Molecular detection and diversity of spotted fever group Rickettsia isolated from ticks in Iran

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Abstract

Background: This study aimed to identify *Rickettsia* spp. and their circulation in ticks collected from sheep in the Khuzestan province, Southwest Iran.

Methods: Four hundred and forty adult hard ticks were collected from the parasite-infected sheep from nine cities of Khuzestan province. DNA samples were extracted and subjected to the single-round polymerase chain reaction amplification of citrate synthase gene (*gltA*).

Results: The tick species were identified as *Hyalomma marginatum*, *Hyalomma anatolicum*, *Hyalomma dromedarii, Hyalomma schulzei*, *Rhipicephalus bursa*, and *Rhipicephalus turanicus*. The presence of *Rickettsia* spp. was observed in half of the collected ticks (50%). For a definitive identification of *Rickettsia* spp., the amplicons (n: 10) were directly sequenced. Sequencing and phylogenetic analyses revealed the definitive presence of *Rickettsia aeschlimannii* (60%), *Rickettsia massiliae* (30%), and *Rickettsia conorii* (10%) in the infected ticks.

Conclusions: The results indicated a significant incidence of tick-borne *Rickettsia* spp. and their unequivocal circulation in Southwest Iran. Hence, the prevalence of spotted fever group *Rickettsia* should be detected in suspected humans and domestic animals in wider areas of Iran.

Background

One mode of transmitting infectious diseases is by vectors. By definition, vectors are living organisms that can transmit contagious pathogens between humans or from animals to humans. Accordingly, vector-borne diseases are human diseases caused by parasites, viruses, and bacteria transmitted by vectors [1].

Infectious diseases have grown dramatically in recent years for various reasons. According to the reports by the US Centers for Disease Control and Prevention (CDC), the number of reports of vector-borne diseases has tripled over 13 years (2004 to 2016)[2]. According to the latest figures from the World Health Organization (WHO), these diseases now account for more than 17% of all infectious diseases, killing more than 700,000 people worldwide each year; hence, more than half of the world's population are at risk for these diseases [1, 3]. As one of the most important and known vectors, ticks can transmit different types of bacteria, protozoa, and viruses that cause a wide range of diseases in humans and animals. Rickettsial diseases caused by bacterial agents from the order Rickettsiales are among these diseases [4].

Ehrlichia spp., *Anaplasma* spp., and several *Rickettsia* spp. are tick-borne pathogens in the order Rickettsiales. *Rickettsia*, the most significant genus, has been classified as four groups based on whole genome analysis data. Among them, the spotted fever group (SFG) and the transitional group (TRG) species are transmitted by ticks of the family Ixodidae and are considered zoonotic [5]. The presence of a high variety of ticks that can transmit *Rickettsiae* to several countries in the Middle East makes it highly prone to cause diseases. Despite clear evidence, no studies have reported any rickettsial disease in Iran [6]. However, the presence of SFG species in serum samples from humans and animals based on the results of IFA (indirect immunofluorescent antibody) and ELISA (enzyme-linked immunosorbent assay) tests has been confirmed [7].

Rickettsiae are the most prominent members of the *Rickettsiaceae* family that belong to the order *Rickettsiales*. This genus includes Gram-negative, aerobic, and intracellular coccobacilli that are transmitted by arthropods, including ticks, mites, fleas, and lice, and its life cycle involves both vertebrate and invertebrate hosts. Members of the genus *Rickettsia* can be classified as several groups, including the SFG, the typhus group, R. bellii, and R. Canadensis [8, 9]. The genus *Rickettsiae* includes 31 different species. Among tick-borne diseases, rickettsiosis is caused by *Rickettsiae* bacteria

belonging to the group of *Rickettsiae* that cause spotted fever [10] diseases such as typhoid [11]. Co-morbidity in humans and animals is a significant point concerning this pathogen. Accordingly, the need to study the distribution and diversity of this bacterium species at different geographical levels, and to predict outbreaks of vector-borne diseases can be compelling. This study aimed to identify the genetic species and to investigate the distribution of *Rickettsiae* in the ticks collected from sheep in Khuzestan province of Iran.

Methods

Study area

The tick samples of this study were collected from the sheep flocks reared in different cities of Khuzestan province. Of note, Khuzestan province is one of the 31 provinces of Iran. It is located in the southwest of the country, bordering Iraq's Basra province and the Persian Gulf. Its capital is Ahwaz and covers an area of 63,238 km^2 [12]. The climate of Khuzestan is generally very hot and occasionally humid, particularly in the south, while winters are cold and dry. During the summer, temperature routinely exceeds 45°C almost daily and in the winter, it can drop below freezing, with occasional snowfall, all the way south to Ahvaz. This province is possibly one of the hottest places on earth with the maximum temperature in summer soaring up to more than 45°C and reaching up to 55℃ at 2 meters height [13]. Khuzestan has desert conditions and experiences many sandstorms. Given the geographical and climatic conditions and the method of livestock farming, especially large herds in this province, it is not surprising that this region is one of the susceptible provinces to arthropod-borne diseases in Iran and the Middle East.

Tick collection

Ticks were collected from the herds of nine cities of Khuzestan province, in some of which sheep and goats are raised together. During spring 2018, 440 adult hard ticks were collected from the parasite-infected sheep (Fig. 1). The collected ticks were transferred to the Central Veterinary Laboratory, Iran Veterinary Organization (Tehran, Iran), and stored in vials containing 70% ethanol at room temperature until DNA extraction. The ticks were identified to the species or genus level using the taxonomic keys [14].

DNA extraction

Ticks were immersed in distilled water for 10 min and dried on sterile filter paper., Then the ticks were pooled into the groups of 5–30, each of which (36 pooled samples) were crushed individually in into its components using a sterile tissue homogenizer. Genomic DNA was extracted using High Pure PCR Template Preparation Kit (Roche, Germany) according to the manufacturer's instructions.

PCR amplification and electrophoresis

Polymerase chain reaction (PCR) amplification was performed using the primers mentioned in Table 1. The reactions were performed (Thermal cycler, Eppendorf, Germany) using the Taq PCR Master Mix Kit (Qiagen, Germany) for each organism (*Rickettsia* spp.) as follows: initial denaturation at 95°C for 6 min; 35 cycles of denaturation at 94°C for 40 sec, annealing at 48°C for 30 sec, and extension at 72°C for 40 sec; and final extension at 72°C for 10 min. The PCR products were electrophoresed on 2% agarose gel.

Table 1
Primer sequences used for PCR

Target gene	Primer name	Oligonucleotide sequences (5' to3')	Annealing temperature (°C)	PCR product size (bp)	Reference
gltA	Ricspp –F	GCT ATT ATG CTT GCG GCT GT	51	806	[31]
	Ricspp-R	TGC ATT TCT TTC CAT TGT GC			

Sequencing and phylogenetic analysis

Ten PCR products of *Rickettsia* spp. were randomly sequenced (Bioneer®, South Korea). Ambiguous sites within the selected nucleotide sequences were coded using the standard International Union of Pure and Applied Chemistry (IUPAC) for combinations of two or more bases. Contigs of all samples were aligned using Clustal W and assembled using Sequencher™v.4.1.4 Software. To authenticate phylogenetic associations among the identified *Rickettsia* spp., a phylogenetic tree was constructed using the MEGA 5.05 software based on the maximum likelihood method with the Kimura 2-parameter model. The distance scale was estimated 0.05. R. philipii (accession number, NC016930) was considered as an out-group species. Bootstrap values higher than 60% supported the topology on each branch [15–17].

Results

Tick identification

The ticks collected were identified as *Hyalomma marginatum* (28.86%), *Hyalomma anatolicum* (30.00%), *Hyalomma dromedarii* (4.09%), *Hyalomma schulzei* (4.54%), *Rhipicephalus bursa* (13.41%), and *Rhipicephalus turanicus* (19.10%). The highest number of ticks was obtained from Shushtar city (107 cases) and the lowest number of registered ticks was related to Ramhormoz and Bavi cities (4 and 6 cases, respectively). It is noteworthy that the lowest and the highest diversities of tick species found during this study were related to *Hyalomma dromedarii* (4.09%) and *Hyalomma anatolicum* (30.00%), respectively. Furthermore, the lowest diversity of tick species was related to those two cities (Ramhormoz and Bavi), and the highest diversity was observed in Karun city (Table 2).

Table 2
Tick abundance around different cities of Khuzestan province

City	Type of tick						
	Hyalomma marginatum	*Hyalomma anatolicum*	*Hyalomma dromedarii*	*Hyalomma schulzei*	*Rhipicephalus bursa*	*Rhipicephalus turanicus*	Total
Ahvaz	6	10	2	-	18	16	52
Ramshir	20	19	6	4	6	-	55
Ramhormoz	4	-	-	-	-	-	4
Shushtar	13	21	-	5	18	50	107
Hoveyzeh	32	18	4	5	7	-	66
Shush	11	33	-	-	-	7	51
Bavi	-	6	-	-	-	-	6
Karun	35	22	6	6	10	11	90
Masjed-e-Soleyman	6	3	-	-	-	-	9
Total	127	132	18	20	59	84	440

PCR amplification of citrate synthase gene (gltA)

Rickettsia spp. were found in half of the collected ticks (50%). For the definitive identification of the species, amplicons (n: 10) were directly sequenced to explore the presence of *Rickettsia* spp. Sequencing and phylogenetic analyses revealed the presence of *Rickettsia aeschlimannii* (60%), *Rickettsia massiliae* (30%), and *Rickettsia conorii* (10%) in the infected ticks.

As abovementioned, *Rickettsia* spp. were found in 50% of the samples (Fig. 2). In Masjed-e-Soleyman city, there were no positive reports of the presence of *Rickettsia* spp., and the highest number of positive samples was from Shushtar city.

Phylogenetic analysis of detected Rickettsia

Among the positive samples detected by PCR for the presence of *Rickettsia* spp., 10 samples were randomly sent for sequencing. After editing and trimming using the software, all of them were 100% compatible with the species pre-registered in the NCBI database. Specifications performed by sequencing the bacterial genes proved the presence of three different species of *Rickettsia* in Khuzestan province. Comparison of the *gltA* gene sequences of the collected samples with the genes already registered in the GeneBank database demonstrated the existence of 6 samples of *Rickettsia aeschlimannii*, 3 samples of *Rickettsia massiliae*, and one sample of *Rickettsia conorii*. These sequences showed high similarity (100%) to *Rickettsia aeschlimannii* (accession numbers MH932014, MT293342, MH932015), *Rickettsia massiliae* (accession numbers MN223696 and HM149282), and *Rickettsia conorii* (accession number AE006914).

The evolutionary history was inferred using the maximum likelihood method with the Kimura 2-parameter model. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% of bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are

shown below the branches. Initial tree(s) for the heuristic search were obtained automatically as follows. When the number of common sites was less than 100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 21 nucleotide sequences. All positions including gaps and missing data were eliminated. There was a total of 186 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [15–17] (Fig. 3).

Discussion

Ticks play an important role in the development of many diseases between humans and animals [18]. According to the results obtained for this study, the frequency of ticks on sheep was significant in Khuzestan province. As far as we know, 46 species of ticks (10 Argasidae and 36 Ixodidae) have been identified in Iran so far [19]. The ticks found in this study included 6 species of two genera, namely, *Rhipicephalus* and *Hyalomma* (Table 2). All the ticks of this study were collected from sheep and phylogenetic characterization was done on them.

Although various methods have been used for the identification of *Rickettsia* spp., PCR-based molecular approaches have been one of the most sensitive, effective, and fast tools for the detection of *Rickettsia* spp. in vectors that affect both humans and animals [20]. In recent years, *gltA* gene has been analyzed in many articles for the characterization of *Rickettsia* spp.[21–26]. In other words, previous studies have concluded that this marker gene (*gltA*) is one of the best genes for the diagnosis and characterization of *Rickettsia* spp. [27]. Given that one of the main objectives of this study was detecting the presence of *Rickettsia* and identifying the confirmed cases (by DNA fingerprinting), we did sequence analysis for the *gltA* gene. The identification of *Rickettsia* spp. via targeting the *gltA* gene in half of the pooled ticks that were randomly collected from sheep could be considered an early warning for diseases caused by this pathogen. Considering the importance of animal husbandry in Khuzestan province, Iran, and the special climatic conditions of this province (which is the basis for the expansion and reproduction of arthropods such as ticks) and also the results of the present study, the need for further study of tick-borne diseases among human populations and animals in this province is becoming more apparent. The importance of this research increases when previous research shows that not many studies on tick-borne diseases have been carried out in this province [19] considering the high livestock population and accordingly high proportion of arthropods in this region.

Phylogenetic analysis indicates that the *Rickettsiae* identified in this study were very similar to three genera of this species. To the best of our knowledge, there have been only a few reports of *Rickettsia* species isolated from ticks in Iran so far. The reported species are *Rickettsia slovaca* and *Rickettsia hoogstraalii* and some cases of *Rickettsia* spp. [19]. The first reports of *Rickettsia aeschlimannii* date back to the 1990s [28]. Our analyses showed that the sequenced *gltA* gene bore the most similarity to *Rickettsia aeschlimannii* which was also confirmed by the phylogenetic analyses of the sequenced genome. Other similarities in DNA sequences was found for *Rickettsia massiliae* and only one sample showed sequence similarity to *Rickettsia conorii*. *Rickettsia massiliae* first was identified in *Rhipicephalus* species in Marseilles, France, in the 1990s [29]. According to the GenBank database, seven subspecies of *Rickettsia conorii* have been recorded so far, and the first case of this species was reported in England in the 1980s [30]. It is noteworthy that the gene sequence and characterization of *Rickettsia* spp. was performed based on only one gene (*gltA*)in this study, however this is the first report on the isolation of Rickettsia spp. (*R. aeschlimannii, R. massiliae, R. conorii*) from ticks in Iran.

Conclusions

Due to the animal and human population densities in Khuzestan province, Iran, as well as the special climate of this region, which is prone to the growth and reproduction of various species of arthropods, it is important to study tick-borne diseases in human and animal populations, especially zoonotic diseases in Khuzestan province. In this study, six species of hard ticks (collected from sheep in this region) were identified as *Hyalomma marginatum*, *Hyalomma anatolicum*, *Hyalomma dromedarii*, *Hyalomma schulzei*, *Rhipicephalus bursa*, and *Rhipicephalus turanicus*. The ticks were identified through phylogenetic analyses based on *gltA* gene. In molecular analysis of *gltA* gene (using PCR technique), half of the pooled ticks were positive for having *Rickettsia* spp., which is a significant finding. Sequencing analyses of *gltA* gene in randomly selected samples for the presence of *Rickettsia* spp. revealed a complete coincidence with three species of *Rickettsia* already registered in the GenBank database. Hence, in this study, the presence of three rickettsial species, namely, *Rickettsia aeschlimannii*, *Rickettsia massiliae*, and *Rickettsia conorii*, in ticks was reported for the first time in Iran.

Abbreviations

PCR
polymerase chain reaction
SFG
spotted fever group
TRG
transitional group
IFA
indirect immunofluorescent antibody
ELISA
enzyme-linked immunosorbent assay.

Declarations

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Authors' contributions

AA and PM did sample collection and correspondence with the laboratory. JS led the project. VJ wrote the manuscript and summarized the topics. AS helped with gene analysis and phylogenetic drawing. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no conflicts of interests.

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Figures

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Figure 1

Ticks collection sites in Khuzestan. The cities from where ticks are collected are darkened.

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Figure 2

Agarose gel electrophoresis of *gltA* gene amplified by PCR. M100: Ladder, DX: Samples, TP: Positive control, T0: Negative control.

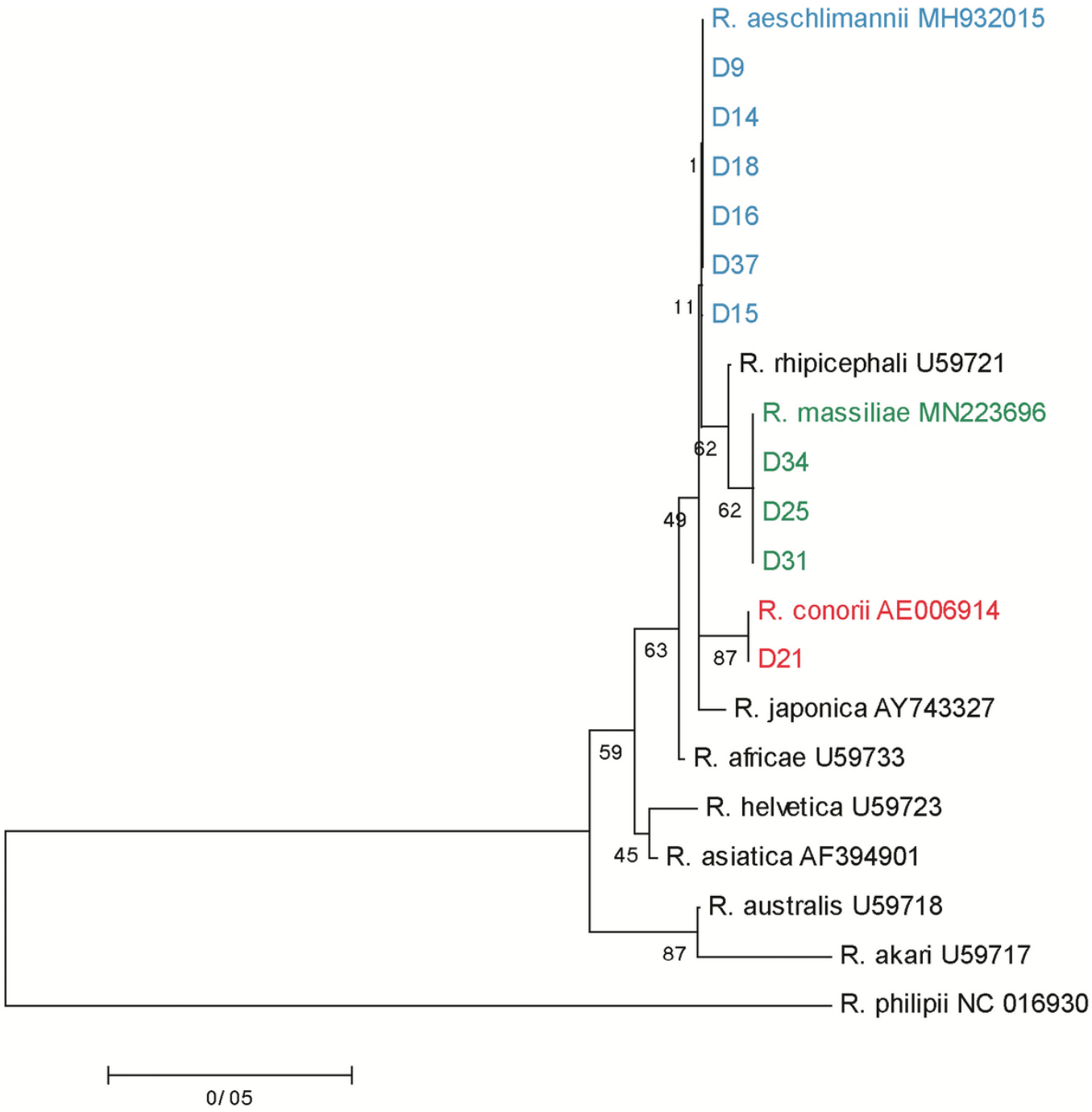

Figure 3

Phylogenetic analysis by maximum likelihood method. DX: Samples.