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Original article

DETECTION OF *EHRLICHIA* SPP. IN TICKS COLLECTED FROM STRAY DOGS IN CENTRAL AND SOUTHEASTERN IRAN

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Summary

Khovand, H., S. R. Nourollahi Fard, M. Khalili, M. Jajarmi & H. S. Hormozzaie, 2022. Detection of *Ehrlichia* spp. in ticks collected from stray dogs in Central and Southeastern Iran. *Bulg. J. Vet. Med.*, **25**, No 4, 648–657.

Ehrlichia is an etiologic agent of ehrlichiosis in humans and some animals. *Rhipicephalus sanguineus* is the main vector of the *Ehrlichia canis* and dogs, red foxes and yellow jackals are reservoirs of the bacterium. This tick has a worldwide distribution and is regarded as one of the commonest species of ticks in Iran. This research aimed to detect *Ehrlichia* spp. in *R. sanguineus* isolated from stray dogs in Central and Southeast Iran (Isfahan and Zabol), by using polymerase chain reaction (PCR) and to evaluate the prevalence of the microorganism in these two areas. Tick samples were collected from stray dogs in Isfahan and Zabol between April and June of 2018. The DNA extraction was performed with commercial kits. PCR was done to determine the 336 bp fragment related to *Ehrlichia* spp. Overall, 15.21% of pools in both areas were positive for *Ehrlichia*, 21.42% and 10% of pools were from Isfahan and Zabol respectively. The results confirmed the presence of *Ehrlichia* spp. in *R. sanguineus* in stray dogs revealing that dogs and their ticks may have a significant role in the epidemiology of the disease.

Key words: ehrlichiosis, Iran, PCR, *Rhipicephalus sanguineus*, stray dogs

INTRODUCTION

Ehrlichia spp. as an obligate intracellular bacterium causing ehrlichiosis in humans and some animal species such as dogs, cats, equine; e.g. *E. canis, E. ewingii*, *E. muris*, *E. ruminantium* and *E. chaffeensis* (Dumler, 2005). Dogs may be infected by *E. canis*, *E. ewingii*, *E. chaffeensis*, *E. equi*, *E. sennetsu*, *E. risticii*, *E. muris* and *E. ruminantium* (Carvalho *et al*., 2008; Cetinkaya *et al*., 2016). The brown dog tick, *Rhipicephalus sanguineus*, is the main vector of *E. canis* and dogs, red

foxes and yellow jackals are considered as the primary reservoirs of the bacterium (Masala *et al*., 2012). *R. sanguineus* is prevalent worldwide and is one of the most common ticks in Iran, usually hosted by domestic dogs. These ticks transmit horizontally *E. canis* to humans (Harrus *et al*., 1997; Rahbari *et al*., 2007; Dantas-Torres, 2010; Khazeni *et al*., 2013).

Canine monocytotropic ehrlichiosis (CME) is one of the major diseases caused by *E. canis*. Donatien & Lestoquard (1935) reported this disease for the first time in Algeria. Moreover, there are some reports for the disease in Southern India, Africa, Singapore, Malaysia and US military dogs in Vietnam (Neer & Harrus, 2006; Masala *et al*., 2012). Clinical signs of acute ehrlichiosis in dogs are fever, loss of appetite, weight loss, hepato/splenomegaly, lymphadenopathy, cardiac failures, respiratory and ocular disorders. The chronic phase of the disease is manifested through the reduction of blood cells, degeneration of bone marrow and haemorrhage (Vargas-Hernández *et al*., 2012). *E. canis* is found worldwide, especially in tropical and semitropical areas (Unver *et al*., 2001). Akhtardanesh *et al*. (2010), Avizeh *et al*. (2010) reported the incidence of the disease by serological methods in dogs in Iran. However, because of the extensive antigenic cross-reactions among *Ehrlichia* and *Ehrlichia*-like organisms, serological tests cannot be a valid technique. Additionally, antibodies against *E. canis* may cross-react with *Neorickettsia helminthoeca*, *Neorickettsia risticii* and *Rickettsia rickettsii* (Harrus & Waner, 2011). Recently, molecular techniques are used for epidemiological studies on ehrlichiosis; their advantages are sensitivity and specificity in the diagnosis and identification of the pathogens (Inokuma *et al*., 2006).

To the best of our knowledge, there is no information on *Ehrlichia* in stray dogs and their ticks in Central and Southeastern Iran, such as Isfahan and Zabol. Thus, this research aimed to detect *Ehrlichia* spp. and to evaluate its prevalence in *R. sanguineus* isolated from stray dogs of Isfahan and Zabol using polymerase chain reaction (PCR). The results of this research can provide data to understand the cycle of this important infectious disease in Iran and the epidemiological role of stray dogs as source of the disease.

MATERIALS AND METHODS

Study areas

This study was conducted in the cities of Isfahan and Zabol that are located in Central and Southeastern Iran (Fig. 1). Isfahan city with the area of 231 km^2 and geographical coordinates of 42’3832° N and 03’5140° E is located in central of Iran. Isfahan has hot summers with a maximum temperature of about 35^oC and arid climate.

The city of Zabol with the area of 344 km^2 is located 480 meters above the sea level in the northeast of Sistan and Baluchestan Province with geographical coordinates of 97’3102° N and 78’6149° E. Zabol city is in Sistan and Baluchistan province, southeastern Iran, with 207 km distance from Zahedan, the capital of the province. In this city, minimum temperature is –12 ^oC and maximum temperature is 53 ^oC.

Sampling method

In this cross-sectional and descriptive-analytical research, tick samples were collected from stray dogs in Isfahan and Zabol. The dogs were in the animal shelters and sampling was conducted between

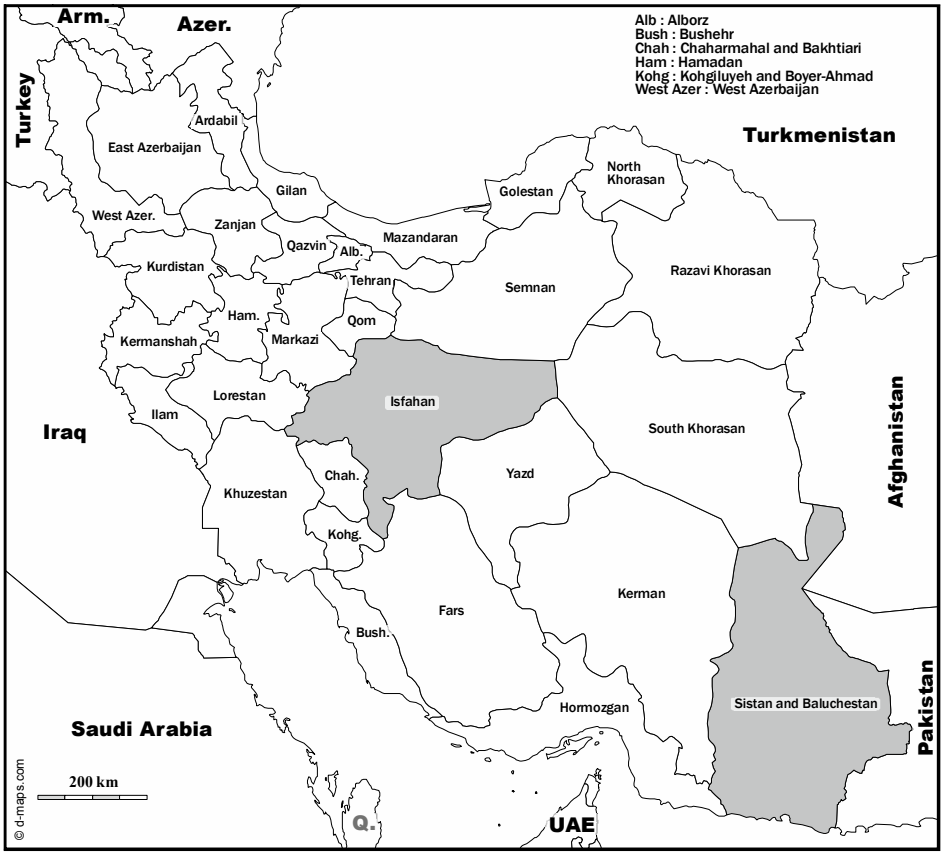

Fig. 1. The studied regions at central and southeastern provinces of Iran.

April and June of 2018, in the peak activity period of ticks. In this study, between 5 to 7 ticks were isolated from various parts of each dog including the ears, neck, shoulders and thighs, regardless of sex, age, breed, and clinical status of the dogs. To avoid damage to the ticks, they were completely isolated from the dogs, so the morphological detection of the ticks would be possible based on their capitulum.

The samples were kept in 70% ethanol at 4 °C and were transferred to the parasitology laboratory of Faculty of Veterinary Medicine, Shahid Bahonar University of Kerman. In the laboratory, the isolated ticks were examined by stereomicroscope (4× zoom) and their genus and species were detected based on their morphological characteristics and the identification key proposed by Walker (2003). Ticks were grouped in pools of 5 or 7 individuals from the same host, the total numbers of pools was 92.

DNA extraction

For the DNA extraction, each pool of ticks was rinsed separately three times with sterile 0.9% physiological solution to remove the alcohol. Then, they were dried for 10 min via a sterile paper.

The ticks were cut into small pieces by a sterile blade and transferred to sterile

microtubes. A commercial kit (Sinapure DNA, Sina Colon Co., Iran), was used for DNA extraction. In brief, 100 µL of prelysis buffer and 30 µL of proteinase K were added to the samples. In the next step, the samples were incubated at 56 °C for 3–5 h. In this period, the samples were vortexed and spun to facilitate DNA extraction. Lysis buffer (400 µL) was added and vortexed (20 s) and precipitation solution (300 µL) was immediately added and vortexed (5 s). The solution was added to silica column and centrifuged (13,000 rpm; 1 min). The samples were respectively rinsed by 400 µL of washing buffer 1 and 2 and centrifuged at 13,000 rpm for 1 min. After adding the elution buffer, the samples were kept at laboratory temperature for 3 min, centrifuged at 13,000 rpm for 1 min and were stored at −20 °C for the next steps.

PCR detection of Ehrlichia spp. in ticks

The purity and concentration of the extracted DNA samples from ticks were assessed by Nanodrop (Epoch, BioTek, USA). PCR was done using a commercial kit (iNtRON, South Korea) to determine the presence of the 336 bp fragment, showing the *Ehrlichia* spp. In this method, 18 µL of distilled water was initially added to microtubes containing 5 µL master mix and 2 µL template DNA was added, so the total volume of reaction was 25 µL.

The microtubes were placed in thermocycler (Bio-Rad, US) and amplified via the following thermal programme: i) primary denaturation at 94 °C for 5 min; ii) 40 cycles including denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s and extension at 72 °C for 40 s and iii) final extension at 72 °C for 5 min. In the final step, PCR end-products were electrophoresed by 2% agarose gel containing DNA Green Viewer (Parstous, Iran) and were observed and imaged using Gel Doc 1000 (Vilber Lourmat, France).

RESULTS

A total of 560 ticks were isolated from different parts of the body of stray dogs in the animal shelters in Isfahan and Zabol. Of these, 210 belonged to Isfahan and 350 belonged to Zabol. All ticks were morphologically detected as *R. sanguineus* regarding specific properties including red-brown colour, elongated body shape and hexagonal basis of capitulum (Table 1).

According to the PCR test, overall 15.21% (14 pools out of 92) of pools in the two areas were detected as positive for *Ehrlichia* spp. That is, 21.42% (9 out of 42) and 10% (5 out of 50) positive pools of ticks were found out in Isfahan and Zabol respectively (Fig. 2).

Table 1. Details of the ticks harbouring *Ehrlichia* spp

Study place	Tick species	Number of ticks	Number of pools	Positive pools	Prevalence (%)
Isfahan	*R.sanguineus*	210	42	9	21.42%
Zabol	*R.sanguineus*	350	50	5	10.00%
Total		560	92	14	15.21%

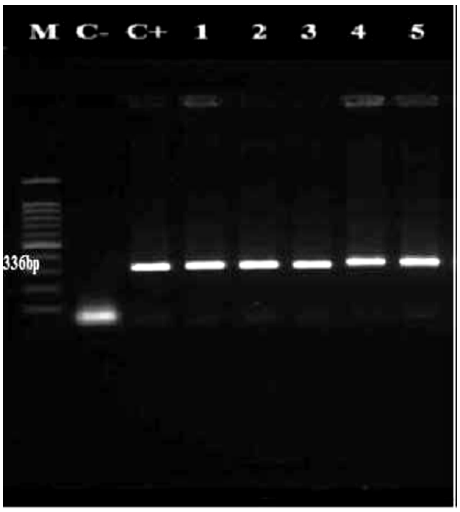

Fig. 2. Agarose gel containing positive samples for *Ehrlichia* spp. M: Marker (100 bp), C−: negative control; C+: positive control; lanes 1, 2, 3, 4 and 5: positive samples (336 bp).

DISCUSSION

The primary objective of this research was molecular detection of *Ehrlichia* spp. in *R. sanguineus* ticks isolated from stray dogs in two different geographical areas, Isfahan and Zabol. According to previous studies in Iran, *R. sanguineus* is a common tick species (Jafar-bekloo *et al*., 2014; Motaghipisheh *et al*., 2016). However, the other species of Ixodidae ticks can also have a significant role in the epidemiology of *Ehrlichia* spp (Hornok *et al*., 2013; Wright *et al*., 2014; Andersson *et al*., 2018; Taira *et al*., 2019).

In the present work, Isfahan had a higher frequency of *Ehrlichia* spp. than Zabol. Previous research has clearly shown the prevalence of *Ehrlichia* spp. in *R. sanguineus* isolated from dogs. In Ardebil, northwestern Iran, Khazeni *et al*. (2013) reported a prevalence of *E. canis* in *R. sanguineus* up to 16.6%. In Kerman, Motaghipisheh *et al*. (2016) reported only 6% of *R.sanguineus* as positive. In Mexico, according to Pat Nah *et al*. (2015) study, 18.5% of *R. sanguineus* belonging to dogs carried *E. canis*. In Turkey, Cetinkaya *et al*. (2016) showed that only 20 (15.75%) of *R. sanguineus* were positive for *E. canis*. In a study in Southeastern Asia, the prevalence of *E. canis* in *R. sanguineus* in Thailand and Taiwan was 3% and 1%, respectively (Foongladda *et al.,* 2011; Yuasa *et al.,* 2017). The highest and lowest prevalence of this pathogen in ticks was reported in Malaysia, which was 51.5% and 0.7% respectively (Koh *et al*., 2016; Low *et al*., 2018).

Differences in the prevalence of ticks and microorganisms in present and previous studies might be associated with many factors, such as tick species, geographical variation, climatic diversity, reservoirs and the methodology (Aktas *et al*., 2009). These results showed that *R. sanguineus* is one of the most common vectors and has a very important role in the transmission of *Ehrlichia* spp. to dogs. Maazi *et al*. (2014) investigated the blood collected from dogs in different areas of Tehran and Alborz provinces to determine *E. canis* by serological and molecular methods. Results showed that out of 40 serologically positive dogs, 9 dogs (22.5%) had the DNA of *E. canis*. Motaghipisheh *et al.* (2016) detected the DNA of *Ehrlichia* spp. in 9% of dogs by the PCR method. In southern Iran and in Fars Province *Ehrlichia* spp. were observed only in 3 dogs of 98 dogs (3.06%) with clinical symptoms of ehrlichiosis (Derakhshandeh *et al*., 2017). *E. canis* has been reported from countries such as the US, Algeria, Mexico, Brazil, Nigeria and Paraguay (Santamaria *et al*., 2014; Dahmani *et al*., 2015; Almazán *et al*., 2016; Ribeiro *et al*.,

2017; Daramola *et al*., 2018; Pérez-Macchi *et al*., 2019).

The presence of *Ehrlichia* spp. in this study indicates that in the transmission of this pathogen to dogs, *R. sanguineus* can be considered a potential risk for the emergence of the disease in humans in the cities Isfahan and Zabol. In Iran, the incidence of human ehrlichiosis was first reported in the northern parts of the country, especially in Mazandaran Province (Babamahmoodi, 2004). Serological and molecular incidence of human cases of the disease has so far been reported in some countries. In Columbia, Botero *et al.* (2014) confirmed a case of human monocytotropic ehrlichiosis caused by *E. chaffeensis* by serological methods, in which the patient showed the clinical signs including fever, minor rashes, thrombocytopaenia, hepato/splenomegaly and multiple organs failure.

Human infections associated with *E. chaffeensis* have been reported from Costa Rica, suggesting the necessity of further studies on the ecology of animal reservoirs and arthropod vectors to determine the risk factors of human monocytotropic ehrlichiosis in this country (Rojas *et al*., 2015). In a retrospective study from 41 American states during an 18-month period between 2013 and 2014, 109 cases of human ehrlichiosis were diagnosed: 2.4% and 0.2% of the patients were diagnosed as positive for *E. chaffeensis* and *E. ewingii*. The places of incidence of the cases were in association with *Amblyomma americanum* (Harris *et al*., 2016). In another study in Costa Rica, *E. canis* infection was also detected from blood bank by serological and molecular methods and the results indicated a high prevalence of the infection among dogs and their ticks (Bouza-Mora *et al*., 2017).

In a study conducted in Colombia, the prevalence of *Ehrlichia* spp. infection in human blood samples was 74.2% by serological tests (Eraso-Cadena *et al*., 2018). Although *E. canis* is well known as a pathogen in dogs, it has also been reported from domestic ruminants (Zhang *et al*., 2015). According to the studies carried out in Iran, the positive cases of *Rickettsia*, especially *Ehrlichia* spp. and *Anaplasma* spp. were also confirmed and detected in ticks collected from ruminants such as cows, sheep and goats, showing the potential of the disease to involve the other domestic animals like ruminants (Jafar-bekloo *et al.,* 2014; Tajedin *et al*., 2016; Jafar-Bekloo *et al*., 2018). The disease has been detected in other animals by different types of serological and molecular methods (Bastos *et al*., 2015; Tsachev *et al*., 2018).

CONCLUSION

This study showed the presence of *Ehrlichia* spp. in ticks collected from stray dogs in central and southeastern Iran. Dogs are asymptomatic source of *Ehrlichia* spp. which may cause tick-borne diseases in humans and other animals. So, dogs and their ticks may play an important role in the epidemiology of the disease in these areas. It should be mentioned that treatment, control and prevention of ehrlichiosis need to increase the level of awareness in physicians, health workers, veterinarians and all people. It is suggested that future studies should be focused on species determination of *Ehrlichia* spp. isolates in Iran to understand the distribution pattern of the disease in the country.

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