Original Article

Seroprevalence and Risk Factors of *Ehrlichia canis* Infection among Companion Dogs of Mashhad, North East of Iran, 2009–2010

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Abstract

Background: The aims of this study were to determine the seroprevalence of canine ehrlichiosis and risk factors of this disease in companion dogs' population of Mashhad, North East of Iran. Canine Monocytic Ehrlichiosis (CME) is a zoonotic disease transmitted by ticks, *Rhipicephalus sanguineus*, and caused by an obligate intracellular bacterium, *Ehrlichia canis*.

Methods: During September 2009 until November 2010, 250 companion dogs from Mashhad, North-East of Iran, were examined for serum antibody detection against *E. canis* by means of immunofluorescence assay test (IFAT) and factors associated with a positive antibody response.

Results: There was a very low prevalence of anti-*E. canis* antibodies (0.8%, 2/250) among studied dogs. The antibody titers for two seropositive dogs were 1:80 and 1:160, respectively. One (0.4%) of seropositive dogs was infested with, *R. sanguineus.* In blood smears from one of infested dogs (0.4%), typical morulae of *E. canis* was observed in lymphocytes. The results confirm that the lowest occurrence of reactive dogs indoors probably related to low tick infestion.

Conclusion: This is the first report that describes serological evidences of canine monocytic ehrlichiosis in North-East of Iran. Results suggested that *E. canis* infection in owned pet dogs from North of Khorasan was not endemic from 2009 to 2010. Additional molecular studies are necessary to confirm E. *canis* infection and to identify the local strains of the organism.

Keywords: *Ehrlichia canis*, Indirect Immunofluorescence Assay, Prevalence, Dog, IFA

Introduction

Canine Ehrlichiosis, a tick borne disease, was first recognized by Donatien and Lestoquar (1935) and has since been reported in dogs geographical widespread (Bretischwerdt 1995). At the end of 1960 an epidemic outbreak of the disease with high mortality has been reported in American military dogs and south Asia. This severe form was initially given the name Canine Tropical Pancytopenia (William 1981).

Ehrlichia species are bacteria of the family *Anaplasmataceae*. *Ehrlichia canis* is a gram negative highly pleomorphic, obligate intracellular bacterium which is enveloped with a rippled thin outer membrane (Marvomatis et al. 2006). It is considered to be the major causative agent of Canine Monocytic Ehrlichiosis (CME) in dogs (Huxsoll et al. 1969).

Rhipicephalus sanguineus, a brown-dog tick, kennel tick or pan-tropical dog tick belonging to Ixodidae family is a ubiquitous tick responsible for transmitting *E. canis*, (Jeremy et al. 2013). It is a one-host tick that feeds on dogs in all three stages of life cycle. Ticks acquire *E. canis* by feeding

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on infected dogs and transmit infection for at least 155 days afterward to other dogs (Groves et al. 1975, Breitschwerdt et al. 1995). They can also act as vector of important pathogens of humans such as *Coxiella burnetii*, *Rickettsia conorii*, *R. rickettsii* and *Bartonella henselae* being of zoonotic concern (Wikswo et al. 2007, Dantus et al. 2008).

This tick species is known to be a vector of *E. canis*, *Babesia canis*, *B. gipsony*, *Hepatozoon canis*, and *Anaplasma platys* in dog (Gal et al. 2007, Anonymous. 2012).

Three clinical stages have been proposed for CME, acute, subclinical and chronic. The acute phase is characterized by fever, anorexia, lymphadenomegaly, epistaxis and petechia (Neer and Harrus 2006). During the subclinical phase dogs appear healthy and have the potential to remain persistent carrier (Waner et al. 1996). In chronic cases, infected dogs fail to mount an effective immune response. Bone marrow involvement leads to pancytopenia (Moriera et al. 2005).

The disease can be diagnosed by the detection of *E. canis* morulae in monocyte in blood smears or serologically detection of specific antibodies by the use of IFA test, dot-ELISA and Western blot immunoassay or by the detection of *E. canis* in tissue and blood by means of PCR (Matthewman et al. 1993, Futch and Corstvet. 1996, Mylonakis et al. 2003). IFA is considered the "Gold standard" serological diagnostic technique for *E. canis* (Ristic et al. 1972). The objectives of this study were to determine the seroprevalance of canine ehrlichiosis and risk factors of this disease in companion dogs' population of Mashhad, North Khorasan of Iran.

Materials and Methods

The study was performed on total 250 owned pet dogs (119 females and 131 males) between September 2009 until November 2010 referred to Veterinary Teaching Hospital of Ferdowsi University of Mashhad for their annual vaccination, as well as with clinical illness.

The following details were obtained for each dog: sex, breed, age, body temperature, location of dog's home, appetite status, examination of lymph node, CRT, infestation by tick, epistaxis and reason for referred to the hospital. After physical examination blood samples were taken in EDTA and non-anticoagulant tube. Blood with EDTA were examined for hematology and complete blood count.

Sera were separated by centrifuge and stored at -20 ºC until assayed. For each case blood smear was prepared and stained with Giemsa and direct microscopic examination was performed to detect Morula on white blood cells especially on monocytes and lymphocytes. Hematocrite and white blood cell count were recorded for all dogs.

Anti- *E. can*is antibodies were detected by Flu Ehrlichia immune fluorescence kit (Flu EHRLICHIA Canis, Megacor, Austria) with following method:

Sera were added to the slides after dilution (1:40) in phosphate-Buffered Saline (PBS) PH 7.2. Positive and negative control sera were also tested. Slides were Placed in humid chamber and incubated for 30 minute at 37 ºC after that, those were washed twice in PBS. Then we added one drop anti-Dog FITC (conjugate) to each slides and those were returned to humid chamber and incubated for 30 minute at 37 ºC. Incubation was performed in the dark place to protect photosensitive conjugate. After these steps, slides were washed as described before and were air-dried then 2–3 drops of mounting fluid were added to each slides and a cover slip was placed. The slides were analyzed at ×400 magnification with IFA microscopy and were compared each wall with negative and positive control. Each serum sample at titer 1:40 or more

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was considered positive. A positive reaction appears as bright sharp regularly stained inclusion bodies in cytoplasm of infected cell. The size, appearance and density of the inclusion were compared with positive control. Sera were positive at the 1:40 were prepared serial dilution 1:80 1:160 1:320 1:640 1:1360 and tested again with IFA.

All data were collected and because of low seropositive cases for *E. canis*, statistical analysis was not performed.

Results

Complete blood count showed 67 anemic (26.8%), 40 thrombocytopenic (16%) dogs. Furthermore, 101 dogs in study population were diagnosed with abnormal leukogram findings including 20 leukopenia (8%), 24 leukocytosis (9.6%), 24 neutropenia (9.6%), 33 neutrophilia (13.2%). 7 dogs (2.8%) showed anemia and thrombocytopenia synchronously. 1 dog (0.4%) had morulae (Fig. 1).

In physical examination, 12 dogs (4.8%) were infested with tick. All ticks were *R. sanguineus*. 15 dogs (6%) had lymph node enlargement, 6 dogs (2.4%) had fever and 4 dogs (1.6%) had epistaxis (Table 1).

Two (0.8%) of the 250 dogs have been examined were found to be seropositive by the IFA. Both of them were adult (above 1 year) and the number of platelets, leukocytes and neutrophils were normal. Morula was found in lymphocytes of one the seropositive dog. This dog showed inappetance and depression, had large submandibular lymph node and infested with *R. sanguineus* on physical examination.

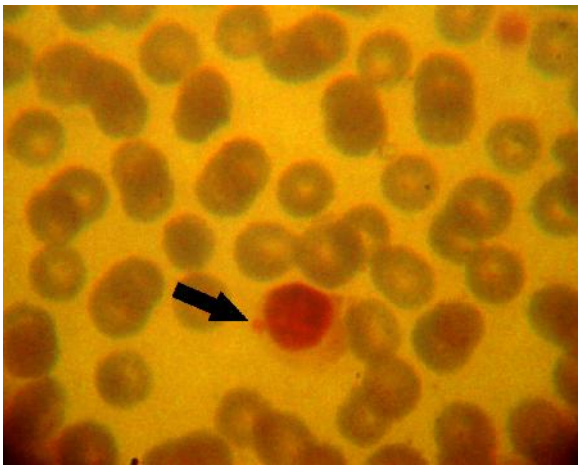

Fig. 1. A morulae of *Ehrlichia canis* (arrowed) in a blood smear from one of seropositive dogs (Morulae in cytoplasm of lymphocyte)

Table 1. Signalment and antibody titer in seropositive dogs

Variable	Dog number 171	Dog number 235	Reference Ranges
Age	13 years old	9 years old	-
Sex	Female	Male	-
Breed	German shepherd	Mixed Terrier	-
Hematocrit	37	42	37-55 %
Thrombocyte	2.5	3.34	1.6-4.3×10^6/µl
Total WBC count	8000	7300	6000-17000/µl
Total Neutrophil count	6500	5986	3000-11500/µl
Morulae	-	In lymphocyte	-
Body Temperature	38.5	39	-
Appetite status	Normal	Inappetite	-
Lymph nodes	Normal	Submandibular L.n enlarged	-
Epistaxis	-	-	-
IFA titer	1:80	1:160	-

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Discussion

This study is the first investigation of the seroprevalence of *E. canis* antibodies among dogs in North Khorasan of Iran. The results revealed low prevalence of *E. canis* (2 dogs, 0.8%).

Prevalence of ehrlichiosis was also reported from other regions of Iran: Jafari et al. (1997) in Shiraz (South west of Iran) have examined 180 dogs. Seventeen dogs (9.44%) were found positive for the presence of *E. canis* in their white blood cells.

Akhtardanesh et al. (2009) used IFA and ICA to detect antibodies against *E. canis* in 123 apparently healthy dogs. The overall seroprevalence was 14.63 %. Seventeen (13.8%) dogs in IFA test and 13 dogs (10.6%) in ICA were seropositive for CME. In blood smears from three infected dogs (16.6%) morulae were observed in monocytes.

Avize et al. (2010) have reported seroprevalence of CME in 198 companion dogs of different ages by means of IFA and ICA 9.6 % in Ahvaz (West of Iran). Morulae of *E. canis* were observed in monocyte of four infected dogs (2.02%).

Blood samples from 980 dogs (510 domestic dogs and 475 wild dogs) in West Azerbaijan and 820 dogs (520 domestic dogs and 300 wild dogs) in East Azerbaijan of Iran were obtained by Asri and others in 2001 and tested by IFA for diagnosis of Canine Ehrlichiosis. Sixty seven percent of wild dogs and 38 % of domestic dogs in West Azerbaijan and for East Azerbaijan 58 % and 39 % were serologically positive for *Ehrlichia*. The main variants have been diagnosed were *E. canis* (75%), *E. platys* (20%) and *E. equi* (5%).

In our study because of low seroprevalence of *E. canis*, we could not reach any correlation between age and CME but in many investigations the prevalence has been significantly differed among age groups.

In Shiraz (Jafari et al. 1997) the animals of all ages seemed equally susceptible to disease. In Ahvaz (Avize et al. 2007) prevalence rate have been significantly higher in adult dogs than juniors. The prevalence rate was 16.8 % in above 3 years old and 11.86 % in 1–3 years old compared with dogs less than 1 year old (1.41%). In Kerman (Akhtardanesh et al. 2009) high association was observed between age and seropositive dogs. Possible explanations for more infection in older group include the immunologic status of the host or more exposure to the vector ticks (Rodriguez-Vivas RI et al. 2005).

German shepherd dog has been reported to be more susceptible to CME (Nyindo et al. 1980, Harrus et al. 1997). In Shiraz (Jafari et al. 1997) 21.1 % of infected dogs were German shepherd. Some research showed higher prevalence in male dogs (Batmaz et al. 2001, Costa et al. 2007). In some studies no significant difference was proved between sex and various breeds with presence of *E. canis* antibodies (Waner et al. 2000a, Rodriguez-Vivas et al. 2005, Hernandez et al. 2005, Solano-Gallego et al. 2006, Akhtardanesh et al. 2009, Avize et al. 2009, Roqueplo C et al. 2009).

The clinical signs of CME may vary among and within geographic locations (Harrus et al. 1997a,b). The probable reasons include *E. canis* strain pathogenicity, dose of infectious organism, breed of dog, immuno status of the host and co-infection with other tick-borne parasites (Rodriguez-Vivas et al. 2005, Neer and Harrus 2006).

Thrombocytopenia is the most common hematological finding in patients with acute CME. This change is found in all stage of disease but is more severe in chronic phase as a result of bone marrow hypoplasia (Troy et al. 1980). Death may occur as a

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consequence of hemorrhages and secondary infections (Hendricks and Bob 2004). In our study because of low prevalence of *E. canis* we could not show any relationship between seropositive dogs and hemathologic changes but platelet and leukocyte count in both seropositive dogs were normal. In Ahvaz (Avize et al. 2009) the prevalence of ehrlichiosis was higher in dogs with thrombocytopenic although the difference was not significant and correlation was not observed between seronegative and seropositive dogs for hemathologic changes. In Kerman (Akhtardanesh et al. 2009) thrombocytopenia, leukopenia and anemia were just observed in dogs with high IFA titer (>1:320).

Rodriguez-Vivas RI et al. (2005) have found that the presence of thrombocytopenia, platelet-related bleeding and a seropositive response to *E. canis* in a patient increase the index of possibility for infection. The only known vectors of *Ehrlichiae* are *ixodid* ticks (Rikishia 1991).

Rhipicephalus sanguineus and possibly the American dog tick, *Dermacentor variabilis* are the vector for E. *canis* (Groves et al. 1975, Johnson et al. 1998). *Rhipicephalus sanguineus* is widely distributed in the world but it is mainly in tropical and subtropical regions and also well adapted to the indoor environment where owned dogs are kept (Uspensku and Ioffe-Uspensky 2002, Dantas-Torres 2010). Dogs may acquire ticks in the city areas in parks and housing estates (Siuda 1993). Infestation by *R. sanguineus* has significant risk factor for *E. canis* seropositivity in Brazil (Trapp et al. 2002). In this study, 4.8 % (12 dogs) were infested by *R. sanguineus*. One of the seropositive dogs also had this tick on his trunk. *R. sanguineus* was also the most common species in North-East of Iran (Razmi et al. 2003).

Diagnostic method can affect on prevalence results of *E. canis*. As said above the indirect immunofluorescence antibody (IFA) test is considered the serological "gold standard" for diagnosis of CME (Ristic et al. 1972). Serological cross-reactivity occurs with other members of *Ehrlichiae* like *E. equi* (Baneth G et al. 1996), *E. ristici* (Ristici et al. 1999), *E. ewingii* (Anderson et al. 1992), *E. chaffeensis*, *Neorickettsia helminthoeca* (Rikisha 1991).

In this study, IFA test was used and seropositive titers were 1:80 and 1:160. IFA test is more susceptible than other test but supplementary test such as PCR and western immunoblotting is needed for detection of active infection and distinguished between infections with different type of species.

Possible explanations for low seroprevalence of *E. canis* in this study are:

1. Selected population: exposures to tick in domestic dogs are lower because of location and observance of health condition by owners. The life conditions of dogs affected the seroprevalence of *E. canis* (Roqueplo et al. 2009). Lim et al. (2010) indicate that risk of exposure to vector-borne disease in rural hunting dogs can be quite high in Korea. Ploneczka et al. (2003) showed that dogs in non-urban areas (9.9%) or they have living in outdoor (12.7%) had a higher prevalence of *E. canis*. Rural dogs had more parasite infestation than urban dogs (Dagnone et al. 2002, Carvalho et al. 2008).

2. Weather conditions: The prevalence of *E. canis* is largely dependent on the distribution of the vector, *R. sanguineus*, which occurs mainly in tropical and subtropical regions but it has worldwide distribution (Jeremy et al. 2013).

Jafari et al. (2008) have determined the prevalence of canine ectoparasite infestation in pet dogs from the Shiraz area of southern Iran. Overall, 160 dogs were examined for ectoparasites, and 142 *R. sanguineus* ticks were found on 13 dogs. A

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significant correlation was observed between increases in temperature and decreases in humidity and increased ectoparasite infestation. The number of dogs infested with ectoparasites in summer and spring was significantly higher than in winter (P= 0.007). Morales-Soto and Cruz-Vazquez (1998) found *R. sanguineus* along the year in Cuernavaca, Mexico but the peaks of tick were found in April, July and November and the lower prevalence were in January. So season of sampling can affect seroprevalance of *E. canis*.

3. Type of serological test: IFA detects antibodies as early as 7 days after initial infection but some dogs may be negative until 28 days after infection or in acute phase of disease and also in chronic phase because of injury to immune system (Groves et al. 1975) when *E. canis* antibody titers results are negative, a follow up examination in 2 to 3 weeks or serotesting for other agents is recommended (Neer and Harrus 2006).

The CME has a worldwide distribution and a significant seroprevalence in dogs from southeast Asia, Africa, Europe, Central and South America was reported (Cardenas et al. 2007). Antibodies against *E. canis* were detected in neighbors and close countries to Iran as 44.4 % in Saudi Arabia (Sacchini et al. 2007), 21 % in Turkey (Batmaz et al. 2001), and 33 % in Egypt (Botros et al. 1995).

In our study, seroprevalence of *E. canis* was estimated less than 1 %. So CME is not endemic in Mashhad, but in Kerman, Ahvaz and Azerbaijan is considered endemic (Asri et al. 2001, Akhtardanesh et al. 2009, Avizeh et al. 2010).

Besides, *E. canis* is a human health hazard and causes clinical signs of disease (Perez et al. 2006). Human Ehrlichiosis is caused by *E. chaffeensis*, *A. phagocytophilum* and *E. ewingii* (Dumler et al. 2007). Co-infection of *E. canis* and *A. phagocytophilum* is possible (Amusategui et al. 2007). *A. phagocytophilum* was reported in *Ixodes ricinus* in North of Iran (Bashirbod et al. 2004).

It is possible that more tick-transmitted pathogens can infect dogs, including *E. canis*, *A. phagocytophilum*, *B. canis*, *Hepatozoon canis*, *Bartonella* spp. (Baneth et al. 1998, Breitschwerdt et al. 1998, Yabsley et al. 2008). So in dogs with clinical signs of thrombocytopenia, leukopenia, fever and epistaxis if they have negative result for *E. canis*, consider the possibility of infectious with other organisms.

There is significant correlation between ehrlichiosis with leptospirosis, leishmaniasis and babesiosis. So in dogs that have one of these diseases, *E. canis* infectious should be considered (Matthewman et al. 1993, Suksawat et al. 2001, Hernandez et al. 2005, Roura X et al. 2005, Tabar et al. 2009).

Conclusion

Ehrlichia canis infection in owned pet dogs from North of Khorasan was not endemic from 2009 to 2010.

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