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'*Candidatus* Bartonella dromedarii' in the dromedary camels of Iran: Molecular investigation, phylogenetic analysis, hematological findings, and acute-phase proteins quantitation

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ABSTRACT

The genus *Bartonella* is comprised of Gram-negative coccobacilli, aerobic, and facultative intracellular bacteria which are transmitted by hematophagous vectors (e.g., fleas, lice, sandflies, and ticks). Each species of *Bartonella* infects one or few related mammals as reservoir host(s). If a *Bartonella* spp. infects a nonspecific host like humans, it can lead to a more acute disease. *Bartonella* spp. has been detected more recently for the first time in camels in Israel by Rasis and colleagues. However, the epidemiological and public health importance of this new pathogen in camels is not clear. In this study, we aimed to detect the *Bartonella* spp. in the blood samples of Iranian camels, measure their prevalence, and determine their species. Also, the relationship between *Bartonella* spp. infection and different hematological factors and acute-phase proteins (Hp, a1AGP, SAA) was investigated. Finally, the sequences of three DNA regions, i.e.16S rDNA, *rpoB,* and ITS, were determined and phylogenetically analyzed. From the 106 examined blood samples of camels from Fars province (southern area of Iran), 18 samples were positive (17%). The findings also showed that *Bartonella* spp. positive camels had significantly lower Hb, MCH, and MCHC but higher RDW, SAA, and WBC ($P < 0.05$) compared to the control group. Our *Bartonella* strain was genetically similar to the '*Candidatus* Bartonella dromedarii' but different from *Bartonella bovis*. Thus, more studies are required to investigate the phenotypic and genotypic characteristics of '*Candidatus* Bartonella dromedarii'. Also, there is a need to evaluate precisely the risk factors, transmission routes, and zoonotic potential of this species.

1. Introduction

The genus *Bartonella* is comprised of Gram-negative, aerobic, short bacillus, and facultative intracellular bacteria that infect the endothelial cells and erythrocytes of mammals (Antequera-Gomez et al., 2015). More than half of the species of *Bartonella* genus are zoonotic agents. Different species of *Bartonella* are found in different mammalian hosts and arthropods which, naturally, circulate between them (Kosoy et al., 2012). The *Bartonella* genus contains more than 30 species which have a shared genome and can be divided phylogenetically into four lineages (Supplementary data, Table 1).

Each species of the *Bartonella* spp. infects one or few closely related mammals as reservoir host(s) (Ben-Tekaya et al., 2013). *Bartonella* spp. host specificity is regulated by the intraerythrocytic persistence and the prevalence of *Bartonella* spp. vectors (Harms and Dehio, 2012). In the reservoir mammalian species, *Bartonella* spp. usually leads to a subclinical infection with bacteremia; during this period, hematophagous vectors (e.g., fleas, lice, sandflies, and ticks) are able to transmit the infection to another host. Humans could also be infected with some of the *Bartonella* spp. through the infected vector (Vayssier-Taussat et al., 2016).

Acute-phase proteins (APP) are blood proteins, primarily synthesized by hepatocytes as part of the acute phase response (APR) (Ceciliani et al., 2012). The APR is part of the early-defense or innate immune system, which is triggered by different stimuli including trauma, infection, stress, neoplasia, and inflammation (Jain et al., 2011). In camels, the presence of different APPs has been suggested as a marker of 'herd health' (Greunz et al., 2018).

The present study was aimed to characterize the *Bartonella* spp. in the blood samples of camels from Fars province, Iran. Therefore, we measured the disease prevalence, determined the sequences of three loci/genes, namely, 16S rDNA, *rpoB,* and ITS, of *Bartonella* spp., and

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		1	2	3	4	5	6	7	8	9	10	11	12	13
Iranian B. dromedarii (Dromedary camel)	1		1	3	15	10	45	39	54	49	74	85	55	63
KJ909798 B. dromedarii (Dromedary camel)	2	99.88		2	14	9	44	38	53	48	73	84	54	62
KJ909806 B. dromedarii (Dromedary camel)	3	99.65	99.77		12	9	44	38	53	48	71	84	54	62
KJ909802 B. dromedarii (Dromedary camel)	4	98.24	98.36	98.59		7	56	50	64	59	59	95	65	72
KJ909799 B. dromedarii (Dromedary camel)	5	98.83	98.94	98.94	99.17		51	45	61	56	66	92	62	68
DQ356075 B. bovis	6	94.74	94.85	94.85	93.45	94.04		9	56	55	79	90	56	67
KJ909808 B. bovis	7	95.42	95.54	95.54	94.13	94.72	98.95		48	47	71	82	48	59
HM167505 B. capreoli	8	93.66	93.78	93.78	92.49	92.84	93.45	94.37		13	42	53	22	27
KM215709 B. chomelii	9	94.25	94.37	94.37	93.08	93.43	93.57	94.48	98.47		35	46	17	24
AY167409 B. schoenbuchensis	10	91.31	91.43	91.66	92.97	92.18	90.76	91.67	95.07	95.89		48	42	34
CP019789 B. schoenbuchensis	11	90.02	90.14	90.14	88.85	89.20	89.47	90.38	93.78	94.60	94.31		53	45
KM215710 B. chomelii	12	93.54	93.66	93.66	92.37	92.72	93.45	94.37	97.42	98.00	95.07	93.78		31
EF605288 B. melophagi	13	92.61	92.72	92.72	91.55	92.02	92.16	93.08	96.83	97.18	95.97	94.66	96.36	

Fig. 1. The figure illustrates the estimation of *rpoB* nucleotide difference (upper right triangle) and percent identity (lower left triangle) for *Bartonella dromedarii*.

analyzed them phylogenetically. Finally, the relationship between *Bartonella* spp. infection and different hematological findings and acute-phase proteins (serum amyloid A (SAA), alpha-1-acid glycoprotein (a1AGP), and haptoglobin (Hp)) was investigated. Moreover, in this study, changes in cell blood count (CBC), WBC differential count, and three major APPs (Hp, a1AGP, SAA) in naturally-infected camels with *Bartonella* spp., as an inflammatory and infectious disease, were also assessed. In addition, as RBC is one of the main target cells infected with *Bartonella* spp. (Dehio, 2004), RBC indices, containing hematocrit (HCT), hemoglobin (Hb), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and red cell distribution width (RDW), were measured instrumentally.

2. Materials and methods

2.1. Blood sampling

One-hundred and six blood samples (containing EDTA anticoagulant) were collected from the camels (17 males and 89 females, ranging from 1 to 6 years old) of Fars province in Iran, which covers 122,608 Km^2 of semi-dried areas. The sampled camels were not restricted to this province because the camels were locally transported to southern provinces, like Bushehr and Hormozgan, for grazing during the autumn and winter and vice versa during the spring and summer.

2.2. DNA extraction

The DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) was used to extract genomic DNA from 100 µl whole blood samples according to the manufacturer's instruction. The extracted DNA was checked by gel electrophoresis and Nanodrop spectrometer.

2.3. PCR reactions

Three individual PCR tests were conducted for the detection of *Bartonella*16S rDNA, *rpoB*, and ITS. The sequences of ITS primers were forward 5′-CTC TTT CTT CAG ATG ATG ATC C-3′ and reverse 5′-AAC CAA CTG AGC TAC AAG CCC T-3′ (Jensen et al., 2000). Moreover, the primer pair used for *rpoB* was forward 5′-CGC ATT GGC TTC GTA TG-3` and reverse 5′-GTA GAC TGA TTA GAA CGC TG-3′ (Bai et al., 2013). Additionally, the following primer set was used for 16S rDNA amplification: Forward 5′-AGA GTT TGA TCC TGG CTC AG-3′ and reverse 5′-ACG GCT ACC TTG TTA CGA CTT-3′ (Dahmani et al., 2017). PCR was conducted by an MJ mini thermal cycler (BioRad, USA). Each PCR reaction was optimized separately to produce the desired PCR product (16S rDNA, *rpoB,* and ITS).

For 16S rDNAs and ITS DNA regions, the total volume of 20 µl in each PCR tube consisted of a mixture of 1.5 µl of each primer (10 µM), 10 µl Master Mix RED (Ampliqon, Denmark), 4 µl DNase free water, and 3 µl sample DNA. The thermal program for this PCR was an initial denaturation of 95 °C for 5 min, 35 cycles of 94 °C for 30 s, 59 °C for 30 s, and 72 °C for 30 s, and also a final extension of 72 °C for 5 min.

In each *rpoB* gene PCR tube, the total volume of 25 µl contained 1.5 µl of each primer (10 µM), 12.5 µlMaster Mix RED (Ampliqon, Denmark), 6.5 µl DNase free water, and 3 µl sample DNA. Touchdown PCR was performed according to the following cycling program: Initial denaturation of 95 °C for 6 min and first cycle of 94 °C for 1 min, 59 °C for 45 s, and 72 °C for 45 s, followed by 5 cycles at decreasing annealing temperature in decrements of 1 °C per cycle, then 34 cycles of 1 min at 94 °C, 45 s at 54 °C, 45 s at 72 °C, and also a final extension at 72 °C for 5 min.

We used a PCR test for ITS DNA region; it produces different product sizes according to different species of *Bartonella* (Jensen et al., 2000) to discriminate different species by PCR results. For example, ITS PCR product sizes in *Bartonella chomelii, Bartonella capreoli, Bartonella bovis,* and *Bartonella schoenbuchensis* were 231 bp, 238 bp, 198 bp, 317 bp, respectively. Thus, in advance, we could find the possible number of *Bartonella* species from the PCR results before sequencing.

As other co-infections may affect the hematological factors and APPs, the presence of some other pathogens, including *Trypanosoma* spp., hemotropic *Mycoplasma,* and *Anaplasma* spp., existing in this region were also investigated using different PCR tests. *Trypanosoma* sp. ITS region and 16S rDNA of *Mycoplasma* spp. were amplified by primer pairs and thermal programs according to Pourjafar et al. (2013) and Hoelzle et al. (2011), respectively. Also, *Anaplasma* spp. was detected as previously described (Ybañez et al., 2012).

The presence of PCR products and their molecular weight was evaluated by the electrophoresis of 7 µl of each product in 1.5% agarose gel stained with RedSafe™ (Intron Biotechnology, Korea) under UV illumination.

2.4. Sequencing and phylogenetic analysis

All positive PCR products were purified by a PCR product purification kit (Fermentas, Burlington, USA) and sequenced bi-directly using a capillary DNA analyzer (ABI 3730; Applied Biosystems, Foster City, CA, USA). Continuous sequences were assembled using BioEdit as a contig assembly program. Further comparisons were made between the assembled 16S rDNA, *rpoB,* and ITS sequences and other associating sequences using BLAST. In addition, the obtained *rpoB* and ITS sequences were aligned against related sequences obtained from the GenBank® database using MEGA 6.0software. Phylogenetic trees were also made using maximum likelihood and maximum parsimony methods (Tamura et al., 2007). Furthermore, the sequence identity

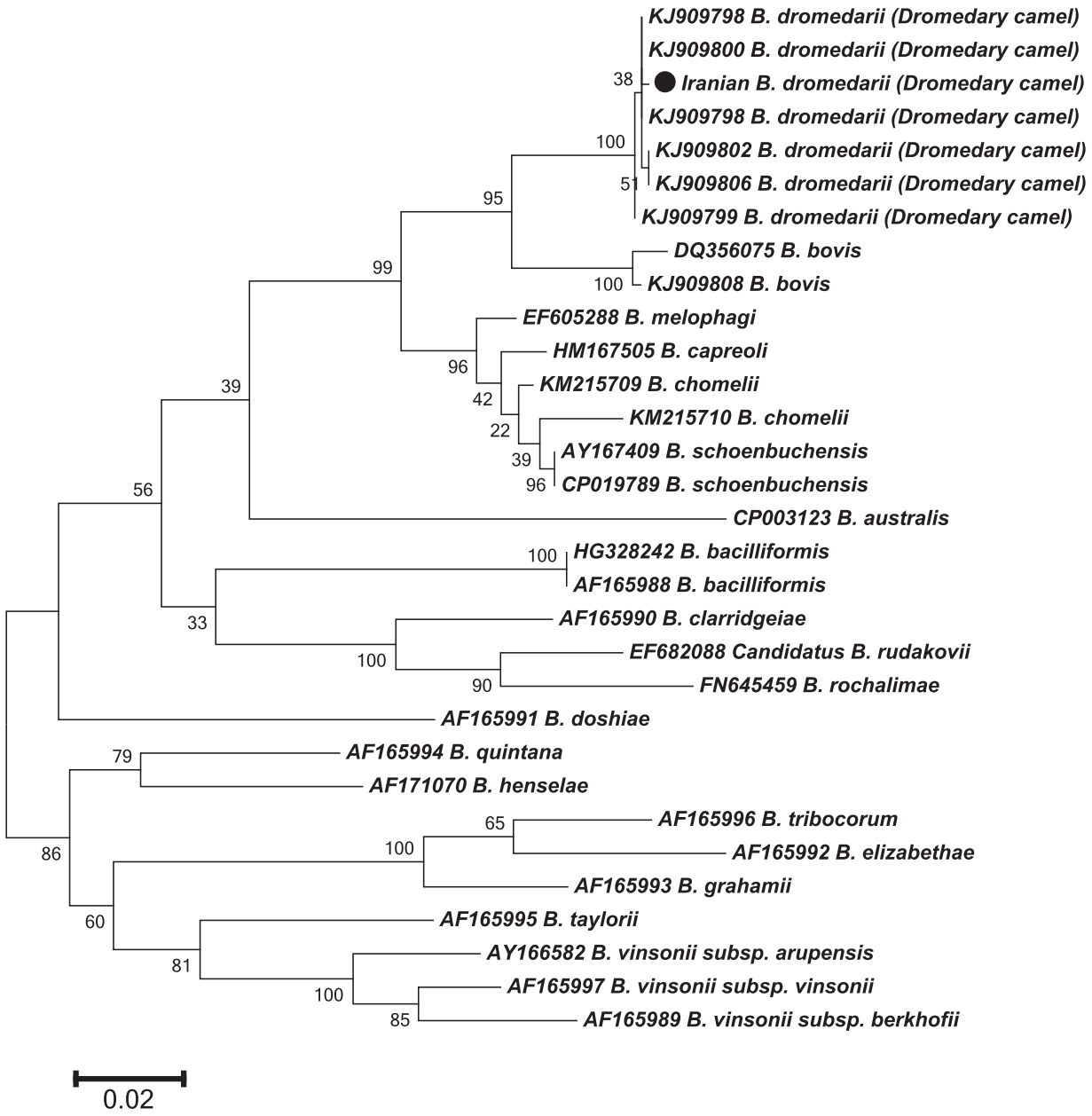

Fig. 2. Phylogenetic analysis of the Iranian strain of *Bartonella dromedarii* (cirf;) and other related sequences existing in the GenBank based on the partial *rpoB* sequences. Maximum likelihood (ML) method was employed for the molecular evolutionary analysis by the MEGA 6.0 software. Scale bar shows nucleotide substitutions; Numbers at the nodes indicate bootstrap values for 10,000 replications.

percentages were calculated using CLC Main Workbench 5 software after the pairwise comparison of aligned 16S rDNA, *rpoB,* and ITS sequence data from different related *Bartonella* species.

2.5. Hematology

An automatic blood cell counter (Exigo, Stockholm, Sweden) was used for complete blood count (CBC). Differential counting of white blood cell (WBC) was performed by microscopic examination using Giemsa staining of blood films (Sigma-Aidrich, Santa Clara, CA, USA).

2.6. APPs measurement

The serum levels of serum amyloid A, alpha-1 acid glycoprotein, and haptoglobin were measured by a quantitative sandwich enzyme immunoassay using commercial camel-specific kits (Shanghai Crystal Day Biotech, Shanghai, China).

2.7. Statistical analysis

According to the normal or abnormal distribution of each data set, a parametric or non-parametric statistical test was performed by IBM SPSS Statistics software (Ver. 22). Independent sample *t*-test and Mann-Whitney test were used from parametric and non-parametric data sets, respectively. Also, chi-square test was conducted to determine whether there was a significant relationship between bartonellosis and both the sex and age of the infected camels. P values fewer than 0.05 were assumed as significant. It must be noted that the results of 17 out of 106 samples were omitted from the statistical analysis because of infections with other pathogens that could potentially interfere with the statistical relationships for the hematological factors and APPs.

Table 1
Mean and SE of different RBC indices and acute phase proteins in *Bartonella* spp. positive, and negative camels. Statistically significant factors ($P < 0.05$) are indicated with a superscripted star. Abbreviations: Haematocrit (HCT), red blood cell (RBC), hemoglobin (Hb), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and Red cell distribution width (RDW), haptoglobin (Hp), alpha-1-acid glycoprotein (a1AGP), and serum amyloid A (SAA).

	HCT (%)	RBC (10^6/µl)	Hb* (g/dl)	MCV (fl)	MCH* (pg)	MCHC* (g/dl)	RDW* (%)	Hp (mg/dl)	a1AGP (mg/dl)	SAA* (µg/ml)
Bartonella positive (n = 14)[a]	27.02 ± 0.93	6.51 ± 0.23	10.17 ± 0.3	41.51 ± 0.91	15.62 ± 0.19	37.86 ± 0.64	27.17 ± 0.62	0.29 ± 0.006	3095.28 ± 334.67	23.49 ± 3.39
Control group (n = 75)[b]	27.73 ± 0.55	6.61 ± 0.16	10.94 ± 0.16	41.45 ± 0.41	16.96 ± 0.33	41.16 ± 0.88	25.05 ± 0.49	0.29 ± 0.003	2698.11 ± 181.1	18.23 ± 1.39
P value	0.515	0.733	0.039	0.955	0.001	0.004	0.012	0.244	0.11	0.028

[a] Four co-infected samples (*Trypanosoma* spp.: 2 samples, hemotropic *Mycoplasma:* 1 sample and *Anaplasma* spp.:1 sample) were excluded from 18 *Bartonella* positive camels.
[b] These camels were negative for *Bartonella* spp., *Trypanosoma* spp., hemotropic *Mycoplasma* and *Anaplasma* spp.

3. Results

3.1. Bartonella PCR test

The ITS PCR product gel picture showed that only an individual *Bartonella* species existed in the positive samples that produced a 156 bp band (Supplementary data, Fig. 1).

3.2. Bartonella prevalence in the camels of the southern area of Iran

From 106 blood samples of apparently healthy camels, 18 samples (4 males and 14 females) were positive, so the prevalence of *Bartonella* spp. in the camels of the southern area of Iran was measured as 17%. Among the infected camels, 12 camels were ≤2 years old and 6 camels were > 2 years old. In the group of *Bartonella* negatives (88 camels), 13 males and 75 females were existed, and 49 camels were ≤2 years old and 39 camels were > 2 years old. Accordingly, the statistical analysis did not show a significant relationship ($P < 0.05$) between *Bartonella* infection and sex or age of camels.

In addition to the *Bartonella* spp. infection, *Trypanosoma* spp. (9 samples), hemotropic M*ycoplasma* (2 samples), and *Anaplasma* spp. (6 samples) were also detected in 17 blood samples, including four co-infections: *Trypanosoma* spp. (2 samples), hemotropic *Mycoplasma* (1 sample), and *Anaplasma* spp. (1 sample). The results of all these infected cases were deleted from the hematological factors and APPs statistical analysis.

3.3. Species of Bartonella in the camels of the southern area of Iran

PCR products of 16S rDNA, *rpoB,* and ITS regions were sequenced to identify the species of the *Bartonella* spp. in the camels from the southern area of Iran. All the readable nucleic acid sequences derived from the PCR amplicons (16S rDNA, *rpoB,* and ITS) in different Iranian camels showed 100% genetic similarity for each molecular markers. Based on the results of the present study on *Bartonella* spp., accession numbers MK358351, MK358352, and MK353805 were provided for *rpoB*, ITS, and 16S rDNA sequences, respectively. Using the NCBI Blastn suite, only the sequences recorded from the first study on camels' *Bartonella,* referred to as "*Bartonella dromedarii*", exactly matched with our sequences (Rasis et al., 2014). Both *rpoB* and ITS sequences of our isolate showed 100% and 95% identities with those of *B. dromedarii* and *Bartonella bovis*, respectively. Also, 16S rDNA sequence of our strain exactly matched with that of *B. bovis* and *B. dromedarii* without variability. Nucleotide difference and the percent identity of our *Bartonella* sp. *rpoB* sequence with related *Bartonella* species are shown in Fig. 1.

3.4. Phylogenetic analysis

RpoB sequence was used to draw the phylogenetic tree of the *Bartonella* spp. in the camels of the southern area of Iran, as shown in Fig. 2.

3.5. Hematological findings and APPs measurement

In order to determine the effects of *Bartonella* spp. infection on different camel hematological factors, like WBC differential count, RBC indices, and acute-phase proteins, we used independent sample *t*-test or Mann-Whitney test. The results of the statistical analysis showed that for some indices, including Hb, MCH, MCHC, RDW, WBC count, and SAA, there was a significant difference between *Bartonella* spp. positive and negative camels ($P < 0.05$) (Tables 1 and 2).

4. Discussion

In this study, the prevalence of *Bartonella* spp. in camels of the southern area of Iran was examined. The present study was the first

Table 2
Mean and SE of total and differential WBC counts in *Bartonella* spp. positive and negative camels. Statistically significant factors ($P < 0.05$) are indicated with superscripted star. The unit for all of the numbers in the table is 10^3/μl.

	WBC*	Neutrophil total	Lymphocyte total	Monocyte total	Eosinophil total	Platelet	Band neutrophil total
Bartonella positive (n = 14)[a]	19.03 ± 1.84	9748.36 ± 1798.28	7750.81 ± 1133.9	364.81 ± 214.49	582.63 ± 180.44	78.23 ± 7.84	327.92 ± 178.02
Control group (n = 75)[b]	15.32 ± 0.81	7610.45 ± 446.1	6141.71 ± 716.77	233.94 ± 59.87	642.77 ± 132.12	88.85 ± 4.63	202.2 ± 43.63
P value	0.029	0.272	0.051	0.963	0.741	0.256	0.878

[a] Four co-infected samples (*Trypanosoma* spp.: 2 samples, hemotropic *Mycoplasma*: 1 sample and *Anaplasma* spp.: 1 sample) were excluded from 18 *Bartonella* positive camels.
[b] These camels were negative for *Bartonella* spp., *Trypanosoma* spp., hemotropic *Mycoplasma* and *Anaplasma* spp.

study to investigate the *Bartonella* spp. in the camels of Iran and the second on *Bartonella* spp. in camels worldwide. The prevalence of *Bartonella* spp. in our study was measured as 17%, which was very close to what was reported in the only previous study done on *Bartonella* spp. by Rasis and colleagues in Israel (Rasis et al., 2014). They found that 9 out of 51 camels (17.6%) were infected with *Bartonella* spp. The similar prevalence of *Bartonella* spp. reported in both Rasis's study and the present study suggests that the prevalence of *Bartonella* spp. infection in camels in the Middle East is around 17–18%, as detected in two different studies in Israel and Iran. In our study, the relationship between *Bartonella* spp. and sex or age in camels was not statistically significant. Other probable risk factors, such as region, season, and, especially, ectoparasites infestation (Ereqat et al., 2016), were not evaluated in our study. Thus, there is a need to evaluate precisely the risk factors of infections in the camels.

The species of *Bartonella* in the camels of Iran was detected as '*Candidatus* B. dromedarii'. In the phylogenetic trees based on *rpoB*, our *Bartonella* strain in dromedary camels grouped with a newly-proposed species, *Bartonella dromedarii*. On the basis of a distinct reservoir and ecological niche, sequence analyses, and the expression of flagella, Rasis designated these isolates as a novel *Bartonella* species and named them *Bartonella dromedarii* (Rasis et al., 2014). In accordance with the findings of Rasis's study, our findings confirmed that the *Bartonella* spp. of camels were genetically different from *B. bovis* and had to be recorded as a novel *Bartonella* species. The phylogenetic tree for *rpoB* gene showed that *Bartonella bovis* was the nearest fully characterized species to our strain, and all the dromedary *Bartonella* spp. strain comprised a distinct monophyletic group different from *B. bovis* (bootstrap test = 95%) (Fig. 2). As expected, additional ruminant-associated *Bartonella* spp. (*B. capreoli, B. schoenbuchensis, B. chomelii*, and *B. melophagi*) were clustered together in the adjacent sister clade, which suggests a common evolutionary ancestor to the *Bartonella* spp. that infects ruminants (bootstrap test = 99%).

The *Bartonella* species are grouped into different lineages according to the reservoir host and different virulence genes (*VirB, Vbh,* and *Trw*) (Supplementary data, Table 1). As our *Bartonella* spp. strain of the camel was grouped into ruminant *Bartonella* species, it is expected that it belongs to lineage 2. It is now necessary that researchers investigate the existence of different virulence genes (*VirB, Vbh,* and *Trw*) in the *Bartonella* spp. of camels and, more importantly, clarify the ability of *Bartonella dromedarii* to infect and cause diseases in humans.

According to the results of the present study, *Bartonella* spp. positive camels, in comparison with the *Bartonella* spp. negative camels, showed a significant increase in WBC and SAA. Hematological changes and APPs (as an indicator of inflammation) had an interrelated pathophysiologic condition and influenced each other on different levels. It should be noted that these results are in line with those of previous studies (El-Deeb and Buczinski, 2015), which proposed SAA as a superior biomarker compared to other APPs.

The significant increase of WBC and SAA in the serum of *Bartonella* spp. of the infected camels of the present study suggests an immune system response to the established bacterial infection in this host. Based on the hematological findings, mild anemia (decreased Hb concentration) was detected in infected animals as a consequence of established infection. Several mechanisms have been identified to play a role in the development of anemia during *Bartonella* spp. infection (Hendrix, 2000). Previous reports implied that the lifespan of the RBCs infected by *B. bacilliformis* was greatly shortened and these RBCs were eliminated from the circulation. Also, the mechanical fragility of the RBC increased in infected camels. The amount of sequestration of RBC by the spleen and liver increased as well. Moreover, an increase was observed in the products of the catabolism of hemoglobin in all the patients studied. The free protoporphyrin levels within the RBCs increased, indicating that there was some interference in the synthesis of hemoglobin (Slater and Welch, 2002). Likewise, in the present study, mild normocytic hypochromic anemia was noticed in the infected populations of camels. The last-mentioned mechanisms may be responsible for the RBCs hypochromia and the decrease in MCH and MCHC indices of the erythrocyte.

Although infected samples with three different pathogens other than *Bartonella* spp. were detected and eliminated from the statistical analysis, conducting an experimental study to determine definitively the effect of specific *Bartonella* sp. infection on the hematological factors and APPs alteration in camels is strongly recommended.

5. Conclusion

We should emphasize that the *Bartonella* sp. of camel is a new species. The genetic characteristics of '*Candidatus* Bartonella dromedarii' are based on16S rDNA, *rpoB,* and ITS molecular markers. The moderate prevalence of '*Candidatus* Bartonella dromedarii' in camels of the southern area of Iran, close contacts between humans and camels, and the zoonosis potential of *Bartonella* spp. indicate the need for further studies on '*Candidatus* Bartonella dromedarii'. More studies are required to examine the existence of flagella and different virulence genes so that we can exactly group *Candidatus* Bartonella dromedarii. Also, its pathogenesis in camels, its routes of transmission, and its zoonosis ability should be investigated in future research. Finally, more studies are needed to determine the epidemiology of the *Bartonella* spp. in camels worldwide and the risk factors involved.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.108404.

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