematocrit and TEC indicating anaemia in these dogs. More than 50% of the dogs had Hb of <7 g/dL and haematocrit of <21% in this study. These abnormalities in dogs with CME could be due to the higher affinity of *E. canis* towards the vascular endothelial cells resulting into vasculitis which is manifested as multiple petechial or ecchymotic haemorrhages in various parts of the body and mucus membranes causing blood loss, anaemia (Sainz *et al.* 2015). Platelet counts were significantly decreased in dogs infected with ehrlichiosis as compared with the healthy dogs. In about 54% of the dogs, the thrombocytopenia was severe ($<50 \times 10^3/\mu L$). Thrombocytopenia in diseased dogs reflected the increased platelet destruction due to development of anti-platelet antibodies (Harrus *et al.* 1996) and consumption of platelets due to haemorrhages in various parts of the body (Kakoma *et al.* 1978). The infected dogs had significantly higher TLC and absolute neutrophil count as compared with the healthy dogs. Leucopenia (<5000/ µL) and leucocytosis (>15000/ µL) were observed in 24.4% and 34.1% of the dogs, respectively. About 22% of dogs had pancytopenia in this study. Similar findings were reported in earlier studies (Foglia Manzillo *et al.* 2006).

There was a significant increase in the levels of serum total bilirubin and ALP in infected dogs as compared with healthy dogs. These findings are attributed to *E. canis* induced inflammation of the sinusoidal endothelium of liver parenchyma (Mylonakis *et al.* 2010). The levels of

Table 1. Comparison of haemato-biochemical parameters between healthy dogs and dogs with ehrlichiosis.

Parameter	Healthy dogs (N = 44)	Dogs with CME		p-value
	(Mean ± SE)	N*	(Mean ± SE)	
Hb (g/dL)	14.5 ± 0.4[a]	41	6.8 ± 0.5[b]	0.0001
Haematocrit (%)	40.9 ± 1[a]	41	21.4 ± 1.5[b]	0.0001
TEC ($\times 10^6$/µL)	6.5 ± 0.2[a]	41	3.3 ± 0.2[b]	0.0001
Platelet count ($\times 10^3$/µL)	378.6 ± 19.5[a]	41	65.2 ± 9.3[b]	0.0001
TLC (cells/µL)	12023 ± 646	41	13448 ± 2076	NS
Neutrophils (cells/µL)	8726 ± 464	35	12966 ± 2172	0.037
Lymphocytes (cells/µL)	3022 ± 186	35	2670 ± 377	NS
Total bilirubin (mg/dL)	0.3 ± 0.03[a]	38	1.3 ± 0.3[b]	0.0001
ALT (U/L)	52.9 ± 2.3	30	66 ± 12	NS
ALP (U/L)	68.1 ± 3.6[a]	28	217.4 ± 53[b]	0.001
Total proteins (g/dL)	6.7 ± 0.1[a]	27	5.2 ± 0.3[b]	0.0001
Albumin (g/dL)	3.1 ± 0.1[a]	24	2 ± 0.1[b]	0.0001
BUN (mg/dL)	14 ± 1.02[a]	29	26.4 ± 2.7[b]	0.0001
Creatinine (mg/dL)	1.1 ± 0.03	30	1.3 ± 0.1	NS

(Hb: Haemoglobin; TEC: Total erythrocyte count; TLC: Total leucocyte count; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; BUN: Blood urea nitrogen; SE: Standard error; NS: Non-significant; N*: Data pertaining to some of the parameters not available; CME: Canine monocytic ehrlichiosis).

total protein and albumin were significantly decreased in dogs with CME when compared with healthy dogs. Hypo-proteinaemia (<5 g/dl) and hypo-albuminaemia (<2 g/dl) were observed in 36.6% and 31.7% of the diseased dogs, respectively. The probable cause for these abnormalities could be due to the loss of plasma proteins from the vascular endothelial pores due to vasculitis caused by *E. canis* (Woody and Hoskins 1991) or due to decreased protein production as a result of concomitant liver disease (Reardon and Pierce 1981) or glomerulonephritis (Codner *et al*. 1992).

As compared with the healthy dogs, there was a significant increase in the levels of BUN in dogs with ehrlichiosis. Although the levels of creatinine were also higher in diseased dogs, but the results were non-significant. Previous studies also reported renal damage in dogs with ehrlichiosis which might be due to concurrent immune mediated glomerulonephritis caused by *E. canis* antigen against the basement membrane of glomerulus (Pinho *et al*. 2016).

CONCLUSION

The present study highlighted important clinico-pathologic alterations alongwith tick infestation, melena, lymphadenopathy, epistaxis, anaemia, thrombocytopenia hypo-proteinaemia and hypoalbuminaemia in dogs with CME. These findings prompt the clinician to consider canine ehrlichiosis as one of the differentials for dogs presented with above-mentioned clinical and haemato-biochemical abnormalities. Further, these alterations should be kept in mind while undertaking therapeutic interventions and to avoid severe clinical and fatal outcomes.

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