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Molecular identification of Anaplasma / Ehrlichia spp based on 16S rRNA gene in Hyalomma ticks in border line of Iran-Pakistan

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Abstract

Background Anaplasmosis / Ehrlichiosis are tick-borne diseases affecting human and livestock in tropical and subtropical regions of the world. Due to infection both animals and humans and monitoring of ticks and the pathogens they carry, an extensive survey was conducted in border line of Iran-Pakistan of Sistan and Baluchistan, southeast corner of Iran in 2016-2017, where animal husbandry is the main activity of people and every week thousands of cattle cross the borders into the countries. The aim of the survey was to determine the prevalence and geographical distribution of Anaplasmosis / Ehrlichiosis agents in Hyalomma spp hard ticks. Ticks were collected, identified and processed for Anaplasma / Ehrlichia spp DNA detection. Results Six Hyalomma species were found in the region, where Hy. anatolicum was the most prevalent species collected on goats, cattle, and camel. Anaplasma / Ehrlichia genomes were found in 68.3% of the specimens. Anaplasma ovis, A.marginale, and E.ewingii DNAs prevalence were 81.82, 9.09, and 9.09% in the infected ticks respectively. DNA sequence and phylogenetic analysis of the 16SrRNA gene confirmed the detection of these three anaplasmosis agents while they had 99-100% identity with the strains previously reported in genbank from different parts of the world. Conclusion Because A.ovis and A.marginale are important livestock pathogens, and E.ewingii is an important human pathogen, both farmers and people in border line of the countries which engaged in livestock need to be made aware of the risks of tick infestation and the tick-borne disease they transmit.

Background

Ticks (Acari: *Ixodidae*) are considered to be second worldwide to mosquitoes as vectors of human diseases [1], but they are the most important vectors of disease-causing pathogens in domestic and wild animals. Ticks transmit a wide variety of pathogens to vertebrates including viruses, bacteria and protozoa.

Anaplasma Theiler 1910,is intracellular, gram negative bacteria and representatives of the order *Rickettsiales* classified into *Anaplasmataceae* family. It is emerging tick-borne pathogen that causes Anaplasmosis in humans and many mammalian species worldwide particularly in tropical and subtropical regions, including Iran [2]. Several species of *Anaplasma* have been detected in domestic animals, including *A. phagocytophilum* Foggie 1949, *A.marginale* Theiler 1910, *A. ovis* Lestoquard 1924, *A. centrale* Theiler 1911, *A. bovis* Donatien and Lestoquard 1936, and *A. platys* Dumler et al. 2001, that *A. phagocytophilum* is human pathogen [3]. The reservoir for *Anaplasma* includes a broad range of wild and domestic mammals. Ehrlichiosis is another tick-borne disease, closely related to anaplasmosis, caused by *Ehrlichia spp*, and transmitted by ticks [4].

Due to the expanse and different climates in Iran, each climate may harbour several different ticks. This situation makes the difference in the epidemiology of anaplasmosis /ehrlichiosis in different regions. The Sistan and Baluchestan province in southeast corner of Iran has a wide border line with Pakistan and Afghanistan where infectious diseases do not respect international boundaries. Animal husbandry is one of the main activities of people in the province. Every week thousands of cattle including sheep, goats,

cows, and buffaloes cross the borders into the countries. It is suggested about one million animals are trafficked yearly to Iran [5].

Nested polymerase chain reaction (PCR) has been displayed to be a highly sensitive and specific test for diagnosis of anaplasmosis /ehrlichiosis [6]. Among deferent markers, 16S-rDNA gene is one the common marker used for the bacterial genotyping [7].

Despite identification of *Anaplasma / Ehrlichia* species in livestock based on the molecular assays, there is many unbeknownst about the vectors of the disease. There have been only a few studies to detect tick anaplasmosis / ehrlichiosis infections in Iran and these studies reported Anaplasma / Ehrlichia infection in ticks in north and counter parts of Iran [8-12]

Literature review has revealed Ixodid ticks play an important role in maintaining Anaplasma/Ehrlicia species in nature. It has been reported that *Ixodes* Latreille , 1795*, Rhipicephalus* Koch, 1844, *Dermacentor* Koch, 1844, and *Amblyomma* Koch, 1844 genera are the main vectors of *Anaplasma / Ehrlichia* bacteria in different regions of the world [13]. Although more is known about *Hyalomma spp* as responsible for the transmission of Crimean-Congo hemorrhagic fever but there is little research on the anaplasmosis/ehrlichiosis and *Hyalomma* spp and whether they are the probable vectors of *Anaplasma / Ehrlichia spp* is still unproven, thus necessitating this study. Our objective was to study the presence and diversity of *Anaplasma / Ehrlichia spp* in *Hyalomma spp* ticks isolated from different domestic animals in Sistan and Baluchestan province, which is one of the animal husbandry poles of the country.

Results

Tick species and abundance

Hard tick specimens were collected in rural areas of Chabahar, Sarbaz and Sib & Suran districts situated in the southeast corner of Iran and were tested for presence of *Anaplasma* by screening nested PCR assays with specific primers against the 16S rRNA gene of the bacteria. A total of 1020 *Hyalomma* ticks were collected in the study areas. These ticks belonged to six species including *Hy. anatolicum* Koch, 1844 (n=462, 228 from cattle and 234 from goat), *Hy. asiaticum* Schulze & Schlottke, 1930 (n=43, 87 from camel, 25 from goat, and 31 from cattle), *Hy. marginatum* Koch, 1844 (n=203, 134 from cattle, 66 from goat and 3 from camel), *Hy. dromedarii* Koch, 1844 (n=188, 46 from goat and 142 from camel),*Hy. schulzei* Olenev, 1931 (n=17, 17 from camel), and *Hy. detricum* Schulze, 1919(n=6, 6 from camel) (Table 1).

Hyalomma anatolicum was the most prevalent species in all three districts of the study area. The number of *Hy. analiticum* ticks were significantly (P<0.01) higher than other five species including the second prevalent species (*Hy. dromedarii*). There were no significant variations between the frequencies of *Hy. marginatum*, *Hy. dromedarii*, and*Hy. asiaticum* (Fig. 1). Due to low number of *Hy. detricum* and *Hy. schulzei*, these two species were excluded from analysis.

Anaplasma / *Ehrlichia* infection in ticks

By use of broad-spectrum EHR primers, 256 out of 1020 collected ticks (25%) were tested for the presence of *Anaplasma*'s or related species *16SrRNA* gene. Result of PCR assays revealed presence of *Anaplasma* / *Ehrlichia* genomes in 68.3% (175 out of 256) of the selected specimens. The species, number and prevalence of *Anaplasma* / *Ehrlichia* in *Hyalomma spp* ticks at each collection site are shown in Table 2. The rate of *Anaplasma spp* / *E.ewingii* infections was similar (67.8-69.2%) in *Hy. anatolicum, Hy. dromedarii, Hy. asiaticum*, and *Hy. marginatum* specimens. This rate was higher in *Hy. detricum* (2 out of 2, 100%) and lower in *Hy. schulzei* (2 out of 5, 40%) than other four species. A subset of positive PCR specimens against *Anaplasma* / *Ehrlichia* genome in ticks were sequenced and the consensus sequences were deposited in Gen Bank. Details of the *Anaplasma* / *Ehrlichia spp* positive samples are listed in Table 3.

Sequence and phylogenetic analysis

Analysis of the sequence data showed that *A.ovis* was the most prevalent (18 out of 22, 81.82%) *Anaplasma* species in the study area. All of the strains of *A.ovis* isolated in this study were identical to each other and to the other Iranian strains and to the strains from China (Accession number: MG869525) and Russia (Accession number: KC484563). In addition of *A. ovis*, two *A. marginale* isolates (9.09%) and two *E. ewingii* isolates (9.09%) were found in the selected ticks. Sequences of *A. marginale* in this study were identical to their counterparts from USA, Tajikistan, and China. It was the same for the isolated strains of *E. ewingii* obtained in this study which were identical with the isolates from Australia, USA, Brazil, Thailand, Iran, China, and Uganda. The sequence similarities between the isolated strains of *A. marginale* and or *E. ewingii* with the available data in Genbank was more than 99% to 100%.

The phylogenetic analysis of *Anaplasma* / *Erhrlichia* species was performed using the sequences obtained in this study in combination with the available data retrieved from Genbank. The bacterial species were clustered in four different clades includingI) *A. ovis*, II) *A. marginale,* III) *A. platys-A. phagocytophilum-A. odocoilei,* IV) *A. centrale-A. capra* (Fig. 2). Interestingly all *E. ewingii* isolates were associated with the branches of clade III. This analysis showed no clear geographical pattern or further association with host among the *A. ovis*, *An. marginale,* or *E. ewingii* isolates.

Discussion

This is the first comprehensive study of *Hyalomma* ticks attached to domestic animals and their associated *Anaplasma* / *Ehrlichia* species conducted in border line of Iran-Pakistan, southeast corner of Iran. The results show that there are six infesting *Hyalomma spp* ticks and that there are at least three anaplasmosis agent that can be transmitted through a tick bite. This result is generally in accordance with observations on hard ticks and pathogens from animals in other parts of the country [8, 14, 15], while there are some distinctive results in this study.

In this study, *Hy. anatolicum*-infested animals were found the most common and the widest geographical range. This species were reported as the most prevalent hard tick previously from most parts of the country including borderline of Iran-Iraq [16], centre [17], south-eastern and north-western [15, 18], north [19], northwest [20, 21], southwest [22], and south [23] of Iran.

Hyalomma marginatum had the second greatest prevalence in the study area. Additional *Hyalomma* tick species collected from animals in this study included species of Hy. asiaticum, Hy. dromedarii, Hy. detricum and Hy. schulzei. There have been reports of these species from different parts of Iran [17, 24].

The present study contributes new information about the risks of high diverse *Hyalomma* infestation of domestic animals in south-eastern Iran. This situation may address the animal traffic from neighbouring countries that may lead to more frequent encounters with these tick species. The ticks in this study were feeding on the animals at the time of collection and were therefore potentially transmitting any *Anaplasma spp* to the animals while feeding. However, the possibility that these tick play a significant role in anaplasmosis transmission to domestic animals where it is endemic requires further investigation. Hyalomma spp ticks in this region carried the *Anaplasma* and *Ehrlichia* agents, including A. ovis, A. marginale, and *E. ewingii*. These Anaplasmataceae pathogens were previously detected using molecular methods in hard ticks in several regions in Iran [11, 14, 15, 25]. The prevalence of *Hyalomma* spp with *Ehrlichia* / Anaplasma *spp* DNA in this study was 68%. This value is higher than the rate of infection in the previous reports from other parts of the country. The prevalence of infection were reported as 4.6% [15], 5.1% [26], 6% [27], 25% [14], 25.8% [10], 26.4% [9], 43.84% [28], and 55.5% [12].

Result of this study and above literature showed that different species of *Hyamlomma* could be one of the primary carriers and reservoirs for *Anaplasma / Ehrlichia spp* in the country. In addition to *Hyalomma spp* ticks, other hard ticks including *Rhipicephalous bursa, R. sanguineous, Dermacentor marginaus, Haemaphysalis erinacei, Ixodes ricinus* [8, 9, 15, 28] are reported as vector of different tick-borne bacteria of the family Anaplasmataceae. However, in other parts of the world, the most important vector of anaplasmosis belongs to different species of *Ixodes* genus; for example Ixodes ricinus in Europe, *I. persulatus* in Eastern Europe and Asia, and *I. scapularis* in North America [29].

In this study a high prevalence of the anaplasmataceae species (81.82%) was of *A. ovis* DNA. This bacterium was isolated from five tick species of *Hy. anatolicum* (31.8%), *Hy. asiaticum* (13.6%), *Hy. marginatum* (18.2%), *Hy. dromedarii* (0.9%),and *Hy. detricum* (0.9%) which were collected from cattle, goat, and camel. *Anaplasma marginale* and *E. ewingii* were found only from *Hy. schulzi* and *Hy. dromedarii* collected on goats and camels respectively. Both *A. ovis* and *A. marginale* are important livestock pathogens whereas *E. ewingii* is an important human pathogen. *Ehrlichia ewingii* mainly infects granulocytes, triggering granulocytic ehrlichiosis in dogs and humans [18, 30]. Granulocytic ehrlichiosis in humans has been described in immunosuppressed as well as immunocompetent patients presenting headache, fever, myalgia, vomiting, nausea, acute renal failure, thrombocytopenia, leukopenia and increased liver enzyme activities [31-33]. Anaplasma ovis is less pathogenic than other Anaplasma species, has got worldwide distribution, and is responsible mostly for small ruminant

anaplasmosis with a low ranking fever [34, 35]. However, it may be an important disease agent for sheep and goats [36, 37]. Fever, anorexia, fatigue, milk reduction and abortion with a low death rate are the common clinical marks of *A. ovis* in infected animals [38]. *Anaplasma marginale* is known as the most important rickettsia disease in cattle. The common clinical signs of the disease are progressive haemolytic anaemia, decrease milk production, abortions, and death. In addition to cattle, other animals including water buffalo, and wild mammals like deer can be infected [39].

Present study revealed circulation of three species of anaplasmatacea in border line of Iran-Pakistan. This finding is in agreement with the results of researchers in border line of Iran-Afghanistan as well as Pakistan who reported different tick-borne pathogens including *Ehrlichia* and *Anaplasma* in ticks [9, 40]. In Pakistan, researchers reported *A. marginale, A. centrale, A. ovis, A. platys*-like organism, *E. minasensis*, and two uncharacterized species: *Ehrlichia* sp. Multan and *Anaplasma* sp. (BL099-6).

Conclusions

In general, farmers and people in border line of Iran-Pakistan who are engaged in livestock need to be made aware of the risks of tick infestation and the tick-borne disease they transmit. Pathogens carried by ticks can infect both animals and humans and monitoring of ticks and the pathogens they carry provides insight into the occurrence and spread of zoonotic diseases. Veterinarians in the region should keep these risks in mind and educate people regarding the risks as well as developing optimal approaches for tick protection protocols that maximize people agreement.

Methods

Sample collection and tick identification

The regions investigated includes three districts of Sib & Suran County (Hiduj district) with the geographical coordinates 27°00'02"N 62°07'01"E, Sarbaz County (Pishin district) with the geographical coordinates 30°35'5.31N 66°59'41.19"E and Chabahar County (Negour district) with the geographical coordinates 25°23'20.84" N 61°8'18.96" E which are located in southeast of Iran with border lines with Pakistan (Fig. 3). The collection of ticks was performed between November, 2017 and March 2018. Totally, 1020 samples were randomly collected from goats, sheep, cattle, and camel. Tick collection was arbitrarily conducted based on the availability of domestic animals for 15 minutes per animal, but efforts were made to obtain a widespread representative sample within the different animal species included in the study. All ticks were transferred to vials and labelled according to their origin of geographic and the animals. The collected ticks were referred 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 morphological characteristics and the method of Estrada-Pena [41].

DNA extraction

After species identification, the ticks were sterilized by immersion in 70% alcohol and washed in distilled water and dried on filter paper in a laminar-flow hood then stored at -80˚C until the DNA extraction. DNA extraction was done using the G-spin Genomic DNA Extraction Kit (iNtRON Biotechnology, South Korea) and carried out according to the manufacturer instructions by grinding of individual ticks in an Eppendorf microtube after isolated tick incubation in the liquid nitrogen tank. The extracted DNA was suspended in sterile distilled water and were then stored at -20˚C prior to molecular investigation.

Molecular detection of *Anaplasma /Ehrlichia spp*

In this study we followed the method of Li et al [42] to differentiate species of *Anaplasma* genera based upon genetic analyses of *16S* rRNA. The *Anaplasma/Ehrlichia spp 16S* rRNA gene was amplified using the nested PCR protocol and the species-specific primers already designed by Rar et al [43] in (Table 4). The forward and reverse primers for outer reactions were Ehr1and Ehr2 and for inner reactions were Ehr3 and Ehr4. PCR reactions were performed in 25µL reaction mixture containing 12.5µL of the Hot Start Taq 2X Master Mix, 1µL of each of the forward and reverse primers, 2µL of DNA template and 7.5µL of nuclease-free H_2O to bring the volume to 25µL. PCR reactions were performed in a DNA thermocycler (Eppendorf, Germany) and PCR condition was done to 15 min at 95˚C for initial denaturation step, 60 s at 94˚C in each cycle for denaturing step, 60 s at 57˚C for annealing and 60 s at 72˚C for extension step followed by 35 cycles and then a final extension for 10min at 72° C. 2µL of the products of the first round of PCR was used as the template for the second round of PCR, which was carried out under the same conditions and reaction mixture as the first round except that were used as the primers [43].

To assess the presence of specific band for *anaplasma* spp., PCR amplification was electrophoresed in 1.5% agarose gel and size of each PCR product was estimated using a 100 base pair (bp) ladder run on the same gel as the marker and then visualized under a UV transilluminator. Two negative controls including double distilled water and DNA template of non-infected tick and a positive control (*Anaplasma* DNA) were included in each PCR assay.

DNA sequencing and Phylogenetic analysis

The positive PCR products were purified and bidirectional DNA sequencing was performed using the same inner PCR primers used for nested PCR amplifications. The acquired sequences in this study were edited and assembled using Chromas and Bioedit softwares to construct consensus sequences and analysed using blast in NCBI (Nucleotide collection) database (https://www.ncbi.nlm.nih.gov/). The consensuses of confident sequences were aligned with other *Anaplasma* corresponding sequences available in Genbank using multiple-sequence alignments available in CLUSTAL Omega (https://www.ebi.ac.uk/Tools/msa/clustalo). Also available gene sequences of *Erlichia ewingii,* and *Spiroplasma* sp as an out group, were obtained from Genbank and combined with the *Anaplasm* sequences for phylogenetic analysis. All DNA sequences used for alignment were cut to get a consistent region (470 bp). The obtained sequences in the current study were submitted to Genbank (Table 3). Phylogenetic and molecular evolutionary analyses were conducted with MEGA 7 software [44]. For

phylogenetic analysis, three representative sequences of *A. ovis*, one representative sequence of *A.marginale*, and one representative sequence of *E. ewingii* [45], obtainedfrom this study were combined with a subset of available representative sequences of all *Anaplasma spp* and *E. ewingii* . Details of the sequences used for this study have been shown in Table 5. 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 1,000 replicates.

List Of Abbreviations

A. ovis : Anaplasma ovis

A. marginale : Anaplasma marginale

A. phagocytophilum : Anaplasma phagocytophilum

A.centrale : Anaplasma central

A. bovis : Anaplasma bovis

A. platys: Anaplasma platys

bp: base pair

Ch : Chabahar

DNA: Deoxyribonucleic acid

E. ewingii :Ehrlichia ewingii

E. minasensis : Ehrlichia minasensis

Hy.schulzei : Hyalomma schulzei

Hy.anatolicum : Hyalomma anatolicum

Hy.marginatum : Hyalomma marginatum

Hy.detricum : Hyalomma detricum

H.dromedarii : Hyalomma dromedarii

Hy.asiaticum : Hyalomma asiaticum

I. scapularis : Ixodes scapularis

I. persulatus : Ixodes persulatus

I. ricinus : Ixodes ricinus

PCR: Polymerase chain reaction

Sar : Sarbaz

S & S : Sib and Suran

UV: Ultraviolet

μL : Microliter

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

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

NC performed the whole study and writing the manuscript, FK accomplished phylogenetic analysis, MK helped in molecular analysis of data, JN was a major contribution in designing and sample collection, and MAO analyzed and interpreted the data, and was a major contributor in editing the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1. Details of tick specimens collected from animals in the study area.

Location	Tick species	No of ticks on animal			Subtotal	Total
		Cattle	Goat	Camel		
Chabahar	*Hy.marginatum*	41	36	0	77	312
	Hy.anatolicum	134	59	0	193	
	Hy.asiaticum	17	25	0	42	
Sarbaz	*Hy.marginatum*	19	13	0	32	322
	Hy.anatolicum	43	132	0	175	
	Hy.dromedarii	0	46	52	98	
	Hy.schulzei	0	17	0	17	
Sib	*Hy.asiaticum*	14	0	87	101	386
&	*H.dromedarii*	0	0	90	90	
Suran	*Hy.detricum*	0	0	6	6	
	Hy.marginatum	75	17	3	95	
	Hy.anatolicum	51	43	0	94	
Total		394	388	238	1020	1020

Table 2. Details of *Anaplasma* / *Ehrlichia* infection in different *Hyalomma* species collected from Sistan and Baluchestan Province, southeast corner of Iran, 2016-2017.

Tick species	No. of collected samples	No. of specimen tested (%)	*Anaplasma* positive (%)	No. of specimens sequenced
Hy. anatolicum	462	115 (24.1)	78(67.8)	7
Hy. asiaticum	143	35(24.4)	24(68.5)	3
Hy. dromedarii	188	47(25)	32(68.1)	4
Hy. marginatum	204	52(25.4)	36(69.2)	4
Hy. detricum	6	2(33.3)	2(100)	2
Hy. schulzei	17	5(29.4)	2(40)	2
Total	1020	256(25.1)	175(68.3)	22

Table 3. Details of infected ticks to *Anaplasma spp /Ehrlichia ewingii* in three districts of Sistan and Baluchestan Province, southeast corner of Iran, 2016-2017.

Tick species	Tick sex	Host	Location	Infection	Genbank ID number
Hy. anatolicum	Female	Cattle	Chabahar	*A.ovis*	MK310471
Hy. anatolicum	Female	Cattle	Sib and Suran	*A.ovis*	MK310472
Hy. anatolicum	Female	Cattle	Sib and Suran	*A.ovis*	MK310473
Hy. anatolicum	Male	Goat	Sib and Suran	*A.ovis*	MK310474
Hy. anatolicum	Female	Goat	Sarbaz	*A.ovis*	MK310475
Hy. anatolicum	Female	Goat	Sarbaz	*A.ovis*	MK310476
Hy. asiaticum	Female	Cattle	Sib and Suran	*A.ovis*	MK310477
Hy. asiaticum	Male	Goat	Sib and Suran	*A.ovis*	MK310478
Hy. asiaticum	Female	Cattle	Chabahar	*A.ovis*	MK310479
Hy .dromedarii	Female	Goat	Sarbaz	*A.ovis*	MK310480
Hy. dromedarii	Male	Camel	Sarbaz	*A.ovis*	MK310481
Hy. marginatum	Male	Cattle	Sib and Suran	*A.ovis*	MK310482
Hy. marginatum	Male	Goat	Chabahar	*A.ovis*	MK310483
Hy. marginatum	Female	Cattle	Chabahar	*A.ovis*	MK310484
Hy. detricum	Male	Camel	Sib and Suran	*A.ovis*	MK310485
Hy. detricum	Male	Camel	Sib and Suran	*A.ovis*	MK310486
Hy.schulzei	Female	Goat	Sarbaz	*A.marginale*	MK310487
Hy. schulzei	Male	Goat	Sarbaz	*A.marginale*	MK310488
Hy. anatolicum	Male	Cattle	Chabahar	*A.ovis*	MK310489
Hy. dromedarii	Female	Camel	Sib and Suran	*E.ewingii*	MK310490
Hy. dromedarii	Male	Camel	Sib and Suran	*E.ewingii*	MK310491
Hy. marginatum	Female	Cattle	Chabahar	*A.ovis*	MH480603

Table 4. Details of the primers used in nested PCR assay for detection of *Anaplasma spp* in hard ticks collected on livestock in Sistan and Baluchestan Province, Iran.

Target gene	Primer name	Oligonucleotide sequences (5'- 3')	Final PCR product size (bp)
16SrRNA	EHR1	GAACGAACGCTGGCGGCAAGC	524
	EHR2	AGTA(T/C)CG(A/G)ACCAGATAGCCGC	
	EHR3	TGCATAGGAATCTACCTAGTAG	
	EHR4	CTAGGAATTCCGCTATCCTCT	

Table 5. Details of the bacterial species used for phylogenetic analysis in this study.

Bacterial species	Origin	Genbank ID number	Reference
A. *A. ovis*	China	MG869525	[46]
	Russia	KC484563	Direct submission
	Iran-East Azarbaijan	MH538097	Direct submission
	Iran-Borderline of Iran-Afghanistan	KM056396	Direct submission
		KF766097	[28]
	Iran-Ardabil	KM517580	Direct submission
	Iran-North Khorasan	JF514506	[8]
	Iran-Mazandaran	MF979832	Direct submission
	Iran-Hamedan	MF979850	Direct submission
	Iran- Kohgiluyeh & Boyer-Ahmad	MG018481	Direct submission
	Iran-Saveh	KX760106	Direct submission
	Iran-Golestan	MK310475	This study
	Iran- Sarbaz	MK310472	This study
	Iran-Sib and Suran	MH480603	This study
	Iran- Chabahar	MK310471	This study
	Iran- Chabahar		
B. *A. bovis*	Iran-Mazandaran	KP017262	Direct submission
C. *A. marginale*	Australia	CP006847MK310488	[47]
	Iran-Sarbaz	MK016525	This study
	Iran-Zabol	MG757665	Direct submission
	Iran-Khozestan	CP023731	Direct submission
	Brazil	MF289480	Direct submission
	China	KT264188	Direct submission
	Thiland	KU686794	Direct submission
	Uganda		Direct submission
A. platys	Iran-Zabol	MK016523	Direct submission
A. phagocytophilum	South Korea	MF787270	Direct submission

A. odocoilei	USA	KT870132	Direct submission
A. centrale	Iran-Mazandaran	JF514509	Direct submission
A. capra [42]	China	KY242456	Direct submission
E. ewingii	Iran-Sib and Suran USA Tajikistan China	MK310490U96436 KM995821MH879869	This study [48] Direct submission Direct submission
Spiroplasma chrysopicola [49]	USA	AY189127	[50]

Figures

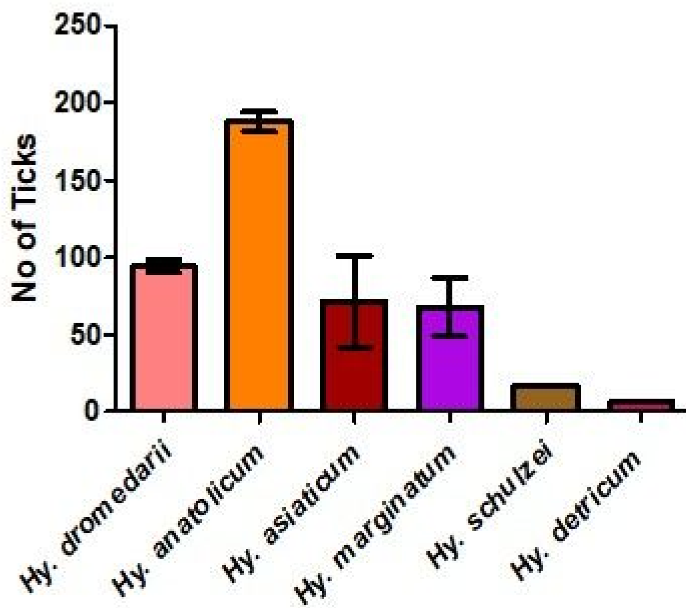

Figure 1

Hyalomma spp abundance collected from three districts of Sistan & Balochistan, southeat corner of Iran, 2016-2017. Bars indicate mean ± SEM.

Figure 2

Phylogenetic relationships inferred from 470 bp of 16S rRNA genes of Anaplasma/Ehrlichia species detected in this study and other available data in Genbank. The representative sequences obtained in this study are marked with black circles. The tree was computed by maximum likelihood (MEGA7.0 software). Bootstrap values are shown on nodes.

Figure 3

Map showing the locations where ticks were collected in Sistan and Baluchistan. Ch: Chabahar, Sar: Sarbaz, S & S: Sib and Suran.