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Molecular identification of anaplasmosis in goats using a new PCR-RFLP method

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Summary

In this study blood samples were collected from 193 goats in north and northeastern Iran with the aim to develop a PCR-RFLP assay, as a specific and sensitive diagnostic tool enabling direct and concurrent identification of two *Anaplasma* species (*A. ovis*, *A. marginale*) in goats. A polymerase chain reaction (PCR) for amplification of a fragment of the major surface protein 4 (msp4) gene from *A. ovis* and *A. marginale* was developed. The results revealed that 123 out of 193 blood samples were positive for *Anaplasma* spp. infection. All 43 positive samples detected by microscopic examination were approved as positive by PCR, whereas no rickettsials were seen through light microscopy in the other 80 PCR positive cases. All positive samples were *A. ovis* as confirmed by restriction fragments length polymorphism (RFLP) method. Our results showed that PCR-RFLP of the msp4 gene could be a useful method for the detection of *A. ovis* in goats.

Key words: *Anaplasma*, Goat, Iran, PCR-RFLP

Introduction

Anaplasma species are obligate intraerythrocytic parasites in the order *Rickettsiales* which infect domestic and wild ruminants transmitted biologically by certain tick species and mechanically by other blood sucking arthropods and fomites (Shompole *et al*., 1989). *Anaplasma ovis* is transmitted by *Rhipicephalus*. *bursa*, *Haemaphysalis*. *sulcata* and probably other ticks in the old world and by *Dermacentor andersoni* in the new world (Friedhoff, 1997; Walker *et al*., 2003).

Hyalomma marginatum and *R. sanguineus* are the most abundant tick species in Golestan and Khorasan Razavi provinces, respectively (Nabian *et al*., 2007; Razmi *et al*., 2007). *Anaplasma ovis* and *A. marginale*, infect goats (Shompole *et al*., 1989). *Anaplasma marginale* (the type species for cattle) also causes latent anaplasmosis in sheep and goats (Maas and Buening, 1981). Experimental inoculation of goats with *A. ovis* induces an acute disease characterized by depression, anorexia, fever, and progressive anemia (Splitter *et al*., 1956). Anaplasmosis occurs in tropical and subtropical regions worldwide (~40° N to 32° S), including Asia (Lew and Jorgensen, 2005). Based on the intraerythrocytic location of inclusion bodies, as a conventional diagnostic method, *A. ovis* is differentiated from *A. marginale* (Splitter *et al*., 1956; Shompole *et al*., 1989).

PCR, as a more sensitive and specific technique than other conventional methods, has been increasingly applied to diagnose anaplasmosis in blood and tick vectors (Lew *et al*., 2002; Lew and Jorgensen, 2005).

The msp4 gene and its protein sequences, which are involved in interactions with both vertebrate and invertebrate hosts and evolve more rapidly

than other genes, are used to characterize *Anaplasma* spp. (de la Fuente *et al*., 2002; de la Fuente *et al*., 2005; de la Fuente *et al*., 2007; Hornok *et al*., 2007; Chochlakis *et al*., 2009; Psaroulaki *et al*., 2009).

Bazargani *et al*. (1985) showed that goat can also be a susceptible host for *Anaplasma ovis*. The prevalence of *A*. spp. infection was studied in goats in the Mashhad area of Iran from 1999 to 2002 (Razmi *et al*., 2006). Although detection of intraerythrocytic *Anaplasma* inclusion bodies in blood smears is useful when parasitemias exceed 0.1 to 0.2%, differentiation of morphological characteristics of *Anaplasma* organisms in low-level parasitemias are not enough to distinguish *Anaplasma* species from each other and from Howell-Jolly bodies (Shompole *et al*., 1989; Ndung'u *et al*., 1995). Therefore, in this study, the use of a PCR for the amplification of a fragment of the msp4 gene from *A. ovis* and *A. marginale* is described. We developed a RFLP assay as a specific and sensitive diagnostic tool enabling direct, concurrent identification of these two *Anaplasma* species.

Materials and Methods

Parasite isolate

Inclusion bodies of *Anaplasma* were diagnosed from blood smears of goats at our laboratory (Department of Parasitology, Faculty of Veterinary Medicine, University of Tehran, Iran). Genomic DNAs extracted from these blood smears were confirmed for *A. ovis* by msp4 gene sequence analysis and used as positive controls for *A. ovis* specific PCR. Venous blood sample, taken from a healthy goat without previous contact with ticks, was used as negative control.

Sample collection

The study was conducted in 2008. Twenty five suspicious farms on the basis of the history of the outbreak of caprine anaplasmosis were selected in Golestan and Khorasan Razavi provinces, in north and northeastern Iran. Blood samples were collected from the jugular vein of 193 goats (170 females and 23 males). The blood samples were collected in vacutainer tubes containing EDTA and held at 4°C until arrival at the laboratory. The blood was used to prepare thin blood smears for microscopic examination and to extract DNA for PCR analysis. Blood smears were fixed with methanol for five minutes, stained with Giemsa at a dilution of 5% in buffer solution for 20 min, and then examined for the presence of *Anaplasma* inclusion bodies under light microscopy. The blood smears were recorded as negative for *Anaplasma* spp. if no inclusion bodies were observed in 200 oil-immersion fields.

DNA extraction

DNA was extracted from the blood samples using the Molecular Biological System Transfer kit (MBST, Germany-Iran) and following the manufacturer's recommendation. 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

For the detection of *Anaplasma* spp. (*A. ovis* and *A. marginale*) PCR method was used. One pair of oligonucleotide primers was designed based on the msp4 gene sequence of *Anaplasma* spp.

Primers for the PCR were forward strand primer 5'-TTGTTTACAGGGGGCCTGTC-3' and reverse strand primer 5'-GAACAGGAATCTTGCTCCAAG-3' The PCR was performed in an automated DNA thermal cycler (Primus Mwg-Biotech) 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 $MgCl_2$, 250 µM of each of the four deoxynucleotide triphosphates, 1.5 unit Taq DNA polymerase (Fermentas) and 20 pg of each primer. 2 µl of DNA suspension (30-50 ng) was used as the template in the PCR. The reaction was performed under the following conditions: an initial denaturation step at 94°C for 5 min was followed by 30 cycles at 94°C for 30 s, 55.5°C for 30 s and 72°C for 45 s with a final extension step of 72°C for 5 min. PCR products were electrophoresed on 1.5% agarose gels,

stained by ethidium bromide and DNA bands were visualized at 254 nm by UV trans illuminator. Expected PCR products for the *Anaplasma* spp. were 831 bp.

Sequencing of DNA

The PCR products for 20 samples were purified and subjected to sequence by di dexy chain termination method and BLAST analysis was used for identification of the genomic DNA of these parasites.

RFLP method

The RFLP method was used for differentiation of two species of *Anaplasma* (*A. ovis* and *A. marginale*) in goat. The enzyme Hpa II was found to differentiate between these two species. The amplified products were digested with the restriction enzyme (Fermentas) as described by the supplier. The digestion reaction was setup in 15 µl volumes within a 500 µl PCR tube. 12 microliters of the PCR product was used for digestion. The digestion mixture consisted of 1.5 µl of the 10 x buffer, 12 µl PCR products and 0.2 µl (2 U) of restriction enzyme made up to 15 µl with autoclaved triple-distilled water. The digestion mix was incubated at 37°C for two h. The analysis of the enzymatic reaction PCR-amplified DNA fragments was performed by electrophoresis on the ethidium bromide-stained 2% agarose gel. The restriction analysis patterns for *Anaplasma* spp. are listed in Table 1.

Table 1: The pattern of RFLP of PCR products of *A. ovis* and *A. marginale* using of HpaII

Species	HpaII
A. ovis	121, 183, 227, 300
A. marginale	92, 152, 587

Results

The specificity of the *Anaplasma* spp. primer pair was tested with DNA from *A. ovis*. The expected fragment was generated from *A. ovis* (Fig. 1). The PCR products for *A. ovis* were purified and subjected to sequence by di dexy chain termination method and deposited in GenBank at accession number EU925811 (http://www.ncbi.nlm.nih.gov). The restriction enzyme Hpa II was found to differentiate the two species of presently known *Anaplasma* spp. in Iran (*A. ovis and A. marginale*). The enzyme digestion of *A. ovis* specific product yielded 300-, 183- and 121-bp sized fragments on a 2% agarose gel electrophoresis (Fig. 2). We did not have genomic DNA of *A. marginale*, so we could not show the enzyme digestion of this species. But it seems that the enzyme *HpaII* produce a pattern which could clearly differentiate the two species. Blood samples were collected in EDTA from 193 goats in north and northeastern Iran. Thin blood smear examination of goats showed that 22.3% (43/193) were positive for *Anaplasma* inclusion bodies. Using PCR, 63.7% (123/193) goats were positive. Infectious prevalence of *Anaplasma ovis* in goats collected from north and northeastern Iran are listed in Table 2. All of the positive samples by thin blood smears were also determined to be positive by PCR, whereas no inclusion bodies were seen by light microscopy in 80 PCR positive animals. Using RFLP of PCR products, all the positive blood samples were positive for *A. ovis*. The PCR products for 20 samples were also purified and subjected to sequence and BLAST analysis for identification of the genomic DNA of the parasites. After sequencing, all the samples were positive for *A. ovis*.

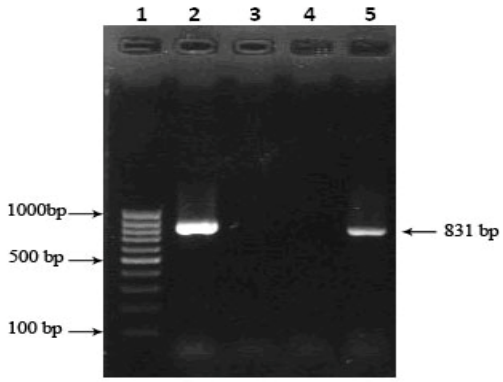

Fig. 1: Agarose-gel electrophoresis of amplification product obtained from *Anaplasma ovis* using *Anaplasma* specific primers. Lane 1: 100 bp ladder (Fermentas), Lane 2: *A. ovis* (positive control), Lane 3: no infected goat blood (negative control), Lane 4: no DNA control and Lane 5: *Anaplasma* spp. (goat) from collected samples in field

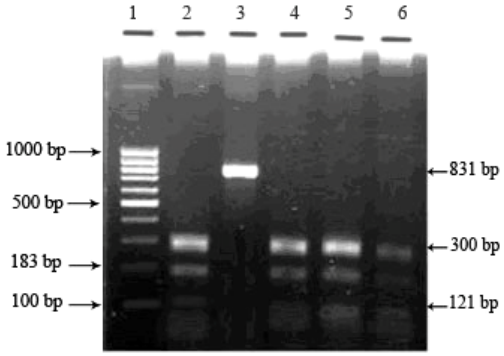

Fig. 2: RFLP by HpaII restriction enzyme. Lane 1: 100 bp DNA ladder marker (Fermentas), Lane 2, 4, 5, 6: digested PCR product of *A. ovis* by *HpaII* restriction enzyme (121, 183, 300 bp) and Lane 3: 831 bp PCR product

Table 2: Prevalence of infection by *Anaplasma ovis* in goats collected from north and north eastern Iran by PCR-RFLP

Goats	Examined (n)	*A. ovis* positive (n)
Male	23	10 (43.5%)
Female	170	113 (66.5%)
Total	193	123 (63.7%)

Discussion

The diagnosis of rickettsial infections in vertebrate hosts has been mainly carried out by microscopic examination of blood smear (Lew and Jorgensen, 2005). Because the *Anaplasma* organisms lack distinguishing morphological characteristics, and in low-level parasitemias they cannot be differentiated from Howell-Jolly bodies, the low sensitivity of this method does not permit its use in epidemiological investigations (Shompole *et al*., 1989; Ndung'u *et al*., 1995). Microscopic examination of Giemsa-stained blood smears, which is used to confirm acute *Anaplasma* infection during which rickettsemia reaches levels of $\geq 10^9$ infected erythrocytes/ml, is insufficiently sensitive to detect persistently infected animals. The rickettsemia levels during persistent *Anaplasma* infection range from 10^3 to 10^7 infected erythrocytes/ml and only a subset of persistently infected cattle can be detected using microscopic examination (Shkap *et al*., 2002).

Although inoculation of an infected animal's blood into splenectomized animals is an effective method to detect *Anaplasma* organisms, because of the expense and time-consumption it cannot be used as a routine procedure (Shompole *et al*., 1989).

Cross-reactivity, false positive and false negative results have made serological diagnosis of anaplasmosis unable to differentiate *A. marginale* from *A. ovis* (Kuttler, 1965; Molloy *et al*., 1999; Passos *et al*., 1998; Torioni de Echaide *et al*., 1998).

Although RFLP-based assays for the detection of hemoparasites from other animals have been published previously (Gubbels *et al*., 2000; Bazarusanga *et al*., 2007; Jefferies *et al*., 2007; Ravindran *et al*., 2007; Heidarpour Bami *et al*., 2009; Psaroulaki *et al*., 2009), there is no RFLP-based assay available for detection of caprine anaplasmosis. In this study molecular techniques (PCR-RFLP) which may offer high sensitivity and specificity compared to morphological examinations and serological tests were used to detect and differentiate *A. ovis* from *A. marginale*, (Papadopoulos *et al*., 1996).

The results of this study for diagnosis of *A. ovis* in goats by PCR-RFLP analysis revealed that the traditional Giemsa staining method is not applicable for identification diagnosis of persistently infected goat which is in accordance with Noaman *et al*. (2009) study on cattle.

Our results showed that *Anaplasma*-like structures could be detected in erythrocytes of 22.3 percent of the total blood samples. Due to the very low amount of the *Anaplasma* infected erythrocytes in goats, and the difficult differentiation between *Anaplasma* organisms and structures like Heinz bodies, Howell-Jolly bodies or staining artifacts, often seen in Giemsa stained blood smears, DNA from corresponding blood samples were analyzed by PCR. *A. ovis* was detected in 123 out of 193 blood samples using PCR-RFLP method.

The results obtained from blood samples collected from goats in north and northeastern Iran showed that the PCR used in this study is more sensitive than detection by light microscopy, which is performed routinely in laboratories in Iran. Similar results have been reported by Figueroa *et al*. (1996), Shkap *et al*. (2002) and Hofmann-

Lehmann *et al*. (2004).

Since the prevalence of *A. ovis* in goats detected in our survey using PCR-RFLP (63.7%) was higher than the blood smear investigation method (22.3%) and higher than the results reported by Razmi *et al*. (2006) from the Mashhad area (38.9%) which was performed by conventional Giemsa-stained blood smears, it is concluded that the observed prevalence of using non molecular methods may be reconsidered by more accurate molecular methods to achieve the real prevalence of anaplasmosis. The prevalence of 29% of anaplasmosis in Fars performed by Sparagano *et al*. (2004) being less than the prevalence of caprine anaplasmosis in our study (63.7%) can be due to numerous factors like geographical and climatic differences, the role of different tick vectors and the method by which the two investigations were performed.

The present study is the first molecular based report of remarkable number of carrier goats in Iran which can serve most probably as the reservoir of infection for vector ticks.

Our results showed that PCR-RFLP of the msp4 gene could be a useful method for the detection of *A. ovis* in goats. *A. ovis* infection is present in goats of north and northeastern Iran. Further studies are needed to confirm the presence of *A. ovis* and its capacity to cause disease in sheep, wild animals and ticks in several parts of Iran.

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