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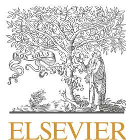
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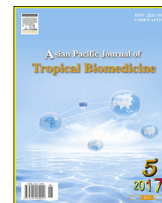

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Molecular detection of *Anaplasma marginale* and *Anaplasma ovis* in sheep and goat in west highland pasture of Iran

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ABSTRACT

Objective: To determine the prevalence of *Anaplasma marginale* (*A. marginale*) and *Anaplasma ovis* from sheep and goat in different highland pasture in west of Iran.

Methods: From July 2015 to October 2015, 370 blood samples of sheep and goat were collected from different regions in Hamedan province, Iran. The DNA extracted from blood and subsequently, 16S rRNA and MSP4 genes were analyzed by nested-PCR, semi nested-PCR and RFLP methods.

Results: In the PCR assessment, overall 27.5% (102/370) of sheep and goat were positive for *Anaplasma ovis* and *A. marginale* infection, which was lower than reports from tropical and subtropical regions of Iran. Statistical analysis (the *Chi*-square test) did not show any significant relation between infection and variables such as location, tick infestation age and sex ($P > 0.05$). No significant correlation between the altitude and the *Anaplasma* species infection was found (Mann–Whitney test: $P > 0.05$). However, *Anaplasma* infection in goat significantly is more than the sheep ($P = 0.008$).

Conclusions: The ecological changes affect the frequency and distribution of *Anaplasma* species. Furthermore, our results indicate that sheep as potential reservoirs of *A. marginale*.

1. Introduction

Anaplasmosis is generally considered to induce only mild clinical symptom, which caused by intraerythrocytic rickettsia of genus *Anaplasma* [1]. The infection with *Anaplasma ovis* (*A. ovis*) and *Anaplasma marginale* (*A. marginale*) is mostly recognized as such, and may cause depression, debility, decreased milk production, weight loss, abortion and severe anemia and jaundice in endemic areas [2]. This rickettsia is transmitted in a variety of ways and two important factors; geography and climate are determining which ticks or biting flies are responsible for local cases of infection [3,4], but in some areas as a result of global warming, the distribution of anaplasmosis may be expected to continue to change, which can influence the movement of biological vectors [5]. Anaplasmosis mainly occurs in tropical and subtropical areas, and the accurate information of *Anaplasma* has not been available in mountainous regions in Iran [6]. Hence the present survey was conducted to determine prevalence *A. marginale* and *A. ovis* of sheep and goat in different highland pasture in west of Iran.

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All experimental procedures involving animals were conducted in accordance to international, national and institutional guidelines for the care and use of animals and approved by Institutional Ethical Committee of Science and Research Branch, Islamic Azad University, Tehran.

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2. Materials and methods

2.1. Ethics statement

All experimental procedures involving animals were conducted in accordance to international, national and institutional guidelines for the care and use of animals and approved by Institutional Ethical Committee of Science and Research Branch, Islamic Azad University, Tehran.

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2.2. Study area

The study was carried out in nine areas of the Hamedan province, in west of Iran, located in a temperate mountainous region to the east of the Zagros and highest points reach an altitude of 3 580 m above sea level, and the lowest point descends to the altitude of 1 420 m. Hamadan province has a dry summer continental climate in transition with a cold semi-arid climate with snowy winters. In fact, it is one of the coldest provinces in Iran. The temperature may drop below 30 °C on the coldest days. The annual average precipitation and temperature are 300 mm and 11 °C, respectively.

2.3. Sampling

From July 2015 to October 2015, a total of 370 blood samples were collected of small ruminant (sheep: 206, goat: 164) from thirty-seven farms from different regions of Hamedan province. The blood samples were taken from each animal through the jugular vein in a sterile venoject tube, immediately two ml of each sample collected in a sterilized test tube containing one ml 96% ethanol alcohol.

2.4. DNA extraction

DNA was extracted using the DNA isolation kit (MBST, Iran) according to the manufacturer's instructions. Briefly, 50 mg of fixed blood samples was first air dried and subsequently lysed in 180 µL lysis buffer and the proteins was degraded with 20 µL proteinase K for 10 min at 55 °C. In the next step, 360 µL binding buffer was added and after vortexing, incubation for 10 min at 70 °C, then 270 µL ethanol (96%) was added to the solution and after vortexing, the complete volume was transferred to the spin column in a 1.5 mL tube. The spin column was first centrifuged at 8 000 rpm for 1 min, and then washed twice with 500 µL washing buffer at 8 000 rpm for 1 min. Finally, DNA was eluted from the carrier using 150 µL elution buffer. The extracted DNA was analyzed on agarose gel before use. In addition, the amount of extracted DNA and its purity was measured by OD260 and the ratio of OD260 to OD280, respectively.

2.5. Polymerase chain reaction of 16S rRNA and MSP4 genes

The *Anaplasma* spp. 16S rRNA and MSP4 genes were amplified by PCR. The PCR was performed in 20 µL reaction mixture containing 10 mm Tris–HCl (PH 9.0), 30 mm KCl, 1.5 mm $MgCl_2$, 250 mm each dNTP, 0.5 mm each sense and antisense primers (16S rRNA: P1/P2; MSP4: M-OM F/M-OM R), 1 IU Taq DNA polymerase (Cinnagen, Iran) and 2 µL of DNA in automated thermocycler (Astec, Germany) for 35 cycles. After an initial denaturation step of 5 min at 95 °C, each cycle consisted of a denaturing step of 30 s at 94 °C, an annealing for 30 s at 58 °C and an extension step of 30 s at 72 °C for 16S rRNA and an annealing step of 30 s at 60 °C for MSP4 gene. Finally, PCR was completed with the additional extension step for 5 min. The PCR products were analyzed on 1.5% agarose gel in 0.5 TAE buffer and visualized using ethidium bromide and UV transilluminator. The details of primers designed are presented in Table 1.

2.6. Semi-nested PCR of 16S rRNA gene

This technique was performed in 20 µL reaction mixture containing 10 mm Tris–HCl (PH 9.0), 30 mm KCl, 1.5 mm $MgCl_2$, 250 mm each dNTP, 0.5 mm each sense and antisense primers (16S rRNA: P1/P5 for *A. marginale*/*A. ovis*), 1 IU Taq DNA polymerase (Cinnagen, Iran) and 1 µL of PCR products in automated thermocycler (Astec, Germany) for 35 cycles. After an initial denaturation step of 5 min at 95 °C, each cycle consisted of a denaturing step of 30 s at 94 °C, an annealing for 30 s at 58 °C and an extension step of 30 s at 72 °C for 16S rRNA. Finally, PCR was completed with the additional extension step for 5 min at 72 °C. The PCR products were analyzed on 1.5% agarose gel in 0.5 TAE buffer and visualized using ethidium bromide and UV transilluminator.

2.7. PCR-RFLP

The primary PCR product of 16s rRNA (781 bp), was amplified using P1/P5 primers (semi-nested PCR product: 120 bp) for *A. marginale*/*A. ovis*. The PCR product was then cut with 0.1 µL restriction endonuclease Bst1107I (Fermentas, Germany, 10 IU/µL) in 2.5 µL 10× corresponding buffer and 12.5 µL H_2O for 1 h by 37 °C. The restriction endonuclease Bst1107I recognizes the sequence (GTATAC) in semi-nested PCR product of *A. marginale* and cut it in the position 68, whereas the used restriction enzyme cannot cut the semi-nested PCR product of *A. ovis* in same position with different sequence (GTACGC).

2.8. Nested PCR of MSP4 gene

This technique was used for confirmation of PCR product and RFLP method in16S rRNA gene. This was performed in

Table 1

The sequence details of primers used in PCR and semi-nested PCR of 16S rRNA gene and MSP4 gene.

Primer	Genbank no.	Nucleotide sequences	Nucleotide positions	Target organism	PCR product (bp)
P1	M60313	5′agagtttgatcctggctcag3′	1–20		781
P2	M60313(16S rRNA gene)	5′agcactcatcgtttacagcg3′	796–815	*Anaplasma* spp.	
P1	M60313	5′agagtttgatcctggctcag3′	1–20	*A. marginale*	120
P5	M60313 (16S rRNA gene)	5′ctatgcattactcacccgtctgccactaacc atacacgcagcaagctgcg3′	71–120	and *A. ovis*	
M-OM F	HM640938.1	5′gggagctcctatgaattacagagaattgtttac3′	1–33	*A. marginale*	867
M-OM R	HQ456350.1 (MSP4 gene)	5′ccggatccttagctgaacaggaatcttgc3′	839–867	and *A. ovis*	
M-MA F	HM640938.1	5′ctgaaggggggagtaatggg3′	113–131	*A. marginale*	344
M-MA R	HM640938.1 (MSP4 gene)	5′ggtaatagctgccagagattcc3′	435–456		
M-OV F	HQ456350.1	5′tgaagggagcggggtcatggg3′	114–134	*A. ovis*	347
M-OV R	HQ456350.1 (MSP4 gene)	5′ggtaattgcagccagggactct3′	438–460		

20 μL reaction mixture containing 10 mm Tris–HCl (PH 9.0), 30 mm KCl, 1.5 mm $MgCl_2$, 250 mm each dNTP, 0.5 mm each sense and antisense primers (MSP4: M-MA F/M-MA R for *A. marginale*; M-OV F/M-OV R for *A. ovis*), 1 U Taq DNA polymerase (Cinnagen, Iran) and 1 μL of PCR product (867 bp) in automated thermocycler (Astec, Germany) for 30 cycles. After an initial denaturation step of 5 min at 95 °C, each cycle consisted of a denaturing step of 30 s at 94 °C, an annealing for 15 s at 60 °C and an extension step of 30 s at 68 °C for *A. ovis* and an annealing step of 15 s at 58 °C for *A. marginale*. Finally, PCR was completed with the additional extension step for 5 min at 68 °C. The PCR products were analyzed on 1.5% agarose gel in 0.5× TAE buffer and visualized using ethidium bromide and UV transilluminator.

2.9. Sequencing

For validation of PCR product, two PCR amplicons of MSP4 gene from each species were purified and subjected to sequencing using the dideoxy chain termination method by Bioneer Company, South Korea. In addition, it should be noted that all positive control samples used during this study were previously sequenced and was provided by the Department of Veterinary Parasitology, University of Tehran.

2.10. Statistical analysis

Data management was performed using SPSS V21.0 (SPSS Inc., Chicago, Illinois, USA) statistical software. The *Chi*-square test was used to determine the relation between infection and variables such as location, tick infestation age and sex (statistically significant differences were determined at $P < 0.05$). Mann–Whitney test was performed for determining the correlation between altitude of the sampled points and *Anaplasma* species infection.

3. Results

In the PCR assessment of DNA samples based on 16S rRNA gene by semi-nested PCR and PCR-RFLP, in total, 27.5% (102/370) and separately 21.8% (45/206) of sheep and 34.7% (57/164) of goat were positive for *Anaplasma* infection. All positive samples by amplifying of 16S rRNA gene, showed the expected 781 bp PCR product then for validation the positive PCR products of before step, again amplified by semi-nested PCR and showed the expected 120 bp PCR product in *A. marginale/ovis* positive samples. Subsequently, RFLP method by the restriction endonuclease Bst1107I to differentiate between *A. ovis* and *A. marginale* was used and cut semi-nested PCR product (120 bp) of *A. marginale* in the expected position 68 bp, whereas the used restriction enzyme cannot cut the semi-nested PCR product of *A. ovis* in same position with different sequence.

Moreover, PCR by specific primers for *A. marginale/ovis* in MSP4 gene, showed the expected 867 bp PCR product and following that in the next step by nested PCR, *A. marginale* showed the expected 344 bp PCR product and *A. ovis* showed the expected 347 bp PCR product. Finally, it can be said that semi-nested PCR and RFLP of 16S rRNA gene, was confirmed by PCR and nested PCR of MSP4 gene.

The results of sheep blood samples in the studied areas were showed 43 (20.8%) positive infection as *A. ovis* and 2 (0.54%) positive infection as *A. marginale*. Furthermore, in goat, 57 (34.7%) of blood samples were positive only by *A. ovis*.

According to the data in Table 2 and the statistical analysis (the *Chi*-square test) did not show any significant relation

Table 2

Prevalence details of *A. ovis* and *A. marginale* in domesticated small ruminants in Hamedan Province.

Group	Sheep					Goat				Total		
	No. tested	No. infected (%)			*P*	No. tested	No. infected (%)		*P* value	No. tested	No. infected (%)	*P*
		Total infection	*A. ovis*	*A. marginale*			Total infection	*A. ovis*			Total infection	
Bioclimatic zone					0.626				0.290			0.769
Hamedan	21	5 (23.8)	3 (14.2)	2 (9.5)		38	12 (31.6)	12 (31.6)		59	17 (28.8)	
Razan	23	5 (21.7)	5 (21.7)	0		18	4 (22.2)	4 (22.2)		41	9 (22.0)	
Tuyserkan	17	5 (29.4)	5 (29.4)	0		12	5 (41.7)	5 (41.7)		29	10 (34.5)	
Nahavand	25	5 (20.0)	5 (20.0)	0		17	6 (35.3)	6 (35.3)		42	11 (26.2)	
Kabudrahang	36	4 (11.1)	4 (11.1)	0		13	7 (53.8)	7 (53.8)		49	11 (22.4)	
Malayer	28	6 (21.4)	6 (21.4)	0		48	20 (41.6)	20 (41.6)		76	26 (34.2)	
Asadabad	23	5 (21.7)	5 (21.7)	0		8	3 (37.5)	3 (37.5)		31	8 (25.8)	
Bahar	18	7 (38.9)	7 (38.9)	0		5	0	0		23	7 (30.4)	
Famenin	15	3 (20.0)	3 (20.0)	0		5	0	0		20	3 (15.0)	
Tick					0.359				0.485			0.343
Absent	104	20 (19.2)	20 (19.2)	0		94	31 (30.4)	31 (30.4)		198	51 (25.3)	
Present	102	23 (22.5)	23 (22.5)	2 (1.2)		70	26 (37.1)	26 (37.1)		172	49 (28.5)	
Age					0.940				0.294			0.563
<1 years	60	12 (20.0)	12 (20.0)	1 (1.7)		56	13 (23.2)	13 (23.2)		116	25 (21.6)	
2 years	87	21 (24.1)	21 (24.1)	1 (1.1)		60	24 (40.0)	24 (40.0)		147	45 (30.6)	
3 years	40	8 (20.0)	8 (20.0)	0		34	15 (44.1)	15 (44.1)		74	23 (29.7)	
4 years	16	3 (18.8)	3 (18.8)	0		10	4 (40.0)	4 (40.0)		26	7 (26.9)	
>4 years	3	1 (33.3)	1 (33.3)	0		4	1 (25.0)	1 (25.0)		7	2 (28.6)	
Sex					0.447				0.722			0.462
Male	95	23 (24.2)	23 (24.2)	1 (1.0)		73	26 (35.6)	26 (35.6)		168	49 (29.2)	
Female	111	22 (19.8)	22 (19.8)	1 (0.9)		91	31 (34.0)	31 (34.0)		202	52 (25.7)	
Total	206	45 (21.8)	43 (20.8)	2 (1.0)	–	164	57 (34.7)	31 (34.0)	–	370	102 (27.5)	0.008*

*: Statistically significant test.

between infection and variables such as location, tick infestation age and sex ($P > 0.05$). No significant correlation between the altitude and the *Anaplasma* species infection was found (Mann–Whitney test: $P > 0.05$). However, *Anaplasma* infection in goat significantly is more than the sheep ($P = 0.008$).

4. Discussion

Iran has a diverse climate in different areas. Skerman and Hillard [7] described four zoogeographical zones of Iran, which consist of the Caspian sea zone at the north (Zone I), the mountainous area which extends from northwest to the southeast (Zone II), the semi-desert area in the central part of Iran (Zone III) and the Persian Gulf lowland (Zone VI).

The most studies of anaplasmosis in ruminants which have been documented in Iran were sampled from tropical and subtropical regions (Zone I, III and VI) and some of them were sampled from husbandries which have a history of outbreaks of anaplasmosis [8,9].

Our survey was conducted in the western mountainous regions (Zone II) of Iran, in this study *A. ovis* and *A. marginale* were identified for the first time in this region, but their overall prevalence (27.5%) was lower than reported by other authors. For instance, our result demonstrated the lower prevalence of *A. ovis* in sheep (21.8%) than the results reported by Razmi *et al.* [10], Hosseini-Vasoukolaei *et al.* [11] and Jalali *et al.* [8] who recorded 80.3%, 43.08% and 87.4% prevalence, respectively. Additionally, *A. ovis* infection in goat (34.7%) was lower than the results reported by Razmi *et al.* [10] and Ahmadi-Hamedani *et al.* [12] who recorded 38.92% and 63.7% prevalence, respectively. Based on available information on the prevalence of *A. marginale* infection in sheep of Iran, there is only one report on this case by Jalali *et al.* [13] from Ahvaz who recorded 43.7% infection.

This difference may have been the result of different sampling methods, geographical differences and climate differences and absence of mechanical and biological vectors.

According to previous report in these areas [14], biological vectors of *A. ovis* and *A. marginale* such as; *Dermacentor* sp., *Ixodes* sp. [14] and *Rhipicephalus annulatus* [15] were absent in collected ticks of sheep. As well as our study is in line with previous research, and population of these tick species within the region are very low.

Generally, due to this information can be mentioned three effective factors about this issue: 1 – tick ecology will change with the climate change, 2 – tick abundance has a strong negative association with altitude [16,17] and 3 – temperature reduction caused a negative impact on transovarial transmission of *Anaplasma* and infection of tick offspring does not occur even, when females are exposed to low temperature [18], so low infection to be expected.

Another issue on this study is significantly high prevalence of *Anaplasma* infection in goat than sheep ($P = 0.008$). It can be suggested that this significant difference is related to the susceptibility of goat to anaplasmosis.

In general, it can be concluded that although increasing of altitude has a negative impact in *Anaplasma* infections, but it is expected that the ecological change of vectors caused by global warming, could change anaplasmosis distribution in highland pastures. In addition to, *A. marginale* is a bacterium detected mainly in cattle, but in our study was detected in sheep which make sheep as potential reservoirs of this organism.

Conflict of interest statement

We declare that we have no conflict of interest.

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