

Molecular detection of *Anaplasma phagocytophilum* in carrier cattle of Iran - first documented report

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ABSTRACT

Background and Objectives: *Anaplasma phagocytophilum* is a zoonotic, tick borne rickettsial pathogen. *A. phagocytophilum* has been detected in North America, Europe, Africa and Asia by molecular methods. In Iran we have little information about the distribution of this agent in human and animals.

Materials and Methods: From March 2007 to July 2007, one hundred and fifty blood samples and corresponding blood smears of cattle without any signs of disease were prepared from a region in Isfahan, Iran with previous history of tick borne disease outbreak. The blood smears were first stained with Giemsa and analyzed for the presence of *A. phagocytophilum* in the neutrophils. The extracted DNA from blood cells were analyzed by *A. phagocytophilum* specific nested PCR using primers derived from the 16S rRNA gene.

Results: All blood smears were negative for *A. phagocytophilum* like structures by Giemsa staining, but 2 out of 150 blood samples (1.33%) were positive for *A. phagocytophilum* specific nested PCR using specific primers derived from 16S rRNA gene.

Conclusion: This study is the first detection of *A. phagocytophilum* in carrier cattle in Iran. The present study showed that *A. Phagocytophilum* is detectable in cattle without any sign of infection but maintained a persistent sub-clinical state in the cattle reservoir, which can be inferred as possible risk for management of public health.

Keywords: *Anaplasma phagocytophilum*, 16S rRNA gene, nested-PCR, carrier cattle, Iran.

INTRODUCTION

Anaplasma phagocytophilum is an obligate Gram-negative intracellular bacterium, which has been reported in different mammals such as sheep, goat, cattle, horse, dog, cat, roe deer, reindeer and human (1). HGE (human granulocytic anaplasmosis) was first reported in human in the United States in 1994, (2,3) and has been considered as an emerging pathogen of public health importance (4).

A. phagocytophilum is transstadially transmitted by the tick vectors. *Ixodes ricinus* has been found to be the

main vector of *A. phagocytophilum* in Europe (5-6). *A. phagocytophilum* has been also detected in *Ixodes ricinus* in Iran (7). However, other ticks have also been associated with *A. phagocytophilum* transmission (1). In Europe, not only large animals (horses, cattle, sheep, goats, dogs, cats) but also small rodents have been shown to harbor *A. phagocytophilum* and act as potential reservoirs (8-10).

In human, the disease usually presents as an acute, sometimes fatal febrile syndrome, illness characterized by headache, chills, myalgias, arthralgia, malaise, and hematological abnormalities, such as thrombocytopenia, leukopenia, and elevated hepatic aminotransferase levels (11).

Cattle tick borne fever (TBF) caused by *A. phagocytophilum* is characterized by high fever,

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reduced milk yield, inclusions in circulating neutrophils, leukopenia, abortions and reduced fertility. In cattle, the incubation period after experimental inoculation is 4-9 days and the fever period may last for 1-13 days. *A. phagocytophilum* infection normally gives mild to moderate clinical signs and is seldom fatal unless complicated by other infections (1). Clinical signs in cattle may include depression, decreased appetite, coughing, nasal discharge, respiratory signs, swelling of the hind limbs and stiff gate. However, the most serious problem associated with TBF, especially in sheep, is the ensuing immunosuppression, which may predispose to secondary infections (6). Infection by *A. phagocytophilum* results in significant disruption of normal neutrophil function, including endothelial cell adhesion and transmigration, motility, degranulation, respiratory burst, and phagocytosis (12). *A. phagocytophilum* subverts the host's innate immune responses by evading elimination by neutrophils, causing persistent infection through antigenic variation and by modulating lymphocyte responses (13).

Several hard tick species are distributed in Iran and are able to infest animals and human, but there is little information available concerning vectors and animal reservoirs of *A. phagocytophilum* in Iran. Extensive knowledge about the functional transmission of vectors and the state of persistently infected animals make it difficult to calculate the potential threats of the pathogens in human health management. Therefore, the aim of the present study was to determine whether *A. phagocytophilum* is detectable in naturally infected dairy cattle in farms with history of outbreak of tick borne diseases in the center of Iran, where the potential vector, *Ixodes ricinus*, is not documented.

MATERIALS AND METHODS

Collection of blood samples. From March 2007 to July 2007, 60 farms in Isfahan province, central part of Iran, were selected for the study based on their history of outbreak of tick borne diseases. Blood samples were collected from jugular vein of 150 cattle (14). Five hundred micro liters of each collected blood sample was fixed with 1 ml 96% ethanol in 1.5 ml sterile eppendorf tube. Additionally, two thin blood smears were prepared immediately after each blood collection. The blood smears were air dried, fixed in methanol, stained with Giemsa and analyzed for the presence of *A. phagocytophilum* in the neutrophils.

All smears were carefully examined by examining at least 100 microscopic fields per slide.

DNA extraction. DNA was extracted using the DNA isolation kit (MBST, Iran) according to the manufacturer's instructions. Briefly, 5 mm³ pieces of fixed blood samples was first air dried and subsequently lysed in 180 µl lysis buffer 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 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. The amount of extracted DNA and its purity was measured by OD²⁶⁰ and the ratio of OD²⁶⁰ to OD²⁸⁰ respectively. In addition the extracted DNA was analyzed on agarose gel before use.

Polymerase chain reaction, nested PCR and Specific nested PCR. Approximately 100 to 500 ng DNA was used for the PCR analysis. The PCR was performed in 100 µl total volume including one time PCR buffer, 2.5 U *Taq* Polymerase (Cinnagen, Iran), 2 µl of each primer (P1/P2, 20 µM, Cinnagen, Tehran, Iran), 200 µM of each dATP, dTTP, dCTP and dGTP (Fermentas) and 1.5 mM MgCl₂ in automated thermocycler (MWG, Germany) with the following program: 5 min incubation at 95°C to denature double strand DNA, 35-38 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 PCR products were analyzed on 2% agarose gel in 0.5 × TBE buffer and visualized using ethidium bromide and UV-transilluminator. To control the specificity of the PCR products for the 16S rRNA gene of *Anaplasma* spp., nested PCR technique was used, in which the additional primers (P3/P4) from the same gene were designed upstream from forward primer (P1) and downstream from reverse primer (P2) respectively.

For identification of *A. phagocytophilum*, an additional specific primer (P5) was designed from the hyper variable region (V1) of the 16S rRNA gene and the specificity was determined using primers P5/P4 by nested PCR. Nested PCR was performed with the PCR product isolated from agarose gel using the MBST-Kit (Iran) according to the manufacturer's

instructions. Briefly, the DNA bands were cut from the gel under UV and dissolved in the solubilization buffer at 60°C. The dissolved agarose was, after adding of ethanol, transferred into the MBST-column. After washing, the bound DNA was eluted

with 30 µl TE-buffer. One to five micro liter of the eluted DNA was amplified with the primers P3/P4 or P5/P4 separately. In addition, nested PCR was performed directly with 1 µl PCR product as well. The primers are listed in the Table 1.

Table 1. Primers and the gene accession numbers used for amplification of *Anaplasma phagocytophilum* and size of PCR products.

Primer	Accession No. in GenBank	Nucleotid sequences	Positions	PCR- product
P1	M73224	5' agagtttgatcctggtcag 3'	1-20	781bp
P2	M73224	5' agcactcatcggttacagcg 3'	781-762	
P3	M73224	5'gcaagcttaacacatgcaagtc3'	35-56	543bp
P4	M73224	5'gttaagccctggtatttcac 3'	577-558	
P5	M73224	5'ctttatagcttgctataaagaa 3'	69-90	509 bp
P4	M73224	5'gttaagccctggtatttcac 3'	577-558	

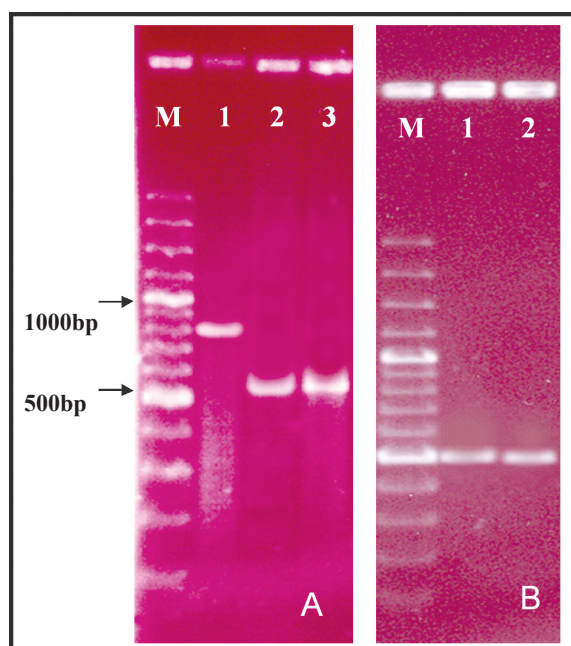


Fig. 1: DNA isolated from blood was analysed by PCR and specific nested-PCR. **A:** DNA was amplified with primer P1/P2 resulting in PCR product of 781bp in length (lane 1). The PCR product amplified using P1/P2 (781bp line 1) was re-amplified with P3/P4 resulting in PCR product of 544 (lane 2, 3). **B:** Specific nested-PCR for *A. phagocytophilum*, the PCR product amplified using P1/P2 (781bp) was re-amplified with P5/P4 resulting in PCR product of 509bp (lane 1, 2). M= 100 bp molecular marker.

RESULTS

Analysis of blood smears. All smears were carefully examined (at least 100 fields per slide) and screened for *A. phagocytophilum* inclusions in neutrophils but no inclusions were seen and all blood smears were negative for *A. phagocytophilum* like structures.

Analysis of blood samples by PCR, nested PCR and specific nested PCR. Before PCR analysis, the purity of the DNA was measured and the ratio of OD²⁶⁰ to OD²⁸⁰ was from 1.7 to 1.9. The PCR analysis was performed using primers derived from 16S rRNA gene published in GenBank under accession number M73224 (Table 1). The nucleotide sequence of 16S rRNA gene is conserved in *Anaplasma* spp. and the primers P1, P2, P3 and P4 can amplify the corresponding gene in all *Anaplasma* spp. 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 (58 samples) 38.76% of the blood samples (Fig. 1A).

To confirm that 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 amplified nested PCR product had an expected PCR product with 543 nucleotides in length (Fig. 1A).

Detection of *A. phagocytophilum* was performed using specific primer (P5) designed from the nucleotide sequences of *A. phagocytophilum* hyper- variable region of the above mentioned gene. Amplification of PCR products with primers P5/P4 had an expected PCR product with 509 nucleotides in length (Fig.1B). Nested PCR analysis of the DNA isolated from 58 positive *Anaplasma* spp. samples with primers P5/P4 revealed expected PCR product in (2 samples) 1.33% of the blood samples.

DISCUSSION

Human granulocytic anaplasmosis, which is emerging as a potentially fatal infectious disease, is caused by obligatory intracellular gram-negative bacteria in the family *Anaplasmataceae* (15). Members of this family also cause economically devastating diseases such as bovine anaplasmosis in livestock (2, 16, 17). In the family *Anaplasmataceae*, five *Anaplasma* species are officially recognized (15). All of them are sustained in nature through an enzootic cycle between bloodsucking ticks and vertebrate hosts, primarily wild mammals.

In North America, Europe and Africa, *A. phagocytophilum* have been detected in human and confirmed by serological and molecular methods (18). In Asia serological evidence of human infection with *A. phagocytophilum* was reported in Korea (19) and the first Molecular detection of *A. phagocytophilum* in wild deer and cattle was reported by Kawahara (2006) and Ooshiro (2008) from Japan respectively (18, 20). 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 (15, 21-23). Our results showed that 2 of total 150 blood samples were *A. phagocytophilum* positive by specific primers based on 16S rRNA gene. This report is the first detection of this agent in carrier cattle in Iran. In a previous study we could show that 58 samples within 150 examined blood samples were *Anaplasma marginale* positive (14). In this study 2 samples from these studied samples could

be amplified with the specific primers for *Anaplasma phagocytophilum*. This means 3.45% of *Anaplasma* positive bloods had mix infection. This data brings evidence that in carrier cattle due to the low amount of infected cells, it is impossible to recognize infected blood cells by the traditional Giemsa staining method. Furthermore, it is known that in the case of mixed infection due to the competition between amplified DNA segments with high sequence homology, the DNA with the lower concentration compared to the DNA with the higher concentration mostly cannot be amplified (competitive inhibition). Therefore, in such cases specific PCR-RFLP can not exclude a second infection.

Ooshiro et al. 2008 in Japan revealed that 12 of 15 cattle tested were positive for infection by *A. phagocytophilum* in specific PCR (20). *A. phagocytophilum* have been detected by PCR in mammals and ticks in nearly all European countries (5, 24). Mammals are presumed to play a crucial role in the maintenance and propagation of *A. phagocytophilum* in nature (10). *A. phagocytophilum* has been found to persist in species such as sheep, horse, dog, red deer, and cattle. Movement of persistently infected individuals may contribute to the spread of variants between geographical areas (1).

In Iran *Ixodes ricinus* was only found in the forest area next to the Caspian Sea. In central part of Iran (Isfahan province) *Hyalomma anatolicum*, *Hyalomma marginatum*, *Rhipicephalus sanguinus* and *Rhipicephalus bursa* are dominant species of tick on cattle respectively. *Ixodes ricinus* is the main vector of *A. phagocytophilum* in Europe (5). *A. phagocytophilum* has been also detected in *Ixodes ricinus* in Iran (7). However, *A. phagocytophilum* has also been associated with other ticks, such as *Haemaphysalis punctata*, *I. persulcatus*, *I. trianguliceps* and *Rhipicephalus sanguinus*, but the epidemiological importance of these findings remains to be determined (1).

In Asia, *A. phagocytophilum* have been detected by PCR in *Haemaphysalis longicornis* (18), *Hyalomma marginatum*, *Rhipicephalus turanicus*, and *Boophilus kohlsi* (25), therefore, *Hyalomma marginatum* and *Rhipicephalus sanguinus* might be important vector ticks of this *Anaplasma* spp. in central part of Iran.

Most human and animal infections with *A. phagocytophilum* have no clinical manifestations and the severity of the infection is influenced by several factors such as variants of *A. phagocytophilum* involved, other pathogens, age, immune status, condition of the host, and factors such as climate and management.

The results of the present study confirm the presence of *A. Phagocytophilum* and possible risk of transmitting this infection to humans in central part of Iran. To control the anaplasmosis we still have to determine the transmitting vectors, animal reservoirs and pathogenesis of *A. phagocytophilum* in human and animals in Iran.

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