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Original article

MOLECULAR DETECTION OF *ANAPLASMA* SPP. IN CATTLE OF TALESH COUNTY, NORTH OF IRAN

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Summary

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Anaplasmosis is generally caused by intraerythrocytic rickettsia of *Anaplasma* genus and transmitted biologically and mechanically. The current study was designed to determine the prevalence of *Anaplasma* spp. in cattle in Talesh; one of the rainy Iranian counties in Gilan province, Iran. From May to November 2015, one hundred and fifty blood samples of cattle were collected from different regions in Talesh. DNA was extracted from blood samples and subsequently, 16S rRNA and MSP4 genes were analysed by Nested-PCR method for differentiation of *Anaplasma spp.* The results showed that 40.66% of blood samples were positive for *Anaplasma spp.* and that 24.66%, 35.33%, 9.33% and 12% of positive samples were infected with *A. phagocytophilum, A. bovis, A. marginale* and *A. centrale* respectively. Statistical analysis by Chi-square test did not show any significant relationship between the presence of *Anaplasma* species and variables sex, age and tick infestation (P>0.05). The 4 species of *Anaplasma* reported in this study are of potential importance for animal and public health.

Key words: *Anaplasma*, cattle, tick-borne, Talesh, Iran

INTRODUCTION

Anaplasmosis is generally caused by intraerythrocytic rickettsia of genus *Anaplasma* (Dumler *et al*., 2001). This bacteria is transmitted biologically by ticks and mechanically by *Stomoxys* and *Tabanus* or blood contaminated instruments (Kocan *et al*., 2010). Anaplasmosis is important in two respects: first, economic losses from reduced milk production, abortion and weight loss iinduced by *Anaplasma ovis*, *Anaplasma marginale*, *Anaplasma centrale* and *Anaplasma bovis* (Rymaszewska & Grenda, 2008). Second comes the zoonotic importance of *Ana-*

plasma phagocytophilum (HGA) which causes a variety of clinical syndromes from mild in healthy subjects to severe in immunocompromised or elderly people (Murray *et al*., 2005; Robinson *et al*., 2009).

Anaplasmosis generally occurs in subtropical and tropical areas but as a result of global warming, the distribution of disease and particularly anaplasmosis may be expected to change, due to the location changes of biological vectors (Jonsson & Reid, 2000; Kocan *et al*., 2010). Hence the present survey was conducted to determine the prevalence of *Anaplasma* spp. in cattle in Talesh county, one of the rainy Iranian counties in Gilan province, Iran.

MATERIALS AND METHODS

Study area

Talesh is located on the southwestern coast of the Caspian Sea (37° 21'54" N, 50° 5' 34" E). Talesh County covers an area of 2,373 square kilometers area of Gilan Province. The annual average precipitation and temperature are 1,360 mm and 24.9 °C respectively (Fig. 1).

Fig. 1. Map of studied areas, sampling regions in Talesh county (Gilan province, Iran).

Sampling

From May to November 2015, one hundred and fifty blood samples (each 2 mL) were collected from the jugular vein of cattle (75 male; 75 female) in a sterile test tube containing 1 mL 96% ethanol from twelve different farms in the different regions of Talesh county.

All applicable international, national, and institutional guidelines for the care and use of animals were followed.

DNA extraction

DNA of blood samples was extracted using the DNA extraction minikit (YTA, Iran, Cat No: FABGK001) following the manufacturer protocol. Twenty µL Proteinase K and 200 µL BG Buffer were added to 50 mg of fixed blood sample, mixed thoroughly by vortexing and incubated for 15 min at 60 °C. In the next step, 200 µL ethanol (96%) was added to the sample and mixed thoroughly by vortexing for 30 s. The complete volume of solution was transferred into the BG column and was centrifuged at 8,000 rpm for 1 min. Then BG column was placed to a new collection tube. The BG column was immediately washed twice with 500 µL and 750 µL, respectively W1 Buffer, centrifuged at 14000 rpm and then the flow-through was discarded. Subsequently 100 µl of Elution Buffer was added to the membrane center of BG column and centrifuged for 2 min at 14000 rpm to elute the DNA. The extracted DNA was analysed in agarose gel and also by spectrophotometry. The ratio of OD_{260} to OD_{280} and the amount of extracted DNA and its purity were measured.

PCR of 16S rRNA gene

The *Anaplasma* spp. 16S rRNA gene was amplified by PCR in 50 µL reaction volume, containing 10 mM Tris-HCl

Table 1. Sequence details of primers used in PCR and Nested-PCR of 16S rRNA and MSP4 genes

Primer	Genbank No.	Nucleotide sequences 5' → 3'	Nucleotide positions	Target organism	PCR product
P1	M60313 (16S rRNA)	AGAGTTTGATCCTGGCTCAG	1-20	*Anaplasma* spp.	781 bp
P2		AGCACTCATCGTTTACAGCG	796-815		
P6	M73224 (16S rRNA)	CTTTATAGCTTGCTATAAAGAA	69-90	*A. phagocytophilum*	509 bp
P4		GTTAAGCCCTGGTATTTCAC	575-594		
P7	Af283007 (16S rRNA)	CAAATCTGTAGCTTGCTACGGA	65-86	*A.centrale*	513 bp
P4		GTTAAGCCCTGGTATTTCAC	575-594		
P8	AB196475 (16S rRNA)	CTCGTAGCTTGCTATGAGAAC	68-89	*A.bovis*	509 bp
P4		GTTAAGCCCTGGTATTTCAC	575-594		
M-OM F	HM640938.1 (MSP4)	GGGAGCTCCTATGAATTACAGAGAATTGTTTAC	1-33	*A.marginale/A. ovis*	867 bp
M-OM R		CCGGATCCTTAGCTGAACAGGAATCTTGC	839-867		
M-MAF	HM640938.1 (MSP4)	CTGAAGGGGGAGTAATGGG	113-131	*A. marginale*	344 bp
M-MAR		GGTAATAGCTGCCAGAGATTCC	435-456		

(pH 9.0), 30 mM KCl, 1.5 mm $MgCl_2$, 250 µm of each dNTP, 0.5 µm of each forward and reverse primers (P1/P2) (Table 1), 1.0 U Taq polymerase (Cinnagen, Iran) and 2 µL of DNA in automated thermocycler (Astec, Germany) for 37 cycles. Following an initial denaturation step of 5 min at 95 °C, each cycle consisted of a denaturing step of 45 s at 94 °C, annealing for 45 s at 58 °C and extension for 45 s at 72 °C and was completed with the final extension step for 5 min. The PCR amplicons (781 bp) were analysed in 1.5% agarose gel stained with ethidium bromide and UV condition (Fig.2A).

Nested-PCR of 16S rRNA gene

This technique was performed for detection of different *Anaplasma* species in 25 µL reaction volume, containing 10 mM Tris-HCl (PH 9.0), 30 mM KCl, 1.5 mM $MgCl_2$, 250 µm of each dNTP, 0.5 µm of each forward and reverse primers specific for each species (Table 1), 1.0 U Taq polymerase (Cinnagen, Iran) and 1 µL (diluted 1:200) of PCR products (781 bp) in automated thermocycler (Astec, Germany) for 35 cycles. Following an initial denaturation step of 5 min at 95 °C, each cycle consisted of a denaturing step of 45 s at 94 °C, annealing for 45 s at 57 °C, extension step of 45 s at 72 °C and a final extension step for 5 min at 72 °C. The PCR amplicons (*A. bovis*: 509 bp; Fig. 2B, *A. phagocytophilum*: 509 bp; Fig. 3A), *A. centrale*: 513bp; Fig. 3B) were analysed in 1.5% agarose gel stained with ethidium bromide.

PCR of MSP4 gene

For primers design limitations that existed in the 16s rRNA gene for differentiation between *A. ovis* and *A. marginale*, we used the MSP4 gene. This technique in first step was performed for detection of *A. marginale/ A. ovis* in 25 µL reaction volume, containing 10 mM Tris-HCl (PH 9.0), 30 mM KCl, 1.5 mM $MgCl_2$, 250 µm of each dNTP, 0.5 µm of each forward and reverse primers specific for *A. mar-*

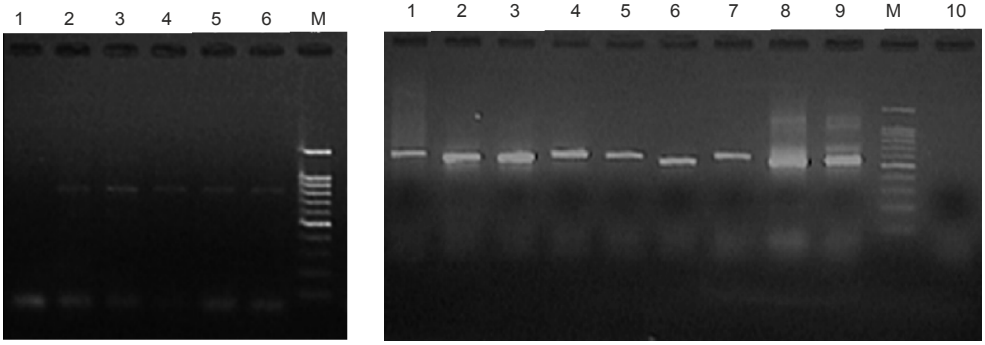

Fig. 2. A. PCR products of *Anaplasma* spp. 16S rRNA gene (781 bp), lane 1: negative control, lane 2: positive control, lanes 3 to 6: *Anaplasma* spp., M: 100 bp DNA ladder **B.** Nested-PCR products of *A. bovis* in 16S rRNA gene (509bp), Lanes 1 to 8: *A. bovis*, lane 9: positive control; lane 10: negative control.

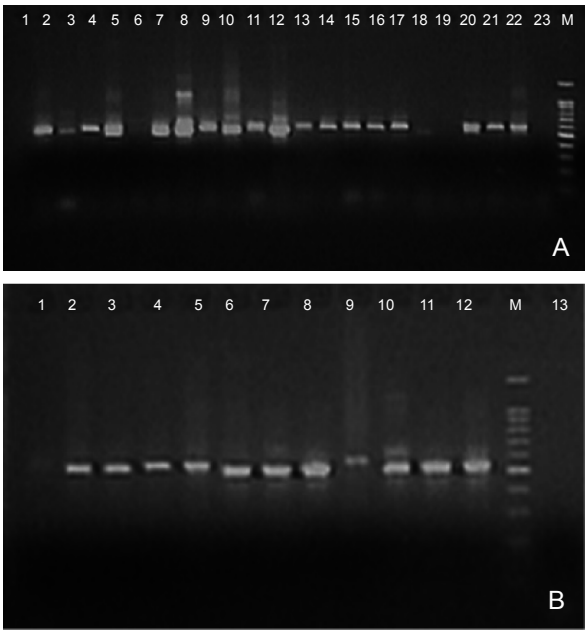

Fig. 3. A. Nested-PCR products of *A. phagocytophilum* in 16 srRNA gene (509 bp), lanes 2–5, 7–17, 20 and 21: *A. phagocytophilum*, lane 22: positive control, lane 23: negative control, M: 100 bp DNA ladder. **B.** Nested-PCR products of *A. centrale* in 16 srRNA gene (513 bp), lanes 2–11: *A. centrale*, lane 12: positive control, lane 13: negative control.

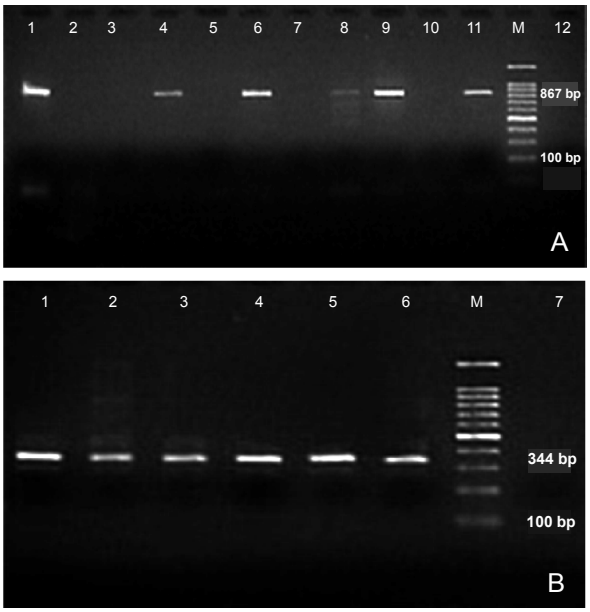

Fig. 4. A. PCR products (867 bp) of *A. marginale/A. ovis* in MSP4 gene, lanes 1, 4, 6, 8 and 9: *A. marginale*, lane 11: positive control, lane 12: negative control, M: 100 bp DNA ladder.
B: Nested PCR products (344 bp) of *A. marginale* in MSP4 gene, lanes 1 to 5: *A. marginale*, lane 6: positive control, lane 7: negative control, M: 100 bp DNA ladder.

ginale/A. ovis (Table 1), 1.0 U Taq polymerase (Cinnagen, Iran) and 2 µL DNA in automated thermocycler (Astec, Germany) for 32 cycles. Following an initial denaturation step of 5 min at 95 °C, each cycle consisted of a denaturing step of 30 s at 94 °C, annealing for 30 s at 60 °C and extension step of 30 s at 68 °C and with the final extension step for 5 min at 68 °C. The PCR amplicons of *A. marginale/ A. ovis*: 867 bp (Fig. 4A) were analysed in 1.5% agarose gel stained with ethidium bromide.

Nested-PCR of MSP4 gene

This technique was performed for differentiation between *A. ovis* and *A. marginale* by Nested PCR in 25 µL reaction volume, containing 10 mM Tris-HCl (PH 9.0), 30 mM KCl, 1.5 mM $MgCl_2$, 250 µm of each dNTP, 0.5 µm of each forward and reverse primers specific for *A. marginale* (Table 1), 1.0 U Taq polymerase (Cinnagen, Iran) and 1 µL (diluted 1:200) of PCR products (867 bp) in automated thermocycler (Astec, Germany) for 35 cycles. Following an initial denaturation step of 5 min at 95 °C, each cycle consisted of a denaturing step of 15 s at 94 °C, annealing for 20 s at 58 °C, extension step of 15 s at 72 °C and a final extension for 5 min at 72° C. The PCR amplicons of *A. marginale*: 344 bp (Fig.4 B) were analysed in 1.5% agarose gel stained with ethidium bromide.

Positive controls

All positive control samples used in this study were previously sequenced and was provided by the Department of Veterinary Parasitology, University of Tehran.

Statistical analysis

The chi-square test was used to analyse the statistical significance between *Anaplasma* species presence and variables such as sex, age and tick infestion at 95% confidence level. using SPSS v21.0 statistical software.

RESULTS

PCR and Nested PCR assessment of DNA samples based on 16S rRNA and MSP4 genes showed that 40.66% (61/150) of blood samples were positive for *Anaplasma* spp. In particular, 24.66% (37/150), 35.33% (53/150), 9.33% (14/150) and 12% (18/150) of positive samples were infected with *A. phagocytophilum, A. bovis, A. marginale* and *A. centrale* respectively.

All positive samples of *Anaplasma* species except 1 sample of *A. centrale,* 3 samples of *A. phagocytophilum* and 22 samples of *A. bovis* had mixed infection with other *Anaplasma* species. Also, *A. marginale* was the only species who was always seen with other species (Table 2).

Statistical analysis performed using the Chi-square test to assess the relationship between the presence of *Anaplasma* species and sex, age and tick infestation of cattle did not show any statistically significant relationships (P>0.05).

DISCUSSION

The 16S rRNA gene was selected for PCR because of high copy numbers in orde5r to increase the accuracy of detection of *Anaplasma* genome in infected animals with low parasitaemia. Since the nucleotides of the hypervariable V1 region of the 16s rRNA gene in *A. marginale* and *A. ovis* are not significantly different (they differ only in two base pairs), the design of a specific primer for any of them is impossible, and therefore, the MSP4 gene was used to differentiate between *A. marginale* and *A. ovis* species (Noaman, 2013).

Numerous researches have been conducted on anaplasmosis distribution among ruminants in Iran and in fact, studies in cattle anaplasmosis have been limited in some areas (Razmi *et al*., 2006; Ahmadi-Hamedani *et al*., 2009; Noaman & Shayan, 2009; 2012; Jalali *et al*., 2013; Hosseini-Vasoukolaei *et al*., 2014; Noaman & Bastani, 2016; Yousefi *et al*., 2017a,b; Yousefi, 2018). Based on available information no studies have been carried out to investigate the prevalence of *A. marginale*, *A.centrale*, *A. phagocytophilum* and *A.bovis* in ruminants from Gilan province.

Our results on *A. phagocytophilum* (24.66%) demonstrated a high prevalence compared to previous studies in Iran, as reported by Bashiribod *et al.* (2004) among *I. ricinus* (5.1%), by Noaman & Shayan (2009) in cattle (1.33%) and Yousefi *et al.* (2017a,b) in sheep and goats (1.08%). However, the prevalence of *A. phagocytophilum* in some reports on cattle from other countries as China (35%; Yang *et al*., 2013) and Turkey (24.8%; Aktas & Özübek, 2015) were similar to our results. Our results showed 35.33% prevalence of *A. bovis* which is higher than the results reported by Noaman & Shayan (2012) from cattle, Hosseini-Vasoukolaei *et al.* (2014) from *Boophilus annulatus* and sheep – 2.66%, 25% and 1.54% respectively. The results of the present study about *A. centrale* prevalence (12%) are different, when compared to the low prevalence (1.33%) found out in the study conducted in Esfahan (Noaman, 2013). On the other hand, contrary to pre-

Table 2. Prevalence details of *Anaplasma* species among cattle in Talesh county, Gilan province

Variables	No. tested	No. infected(%)							
		Anaplasma bovis	*Anaplasma centrale*	*Anaplasma marginale*	*Anaplasma phagocytophilum*	Co-infection with two species	Co-infection with three species	Co-infection with four species	Total infected
Sex									
Male	75	28(37.33)	8(10.66)	9(12)	15(20)	9(12)	10(13.33)	1(1.33)	29(38.66)
Female	75	25(33.33)	10(13.33)	5(6.66)	22(29.33)	6(8)	8(10.66)	2(2.66)	32(42.66)
Age									
< 1 years	32	9(28.12)	0	2(6.25)	0	3(9.37)	4(12.5)	0	10(31.25)
2 years	35	12(34.28)	8(22.85)	0	8(22.85)	2(5.71)	4(11.42)	0	14(40)
3 years	31	11(35.48)	2(6.45)	3(9.67)	11(35.48)	1(3.22)	3(9.67)	0	13(41.93)
4 years	26	9(34.61)	6(23.07)	5(19.23)	8(30.76)	7(26.92)	7(26.92)	3(11.53)	12(46.15)
>4 years	26	12(46.15)	2(7.69)	4(15.38)	10(38.46)	2(7.69)	0	0	12(46.15)
Tick infestation									
Infested	97	35(36.8)	9(9.27)	4(4.12)	21(21.64)	8(8.24)	3(3.09)	3(3.09)	36(37.11)
Uninfested	53	18(33.96)	9(16.98)	10(18.86)	16(30.18)	7(13.2)	15(28.30)	0	25(47.16)
Total	150	53(35.33)	18(12)	14(9.33)	37(24.66)	15(10)	18(12)	3(2)	61(40.66)

vious studies, the prevalence of *A. marginale* in this study was low (9.33%).

In discussing the high frequency of *Anaplasma* species evidenced by our results compared to those of others, it must be said that Iran has a diverse climate in different areas and that of Gilan province can be classified as humid subtropical climate (Skerman & Hillard, 1966). Due to the fact that the climate type will determine the type of insect fauna such as ticks, the presence of these vectors and their populations can affect the spread of different tick-borne diseases especially the prevalence of *Anaplasma* species. Further studies are required in diffrent areas to ascertain our knowledge and to verify *Anaplasma* species isolates, especially zoonotic species such as *A. phagocytophilum* in other animals, human and *Anaplasma* vectors in Iran.

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