Veterinary Research Forum
Vol: 1, No: 2, September, 2010, 117 - 122

Molecular Detection of *Anaplasma bovis* in Cattle from Central Part of Iran

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Received: 5 April 2010, Accepted: 21 June 2010

Abstract

Anaplasma bovis is a leukocytotropic agent of bovine anaplasmosis and there is no available information about molecular study on this agent in cattle of Iran. In this study a total 150 cattle blood samples were collected from central part of Iran. The presence of *A. bovis* examined using light microscopic detection and species-specific nested polymerase chain reaction (nested-PCR) based on 16S rRNA gene. Of the 150 cattle, 4 (2.66 %) was positive for *A. bovis* by nested-PCR. These data is the first *A. bovis* DNA presence in cattle from central part of Iran.

Key words: *A. bovis,* Nested-PCR, 16S rRNA gene, Iran.

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Introduction

Anaplasmosis is a tick-born disease of cattle and other ruminants caused by species of the genus *Anaplasma* (Rickettsiales: Anaplasmataceae).[1] Four species, including of *Anaplasma marginale*, *A. centrale*, *A. bovis* and *A. phagocytophilum* are well recognized in cattle.[2] Among these species, *A. bovis* and *A. phagocytophilum* are considered as leukocytic *Anaplasma* as these agents infect monocytes and granulocytes, respectively.[1]

A. bovis is a monocytotropic specie and its morulae within monocytes of infected cattle was first described in 1936 during experiments of *Theileria* sp. transmission, in which *Hyalomma* sp. ticks from Iran were fed on French cattle. *A. bovis* infection in cattle has been documented as asymptomatic but the agent can cause fever, anemia, weight loss and rarely abortion and death in cattle of tropical and subtropical regions of the world. Survivors are lifelong carriers.[3] *A. bovis* DNA has been detected in wild deer, ticks and cattle in Asia.[4-7]

Several hard tick species distribute in Iran and they are the most important ectoparasites of cattle in central part of Iran. Although more is known about ticks as responsible vectors for the transmission of several rickettsial pathogens to cattle, there is little knowledge about leukocytic anaplasmosis in cattle of Iran. There is only one report regarding the *A. phagocytophilum* carrier cattle analyzed by PCR (Polymerase Chain Reaction) in Iran.[8] As regards the *A. bovis* infection in cattle, there is still no information in Iran. The present study was carried out to determine whether *A. bovis* is detectable by molecular techniques in naturally infected dairy cattle in central part of Iran.

Materials and Methods

Collection of blood samples. From March to July 2007, 60 non-industrial cattle farms in Isfahan province, central part of Iran, were selected for the study. Blood samples were collected from jugular vein of 150 cattle. Five hundred micro liters of each collected blood samples was fixed with 1 ml 96 % ethanol in 1.5 ml sterile eppendorf tubes. Additionally, two thin blood smears were prepared immediately after each blood collection. The blood smears were air dried, fixed in methanol, stained by Giemsa and observed for the presence of *A. bovis.* The preferred habitat of *A. bovis* is the cytoplasm of cattle monocytes where it forms microcolonies or morulae containing up to 20 individual organisms.[1] All smears were carefully examined for the presence of *A. bovis.* In each blood smear 100 microscopic fields were examined.

DNA extraction. DNA was extracted using the DNA isolation kit (Molecular Biology System Transfer, Iran) according to the manufacturer's instructions. Briefly, 5 mm^3 of fixed blood samples was first air dried and subsequently lysed in 180 µl lysis buffer (Molecular Biology System Transfer, Iran) and the proteins were degraded with 20 µl proteinase K for 10 min at 55°C. After addition of 360 µl binding buffer and incubation for 10 min at 70°C, 270 µl ethanol (96 %) was added to the solution and after vortexing the whole 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. The amount of extracted DNA and its purity was measured by OD^{260} (Optical density) and the ratio of OD^{260} to OD^{280}, respectively.

PCR and nested-PCR (Nested Polymerase Chain Reaction). Before PCR analysis the purity of the DNA was measured and the ratio of OD^{260} to OD^{280} was from 1.7 to 1.9. PCR and nested-PCR reactions were performed using 16S rRNA gene and base external (P1/P2) and internal (P3/P4) primer sets to amplify *Anaplasma* species.[9,10] To confirm the

PCR products were *Anaplasma* spp. specific, the PCR products were amplified with the primers P3/P4, which were designed from the region flanked by the primers P1/P2. All PCR products could be amplified with the above mentioned primers (P3/P4), which denoted that the first PCR product belongs to the 16S rRNA gene of *Anaplasma* spp. The primers sequences are given in Table 1. The PCR products were used as templates for the *A. bovis* nested-PCR.

Since the *A. marginale* is the most prevalent *Anaplasma* spp. in cattle of Iran, the PCR products were first cut with the restriction endonucleasis Bst 1107I, as previously described by Noaman and Shayan.[10]

To detect and discriminat of *A. bovis*, an additional forward specific internal primer, AB1f used.[5] *A. bovis*-specific nested-PCR reactions performed using the 16S rRNA gene base primer set, AB1f /P4. Specific nested-PCR reactions performed directly with 1 µl of the primary PCR product.

The nested-PCR for *A. bovis* was performed in 50 µl total volume including ×PCR buffer, 1.25U Taq Polymerase (Cinnagen, Iran), 1µl of each primer (20 µM, Cinnagen, Iran), 200 µM of each dNTPs (Fermentas, European Union), 1.5 mM $MgCl_2$ and 1µl DNA template in automated Thermocycler (MWG, Germany) with the following program: 5 min incubation at 95 °C to denature double strand DNA, 35 cycles of 45 s at 94 °C (denaturing step), 45 s at 56 °C (annealing step) and 45 s at 72 °C (extension step). Finally, PCR was completed with the additional extension step for 10 min. The nested-PCR products were analyzed on 2% agarose gel in 0.5 × TBE (Tris-Borate-EDTA) buffer and visualized using ethidium bromide and UV-transeluminator. Positive controls of PCR were not used for *A. bovis*, because DNA from these agents was not available in the laboratories of Iran. The specificity of the reactions was also tested with DNA extracted from *Theileria annulata, A. marginale and A. ovis*.

Results

Blood smear examination. All smears were carefully examined at least 100 fields per slide and screened for *A. bovis* inclusions in monocytes but no inclusions were observed and all blood smears were negative for *A. bovis* like structures. Some blood smears were found to be infected with *Theileria* spp. and *Anaplasma* spp. in erythrocytes.

PCR and nested-PCR evaluation. PCR analysis of the DNA isolated from 150 blood samples with primers P1/P2 revealed an expected PCR product with 781 nucleotides in length in 38.8 % of blood samples (Fig. 1). The amplified nested-PCR product had an expected PCR product with 543 nucleotides in length (Fig. 1).

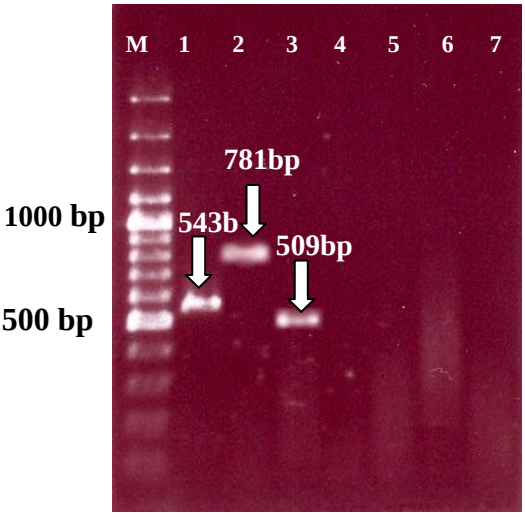

Fig 1. PCR and nested-PCR of *A. bovis*. PCR product of 543bp (lane 1), PCR product of 781bp (lane 2), specific nested-PCR for *A. bovis* (lane 3), *Theileria annulata* (lane 4), *A. marginale* (lane 5), *A. ovis* (lane 6), negative control (lane 7) and M (Marker 100 bp).

The restriction endonucleasis Bst 1107I recognizes the sequence (GTATAC) in corresponding PCR product of *A. marginale* and cut it in the position 68,

whereas the used restriction enzyme can not cut the corresponding PCR product of other *Anaplasma* spp.[10] Analysis of all 58 Anaplasma positive PCR products with the restriction endonucleasis Bst 1107I showed that all PCR products could be cut in two expected DNA fragments.

Amplification of PCR products with primers AB1f /P4 had an expected PCR product with 509 nucleotides in length (Fig. 1 and 2). nested-PCR analysis of the primary PCR products with primers AB1f /P4 revealed expected PCR product in (4/150) 2.66 % of the blood samples. To confirm the result of *A. bovis*-specific nested-PCR, the PCR products from four positive samples were reacted using *A. phagocytophilum and A. centrale (Amori strain)* specific primer sets, P6/P4 and P7/P4 respectively (Fig. 2).

Discussion

A. marginale and *A. phagocytophilum* have long been recognized as bovine anaplasmosis agents. Recently, they have been detected in cattle of Iran by molecular approaches.[8,9] However, there is no molecular evidence for *A. bovis* in cattle of Iran.

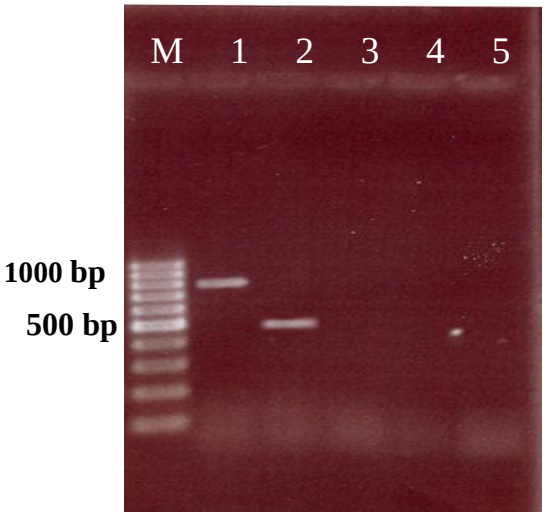

Fig 2. PCR product of *Anaplasma bovis.* PCR product of 781bp (lane 1), *A. bovis*-specific nested-PCR product of 509bp (lane 2), *A. phagocytophilum* (lane 3), *A. centrale (Amori strain)* (lane 4), negative control (lane 5) and M (Marker 100 bp).

Table 1. Designed primers set based on 16S rRNA gene

Primer	Publication references and Accession No. in GenBank	Nucleotid sequences	Nucleotid epositions	Target organism
P1	M60313	5'agagtttgatcctggctcag3'	1-20	
P2	M60313	5'agcactcatcgtttacagcg3'	781–762	*Anaplasma genus*
P3	M60313	5'gcaagcttaacacatgcaagtc3'	35-56	
P4	M60313	5'gttaagccctggtatttcac3'	577-558	*A.genus*
AB1f	AB196475 Kawahara *et al*. 2006	5'ctcgtagcttgctatgagaac3'	69-89	*A. bovis*
P6	M73224	5'ctttatagcttgctataaagaa3'	69-90	*A. phagocytophilum*
P7	AF283007 Inokuma *et al*. 2001	5'caaatctgtagcttgctacgga3'	65-86	*A. centrale (Amori strain)*

The 16S rRNA gene of *Anaplasma* spp. has a small hyper variable region; its nucleotide sequence has been used for the differentiation of *Anaplasma* spp. from each other.[1,11-13] Because of highly sequence similarity among hyper variable region (V1) of 16S rRNA gene, designing of species-specific primers based on 16S rRNA of *A. marginale*, *A. centrale (South Africa strain)* and *A. ovis* is impossible.[10] However hyper variable regions of the 16S rRNA gene of *A. bovis*, *A. phagocytophilum* and *A. centrale (Amori strain)* have sufficient differentiation in alignments of sequences to design species-specific primers. In this study we therefore use species-specific primer based on 16S rRNA for molecular detection of *A. bovis*, as previously described by Kawahara *et al*.[5]

Although Donatine and Lestoquard (1936) have reported the presence of *A. bovis* morulae within monocytes of French cattle,[3] there is a little information concerning animal reservoirs of *A. bovis* in Asia. *A. bovis* DNA has recently been detected from wild deer and cattle in Japan.[5, 6]

In the present study, *A. bovis* (2.66 %) identified. So far, there is no report of infected cattle with this pathogen in Iran. The present data indicates that the cattle in central part of Iran were infected with *A. bovis.* This study is the first molecular detection of this agent from cattle of Iran.

Pathogenicity of *A. bovis* with subclinical infection is already recognized.[14] In the present study, no clinical signs were recorded in the positive cattle. Jilintai *et al*. showed that 15 % of tested animal were positive for *A. bovis* with no clinical symptoms and morula in blood smears.[7] Monocytes generally comprise of less than 1% of all leukocytes in circulating blood, and therefore few infected cells would be present on a blood smear. In this study, despite careful observation of blood smears of these cattle, morulae were not detected in blood smears.

The results of present study confirm the presence of *A. bovis-DNA* in central part of Iran. Additional molecular studies are needed to determine the vectors of this agent in Iran.

Acknowledgements

This work was supported by Veterinary department of Isfahan research center for agriculture and natural resources and Veterinary Faculty of Tehran University. The authors declare that there is no conflict of interests.

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