Morphological and Molecular Investigation of *Anaplasma* Infection in Dromedary Camel (*Camelus dromedarius*) in Bushehr Province, Iran

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**Abstract**

**BACKGROUND:** Anaplasmosis is caused by an obligate intracellular, gram-negative microorganism, which belongs to the family Anaplasmataceae and can be transmitted by ticks and other arthropods.

**OBJECTIVES:** The present study aimed to investigate the status of *Anaplasma* spp. infection by microscopy and molecular methods in dromedary camels in Bushehr province, Iran.

**METHODS:** A total of 139 blood samples were collected from dromedary camels in Bushehr province. Giemsa staining and nested-polymerase chain reaction (PCR) were conducted to detect *Anaplasma* infection in the dromedary camels.

**RESULTS:** We found that 27 (19.4%) out of the total 139 blood samples were suspected for the presence of *Anaplasma* spp. by morphological study. The PCR and nested-PCR sequencing results showed 111 (80%) and 134 (96%) samples positive for *Anaplasma* spp. and BLAST search in NCBI GenBank presented 100% identity with *Candidatus Anaplasma camelii*.

**CONCLUSIONS:** The molecular results presented the high frequency of *Candidatus Anaplasma camelii* in camels, in Bushehr city.

**KEYWORDS:** Anaplasma, *Candidatus Anaplasma camelii*, Dromedary camel, Molecular study, Nested-PCR

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Investigation of Anaplasma in Camel in Iran

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Introduction

*Anaplasma* spp. are obligate intracellular organisms that cause anaplasmosis in animals and humans. The members of the *Anaplasmataceae* family can be transmitted by ticks and other arthropods. The genus *Anaplasma* entails diverse species, such as *A. marginale*, *A. centrale*, *A. phagocytophilum*, *A. bovis*, *A. ovis*, and *A. platys* (Rymaszewska et al., 2008). Up to now, *A. phagocytophilum*, *A. ovis*, and newly diagnosed *Candidatus Anaplasma camelli* (genetically close to *A. platys*) have been detected in camels by molecular methods (Bahrami et al., 2018; Bastos et al., 2015; Li et al., 2015; Noaman, 2018).

*A. phagocytophilum* propagates in polymorphonuclear leukocytes causing granulocytic anaplasmosis in humans, tick-borne fever in ruminants, in addition to canine and equine granulocytic anaplasmosis (Rymaszewska et al., 2008). *A. ovis* is an intraerythrocytic pathogen of small ruminants (de la Fuente et al., 2004). *A. platys* has been reported as an intraerythrocytic rickettsia that causes canine cyclic thrombocytopenia with different clinical symptoms, including fever, anorexia, lethargy, respiratory distress, mucous hypersecretion, purulent ocular discharge, splenomegaly, and muzzle hyperkeratosis (Sainz et al., 1999).

Bastos et al. (2015) reported a new species close to *A. platys* in Saudi Arabia and named it *Candidatus Anaplasma camelli*. Limited information is available concerning the presence of *Anaplasma* species in Iranian dromedaries. A microscopic study demonstrated *Anaplasma* spp. infection in camels in Iran (Ghazvinian et al., 2017). Some molecular evaluations have revealed *A. phagocytophilum*, *A. ovis*, and *Candidatus A. camelli* in Iranian camels (Bahrani et al., 2018; Noaman, 2018; Sharifiyazdi et al., 2017).

Bushahr province is one of the main camel breeding areas in Iran. Therefore, we investigated the presence of *Anaplasma* spp. in the dromedarian camels in Bushehr province, Iran.

Materials and Methods

Sampling and Morphological Investigation

Blood samples were collected from 139 healthy or anemic dromedaries throughout Bushehr province with a mean annual rainfall of 237-350 mm in the South of Iran between 28° 58' 59.99" N latitude and 50° 48' 59.99" E longitude. A thin layer of blood was spread on a clean dry microscopic glass slide, allowed to air dry, and stained by Giemsa staining. In summary, the smears were immersed in pure methanol for fixation and allowed to air dry for 30 sec. The slide was flooded with 10% Giemsa stain solution for 45 min.

DNA Extraction and PCR

Total DNA was extracted from the blood samples employing a DNA extraction kit (MBST, Tehran, Iran) according to the manufacturer’s instructions. The DNA extraction was evaluated using common primer pairs (camel-act F: 5'-tacctagatgccctggtgg-3' and camel-act R: 5'-ggtctctgtaagagctcctaatg-3' derived from the β actin gene of camels. Nested polymerase chain reaction (PCR) was performed according to the method of Noman (Noaman et al., 2009). Briefly, the samples infected with *Anaplasma* were assessed for the presence of the 16S rRNA gene of this rickettsia by PCR.

Two sets of primers were designed based on the 16S rRNA gene of *Anaplasma* (M60313). The first DNA amplification was carried out using primers F1 (5'-agatgttagctcaagctctag-3') and R1 (5'-agacg-catgtaatcagctc-3') of 16S rRNA sequences. To control the specificity of the PCR products for the 16S rRNA gene of *Anaplasma* spp., nested PCR was applied in which the additional primers F2 (5'-geaatggttagctcagcctaat-3') and R2 (5'-gttgaacectcggttattacct-3') belonged to the same gene.

In PCR and nested PCR, about 20 ng of DNA solution was utilized in a total volume of 100 μL, including 10x PCR buffer, 2.5 U Taq DNA polymerase (Sinaclon, Iran), 20 μM of each primer (Sinaclon, Iran), 100 μM dNTPs (Fermentas), and 1.5 mM MgCl2 (Sinaclon, Iran). The reaction was completed in a thermal cycler (Bio-Rad) with the following program: 5 min incubation at 95°C followed by 35 cycles of 45 sec at 95°C (denaturation), 45 sec at 59°C or 55°C (annealing), 45 sec at 72°C (extension), and a final extension at 72°C for 5 min. Positive (available from previous work) and negative controls were used in each PCR. The annealing temperature for PCR using primers derived from the β-
The β-actin gene of camelids was 50°C. Next, the PCR products were electrophoresed on 1.5% agarose gel, stained with Cybersafe, and visualized under UV light. Purified nested PCR products were sequenced by Kowsar Company (Tehran, Iran). The resultant nucleotide sequences were analyzed by the basic local alignment search tool (BLAST) on the National Center for Biotechnology Information (NCBI) database website (http://www.ncbi.nlm.nih.gov/blast). A phylogenetic tree was created with MEGA 6 software (USA) applying the maximum likelihood method with bootstrap analysis (1,000 replicates).

Results

Anaplasma spp. was detected in 27 (19.4%) of 139 blood smears by microscopy. For molecular characterization, first, the efficient isolation of DNA was ensured from each sample using the specific primers designed from the β-actin gene of camelids. Each sample was analyzed by PCR and nested PCR by two pairs of primers designed based on 16S rRNA and sequencing in the presence of Anaplasma spp. The PCR product was observed at 781 bp in PCR and 543 bp in nested PCR (Figure 1). In the present study, Anaplasma spp. infection was detected in 111 (80%) specimens by PCR and in 134 (96%) cases by nested PCR. The similarity between all the sequenced Anaplasma spp. was identified to be 100% with Candidatus A. camelii from Iran (KX765882) and Saudi Arabia (KF843825.1-KF843823). The phylogenetic tree represented two major branches for Anaplasma spp., one of which contained Candidatus A. camelii and A. platys and the other included other Anaplasma spp. Wolbachia was in a separate branch (Figure 2).

![Figure 1](image1.png)

**Figure 1.** PCR products and nested-PCR products used to detect Anaplasma. Lane 1= Nested-PCR product; Lane 2= positive control; Lane 3= DNA marker; Lane 4, 5= PCR product; Lane 6= positive control; Lane 7= negative control.

![Figure 2](image2.png)

**Figure 2.** Molecular Phylogenetic analysis based on the 16S rDNA gene of Anaplasma obtained from dromedary camel in Bushehr province (showed with ●) by using Maximum Likelihood method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Evolutionary analyses were conducted in MEGA6.
Discussion

Anaplasmosis in camels is more commonly referred to as a subclinical disease and sometimes has symptoms, such as weakness, anorexia, and anemia. In the current investigation, microscopy and molecular techniques were used to detect *Anaplasma* spp. in dromedary camels in Bushehr province, Iran.

We determined the frequency of *Anaplasma* spp. microscopically in 19.4% of blood smears. The frequency of *Anaplasma* spp. infection using microscopy method has been reported as, 40.5% in Saudi Arabia (Ismael et al., 2016), 17% in Tunisia (Ismael et al., 2016; Selmi et al., 2019), and 17.4% in Iran (Ghazvinian et al., 2017). Some other studies did not detect *Anaplasma* in blood smears in Iran (Sazmand et al., 2019; Sharifiyazdi et al., 2017) and China (Li et al., 2015). The discrepancy in the results may be attributed to climatic conditions or misdiagnosis with Howell-Jolly body or other pathogens, such as *Mycoplasma*. Considering the similarity of *Anaplasma* spp. to Howell-Jolly body or other pathogens that lead to misdiagnosis, microscopic examination is not an appropriate method especially for the detection of reservoir animals.

Due to the disadvantages of microscopic techniques, the best method of diagnosis is molecular methods. The 16S rRNA gene with a high copy number was found to be convenient and appropriate for the detection of *Anaplasma* spp. even in small amounts. The molecular examinations (PCR and nested PCR) showed that 80% and 96% of camels were infected with *Anaplasma* species. Comparison between the results of microscopy and molecular methods revealed that the microscopic method was not enough sensitive (19.4% vs. 96% infection rate).

Similar molecular studies indicated the infection rate of *Anaplasma* spp. in camels as 15% and 6% in Iran (Sazmand et al., 2019; Sharifiyazdi et al., 2017), 30% in Saudi Arabia (Bastos et al., 2015), 7.2% in China (Li et al., 2015), 17.7% in Tunisia (Belkahia et al., 2015), 39.62% in Morocco (Lbacha et al., 2017), 13.3% in Pakistan (Azmat et al., 2018), and 68.67% in Kenya (Kidambasi et al., 2019). The infection rate of *Anaplasma* in the present study was higher than previous reports from Iran (Sazmand et al., 2019; Sharifiyazdi et al., 2017) and other parts of the world (Azmat et al., 2018; Bastos et al., 2015; Belkahia et al., 2015; Lbacha et al., 2017; Li et al., 2015). The reason may be that we sampled the camels with the symptoms of anemia.

In this study, sequence analysis showed the highest identity (100%) between our sequences and the *Candidatus A. camelii* sequence from Saudi Arabia. There is a notable nucleotide difference in the hypervariable region of the 16S rDNA gene between the obtained sequence in the present study and *A. platys*. In line with other researchers, we believe that *Candidatus A. camelii* is an independent species. However, more extensive research is required on other *Anaplasma* genes. Genetic analysis of the 16S rDNA gene of dromedary camels in the hypervariable region revealed 100% identity with *Candidatus A. camelii* that was previously reported from Fars province, Iran (Sharifiyazdi et al., 2017) and Saudi Arabia (Bastos et al., 2015).

*A. phagocytophilum* and *A. ovis* were also reported in camels in Iran (Bahrami et al., 2018; Noaman, 2018). A phylogenetic tree of the achieved sequences in the current study with sequences submitted in GenBank revealed two major branches for *Anaplasma* spp. One branch included *Candidatus A. camelii* and *A. platys* and the other entailed other *Anaplasma* spp. Wolbachia was in a separate branch. Li et al. (2015) also generated a separate cluster from *A. platys* and *Candidatus A. camelii* in phylogenetic trees based on 16S rDNA.

Conclusion

The molecular results showed the high frequency of *Candidatus A. camelii* in camels in Bushehr province. Furthermore, molecular examination (96%) demonstrated a higher and more accurate frequency than microscopic examination (19.4%). We believe that *Candidatus A. camelii* is a different species from *A. platys* in dromedarian camels.

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Conflict of Interest

There is no conflict of interest.

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بررسی مورفولوژیک و مولکولی عفونت آنانالاسما در شتر یک‌گوهانه استان بوشهر، ایران

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چکیده
زمینه مطالعه: آنانالاسوموز توسط یک جرم داخل سلولی اجباری گرم منفی متعلق به خانواده آنانالاسمانیا ایجاد می‌شود که می‌تواند توسط کنه و سایر بندپایان منتقل شود.

هدف: هدف مطالعه حاضر تعیین وضعیت آنانالاسما در شترهای یک‌گوهانه استان بوشهر با روش مولکولی و مایکروسکوپی است.

روش کار: در تحقیق حاضر ۱۲۹ نمونه خون از ۱۷۹ شترهای استان بوشهر جمع آوری شد. از آنجایی که در ۱۲۹ نمونه آنانالاسما در شترهای یک‌گوهانه استان بوشهر کننده‌ای محسوس نبود به منظور بررسی حضور عفونت آنانالاسما در شترهای یک‌گوهانه استفاده شد. همچنین نسبت به شناسایی مولکولی گوئن انانالاسما اقدام گردید.

نتایج: در این بررسی در ۲۸ (۱۹.۷٪) نمونه خون از مجموع ۱۲۹ گشتراش خون با روش مایکروسکوپی اجرام آنانالاسما مشاهده گردید. نتایج PCR و جواز آنانالاسما را در ۱۲۴ (۹۶.۷٪) نمونه (۱۷۹) نمونه تایید کرد. نتایج تعیین توالی و اتیوم آنانالاسما ۱۰۰% با نمونه های تایید شده کامبیاوت‌های آنانالاسما کاملاً بود.

نتیجه‌گیری نهایی: نتایج مولکولی نشان داد فراوانی کاندیداتوس آنانالاسما کاملاً در شترهای یک‌گوهانه در شهرستان بوشهر بالا بود.

واژه‌های کلیدی: آنانالاسما، آنانالاسوموزیس، کاندیداتوس آنانالاسما، شتر یک‌گوهانه، مطالعه مولکولی