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Molecular detection and phylogenetic analysis of *Borrelia* spp. from sheep and goats blood samples in West Azerbaijan province, Iran

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Abstract

Borrelia species are spirochetes transmitted by ticks that are important in human and animals. In most countries, there is still no molecular epidemiology of borreliosis in ruminants. This study was aimed to evaluate the existence of *Borrelia* spp. DNA in the blood samples of small ruminants using polymerase chain reaction (PCR) method in West Azerbaijan Province, Iran. To detect *Borrelia* spp. DNA, about 1,018 ruminants (456 goats and 562 sheep) blood samples were examined from different bioclimatic regions in West Azerbaijan province, Iran. The DNA extracting and PCR were conducted. In sheep, the following prevalence rates were respectively obtained for the *16S rRNA, 5S - 23S rRNA* and *ospA* genes: 3.55% (20/562), 2.13% (12/562) and 0.88% (5/562). And so, the prevalence rates of the genes in goats were 0.87% (4/456) for *5S - 23S rRNA* gene, 1.75% (8/456) for *16S rRNA* gene and 0.65% (3/456) for *ospA* gene. The prevalence of *Borrelia* spp. was significantly different in small ruminants based on the farms and localities. The sheep and goats in humid areas (north of West Azerbaijan) were infected statistically more than those in sub-humid areas (south of West Azerbaijan). It is demonstrated that host species like sheep and goats may have a key role in natural Lyme disease cycles and other borreliosis diseases in Iran.

Introduction

Borrelia species are bacterial pathogen in the Spirochaetaceae family, causing tick-borne Lyme borreliosis (LB). It is a mammal multi-organ disease prevalent in the northern hemisphere.[1,2] Ticks are the second most widespread pathogen vectors worldwide, followed by mosquitoes. The diseases caused by ticks are increasingly threatening human and animal health, besides causing economic losses.[3-5] *Borrelia* spp. are distributed worldwide and maintained in nature within different mammalian and arthropod vectors, reptilian or avian hosts.[6] Regarding domestic animals, studies on LB mainly focus on horses and dogs[7] and only a few studies are available on sheep and cattle.[8] The LB was first reported in cattle in the United States in the 1980s. Weight loss, lameness and abortion are the predominant clinical signs of infection. Some cases of infection of *Borrelia burgdorferi* sensu lato were reported in sheep with anorexia, lameness and poor body conditions.[7,9] Most ruminants are incompetent reservoirs.[9] However, they are important in the dynamic circulation and ecology of *B. burgdorferi* sensu lato, particularly by functioning as spreaders of infected ticks as maintenance hosts for tick populations[6,10] or as a potential host of ticks which can infect themselves by co-feeding, as found in sheep.[11]

Some indirect and direct methods are available to identify the existence of *Borrelia* spp. The indirect methods, primarily serological ones, are mainly based on antibody detection.[1,12] The higher sensitivity and specificity of the direct DNA identification of bacteria have been proved by polymerase chain reaction (PCR).[13] There is little molecular information on the existence of *Borrelia* spp. in goat and sheep blood analysis in Iran. Regarding the livestock industry and animal health, *Borrelia* spp. must be identified. To detect *Borrelia* spp. by PCR, some primers were allocated to amplify various parts of the bacterium genome. The primers designed to detect the genes within the chromosome include *rrf* (gene encoding *5S rRNA*), *16S rRNA* sequence, *rrl* (gene encoding *23S rRNA*) inter-genic gap and *23S rRNA* sequence.[12,14] The *OspA* gene is among the sequences within plasmids.

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The present epidemiological study was aimed to identify *Borrelia* spp. in the blood samples of small ruminants by molecular analysis used for *5S 23S rRNA, 16S rRNA* and *ospA* genes in West Azerbaijan province, Iran.

Materials and Methods

Study areas. West Azerbaijan province, located in the northwest of Iran, has a diverse climate and geographical areas such as mountainous areas, relatively flat terrains and the coastline of Urmia Lake. The climate of this province is mainly influenced by the rainy winds of the Mediterranean Sea and Atlantic Ocean (Fig. 1).[15]

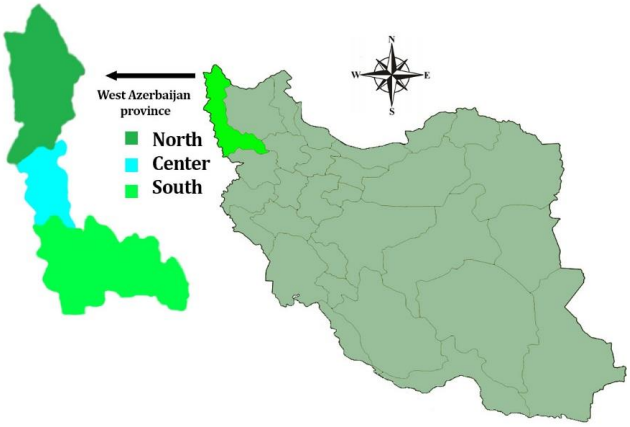

Fig. 1. Schematic map of the study areas, West Azerbaijan province, Iran.

Collection of samples. In this study, 1,018 blood samples were randomly collected from goats (n = 456; 364 females and 92 males) and sheep (n = 562; 385 females and 177 males) belonging to 30 flocks in three various geographical areas of West Azerbaijan province (south, north and center) during four seasons in 2021. Ten flocks were randomly chosen from each area (five flocks of goats and sheep), and 53 animals were sampled from each flock. The samples were taken from seemingly healthy animals. The venous blood samples were put in sterile tubes containing anticoagulant. Then, the collected blood samples were put on ice and transferred immediately to the Microbiology Laboratory at the Faculty of Veterinary Medicine, Urmia University, Urmia, Iran.

DNA extraction. The DNA was extracted from the whole blood using Blood Genomic DNA Extraction Mini Kits (Favorgen, Ping Tung, Taiwan) based on the manufacturer's instructions. The quantity and quality of the extracted DNA were assessed by NanoDrop 2000c (Thermo Scientific, Waltham, USA). The extracted DNA was kept at –20.00 ˚C for subsequent use in PCR. The negative control of DNA extraction process was the elution buffer from the extraction kit during the DNA extraction process.

Molecular detection of *Borrelia* spp. For molecular diagnosis of *Borrelia* species, Nested-PCR targeting *16S rRNA*, *5S - 23S rRNA* and *ospA* genes which were made with Amplifx Software (version 1.5.4; University of Marseille, Marseille, France) was used (Table 1). To perform the first stage of the Nested-PCR, *Taq* DNA Polymerase Master Mix RED (Amplicon, Odense, Denmark) was used.

Table 1. Primer sequences for detection of *Borrelia* spp. gene by Nested-PCR being designed with Amplifix Software.

Genes	Primer	Nucleotide sequence (5′-3′)	Amplicon size (bp)	PCR condition
16SrRNA	OuterF OuterR	GCGAACGGGTGAGTAACG CCTCCCTTACGGGTTAGAA	912	Initial denaturation: 95.00 ˚C for 4 min. 32 cysles of: Denaturation at 95.00 ˚C for 90 sec. Touchdown at 63.00 - 66.00 ˚C (5) for 90 sec, Extension at 72.00 ˚C for 90 sec. Final extension: 72.00 ˚C for 7 min.
	InnerF InnerR	GAGGCGAAGGCGAACTTCTG CTAGCGATTCCAACTTCATGAAG	597	Initial denaturation: 95.00 ˚C for 4 min. 38 Cycles of: Denaturation at 95.00 ˚C for 60 sec, Annealing at 63.00 ˚C for 60 sec, Extension at 72.00 ˚C for 60 sec. Final extension at 72.00 ˚C for 7 min.
5S–23SrRNA	5S rRNA 23S rRNA	ACGGTCCTAAGGTAGCGAAATTCC ACTTGCCACCGCAGATCACTAA	481	Initial denaturation: 95.00 ˚C for 4 min. 32 cysles of: Denaturation at 95.00 ˚C for 55 sec, Touchdown at 63.00 - 66.00 ˚C (5) for 55 sec, Extension at 72.00 ˚C for 55 sec. Final extension: 72.00 ˚C for 7 min.
	5S rRNA (N) 23S Rrna(N)	CGCACGAATGGTGTAACGATTTGG TTGCGCACCTCCGTTACTCTTTAG	317	Initial denaturation: 95.00 ˚C for 4 min. 30 Cycles of: Denaturation at 95.00 ˚C for 15 sec, Annealing at 66.00 ˚C for 15 sec, Extension at 72.00 ˚C for 15 sec. Final extension at 72.00 ˚C for 7 min.
ospA	N1 C1	CAAAGACGGCAAGTACGATC GCTGACCCCTCTAATTTGGT	636	Initial denaturation: 95.00 ˚C for 4 min. 32 cysles of: Denaturation at 95.00 ˚C for 90 sec, Touchdown at 57.00 - 62.00 ˚C (5) for 90 sec, Extension at 72.00 ˚C for 90 sec. Final extension: 72.00 ˚C for 7 min.
	N2 C2	ACTTGAAGGCGTAAAAGCTGAC TTAGCTTTTCCAGATCCATCGC	252	Initial denaturation: 95.00 ˚C for 4 min. 38 Cycles of: Denaturation at 95.00 ˚C for 55 sec, Annealing at 60.00 ˚C for 55 sec, Extension at 72.00 ˚C for 60 sec. Final extension at 72.00 ˚C for 7 min.

The PCR reaction was carried out in 25.00 µL volume comprising 3.00 µL of extracted DNA, 1.00 µL of each primer, 12.50 µL of the master mix and 7.50 µL of DNAse free distilled water. For step two of Nested-PCR, 2.50 µL of the previous PCR product was used as a template and the other materials and conditions were same as the previous step. The PCR products for both stages were electrophoresed on a 2.00% agarose gel containing safe stain (Lab net, Chicago, USA) and then,visualized using Genius Gel Documentation (Syngene Bio-Imaging, Cambridge, UK; Fig. 2). The *Borellia* spp. positive PCR products were sent to Pishgam Biotech Co. (Tehran, Iran) to sequence the DNAs.

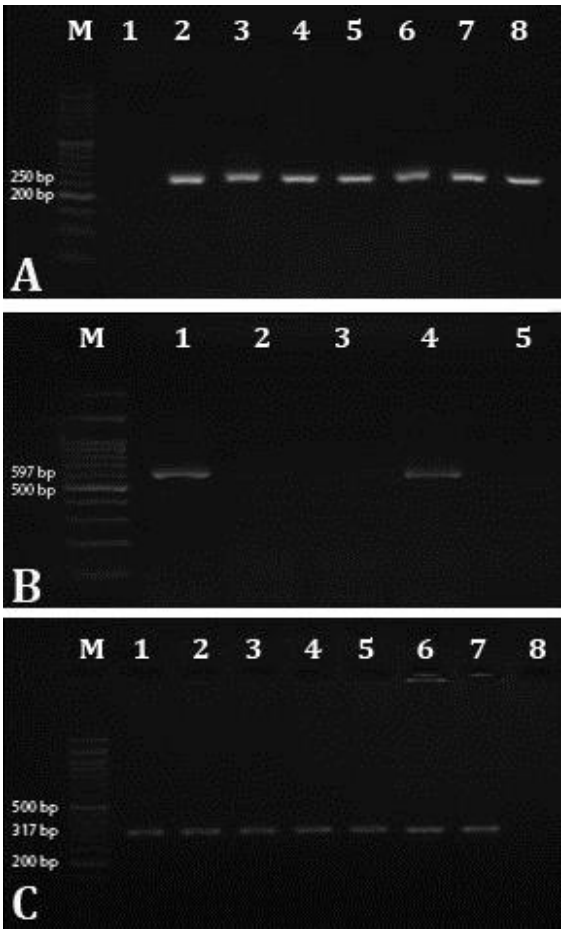

Fig. 2. A) Agarose gel electrophoresis of amplified fragment of *Borrelia* spp. *ospA* gene (252 bp) using Nested-polymerase chain reaction (PCR). Lane M: 50-bp molecular ladder (Smobio Technology Inc., Hsinchu, Taiwan); Lanes 2 - 8: Positive blood samples for *Borrelia* spp.; Lane 1: Negative control. **B)** Agarose gel electrophoresis of amplified fragment of *Borrelia* spp. *16S rRNA* gene (597 bp) using Nested-PCR. Lane M: 100-bp DNA ladder (Smobio Technology Inc); Lanes 1 - 4: Positive blood samples for *Borrelia* spp.; Lane 5: Negative control. **C)** Agarose gel electrophoresis of amplified fragment of *Borrelia* spp. *5S - 23S rRNA* gene (317 bp) using Nested-PCR. Lane M: 50-bp DNA ladder (Smobio Technology Inc); Lanes 1 - 7: Positive blood samples for *Borrelia* spp.; Lane 8: Negative control.

Nucleotide diversity and phylogenetic tree construction. Sequences were uploaded to the National Center for Biotechnology Information (NCBI) to seek the most analogous reference sequences. Furthermore, the Country of Origin Information (COI) positions were identified using NCBI's BLAST. For phylogenetic analysis, all *Borrelia* spp. COI sequences accessible in the GeneBank® were utilized. Using the Clustal alignment program, the alignment was manually adjusted to eliminate any related errors before being released as MEGA and FASTA files. All of the acquired nucleotide sequences were entered into the GeneBank® and given accession numbers. Following that, the phylogenetic association was investigated and developed using the MEGA Software (version 10.0; Biodesign Institute, Tempe, USA) by the maximum-likelihood method. One thousand bootstraps were used to test the reliability of an inferred tree. BioEdit (version 7.0; Ibis Therapeutics, Carlsbad, USA) and BLAST Software (https://blast.ncbi.nlm.nih.gov/Blast.cgi) were used to analyze DNA sequence polymorphism to assess nucleotide diversity (Fig. 3).[16]

Statistical analysis. The obtained data were analyzed using one-way ANOVA method in SPSS Software (version 22.0; IBM Corp., Armonk, USA). A p value less than 0.05 was considered statistically significant.

Results

The positive rate of *Borrelia* spp. DNA was 3.55% and 1.75% of blood samples using *16S rRNA* and *5S - 23S rRNA* genes, respectively, for both sheep and goat. *Borrelia* spp. DNA was identified based on *16S rRNA* and *5S - 23S rRNA* genes under accession numbers of OP293343.1 and OP888467. Also, the results of the molecular prevalence of *Borrelia* spp. DNA were presented 20 (n = 562; mean: 3.50%; 95.00% confidence interval (CI): 2.30 - 5.40%) based on *16S rRNA* and 12 (n = 562; mean: 2.10%; 95.00% CI: 1.20 - 3.00%) positive cases on *5S - 23S rRNA* genes in sheep. In goats, the prevalence of *Borrelia* spp. DNA was detected eight (n = 456; mean: 1.70%; 95.00% CI: 0.80 - 3.40%) and four (n = 456; mean: 0.80%; 95.00% CI: 0.30 - 2.20%) depending on both genes, respectively (Table 2).

The *ospA* gene, which is a plasmid gene, was also assessed through PCR analysis (accession No. OP913461). But, compared to the previous genes, less was detected (five [n = 562; mean: 0.80%; 95.00% Cl: 0.30 - 2.00%] *Borrelia*-positive sheep). In goats, the prevalence of *Borrelia* spp. DNA was detected three (n = 456; mean: 0.60%; 95.00% Cl: 0.20 - 1.90%; Table 2).

The blood collection period was significantly different (p < 0.05). High positive samples were observed in summer and spring seasons in host that adapted to ticks exposure. Furthermore, the frequency of the positive blood samples for *Borrelia* spp. was higher in the north of West Azerbaijan province than other regions.

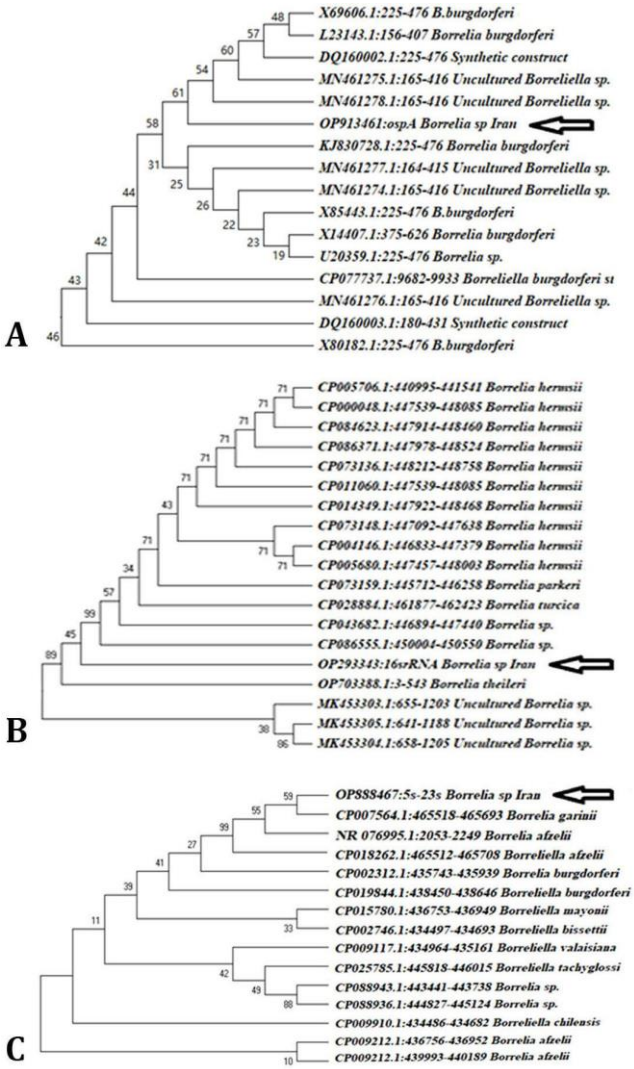

Fig. 3. The phylogenetic tree of **A)** *ospA*, **B)** *16S rRNA* and **C)** *5S-23S rRNA* genes sequences of our achieved *Borrelia* species and those deposited in GenBank from various accession numbers. Accession numbers are presented after isolate names. The *ospA, 16S rRNA* and *5S-23S rRNA* genes sequences attained in this work are represented by arrows. To infer the tree, the neighbor-joining technique of MEGA Software (version 10.0; Biodesign Institute, Tempe, USA) was used and bootstrap values are displayed at each branch point. The bootstrap support is reflected by the numbers above the branch from 1,000 replications. All the alignment sites comprising missing or insertions-deletions data were removed from the analysis (option "complete deletion").

Discussion

Borreliosis, often known as Lyme disease, is one of the most widespread arthropod-transmitted diseases affecting humans, livestock farms, and domestic and wild animals.[17,18] As a result of climate change, ticks can spread to new regions including higher elevations and mountain ranges as Lyme disease carriers or vectors.[19,20]

To date, there is still little knowledge on the distribution and prevalence of *Borrelia* spp. among ruminants in Iran. Therefore, in an attempt to fill this gap, the present study was the first report of the presence of *Borrelia* spp. in sheep and goats in West Azerbaijan province, Iran.

In a study done in Tunisia by Ben Said *et al.*, the prevalence rates of borreliosis were lower in rams and bucks (10.00 - 35.70%) than ewes and does (5.20 - 28.80%). However, there was no significant difference between the sexes. Their results are consistent with the present study. In the current study, the prevalence rates of *Borrelia* DNA in the sex/species mentioned above were 1.37 - 3.30% and 3.26 - 3.95%, respectively. Based on statistical analysis, the present study results showed no significant difference between them.[8]

Based on the results of present study, *Borrelia* prevalence in ages older than 6 years was 4.21% in sheep and 1.14% in goats. While in ages between 3 and 6, it was 2.77% in sheep and 1.95% in goats. In younger ages (< 2 years old), the prevalence of *Borrelia* was 5.66% in sheep and 1.76% in goats. However, Ben Said *et al.* showed that older goats were more likely to be infected (42.10%) than younger ones (21.20%). In addition, younger sheep had higher contamination rates (14.30%) than older sheep (3.50%), which was in accordance with the present study. The statistical analysis of the present study showed no significant difference between groups.[8]

According to Furuno *et al.*, *Borrelia* spp. were most prevalent during the summer and less prevalent during the winter in Japan.[16] Our study was the first molecular study that investigated the effect of season on the prevalence of *Borrelia* in sheep and goats. The present study results were consistent with the study of Furuno *et al.*[16] Their study showed a significant difference between the prevalence of *Borrelia* spp. in different seasons among sheep. However, the effect of seasons on the prevalence of *Borrelia* spp. was not significant in goats.

The Mediterranean and Atlantic Ocean wet winds significantly impact the climate of West Azerbaijan province. Regarding the geographic location, the results showed that the northern part of West Azerbaijan province had the highest frequency of *Borrelia* DNA in both sexes (4.23% in sheep and 3.52% in goats). Aouadi *et al.* found that the prevalence of *Borrelia* spp. was 5.80% in sheep blood samples and 10.80% in goat blood samples.[21] In another study, Ben Said *et al.* showed that the prevalence rates for sheep and goats were 6.20% and 30.40%, respectively.[8] A decrease in the humidity related to the amount of rainfall is directly proportional to the reduction in the tick population in the livestock population and the amount of rainfall is inversely proportional to the temperature of the environment; both of which may contribute to the relatively low level of contamination observed in this study.

Table 2. Prevalence of *Borrelia spp.* DNA in the blood samples collected from sheep and goat farms in different seasons and regions of West Azerbaijan province, Iran.

Parameters	Sheep (n = 562)			Goat (n = 456)		
	16SrRNA	*5S - 23S rRNA*	*ospA*	*16SrRNA*	*5S - 23S rRNA*	*ospA*
Genus						
female	13/385 (3.37%)	8/385 (2.07%)	3/385 (0.77%)	5/364 (1.37%)	2/364 (0.54%)	2/364 (0.54%)
male	7/177 (3.95%)	4/177 (2.25%)	2/177 (1.12%)	3/92 (3.26%)	2/92 (2.17%)	1/92 (1.08%)
***p*-value**	0.73	0.89	0.68	0.21	0.13	0.56
Age (year)						
< 2	6/106 (5.66%)	3/106 (2.83%)	1/106 (0.94%)	2/113 (1.76%)	1/113 (0.88%)	1/113 (0.88%)
3 - 6	10/361 (2.77%)	7/361 (1.93%)	3/361 (0.83%)	5/256 (1.95%)	3/256 (1.17%)	1/256 (0.39%)
> 6	4/95 (4.21%)	2/95 (2.10%)	1/95 (1.05%)	1/87 (1.14%)	0/87 (0.00%)	1/87 (1.14%)
***p*-value**	0.34	0.85	0.97	0.88	0.59	0.70
Season						
Spring	7/152 (4.60%)	4/152 (2.63%)	1/152 (1.92%)	4/123 (3.25%)	2/123 (2.11%)	0/123 (0.00%)
Summer	10/143 (6.99%)	7/143 (4.89%)	3/143 (2.09%)	3/114 (2.63%)	2/114 (2.11%)	2/114 (2.11%)
Autumn	2/134 (1.49%)	1/134 (0.74%)	1/134 (0.74%)	1/107 (0.93%)	0/107 (0.00%)	1/107 (0.93%)
Winter	1/133 (0.75%)	0/133 (0.00%)	0/133 (0.00%)	0/112 (0.00%)	0/112 (0.00%)	0/112 (0.00%)
***p*-value**	0.01*	0.02*	0.29	0.21	0.29	0.28
Region						
North	10/236 (4.23%)	5/236 (2.11%)	2/236 (0.84%)	5/142 (3.52%)	2/142 (1.41%)	2/142 (1.41%)
Center	4/117 (3.41%)	3/117 (2.56%)	1/117 (0.85%)	2/122 (1.63%)	1/122 (0.81%)	0/122 (0.00%)
South	6/209 (2.87%)	4/209 (1.91%)	2/209 (0.95%)	1/192 (0.52%)	1/192 (0.52%)	1/192 (0.52%)
***p*-value**	0.73	0.92	0.99	0.11	0.68	0.35
Total	20/562 (3.55%)	12/562 (2.13%)	5/562 (0.88%)	8/456 (1.75%)	4/456 (0.87%)	3/456 (0.65%)

* Indicates significant difference between the data at $p < 0.05$.

Temperatures between 10.00 - 20.00 ˚C with relative humidity between 50.00 - 70.00% lead to a higher number of ticks. The difference between this study and others was likely because adult ticks prefer to stay near the ground surface during hot and dry seasons to find suitable habitats. Since Africa is a hot and dry continent, the study area geography and climate may be to blame.[22] The statistical analysis of the present study showed no significant difference among geographical groups.

The frequency of *Borrelia* in blood samples of sheep in summer, spring, autumn and winter was 6.99%, 4.60%, 1.49% and 0.75%, respectively. While, the frequency of *Borrelia* in goats blood samples was 3.25%, 2.63%, 0.93% and 0.00%, respectively. In our study there was only a seasonal significant difference in contamination of sheep with *Borrelia* spp.

Finally, our findings also highlighted a considerable variation in *Borrelia* spp. prevalence between sheep and goats. It was found that *Borrelia* spp. DNA was significantly lower in sheep than goats. Our findings were consistent with those of a similar study conducted in Tunisia[8] with the exception that the highest prevalence was found in goats. This difference could be attributed to different sample sizes used to analyze various animal species. Different prevalence rates of *Borrelia* were found in both sheep and goats in a separate investigation by Aouadi *et al*. in Algeria, which was 5.80% in sheep and 10.80% in goats. In contrast, in the current research, the prevalence was 3.55% in sheep and 1.75% in goats. Both housekeeping and location could play a role in this difference.[21]

To clarify the genetic identity of *Borrelia* spp. spirochetes, their differential reactivity can be considered with PCR primers being specific for genospecies, targeting the *5S - 23S rRNA* inter-genic spacer amplicon gene. It is essential to further classify genetic heterogeneity by analyzing longer sequence data within *Borrelia* spp. identified previously as the same genospecies of uncharacteristic strains of *Borrelia* spirochetes.[16,23] Furthermore, the *16S rRNA* gene was found at a greater positive rate in blood samples studied for *Borrelia* spirochetes compared to the other genes in former studies.[16] We found a lower detection rate of these genes as *16S rRNA* > *5S -23S rRNA* > *ospA*.

Considering the results of Nested-PCR, 562 sheep blood samples investigated with primers targeting the *16S rRNA*, *5S-23S rRNA* and *ospA* genes, represented 20 (3.55%), 12 (2.13%) and 5 (0.88%) positive samples. In 456 goat blood samples examined with primers targeting the *16S rRNA*, *5S-23S rRNA* and *ospA* genes, positive samples of eight (1.75%), four (0.87%) and three (0.65%) were revealed from three regions of West Azerbaijan province in Iran.

Borrelia spp. infection molecular prevalence was reported in goats and sheep for the first time in this study in Iran. In this study, the prevalence of *Borrelia* spp. infestation in sheep and goats was recorded by molecular method for the first time in Iran. The present work aimed to comprehend the role of goats and sheep as potential spreaders and carriers of Lyme disease in Iran. The PCR assay and sequencing were used to assess the exposure of

goats and sheep to *Borrelia* spp. The sequences of the *5S - 23S rRNA*, *16S rRNA* and *ospA* genes achieved from positive DNA samples were analyzed and they were considered as hosts.

In the present work, *Borrelia* spp. DNA was found in goats and sheep in West Azerbaijan province, Iran. According to the phylogenetic and sequence analyses, those isolates were related closely to the consistent genotypes in terms of the *16S rRNA* gene with higher sequence similarities (100%, accession No. OP703388; 98.00 - 100%, accession No. CP086555; Fig. 3B). Hence, it was indicated that the *Borrelia* spp. genetics was diverse in different geographical and host locations. The existence of a new *Borrelia* species related to the relapsing fever group was represented by sequencing the cloned PCR products from the *16S rRNA* gene of *Borrelia* spp. from West Azerbaijan province, Iran.

There was a similarity of 100 and 99.80% in the *16S rRNA* gene sequence of *Borrelia* spp. achieved from goats and sheep to the gene of *Borrelia theileri*, respectively (accession No. CP703388 and OP086555). The *B. theileri* is related to the relapsing fever *Borrelia* spp. group, and is the bovine borreliosis causative agent. It was first identified in cattle and then, in deer, goats and sheep from Africa, Mexico, Australia and South America.[24,25]

In conclusion, in the present study, the first insight was provided into the existence of *Borrelia* spp. by extraction of DNA from the blood through PCR in West Azerbaijan province, Iran. Infestation with *Borrelia* spirochetes was identified from goat and sheep blood samples taken from different districts in the West Azerbaijan province in Iran. *Borrelia* spp. was highly prevalent in the province sampled areas. More studies are required on the occurrence of *Borrelia* spp. groups for both relapsing fever and Lyme disease to prove the existence of these various *Borrelia* species in goats and sheep in Iran. We found that *Borrelia* infestation in goats and sheep could present a potential concern for public health. Future studies can warrant further widespread and detailed control of ticks population and screening for *Borrelia* in various hosts.

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Conflict of interest

This manuscript has not been published and is not under consideration for publication elsewhere. There is no conflict of interest to disclose.

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