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Molecular and serological detection of *Ehrlichia canis* in naturally exposed dogs in Iran: an analysis on associated risk factors

Detecção molecular e sorológica de *Ehrlichia canis* em cães naturalmente expostos no Irã: uma análise dos fatores de risco associados

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Abstract

The general aim of this study, which was conducted for the first time in Iran, was to evaluate the seroprevalence and geographical distribution of *Ehrlichia canis* in a dog population in Iran, followed by molecular confirmation using PCR and sequencing. Blood samples were collected from 240 dogs in different areas of Alborz and Tehran Provinces and initially analyzed using the immunofluorescent antibody (IFA) test to detect anti-*Ehrlichia canis* IgG antibodies. Subsequently, nested PCR was performed based on a fragment of the 16S rRNA gene of *E. canis* on serologically positive samples. The results showed that 40/240 dogs (16.6%) presented anti-*Ehrlichia canis* IgG antibodies and that nine of the blood samples from the 40 seropositive dogs (22.5%) contained *E. canis* DNA, which was confirmed by sequencing. The seroprevalence of *E. canis* tended to be higher in purebred, one to three-year-old male dogs living in the Plain zone, in rural areas; however, this difference was not statistically significant.

Keywords: Ehrlichiosis, *Ehrlichia canis*, IFA, nested PCR, dogs, Iran.

Resumo

O objetivo geral deste estudo, que foi feito pela primeira vez no Irã, foi avaliar a soroprevalência e distribuição geográfica de *Ehrlichia canis* em população de cães no Irã, seguida da confirmação molecular por meio de PCR seguida de sequenciamento. Amostras de sangue de 240 cães de diferentes áreas das Províncias de Alborz e Teerã foram coletadas e, inicialmente, analisadas pelo Reação de Imunofluorescência (IFA) para detecção de anticorpos IgG anti-*Ehrlichia canis* Subsequentemente, uma reação do tipo nested PCR baseada em um fragmento do gene 16S rRNA de *E. canis* foi realizada nas amostras sorologicamente positivas. Os resultados mostraram que 40/240 cães (16,6%) apresentaram anticorpos IgG anti- *Ehrlichia canis* e nove (22,5%) das amostras de sangue dos 40 cães soropositivos continham DNA de *E. canis*, confirmado por sequenciamento. A soroprevalência de *E. canis*, embora não estatisticamente significativa, mostrou uma tendência em se apresentar maior em cães machos com 1-3 anos, de raça pura, que vivem em zonas planas e áreas rurais.

Palavras-chave: Erliquiose, *Ehrlichia canis*, IFA, nested PCR, cães, Irã.

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Introduction

Ehrlichia canis, a pleomorphic Gram-negative obligate intracellular bacterium, is classified in the family Anaplasmataceae (HUXSOLL et al., 1970) and is primarily transmitted by the brown dog tick *Rhipicephalus sanguineus*, with worldwide distribution (GROVES et al., 1975).

This organism presents tropism for mononuclear leukocytes and causes canine monocytic ehrlichiosis (CME), which is an important emerging canine disease. Although CME is a cosmopolitan tick-borne infection, it has been predominantly reported in tropical and subtropical regions, where associated vector ticks have highly prevalent geographical distribution (NEER et al., 2002).

The emergence of several strains of *E. canis* has led to use of faster and more accurate diagnostic techniques such as serological tests, e.g. the immunofluorescent antibody (IFA) test, and molecular assays, e.g. the polymerase chain reaction (PCR). Consequently, several strains of *E. canis* such as the Thai, Turkish, Venezuelan and Greek strains have been detected using PCR in different geographical regions. The seroprevalence of *E. canis* among dogs in the Middle East was reported to be 21% in Turkey (BATMAZ et al., 2001) and 30% in Israel (BANETH et al., 1996). Recent surveys in the cities of Kerman and Ahvaz in Iran revealed that 14.63% and 9.6% of dogs were seropositive for *E. canis* respectively (AKHTARDANESH et al., 2010; AVIZEH et al., 2010).

The purpose of the present preliminary study, which was conducted for the first time in Iran, was to evaluate the seroprevalence and geographical distribution of *E. canis* infection and the risk factors associated with this, along with molecular detection and identification of its 16S rRNA partial sequence in dog populations in Alborz and Tehran provinces.

Materials and Methods

Study site

This study was carried out in Alborz and Tehran provinces, which are located in the north of the central plateau of Iran (Figure 1). They are adjacent and lie between longitudes 50°10' and 53°10' E and latitudes 34° 52' and 36° 21' N, at altitudes ranging from 790 to 5678 meters above sea level. The hottest months of the year are from mid-July to mid-September (30-35 °C) and the coldest months are experienced around December-January (–15 to +1 °C). The average annual rainfall is approximately 400 mm. Both of these provinces consist of two different geographical zones: Foothill and Plain. The Foothill zone consists of Shemiran, Damavand and northern parts of Karaj and Savojbolagh counties, at altitudes ranging from 1500 to 2500 meters above sea level. The climate of this zone is cold and semi-humid. The Plain zone consists of Mallard, Shahriar, Eslamshahr, Robat Karim, Rey, Varamin, Nazarabad and other parts of Karaj and Savojbolagh counties, which have altitudes ranging from 900 to 1500 meters above sea level, with a warm and dry climate.

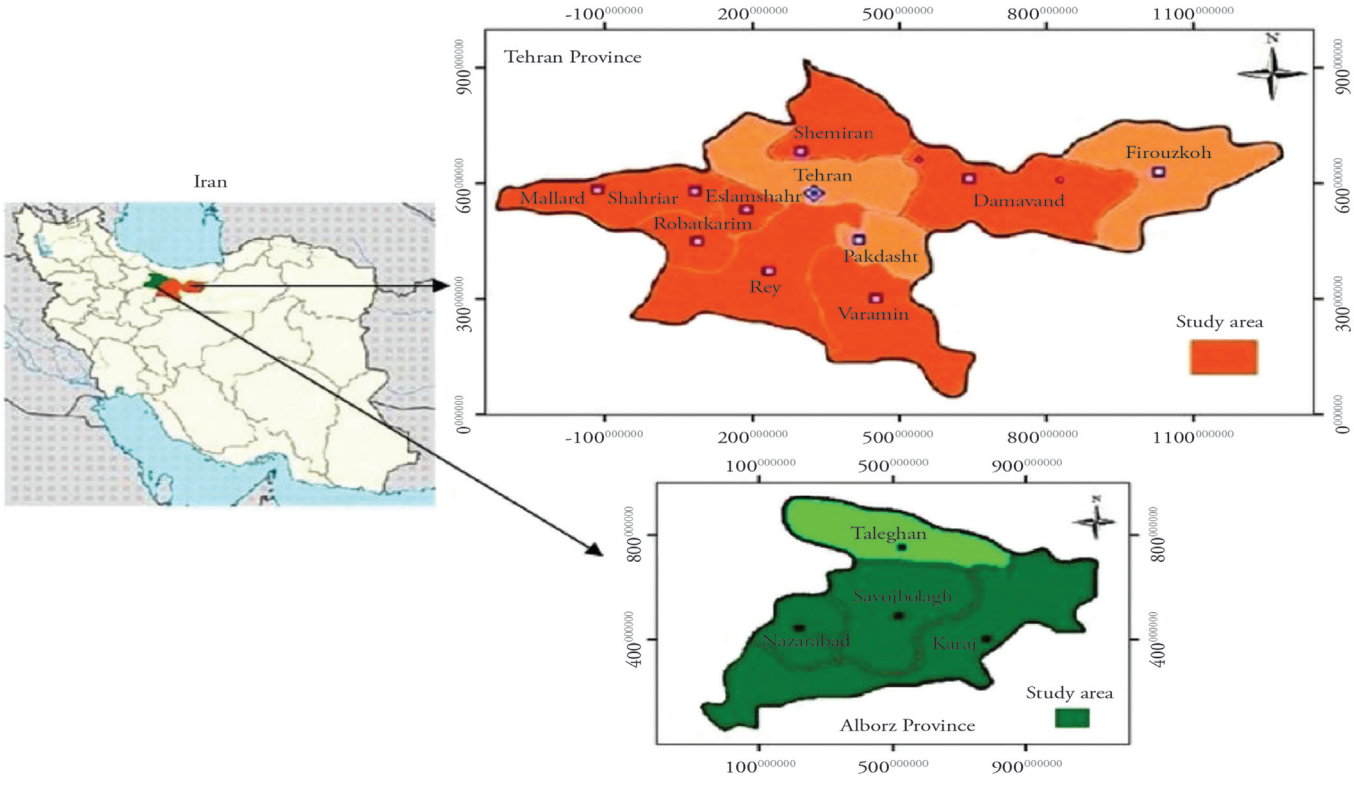

Figure 1. Map of Iran showing the two provinces and eleven localities (Damavand, Eslamshahr, Rey, Robatkarim, Shahriar, Shemiran, Mallard, Varamin, Savojbolagh, Karaj, Nazar Abad) where this survey was conducted.

Sample collection

In this study, 240 dogs living in two zones (Foothill and Plain) of Alborz and Tehran provinces were selected randomly. Information on each dog regarding age, breed, sex, geographical zone (Foothill or Plane), type of residential area (urban or rural), tick presence, history of tick infestation and anti-ectoparasite treatment was recorded through questionnaires. Ticks were recovered manually from dogs during blood collection and were stored in 70% ethanol for morphological identification. Two blood samples were taken from the cephalic or jugular vein of each dog by means of puncture. One sample was collected in an anticoagulant-free tube; this was then allowed to clot and was finally centrifuged (800 × g for 30 min) to separate the serum. Serum was stored at –20 °C until serological testing. Another sample was collected in tube containing EDTA as the anticoagulant, for hematological and cytological analysis and DNA extraction. Complete blood counts were performed using an automated hematological analyzer. Buffy coat films were then prepared, stained with Wright-Giemsa, and examined microscopically to detect *E. canis* morulae as previously described (RODRIGUEZ-VIVAS et al., 2005). The samples were kept frozen at –20 °C until DNA extraction.

Serological testing

Serum samples were screened for detection of IgG antibodies against *E. canis* using a micro-immunofluorescent assay (IgG MIF kit, Fuller Laboratories, Fullerton, CA, USA). Serum samples were diluted to 1:80 in phosphate-buffered saline (PBS) (pH: 7.2) and the procedure was performed as described by the provider. Positive and negative controls were included in the kit.

DNA extraction

DNA was extracted from 150 µL of the whole blood of serologically positive samples by using a DNA isolation kit (MBST Inc., Tehran, Iran), in accordance with the manufacturer's instructions. The extracted DNA was eluted in 100 µL of elution buffer and stored at –20 °C.

PCR amplification

Nested PCR was performed to detect *E. canis* DNA. In the outer reaction, a portion of the 16S rRNA of *Ehrlichia* species was amplified by using ECC (5-AGAACGAACGCTGGCGGCAAGC-3) and ECB (5-CGTATTACCGCGGCTGCTGGCA-3) primers (DAWSON et al., 1994). The PCR amplification was performed in a 50 µL reaction mixture containing 5 µL of extracted DNA template, 0.2 mM of dNTPs, 1.5 mM of $MgCl_2$, 0.4 µM of each primer, 1x of *Taq* buffer and 2U of *Taq* polymerase (Cinnagen, Iran) under the conditions previously described (DAWSON et al., 1994) with some modifications: initial denaturation at 94 °C for 3 min, then 38 cycles at 94 °C for 45 s, 66 °C for 45 s, 72 °C for 45 s and final extension at 72 °C for 10 min. The nested reactions were performed using 1 µL of the outside reaction as the template, with the species-specific primers ECF (5-CCGAGGGGGAAAGATTTATCGCTAT-3) and ECR (5-AAGGCCTTCTTCACTCACGCGG-3) for *E. canis*, under the same conditions as above but with 30 cycles of PCR amplification and with an annealing temperature of 60 °C for 45 s. A 224 bp sequence of the 16S rRNA gene was generated. The species-specific primers were designed from the hypervariable region of the 16S rRNA gene by means of a primer design program (National Center for Biotechnology Information). *E. canis* DNA (kindly supplied by Kawsar Tech Exploration Co., Tehran, Iran) and distilled water were included as positive and negative controls, respectively. The PCR products were analyzed using 1.5% agarose gel electrophoresis, stained with ethidium bromide and photographed. To prevent contamination, DNA extraction and PCR were performed in separate areas.

DNA sequencing and BLAST analysis

The nested PCR products were purified using the PCR purification kit (MBST Inc., Tehran, Iran) in accordance with the manufacturer's instructions and were sequenced with the same primers used in the nested PCR by means of an automated DNA sequencer (ABI 3130, Applied Biosystems). The DNA sequences obtained were aligned using the software programs BioEdit (HALL, 1999) and ClustalW (THOMPSON et al., 1994) and were blasted in GenBank (http://www.ncbi.nlm.nih.gov/BLAST).

Statistical analysis

The seroprevalence of *E. canis* with regard to risk factors such as geographical zone (Foothill or Plain), gender (female or male), age (1-3, 3-5 or more than 5 years old), breed (purebred or mongrel), tick infestation (observed or not observed) and animal residence area (urban or rural) was analyzed by means of the Phi and Cramer's V coefficients and the T-test and Pearson correlation statistical tests at the significant levels of $p < 0.05$ and $p < 0.01$.

Results

The population of this study consisted of 154 males (64.16%) and 86 females (35.84%), with ages ranging from six months to eleven years (mean 2.95 years) (Table 1). Fifty-three of these animals were purebred and 187 were mongrels (Table 1). Tick infestation was observed in 82 dogs (34.2%) at the time of sampling, although all of the dogs had histories of tick infestation and most of them (86%) had never received any previous anti-ectoparasite treatment (Table 1). The ticks recovered were identified in accordance with the keys provided by Wall and Shearer (2001) as *R. sanguineus* (91%), *R. bursa* (6%) and *R. turanicus* (3%). No mixed infestations with two or more tick species were detected.

In the serological analysis, IgG antibodies against *E. canis* were detected in 40 dogs (16.6%), of which 28 were male and 12 were female. The results from IFA and PCR are summarized in Table 2 based on the geographical zones, sex, breed, age, animal residence area and tick infestation. The majority of the seropositive dogs were male and lived in the Plain zone, in rural areas. Furthermore, the

highest prevalence was seen in purebred dogs and in the age group of 1-3 years. Sixteen dogs (40%) among the seropositive animals had tick infestations at the time of blood sampling. However, no significant differences among these results were found.

In cytological examinations, inclusion bodies were seen in five dogs (2.1%), but only one of them was positive for *E. canis* using the IFA and PCR assays. The seropositive dogs had an average count of 214,000 platelets/µl, 5.48 red blood cells (RBC) × 10^6/µl and packed cell volume (PCV) of 32.16%, whereas these value were 347,500/µl, 7.15 × 10^6/µl and 42.43% in the seronegative dogs. The mean PCV percentage among the IFA-positive purebred dogs was 37.73% while it was 30.53% among the mongrels. Furthermore, the mean RBC count and PCV of the IFA-positive dogs that lived in rural areas were 5.98×10^6/µl and 35.00%, in contrast with 4.85×10^6/µl and 28.67% in urban regions, respectively (Table 3). Thrombocytopenia (platelet count <200,000/µl) and anemia (RBC count < 5.5 × 10^6/µl, PCV < 35%) were observed in 60 (25%) and 50 (20.8%) of the dogs. Among the 40 seropositive animals, 26 (65%) had thrombocytopenia and 29 (72.5%) had anemia.

A total of 40 seropositive dogs were screened using the nested PCR technique and, among them, nine animals (22.5%) were positive for *E. canis*. Of those animals, six were males and three had tick infestation. Sequence analysis on these amplicon sequences showed high similarity (99-100%) with the 16S rRNA gene sequences of *E. canis* strains that are available in the GenBank database. The 16S rRNA sequences from this study were deposited in the GenBank database under accession numbers JX556420 and JX556421 for *E. canis* Irani1 and Irani2, respectively.

Table 1. Characteristics of the population studied (n = 240) in Alborz and Tehran provinces, Iran.

		N. dogs	(%)
Geographical zone	Foothill	72	30
	Plain	168	70
Sex	Male	154	64.2
	Female	86	35.8
Breed	Purebred	53	22.1
	Mongrel	187	77.9
Age group	1-3 years	131	54.6
	3-5 years	70	29.2
	More than 5 years	39	16.3
Residence area	Urban	135	56.3
	Rural	105	43.8
Tick infestation	Yes	82	34.2
	No	158	65.8
Total		240	100

Discussion

The results from this study demonstrated that the seroprevalence of *E. canis* in dogs in the Alborz and Tehran provinces of Iran is 16.6% using the IFA test, which is in consistent with the findings in Kerman (14.63%; AKHTARDANESH et al., 2010) but significantly higher than the findings in Ahvaz (9.6%; AVIZEH et al., 2010). The higher seroprevalence found in Turkey (21%) and Israel (30%) (BATMAZ et al., 2001; BANETH et al., 1996, respectively) might be associated with different climatic conditions, the severity of tick infestation and use of preventive or therapeutic anti-ectoparasite applications.

In this study, 22.5% of the seropositive dogs had *E. canis* DNA. These results revealed differences between the IFA and PCR findings, i.e. 77.5% of the seropositive dogs were PCR-negative, possibly due to previous exposure, cross-reactivity between *Ehrlichia* and *Anaplasma* species (WANER et al., 2001) or detection of levels of *Ehrlichia* below the limits of PCR tracing, particularly during the chronic phase of the disease (AGUIRRE et al., 2004). Dawson et al. (1996) and Murphy et al. (1998) observed similar results among dogs in Virginia and Oklahoma, USA, respectively.

Analysis on variables such as sex, breed and age did not show any significant differences ($p < 0.05$) among them, although the prevalence was higher in male dogs (18.2%), which is in agreement with Rodriguez-Vivas et al. (2005) and Solano-Gallego et al. (2006)

Table 2. Analysis on risk factors among positive and negative dogs tested using IFA and PCR.

Variables*		IFA		PCR	
		N. positive (%)	N. tested	N. positive (%)	N. tested
Geographical zone	Foothill	8 (11.1)	72	0 (0)	8
	Plain	32 (19)	168	9 (28.1)	32
Sex	Female	12 (14)	86	3 (25)	12
	Male	28 (18.2)	154	6 (21.4)	28
Breed	Purebred	9 (17)	53	2 (22.2)	9
	Mongrel	31 (16.6)	187	7 (22.6)	31
Age Group	1-3 years	24 (18.3)	131	7 (29.2)	24
	3-5 years	11 (15.7)	70	2 (18.2)	11
	More than 5 years	5 (12.8)	39	0 (0)	5
Residence area	Urban	18 (13.3)	135	4 (22.2)	18
	Rural	22 (21)	105	5 (22.7)	22
Tick infestation	Yes	16 (19.5)	82	3 (18.8)	16
	No	24 (15.2)	158	6 (25)	24
Total		40	240	9	40

* There was no significant difference in comparison of risk factors among positive and negative dogs according to the Phi and Cramer's V coefficients. N - Number; % - percentage.

but not with Batmaz et al. (2001). Furthermore, the frequency of seropositivity was higher in dogs aged 1-3 years (18.3%) than in other age groups. Also, dogs with tick infestation were at greater risk (odds ratio = 1.2) of seropositivity for canine ehrlichiosis, which is in agreement with Trapp et al. (2006) and Costa et al. (2007).

In agreement with previous reports (BANETH et al., 1996; NEER et al., 2002), hematological findings such as thrombocytopenia and anemia were significantly more frequent in seropositive dogs than in seronegative dogs ($P < 0.05$). Thrombocytopenia that is observed in the acute, chronic or subclinical stage of the disease may be due to destruction and consumption of platelets, increased hepatic or splenic platelet sequestration, decreased platelet production following bone marrow hypoplasia (WOODY; HOSKINS, 1991) and production of antiplatelet antibodies (GAUNT et al., 2010). Several mechanisms such as the effects of the mononuclear phagocytic system, cell lysis mediated by the complement system and suppression of erythropoiesis at the bone marrow (CARLOS et al., 2011) may lead to anemia.

Table 3. Comparison of mean age, platelet, RBC and PCV values of the dogs studied.

Variable	Breed		T-value	Sig.
	Mongrel	Purebred		
Age (Y)	2.52	3.61	- 1.69	0.09
Platelet (103/µl)	207.19	237.11	- 0.49	0.62
RBC (106/µl)	5.20	6.43	- 1.86	0.07
PCV (%)	30.53	37.73	- 1.99	0.05*
	Sex			
	Male	**Female**		
Age (Y)	2.67	3.00	- 0.54	0.58
Platelet (103/µl)	209.82	223.50	- 0.24	0.80
RBC (106/µl)	5.55	5.29	0.42	0.67
PCV (%)	32.93	30.34	0.75	0.45
	Residence			
	Rural	**Urban**		
Age (Y)	3.13	2.32	1.50	0.14
Platelet (103/µl)	193.68	238.66	- 0.88	0.38
RBC (106/µl)	5.98	4.85	2.05	0.04*
PCV (%)	35.00	28.67	2.10	0.04*
	Tick infestation			
	No	**Yes**		
Age (Y)	3.14	2.20	1.72	0.09
Platelet (103/µl)	202.08	231.68	- 0.57	0.57
RBC (106/µl)	5.39	5.60	- 0.36	0.71
PCV (%)	31.89	32.55	- 0.20	0.83
	PCR			
	Negative	**Positive**		
Age (Y)	2.90	2.42	- 0.71	0.47
Platelet (103/µl)	232.76	169.00	- 1.05	0.29
RBC (106/µl)	5.57	5.17	- 0.57	0.56
PCV (%)	32.73	30.67	- 0.72	0.59
	IFA			
	Negative	**Positive**		
Age (Y)	2.98	2.77	- 0.72	0.46
Platelet (103/µl)	347.51	213.92	- 4.34	0.00**
RBC (106/µl)	7.14	5.47	- 7.50	0.00**
PCV (%)	42.42	32.15	- 8.43	0.00**

As shown in Table 3, the mean PCV among IFA positive samples was significantly higher in the purebred dogs than in the mongrels ($P < 0.05$). Furthermore, the IFA-positive dogs that lived in rural areas had significantly higher RBC counts and PCV ($P < 0.05$), compared with those in urban regions. Based on these results, it can be concluded that dog breed and residence area may affect the severity of anemia in seropositive animals. The Pearson coefficient matrix between the main variables showed some associations between the factors (Table 4), among which, RBC and PCV had significant positive correlations with age ($R^2 = 0.33$ and 0.31, respectively; $P < 0.05$). In other words, for older dogs, the RBC and PCV values were also greater. Because of the limited number of confirmed positive samples, this finding and also the positive effect of age on RBC and PCV values, as found in this statistical analysis, need further studies.

The seroprevalence was higher in dogs living in the Plain zone and in rural areas than it was in the Foothill zone and in urban areas, which was also reported by Lim et al. (2010) in Korea and Costa et al. (2007) in Brazil. This may be explained by a number of factors, such as suitable environmental conditions for tick survival (for example, warmer temperatures that lead to higher tick infestation rates) and the lack of ectoparasite control programs in rural areas and in the Plain zone.

The influence of climatic conditions on the dynamics of tick populations has also been shown (SONENSHINE, 1993; RANDOLPH, 2008). In cold climates (< 17 °C), the number of tick generations produced within a year becomes smaller because of delay in oviposition, with interference in egg eclosion and an increased parasitic phase (SONENSHINE, 1993). In the present study, the Plain zone had an optimal temperature for development of ticks, thus showing a higher number of infested dogs (45.8%) with higher seroprevalence (19%), while the Foothill zone, with cold temperatures, had a lower percentage of infested animals (6.9%) with lower seroprevalence (11.1%). Thus, dogs living in the Plain zone had a higher risk of exposure to *E. canis* and higher seroprevalence ($p < 0.05$), in comparison with dogs living in the Foothill zone.

Apart from climatic conditions, certain epidemiological factors such as vector distribution, animal behavior, age of the population studied (RODRIGUEZ-VIVAS et al., 2005; TRAPP et al., 2006; LIM et al., 2010), management practices and habitat where the animals live (SAINZ et al., 1995) may affect the prevalence of canine ehrlichiosis. The prevalence of *E. canis* in dogs that lived in rural areas was 21% in this study, which was much higher than

Table 4. Analysis on correlation between age, platelet, RBC and PCV values of the population sampled (Pearson coefficient).

Variable	Age	Platelet	RBC	PCV
Age	1.00			
Platelet	- 0.13	1.00		
RBC	0.33*	0.07	1.00	
PCV	0.31*	0.03	0.97**	1.00

(*R2 = 0.33 and 0.31; **P < 0.05)

among urban animals ($p < 0.05$). The results suggest that dogs living in rural areas are more exposed to tick vectors than those living in urban areas, which may be related to better sanitary conditions with regular anti-ectoparasite treatments among urban dogs and poor living conditions among rural dogs.

The nucleotide sequences of the 16S rRNA partial gene from all the nine positive dogs were identical. Eight of them were quite similar [*E. canis Irani1*: JX556420] and the other one had slight differences [*E. canis Irani2*: JX556421]. These sequences were 100% identical to *E. canis* strains from dogs in Brazil [EF195134, EU376116], Venezuela [AF373614], India [JN967645], Japan [AF308455], Taiwan [GU810149], USA [M73221, M73226], and had 99% similarity with strains reported from Peru [DQ915970], China [AF156785], Tunisia [EU781695], Italy [GQ857078] and Spain [AY394465]. Further molecular investigations and phylogenetic analyses are needed to better understand the genetic diversity and affinity of *E. canis* from dogs in Iran, compared with strains reported from other geographical areas.

Conclusions

The present study amplified and sequenced part of the 16S rRNA gene of *E. canis*, and conducted molecular confirmation of *E. canis* for the first time in Iran. The risk of exposure to *E. canis* among dogs living in the Plain zone and in rural areas was higher than the risk among dogs living in the Foothill zone and in urban areas in the areas of Iran that were studied, although the difference was not statistically significant. Future studies should emphasize larger populations of dogs in other parts of Iran, for molecular detection of *E. canis* and other Anaplasmataceae agents, as well as providing analysis on differences between risk factors.

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