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Scientific Research

Molecular Detection of *Anaplasma* and *Ehrlichia* Infection in Ticks in Borderline of Iran-Afghanistan

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Abstract

Anaplasmosis, a disease caused by various species of *Anaplasma*, poses important economic constraints to animal breeders. Ehrlichiosis is a worldwide zoonosis illness and mostly occurs in tropical and subtropical regions that are close to the vector's distribution. Tick-borne pathogens lead to over 100,000 cases of illness in the world each year. Besides the costs of the additional veterinary care, anaplasmosis causes abortion in animals, reduction of milk production, body weight, and frequently leads to death. In this study, we investigated on infection of ticks to *Anaplasma* and *Ehrlichia* pathogens in Zabol and Zahak County in Sistan and Baluchestan Province where is bordered with Afghanistan. Totally from June 2013 to May 2014, 369 ticks were caught from goats, cows and sheep. Molecular studies on 53 of these samples which represented all specimens, showed that *Ehrlichia*'s DNA and *Anaplasma*'s DNA was found in 14 (26.4%) out of the 53 selected specimens. The results showed the infection of *Rhipicephalus sanguineus* and *Hyalomma anatolicum* with *Anaplasma ovis*. Also we saw infection of *H. anatolicum* and *H. asiaticum* ticks to *Ehrlichia* spp. This study has been intended to do a comprehensive survey of *Ehrlichia* and *Anaplasma* distribution in ticks caught from east of Iran; it was designed to investigate the presence of *Anaplasma* spp. and *Ehrlichia* spp. in Zabol and Zahak Counties, Iran. These results show that these pathogens should be controlled in such regions.

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Keywords

***Anaplasma*, *Ehrlichia*, Tick, Iran**

1. Introduction

Ticks are vectors of bacterial, viral and protozoan agents [1]. Investigating on these pathogens in ticks requires the identification of ticks. Ticks are living in most places. There are some investigations about ticks in Iran [2]-[4]. Tick-borne pathogens lead to over 100,000 cases of illness in the world each year [5]. *Ehrlichiachaffeensis*, *E. canis*, *E. ewingii*, and *Anaplasma phagocytophilum* are the most important tick-borne pathogens of human and animals belong to the family *Anaplasmataceae*. Ehrlichiosis is a worldwide zoonosis problem and mostly occurs in tropical and subtropical regions that are close to the vector's distribution [6] [7].

Anaplasmosis, a disease caused by various species of *Anaplasma*, poses important economic constraints to animal breeders. Besides the costs of the additional veterinary care, anaplasmosis causes abortion in animals, reduction of milk production, body weight, and frequently leads to death [8]. Members of the genus *Anaplasma* are obligatory intracellular gram negative bacteria that infect blood cells of mammals. Six *Anaplasma* species are currently recognized [9]. Vertebrates are main reservoirs of the *Anaplasma* bacteria, however in many cases bacteria from the genus *Anaplasma* cause diseases in domestic animals and human. *Anaplasma ovis* invades and reproduces within erythrocytes. This bacterium induces acute anemia in sheep and goats [10]. Anaplasmosis in cattle is caused by *A. bovis*, *A. marginale*and *A. centrale* infecting monocytes and red blood cells [11] [12]. *Anaplasma bovis* is reported mostly from cattle, but also detected in small ruminantswhich could be a reservoir of this bacterium [13]. Ixodid ticks play an important role in maintaining *Anaplasma* species in nature. It is evidenced that various species of *Ixodes*, *Dermacentor*, *Rhipicephalus* and *Amblyomma* genera are the main vectors of the *Anaplasma* bacteria in different regions of the world. *Rhipicephalus sanguineus*, a common tick vector for *Anaplasma*, has been reported from India, the United States, all regions of Africa, and around the Mediterranean Basin [14]. Animal husbandry is one of the main activities of people in Estern Iran, and anaplasmosis is one of the major veterinary health problems there [15]. However, there have been only a few studies to detect tick anaplasmosis infections in the country [16]-[19]. We previously studied on*anaplasma*l infection of human, livestock and ticks in Mazandaran Province, Iran. Our results showed the presence of *A. ovis* in two *Rhipicephalus sanguineus* and two *Ixodesricinus* ticks, one human and 4 sheep samples. Also one *Boophilusannulatus* tick and one sheep sample were infected with *A. bovis*. Furthermore, one sample of sheep was infected with *A. centrale*. That was the first report of tick infection to *A. ovis*, *A. bovis* and human infection to *A. ovis* in Iran [17]. Khazeni *et al*., investigated on the infection of ticks caught from Ardebil Province, Iran and found that *Ehrlichia* spp. and *Anaplasma* spp. were found in 43.84% of all the specimens containing *Anaplasma ovis* and *Ehrlichia* spp. and *Ehrlichiacanis* [18] Khazeni *et al*., reported *E. canis* in vector from Iran for the first time [18]. The aim of the present investigation was to study prevalence and genetic diversity of infection of ixodidae ticks to infectious bacteria due to our observations of ranchers' complaint about their livestock diseases and the lack of documented information about *Anaplasma* and *Ehrlichia sp*ecies in ticks in eastern Iran. We conducted the present study to distinguish the rate of *Anaplasma* and *Ehrlichia* infections in Zabol and Zahak counties which have been located in borderline of Iran-Afghanistan in eastern Iran.

2. Materials and Methods

2.1. Study Area

This study was conducted in Zabol and Zahak counties in Sistan and Baluchestan Province (Located in Eastern Iran) which is bordered with Afghanistan. Zahak and Zabol Counties with 30˚N latitude and 61˚E longitude are located in the east of the Province. Zabol County has a population about 320,000 and Zahak County has a population about 70,000.

2.2. Sample Collection

From June 2013 to May 2014, we collected tick samples from goats, cows and sheep from Hossein Abad, Heydar

Abad, Fathollah and Bagher Abad which are located in Zabol County and Khomak, Bonjar and Hassankhoon which are located in Zahak County (**Figure 1**). Ticks were mostly found on shoulders and ears of the livestock.

Specimens were collected using a forceps and kept in labeled holding tubes individually. Specimens were transferred into the labeled holding tubes individually. In some cases it was impossible to collect all ticks due to lack of time. We did not ask the origin of the sheep, goats and cows.

Specimens were transferred to the Entomology Laboratory, School of Public Health, Tehran University of Medical Sciences. All specimens were identified based on morphological characteristics and the keys given by Janbakhsh (1957) and Walker (2003) based on shape of capitulum, scutum, eyes, festoone and hypostome, spiracle, genital groove, spure of coxa, adanal shield and another characters [20] [21].

2.3. DNA Extraction

DNA was extracted using Gspin™ Genomic DNA Extraction kit (iNtRON). Extraction was carried out according to the manufacturer instructions by grinding of individual ticks in an eppendorf microtube after maintaining 5 minutes in the liquid nitrogen tank and using glass pestle. 400 µl of G-buffer per 20 - 30 mg of tissue was added and incubated at 70˚C for 5 - 10 min and then mixed well. At the next step, 400 µl of Binding buffer was added and transferred to the G-spine columns in the next step, centrifuged for 1 min at 13,000 rpm. Then, 500 µl of washing buffer and 100 µl of elution buffer were added respectively and centrifuged for 1 min at 13,000 rpm. After adding elution buffer, samples were incubated for 1 min at room temperature and then they were kept at 4˚C for further use.

2.4. Detection of *Ehrlichia* and *Anaplasma* by Nested-PCR

By using EHR1, EHR2, EHR3 and EHR4 primers, detection of *Ehrlichia* and *Anaplasma* was performed by nested-PCR, 16 s rRNA amplification, [18] [22]. These primers are able to detect the infection of both *Ehrlichia* and *Anaplasma*.

As positive control we used *Anaplasma* DNA obtained from Department of Medical Entomology, School of Public Health, Tehran University of Medical Sciences [18], and double distilled water as negative control was used.

First round of PCR amplifications were done in a Maxime PCR premix kit (iTaq). For primary reactions 5 µl of purified DNA was used as a template in mentioned PCR premix kit. PCR cycles were consisted of 5 minutes at 94˚C, 35 cycles at 94˚C for 1 minute (denaturation of DNA), 60˚C for 1 minute (annealing of primers), 72˚C for 1 minute (extension of the primers), and a final extension at 72˚C for 7 minutes. Second round of PCR assay was performed using species-specific primers (Ehr 3, Ehr 4) (**Table 1**), [18] [23] and 3 µL from the initial PCR product was used as template. The PCR products were loaded in 1% agarose gel, stained with ethidium bromide and then visualized under UV light.

2.5. Nucleotide Sequencing

Sequencing was performed using an ABI 3730 sequencer machine. Obtained sequences were checked to correct

Figure 1. Geographical location of Sistan and Baluchestan Province and Zabol and Zahak Counties, Iran. Zahak and Zabol Counties are bordered with Afghanistan.

Table 1. Details of the primers were used for *Ehrlichia* spp. and *Anaplasma* spp. detection in ticks collected on livestock, Zahak and Zabol County, Sistan and Baluchestan Province, Iran.

First round PCR	**EHR1 (Forward)**	**5'-GAACGAACGCTGGCGGCAAGC-3'**
	EHR2 (Reverse)	5'-AGTA(T/C)CG(A/G)ACCAGATAGCCGC-3'
Second round PCR	EHR3 (Forward)	5'-TGCATAGGAATCTACCTAGTAG-3'
	EHR4 (Reverse)	5'-CTAGGAATTCCGCTATCCTCT-3'

ambiguities. Determination of sequence homologies was done in GenBank by BlastN and aligned with ClustalW was checked using basic local alignment search tool (BLAST) analysis software (www.ncbi.nlm.nih.gov/BLAST).

3. Results

The prevalence of ticks was low, so a total of 369 ticks were collected and these ticks were morphologically identified at the level of species.

Detection of *Ehrlichia* and *Anaplasma*

Fifty three out of 369 collected ticks were tested for the presence of *Anaplasma* and *Ehrlichia's* DNA. *Ehrlichia's* DNA and *Anaplasma's* DNA were found in 14 (26.4%) out of the 53 selected specimens (generated characteristic 524 bp products, **Figure 2**). Nested PCR detected ehrlichial DNA in 14 samples of 53 samples which had been selected by species and region differences and represented the whole regions, whole tick species and whole livestock. Details of positive samples are listed in **Table 2**. Out of 53 ticks which used for DNA extraction, 14 samples were infected with *Anaplasma* or *Ehrlichia* spp. For detection of infection, 7 of these samples were sent for sequencing. The results demonstrated that a female *Rhipicephalus sanguineus* which had been caught of a sheep from Zabol County was infected with *Anaplasma ovis*. Two female *Hyalomma anatolicum* ticks which had been caught from Zahak County were infected with *Ehrlichia* spp. One of these ticks had been caught from cow and the other had been caught from sheep. A male *Hyalomma asiaticum* which had been caught from a cow in Zaboldistrict, was also infected with *Ehrlichia* spp. Two *Hyalomma anatolicum* which one of them was male and the other was female and were caught from goat, were infected with *Anaplasma ovis*. Both of these infected ticks were caught from Zahak district. Also a male *Hyalomma anatolicum* which had been caught from Zabol district was infected with *Anaplasma ovis*. This tick was caught from a cow. Totally 3 of positive samples were caught from sheep, 3 of them were caught from goats and 8 of them were caught from cows. Two genus of ticks: *Rhipicephalus* and *Hyalomma* were found to be infected with *Anaplasma* or *Ehrlichia* genus. Eight of positive samples had been caught from Zahak and 6 of them had been caught from Zabol district. Alignment of sequenced *Anaplasma ovis samples* (KM056396-KM056399) showed 99% identity with each other. Also 3 of the *Ehrlichia* spp. samples were completely identical to each other (100%).

Comparison of the sequences with available data in GenBank showed that the sequences were highly similar to ITS2 region of *Anaplasma ovis* and *Ehrlichia* spp. with 100% identity. Obtained sequences from this study were submitted to the GenBank, under the accession numbersKM056396-KM056402.

4. Discussion

This study provides primary data, regarding the existence of *A. ovis* and *Ehrlichia* spp. in ticks in Zahak and Zabol Counties which is located in east of Iran and bordered with Afghanistan. Nested PCR enhances the sensitivity of detection of target nucleotide sequences [24]. This technique has been shown to be sensitive for direct identification of ehrlichiae in ticks [25]. Nested PCR with subsequent sequencing had been shown that hard ticks had been containing *Anaplasma ovis*, *Ehrlichia* spp. and other anaplasmal or ehrlichial DNA [17] [18] [26].

The main vectors of the *Anaplasma* bacteria are ticks, especially the genera *Ixodes*, *Dermacentor*, *Rhipicephalus* and *Amblyomma* [17]. Our results demonstrated the presence of *A. ovis* in *Rhipicephalus sanguineus* and *H. anatolicum*; so they might be vectors of *A. ovis* in this region. We report the infection of *R. sanguineus* and *H. anatolicum* to *A. ovis*in Zahak and Zabol Counties. We also demonstrated the infection of *H. anatolicum* to *Ehrlichia* spp. in Zahak and infection of *H. asiaticum* to *Ehrlichia* spp. in Zabol County, Iran.

Table 2. Details of infected ticks to *Anaplasma*/*Ehrlichia* in two studied districts.

Code	Tick species	*M*/*F*	Caught from	Cought region	Infection	Accession number
T10	*Rhipicephalus sanguineus*	♀	Sheep	Zabol	*Anaplasma ovis*	KM056396
T1	*Rhipicephalus sanguineus*	♀	Goat	Zahak		-
T12	*Hyalomma anatolicum*	♀	Cow	Zahak	*Ehrlichia* spp.	KM056400
BZ15	*Hyalommaasiaticum*	♂	Cow	Zahak		-
BZ1	*Hyalomma anatolicum*	♀	Goat	Zahak	*Anaplasma ovis*	KM056398
BZ8	*Hyalomma anatolicum*	♂	Cow	Zabol		-
BZ4	*Hyalomma asiaticum*	♂	Cow	Zabol	*Ehrlichia* spp.	KM056401
BZ2	*Hyalomma anatolicum*	♂	Goat	Zahak	*Anaplasma ovis*	KM056397
T15	*Hyalomma anatolicum*	♂	Cow	Zabol	*Anaplasma ovis*	KM056399
T18	*Hyalomma anatolicum*	♀	Sheep	Zahak	*Ehrlichia* spp.	KM056402
BZ10	*Hyalomma* spp.	♀	Cow	Zabol		-
T20	*Hyalomma anatolicum*	♀	Cow	Zahak		-
BZ9	*Hyalomma asiaticum*	♂	Cow	Zabol		-
T19	*Hyalomma anatolicum*	♀	Sheep	Zahak		-

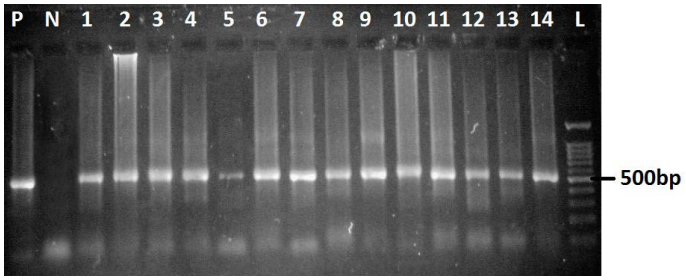

Figure 2. 16 s rRNA amplification of *Ehrlichia* spp. and *Anaplasma* spp. in ticks using nested-PCR. Lanes P: positive control, Lane N: Negative control, Lanes 1-14 represent all the positive samples which are infected to *Ehrlichia* spp. and *Anaplasma* spp. (524 bp).

Since sheep are reservoirs of *A. ovis*, human infection with this pathogen may occur, but transmission of *A. ovis* to human is uncertain. Previously, sequence analysis of PCR products confirmed the presence of *A. ovis* in *Rhipicephalus sanguineus* and *Ixodesricinus* ticks, human and sheep samples in Ghaemshahr, which is located in north of Iran. High level of infestation of *R. sanguineus* to domestic ruminants, and the prevalence of *Anaplasma*in these specimens showed that they are the most abundant vector of *Anaplasma* species in Ghaemshahr county [17]. In another study the infection rate of *A. ovis* in ticks collected from dogs' ears, neck, shoulder and toes reported from Meshkin-Shahr (Ardebil Province, Iran) was as much as 56.6 percent. In addition, 21.17% of nymphs and at least 53.42% of adult ticks were positive for *A. ovis* and *Ehrlichia* spp. [18].

Rhipicephalus sanguineus is widely spread not only in Iran but also in all over the world [27].

The results demonstrated the presence of *Ehrlichia* spp. in *H. anatolicum* and *Hyalomma asiaticum* while *Rhipicephalus sanguineus* was infected with *Anaplasma ovis*.

Almost 57% of the infected specimens collected on cow, the other belong to sheep or goat. The only specimens which infected with *Anaplasma ovis* collected on sheep. Based on our results, female ticks were more infected with *Anaplasma* spp. and *Ehrlichia* spp. in comparison with male ticks, while in Khazeni *et al*. investigation, this is contrary [18].

Zahak is more infected with *Anaplasma* spp. and *Ehrlichia* spp. than Zabol County. According to our previous study [18] dominant tick species which infected with *Ehrlichia* was *Rhipicephalus sanguineus* while Satta *et al*.

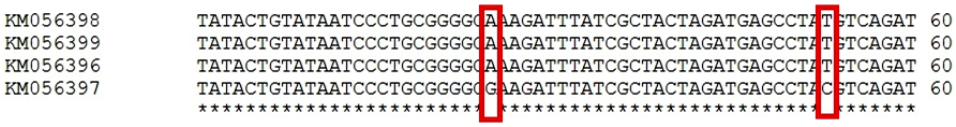

Figure 3. The differences between 2 *A. ovis* haplotypes. The sequence "KM056397" has an identity of 99% with three other sequences (KM056396, KM056398, KM056399).

demonstrated that dominant [28], Whereas Meng *et al*. [29] determined that dominant tick species which infected to *Ehrlichia* was *Hyalomma asiaticum*. Khazeni *et al*. Identified *Rhipicephalus sanguineus*, *Hyalomma asiaticum*, *Hyalomma marginatum*, *Hyalomma anatolicum*, *Dermacentorniveus*, *Dermacentormarginatus* as the infected specimens while in this study we could show *Rhipicephalus sanguineus*, *Hyalomma asiaticum*, *H. anatolicum* and a *Hyalomma* spp. as the infected specimens.

Sequence alignments of 3detected *Ehrlichia* (KM056400-KM056402) in this study showed 100% identity with some of the submitted ones in Genbank. We could find one haplotype in these 3 sequences; but we found 2 haplotypes in *A. ovis* in our study. Three of sequenced samples (KM056396, KM056398, KM056399) were identical (100%) to each other and to the submitted ones. The other *A. ovis* sequenced sample (KM056397) which is detected from one *H. anatolicum*, had an identity of 99% with other samples and the submitted samples in Genbank. The difference between this sequence (KM056397) with the three other sequences (KM056396, KM056398, KM056399) was as much as 2 nucleotides (**Figure 3**).

In Iran, *A. ovis* was previously identified in sheep [15] [19]; however *R. sanguineus* and *I. ricinus* are dominant tick species in sheep in north part of this country [27] [30]. Based on a study which was done in Turkey, *A. ovis* 16S rRNA gene fragment was detected in two *R. sanguineus* ticks [31]. On the other hand, there are few studies on the infectivity of animal blood samples to *Anaplasma* in Iran. One of our laboratory studies, demonstrated that 39% of blood samples of humans and livestock were infected with *Anaplasma* in north of Iran [17]. In Khorasan Province, north east of Iran, about 80% of sheep and 38% of goats blood smears were infected with *A. ovis* [15]. In current study, *A. ovis* DNA was detected in ticks which had been collected from sheep, goats and cows. We could also detect *Ehrlichia* spp. DNA in ticks which had been collected from cows and sheep. These results make these livestock as potential reservoirs of these pathogens.

This study has been intended to do a comprehensive survey of *Anaplasma* and *Ehrlichia* distribution in ticks collected from east of Iran; it was designed to investigate the presence of *Anaplasma* spp. and *Ehrlichia* spp. in Zabol and Zahak County, Iran. It is recommended to investigate the competency of vectors to *Rhipicephalus sanguineus*, *Hyalomma asiaticum* and *H. anatolicum*.

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