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Response to Reviewers: Dear Editor and Reviewers, we have corrected the manuscript according to the suggestions made. The insertions have been made as requested by reviewer 2 on lines 59 & 72. The manuscript has also been checked for spacing errors and these have been corrected on lines 18; 34; 78; 82; 83; 84 and 88 accordingly.

We thank you for helping to improve the quality of this manuscript.

A Case of Canine Borreliosis in Iran Caused by *Borrelia persica*

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Abstract:

Tick-borne relapsing fever is an endemic disease in Iran, with most cases attributed to infection by *B. persica*, which is transmitted by *Ornithodoros tholozani* soft ticks. Here, we report spirochetemia in blood of a puppy residing in Tehran, Iran. The causative species was identified by use of highly discriminative IGS sequencing; the 489 bp IGS sequence obtained in our study showed 99% identity (100% coverage) when compared with *Borrelia persica* sequences derived from clinical cases or from *Ornithodoros tholozani* ticks. Our IGS sequence also showed 99% similarity over 414 bp (85% coverage) with a strain from a domestic dog, and 96% over 328 bp (69% coverage) with a strain from a domestic cat. Pet-keeping in cosmopolitan cities like Tehran has become increasingly popular in recent years. Animals are often transported into the city in cages or cardboard boxes that might also harbour minute tick larvae and/or early stages of the nymphs bringing them into the urban environment. This may pose a threat to household members who buy and keep these puppies and as a result may come into close contact with infected ticks.

Keywords:

Canine borreliosis; *Borrelia persica*; tick-borne relapsing fever; companion animals; vector-borne infection.

Introduction:

Tick-borne relapsing fever is an established endemic disease in Iran (Karimi, 1981; Masoumi Asl et al., 2009). The causative agents are spirochetes belonging to the genus *Borrelia*, transmitted via Argasid soft ticks belonging to the genus *Ornithodoros* (Goubau, 1984). In Iran, the main cause of tick-borne relapsing fever is *B. persica*, transmitted by *Ornithodoros tholozani* ticks (Karimi, 1981; Karimi et al., 1979). Other *Borrelia* species including *B. microti*, *B. latyschewii*, *B. balthazardi*, and a species with great similarity with African borreliae have also been reported from Iran (Karimi, 1981; Karimi et al., 1979; Naddaf et al., 2012; Naddaf et al., 2015; Piazak et al., 2000). Identification has historically been based upon knowledge of *Borrelia* species in a specific soft tick within a geographical setting, and supported by *in vivo* pathogenicity tests (Assous and Wilamowski, 2009; Karimi, 1981). *Borrelia microti* spirochetes were demonstrated in blood of *Meriones* sp. rodents from South Iran (Rafyi, 1947), but a vertebrate reservoir for *B. persica* remains to be elucidated.

We report spirochetemia in blood of a puppy residing in Tehran and identify the causative species by the highly discriminative intragenic spacer (IGS) sequencing.

Materials and Methods:

A two week-old female mixed breed puppy with sign of anorexia, diarrhea, and vomiting was referred to the Small Animal Teaching Hospital, Faculty of Veterinary Medicine, University of Tehran. The puppy was in poor health and upon physical examination was feverish, dehydrated, with inflamed submandibular lymph nodes and pale oral, nasal, and eye mucosa. On examination of the animal's body the owner found an unattached tick. About 3 ml of blood was obtained from the puppy. Complete blood tests were performed and a Giemsa-stained thin blood smear prepared. For molecular identification of *Borrelia* species, about 1 ml of blood sample was sent to Department of Parasitology, Pasteur institute of Iran. DNA extraction was performed from 200µl serum sample using a Miniprep DNA extraction kit (Qiagen, Germany) according to the manufacturer's instructions, and partial sequence of IGS was targeted using the primers and conditions detailed by others (Cutler et al., 2010; Picken, 1992). Final reaction volumes of 25 µl contained 10 pmol of each primer, 2.5 mM $MgCl_2$, 10 mM Tris-HCl, 50 mM KCl, and 200 mM deoxynucleotides. Negative controls containing all reagents except DNA were included in all amplifications. PCR products were resolved on 1.5% agarose gels in TAE (Tris-acetate-EDTA) buffer, and amplicons of the expected size were purified using a gel purification kit (Bio Basic, Ontario, Canada) according to the manufacturer's instructions and sequenced in both directions using the same primers at concentrations of 10 pmol at the Pasteur Institute of Iran. The sequences were checked manually by Chromas software, version 2.4 (http://www.technelysium.com.au) and compared with similar sequences deposited in GenBank database using BLAST analysis. The data for IGS sequence was submitted to GenBank database with accession No KR816159.

Results:

Microscopic examination of a Giemsa-stained slide revealed spirochetes in the thin smear with mean concentration of 7 spirochetes in 10 microscopic fields of view at magnification of 1000X (Fig 1). The results of hematology tests are given in Table 1. The 489 bp IGS sequence revealed 99% sequence identity (100% coverage) with those of 11 other deposited sequences of *B. persica* (Acc. Nos. HM13126, HM194474, HM194750- HM194752, and HM194754- HM194755) originating from either human blood samples or *O. tholozani* ticks from Israel and the Palestinian Authority (Safdie et al., 2010). Our IGS sequence also showed 99% similarity over 414 bp (85% coverage) with a strain from a domestic dog, and 96% over 328 bp (69% coverage) with a strain from a domestic cat (unpublished data, Acc. Nos DQ768103 and DQ768102). The puppy received intravenous dextrose (3.33%) and sodium chloride (0.9%) solution (20 ml/ every 12 hours), Metoclopramide (2 mg/kg/day) by continuous IV infusion, and Vitamin B complex (1 ml/day) for three days. The animal also received Vitamin B12 (0.5 mg/dog, IM every week) for two weeks. The *Borrelia* infection was treated with ampicillin 20 mg/kg, 3 times a day for 10 days. The dog recovered after treatment and was found healthy in a two-month follow-up.

Discussion:

Demonstration of a high spirochetemia in a puppy suggests that young domestic animals may serve as transient hosts for *B. persica* in Iran. *Borrelia persica* is the main cause of tick-borne relapsing fever in west, northwest and foothills of Alborz Mountains. In these regions, the *O. tholozani* tick vector for *B. persica* is commonplace in animal shelters and adjacent human dwellings. As *B. persica* undergoes transovarial transmission within *O. tholozani* ticks (Barbour, 2004; Karimi, 1981), this tick serves as the primary reservoir for this spirochete. However, detection of borrelial DNA in blood of animals such as dogs, sheep, and goats, and within

94 engorged *O. tholozani* ticks, suggests vertebrates serve as additional potential reservoirs of *B.*
95 *persica*. Our results identified *B. persica* as the causative agent of borreliosis in this puppy with
96 IGS sequence demonstrating 99% sequence identity with those of other *B. persica* deposited in
97 Genbank database. An earlier case report of borreliosis was reported in a puppy from Tehran, but
98 the causative species remained unidentified (Rostami et al., 2011). Two further *B. persica* IGS
99 sequences derived from a domestic cat and a domestic dog from Israel, where *B. persica* is also
100 endemic, were found in GenBank (unpublished data, Acc. Nos DQ768102 and DQ768103).
101 Natural infections of dogs with borrelial species have been reported from United States with *B.*
102 *turicatae* in Florida and Texas (Breitschwerdt et al., 1994; Schwan et al., 2005; Whitney et al.,
103 2007) and *B. hermsii* in Washington (Kelly et al., 2014) detected from domestic Canids.
104 Moreover, Lyme Borreliosis, which is the most frequent vector-borne disease in humans in the
105 Northern Hemisphere, is a clinically apparent disease in dogs (Krupka and Straubinger, 2010).
106
107 Keeping pet animals in big cities like Tehran has become increasingly popular and animal traders
108 offering puppies or kittens are frequently seen alongside highways. These animals are often born
109 in suburbs of the city or nearby rural areas in southern foothills of Alborz Mountains, where *O.*
110 *tholozani* ticks are frequently encountered. In such a situation, transmission of the pathogen to
111 animals is very likely and as with the case described herein, can cause high spirochetemia in
112 young animals like puppies, providing a potential source of infection for uninfected ticks.
113 Similarly, animal cages or cardboard boxes used to transport animals to cities for sale may
114 harbour minute larval ticks and/or early stage nymphs that might be transferred with them. This
115 may pose a threat to members of a household, particularly apartments, who buy these puppies
116 and as a result may unexpectedly come into close contact with infected ticks.

Acknowledgement:

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121 Table 1. Results of hematology test for the puppy infected with *Borrelia persica*

Test	Results	Hematology Reference intervals (dog)
Hematocrit (Hct)	21.6%	35–57%
Hemoglobin (Hb)	7.2 g/dL	11.9–18.9 g/dL
Red blood cell count (RBC)	2.9×10^6 cell/µL	$4.95–7.87\times10^6$/µL
Mean corpuscular volume (MCV)	72.7 fL	66–77 fL
Mean Corpuscular Hemoglobin (MCH)	24.2, pg	21.0–26.2 pg
Mean corpuscular Hgb concentration (MCHC)	33.3 g/dL	32.0–36.3%(g/dL)
Platelet count	260×10^3/µL	$211–621\times10^3$/µL
Red Cell Distribution Width (RDW)	17.6%	14 – 19%
White blood cell (WBC)	31.6×10^6	$5.0–14.1\times10^3$/µL
Neutrophils	94%	60-77%
Bands	3%	0-3%
Lymphocytes	3%	12-30%

122

123

Figure 1. Spirochetes in a Giemsa-stained thin blood smear from the puppy (magnification x1000).

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Figure 1: Spirochaetes in a Giemsa-stained thin blood smear
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