Hyalomma spp. ticks and associated Anaplasma spp. and Ehrlichia spp. on the Iran-Pakistan border

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Abstract
Background: Anaplasmosis and ehrlichiosis are tick-borne diseases affecting humans and livestock, particularly in tropical and subtropical regions. Animal husbandry is the main activity of people on the borders of Iran and Pakistan, with thousands of cattle crossing the border each week.

Methods: PCR and sequencing of the 16S rRNA gene was used to determine the percentage and geographical distribution of the pathogens carried by Hyalomma spp. (n = 306) collected from 126 goats, cattle and camels in the region between November 2017 and late March 2018.

Results: In total, 1124 hard ticks including 1020 Hyalomma spp. ticks belonging to six species (Hyalomma anatolicum, Hyalomma asiaticum, Hyalomma marginatum, Hyalomma dromedarii, Hyalomma schulzei, and Hyalomma detritum) were found on the borders of Iran and Pakistan, with H. anatolicum being the most prevalent tick species. Anaplasma spp. and/or Ehrlichia spp. DNA was found in 68.3% of the engorged tick specimens (n = 256). Sequencing of a subset (12.6%) of PCR-positive samples revealed Anaplasma ovis, Anaplasma marginale, and Ehrlichia ewingii DNA in 81.8%, 9.1%, and 9.1% of the ticks, respectively. To our knowledge, this is the first report of E. ewingii, an important human pathogen, in Iran.

Conclusions: Based on molecular analysis, three pathogenic Anaplasmataceae were detected in six Hyalomma spp. parasitizing cattle, goats and camels, confirming the presence of these pathogens along the Iran-Pakistan border.

Keywords: Anaplasmosis, Ehrlichiosis, Hard ticks, Iran, Pakistan, Tick-borne diseases

Background
Ticks are considered to be the second most common vector of human diseases worldwide after mosquitoes, but they are the most important vectors of disease-causing pathogens in domestic and wild animals. Indeed, ticks transmit a wide variety of pathogens, including viruses, bacteria and protozoa, to their vertebrate hosts [1].

For instance, anaplasmosis is caused by a number of established and emerging tick-borne pathogens, including Anaplasma phagocytophilum, Anaplasma marginale, Anaplasma ovis, Anaplasma centrale, Anaplasma bovis, Anaplasma capra, Anaplasma platys, and ‘Candidatus Anaplasma camelii’, which are found worldwide, particularly in tropical and subtropical regions, including Iran [2–6]. Anaplasma spp. may infect humans and a broad range of wild and domestic mammals, including horses, dogs, cats, deer, goats, sheep, cattle, camel, and other ruminants [4, 6, 7]. Ehrlichiosis caused by Ehrlichia spp. is another tick-borne disease [8], and some Ehrlichia spp. have been identified as pathogens in humans. For example, Ehrlichia chaffeensis causes human monocytic ehrlichiosis and Ehrlichia ewingii is an agent of human granulocytic ehrlichiosis [9]. The zoonotic

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nature of the human ehrlichiosis is supported by reports of natural infections with the same *Ehrlichia* spp. in dogs, deer, horses, and rodents [10]. Granulocytic ehrlichiosis in humans has been described in immunocompromised and immunocompetent patients, causing headache, fever, myalgia, vomiting, nausea, acute renal failure, thrombocytopenia, leukopenia and increased liver enzyme activities [11–13].

Iran has a variety of climates: a mild cold climate in the high mountains; continental and arid climates in the plateau; a mild and damp climate on the Caspian coast; and a hot desert climate on the southern coast and in the south-east. Each climate may provide conditions suitable for the development of different tick species, which probably explains the difference in the epidemiology of anaplasmosis and ehrlichiosis in different regions [14]. Sistan and Baluchistan Province in the south-east corner of Iran has a long border with Pakistan and Afghanistan, where infectious diseases do not respect international boundaries [15]. Animal husbandry is one of the main activities of the people in the province, and every week thousands of head of livestock, including sheep, goats, cows, camels, and buffaloes, cross the borders between the countries [16].

Although *Anaplasma* spp. and *Ehrlichia* spp. have been identified by molecular assays in livestock in Iran, their presence in their vectors has been much less studied. There have been only a few studies on the detection of *Anaplasma* spp. and *Ehrlichia* spp. in ticks in Iran, which reported the presence of infected ticks in the north and other regions of Iran [14, 17, 18].

Ixodid ticks play an important role in maintaining *Anaplasma* spp. and *Ehrlichia* spp. in nature [19, 20]. Nonetheless, while *Hyalomma* spp. have been suggested as vectors of *A. marginale* [21], there has been little research on their possible involvement in *Anaplasma* spp. and *Ehrlichia* spp. transmission. Here, we report the occurrence of *Hyalomma* spp. infesting various domestic animals in the Iranian Province of Sistan and Baluchistan, with notes on *Anaplasma* spp. and *Ehrlichia* spp. infections in these ticks.

**Methods**

**Sample collection and tick identification**

The regions investigated include three districts of Sib & Suran County (Hiduj district; geographical coordinates 27°00′02″N, 62°07′01″E), Sarbaz County (Pishin district; geographical coordinates 30°35′5.31″N, 66°59′41.19″E) and Chabahar County (Negour district; geographical coordinates 25°23′20.84″N, 61°8′18.96″E), all of which are located in the south-east of Iran, close to the border with Pakistan. The collection of ticks was performed in three randomly selected major animal husbandry farms in each district, between November 2017 and late March 2018, i.e. at the season when adult ticks are most active in the region. In total, 1124 ticks were collected from goats (*n* = 80), cattle (*n* = 34), and camels (*n* = 12). Tick collection was arbitrarily conducted, depending upon the availability of the domestic animals for a 15-min examination per animal, but efforts were made to obtain a widespread representative sample from the different animal species included in the study. All ticks were transferred to vials and labelled according to their geographical origin and the animal from which they were obtained. The collected ticks were subsequently transferred to the Entomology Laboratory in the School of Public Health at the Tehran University of Medical Sciences and were identified to species level, based on microscopic observation of external morphological characteristics according to the identification keys [22–24].

**DNA extraction**

After species identification, the ticks were sterilized by immersion in 70% alcohol, washed in distilled water, dried on filter paper in a laminar-flow hood, and then stored at −80°C until the DNA extraction.

Individual ticks were frozen in liquid nitrogen and then ground in an Eppendorf microtube. The DNA was then extracted using the G-spin Genomic DNA Extraction Kit (iNtRON Biotechnology, South Korea), according to the manufacturer’s instructions. The precipitated DNA samples were quantified using a spectrophotometer (Thermo Scientific™ NanoDrop™ One, Wilmington, DE, USA), and their integrity was assessed using 1% agarose gel electrophoresis. Then, the extracted DNA was suspended in sterile distilled water and stored at −20°C. Of the ticks collected, all the engorged ticks were analysed, and a subset (*n* = 50) of the unfed ticks were also tested for the presence of *Anaplasma* spp. and *Ehrlichia* spp.

**Molecular detection of *Anaplasma* spp. and *Ehrlichia* spp. in ticks**

The 16S rRNA gene of *Anaplasma* spp. and *Ehrlichia* spp. was amplified using the nested PCR protocol designed by Rar et al. [25]. The forward and reverse primers for initial reactions were Ehr1 (5′-GAA CGA ACG CTG GCG GCA AGC-3′) and Ehr2 (5′-AGT A(T/C)C G(A/G)A CCA GAT AGC CGC-5′) and for nested reactions were Ehr3 (5′-TGC ATA GGA ATC TAC CTA GTA G-3′) and Ehr4 (5′-CTA GGA ATT CCG CTA TCC TCT T-3′). The size of final PCR products was 524 base pairs (bp). PCR reactions were performed in a 25 μl reaction mixture, containing 12.5 μl of the Hot Start Taq 2 × Master Mix, 1 μl of each of the forward and reverse primers, 2 μl of DNA template
and 7.5 μl of nuclease-free water to bring the volume to 25 μl. PCR reactions were performed in a DNA Mastercycler Personal PCR machine (Eppendorf, Germany) and PCR conditions were: 15 min at 95 °C for initial denaturation, then 60 s at 94 °C for denaturing, 60 s at 57 °C for annealing, and 60 s at 72 °C for extension for 35 cycles, and then a final extension for 10 min at 72 °C. The products (2 μl) of the first PCR were used as the template for the second PCR, which was carried out under the same conditions and reaction mixture as the first, except that nesting primers were used [25]. Two negative controls (one of double-distilled water and one of an un-infected tick DNA template) and a positive control (a confirmed sequenced A. bovis DNA isolated from a tick) were included in each PCR run. To ensure the presence of viable DNA, the barcoding region of the tick COI gene was used as endogenous control [26].

To assess the presence of expected bands for Anaplasma spp. and Ehrlichia spp., the PCR products were electrophoresed in a 1.5% agarose gel, and the size of each PCR product was estimated using a 100 bp ladder and visualized with a UV transilluminator.

DNA sequencing and phylogenetic analysis
The positive PCR products were purified, and bidirectional DNA sequencing was performed by the Sanger method, using the same inner PCR primers that were used for nested PCR amplification. The sequences obtained were edited and assembled using Chromas (http://www.technelysium.com.au/chromas.html) and BioEdit [27] software to construct consensus sequences, which were then analysed using the NCBI Blast database (Nucleotide collection) (https://www.ncbi.nlm.nih.gov/). The consensus high-confidence sequences were aligned with other sequences that were available in GenBank, using multiple sequence alignments available in CLUSTAL Omega (https://www.ebi.ac.uk/Tools/msa/clustalo). For phylogenetic analysis, the representative sequences of Anaplasma spp. and Ehrlichia spp. obtained in this study were combined with a subset of available representative sequences of all Anaplasma spp. and E. ewingii, using Spiroplasma sp. sequences as an outgroup [28-31]. Details of these sequences are shown as supplementary data (Additional file 1: Table S1). All the DNA sequences used for alignment were cut to obtain a consistent region (470 bp), and phylogenetic analyses were performed using the MEGA 7 software [32]. The data were aligned and the maximum likelihood method was employed to construct a phylogenetic tree. The same program was utilized to evaluate the stability of the obtained tree through bootstrap analysis with 1000 replicates.

Results
Tick species and abundance
A total of 1124 hard ticks were collected which were classified into three genera: Hyalomma (n = 1020, 90.7%), Rhipicephalus (n = 68, 6.1%), and Dermacentor (n = 36, 3.2%). All of the 1020 ticks collected were morphologically identified as belonging to one of six Hyalomma spp., with Hyalomma anatolicum being the most common species in all three districts. In detail, the tick species were found to be H. anatolicum (n = 462; 228 from cattle and 234 from goats), Hyalomma asiaticum (n = 143; 87 from camels, 25 from goats, and 31 from cattle), Hyalomma marginatum (n = 203; 134 from cattle, 66 from goats and three from camels), Hyalomma dromedarii (n = 188; 46 from goats and 142 from camels), Hyalomma schulzei (n = 17 from goats), and Hyalomma detritum (n = 6 from camels) (Table 1). The average number of ticks on camels, cattle, and goats were 19.8, 11.6, and 4.9, respectively. The collected tick specimens were mix of unfed (75%), partially fed (15.1%), and fully engorged (9.9%). Unfed were very small, often black or brown colour, partially engorged (partially swollen), which means the tick has had a partial blood meal, and engorged (swollen, usually blue-grey in colour) (Table 1).

Anaplasma spp. and Ehrlichia spp. infections in ticks
Using generic EHR primers, a subset (n = 50) of non-engorged tick specimens, and all of the 255 fully or partially engorged tick specimens, comprising 63 males (25%) and 192 females (75%), were tested for the presence of Anaplasma spp. and Ehrlichia spp. The tested specimens were representatives of the ticks collected from different animals in the three regions. The positive results were obtained with 68.6% (175 out of 255) of the engorged specimens, whereas non-engorged specimens were all negative. The species, number, and percentage of pathogen infection in Hyalomma spp. ticks at each collection site are shown in Table 2. A sub-set of positive PCR amplicons was sequenced and the consensus sequences were deposited in GenBank (accession numbers shown in Table 3). Details of the Anaplasma spp. and Ehrlichia spp.-positive samples are provided in Table 3.

Sequence and phylogenetic analysis
Analysis of the sequence data showed that the highest percentage of sequences obtained corresponded to A. ovis (18 out of 22, 81.8%). All of the strains of A. ovis detected in this study were identical, both to each other and to the other Iranian strains, and to the strains from China (GenBank: MG869525) and Russia (GenBank: KC484563). In addition to A. ovis, two A. marginale (9.1%) and two E. ewingii (9.1%) positive samples were detected. Sequences of A. marginale
found in this study were identical to sequences from the USA, Brazil, Cuba, Iraq and China (GenBank: CP000030, CP023731, MK804764, MH551233, and KU586075, respectively). Similarly, the detected strains of *E. ewingii* obtained in this study were identical or highly similar to the isolates from Australia, the USA, Brazil, Thailand, and China (GenBank: NR_044747, NR_044747, HQ908082, NR_044747, and MN148615, respectively). The detected strains of *A. marginale* and *or* *E. ewingii* showed sequence similarities of 99–100% with sequences available in GenBank.

Phylogenetic analysis revealed that the *Anaplasma/Ehrlichia* spp. detected in this study clustered into four different clades, including (I) *A. ovis*, (II) *A. marginale*, (III) *A. platys-A. phagocytophilum-A. odocoilei*, and (IV) *A. centrale-A. capra* (Fig. 1). Both *E. ewingii* sequences obtained herein were associated with the branches of clade III.

**Discussion**

This is the first comprehensive study of the *Hyalomma* spp. ticks from domestic animals and their associated *Anaplasma* spp. and *Ehrlichia* spp. along the Iran-Pakistan border. The results show that domestic animals in this area are infested with at least six *Hyalomma* spp., in addition to ticks belonging to other genera, which were not identified to species level herein. Moreover, at least two *Anaplasma* spp. and one *Ehrlichia* sp. were detected in the collected ticks. These results are generally in agreement with previous observations in Pakistan and in other parts of Iran [17, 33–36].

Herein, more than 90% of livestock-infesting adult ticks belonged to the genus *Hyalomma*, which agrees with the
result of other researchers in Baluchistan of Pakistan where 525 out of 529 (99.2%) of the ticks belonged to this genus [37]. Among Hyalomma spp., H. anatolicum was the most common species and presented the widest geographical range. This three-host tick species was previously reported to be the most prevalent hard tick from different parts of Pakistan and Iran [17, 18, 33–35, 38–43].

The ecology of different Hyalomma spp. ticks may influence the risk of tick-borne diseases in the region [39, 41, 44–46]. The intense animal movements across Iranian regions and neighbouring countries may also facilitate the spread of these ticks. The infected ticks found in this study were feeding on the animals at the time of collection and were, therefore, potentially transmitting (or ingesting) Anaplasma spp. and E. ewingii while feeding. Therefore, the possibility that these ticks play a role in the transmission of these agents to domestic animals in the study area requires further investigation.

Except for E. ewingii, the pathogens found herein have previously been detected in ticks in several regions of Iran [14, 18, 33, 41–43]. However, we found the prevalence of Hyalomma spp. ticks carrying Ehrlichia spp. or Anaplasma spp. to be 68%, which is higher than previously reported from other parts of the country, including 4.6% in the south-eastern and north-western regions [33], 25% in the north [35], 43.84% in Meshkin-Shahr, Ardebil Province [43], and 55.5% in the four provinces of East Azerbaijan, Gilan, South Khorasan and Yazd [14]. These differences in tick infection rates could be related to several factors, including sampling methods, diagnostic methods, season, tick species, and feeding status of the ticks. Here, although the nested PCR technique used is known to be highly sensitive and specific, more nested PCR-positive samples should have been sequenced to determine the actual prevalence of Anaplasma spp. and Ehrlichia spp. infection in the ticks.

The results of this study, taken together with the above literature, have shown that different Hyalomma spp. could be involved in the transmission of pathogens to cattle, goats and camels in different regions of Iran. In addition to the Hyalomma spp. ticks, other ticks, including Rhipicephalus bursa, Rhipicephalus sanguineus sensu lato, Dermacentor marginatus, Haemaphysalis erinacei, and Ixodes ricinus have been reported as proven or putative vectors of different bacteria of the family Anaplasmataceae in Iran [17, 18, 33, 43].

The fact that no Anaplasma spp. or Ehrlichia spp. DNA was detected in the non-engorged specimens may suggest that there is little or no transovarial transmission of

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Table. Details of Anaplasma spp. and Ehrlichia spp. infections in Hyalomma spp. from three districts of Sistan and Baluchistan Province, south-east corner of Iran, 2017–2018

| Tick species          | Tick sex | Host       | District          | Pathogen species | GenBank ID number |
|-----------------------|----------|------------|-------------------|------------------|------------------|
| Hyalomma anatolicum   | Female   | Cattle     | Chabahar          | Anaplasma ovis   | MK310471         |
| Hyalomma anatolicum   | Female   | Cattle     | Sib and Suran     | Anaplasma ovis   | MK310472         |
| Hyalomma anatolicum   | Female   | Cattle     | Sib and Suran     | Anaplasma ovis   | MK310473         |
| Hyalomma anatolicum   | Male     | Goat       | Sib and Suran     | Anaplasma ovis   | MK310474         |
| Hyalomma anatolicum   | Female   | Goat       | Sarbaz            | Anaplasma ovis   | MK310475         |
| Hyalomma anatolicum   | Female   | Goat       | Sarbaz            | Anaplasma ovis   | MK310476         |
| Hyalomma asiaticum    | Female   | Cattle     | Sib and Suran     | Anaplasma ovis   | MK310477         |
| Hyalomma asiaticum    | Male     | Goat       | Sib and Suran     | Anaplasma ovis   | MK310478         |
| Hyalomma asiaticum    | Female   | Cattle     | Chabahar          | Anaplasma ovis   | MK310479         |
| Hyalomma dromedani    | Female   | Goat       | Sarbaz            | Anaplasma ovis   | MK310480         |
| Hyalomma dromedani    | Male     | Camel      | Sarbaz            | Anaplasma ovis   | MK310481         |
| Hyalomma marginatum   | Male     | Camel      | Chabahar          | Anaplasma ovis   | MK310482         |
| Hyalomma marginatum   | Female   | Camel      | Chabahar          | Anaplasma ovis   | MK310483         |
| Hyalomma marginatum   | Female   | Camel      | Sib and Suran     | Anaplasma ovis   | MK310485         |
| Hyalomma detritum     | Male     | Camel      | Sib and Suran     | Anaplasma ovis   | MK310486         |
| Hyalomma schulzei     | Female   | Goat       | Sarbaz            | Anaplasma marginale | MK310487 |
| Hyalomma schulzei     | Male     | Goat       | Sarbaz            | Anaplasma marginale | MK310488 |
| Hyalomma anatolicum   | Male     | Cattle     | Chabahar          | Anaplasma ovis   | MK310489         |
| Hyalomma dromedani    | Female   | Camel      | Sib and Suran     | Ehrlichia ewingii | MK310490         |
| Hyalomma dromedani    | Male     | Camel      | Sib and Suran     | Ehrlichia ewingii | MK310491         |
| Hyalomma marginatum   | Female   | Cattle     | Chabahar          | Anaplasma ovis   | MKH80603         |
the bacteria in *Hyalomma* spp. ticks. Moore et al. [47] showed some evidence of transovarial transmission of *A. ovis* in *Dermacentor nuttalli*, although they did not detect any *A. ovis* DNA in larvae or nymphs. Another study [48] demonstrated the transovarial transmission of *A. marginale* in *Rhipicephalus microplus*, and that infected larvae can transmit the infection to susceptible hosts.

In this study, *A. ovis* was detected in five tick species: *H. anatolicum*, *H. asiaticum*, *H. marginatum*, *H. dromedarii*, and *H. detritum*, which were collected from cattle, goats, and camels. On the other hand, *A. marginale* and *E. ewingii* were found only in *H. schulzei* and *H. dromedarii* collected from goats and camels, respectively. Both *A. ovis* and *A. marginale* are important livestock pathogens [49], whereas *E. ewingii* is an important human pathogen [10, 50, 51] and is reported for the first time in Iran. However, the presence of *E. ewingii* should be confirmed by sequencing other genes (e.g., groEL), which was not possible in this study.

![Fig. 1](image)

**Fig. 1** Phylogenetic relationships inferred from 470 bp of 16S rRNA gene sequences of *Anaplasma* spp. and *Ehrlichia* spp. detected in this study and others available in GenBank. The representative sequences obtained in this study are marked with red circles. The tree was computed by maximum likelihood (MEGA 7.0 software). Bootstrap values (> 50%) are shown on nodes. For sequences from Iran, the county or province name is also provided.
Conclusions

In conclusion, we confirmed the presence of *A. ovis*, *A. marginale* and *E. ewingii* in *Hyalomma* spp. ticks collected from cattle, goats and camels on the Iran-Pakistan border. Further research is needed to confirm the role of these *Hyalomma* spp. and other ticks in the transmission of these pathogens in this region.

Abbreviations

PCR: Polymerase chain reaction; Rps: Base pair; COI: Cytochrome oxidase subunit one; UV: Ultraviolet; NCBI: National Center for Biotechnology Information.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13071-021-04956-3.

Additional file 1: Table S1. Details of the bacterial species used for phylogenetic analysis in this study. *D. Dermacentor*, *Rh. Rhipicephalus*, *Hy.*, *Hyalomma*, *Ha.*, *Haemaphysalis*.

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Authors' contributions

NC performed the whole study and wrote the manuscript. FK performed the phylogenetic analysis. MK helped in molecular analysis of data. JN made major contributions to study design and sample collection. MAO was the guarantor and analysed the data, and was the major contributor in the editing of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analysed during this study are included in this published article. The sequence data are available at NCBI database.

Declarations

Ethics approval and consent to participate

All procedures were performed in accordance with the terms of the Iran Animals (Scientific Procedures) Act Project License and were approved by the Tehran University of Medical Sciences Ethical Review Committee, reference number: IR.TUMS.SPH.REC.1395-926. Due to social concerns, consent to participate was obtained verbally from the owners of the animals used in this study, which was approved by the committee.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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