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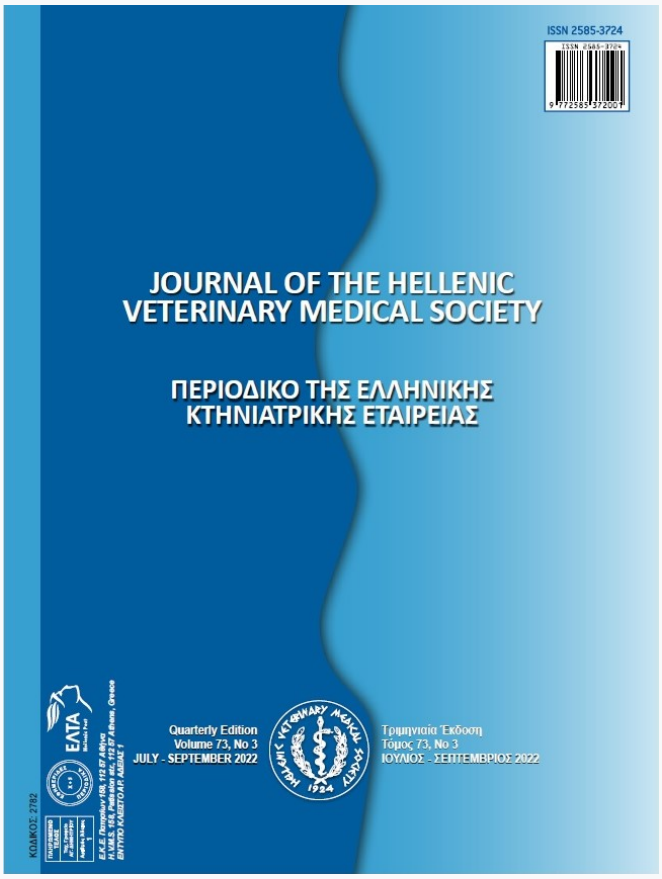

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Research article
Ερευνητικό άρθρο

Application of a nested polymerase chain reaction assay to detect *Anaplasma bovis* and *Anaplasma phagocytophilium* in cattle in Lorestan province, Iran

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ABSTRACT: Anaplasmosis is an important tick-borne disease caused by *Anaplasma* species in cattle and other ruminants. This study aimed to determine the leukocytotropic species of bovine anaplasmosis (*Anaplasma bovis* and *A. phagocytophilium*) among cattle of Lorestan province, Iran using nested polymerase chain reaction (nPCR). Two hundred blood samples were taken from the jugular vein from healthy cattle, randomly. The extracted DNA from blood cells was screened using the universal primers, which amplify a DNA fragment (1468bp) from the 16S rRNA gene of eubacteria. The PCR products from positive samples were examined for *A. bovis* and *A. phagocytophilium*16S rRNA gene by species-specific nPCR.Out of the 200 cattle examined, 10 (5%) and 4 (2%) were found nPCR positive for *A. bovis* and *A. phagocytophilium*, respectively. No morulae were observed in the blood smears and clinical signs were not seen in infected cattle. Findings in the present study corroborate the low infection rate of *A. bovis* and *A. phagocytophilum* in Lorestan province, Iran. Identifying interactions among animal reservoirs, vectors, and pathogens in different climatic zones in Iran are necessary to be performed in future investigations.

Key words:16S rRNA gene, *Anaplasma bovis*, *Anaplasma phagocytophilium*, nested-PCR, Cattle, Iran

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INTRODUCTION

The climate of Iran is divided into four zones and different geographic areas with various climatic conditions that have already resulted in the development of arthropod vectors and an exceeding rise in tick populations. These climatic changes have a widespread influence on ecosystems. Humidity, precipitation, and variation in temperature that occur under different climatic conditions affect the ecology and biology of vectors and intermediate hosts and may increase the risk of infection transmission (Noaman, 2020). Tick spread is also closely connected to climate, and there is a lot of concern that tick-borne diseases rate, such as anaplasmosis, may be increasing in Iran (Noaman et al., 2017). Anaplasmosis is an important tick-borne disease caused by *Anaplasma* species in cattle and other ruminants (Rickettsiales: Anaplasmataceae). Six species with differences in host cell tropism include *Anaplasma bovis, A. centrale, A. marginale*, *A. platys,* and *A. phagocytophilium* recognized in cattle. Of these species, leukocytic anaplasmas generally are attributed to two species *A. bovis* and *A. phagocytophilium* which infect monocytes and granulocytes, respectively (Noaman et al., 2017).

Anaplasma bovis was first described in 1936 in *Hyalomma* sp. ticks from Iran. Although *A. bovis* often causes subclinical infection in tropical and subtropical regions of the world, some conditions cause severe clinical infection characterized by fever, weight loss, anaemia, abortion, and death in cattle. Infected animals are lifelong carriers (Donatien and Lestoquard, 1936).

A. phagocytophilum is a tick-borne infective pathogen. Although the organism first recognized as a veterinary agent, in 1994, the first human case was reported and *A. phagocytophilum* considered as an organism with public health importance (Walker and Dumler, 1996). Disease in cattle (Tick-borne fever) caused by *A. phagocytophilum* is characterized by reduced milk yield, fever, reduced fertility, abortions, leukopenia, and inclusions in circulating neutrophils. Mostly *A. phagocytophilum* causes mild or subclinical infections but some genotypes rarely causing death complexed with other infections (Noaman, 2019). Tick vector scan play an important role in the transstadial transmission of *A. phagocytophilum*. *Ixodes ricinus* was identified as the main vector of *A. phagocytophilum* in Europe (Woldehiwet, 2010). Molecular identification of *A. phagocytophilum* in *Ixodesricinus* has been recorded in the Caspian zone of Iran (Bashiribod, 2004). However, other hard tick species can also play a role in transmitting *A. phagocytophilum* (Atif, 2015).

Ixodidae (hard ticks) are widely distributed throughout four climatic zones of Iran, and they are the most common arthropods of livestock that are associated with haemoparasites transmission in cattle in the western parts of Iran. Although most of the information is about erythrocytic anaplasmosis, little information is available about other agents of anaplasmosis in cattle in Iran. In carrier cattle from the central part of Iran, *A. bovis* and *A. phagocytophilum* were recognized by molecular methods for the first time (Noaman and Shayan, 2009; 2010). Because of the change in climatic conditions and vector population, regular monitoring for the determination and prevalence rate of pathogens is important. To improve this information, a study was conducted to detect *A. bovis* and *A. phagocytophilum* in naturally infected dairy cattle in Lorestan province by molecular techniques.

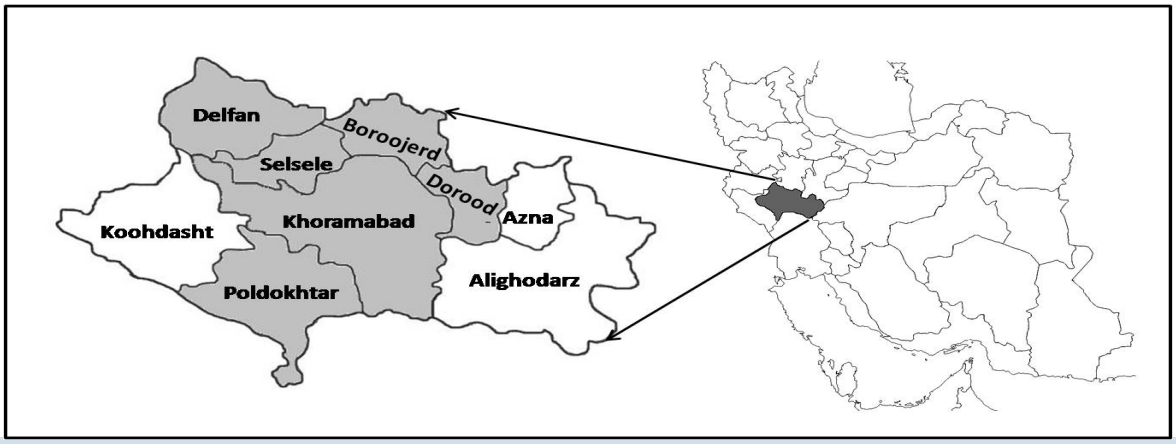

Figure 1. The geographical location of Lorestan province in Iran and sampling site in Lorestan province.

MATERIALS AND METHODS

Blood sample collection and analysis

The study was conducted in six counties in Lorestan province, Iran. The province has a dry and moderate climate, changing from -36°C to 47.4°C in winter and summer, respectively. The mean temperature has been listed as 5.9°C, and the annual rainfall on the mean has been listed as 550 mm yearly. Stratified random sampling was used for villages and flocks selection (Figure 1). Blood samples via jugular vein were collected from the 200 healthy cattle. After each blood collection, two thin blood smears were prepared immediately. Each blood sample (50 µl) was taken in tubes including EDTA (anticoagulant ethylene diamine tetra-acetic acid). Blood samples were kept at −20◦C before DNA extraction. After drying, fixation with methanol, and staining with Giemsa, blood smears were analyzed for the presence of *A. bovis* and *A. phagocytophilum* in the monocytes and neutrophils, respectively. In each smear at least 50 microscopic fields were examined carefully.

DNA extraction

According to the manufacturer's guidelines, DNA extraction was performed by MBST kit [Molecular Biology System Transfer, Iran]. Briefly, 180 µl lysis buffer was used for lysing 50 µl of blood samples and the denaturation of the proteins was performed at 55°C in 10 min by 20 µl proteinase K. Then 360 µl Binding buffer was added and sample incubation at 70°C for 10 min, 270 µl ethanol (96%) was added to the solution and after vortexing, the complete volume was passed to the MBST-column. The centrifuged MBST-column was rinsed twice with a 500 µl washing buffer. In the end, for eluting DNA from the carrier, a 100 µl Elution buffer was added. The purity of extracted DNA was measured by optical density260 (OD260) and the ratio of OD260 to OD280, respectively. Before use, the extracted DNA was analyzed on an agarose gel.

Molecular analysis (PCR and specific nPCR)

One set of universal primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and Rp2 (5'-ACGGCTACCTTGTTACGACTT-3') were used for the first PCR, in 50 µl total volume including 2 µl of each primer (fD1/ Rp2, 20 µM, Cinaclone), one time PCR buffer, 200 µM of each dATP, dTTP, dCTP and dGTP (Cinaclone), 2.5 U Taq Polymerase (Cinnagen, Iran), 1.5 mM MgCl2 and 100-500 ng extracted DNA in an automated thermocycler (T100 Thermal Cycler, Bio-Rad) under the designed program: first denaturation (5 min incubation at 94°C to denature double-strand DNA), followed by 40 cycles of denaturing step (45 s at 94°C), annealing step (45 s at 55°C) and extension step (1.5 min, at 72°C) (Weisburg et al., 1991).This approach has been widely used to both identify newly discovered bacteria as well as to amplify the 16S rRNA gene from all known bacteria to obtain a larger segment of the 16S rRNA gene.

To specific detection of *A. bovis* 16S rRNA gene, amplification was performed using a primer set (5' CTCGTAGCTTGCTATGAGAAC 3'/5' TCTCCCGGACTCCAGTCTG 3') as described by Kawahara et al. (2006), which produce an expected amplicon of 551 bp.

Also, the *A. phagocytophilum* 16S rRNA gene was amplified using a primer set (5' GTCGAACGGATTATTCTTTATAGCTTGC 3'/5' CCCTTCCGTTAAGAAGGATCTAATCTCC 3') as described by Barlough et al. (1996) which produce an expected amplicon of 926 bp.

Specific internal primer sets to detect *A. bovis* and *A. phagocytophilum* targeting the hypervariable region (V1) of the 16S rRNA.2 µl of the primary PCR product was used to specific nPCR reactions one by one.

The nPCR for *A. bovis* was performed in a total reaction volume of 50 µl, containing 1× PCR buffer, 1 µl of each primer (20 µM, CinnaClone), 1.25U Taq Polymerase (CinnaClone, Iran), 1.5 mM $MgCl_2$, and 200 µM of each dATP, dTTP, dCTP, and dGTP (CinnaClone, Iran) in automated Thermocycler (T100 Thermal Cycler, Bio-Rad, USA) using the following program: first denaturation (5 min incubation at 94°C to denature double-strand DNA), followed by 35 cycles of denaturing step (at 94°C, 45 s), annealing step (at 56°C, 45 s), extension step (at 72°C, 45 s) and final extension step at 72°C for 10 min.

In specific nPCR for amplification of *A. phagocy,* only the annealing temperature was 50°C and other nPCR requirements were the same as those described for the *A. bovis*. The nPCR products were run on 1.5% agarose gel in 0.5 times Tris-Borate-EDTA buffer and stained using ethidium bromide and visible by UV-transilluminator.

RESULTS

Blood smears analysis

All smears were carefully examined at least 50

fields per slide and screened for *A. bovis* and *A. phago* inclusions in monocytes and neutrophils but no inclusions were seen and all blood smears were negative for *A. bovis* and *A. phagocytophilum* like structures.

Analysis of blood samples by PCR and Specific nested PCR

Amplification of DNA samples from 200 blood samples using outer primers in the first PCR showed a PCR product with approximately 1468bp in length from the 16S rRNA gene as expected (Figure 2).

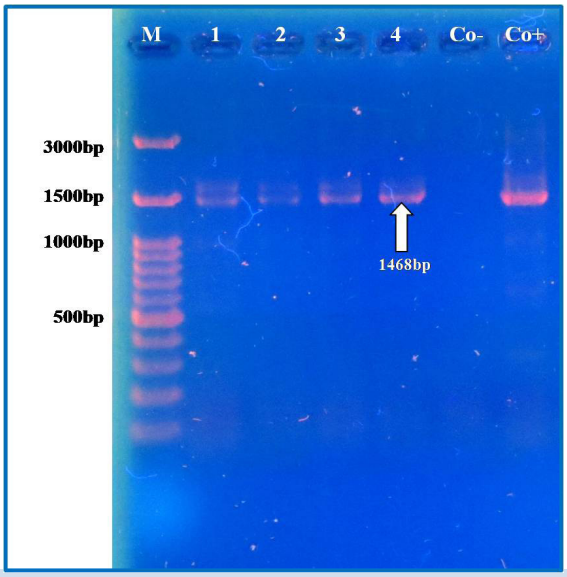

Figure 2. PCR for *Anaplasma* spp. The expected sizes (1468 bp) are indicated (lanes 1 to 4). M= Marker, Co- and Co+ are negative and positive controls, respectively.

Amplification of *A. bovis* was performed using inner specific primers obtained from the nucleotide sequences of the *A. bovis* hypervariable region of the 16S rRNA gene.

An expected amplification product with 551 base pair molecular weight was amplified in specific *A. bovis* nPCR. nPCR analysis of the primary PCR products with these primers showed the expected PCR product in 5% (10 samples out of 200 samples) of the blood samples (Figure 3).

A. phagocytophilum was detected using specific primers obtained from the nucleotide sequences of the *A. phagocytophilum* in the hypervariable region of the 16S rRNA gene. The amplification of PCR products with primers revealed an expected amplification product with 926 base pair molecular weight (Figure 4). nPCR analysis of the primary PCR products with these primers demonstrated the expected amplification product in 2% (4 samples out of 200 samples) of the blood samples.

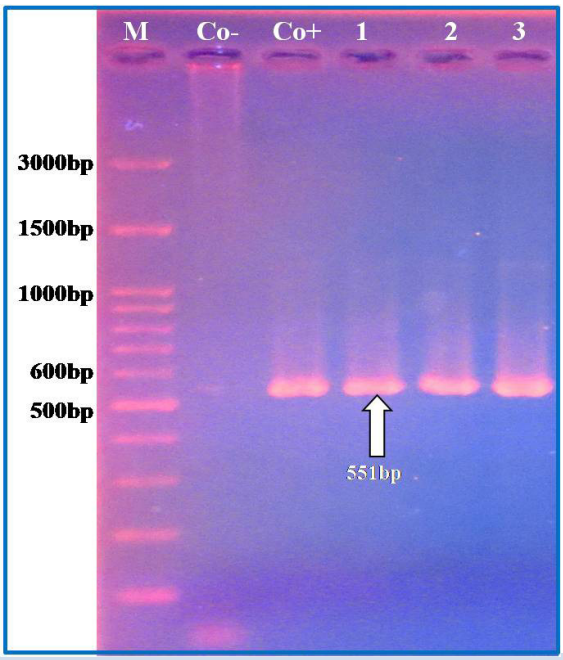

Figure 3. nPCR for detecting *A. bovis.* The expected sizes (approximately 551 bp) are indicated (lanes 1 to 3). M= Marker, Co- and Co+ are negative and positive controls, respectively.

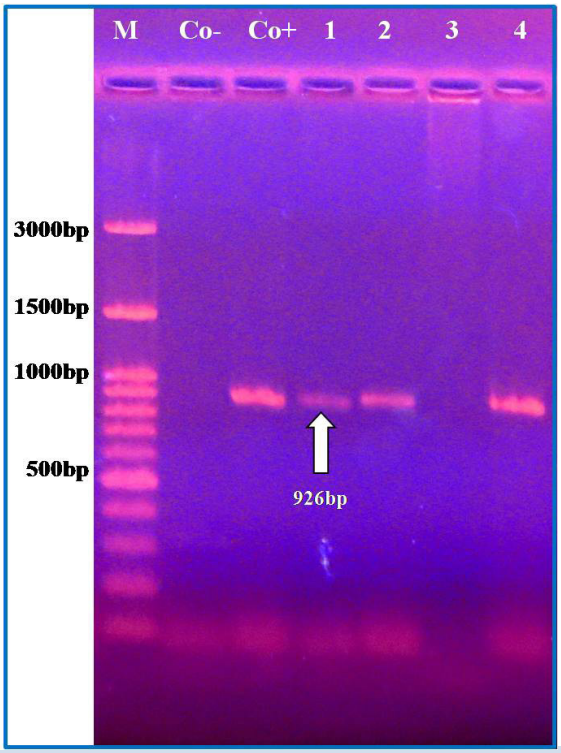

Figure 4. nPCR for detecting *A. phagocytophilum.* The expected sizes (approximately 926 bp) are indicated (lanes 1, 2, and 4). M= Marker, Co- and Co+ are negative and positive controls, respectively.

DISCUSSION

Knowledge of the detection of *Anaplasma* species in ruminants in the various regions of Iran is limited and there is no information about *A. bovis* and *A. phagocytophilium* in the west of Iran (Lorastan province). Until now, five species of *Anaplasma* had been detected in Iranian cattle and sheep by molecular methods includes *A. bovis, A. centrale, A. marginа*, and *A. phagocytophilium* (Noaman, 2017).

In this study, no inclusions were seen and all thin blood smears were negative for *A. bovis* and *A. phagocytophilum* like structures. The sensitivity and specificity of the thin blood smear are lower for blood protozoan infections compared to the buffy-coat technique. In fact, leukocytes can be 10-20 times more concentrated in the buffy-coat than in whole blood (Spielman et al., 1988). Therefore, to obtain an acceptable detection, microscopic analysis of the stained buffy-coat would be required, which we hope to accomplish in future work.

Various primer sets are being used for amplification of 16S rRNA genes and were considered to be universal. However, primers may differ in specificity and sensitivity. Moreover, amplification primer sets need to be chosen depending on the type of biological sample as different types of bacteria can be present across various biological specimens, such as blood, saliva or stool samples (Palkova et al., 2021). New studies showed that amplification of different variable regions of 16S rRNA genes may result in different outcomes and that results may be highly variable depending on the primer set used (Yang et al., 2016). Differences in bacterial composition can be observed even within the analysis of the same 16S rRNA variable regions with alternative primer sets. Furthermore, not only the quality but also the quantity of the detected bacterial genera differed between primers sets. In our study, the universal primer set (fD1 and Rp2) for amplifying the 16S rRNA genes containing V regions was used. The primer set has been successfully applied to identify and classify known and several previously unknown *Anaplasma*, *Ehrlichia*, and *Rickettsia* spp., similarly to our study (Byaruhanga et al., 2018; Guo et al., 2018; Noaman, 2020; Sakamoto et al., 2010; Ybañez et al., 2013).

Small hypervariable region the 16S rRNA gene of *Anaplasma* spp. has a nucleotide sequence for the differentiation of *Anaplasma* spp. from each other (Dumler et al., 2001; Liu et al., 2005; Noaman, 2013). Although hypervariable regions of the 16S rRNA gene of *A. bovis* and *A. phagocytophilum* have sufficient different nucleotide sequences for construction species-specific primers and distinction of these species from other *Anaplasma* spp., designing of species-specific primers based on 16S rRNA of *A. centrale (South Africa strain), A. marginale*, and *A. ovis* is impossible because of high sequence resemblance among hypervariable region (V1) of 16S rRNA gene (Noaman et al., 2016). Therefore, in this study, we used specific primers based on 16S rRNA for molecular identification of *A. bovis* and *A. phagocytophilum.*

In this study*, A. bovis* were identified by specific nPCR in 5% (10/200) of the blood samples. Although Donatine and Lestoquard (1936) have reported the presence of *A. bovis* in the blood of French cattle during experiments of *Theileria* sp. transmission by Iranian *Hyalomma* sp. ticks, there is little data concerning reservoirs of *A. bovis* in Asian countries (Donatien and Lestoquard, 1936). *A. bovis* DNA has recently been detected in wild deer (Kawahara et al., 2006) and cattle in Japan (Ooshiro et al., 2008).

Using specific primers based on the 16S rRNA gene, Noaman et al. suggested that the infection rate of cattle blood samples for *A. bovis* was 2.66% in central Iran (Noaman and Shayan, 2010).

Variable values were reported for *A. bovis* prevalence in cattle from several countries: 4.4% in Algeria (Rjeibi et al., 2018), 4.2% in South Korea (Park et al., 2018), 3.9% in Tunisia (Belkahia et al., 2015) and in the neighbouring country, Pakistan, reported 7.78% prevalence rate of *A. bovis* infection in the cattle population using molecular methods (Iqbal et al., 2019). The prevalence rate is dependent on the vectors, type of species infected, and the diagnostic method used (Iqbal et al., 2019).

The present data show the low infection rate of cattle in the western parts of Iran with *A. bovis.* Pathogenicity of *A. bovis* is already characterized and mainly related to subclinical infection; but in clinical forms, clinical signs include lymphadenopathy, fever, loss of conditioning, and depression (Harrison et al., 2013). In the present study, infected cattle had no clinical signs. During a molecular survey, Jilintai et al. (2009) found *A. bovis* infection in 15% (12/78) of cattle while none of them had clinical signs and morula in the monocytes from blood smears (Jilintai et al., 2009). Nearly under 1% of all white blood cells in circulation are monocytes, therefore, few infected cells could be present on a blood smear and despite

the careful search of blood smears of these, morulae in monocytes were not detected in blood smears.

Anaplasma bovis has since been described in buffalo and cattle from Asia (Middle East), South America, and Africa, also *Amblyomma variegatum, Rhipicephalus appendiculatus*, and *Hyalomma* spp. have been verified to be vectors of *A. bovis* in African countries (Harrison et al., 2013). *Hyalomma* species are the most common hard-tick of cattle in the Zagros region in Iran (Noaman et al., 2017) therefore, *Hyalomma* spp. maybe a prominent tick species concerning the epidemiology *of A. bovis* infection in cattle in this area.

Our results indicated that the infection rate of *A. phagocytophilum* in blood samples was 2% (4/200) by nPCR based on the 16S rRNA gene. These findings are in agreement with the observations made in the southwest of Iran, which reported a prevalence of 2% in cattle (Noaman and Moradi, 2019). The overall prevalence of *A. phagocytophilum* in cattle in Lorestan was lower than the prevalence reported in cattle in different zones of Iran (15.5%) and Gilan province, north of Iran (24.6%) but higher than those reported in the center(1%) of Iran (Noaman and Moradi, 2019; Noaman et al., 2016; Salehi-Guilandeh et al., 2018). The prevalence rate of *A. phagocytophilum* in cattle was also reported in Iran's neighbouring countries inclusive of 2.66% in Pakistan (Iqbal et al., 2019), 30.8% in Turkey (Aktas and Özübek, 2015), and 40% in Iraq (Ayyez et al., 2019) as well as other Asian countries, including 2.1% in South Korea (Seo et al., 2018), 2.4% in China (Yan et al., 2020), and 2% in Japan (Ybañez et al., 2013).

However, some disagreement among reports could originate from the different locations with various climatic conditions, different seasons, tick generation, various diagnostic methods (with different specificity and sensitivity), and the difference in the number of samples (Yan et al., 2020).

Ooshiro et al. (2008) and Kawahara et al. (2006) reported the first molecular detection of *A. phagocytophilum* in cattle and wild deer of Japan, respectively. *A. phagocytophilum* has been found in ticks and mammals by molecular techniques in nearly all European countries (Woldehiwet, 2010). Mammals are supposed to play the main role in the propagation and maintenance of *A. phagocytophilum* in nature. *A. phagocytophilum* has been recorded to stay in species such as red deer, dog, horse, cattle, and sheep (Noaman, 2022). The movement of persistently infected animals may cause the spread of variants from one place to another (Stuen et al., 2013).

The principal vector of *A. phagocytophilum* in Europe is *ricinus (*Jahfari et al., 2014). Other ticks, such as *I. trianguliceps*, *I. persulcatus*, *R. sanguinus,* and *Haemaphysalis punctata* are associated with *A. phagocytophilum* transmission but the importance of these findings remains to be determined epidemiologically (Stuen et al., 2013). In Iran, *Ixodes ricinus* is only propagated in the forest area by the side of the Caspian Sea zone. *Hy. marginatum, Hy. anatolicum*, *R. bursa*, and *R. sanguinus* are dominant tick species on cattle in Iran and Lorestan province (Noaman et al., 2017). In Asia, *A. phagocytophilum* has been found by PCR in *R. turanicus*, *Boophilus kohlsi, Hy. Marginatum* Keysary et al., 2007), and *Haemaphysalis longicornis (Kawahara et al., 2006)* therefore, *R. sanguinus* and *Hy. marginatum* might be the main vector ticks of this *Anaplasma* sp. in Iran.

CONCLUSIONS

Findings in the present study corroborate the low infection rate of *A. bovis* and *A. phagocytophilum* in Lorestan province, Iran. To infection control, we still have to determine interaction among animal reservoirs, vectors, and pathogens (*A. bovis* and *A. phagocytophilum)* in different climatic zones in Iran.

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CONFLICT OF INTEREST STATEMENT

The authors declared no conflict of interest.

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