

Molecular detection and identification of *Anaplasma* species in sheep from Ahvaz, Iran

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Summary

Ovine anaplasmosis is a tick-borne rickettsial disease, widespread in tropical and subtropical areas. In the present study, a PCR-RFLP method based on major surface protein 4 (MSP4) gene, was utilized for the detection of *Anaplasma* infection in 119 sheep blood samples collected from different parts of Ahvaz in the southwest of Iran. PCR identified *Anaplasma* infections in 87.4% (104/119) of the samples in contrast to the routine blood smear examination, which revealed inclusion bodies in only 33.6% (40/119) of samples. RFLP assessment revealed that all PCR positive samples were *A. ovis*, while for the first time in Iran, a mixed infection with *A. marginale* was seen in 50% (52/104) of *Anaplasma* infected samples. These results suggest higher sensitivity of PCR method over the conventional microscopic technique for diagnosis of anaplasmosis, particularly in carrier animals. It also revealed that ovine anaplasmosis caused by *A. ovis* and *A. marginale* is present and highly prevalent in Ahvaz and appears to be the first report from this region.

Key words: *Anaplasma*, Sheep, PCR-RFLP, Ahvaz, Iran

Introduction

Recently, there has been growing interest in rickettsia from the genus *Anaplasma*, due to its pathogenicity in farm animals (Kocan *et al.*, 2004; de la Fuente *et al.*, 2005; Ahmadi-Hamedani *et al.*, 2009). Anaplasmosis, a disease caused by various species of *Anaplasma*, is especially important for animal breeders, as it can reduce the animal's body weight, cause abortions, reduce milk production and frequently lead to death (Splitter *et al.*, 1955; Sainz *et al.*, 1999; Melendez, 2000; Stuen *et al.*, 2003).

Ovine anaplasmosis is mainly caused by *A. ovis* and *A. marginale*. In the case of *A. ovis*, bacterial inclusions are found 35-40% of the time in the central or submarginal part of the host erythrocyte, and the remaining 60-65% in the marginal part (Shompole *et*

al., 1989). Although *A. ovis* may infect domestic sheep and goats without clinical signs (Splitter *et al.*, 1956), it can predispose animals to other infections resulting in clinical disease and eventually death (Kocan *et al.*, 2004).

Anaplasmosis is transmitted to mammalian hosts biologically by ticks and mechanically by biting flies and blood-contaminated fomites (Aguirre *et al.*, 1994; de Echaide *et al.*, 1998). *Rhipicephalus sanguineus*, *Hyalomma marginatum marginatum*, and *Hyalomma anatolicum anatolicum* have recently been shown to be the main ticks which transmit ovine anaplasmosis in Iran (Noaman, 2012). All of these tick vectors are widespread in Khuzestan province (Rahbari *et al.*, 2007; Nabian *et al.*, 2009).

Diagnosis of *Anaplasma* infection is performed routinely by microscopic

examination of giemsa-stained blood smears in Iran. This method is suitable in detection of anaplasmosis in acute phase, but it is not applicable in identifying pre-symptomatic and carrier animals (Carelli *et al.*, 2007).

Molecular methods, as more sensitive and specific diagnostic tools, have been increasingly used to detect and differentiate *Anaplasma* spp. in carrier animals (Bekker *et al.*, 2002; Molad *et al.*, 2006; Carelli *et al.*, 2007; Ahmadi-Hamedani *et al.*, 2009; Noaman and Shayan, 2010).

The major surface protein 4 (MSP4) gene, a part of the MSP2 protein superfamily, is proven to be useful for detection and genetic characterization of *Anaplasma* spp. (de la Fuente *et al.*, 2005; Brayton *et al.*, 2006).

Anaplasmosis has been reported from some parts of Iran (Razmi *et al.*, 2006), but few of them were detected with molecular methods (Nazifi *et al.*, 2008; Ahmadi-Hamedani *et al.*, 2009; Noaman *et al.*, 2009).

Ahvaz, the capital city of Khuzestan province, is a tropical area in the southwest of Iran, in which parasitic infections and tick-borne diseases are highly prevalent (Al-Amery and Hasso, 2002; Zaeemi *et al.*, 2011). However, the epidemiological aspects of ovine anaplasmosis in the southwest of Iran are poorly understood. *Anaplasma*, like intraerythrocytic organisms, were frequently detected in sheep presented to the Veterinary Clinic, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz. But morphological characteristics of *Anaplasma* inclusion bodies, especially in low-level parasitemias are not enough to distinguish *Anaplasma* species from each other and from Howell-Jolly bodies which may be seen in the anemia of different origins (Shompole *et al.*, 1989; Ndung'u *et al.*, 1995), thus complicating the treatment.

Since there were no previous reports regarding ovine anaplasmosis prevalence in Ahvaz, the current study was designed to characterize the infection with *Anaplasma* and to identify the species involved, employing a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) protocol based on MSP4 gene sequence, and its comparison over the

routine blood smear method in naturally infected sheep of Ahvaz region, Iran.

Materials and Methods

Collection of blood samples

The present study was conducted in Ahvaz, and the surrounding area, which is situated in the southwest of Iran, the tropical endemic area of ovine tick-borne diseases. Sampling was performed during the tick activity season, July to September 2011, in which the temperature and humidity of this area ranges between 26.3 to 47.3°C, and 10 to 48%, respectively. Eight different parts of Ahvaz and the villages in the north, northwest, and south of the city were sampled, based on their history of outbreak of ovine anaplasmosis. Blood was collected from jugular vein of 119 sheep (50 male and 69 female) into sterile tubes with and without anticoagulant (EDTA). The animals used in this study ranged in age from 3 months to 9 years. The blood samples were used to prepare thin blood smears for microscopic examination and the remaining samples were stored at -20°C until performing DNA extraction for PCR analysis.

Microscopic examination

Blood smears were prepared and fixed with methanol for 5 min and stained with 5% giemsa solution for 30 min and then examined for the presence of *Anaplasma* inclusion bodies under oil immersion lens ($\times 100$).

Parasitemia ratio was assessed by counting the number of infected red blood cells on examination of at least 200 microscopic fields. The number of infected cells was then expressed as a percentage.

DNA extraction

DNA extraction was performed by using molecular biological system transfer kit (MBST, Iran), based on the manufacturer's instructions.

Briefly, 100 μ l of blood samples were lysed in 180 μ l lysis buffer and the proteins were degraded with 20 μ l Proteinase K for 10 min at 55°C. After addition of 360 μ l Binding buffer and incubation for 10 min at 70°C, 270 μ l ethanol (96%) was added to the

solution and after vortexing, the complete volume was transferred to the MBST-column. The MBST-column was first centrifuged, and then washed twice with 500 µl washing-buffer. Finally, DNA was eluted from the carrier using 100 µl Elution buffer.

DNA yields were determined with an Eppendorf Biophotometer (Germany), and typical nucleic acids concentration values ranged between 15 and 25 ng/µl. DNA was stored at -20°C until subsequent analysis.

Polymerase chain reaction

A PCR method was used to detect *Anaplasma* spp. (*A. ovis* and *A. marginale*). One pair of oligonucleotide primers, based on the MSP4 gene sequence of *Anaplasma* spp., was employed. Primers were forward strand primer 5'-TTGTTTACAGGGGGCC TGTC- 3' and reverse strand primer 5'- GAACAGGAATCTTGCTCCAAG-3', which were described previously by Ahmadi-Hamedani *et al.* (2009).

The PCR was performed in a total reaction volume of 30 µl containing 3 µl 10 X reaction buffer [100 mM Tris-HCl (pH = 9), 500 mM KCl, 1% Triton X-100], 1.5 mM MgCl₂, 250 µM of each of the four deoxynucleotide triphosphates, 1.5 units of Taq DNA polymerase (Fermentas, Lithuania) and 20 pmol of each primer. 2 µl of DNA suspension (30-50 ng) was used as the template in the PCR.

The amplification was performed in an automated thermocycler (Corbett Research, Australia) under the following program: an initial denaturation step at 94°C for 5 min followed by 30 cycles at 94°C for 30 s, 59°C for 1 min and 72°C for 1 min with a final extension step of 72°C for 5 min.

Then, 10 µl aliquots of the PCR products were stained with cyber green solution and electrophoresed through a 1.5% agarose gel. After electrophoresis, results were visualized by UV transilluminator. Expected PCR products for the *Anaplasma* spp. were 831 bp.

RFLP of PCR products

The *A. ovis* and *A. marginale* were differentiated from each other by PCR-RFLP using *HpaII* enzyme similar to the method of Ahmadi-Hamedani *et al.* (2009).

The enzymatic digestion was carried out in 20 µl reaction mixtures consisting of 10 µl PCR amplicons, 2 µl 10 X corresponding buffer, and 0.1 µl (1 U) restriction enzyme made up to 20 µl with autoclaved triple-distilled water. The digestion mixture was incubated at 37°C for 2 h. Restriction digestions were separated by electrophoresis on 3% agarose and analysed in UV transilluminator. The restriction analysis patterns for *Anaplasma* spp. are listed in Table 1.

Table 1: The DNA fragment size expected for PCR RFLP

Species	<i>HpaII</i>
<i>A. ovis</i>	73, 183, 275, 300
<i>A. marginale</i>	92, 164, 572

Sequencing of PCR amplicons

For confirmation of PCR product, two PCR amplicons of MSP4 gene were purified and subjected to sequencing using dideoxy chain termination method by Bioneer Company, South Korea.

Results

Analysis of blood smears microscopically

Microscopic examination of 119 blood smears obtained from eight different parts of Ahvaz and the surrounding area, revealed that 33.6% (40/119) of sheep were infected with *Anaplasma* like structures. In these samples, the percentage of infected erythrocytes with *Anaplasma* inclusions varied from 0.01 to 1.16% (Figs. 1A, B).

Analysis of blood samples by PCR and RFLP

In PCR assessment of DNA samples, 87.4% (104/119) of sheep were positive (Fig. 2).

All microscopically positive samples were confirmed by PCR. No *Anaplasma* inclusions were seen on blood smears of samples that were negative in PCR. However, there were 64 PCR positive samples, which were negative in microscopic examination.

The results of enzymatic digestion of

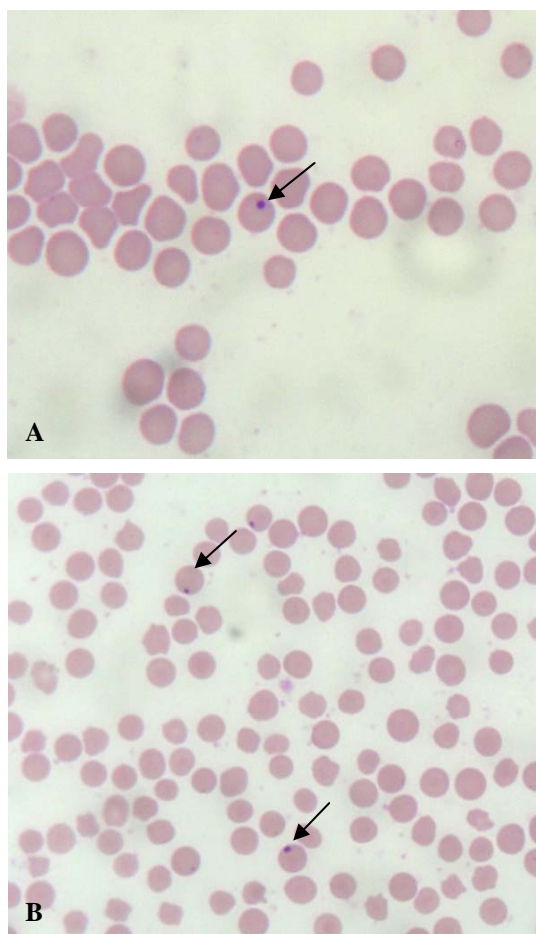


Fig. 1: A and B: *Anaplasma* inclusions in erythrocytes of sheep blood smears stained with giemsa

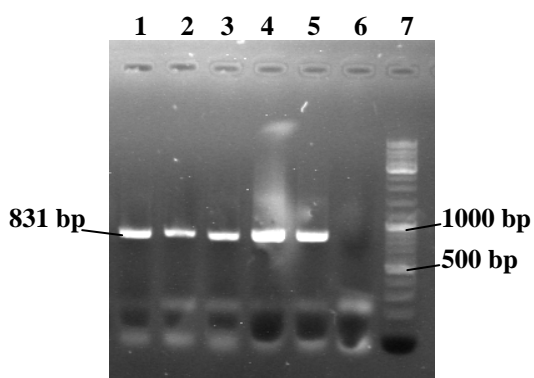


Fig. 2: Agarose-gel electrophoresis of *Anaplasma* spp. PCR products. Lane 1 to 5: *Anaplasma* positive samples, Lane 6: Negative control, and Lane 7: 100 bp DNA ladder

PCR products by *HpaII* to differentiate between two species of *Anaplasma* in sheep, showed that all the positive blood samples in the studied areas were positive as *A. ovis*. Also, mixed infection with *A. marginale* was detected in 50% (52/104) of cases (Fig. 3).

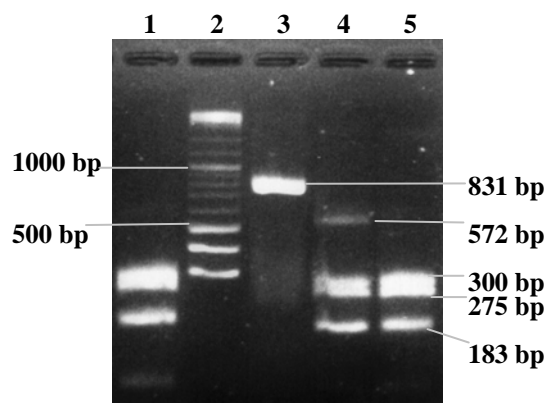


Fig. 3: RFLP analysis of PCR products of *Anaplasma* MSP4 gene by *HpaII* restriction enzyme. Lane 1 and 5: *A. ovis*, Lane 4: *A. ovis/A. marginale*, Lane 3: Undigested PCR product, and Lane 2: 100 bp DNA ladder

Sequencing results of PCR amplicons

The sequencing chromatograms were viewed and edited by Chromas software and submitted to GenBank at accession numbers: JQ621902 and JQ621903. Both of them showed identities of 99-100% with *A. ovis* (GenBank accession number AY702924.1).

The effect of age and sex on the prevalence of anaplasmosis

In order to investigate *Anaplasma* infection rate in sheep of different sex and age, the data was compared in two groups based on sheep sex, male (n=50) and female (n=69), and also in two age groups, less than one year (n=58) and over one-year-old (n=61). No significant difference was seen in the percentage of *Anaplasma* infection between male (88%) and female (86.96%) and also between immature (87.93%) and mature (86.88%) sheep.

Discussion

Ovine anaplasmosis is a tick-borne rickettsial disease widespread in tropical and subtropical areas, which is usually a subclinical or mild condition, but moderate to severe clinical disease is generally characterized by fever and a variable degree of anemia and icterus that may occasionally lead to death (Stoltsz, 2004).

In the present study, microscopic examination of sheep blood smears acquired from different areas in Ahvaz and the

surrounding suburbs, demonstrated that 33.6% of sheep were infected with *Anaplasma* like structures. This method has been frequently employed in previous studies on ovine and caprine anaplasmosis prevalence (Razmi *et al.*, 2006; Ahmadi-Hamedani *et al.*, 2009; Noaman *et al.*, 2009).

The rate of infected erythrocytes with *Anaplasma* inclusions in this study ranged from 0.01 to 1.16%. This was comparable to the results of other researches (Noaman and Shayan, 2010).

However, it is difficult to differentiate the organism from other similar structures like Howell-Jolly bodies, or staining artifacts, especially in carrier animals with low level of rickettsiaemia (Shompole *et al.*, 1989; Ndung'u *et al.*, 1995). This makes microscopic assessment unreliable for the detection of persistent infections (Noaman and Shayan, 2010). Hence, alternative diagnostic techniques, such as serological tests (Knowles *et al.*, 1996; de la Fuente *et al.*, 2004) and nucleic acid-based assays (Molad *et al.*, 2006; Heidarpour Bami *et al.*, 2010; Noaman and Shayan, 2010) can be used for detecting tick-borne parasites in carrier animals.

Anaplasma major surface proteins (MSPs) have been approved for use in interactions of the parasite with both vertebrate and invertebrate hosts, as these genes evolve more rapidly than others (Kocan *et al.*, 2004; Brayton *et al.*, 2006). Therefore, MSP4 gene, which is part of the MSP2 protein superfamily and is shown to be useful for the genetic characterization of *Anaplasma* spp. (de la Fuente *et al.*, 2007), was selected in the current study.

The results of PCR-RFLP analysis of the MSP4 gene in this study revealed that 87.4% of sheep blood samples were *Anaplasma* spp. positive. In RFLP assessment, all PCR products were *A. ovis* and mixed infections with *A. marginale* were also seen in 50% of cases.

The presence of multiple transmission factors and ticks in abundance in the tropical region, selected for the present study, can hereby be strongly correlated with the relatively higher prevalence of *A. ovis* infection in sheep hosts.

There was also no significant difference

in the percentage of *Anaplasma* infection in male and female sheep and also in different age groups. An epidemiological study on *Anaplasma* infection in Mashhad suburb, Khorasan province, Iran which was performed by Razmi *et al.* (2006), also revealed the same results in cattle, sheep, and goats. It has been shown that sheep and goats of all ages are susceptible to *A. ovis* infection, but older animals may suffer from a greater reduction in hematocrit values (Stoltz, 2004).

In a study in the northeast of Iran conducted by Ahmadi-Hamedani *et al.* (2009) using PCR-RFLP of the MSP4 gene, 63.7% (123/193) of examined goats were *Anaplasma* positive, all of which were *A. ovis*. They recommended this method as a useful tool for the detection of *A. ovis* in goats. Similarly, de la Fuente *et al.* (2005) analysed the prevalence of *A. marginale* by PCR and sequence analysis of MSP4 amplicons in Sicily and reported 50% positivity among the tested bovine samples (25/50).

In Iran, PCR analysis of *A. marginale* 16S ribosomal RNA (rRNA) gene on bovine blood samples showed 58 out of the total 150 blood samples to be positive for *Anaplasma* spp. (Noaman and Shayan, 2010). Noaman *et al.* (2009) also detected 50 *A. ovis* positive ovine blood samples using a semi-nested PCR based on 16S rRNA gene followed by RFLP. A molecular surveillance of tick-borne diseases of sheep in the south of Iran, performed by Spitalska *et al.* (2005), showed 29.0% *Anaplasma* spp. positive blood samples.

Recently, PCR amplification of the segment spanning the VI region of the 16S rRNA gene of *Anaplasma* species, followed by reverse line blot (RLB) hybridization assay identified *Anaplasma* infections in 9.0% (35/389) of the bovine samples from Turkey (Aktas *et al.*, 2011).

These findings indicate the superiority of PCR-based assay over the traditional method to diagnose *Anaplasma* infections which are parallel to the findings of the current investigation.

The results obtained from the present study demonstrate that ovine anaplasmosis caused by *A. ovis* and *A. marginale* is present and highly prevalent (87.4%) in the

Ahvaz region, southwest Iran and appears to be the first report of its kind from this area. Therefore, PCR-RFLP of MSP4 gene can detect *A. ovis* and *A. marginale* infection even at low levels frequently exhibited in apparently healthy carrier animals and can serve as a beneficial diagnostic tool under field conditions.

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