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# Concurrent occurrence of *Anaplasma phagocytophilum* and *A. marginale* in bovine peripheral blood samples from southwest of Iran

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**ABSTRACT:** *Anaplasma phagocytophilum* and *A. marginale* are the most important tick-borne bacteria of veterinary and public health significance. *Anaplasma phagocytophilum* causes febrile disease in humans (human granulocytic-HGA) and animals. *Anaplasma marginale* is the aetiological agent of acute anaplasmosis, a bovine syndrome characterized by progressive hemolytic anemia associated with fever, weight loss, abortion, decreased milk production, and in some cases, death of the infected cattle. The present study was designed to investigate the prevalence of *A. phagocytophilum* and *A. marginale* in cattle from Khuzestan province, southwest of Iran. Samples were collected between March to August 2016. Farmed cattle were selected from the four geographic regions of Khuzestan province with the highest population of cattle herds: Behbahan; Dezful; Shushtar; and Ahvaz. Blood samples were collected from the jugular vein of 200 cattle Species specification was accomplished by specific Nested PCR according to amplification of the 16SrRNA gene. To identify *A. marginale*, semi-nested PCR product was cut with restriction endonucleases Bst 1107 I. The prevalence of the *A. marginale* infection (21.5 %) was higher than that of *A. phagocytophilum* (7.5 %), which was found in a mixed infection with *A. marginale*. Overall, in the present study 7.5% of cattle were infected with both *A. phagocytophilum* and *A. marginale*. Despite the healthy appearance of infected cattle, they can transmit *Anaplasma* to ticks and are potential continuous sources for maintaining and disseminating the organisms to the human and animals' population. More epidemiological studies are needed to determine the vectors and reservoir animals for the *Anaplasma* species and to clarify the pathogenicity of *A. marginale* and *A. phagocytophilum* for humans and animals in Iran.

***Keywords:*** *Anaplasma phagocytophilum*, *A. marginale*, Cattle, Khuzestan province, Iran

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## INTRODUCTION

Many species of the genus *Anaplasma* induce different and distinct forms of anaplasmosis in cattle. The Office International des Epizooties (OIE) Animal Health Code categorizes anaplasmosis as a notifiable disease due to its socio-economic impact and international trade restrictions. However, the significance of anaplasmosis is frequently underestimated due to seasonal outbreaks and stability in areas of endemicity. *Anaplasma phagocytophilum* and *A. marginale* are the most important tick-borne bacteria of veterinary and public health significance (OIE 2008).

*Anaplasma phagocytophilum* is a medically and veterinary important emerging tick-borne pathogen. It is an alpha pleomorphic gram-negative bacterium localized in the blood cells (primarily granulocytes) or endothelial cells of blood vessels (Rikihisa 2003). *Anaplasma phagocytophilum* causes febrile disease in humans (human granulocytic- HGA) and animals. *Ixodes ricinus* is the main vector of *A. phagocytophilum* throughout Europe. Additionally, the pathogen has been detected with molecular methods in *I. persulcatus*, as well as in *Dermacentor reticulatus*, *Haemaphysalis concinna*, and *I. ventalloi* ticks (Masuzawa et al. 2008; Paulauskas et al. 2012; Santos et al. 2004; Tomanovic et al. 2013). *Anaplasma marginale* is the aetiological agent of acute anaplasmosis, a bovine syndrome characterized by progressive hemolytic anemia associated with fever, weight loss, abortion, decreased milk production, and in some cases, death of the infected cattle (Wannduragala et al. 1993). Transmission routes include ticks, particularly *Dermacentor spp*., as well as mechanical transmission by biting flies and fomites (iatrogenically). Cattle that survive acute infection by *A. marginale* and *A. phagocytophilum* progress to become subclinical carriers of infection. The carrier animals can serve as reservoirs of infection for naïve cattle despite vaccination with live *A. centrale* bacteria and treatment in countries where domestic ruminants are vaccinated (Coetzee et al. 2006). The main methods for diagnosing anaplasmosis include serological tests and microscopic examination of Giemsa-stained blood smears (Aubry & Geale 2011), although these methods have limitations as the specific detection or as the detection of low levels of parasitemia. Thereby, a specific and sensitive molecular diagnostic method would improve detection and differentiation between species. There is very little information on *A. phagocytophilum* in Iran; therefore, the present study was designed to establish the prevalence of *A. phagocytophilum* and *A. marginale* in cattle of Khuzestan province, Southwest of Iran.

## MATERIALS AND METHODS

### Study sites and collection of samples

Khuzestan province has a border of about 64,236 km2, between 47° and 41′ to 50° and 39′ of eastern longitude from prime meridian and 29° and 58′ to 33° and 4′ of northern latitude from the equator (Statistical book of Khuzestan province 2006). The province has hot and wet summers, mild spring, and cold winters. Samples were collected between March to August 2016. Farmed cattle were selected from the four geographic regions of Khuzestan province with the highest population of cattle herds: Behbahan; Dezful; Shushtar; and Ahvaz. Blood samples were collected from the jugular vein of 200 cattle into sterile vacuum tubes containing EDTA and kept at -20ºC until analyzed.

Polymerase chain reaction and nested-PCR for *A. phagocytophilum* identification

DNA was exploited by the application of the genomic DNA extraction Kit (Cinnagen, Iran). Species specification was accomplished by specific Nested PCR according to amplification of the 16SrRNA gene which conserved for all *Anaplasma* species. PCR protocol and primer selection were adopted according to the previously described by Noaman and Shayan (2009). Briefly, amplification of the 16S rRNA gene was performed in 25µl reaction volumes including 5µl of DNA template, 5 pmol of forward and reverse primers (P1/P2 each 1µl), 12.5µl of master mix (Ampliqon, Denmark) containing 3mM $MgCl_2$, 0.4mM of each dATP, dCTP, dGTP and dTTP and 0.08 U/ml Taq DNA polymerase in reaction buffer. The thermal program of PCR was as follows: 95°C for 5 min, 35 cycles of 94°C for 45s, annealing at 56°C for 45s, and 72°C for 45s, followed by a final extension step at 72°C for 5 min. Amplified products were identified using 2% of agarose gel stained by safe stain and compared with a 100bp ladder after visualization by UV transilluminator. To control the specificity of the PCR products for the 16S rRNA gene of *Anaplasma spp*., the 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). To confirm *A. phagocytophilum*, another specific PCR with primers P5/P4 was used. The primers are listed in Table 1. All the circumstances for nested PCRs including thermal

program were identical to prime PCR. One µl of first PCR was used as the template in nested PCRs.

### Semi nested PCR-RFLP for *A. marginale* identification

To identify *A. marginale*, DNA was amplified using P1/P4 primers. Prime PCR product was used as a template. 10µl of semi-nested PCR product was then cut with 0.1 µl restriction endonucleasis Bst 1107 I (Roche, Germany, 10U/ µl) in 2.5 µl 10 x corresponding buffer and 12.5 µl H2O for 1 h by 37°C. As control 10 µl PCR products were treated with 2.5 µl 10 x corresponding buffer and 12.5 µl H2O without adding of the enzyme.

## RESULTS

A total of 200 cattle was obtained from the four geographic regions of Khuzestan province, southwest of Iran: 61 samples were collected in Behbahan (30.5%), 40 in Dezful (20%), 45 in Shushtar (22.5%), and 54 in Ahvaz (27%) (Fig 1). The prevalence of *Anaplasma spp*. among 200 cattle was 21.5% with P1/P2 and P3/P4 but the overall prevalence of *A. phagocytophilum* was 7. 5% (15/200) with P5/P4. Fig 2. showed amplification of *Anaplasma spp.* and *A. phagocytophilum* in the mentioned methods.

**Table 1.** List of primers used in the present study to detect *Anaplasma spp*., *A. phagocytophilum,* and *A. marginale*.

| Primer | Nucleotide sequence | Positions | PCR product |
|---|---|---|---|
| P1 | 5`-agagtttgatcctggctcag-3` | 1-20 | 781bp |
| P2 | 5`-agcactcatcgtttacagcg-3` | 781-762 | |
| P3 | 5’-gcaagcttaacacatgcaagtc-3’ | 35-56 | |
| P4 | 5’-gttaagccctggtatttcac-3’ | 577-558 | 543bp |
| P5 | 5’-ctttatagcttgctataaagaa-3` | 69-90 | |
| P4 | 5’-gttaagccctggtatttcac-3’ | 577-558 | 509bp |
| P1 | 5`-agagtttgatcctggctcag-3` | 1-20 | |
| P4 | 5’-gttaagccctggtatttcac-3’ | 577-558 | 577bp |

**Figure 1.** Map of Iran and Khuzestan province. Sampling locations were included Ahvaz, Behbahan, Dezful, and Shushtar.

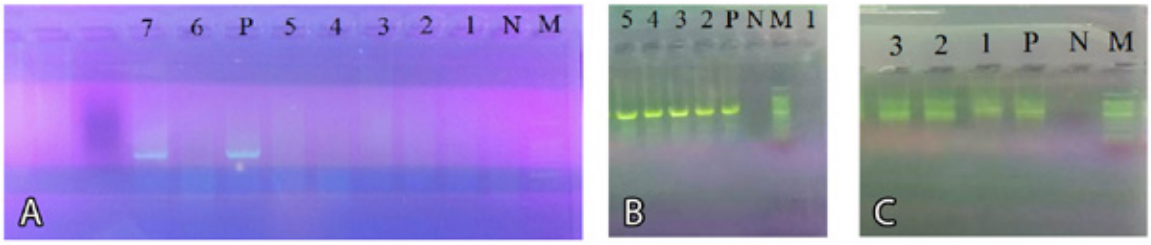


**Figure 2.** A: Agarose gel electrophoresis of PCR of 16SrRNA gene (P1/P2), 1-6: negative samples, 7: positive sample for *Anaplasma spp.* with 781bp of amplified products; B: PCR (P3/P4), 1: negative sample, 2-5: positive samples for *Anaplasma spp.* with 543bp of amplified products; C: Specific PCR (P5/P4), 1-3: positive samples for *A. phagocytophilum* with 509bp of amplified products. P, N, and M represent positive control, negative control, and marker (100bp), respectively.

**Table 2.** Prevalence of *Anaplasma spp.* based on different methods.

| Method | No. examined (%) | Positive (%) | Negative (%) |
|---|---|---|---|
| PCR with P1/P2 primers to detect *naplasma spp.* | 200 (100) | 43 (21.5) | 157 (78.5) |
| PCR with P3/P4 primers to detect *Anaplasma spp.* | 200 (100) | 43 (21.5) | 157 (78.5) |
| PCR with P5/P4 primers to detect *A. phagocytophilum* | 200 (100) | 15 (7.5) | 185 (92.5) |
| PCR with P1/P4 primers to detect *A. marginale* | 200 (100) | 43 (21.5) | 157 (78.5) |

**Table 3.** Prevalence of *A. phagocytophilum* and *A. marginale* in four geographic regions of Khuzestan province, southwest of Iran.

| | Locality | No. examined (%) | Positive (%) | Negative (%) |
|---|---|---|---|---|
| *A. phagocytophilum* | Ahvaz | 54 (27) | 3 (5.5) | 51 (94.5) |
| | Behbahan | 61 (30.5) | 4 (6.5) | 57 (93.5) |
| | Dezful | 40 (20) | 3 (7.5) | 37 (92.5) |
| | Shushtar | 45 (22.5) | 5 (11.5) | 40 (88.9) |
| | Total | 200 (100) | 15 (7.5) | 185 (92.5) |
| *A. marginale* | Ahvaz | 54 (27) | 14 (25.9) | 40 (74.1) |
| | Behbahan | 61 (30.5) | 13 (21.3) | 48 (78.7) |
| | Dezful | 40 (20) | 7 (17.5) | 33 (82.5) |
| | Shushtar | 45 (22.5) | 9 (20) | 36 (80) |
| | Total | 200 (100) | 43 (21.5) | 157 (78.5) |

Amplification of all PCR products with primers P1/P4 resulted in the PCR product of 577 bp. Then the later PCR product was purified and cut with the restriction endonuclease Bst 1107I. The restriction endonuclease Bst 1107I recognizes the sequence (GTATAC) in a corresponding PCR product of *A. marginale* and cut it in the position 68, whereas the used restriction enzyme cannot cut the corresponding PCR product of *A. ovis* (GTACGC) or *A. centrale* (GTACGC). Analysis of all 43 *Anaplasma* positive PCR products with the restriction endonuclease Bst1107I showed that all PCR products could be cut in two expected DNA fragments with 509 bp and 68 bp in length, respectively. Forty-three cattle (21.5%) were infected with *A. marginale* and in 7.5% of cattle co-infection of *A. phagocytophilum* and *A. marginale* was occurred. Table 2. showed the prevalence of *Anaplasma*, *A. phagocytophilum* and *A. marginale* in 200 cattle.

Based on the statistical analysis there was no significant relationship between infection with *A. mar-*

*ginale* and A. *phagocytophilum* and the geographic regions of infected animals. Table 3. showed the number of sampled animals, locality, and molecular detection of *A. marginale* and *A. phagocytophilum.*

## DISCUSSION

The tick-borne, hemoparasitic diseases are among the most devastating to cattle worldwide and include rickettsial diseases. These diseases, enzootic principally in countries with tropical and subtropical climates, place over one-half billion cattle at risk to one or more of the infectious agents. Hence, clinical manifests of *A. phagocytophilum*, if present, are not pathognomonic, therefore the diagnosis of this infection is basically based upon paraclinical aspects of the infection. For this purpose, many diagnostic approaches including microscopy to recognize morulae in leukocytes, different serologic procedures, and tracing DNA of rickettsia from blood, buffy coat, bone marrow, or spleen are well described by researchers are developed (Carrade et al. 2009). Most of the molecular techniques target the major surface proteins (MSPs) (de la Fuente et al. 2007), the heat-shock gene *groEL* (Park et al. 2005), the 23S rRNA (Dahmani et al. 2015) and the 16S rRNA gene (Reinbold et al. 2010). Here, we targeted the 16S rRNA. Based on our results 21.5% of cattle were infected with *A. marginale* while 7.5% were infected with *A. phagocytophilum* which was found in a mixed infection with *A. marginale.* Despite the importance of *A. phagocytophilum*, there is limited information on the occurrence of *A. phagocytophilum* in Iran. For the first time Noaman and Shayan (2009) detected *A. phagocytophilum* in 1.33% of cattle from Iran. Yousefi *et al* (2017) studies showed that 1.08% (4/370) of Iranian domesticated small ruminants were positive for *A. phagocytophilum* infection. In our previous study, the molecular prevalence of *A. phagocytophilum* was noticeably high in rural dogs of Khuzestan province (Hamidinejat et al. 2019). The risk of exposure to the vector-borne pathogen is, among other factors, influenced by the abundance of the vector and the prevalence of the pathogen within the vector population (Medlock et al. 2013). Khuzestan province has wet weather with hot summer. The weather conditions of the province are perfectly suitable for ticks' growth and multiplication. *Anaplasma phagocytophilum* is usually associated with ticks of the genus *Ixodes,* including *I. scapularis*, *I. pacificus* (Parola et al. 2005) and *I. dentatus* (Goethert & Telford 2003) in the USA; *I. ricinus* and *I. trianguliceps* in Europe (Bown et al. 2008); and *I. persulcatus* in Asia (Cao et al. 2003). However, based on our previous study *Ixodes* is not found in Khuzestan province but other ticks including, *Dermacentor reticulatus*, *D. silvarum*, *D. variabilis*, *Haemaphysalis concinna*, *H. megaspinosa*, *H. longicornis*, *Hyalomma* (Hy) *marginatum* and *Hy. detritum* are endemic (unpublished). It should be mentioned that *A. phagocytophilum* has been detected with molecular methods in *D. reticulatus*, *D. silvarum*, *D. variabilis*, *D. occidentalis*, *D. albipictus*, *H. concinna*, *H. megaspinosa*, *H. douglasii*, *H. longicornis*, *H. japonica*, *Hy marginatum*, *Hy. Detritum*, *R. turanicus* and *Boophilus kohlsi* (Cao et al. 2003; Baldridge et al. 2009; Jiang et al. 2011). Mechanical transmission by blood-sucking deer ked (*Lipoptena cervi*) from red deer (*Cervus elaphus*), roe deer (*Capreolus capreolus*), and fallow deer (*Dama dama*) have been reported using PCR (Vichová et al. 2010). It should be mentioned that in protected areas of Khuzestan province Persian fallow deer (*Dama dama mesopotamica)* can be found and they are known as one of Iran's wildlife species. Similarly, there are reports of transplacental (lambs and calves), perinatal, blood transfusions, and nosocomial associated transmissions (Horowitz et al. 1998; Dhand et al. 2007; Zhang et al. 2008; Annen et al. 2012). Variations in the prevalence of *A. phagocytophilum* in ticks may be attributed to several factors, such as the susceptibility of individual tick species, the susceptibility of certain tick populations, and the vector competence of tick species; the transmissibility of the *A. phagocytophilum* variant involved, the susceptibility of different host species, the susceptibility of individual hosts or host populations and the reservoir competence of the host. Especially the availability of different reservoir hosts and the adaptation strategy of *A. phagocytophilum* seem to be crucial factors in this variability. The availability of reservoir hosts depends on factors such as landscape structure and fragmentation (Medlock et al. 2013). Also, effects exerted by changes in climate, demography, and agriculture may influence the tick distribution and density and their hosts (Stuen et al. 2013).

Anaplasmosis caused by *A. marginale*, has the greatest worldwide prevalence. It is found on six continents and is responsible for high morbidity and mortality in cattle in temperate, subtropical, and tropical regions (Kocan et al. 2010). The obtained prevalence of *A. marginale* (21.5 %) was lower than that reported in Kansas (37.6 %) (Reinbold et al. 2010), India (73.1–36.8 %) (Sharma et al. 2015; Singh et al. 2012), Sicily (50 %) (De la Fuente et al. 2005), Brazil

(70.2 %) (Pohl et al. 2013), South African provinces (65-90 %) (Mutshembele et al. 2014), Texas (82 %) (Hairgrove et al. 2015) and Costa Rica (56.9 %) (Shebish et al. 2012). By contrast, this prevalence was higher than those recorded in Turkey (2.8 %) (Aktas et al. 2011) and the Philippines (19.8 %) (Ybanez et al. 2014). The significant prevalence of *A. marginale* warrants further investigation to evaluate the impact of this bacterium on livestock production, since it is a pathogenic species in Iran, causing severe clinical symptoms and very serious economic losses (Sergent et al. 1945). However, at the time of blood sampling (March-August), the 43 cattle infected with *A. marginale* showed no clinical signs. These animals could be considered asymptomatic carriers.

Twenty different tick species are capable of transmitting *A. marginale* and play important roles in maintaining *A. marginale* in cattle (Kocan et al. 2004). In several geographic areas of the world *Dermacentor* species are the principal recognized vectors. Ticks can transmit the infection to susceptible cattle after acquiring the parasite from acutely infected or chronic carrier cattle (Goff et al. 1988). The agro-ecological and geo-climatic conditions of Khuzestan province highly favorable for growth and multiplication of ticks that act as natural vectors of anaplasmosis.

The pathogenesis of disease associated with *Anaplasma spp* infection is influenced by the tick vector as it attaches to host skin, feeds, and inoculates the animal with the bacteria. During feeding, hard ticks secrete bioactive salivary molecules into the skin to promote host bleeding and reduce anti-tick inflammation (Nuttall & Labuda 2004). Saliva may have a complement, cytokine, and antibody inhibitors; histamine-binding proteins; leukocyte modulators; and anti-hemostatics. Thus, inoculation of tick-borne pathogens directly into the skin in the presence of tick saliva is likely to induce local changes in the dermis, and these changes may modulate the early pathogenesis of infection. *Anaplasma phagocytophilum* infection is immunosuppressive; thus, coinfection with *A. marginale* may modulate immunopathologic sequelae of infection, resulting in either enhancement of morbidity, increased mortality rate, or a cross-protective effect. Also, Sergent et al. (1945) have shown that North African strains of *A. marginale* confer immune protection in experimentally infected animals.

## CONCLUSIONS

In the present study 7.5% of cattle were infected with both *A. phagocytophilum* and *A. marginale*. Despite their healthy appearance, they can transmit *Anaplasma* to ticks and are a potential continuous source for maintaining and disseminating the organisms to the human and animals' population. The diagnosis of subclinical infections is important to prevent the spread of anaplasmosis. More epidemiological studies are needed to determine the vectors and reservoir animals for the *Anaplasma* species and to clarify the pathogenicity of *A. marginale* and *A. phagocytophilum* for humans and animals in Iran.

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## CONFLICT OF INTEREST

There is no conflict of interest.

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