Original Article

Molecular Study of *Anaplasma* spp. in Horses, Sheep, and Goats with Phylogenetic Analysis in Northwest Iran

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ABSTRACT

Anaplasmosis, a tick-borne disease with worldwide distribution, impacts ruminants, equines, carnivores, and humans. This study aimed to investigate *Anaplasma phagocytophilum* in horses from Ardabil province and *Anaplasma ovis* in small ruminants from East Azerbaijan province using the Nested Polymerase Chain Reaction (PCR) method. Blood samples were taken from the jugular vein of 100 healthy horses in the Ardabil province and 156 healthy sheep and goats (116 sheep and 40 goats) in the East Azerbaijan province during the spring and summer seasons of 2016 in northwest Iran. The collected blood samples were stored at -20°C until the molecular experiments were conducted. Nested PCR was employed to detect *A. phagocytophilum* in horses and *A. ovis* in small ruminants using extracted DNA and amplifying *16S rRNA* and *msp4* genes. The Chi-square test of independence was used to determine the relationship between Anaplasma spp., infection, and independent variables, including age, gender, animal species, and sampling location. None of the 100 samples collected from horses in the Ardabil province were positive for *A. phagocytophilum*. In the East Azerbaijan province, 11 out of the 156 (7.05%) blood samples collected from sheep and goats tested positive for *A. ovis*. In addition, *A. ovis* infection was not significantly related to the independent variables. Phylogenetic analysis showed that the sequence obtained in this study (MH790273) had 100% homology with the sequence obtained from sheep infected with Anaplasma in Ahvaz province (JQ621903.1). The findings of this study can contribute to the prevention and control of anaplasmosis in farm animals in northwestern Iran.

Keywords: *Anaplasma ovis*, *Anaplasma phagocytophilum*, Nested PCR, Sequencing

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1. Introduction

Anaplasma phagocytophilum and *Anaplasma ovis* are obligate intracellular bacteria transmitted by ticks. *Anaplasma phagocytophilum* can replicate in granulocytic leukocytes (neutrophils and eosinophils), causing granulocytic anaplasmosis in humans, horses, dogs, and cats, as well as tick-borne fever in ruminants (1). In horses, *A. phagocytophilum* can induce both subclinical and acute clinical disease with an incubation period of 1-3 weeks characterized by high fever, lethargy, lack of appetite, staggering or ataxia, reluctance to move, limb edema, and mucous membranes petechiae (2). Although the disease can occur at any age in horses, its severity is higher in horses over four years old. The geographic distribution of infected horses and ticks plays a crucial role in spreading the disease among horses worldwide (3). The main tick vectors of the disease are *Ixodes*, *Dermacentor*, *Rhipicephalus*, *Hyaloma*, and *Hemophysalis* species, varying by geographic region (4). *Anaplasma ovis* mainly affects the erythrocytes of sheep and goats, and it has been detected in wild ungulates, such as roe and red deer. This highly host-specific pathogen causes ovine anaplasmosis in sheep (1). Although *Anaplasma ovis* can be more pathogenic in horses than in sheep, it depends on the strain that causes the disease and on the breed of the animal (5). Even though sheep typically experience a subclinical form of the disease, exposure to various stressors, including hot weather, improper sanitary conditions, severe tick infestations, long transportations, and simultaneous infections, can lead to the clinical form (6). Clinical signs include intermittent fever, anorexia, severe emaciation, impaired fertility, abortion in pregnant animals, and the jaundice of mucous membranes, especially following the acute stage (7). In cases of acute *A. phagocytophilum* infections, the presence of intracellular inclusions known as morulae in neutrophils and eosinophils can be used to make a diagnosis. A definitive diagnosis requires more than three morulae in a blood smear. However, the diagnosis can be challenging based on microscopic methods, particularly in cases of low-level infection and subclinical forms of the disease (3). Similar challenges exist in diagnosing *A. ovis* in sheep and goats. Hence, serological and molecular methods detect subclinical and latent infections more efficiently (8). Molecular methods demonstrate high sensitivity and specificity in identifying Anaplasma species. Nested polymerase chain reaction (PCR) with amplification of the *16S rRNA* and *msp4* genes can detect *A. phagocytophilum* in horses and *A. ovis* in sheep and goats, respectively (9). Several molecular studies have been performed in different parts of Iran to identify various Anaplasma species in farm animals (10-13). However, the lack of information regarding the status of anaplasmosis in horses and small ruminants in northwest Iran, coupled with the importance of identifying carrier animals to prevent the spread of this disease, highlights the necessity for further research on this subject. Therefore, this study was conducted to investigate the infection rate of *A. phagocytophilum* in horses in Ardabil province, Iran, and *A. ovis* in small ruminants in East Azerbaijan province, Iran, using the nested-PCR method.

2. Materials and Methods

2.1 Study Area and Sampling

A cross-sectional study was conducted for the molecular identification of *A. phagocytophilum* in horses in Ardabil province and *A. ovis* in sheep and goats in East Azerbaijan province during the spring and summer seasons of 2016 in northwest Iran. Blood samples were obtained from 100 healthy horses in the cities of Ardabil, Nir, Namin, Sareyn, and Meshginshahr in Ardabil province, and 156 healthy sheep and goats (116 sheep and 40 goats) in the cities of East Azerbaijan province, including Tabriz, Bostan Abad, Azarshahr, Hashtroud, Jolfa, and Charuymaq. Blood Samples were collected from the jugular vein using tubes containing the anticoagulant EDTA-K2. The characteristics of all the sampled animals, including age, gender, and sampling location, were recorded. Subsequently, the samples were quickly transferred to the laboratory under frozen conditions and kept at -20°C until molecular tests.

2.2 DNA Extraction

DNA extraction from whole blood followed by the kit manufacturer's instructions (Yekta Tajhiz Azma Co, Tehran, Iran). Briefly, 20 µl proteinase K and 200 µl BG buffer were added to 200 µl blood. The sample was mixed and incubated at 60°C for 15 min, and then, 200 µl ethanol was added. The mixture was transferred into the BG column, and the effluent was discarded after centrifugation at 8,000 rpm for 1 min. Finally, the columns were rinsed with 200 µl washing buffer and centrifuged at 14,000 rpm for 2 min, and extracted DNA was kept at -20°C until the PCR test.

2.3 PCR Reactions

The DNA samples were analyzed for the presence of the *A. phagocytophilum 16S rRNA* gene and the *A. ovis msp4* gene using nested PCR on a thermocycler (BioRad T100, Germany). For the detection of the *A. phagocytophilum 16S rRNA* gene, the first amplification reaction was performed using F1 and R1 primers (Table 1) and 2 µl of DNA template in a 20 µl reaction mixture containing 0.50 µM of each primer, two units of Taq DNA polymerase, 0.20 µM deoxynucleoside triphosphate (dNTP) mix, 1.50 mM $MgCl_2$, and 1× PCR buffer. The cycling conditions were as follows: 30 cycles of denaturation at 94 °C for 30

sec, annealing at 55 °C for 30 sec, and extension at 72 °C for 1 min, with a final extension at 72 °C for 5 min. The nested amplification was performed using F2 and R2 primers (Table 1) in a 50-µl reaction mixture containing 1 µl of the primary PCR product, 200 mM of each dNTP, 1.25 U of Taq polymerase, and 0.20 mM of each primer. The cycling conditions were the same as the first amplification. Successful amplification was confirmed by 1% agarose gel electrophoresis, and the size of the amplicons was determined by comparing it with the DNA marker. The obtained sequence was used as a positive control after confirming its identity with the corresponding sequences available in GenBank. For *A. ovis msp4* gene, the first-round amplification was performed with F3 and R3 primers (Table 1). The cycling conditions were 30 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. The nested PCR was performed using F4 and R4 primers (Table 1) in a 25-µl reaction mixture containing 1 µl of the first-round PCR product, 1.75 U of Taq DNA polymerase, 0.20 mM of dNTP mix, 1.50 mM of $MgCl_2$, and 2.50 µl of 10 × PCR buffer. The cycling conditions were the same as the first amplification. Successful amplification was confirmed by 1% agarose gel electrophoresis, and sterile distilled water was used as the negative control.

2.4 Sequence Analysis of the *msp4* gene and Phylogenetic Analysis

PCR products were sequenced in both forward and reverse directions (ABI 3130 Genetic Analyzer Applied Biosystems, USA). The obtained sequences were used as positive controls in PCR assays after the confirmation of their identity with the corresponding sequences available at GenBank. Sequence analyses were carried out by nBLAST search in GenBank or by using the Clustal w method. The phylogenetic tree was constructed by the neighbor-joining algorithm of the Mega 4 software using the *msp4* gene sequence of *A. ovis* (outgroup). The *msp4* gene sequence of *A. ovis* obtained in this study was assigned accession number MH790273.

2.5 Statistical Analysis

The Chi-square test of independence was used to determine the relationship between age, gender, species, and sampling location with positive cases of *A. ovis* in sheep and goats and *A. phagocytophilum* in horses. Data were analyzed using SPSS software (SPSS, version 22, IBM statistics®, USA), and $P < 0.05$ was considered significant.

3. Results

3.1 PCR Results for *A. phagocytophilum*

Blood samples collected from 100 healthy horses in rural areas of Ardabil, Nir, Namin, Sareyn, and Meshginshahr were analyzed. The age, gender, and sampling locations of the horses are presented in table 2. Both first-stage and nested PCR targeting the 16S rRNA gene showed that none of the horses were infected with *A. phagocytophilum* (Figure 1).

3.2 PCR Results for *A. ovis* and Phylogenetic Analysis

The nested PCR reaction for the *msp4* gene of *A. ovis* successfully amplified the desired fragments (Figure 2). Out of 156 blood samples from sheep and goats of East Azerbaijan province, 11 (7.05%) tested positive for *A. ovis*. No significant relationship was found between *A. ovis* infection and the age of the studied animals. Among the 156 sheep and goats, 37 were one year old or younger, of which only 1 (2.71%) tested positive. In contrast, 119 samples were over one year old, of which 10 (8.40%) were tested positive (Table 3). In this study, among the positive samples, two cases were male (10.00%), and 9 were female (6.62%), with no significant relationship between gender and *A. ovis* infection (Table 3). As shown in table 3, out of the 156 small ruminants examined, 116 were sheep, and 9 (7.75%) tested positive for *A. ovis*. Additionally, 2 (5.00%) out of 40 goats were infected with *A. ovis*, with no significant relationship between the species and the level of infection with *A. ovis*. While no significant relationship between *A. ovis* infection and sampling location was identified, the infection rate was higher in Hashtroud and Charuymaq districts (Table 3).

Table 1. Primers for PCR analysis in the present study

Pathogen	Target Gene	Primer sequences (5'-3')	Amplicon size (bp)
A. phagocytophilum	*16S rRNA*	F1: CACATGCAAGTCGAACGGATTATTC	932
		R1: TTCCGTTAAGAAGGATCTAATCTCC	
		F2: AACGGATTATTCTTTATAGCTTGCT	546
		R2: GGCAGTATTAAAAGCAGCTCCAGG	
A. ovis	*msp4*	F3: GGGAGCTCCTATGAATTACAGAGAATTGTTTAC	870
		R3: CCGGATCCTTAGCTGAACAGGAATCTTGC	
		F4: AGTCAAGCAGAGAGACCTCG	418
		R4: GAAATGTCGACAAAGCTAGCA	

Sequencing results revealed that the amplified gene fragments of the *msp4* gene of *A. ovis* exhibited a high identity (98-100%) with the sequences in the GeneBank. Furthermore, the sequences obtained in the present study had 100% and 99% identity with the previously recorded sequences of JQ621903.1 and JQ663993.1, respectively, from Iran (14). The phylogenetic tree (Figure 3) shows that the sequence obtained in the present study (MH790273) was closely related to various sequences obtained from different parts of the world, including

Table 2. Characteristics of horses sampled in different regions of Ardabil province

Variables		Frequency (%)
Age	**< 5 years**	**27 (27%)**
	> 5 years	**73 (73%)**
	Total	**100 (100%)**
Gender	**Female**	**22 (22%)**
	Male	**78 (78%)**
	Total	**100 (100%)**
Sampling location	**Ardabil**	**36 (36%)**
	Nir	**32 (32%)**
	Namin	**13 (13%)**
	Sareyn	**7 (7%)**
	Meshginshahr	**12 (12%)**
	Total	**100 (100%)**

Table 3. The infection rate of *A. ovis* in sheep and goats and its relationship with age, gender, species, and sampling location

Variable		Infected (%)	Non-infected (%)	Total (%)	*P* value	Chi-square
Age	**≤ 1 years**	**1 (2.71%)**	**36 (97.29 %)**	**37 (23.72%)**	***P* > 0.05**	**1.39**
	> 1 years	**10 (8.40%)**	**109 (91.60%)**	**119 (76.28%)**		
	Total	**11 (7.05%)**	**145 (92.95%)**	**156 (100.00%)**		
Gender	**Female**	**9 (6.62%)**	**127 (93.38%)**	**136 (87.18%)**	***P* > 0.05**	**0.304**
	Male	**2 (10.00%)**	**18 (90.00%)**	**20 (12.82%)**		
	Total	**11 (7.05%)**	**145 (92.95%)**	**156 (100.00%)**		
Species	**Sheep**	**9 (7.75%)**	**107 (92.25%)**	**116 (74.35%)**	***P* > 0.05**	**0.345**
	Goat	**2 (5.00%)**	**38 (95.00%)**	**40 (25.65%)**		
	Total	**11 (7.05%)**	**145 (92.95%)**	**156 (100.00%)**		
Sampling location	**Tabriz**	**2 (4.35%)**	**44 (95.65%)**	**46 (29.49%)**	***P* > 0.05**	**3.35**
	Bostan Abad	**1 (5.00%)**	**19 (95.00%)**	**20 (12.82%)**		
	Azarshahr	**1 (7.14%)**	**13 (92.86%)**	**14 (8.97%)**		
	Jolfa	**1 (3.57%)**	**27 (96.43%)**	**28 (17.96%)**		
	Hashtroud	**3 (12.00%)**	**22 (88.00%)**	**25 (16.02%)**		
	Charuymaq	**3 (13.04%)**	**20 (86.96%)**	**23 (14.74%)**		
	Total	**11 (7.05%)**	**145 (92.95%)**	**156 (100.00%)**		

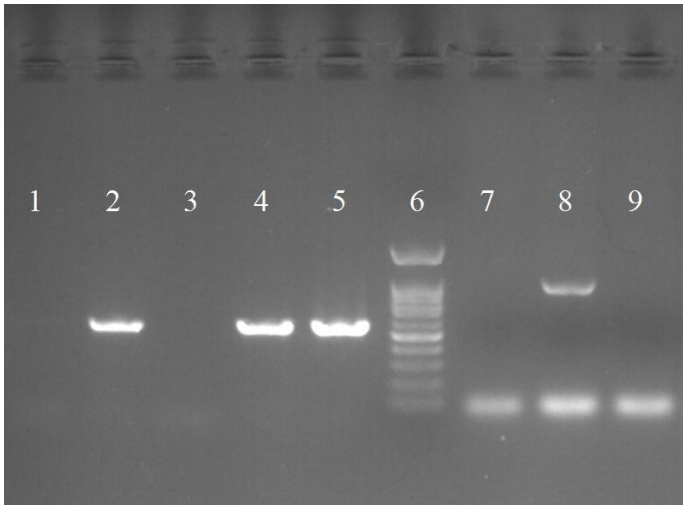

Figure 1. Electrophoresis of Nested PCR reaction products targeting the *16S rRNA gene*. Column 1: negative sample, columns 2, 4 and 5: positive control, column 3: negative control for the second PCR, column 6: DNA marker, column 7: negative control, column 8: positive control, column 9: negative sample for the first PCR.

Mongolia (LC141077.1), Sudan (MG778620.1), China (KX236056.1), Greece (FJ460455.1), Hungary (EF190513.1), Tunisia (KM285222.1), China (KM246804.1), and Iran (JQ663993.1). However, the sequence obtained in this study was relatively different from some sequences recorded from Sudan (KU497705.1), Iran (KY091899.1), Italy (DQ674249.1), and America (AF393742.1). It is also more distantly related to sequences from Tunisia (KC432644.1) and Iran (EU925811.1).

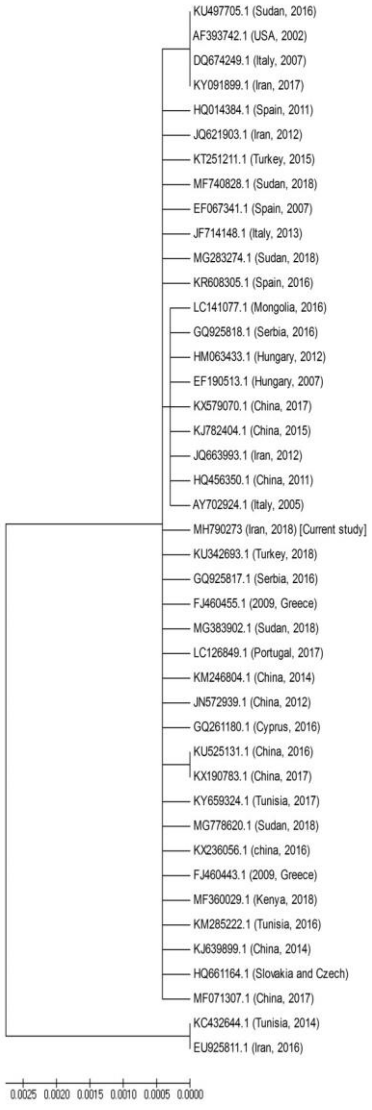

Figure 2. Electrophoresis of Nested PCR reaction products targeting the *msp4* gene. Column 1: DNA Marker, column 2: The fragment obtained from the first reaction is 870 bp, amplified at 60°C with a $MgCl_2$ concentration of 1.5 mM, column 3: The fragment resulting from the second reaction is 418 bp, amplified at 56°C with a $MgCl_2$ concentration of 1.5 mM.

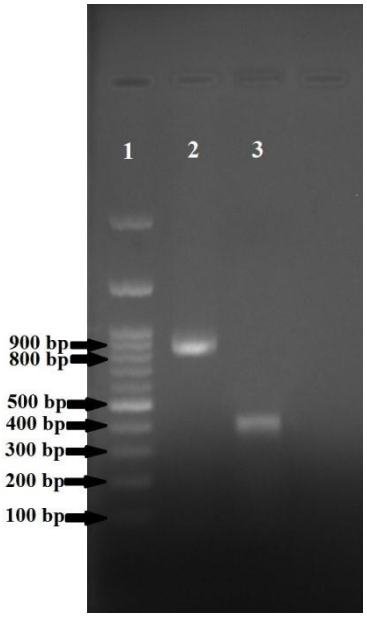

Figure 3. Phylogenetic analysis of *Anaplasm ovis* based on *msp4 gene* complete cds.

4. Discussion

In this study, the nested PCR method was used to amplify the *16S rRNA* gene in blood samples taken from indigenous horses of Ardabil province, showing no infection with *A. phagocytophilum*. There has been no prior study on the prevalence of *A. phagocytophilum* in Iranian horses. This disease was first reported in California, USA, in 1969 and has since been identified on multiple continents, including Europe, Asia, and Africa (4). In contrast to the present study, which did not detect a positive case of *A. phagocytophilum* infection using the PCR method, several studies in neighboring countries of Iran identified this organism in horses (15, 16). In a study conducted on 210 horses in Pakistan, the prevalence of *A. phagocytophilum* was found to be 4.3% using the PCR-restriction fragment length polymorphism (RFLP) method (15). Recently, a study in Iraq reported the infection rate of *A. phagocytophilum* in 160 racing horses of Arabian, Thoroughbred, and mixed breeds, 6.88% by microscopic method and 13.13% by using the PCR method (16). In general, PCR can detect the infection 2-3 days prior to the appearance of clinical signs and up to 129 days after the animal infection with *A. phagocytophilum*. The persistent nature of the infection may result in positive PCR tests if the animal undergoes stress (2). Considering this, the reasons for the negative results of the PCR test in the present study can be attributed to the absence of active infection in the tested horses, the passing of a considerable time since the animal's exposure to *A. phagocytophilum*, or the lack of exposure to tick vectors of the disease. Although there are no molecular studies on the prevalence

of *A. phagocytophilum* in horses in Iran for comparison, some studies have reported its prevalence in ruminants. The prevalence rates of *A. phagocytophilum* in cows from Isfahan and Lorestan provinces were 1.33% and 2.00% (17, 18), while they were 0.48% (1/206) and 1.82% (3/164) in sheep and goats from Hamadan province, respectively (19). The results of these studies demonstrated a low level of *A. phagocytophilum* infection in ruminants. However, two larger-scale molecular studies using the nested PCR method on the *16S rRNA* gene detected *A. phagocytophilum* in 1,851 cows and 1,782 sheep from 4 climatic areas of Iran. The overall prevalence was 15.50% (13) in cows and 8.10% in sheep (10), indicating a higher prevalence when compared to previous studies. It should be noted that the very high prevalence rate of *A. phagocytophilum* in cows from some provinces, such as Kurdistan (47.50%), Semnan (32.70%), Gilan (33.00%), and Mazandaran (20.00%), influenced the overall prevalence rate in the study conducted by Noaman (2020)(13). Out of 222 blood samples examined in that study, there were no positive cases of *A. phagocytophilum* in cows from West Azerbaijan province. These findings were consistent with those of the present study, possibly because of the similar climatic conditions. Anaplasmosis in small ruminants caused by *A. ovis* is a disease distributed worldwide (20). It is one of the most common tick-borne diseases in Mediterranean countries such as Bulgaria, France, Germany, Greece, Hungary, Italy, Portugal, and Romania (6). In Iran's neighboring countries, such as Iraq, Saudi Arabia, Turkey, and Pakistan, *A. ovis* infection has been also reported in small ruminants (21-24). In addition, there have been reports regarding infections in small ruminants in some regions of Iran caused by this organism (8, 14, 25). In the present study, 11 out of 156 (7.05%) blood samples collected from sheep and goats in East Azerbaijan province were positive for the presence of the *msp4* gene related to *A. ovis*, showing a low level of infection with this organism in small ruminants. In a molecular study conducted in West Azerbaijan province, the frequency of *A. ovis* in healthy sheep was only 5% (5/100), which was similar to the findings of the present study (8). The reasons for similarity could be related to common geographical and climatic conditions between the two provinces, the tick population, and the similarity in the technique used for identifying the organism. In Kurdistan province, west of Iran, the infection rate of *A. ovis* using the nested PCR method targeting the *msp4* gene was 10% (20/200), which was similar to the present study (11). Recently, a molecular study with the nested-PCR method targeting the *msp4* gene on 1,842 sheep in nine provinces of Iran reported an overall prevalence of 28.30% of *A. ovis* in sheep (12). However, Khuzestan and Mazandaran provinces, with respectively infection rates of 70% and 58.90%, increased the overall prevalence of *A. ovis* in this study. In contrast, the samples from West Azerbaijan province, neighboring East Azerbaijan province, showed the prevalence of *A. ovis* in sheep to be 5% (10/200), aligning with the results of the present study (12). Nevertheless, some studies have reported a higher prevalence of *A. ovis* in sheep and goats, which contradicts the findings of this study. In a study using a PCR-RFLP method, the prevalence of *A. ovis* was reported to be 63.70% in 193 goats from Khorasan Razavi and Golestan provinces (25). Similarly, the infection rates of *A. ovis* were estimated at 33.60% and 87.40% in 119 sheep in Ahvaz using microscopic and PCR-RFLP methods, respectively (14). Moreover, in a study conducted by the PCR method in eastern Turkey, the overall prevalence of *A. ovis* in sheep and goats was 67.06% (283/422) (23). The observed variations in anaplasmosis prevalence could be related to different factors, such as geographical areas, climatic conditions, different tick vectors, and the presence of reservoir hosts. *Ripicephalus bursa* and other ticks can transmit *A. ovis* to small ruminants. The high prevalence of *A. ovis* in eastern Turkey can be attributed to the domination of *Hyaloma* and *Ripicephalus* ticks in that region (23). In the present study, although the infection with *A. ovis* was not significantly different between the over- and under-one-year-old sheep and goats, with older animals showing a higher infection rate. Although infections with *A. ovis* usually occur in sheep and goats at all ages, adult animals are more likely to be infected, since they spend more time in the pasture for grazing and are more prone to be bitten by the tick vectors. Similarly, a study showed that the prevalence of *A. ovis* was 1.2 times higher in sheep over one year old than in sheep under that age (12). Furthermore, our study found no significant association between the prevalence of anaplasmosis and gender, species, and sampling location, consistent with the findings from other studies (12, 24). Phylogenetic analysis showed that the sequence obtained in the present study (MH790273) had 100% homology with the sequence obtained from sheep infected with Anaplasma in Ahvaz province, Iran (JQ621903.1) (14) and was closely related to the sequences obtained from Mongolia, Sudan, China, Greece, Hungary, and Tunisia. However, our isolates were relatively different from isolates found in Sudan, Iran, Italy, and America. It also completely differed from the sequences obtained from Tunisia (KM285222.1) and Iran (EU925811.1) (Figure 3). Phylogenetic findings have revealed the genetic variations in different isolates, and the existence of these variations can be attributed to the

differences in geographical areas, climate, tick vectors, intermediate hosts, and even the pathogenicity of *A. ovis* (24). In conclusion, the findings of this molecular study showed that *A. phagocytophilum* was not present in horses sampled from different regions of Ardabil province. In contrast, 7.05% of sheep and goats in East Azerbaijan province tested positive for *A. ovis*, highlighting the prevalence of this pathogen in this region. Phylogenetic analysis indicated a close relationship between the obtained *A. ovis* sequence and various global strains, demonstrating the worldwide distribution of this pathogen.

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Authors' Contribution
H. A.: Conceptualization, investigation, writing-original draft, and analysis
M. B.: Investigation, Mega 4 software for drawing the Phylogenetic tree, reviewing, and editing
A. I. B.: Investigation and analysis
Z. A.: Investigation and writing-original draft

Ethics
This study was approved by the Regional Research Ethics Committee of the University of Tabriz, Iran (Approval ID: IR.TABRIZU.REC.1398.036, Approval date: 2020-03-01; Approval ID: IR.TABRIZU.REC.1399.038, Approval date: 2020-09-27).

Conflict of Interest
The authors declared no conflict of interest.

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