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Anaplasma spp. identification in hard ticks of Iran: First report of *Anaplasma bovis* in *Haemaphysalis inermis*

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ABSTRACT — The aim of the present study was to determine the presence of *Anaplasma* spp. in hard ticks in the north of Iran. Tick samples were collected from sheep and goats grazing in Savadkooh, Mazandaran province and identified under a stereomicroscope according to identification keys. Salivary glands of the ticks were dissected and a polymerase chain reaction (PCR) assay, followed by partial sequencing of the 16S ribosomal RNA gene, was used for the detection and identification of *Anaplasma* spp. in the DNA extract of the salivary glands. A total of 618 ticks were collected from 122 sheep and goats from Savadkooh. The identified tick specimens belonged to 5 genera and 11 species including *Rhipicephalus bursa*, *Rh. sanguineus*, *Rh. turanicus*, *Rh. (Boophilus) annulatus*, *Haemaphysalis punctata*, *Ha. concinna*, *Ha. parva*, *Ha. inermis*, *Hyalomma marginatum*, *Dermacentor marginatus* and *Ixodes ricinus*. Eight of these, including *Rh. sanguineus*, *Rh. bursa*, *Ha. punctata*, *Ha. inermis*, *Ha. concinna*, *D. marginatus*, *Rh. turanicus* and *I. ricinus*, were positive for the presence of *Anaplasma*. All of the sequenced samples showed 99-100 % identity to *Anaplasma bovis*. The present paper is the first to detect *A. bovis* in *Rh. sanguineus*, *Rh. bursa*, *Ha. punctata* and *D. marginatus* in Iran; the highest infection rate of *A. bovis* in the collected ticks was found in *Rh. bursa*. This research is also the first report of *A. bovis* in *Ha. inermis* in the world.

KEYWORDS — *Anaplasmosis*; PCR; hard ticks; sheep; goats

INTRODUCTION

Ticks are blood sucking ectoparasites that play a significant role in the transmission of many pathogens to both animals and humans throughout the world. *Anaplasma* is a bacterial genus that includes several tick-borne pathogens causing anaplasmosis in animals and humans. *Anaplasma* spp. are small gram-negative obligate intracellular organisms. The genus *Anaplasma* includes *A. marginale*, *A. centrale*, *A. phagocytophilum*, *A. bovis*, *A. ovis* and *A. platys* (Rymaszewska and Grenda, 2008; Ybanez et al., 2014). *A. marginale* is the main intraery-

throcytic agent of bovine anaplasmosis. *A. centrale* is less pathogenic than *A. marginale*. *A. phagocytophilum* tends to invade and propagate in polymorphonuclear leucocytes causing human granulocytic anaplasmosis (HGA), tick-borne fever (TBF) in ruminants, and canine and equine granulocytic anaplasmosis (Rymaszewska and Grenda, 2008). *A. bovis* mainly occurs in monocytes of cattle but also has been detected in small ruminants, dogs, cats, rabbits and wild mammals which are probably reservoirs of the bacterium. Infection in cattle is usually asymptomatic but can result in clinical signs

including fever, anemia, weight loss and enlargement of prescapular lymph nodes. (Uilenberg, 1997; Goethert and Telford, 2003; Santos and Carvalho, 2006; Sakamoto *et al.*, 2010; Liu *et al.*, 2012; Sasaki *et al.*, 2012; Said *et al.*, 2015). *A. ovis* is an intraerythrocytic pathogen of sheep, goats and wild ruminants (de la Fuente *et al.*, 2004) and is less pathogenic in sheep than in goats. There is a paucity of information about the tick vectors of these rickettsial agents in Iran (Donatien and Lestoquard, 1936; Bashiribod, 2004; Hosseini-Vasoukolaei *et al.*, 2014; Saghafipour *et al.*, 2014). The aim of the present study is thus to determine the presence of *Anaplasma* spp. in the salivary glands of hard ticks collected from grazing sheep and goats of Savadkooh in Mazandaran province.

MATERIALS AND METHODS

Study area

Savadkooh is located in the south of the Caspian Sea with an altitude range of 250 m to 3651 m above the sea level. This region has a short summer with a mild, humid Mediterranean climate and long, freezing cold winters. Changes in altitude and slope result in high variation in weather and vegetation conditions. Vegetation up to 2000 m above sea level is green and forested, whereas above this altitude, there is only low vegetation with arid and cold weather. There are different types of trees, shrubs and grasses in the different areas with a variety of animals using the landscape: cattle, sheep, goats and deer feed and graze in this region, although sheep outnumber the other livestock species.

Tick sampling and identification

The tick samples were collected by examining the sites of predilection for ticks on the bodies of 86 sheep and 36 goats during the first 6 months of 2012. These animals belonged to 10 separate herds located across in the region. Individual ticks were counted on the animals and preserved in separated vials containing 70 % ethanol. Adult ticks were identified under a stereomicroscope according to identification keys (Walker *et al.*, 2003; Estrada-Peña

et al., 2004). Salivary glands of the ticks were dissected according to Edward *et al.* (2009). For each tick, sterilized scalpel blades were used to avoid possible contamination.

DNA extraction and PCR

Total DNA was extracted from the individual salivary glands of each tick using a DNA extraction kit (MBST, Tehran, Iran) and following the manufacturer's instructions.

The successful extraction of DNA was controlled using a primer pair (F1=5'-cacagggaggtagtgacaag-3' and R1=5'-aagaatttcacctatgacag-3') to amplify a fragment of the 18S rRNA gene (AJ003815) of the ticks.

The presence of Rickettsiales of the genus *Anaplasma* was assessed based on the presence of the 16S rRNA gene by PCR as previously reported (Noaman *et al.*, 2009). Two primer pairs that cover the hypervariable region of this locus were used. A first amplification was performed using primers F2 (5'-agagtttgatcctggctcag-3') and R2 (5'-agcactcatcgtttacagcg-3'). To control the specificity of the PCR products, a nested PCR was then performed to amplify an internal 543bp fragment of the same gene using a second pair of primers (F3=5'-gcaagcttaacacatgcaagtc-3' / R3=5'-gttaagcctggtatttcac-3'). These primers were designed by Noaman *et al.* (2009).

Approximately 20 ng of DNA was used for the PCR analysis performed in 100 μ L total volume including 10x PCR buffer, 2.5 U Taq Polymerase (Sinaclon, Iran), 2 μ L of each primer (20 μ M, Sinaclon, Iran), 2 μ L of each dATP, dTTP, dCTP and dGTP, (100 μ M, Fermentas), 1.5 mM MgCl₂ (50mM, Sinaclon, Iran). Reactions were carried out in automated thermal cyclers (Bio-Rad) with the following program: 5 min incubation at 95°C to denature double-stranded DNA, followed by 35 cycles of 45 s at 95°C (denaturation), 45 s at 59°C or 55°C (annealing) and 45 s at 72°C (extension) and an additional extension step at 72°C for 5 min. As a positive control, DNA extracted from *A. marginale* was used. As a negative control, we used distilled water. The annealing temperature for the PCR reaction was 50°C.

TABLE 1: Tick fauna and their infection prevalence with *Anaplasma* in the studied area, Mazandaran Province.

Tick species	Total (%)	Positive <i>Anaplasma</i> (%)	Negative <i>Anaplasma</i>
<i>Rh. turanicus</i>	146 (24)	95(65)	51
<i>Rh. bursa</i>	165 (27)	119(72)	46
<i>Rh. sanguineus</i>	20 (3)	8(40)	12
<i>Ha. punctata</i>	171(27.5)	105(61)	66
<i>Ha. concinna</i>	43(7)	22(51)	21
<i>Ha. parva</i>	18 (3)	0	18
<i>Ha. inermis</i>	14 (2)	6(42)	8
<i>Hy. marginatum</i>	14 (2)	0	14
<i>D. marginatus</i>	14 (2)	4(29)	10
<i>I. ricinus</i>	11 (2)	5(45)	6
<i>Rh. annulatus</i>	2 (0.3)	0	2
Total	618	364 (59%)	254 (41%)

Amplified PCR products were analyzed by electrophoresis on 1.5 % agarose gels, stained with Cybersafe and visualized under UV light. The PCR products were purified using a PCR purification kit (MBST, Tehran, Iran) and were directly sequenced by Kowsar Company (Iran, Tehran). For the analysis, the obtained nucleotide sequences were input to the Basic Local Alignment Search Tool (BLASTn) on the National Center for Bio-technology Information (NCBI) database website.

A phylogenetic tree was constructed with MEGA 6 software, applying the UPGMA method with bootstrap analysis (1,000 replicates).

RESULTS

In the present research a total of 618 ticks (294 female and 324 male) were collected from 122 sheep and goats. The identified tick specimens belonged to 5 genera and 11 species including *Rhipicephalus bursa* (27 %), *Ha. punctata* (27.5 %), *Rh. sanguineus* (3 %), *Ha. concinna* (7 %), *Rh. turanicus* (24 %), *Ha. parva* (3 %), *Hy. marginatum* (2 %), *D. marginatus* (2 %), *I. ricinus* (2 %), *Ha. inermis* (2 %) and *Rh(B). annulatus* (0.3 %).

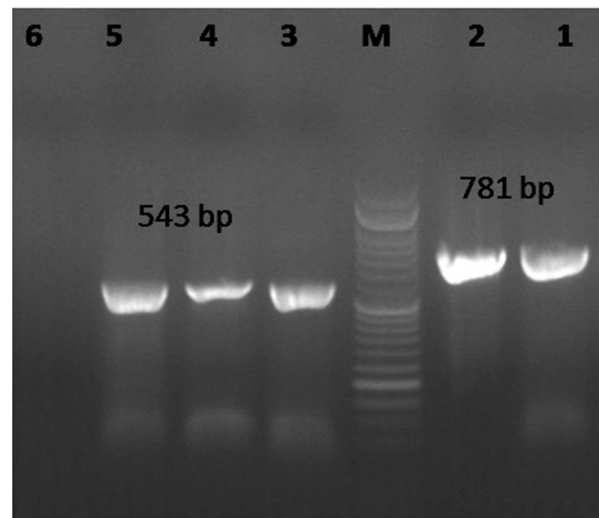


FIGURE 1: PCR products and nested-PCR products used to detect *Anaplasma*. Lane 1= PCR product with F2 and R2 primers; Lane 2= positive control; M=marker; Lane 3, 4, 5= Nested-PCR product with F3 and R3 primers; Lane 6= negative control

Amplification of the 18S rRNA gene of the ticks showed that DNA extracted from the salivary glands was of good quality. The amplicons obtained from the nested-PCR for *Anaplasma* were approximately 781bp and 543bp, respectively (Fig.1). The

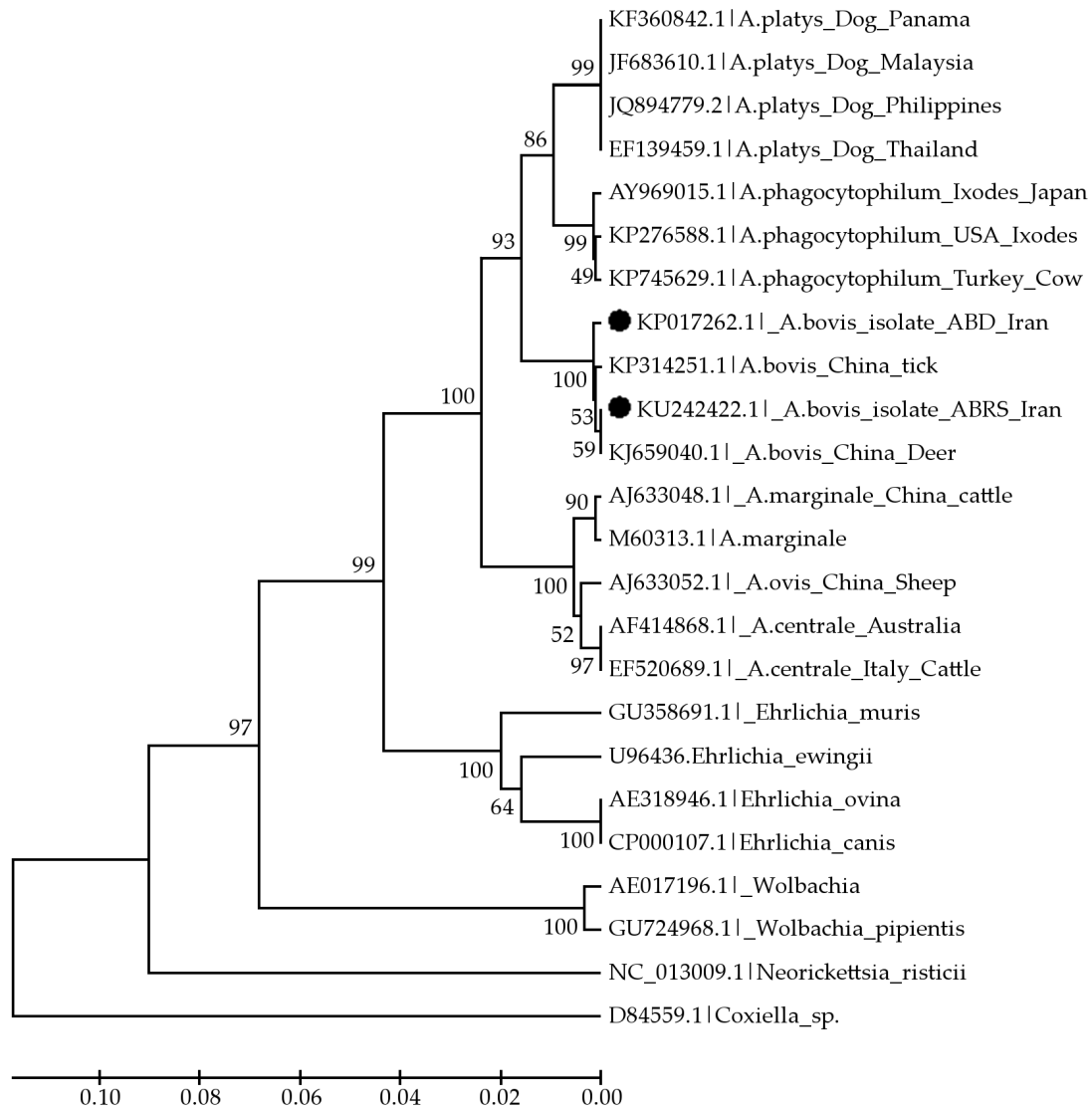


FIGURE 2: The phylogenetic tree of *A. bovis* obtained from the ticks in this study (showed with ●) and known *Anaplasma* species in GenBank. The evolutionary history was inferred using the UPGMA method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 24 nucleotide sequences. There were a total of 488 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

16S rRNA gene of *Anaplasma* spp. was detected in *Rh. sanguineus*, *Rh. bursa*, *Ha. punctata*, *Ha. inermis*, *Ha. concinna*, *D. marginatus*, *Rh. turanicus* and *I. ricinus*. From all the examined tick samples that were collected from different herds, 59 % were infected with *Anaplasma* spp. (Table 1). In total, fifty nested PCR products, chosen in relation to the population size of the herd, were sequenced; all showed 99–100 % identity with *A. bovis* sequences available in GenBank including *A. bovis* detected in ticks from Japan and China (AB983376-KP314251), deer from China and Korea (KJ659040, KJ639885, GU556626), cattle from Japan and Tunisia (JN811556, KM401902), *Ha. longicornis* from South Korea (KC311345), and goats from China (HQ913644). The 16S rRNA nucleotide sequences of *A. bovis* registered under accession numbers KP017262 and KU242422 were found in the *D. marginatus*, *Rh. sanguineus*, *Rh. bursa*, *Ha. inermis* and *Ha. punctata*.

The similarity among the sequenced strains of *Anaplasma* in this study was 100 % except for *A. bovis* from *D. marginatus* showing 99.8 % identity with the others. In this investigation, no infection was detected in the following species: *Ha. parva*, *Hy. marginatum* and *Rh. (B). annulatus*. *Rh. turanicus* and *I. ricinus* were positive in PCR and nested-PCR for the presence of *Anaplasma*, however, the *Anaplasma* sequences obtained from these tick species could not be identified due to incomplete sequencing. Our results showed that the most infected ticks were *Rh. bursa* and *Rh. turanicus* with 72 and 65 % infection rate respectively.

A phylogenetic tree based on the similarity between our sequences with registered sequences in GenBank showed 2 subclades for *Anaplasma* spp.: one subclade including *A. platys*, *A. phagocytophilum* and *A. bovis* and the other with *A. marginale*, *A. ovis* and *A. centrale*. *Wolbachia* and *Ehrlichia* were in separate clades (Fig.2).

DISCUSSION

Ticks transmitting pathogens, such as the Crimean-congo haemorrhagic fever virus, *Anaplasma* spp., *Ehrlichia* spp., and *Babesia* spp. are serious threats to human and animal health. As global warming can

result in climate change and modifications in the distribution of tick species and tick-borne disease agents (Aydin and Bakirci, 2007), regular monitoring of tick species and associated infectious agents is essential for understanding disease risk and for implementing control and prevention strategies.

A. bovis was first described in Iran (Donatien and Lestoquard, 1936) and this was followed by reports in many other countries such as Africa, Brazil, North America, China, Japan and Korea (Goethert and Telford, 2003; Kawahara *et al.*, 2006; Ooshiro *et al.*, 2008; Liu *et al.*, 2012; Doan *et al.*, 2013). Anaplasmosis has been reported to be present in animals in Iran by many investigators (Spitalska *et al.*, 2005; Razmi *et al.*, 2006; Ahmadi-Hamedani *et al.*, 2009; Noaman and Shayan, 2009; Noaman *et al.*, 2009; Noaman and Shayan, 2010; Jalali *et al.*, 2013); however, there are few studies about the tick vectors of this rickettsial agent in the country. In the present work, the 16S rRNA gene was employed as a sensitive molecular tool for the detection of *Anaplasma* DNA (Kang *et al.*, 2011). Unlike other studies which typically used DNA from whole ticks or tick pools, we used extracts from individual tick salivary glands in order to quantify prevalence more exactly. In this study *A. bovis* was detected in all of the collected tick species except *Ha. parva*, *Hy. marginatum* and *Rh. (B). annulatus*. Although one paper reported *I. ricinus* as a vector of *A. phagocytophilum* in Iran (Bashiribod, 2004), *A. phagocytophilum* was not detected in this study. Hosseini-Vasoukolaei *et al.* (2014) showed *A. ovis* in *Rh. sanguineus* and *I. ricinus* and *A. bovis* in *Rh. (B). annulatus* from Ghaemshahr, Iran (Hosseini-Vasoukolaei *et al.*, 2014). We did not detect *Anaplasma* spp. in collected *Rh. (B). annulatus*, but this may be due to the low number of samples of this particular tick species.

Saghafipour *et al.* (2014) isolated *A. ovis* from *Rh. sanguineus* in Qom, Iran, but they could not detect any *Anaplasma* spp. in *Hy. dromedarii*, *Hy. schulzei* and *Hy. marginatum* (Hosseini-Vasoukolaei *et al.*, 2014; Saghafipour *et al.*, 2014). Our results supported the data reported previously by Hosseini-Vasoukolaei *et al.* (2014) and Saghafipour *et al.* (2014) dealing with the absence of any *Anaplasma* spp. in *Hy. marginatum*.

According to Hosseini-Vasoukolaei *et al.* (2014), 43 % of sheep were positive for the presence of *Anaplasma spp* (*A. ovis* and *A. bovis*) in Ghaemshahr that is close to Savadkooh. However, *A. bovis* is mainly associated with cattle. In our study was also detected this bacterial species in ticks infesting small ruminants, suggesting that sheep and goats could be reservoirs for *Anaplasma* in cattle.

Based on numerous studies, the main vectors of *Anaplasma* may include a variety of ticks from the genera *Ixodes*, *Dermacentor*, *Rhipicephalus*, and *Amblyomma* (Rymaszewska and Grenda, 2008). *I. ricinus*, *I. persulcatus*, *I. scapularis*, *Rh. sanguineus*, *Rh. bursa*, *Rh. turanicus*, *A. americanum*, *D. variabilis*, *D. andersoni*, *D. auratus*, *D. silvarum*, *Ha. lagrangei*, *Ha. leporispalustris*, *Ha. longicornis*, *Ha. concinna*, *Ha. punctata*, *Ha. megaspinosa*, *Hy. marginatum*, *Hy. asiaticum*, and *Hy. detritum* have been confirmed as vectors for *Anaplasma* in different studies (Parola and Raoult, 2001; Goethert and Telford, 2003; Kim *et al.*, 2003; Parola *et al.*, 2003; de la Fuente *et al.*, 2004; Stafford III, 2004; Kawahara *et al.*, 2006; Kim *et al.*, 2006; Shpynov *et al.*, 2006; Rymaszewska and Grenda, 2008; Yoshimoto *et al.*, 2010; Jiang *et al.*, 2011; Meng *et al.*, 2012; Bonnet *et al.*, 2013; Palomar *et al.*, 2015). Our study also supported *Rh. sanguineus* as a common tick vector for *Anaplasma* throughout the world (Stafford III, 2004; Rymaszewska and Grenda, 2008; Ghafar and Amer, 2012; Víchová *et al.*, 2014). Thanh Doan *et al.* (2013) detected *A. bovis* in 9.3 % of 535 *Ha. longicornis* by PCR using the 16S rRNA gene. They found 4 genotypes of *A. bovis*, but in the present study only one genotype of *A. bovis* was detected. The phylogenetic analysis presented in this study is consistent with Doan *et al.* (2013) and Ybanez *et al.* (2014), showing that *A. bovis* forms a cluster with *A. platys* and *A. phagocytophilum*.

In conclusion, the present research is the first report of *A. bovis* in a wide range of different tick species feeding on sheep and goats in Iran. Moreover, the present study has detected the presence of *A. bovis* in *Ha. inermis* for the first time in the world. Although the examined ticks were collected from small ruminants rather than cattle, these hosts may function as reservoirs for *A. bovis*, not only for cattle

but also for the wild animals living in the studied areas.

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