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ORIGINAL ARTICLE

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Serodetection of *Ehrlichia canis* infection in dogs from Ludhiana district of Punjab, India

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Abstract Dot-ELISA (enzyme-linked immunosorbent assay) Immunocomb® assay was conducted to detect the presence of antibodies against *Ehrlichia canis* in blood samples of 60 privately owned dogs suspected to be infected with *E. canis* from the Small Animal Clinics, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab (India). Antibodies reactive to *E. canis* were detected in plasma in 48 samples out of 60 samples by Immunocomb® Dot-ELISA. Out of these 39.58% samples were low positive (Titre 1:20–1:40), 31.25% were medium positive (Titre 1:80–1:640) and 29.16% were high positive (Titre >1,280), for the infection. When examined by microscopy, only two samples revealed typical *E. canis* morulae. Haemato-cellular examination revealed thrombocytopenia along with anaemia and leucopenia. Results suggest that *E. canis* infection circulates in dogs in India in low non-detectable numbers by microscopy and is transmitted by the brown dog tick *Rhipicephalus sanguineus*.

Keywords Dogs · Dot-ELISA · *Ehrlichia canis* · Immunocomb · Pancytopenia

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Introduction

Canine ehrlichiosis, caused by a number of small obligatory intracellular pleomorphic rickettsiae of *Ehrlichia* species, is prevalent in dogs around the globe, often in tropical and subtropical areas (Keefe et al. 1982; Matthewman et al. 1993; Baneth et al. 1996; Waner et al. 1996; Harrus et al. 1998; Pretorius and Kelly 1998; Batmaz et al. 2001; Suto et al. 2001). The distribution of disease is related to the distribution of the vector, the brown dog-tick *Rhipicephalus sanguineus*. Among the various *Ehrlichia* species, *E. canis*, parasitizing the circulating monocytes intracytoplasmically in form of clusters called morulae, is most important species of *Ehrlichia* in dogs causing serious and potentially fatal disease called as canine monocytic ehrlichiosis (CME) that requires rapid and accurate diagnosis in order to initiate appropriate therapy leading to a favourable prognosis (McBride et al. 2001). This disease is also invariably referred as tropical canine pancytopenia due to affinity of the parasite to haematopoietic cells of the body that results in leucopenia and thrombocytopenia. The disease was firstly described in Algeria in 1935 (Donatien and Lestoquard 1935). Dogs infected with *E. canis* remained infected for their entire lives, even if they received antibiotic treatment with doxycycline (Wen et al. 1997).

Early diagnosis of *E. canis* infection during the acute phase ensures the best prognosis and usually leads to complete recovery (Troy and Forrester 1990). Currently, the indirect fluorescent antibody test (IFAT) is considered the "gold standard" and is the most widely used method for diagnosis of CME (Waner et al. 2001). However, due to cross-reactive antibodies generated among related organisms which can perplex test results, high level of expertise in distinguishing the etiologic agent, low sample output and lack of standardization of test, expensive microscopy

equipment are some of the considerations that limit the reliability and applicability of IFAT. So keeping these considerations in view, the study is focused towards sensitive, specific and reliable diagnosis of the disease utilizing either recombinant or parasite specific antigen markers up to generic level in which various enzyme-linked immunosorbent assays (ELISAs) have been developed based on the above said approaches. Although the first case of CME was diagnosed in 1992 (Juyal et al. 1992) from Punjab, the epidemiological seroprevalence for *E. canis* specific antibodies in Punjab (India) has not been yet conducted. So the present study was undertaken with the aim to utilize Dot-ELISA using Immunocomb® (Biogal Galed Laboratories) in detecting anti-*E. canis* specific immunoglobulin-G (IgG) antibodies in plasma samples from suspected dogs.

Materials and methods

Animals

Dot-ELISA based Immunocomb® assay was used in the study to survey the presence of antibodies against *E. canis* in blood samples of 60 pet dogs harboring ticks and suspected to be naturally infected with *E. canis* based on the clinical history and symptoms from the Small Animal Clinics, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Punjab (India). The plasma samples were collected from privately owned pet dogs presented to clinics for examination and were frozen at −20°C until assayed. The dogs were examined physically and peripheral blood smears were drawn and stained with Giemsa and examined microscopically. Ticks were manually removed, placed into labeled vials containing 70% ethanol and then identified according to taxonomic keys.

Test performed

The Immunocomb® Dot-ELISA test was performed as per manufacturer's recommendations and *E. canis* IgG antibody titers of plasma samples were determined as described previously (Waner et al. 2000). Briefly, a semi-quantitative Dot-ELISA based Immunocomb® kit (Biogal Galed Laboratories, Israel) containing two main components: a comb shaped plastic card containing 12 teeth and a multi-compartment developing plate was used. In the comb, a test spot containing *E. canis* antigen is attached to the lowest margin of each tooth of the comb. The uppermost spot is positive reference and middle spot is negative reference spot, respectively. Sera were diluted in the supplied buffer in multi-compartment developing plate and incubated with antigen spotted comb for 5 min. Thereafter proper washing was done for 2 min to displace unbound antibodies and the comb was allowed to react for 5 min with an enzyme labeled anti-dog IgG antibody that binds to the antigen–antibody complexes formed at test spots. After two successive washing steps for 2 min, bound antibodies were detected with a precipitating chromogen via an enzymatic reaction. Sera from an *E. canis* infected dog (through microscopic examination revealing morulae) served as positive control whereas sera from normal, healthy dog free from any haemoprotozoan infection constituted negative control in the test. The concentration of *E. canis* antibodies for each sample were detected via a supplied scanning device designed for automatic reading of the color intensity of the reaction spots on the comb and results were recorded as optical density (OD) units. The results were expressed in "S" units on a scale of 0–6 on a color-coded scale provided in the Immunocomb® kit. Three "S" units were calibrated by the manufacturer to a titer of 1:80 according to the IFAT (Waner et al. 2000). A result of greater than or equal to three "S" units in the Immunocomb® test was considered positive.

Haematological findings

The whole blood, immediately after collection was used for determination of haematological parameters as per the description by Jain (1986). Estimation regarding the adequate, inadequate and high number etc. of platelets was done in stained smears.

Results and discussion

Antibodies reactive with *E. canis* were detected in plasma in 48 (80%) of 60 samples by Immunocomb® Dot-ELISA and titre equivalent to three "S" units i.e. 1:80 or more was considered as positive as per Waner et al. (2000). Most of the dogs were showing the clinical signs of *E. canis* infection and were also having tick infestation (*R. sanguineus*). Out of the 48 positive samples, 19 (39.58%) were low positive (Titre 1:20–1:40), whereas 15 (31.25%) were medium positive (Titre 1:80–1:640) and 14 (29.16%) were high positive (Titre >1280), respectively. Considering the cut-off titres to 1:80, the seroprevalence came out to be 48.33%. Twenty-five percent (15/60) samples had medium titres and 23.33% samples (14/60) had high titres. The seroprevalence of CME in the present study is higher than the sero survey conducted in Egypt, Israel, Germany, Turkey and France with 33, 30, 25.1, 20.8 and 9–12% seroprevalence, respectively (Davoust 1994; Botros et al. 1995; Baneth et al. 1996; Gothe 1998; Batmaz et al. 2001).

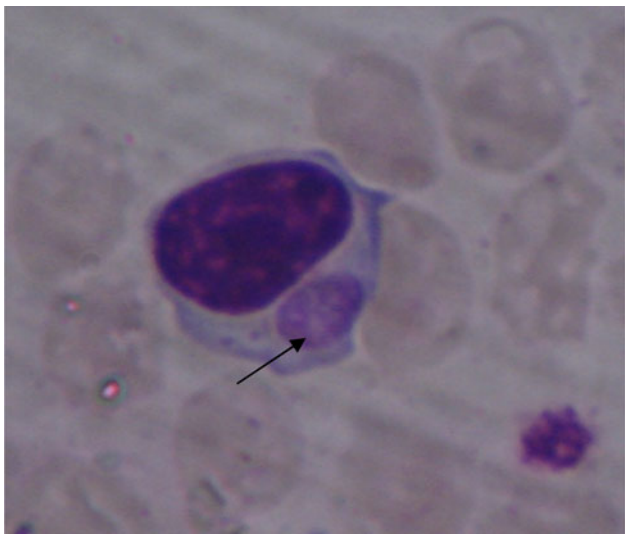

Fig. 1 Inclusion with the monocyte in the peripheral blood of dog has an internal structure consistent with an *E. canis* morula (*arrow*). Wright's–Giemsa stain

Among the 60 samples employed in the test, only two dogs with serological evidence of ehrlichiosis revealed typical *E. canis* morula (arrow) when detected by microscopy (Fig. 1). In the previous studies in India using conventional examination of stained blood smears the prevalence of canine ehrlichiosis have reported between 0.35% in Punjab (Juyal et al. 1994) and 18.9% in Nagpur (Samaradni et al. 2003). Species-specific nested PCR it was found to be more sensitive (50%) for detection of *E. canis* in privately owned dogs in Chennai as compared to microscopy (19%) (Lakshmanan et al. 2007). Microscopic evaluation of stained blood smears is not sensitive as the organisms are not readily demonstrable in smears (Juyal et al. 1994; Lakshmanan et al. 2006, 2007). Limitation of low diagnostic sensitivity in detection of CME has been also also been described by Mylonakis et al. (2003). Neither sex nor age factor was found to be associated with the sero-positivity of *E. canis*.

Inadequate number of platelets, anaemia and leucopenia were observed in seropositive dogs. Haemoglobin of seropositive dogs ranged between 5 and 16 g/dl (reference values 12–18 g/dl). Eighteen seropositive dogs with cut-off titres 1:80 had anaemia (62.07%). Non-regenerative, normochromic normocytic anaemia was observed previously in *E. canis* seropositive dogs (Batmaz et al. 2001). The total leucocyte count ranged between 0.25 and 52 $\times$ 10^3/µl (reference range 6–17 $\times$ 10^3/µl). Ten dogs had severe leucopenia, seven had a leucocytosis and 18 had lymphopenia. On the basis of examination of stained blood smears thrombocytopenia was observed in 19 cases. Mild to severe thrombocytopenia in *Ehrlichia* infected dogs has been reported in literature (Codner and Farris-Smith 1886; Niwetpathomwat et al. 2006). As such 8 cases were leucopenic, anaemic and thrombocytopenic (Pancytopenia) at the same time. Variable leucopenia, anaemia, thrombocytopenia, and hypergamma-globulinaemia has already been reported in subacute phase of ehrlichiosis (Lakkawar et al. 2003).

Results suggest that *E. canis* circulates in dogs in India and it is transmitted by the brown dog tick, *R. sanguineus* (Fig. 2). The high prevalence of *E. canis* indicates that a sizeable portion of canines had been exposed to erhlichiosis, which may have been in one of the stages of disease. Persistent infection allows the canine hosts to serve as reservoir for infection when ticks first feed on infected, then on uninfected host. Thus further studies are needed to know whether serological positive animals should be treated or not regardless of presence or absence of morulae in monocytes when blood is examined microscopically. *E. canis* infects dogs without age or sex predilection and infected carrier dogs may also play an important role acting as reservoirs. The Dot-ELISA based test used in the present study to detect anti-*E. canis* antibodies was found to be highly specific and sensitive when compared to the routinely used microscopic examination in most parts of the country. Though IFAT has been used for diagnosis of CME, there is always a chance of giving false positive interpretation due to cross-reactive antibodies production in animals. Also a high level of expertise, low sample output, lack of standardization, expensive microscopy

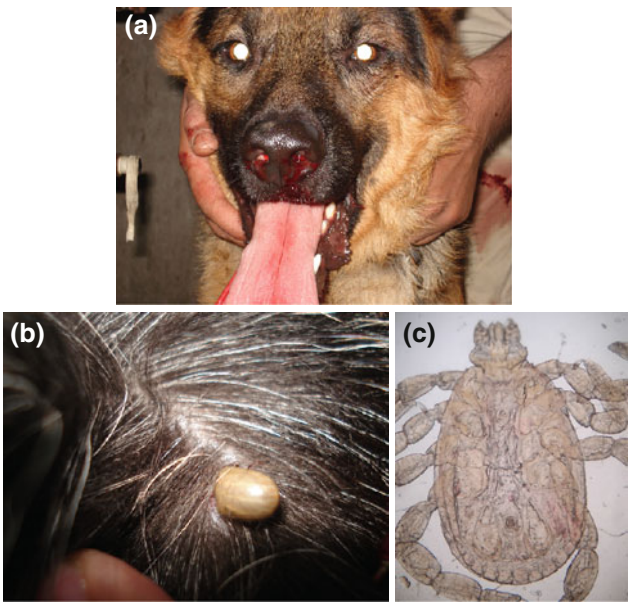

Fig. 2 The dog showing typical clinical signs of *E. canis*, with presence of *R. sanguineus*. **a** Epistaxis in a German Shepherd dog affected with tropical canine pancytopenia. **b** A female of *R. sanguineus* firmly attached to and feeding on the belly of a dog. **c** A microphotograph of *R. sanguineus* male isolated from *E. canis* infected dog

equipment too limit its reliability and applicability. The Dot-ELISA based technique used in the present study requires a minimum of equipment, is easy and quick to perform, involves a single-step dilution and is highly specific and sensitive thus helping in unequivocal diagnosis of CME. As CME may be manifested by a wide variety of clinical signs, so when dogs are admitted with non-specific signs of illness, clinicians should always take into consideration the possibility of *E. canis* infection.

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