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Molecular detection of *Rickettsia* spp. in ticks of dogs

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Abstract

Arthropods especially ticks are important microbial vectors that work specific roles in medical and veterinary problems. The study was conducted on the ticks that carried Rickettsia spp. from the dog in different parts of the province of Hormozgan in Iran. Ticks were sampled from various locations all over the dogs' bodies during the spring and summer of 2022. Ticks were collected and placed in sterile glass bottles containing 95% ethanol. For the extraction of DNA, ticks were initially air-dried on clean paper in a well-ventilated area following a brief ethanol 70% rinse. They were then processed using a commercially available DNA extraction kit (DNA Extraction Kit, MBST, and Iran) to extract DNA. Specific primers targeting the genes of Rickettsia spp. were used for polymerase chain reaction (PCR). PCR was employed to identify Rickettsia spp. The positive PCR product was sent for sequence analysis. The Maximum likelihood method in MEGA V.10 was used to analyze the sequencing data and phylogenetic analysis was conducted. Rickettsiae spp2 (n = 47; 4.25%; 95%Cl: 1.18% to 14.26%) positive for the gltA gene were detected. The identification of Rickettsia spp. in dog ticks is reported for the first time in Iran. These results showed that hard ticks act as vectors of Rickettsia spp. in Iran, which may have important public health implications in their areas of distribution.

Introduction

Rickettsias are rod-shaped, short, obligate intracellular, gram-negative bacteria. A considerable number of obligate intracellular prokaryotic microbes belonging to the Gram-negative group, *Rickettsia* spp., are categorized into four groups: the spotted fever group (SFG), the typhus group, the *Rickettsia canadensis* group, and the *Rickettsia bellii* group. The SFG *Rickettsia* are often transmitted by ticks and are responsible for causing *Dermacentor*-borne necrosis, which is linked to various zoonotic diseases globally (1, 2).

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Typically, these bacteria are spread to humans and animals by infected arthropod vectors (such as fleas, ticks, lice, and mites) or through infectious materials like flea dirt. *Rickettsia* spp. are responsible for various diseases including epidemic typhus fever (ETF), Rocky Mountains spotted fever (RMSF), scrub typhus fever (STF), and murine typhus fever (MTF).

These illnesses can lead to various historically, mainly affecting humans and dogs as the primary agents of severe Rickettsiosis (3). In humans, the disease is marked by a petechial rash, although individuals initially may experience fever, headache, malaise, and myalgia. In dogs, RMSF may appear as fever, lethargy, decreased appetite, and tremors, occasionally accompanied by a maculopapular rash on exposed skin areas (3, 4). Dogs act as reservoirs of zoonotic agents, providing nourishment for numerous arthropods that also bite humans, thereby elevating the risk of zoonotic infections.

Thus, dogs are gaining increasing public attention because they live in intimate contact with humans. A study has demonstrated that pet ownership is significantly correlated with the risk of tick encounters (5).

Ticks rank second only to mosquitoes as transmitters of diseases that affect both humans and animals (6, 7). Worldwide, ticks are acknowledged as vectors and reservoirs for several pathogens such as protozoa, viruses, and bacteria affecting almost all vertebrates including humans (6, 8).

The primary hard ticks that harbor pathogens and cause infections in both domestic and wild animals come from various genera such as *Rhipicephalus*, *Hyalomma*, *Haemaphysalis*, *Ambylomma*, and Ixodes. These blood-feeding external parasites have a significant impact on transmitting various pathogens, including bacteria, protozoa, and viruses, which have the potential to trigger zoonotic outbreaks posing a threat to both human and animal health (9, 10).

Rickettsia spp., transmitted by ticks, is considered to be an important causative agent for FSME and contains many zoonotic agents. Most of the *Rickettsia* spp. of the SFG is transmitted to vertebrate hosts by the hard ticks of the Ixodidae family. Several species of *Rrickettsia* have been described to be pathogenic to humans, and species of *Rickettsia* of uncertain pathogenicity have been observed on ticks (8, 9).

Around the world, *Rickettsia conorii* has been found in different species, including *Rhipicephalus turanicus*, *Rhipicephalus sanguineus*, , *Rhipicephalus pumilio, Rhipicephalus bursa Rhipicephalus evertsi*, *Rhipicephalus simus, Rhipicephalus mushamae, Haemaphysalis leechi*, and *Haemaphysalis punctaleechi*, across various countries such as France, Bulgaria, Turkey, India, African nation (11).

The use of morphological diagnostic keys to identify *Rhipicephalus* and *Hyalomma* species may be limited by several factors. *Rhipicephalus* ticks are recognized as carriers of diverse pathogens, including bacteria (12), viruses, and protozoa. These can include the Crimean-Congo hemorrhagic fever virus, *Anaplasma*, *Babesia*, *Ehrlichia*, and various *Rickettsia* species. These pathogens are linked to diseases like Crimean-Congo hemorrhagic fever, Lyme disease, Rocky Mountain spotted fever, and other tick-borne diseases (13, 14). *Rhipicephalus* ticks are widespread in Africa, Asia, and Europe and include several species (1). These ticks are recognized for transmitting a variety of viral, bacterial, and protozoal pathogens, some of which have the potential to cause serious diseases in both humans and animals. Additionally, they can spread bacterial pathogens like *Rickettsiae*, *ehrlichiae*, and *anaplasmae*, as well as protozoa such as *Theileriae* and *Babesiae* (10, 15, 16). The objective of this study was to establish the Rickettsial DNA from hard ticks of dogs.

Materials and methods

Study areas

Hormozgan Province, covering an estimated area of 7072.06 km^2, is one of the seven coastal provinces of Iran. It occupies about 4.1% of the country's total

land area and can be found on the north shore of the Strait of Hormuz. It shares its boundaries with Sistan-Baluchestan, Kerman, Fars, and Bushehr. Depending on the geography, there are two types of climates: coastal desert and inland desert. Like other central desert regions of the country, this region has a hot and wet climate, while the uplands have a more moderate climate. Regarding rainfall, the province received a typical yearly rainfall of 151 mm last year, which is well above the national average (17).

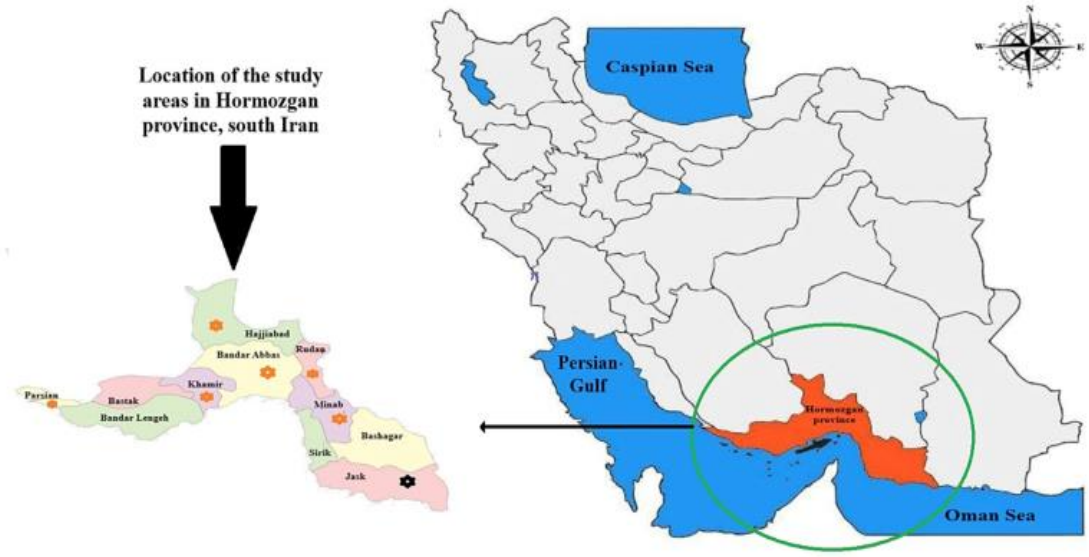

Fig. 1. A diagrammatic representation of the research locations (17).

Sample collection

Ticks were sampled from various locations all over the dog's body surface in Hormozgan province in the spring and summer of 2022. Out of the total number of 1863 dogs related to all epidemiological units, 5% of the units were selected as the goal unit and 3-5 ticks were sampled for each dog, the total number of 233 ticks from 47 dogs, of which 70% of the samples are related to the shelter of stray dogs in Bandar Abbas Municipality and 30% of the samples were taken from nomadic dogs and rural areas. Tick samples were stored in sterile glass bottles containing 75% ethanol. Then, these samples were moved to the Parasitology Laboratory, Faculty of Veterinary Medicine at Urmia University for the recognition of tick species. A magnifying loupe microscope and reliable diagnostic keys were used to identify the ticks. Molecular identification of the bacteria was carried out in the bacteriology laboratory using PCR to detect chromosomal *gltA* genes (18).

DNA extraction

For DNA extraction, the ticks were first air-dried on clean paper in a well-ventilated area. This was done after extraction from 70% ethanol. The DNA was then extracted using a commercially available DNA extractor (MBST, Iran). NanoDrop 2000c (Thermo Scientific, USA) was used to assess the quantity and quality of the extracted DNA. To be used later in the PCR, the DNA extracted was preserved at -20 °C. The elution buffer provided in the kit served as the negative control for the DNA extraction process (19).

Genomic identification of Rickettsia spp.

The DNA was tested to detect the specific bacteria using conventional PCR. The primer sets used for this purpose were collected from a previous study by Ghasemi *et al*. (2022) (20), as shown in (Table

1), and the *gltA* gene was the target gene for the identification of *Rickettsia* spp.

The PCR reaction was conducted using a volume of 25 µL volume, consisting of 4 µL of template DNA, 1 µL of each primer (primer concentration 0.1 nmoL/µL), 12.5 µL of master mix, and the remaining volume was completed with sterile distilled water (Table 1), and run on a thermocycler (Quanta Biotech, UK). The touch-down PCR program typically involves an initial high annealing temperature, which is gradually lowered in subsequent cycles to promote specific amplification and reduce the amount of non-specific amplification.

PCR products were electrophoresed on 1% agarose gels stained for safety (Labnet, Enduro, USA). The gel was then imaged with the Genius gel imaging system (Syngene BioImaging, UK) (21).

Table 1. Primer sequences for identifying of the gene of *Rickettsia* spp.

***Rickettsia* spp. target gene**	**Nucleotide sequence (5′-3′)**	**Amplicon size (bp)**	**PCR condition**	**Ref.**
gltA(F)	GCTCTTCTCATCCTATGGCTATTA	834	Initial denaturation: 95 °C for 4.0 min. 32 cycles of: Denaturation at 95 °C for 90 sec annealing 58 for 90 sec, Extension at 72 °C for 90 sec. Final extension: 72 °C for 7.0 min.	**(21)**
gltA(R)	CAGGGTCTTCRTGCATTTCTT			
gltA(R)	CAGGGTCTTCATGCATTTCTT			

Sequencing

Certain positive samples were forwarded to Pishgam Biotechnology Company (Tehran, Iran) for sequencing using the Sanger method. Subsequently, the resultant sequences were submitted to the NCBI website for analysis and comparison using BLAST. For phylogenetic tree construction, the *gltA* gene with 834 nucleotides was used (22).

Degenerate primers involve combining multiple primer sequences with slight variations, allowing for flexibility in target DNA sequence recognition when the precise nucleotide sequence is unknown but can be predicted from the corresponding amino acid sequence.

When the exact nucleotide sequence of a target DNA is unknown, its corresponding amino acid sequence can be used to infer potential primer sequences. However, due to the genetic code's degeneracy, where more than one codon can encode the same amino acid, these inferred sequences may have multiple variations. To accommodate this uncertainty, researchers often design degenerate primers – a combination of several primer candidates that differ at specific positions, allowing the primers to recognize their target DNA sequence with flexibility (23).

Phylogenetic tree construction and nucleotide diversity

This was done by uploading the sequences to the National Center for Biotechnology Information (NCBI) to find the closest reference sequences and then using BLAST from NCBI to identify the COI. Phylogenetic analysis was performed using the COI sequences of *Rickettsia* spp. available in the GeneBank. Before their release as MEGA and FASTA files, the alignments were manually adjusted to eliminate any associated errors using the Clustal alignment program (24). All obtained sequences were submitted to the GeneBank and accession numbers were assigned. Phylogenetic relationships were then investigated and developed using MEGA software version 10 with a maximum likelihood approach. One thousand bootstraps were utilized to test the reliability of the inferred tree. BioEdit version 7.0.1 and BLASTN software were used to analyze DNA sequence polymorphism to assess nucleotide diversity (15).

Data analysis

Statistical data were analyzed using the chi-square test using SPSS software version 22. P-values below 0.05 were deemed significant.

Results

The 233 tick samples taken from 47 dogs were divided into 5 pools and 47 pooled samples were analyzed using the PCR method. *Rickettsia* spp. DNA's positive percentage in the tick samples was 4.26% as determined by the *gltA* gene. The *Rickettsia* spp. was identified based on *gltA* gene under the accession number (**OQ507338**). Furthermore, the results regarding the molecular prevalence of *Rickettsia* spp. 2 (n = 47; 4.25%; 95%Cl: 1.18%-14.26%) positivity are reliant on the *gltA* gene (Table 2).

Table 2. The prevalence of *Rickettsia* spp. in ticks' samples from Dog in Hormozgan province

Number of ticks	Ticks species	No.	Number of pools	Genus	Positive samples for *gltA* gene
204	*Rhipicephalus sanguineus*	149	30	Male	2/30 (6.66%)
		55	11	Female	0/11 (0.0%)
p-value					0.284
29	*Rhipicephalus turanicu*	25	5	Male	0/5 (0.0%)
		4	1	Female	0/1 (0.0%)
p-value					-
Total		233	47		2/47 (4.25%)

In this study, of 233 ticks, 174 and 59 were male and female, respectively. The identified tick species included *Rh. sanguinus* (149 male and 55 female) and *Rh. turanicus* (25 male and 4 female). The ticks were grouped into 47 pooled samples each of 5 ticks, and were evaluated utilizing the PCR method. The findings showed that 2 samples of the male *Rh. Sanguinus* ticks were infected with *Rickettsia selovaca* bacteria, with a 100% similarity in the DNA sequence.

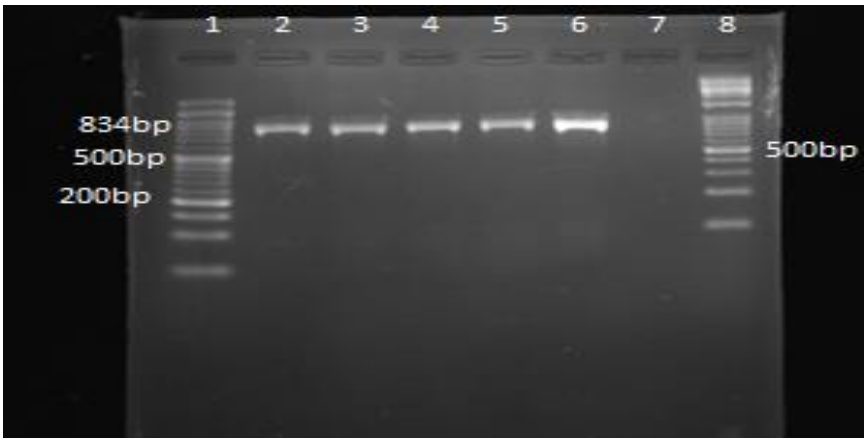

Fig. 2. Analyzed agarose gel electrophoresis image of PCR products for the detection of *Rickettsia* spp. harboring the *gltA* gene in ticks (834bp), Line: marker 50 bp and line 8: marker 100bp DNA (Smobio Technology Inc., Taiwan). Lines 2-6: positive Tick samples, Line 6: negative control.

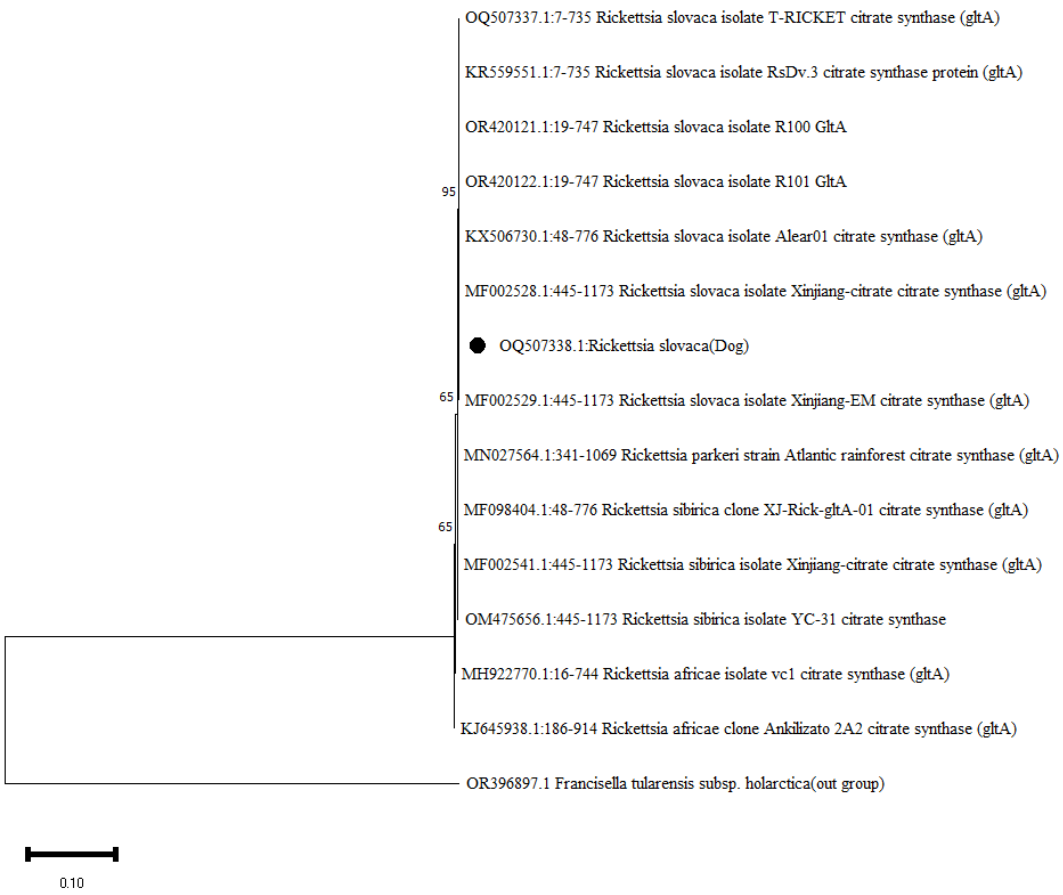

Fig. 3. In the present work, the phylogenetic tree of the *gltA* gene sequences of *Rickettsia* spp. from different accession numbers has been obtained and deposited in the gene bank. The *gltA* gene sequences acquired in this study are denoted by the bold dot. The tree was constructed using the Maximum likelihood approach of MEGA 11. Bootstrap values are indicated at branch points. Bootstrap support of 1000 replicates are indicated by numbers above branches.

Discussion

Ticks are separated into two groups: soft ticks and hard ticks. Hard ticks, or the Ixodidae family, are the most important and primary vectors of *Rickettsia*. In this study, sampling was exclusively conducted on hard ticks. The study indicates that hard ticks are the primary vectors and reservoirs of *Rickettsia* bacteria (13, 25).

The study revealed that Hormozgan province, due to its specific climatic conditions, experiences hot and humid weather for extended periods throughout the year. Consequently, the prevalence of tick infestation in terms of both number and diversity, exhibits an increasing trend, particularly during these seasons. However, only a small proportion of ticks are infected with *Rickettsia*. In ticks, the transmission of bacteria to the next generations also occurs.

Rh. sanguineus emerged as the most prevalent tick species harboring *Rickettsia* bacteria in this study.

The detection rate of *Rickettsia* in *Rh. sanguineus* ticks were 4.25%, while only two positive samples were identified from the pooled dog samples.

Rickettsia bacteria were primarily detected in samples collected from Bandar Abbas County, highlighting the significance of sample size in this context. Among all counties in the province, Bandar Abbas exhibited the highest epidemiological unit, accounting for 36.4% of the total samples. Following sample size, the sampling location held substantial importance. While ticks collected from nutrient-rich pastures, despite their higher abundance, tested negative for Rickettsia bacteria, those sampled from improper stray dog shelters exhibited the highest Rickettsia prevalence.

These two counties, despite being distinct, shared identical climatic and weather conditions. Rickettsia was first reported in the Khuzestan region (26). In the current study, *Rickettsia* bacteria were isolated from ticks of the species *Hyalomma asiaticum*, *Hyalomma anatolicum*, and *Rh. sanguineus*. Ticks are known to transmit *Rickettsia* to farm animals, wildlife, and humans. Regarding human involvement with rickettsiosis due to tick bites, it's important to note that humans are accidental hosts of *Rickettsia*, and transmission occurs in these circumstances. The prevalence of *Rickettsia* in ticks identified in various hosts, in addition to *Rhipicephalus*, *H. anatolicum*, and *H. asiaticum*, was diagnosed in the target population under study.

The prevalence of tick species in Hormozgan province may be due to its adjacency to Kerman province and the role of migration or seasonal migration in different seasons, especially in warm seasons, which can lead to the transfer of physical and/or genetic conditions among the species under discussion. A detailed investigation in this regard can contribute to timely diagnosis and the presentation of appropriate strategies for combating the discussed parasites. In the study by Ifeanyi Charles Okoli (2006) in Nigeria, which is a warm and humid region, *Rh. sanguineus* in the spring season (April-June), with high rainfall, is highly active (27). Considering that Hormozgan is very close to Nigeria in terms of climate, *Rh. sanguineus* should primarily be considered as the reservoir and main vector of *Rickettsia* for epidemiological investigation of *Rickettsia* bacteria, especially in warm and humid areas, particularly in dogs.

In the world, the rate of *Rh. sanguineus* infection with *Rickettsia* varies depending on the health conditions of dogs, geographical location, and climate. For example, in the study by Laia Solano Gallengo *et al*. (2020) in South Africa, the infection rate in dogs was 26-60%. However, all the ticks were sampled from sick dogs (28), while in the present study, random sampling was performed on dogs, and the infection rate of this tick genus was 4%. The conclusion is that geographical location, climatic conditions, and other factors play a crucial part in the occurrence and prevalence of *Rhipicephalus* ticks. Regarding the prevalence of this tick species, they are vectors and reservoirs of specific types of Rickettsia in different geographical locations. According to a study by Eremeeva et al, (2011), this species is a vector of *Rickettsia conori* in the Mediterranean region and a vector of in Mexico (29). Therefore, geographical location is one of the most important items to be considered in the fight against ticks and the diseases caused by them. Of course, the health and responsibility of dogs as the main host of this tick species are of great importance. In relation to the study of various genes present in the genome of Rickettsia, the *gltA* gene is easier to study and identify compared to other genes such as *ompA* and *ompB* due to its conserved nature. For the first time in 1968 in France, *Rickettsia slovaca* was isolated from *Dermacentor marginatum* ticks (30).

This bacterium is known as the causative agent of Tick-borne Lymphadenopathy (TIBOLA) disease. Under the same conditions in Spain, this bacterial species causes a disease called Dermacentor-borne Necrosis Erythema Lymphadenopathy. According to the results of the study, *R. slovaca* was isolated and identified from various ticks such as *Rh.*

sanguineus and *H. dromedarii* in dogs, cattle, and sheep, respectively (31).

According to a study by Ortun et al. (2008) on dogs in the city of Barcelona, Spain, the dogs were carriers of *Rh. sanguineus* ticks, which were exclusively the reservoir and vector of *R. conori*. However, in the present study, the mentioned ticks were vectors of *R. slovaca*, indicating that the maintenance conditions and geographical location play a fundamental role in the type of bacteria transmitted by *Rhipicephalus* ticks (32).

Regarding the tick samples isolated from herd dogs and dogs in the shelter for stray dogs belonging to the Bandar Abbas municipality, 5 tick samples were taken from each of the 160 dogs under hygienic conditions. In the parasitology laboratory, the genus and species of ticks were identified as *Rh. sanguineus* and *Rh. turanicus* (87.5% and 12.5%, respectively). After DNA extraction and molecular tests, it was determined that *Rh. sanguineus* had an infection rate of about 4% with *Rickettsia*.

R. conori can be transmitted through *Rh. sanguineus* and cause disease, with dogs primarily considered as reservoirs for *R. conori*. However, different breeds of dogs have varying levels of susceptibility to *R. conori* (33). In this study, *Rh. sanguineus* and *Rh. turanicus* were isolated from dogs, with only 4 % of cases found to be contaminated with *R. slovaca*. Therefore, considering that both *R. slovaca* and *R. conori* were isolated and identified from *Rh. sanguineus* in two completely different geographical locations, it can be concluded that ticks in different places and climates can serve as reservoirs and carriers of different species of a bacterial family. To understand the natural status of *Rickettsia* in ticks in Japan, Rickettsial genes, the *gltA* gene, were amplified from ticks. The prevalence of Rickettsial *gltA* genes among *Haemaphysalis flava, H. kitaokai,* and *I. persulcatus* was 62, 57, 24, 24, 19, 13, and 10 respectively (34).

Conclusion

This is an initial investigation of *Rickettsia* spp. identified by sequencing of positive samples of *Rickettsia* spp. in ticks collected from Hormozgan province, Iran. These studies have documented the presence of important pathogens, particularly in veterinary medicine. The findings indicate that ticks belonging to the genera *Rh. sanguineus, H. egebiom, H. asiaticum, H. anatolicum* and *Rh. turanicus* can transmit *Rickettsiae*. Especially because of its public health significance, the data on the *Rickettsia* group can be used in future work for the development of tick control mechanisms. The molecular tool presented can be utilized to examine the genetic diversity of Rickettsial spirochetes present in various reservoir hosts and tick vectors. This will improve our comprehension of the significance of genetic variability concerning the epidemiological traits of Rickettsial spirochetes in Hormozgan province, Iran.

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Ethical approval

This work was conducted in accordance with the guidelines and standards of the Animal Research Ethics Committee of the Urmia University. However, the tick samples gathered from live animals and there was no need to receive the code of ethics.

Conflict of interest

There is no conflict of interest in conducting this research.

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