Original Article

Anaplasma Infection in Ticks, Livestock and Human in Ghaemshahr, Mazandaran Province, Iran

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Abstract

Background: Anaplasmosis is an important issue for animal breeders in terms of economic losses as well as a health concern to human. Ticks are considered as the main vector of this disease. Lack of documented information about *Anaplasma* species in Iran was the scope of this study to determine the population of ticks and the presence of *Anaplasma* in ticks, domestic ruminants and also human beings in northern Iran.

Methods: A total of 101 unengorged hard ticks, 78 domestic ruminants and 40 human blood samples collected from Ghaemshahr, Mazandaran Province, northern Iran were tested by nested PCR against 16s rRNA gene of *Anaplasma* species.

Results: Positive PCR was found in 50 ticks, 28 sheep, 2 cattle, one goat, and 10 human specimens. Sequence analysis of the PCR products confirmed presence of *A. ovis* in two *Rhipicephalus sanguineu*s and two *Ixodes ricinus* ticks, one human and 4 sheep samples. Moreover one *Boophilus annulatus* tick and one sheep sample were infected with *A. bovis*. Furthermore one sample of sheep was infected with *A. centrale*.

Conclusion: This study is the first report of tick infection to *A. ovis*, *A. bovis* and human infection to *A. ovis* in Iran. The result of this study is a survey of *Anaplasma* infections from ticks, domestic animals and human in Iran which help to have appropriate prevention measures for anaplasmosis.

Keywords: *Anaplasma*, Human, Iran, Livestock, Tick

Introduction

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 (Stuen et al. 2003).

Members of the genus *Anaplasma* are obligatory intracellular gram negative bacteria that infect blood cells of mammals. Six *Anaplasma* species are currently recognized (Dumler et al. 2001). 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 (Splitter et al. 1956). Anaplasmosis in cattle is caused by *A. bovis* infecting monocytes (Uilenberg 1993), or by *A. marginale* and *A. centrale* which parasitize and replicate in red blood cells (Kuttler 1966). *Anaplasma bovis* is reported mostly from cattle, but also detected in small ruminants

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which could be a reservoir of this bacterium (Goethert et al. 2003).

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 (Stafford 2007).

Agriculture and animal husbandry is the main activities of people in Mazandaran Province, northern Iran, and anaplasmosis is one of the major veterinary health problems there. However, there have been only a few studies to detect tick anaplasmosis infections in the country (Bashiribod et al. 2004, Spitalska et al. 2005). These two studies reported *A. phagocytophilum* and *Ehrlichia ovina* infection in ticks in north and counter parts of Iran.

Due to the lack of documented information about *Anaplasma* species in ticks, animals and also human beings in the country and having found clinical features and laboratory findings similar to anaplasmosis in some shepherds in suburban areas of Ghaemshahr County in northern Iran during recent years, we conducted the present study to understand more about the *Anaplasma* infections in Iran.

Materials and Methods

Sampling

The study was carried out in Ghaemshahr County in north of Iran, in which a number of suspected cases of anaplasmosis were reported (Mahmoudi 2004). The collection site was villages of suburban forest area in Ghaemshahr in which the climate is subtropical with cold winters and moderate summers.

About 425 domestic ruminants (361 sheep, 54 goats and 10 available cattle) from 18 herds of nine villages in Ghaemshahr were inspected for tick infestation. The whole body of each animal was inspected and the ticks were manually removed from animals' body. The sampling was done through 2008 in a period corresponding to seasonal tick activity. Collected ticks from infested animals were kept in dry plastic tubes containing few fresh grass leaves covered by a lid containing several minute holes. Tubes were labeled and conditioned under room temperature for a few days, and then they were dispatched to the laboratory. The purpose of this procedure was to maintain ticks alive inside the tubes until the laboratory taxonomic identification. Ticks species identification was done by using the criteria key described by Hoogstraal (Hoogstraal 1979). Totally 323 ticks were collected from which 101 unengorged ticks were selected for pathogen detection by PCR examination.

Blood samples were additionally taken from both domestic ruminants and corresponding voluntary shepherds. Blood samples were taken from jugular vein of sheep and goat and from caudal vein of cattle. A total of 78 blood samples including 65 from sheep, nine from cattle and four from goats were collected, from which 38 were from animals with tick infestation and 40 from animals without any tick infestation.

Forty samples were taken from median cubital vein of corresponding voluntary shepherds from different age groups. All animals and humans had not any clinical signs. All tissue had been obtained with consent given according to the institutional guidelines.

DNA extraction and PCR amplification-sequencing

DNA extraction carried out from ticks and blood samples using the G-spin™ Genomic DNA Extraction Kit (iNtRON Biotechnology, Korea) and i-genomic Blood

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DNA Extraction Mini Kit (iNtRON Biotechnology, Korea), respectively following the manufacturer protocol.

Anaplasma DNA was detected by means of nested PCR with primers derived from the 16S rRNA gene of *Anaplasma* species. PCR was performed in 20 µl reaction mixture containing 10 mm Tris-HCl (Ph 9.0), 30 mm KCl, 1.5 mm MgCl2, 250 mm each dNTP, 0.5 mm each sense and antisense primers, 1 U Taq DNA polymerase and 2 µl of DNA. The conditions for PCR included an initial denaturation at 94 ˚C for 3 min followed by 35 cycles of amplification (1 min denaturation at 94 ˚C, 1 min annealing at 57 ˚C and 1 min elongation at 72 ˚C) (Rar et al. 2008). The primer pair used for PCR were Ehr1 (5´-GAACGAACGCT GGCGGCAAGC-3´) and Ehr2 (5´-AGTA [T/C]CG[A/G]ACCAGATAGCCGC-3´).
Nested PCR was performed with 2 µl of the first PCR reaction using primer pair Ehr3 (5´-TGCATAGGAATCTACCTAGTAG-3´) and Ehr4 (5´-CTAGGAATTCCGCTATCC TCT-3´) and resulted in a 524-bp product. The PCR procedure was with exception of annealing temperature (60 ˚C), the same as described above (Rar et al. 2008).

As positive control we used *Anaplasma* DNA obtained from Department of Parasitology, Faculty of Veterinary Medicine, University of Tehran (Noaman et al. 2009), and double distilled water as negative control was used. The PCR analysis was performed on agarose gel using ethidium bromide and UV condition. The PCR products were directly subjected to sequencing by Seqlab (GmbH, Germany).

The nucleotide sequences were then blasted by BLASTN (http://www.ncbi.nlm.nih.gov/BLAST) program.

Results

Blood and ticks infected by *Anaplasma* species

Overall 102 (24%) out of 425 inspected animals and in particular 28.25% (88/361) sheep, 22.22% (12/54) goats and 20% (2/10) cattle were infested with ticks. Altogether 323 ticks were collected categorized into four genera and six species: *Rhipicephalus sanguineus* (266, 82.35%), *R. bursa* (1, 0.31 %), *Ixodes ricinus* (49, 15.17%), *Boophilus annulatus* (4, 1.24%), *Haemaphysalis punctata* (1, 0.31%) and *H. numidiana* (2, 0.62%). *Rhipicephalus sanguineus* with 82.35% was observed as the most abundant tick species found in the study area. Totally 101 unengorged ticks were examined by nested PCR for presence of *Anaplasma* species.

A 524-bp 16S rRNA gene fragment of *Anaplasma* species was identified in 49.5% of examined ticks (50 out of 101), including *R. sanguineus* (34/54, 62.96%), *I. ricinus* (14/39, 35.9%), *B. annulatus* (1/4, 25%), and *H. punctata* (1/1, 100%).

Similarly, blood samples from ruminants also contained *Anaplasma* DNA. Genome of *Anaplasma* species was detected in 39.74% (31/78) blood samples, and in particular 43.08% (28/65) sheep, 22.22% (2/9) cattle, and 25% (1/4) goats were infected (Table 1, Fig. 1).

Thirty eight out of 78 tested blood samples, belonged to the ruminants infested with ticks. Nineteen out of 38 (50%) were infected with *Anaplasma*, and in 16 cases, *Anaplasma* DNA was detected in both the tick and its host simultaneously. Of 78 blood samples, 40 were collected from ruminants without tick infestation; in 12/40 (30%) infection to the bacteria were revealed.

This study also is the first which tested human blood by molecular methods for the presence of *Anaplasma* species In Iran.

Blood samples from 40 people between 15 and 78 years old were examined by nested PCR with the same primers. DNA of *Anaplasma* species was detected in 25% (10/40) blood samples, and in particular six of 22 (27.27%) female and four of 18 (22.22%) male. Most of the infected cases

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were from shepherds older than 40 years old and in contact with livestock for most of their lives (Table 1, Fig. 1).

Sequencing

A number of positive PCR samples against *Anaplasma* in ticks separated from sheep (5), sheep blood (6), and human blood (1) were sequenced by the forward and reverse primers and the consensus sequences were submitted to GenBank.

All nucleotide sequences were registered in GenBank under accession numbers JF514503, JF514504, JF514505, JF514506, JF495135, JF514507, JF514510, JF514511, JF514512, JF514513, JF514508 and JF514509.

Among the specimens, two *R. sanguinus* (JF514510, JF495135), two *I. ricinus* (JF514503, JF514511), three sheep (JF514504, JF514505, JF514506) and one human (JF514507) with 484 bp length were 100% identical to *A. ovis* present in GenBank database. One specimen originated from sheep (JF514512) was identical to *A. ovis* except for one nucleotide transmission (C/T).

Nucleotide sequences of *A. bovis* determined in one female *B. annulatus* tick (JF514508) and one sheep (JF514513) were identical to each other. Also one blood sample from a female sheep was infected with *A. centrale* (JF514509).

The present study for the first time demonstrated the presence of *A. ovis* in human, *R. sanguineus* and *I. ricinus*, and *A. bovis* in *B. annulatus* in Iran.

Table 1. Results of nested PCR for detection of *Anaplasma* species in ticks, domestic animal and human blood samples collected from Ghaemshahr, Iran, 2008

	Male	Total
Ticks		
Rhipicephalus sanguineus	11/ 16 (68.75)	34/ 54 (62.96)
Rhipicephalus bursa	0	0/1 (0)
Ixodes ricinus	1/6 (16.67)	14/39 (35.9)
Boophilus annulatus	0/1 (0)	1/4 (25)
Haemaphysalis punctata	1/1 (100)	1/1 (100)
Haemaphysalis numidiana	0	0/2 (0)
Domestic ruminants		
Sheep	9/15 (60)	28/65 (43.08)
Goat	0	1/4 (25)
Cattle	0	2/9 (22.22)
Human age groups		
< 20 years	0/3 (0)	1/5 (20)
20-30 years	1/4 (25)	1/7 (14.29)
30-40 years	0/3 (0)	0/8 (0)
40-50 years	1/5 (20)	3/9 (33.33)
50-60 years	1/2 (50)	3/6 (50)
> 60 years	1/1 (100)	2/5 (40)

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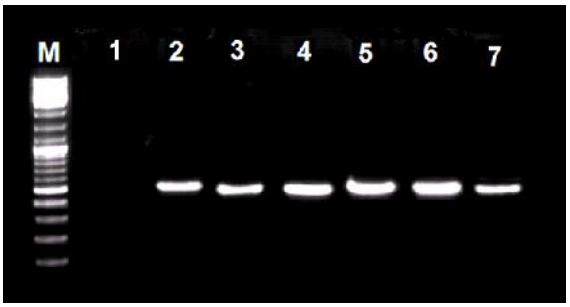

Fig. 1. Nested PCR amplicons of a 524-bp *Anaplasma* 16S rRNA gene fragment from biological samples (tick and blood). M, 100-bp size marker (Fermentase, Germany), lane 1, negative (water) control, lane 2, positive control, lane 3–7, *Anaplasma* positive samples of tick, sheep, cattle, goat and human blood, respectively

Discussion

The main vectors of the *Anaplasma* bacteria are ticks, especially the genera *Ixodes*, *Dermacentor*, *Rhipicephalus* and *Amblyomma.*

During our study six species of hard ticks were identified: *R. sanguineus*, *R. bursa*, *I. ricinus*, *B. annulatus*, *H. punctata*, and *H. numidiana.* Half of these species are known as *Anaplasma* vectors. Out of 323 collected ticks from animals, a significant number of 266 *R. sanguineus* were identified. This species stands out as being the most prevalent tick species comprising 82.35% of the ticks collected from domestic ruminants in Ghaemshahr. A total of 101 unengorged ticks were assayed by nested PCR for presence of *Anaplasma* species. Most of (34/50, 68%) the infected ticks belonged to *R. sanguineus* species. Therefore *R. sanguineus* with a high level of infestation of domestic ruminants, and the prevalence of *Anaplasma* DNA (62.96%) could be the most abundant vector of *Anaplasma* species in Ghaemshahr. The nucleotide sequence determination deriving from one male and one female *R. sanguineus* were identical to that of the gene of *A. ovis* (JF495135, JF514510). This is in agreement with the results of other researches indicating the role of *R. sanguineus* in *Anaplasma* transmission. Previous study in Africa had shown presence of *A. platys* DNA in a female *R. sanguineus* (Sanogo et al. 2003). In a study in Turkey *A. ovis* 16S rRNA gene fragment was detected in two *R. sanguineus* ticks (Aktas et al. 2009).

In the present study *A. ovis* 16S rRNA was detected in two *I. ricinus* ticks making this tick species as a probable vector of *A. ovis* in Ghaemshahr. The tick *I. ricinus* has been reported from Europe and Northern Africa (Sarih et al. 2005), and was evidenced to be the vector of *Anaplasma* and in particular *A. phagocytophilum* in Europe (Parola et al. 2005, Silaghi et al. 2008, Aktas et al. 2009, Portillo et al. 2011). In Iran, *I. ricinus* ticks were found only in Caspian Sea regions in north part of Iran (Rahbari et al. 2007, Razmi et al. 2007). In Ghaemshahr, Iran *A. phagocytophilum* 16S rRNA was detected in 5 (5.1%) of tested *I. ricinus* ticks. It showed a high probability of human granulocytotropic ehrlichiosis (HGE) in some stock-farmer patients from Ghaemshahr suburban areas with similar clinical and laboratory findings to HE (Bashiribod et al. 2004).

In Brazil, *A. marginale* DNA was detected in *B. microplus* ticks. *B. annulatus* could experimentally transmit this bacterium to calves (Shimada et al. 2004). We detected a 524 bp 16S rRNA gene fragment of *A. bovis* in one female *B. annulatus* collected from sheep in Ghaemshahr.

We could detect *Anaplasma* sp. in one *H. punctata* tick collected from a sheep. *H. punctata* was experimentally able to transmit *Babesia major* to calves. *Anaplasma phagocytophilum* and *A. bovis* was detected in *H. megaspinosa* and *H. longicornis* in Japan and Korea, respectively (Oh et al. 2009, Yoshimoto et al. 2010).

We examined two groups of blood samples obtained from animals with and without tick infestation. Fifty percent (19/38) of samples from animals with tick infestation were *Anaplasma* positive compared to 30% (12/

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40) positive samples taken from animals without any ticks on their bodies. These results demonstrate the importance of ticks in *Anaplasma* transmission to domestic animals in the studied area.

A few studies on the infectivity of animal blood samples to *Anaplasma* have been done in Iran; in Khorasan Province, north east of Iran 80.3% of sheep and 38.92% of goats blood smears were infected with *A. ovis* (Razmi et al. 2006). In Isfahan Province in the center of Iran, 16S rRNA gene fragment of *A. marginale* was detected in 50 % of cattle without any clinical signs (Noaman et al. 2009). In the present study 39.7% of blood samples were infected with *Anaplasma*. These blood samples were obtained from domestic ruminants without any clinical signs showing their role as reservoirs of *Anaplasma*.

During our study, *A. ovis* and *A. bovis* DNA was detected in sheep blood samples. *A. bovis* is a bacterium detected mainly in cattle and small mammals (Goethert et al. 2003), but in our study was detected in sheep. These results make sheep also as potential reservoirs of this bacterium.

Of 40 blood samples taken from people from different age groups 15–78 years, 10 (25%) were infected. There was not any significant difference between males and females showing equal cooperation of women and men in livestock husbandry in studied area. Most of the infected cases were from shepherds older than 40 years old and in contact with livestock for most of their lives. One blood sample obtained from a 55-year-old woman without any clinical symptoms was infected with *A. ovis*.

Some researches in Cyprus showed *Anaplasma* spp. infections in humans (Psaroulaki et al. 2008, Chochlakis et al. 2009). In another laboratory testing of human blood samples by universal primers against all *Anaplasma* species in Cyprus, *A. ovis* 16S rRNA was found in one human blood sample taken from a 27-year-old woman with thrombocytopenia and elevated levels of transminses (Chochlakis et al. 2010).

In Iran, *A. ovis* was identified in sheep (Spitalska et al. 2005, Razmi et al. 2006). Since sheep are reservoirs of *A. ovis*, infection of humans with this pathogen may occur, but transmission of *A. ovis* to humans is uncertain. *R. sanguineus* and *I. ricinus* are dominant tick species in sheep in northern Iran (Rahbari et al. 2007, Hosseini Vasoukolaei et al. 2010). Results revealed that *R. sanguineus* highly infested domestic ruminants and *A. ovis* DNA was detected in this tick species. *Ixodes ricinus* role as a vector of *A. phagocytophilum* to human has been suggested (Bashiribod et al. 2004). However, potential role of *R. sanguineus* and *I. ricinus* as vectors of *A. ovis* to human is unknown.

Conclusion

This study is the first to report molecular detection of *A. ovis* from human in Iran, whereas does not show *A. ovis* human pathogenicity. However, further epidemiological, clinical and pathological investigations are needed to understand *A. ovis* potential to infect human.

Infection of ticks, ruminants and humans to *Anaplasma* species demonstrated the potential endemicity and circulation of the pathogen among different tick species found in the region. Ticks of *R. sanguineus* are probably the main vector of *Anaplasma* in northern part of Iran. Application of control measures with emphasizing on control of *R. sanguineus* could prevent *Anaplasma* transmission in the region.

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