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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 Anaplasmatacea 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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Introduction

Anaplasma spp. are obligate intracellular organisms that cause anaplasmosis in animals and humans. The members of the *Anaplasmatacea* 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 camelii* (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 leucocytes 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 cameli*. 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. cameli* in Iranian camels (Bahrami *et al.*, 2018; Noaman, 2018; Sharifiyazdi *et al.*, 2017).

Bushehr 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 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′-ttacaatgagctgcgtgtgg-3′ and camel-act R: 5′-gagtccatcacgatgccagt-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′-agagtttgatcctggctcag-3′) and R1 (5′-agcactcatcgtttacagcg-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′-gcaagcttaacacatgcaagtc-3′) and R2 (5′-gttaagccctggtatttcac-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 $MgCl_2$ (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-

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 (Figure1). 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).

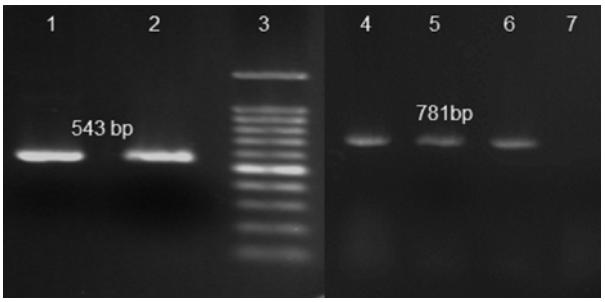

Figure 1. PCR products and nested-PCR products used to detect Anaplasma. Lane 1= Neated-PCR product; Lane 2= positive control; Lane 3= DNA marker; Lane 4, 5= PCR product; Lane 6= positive control; Lane 7= negative control

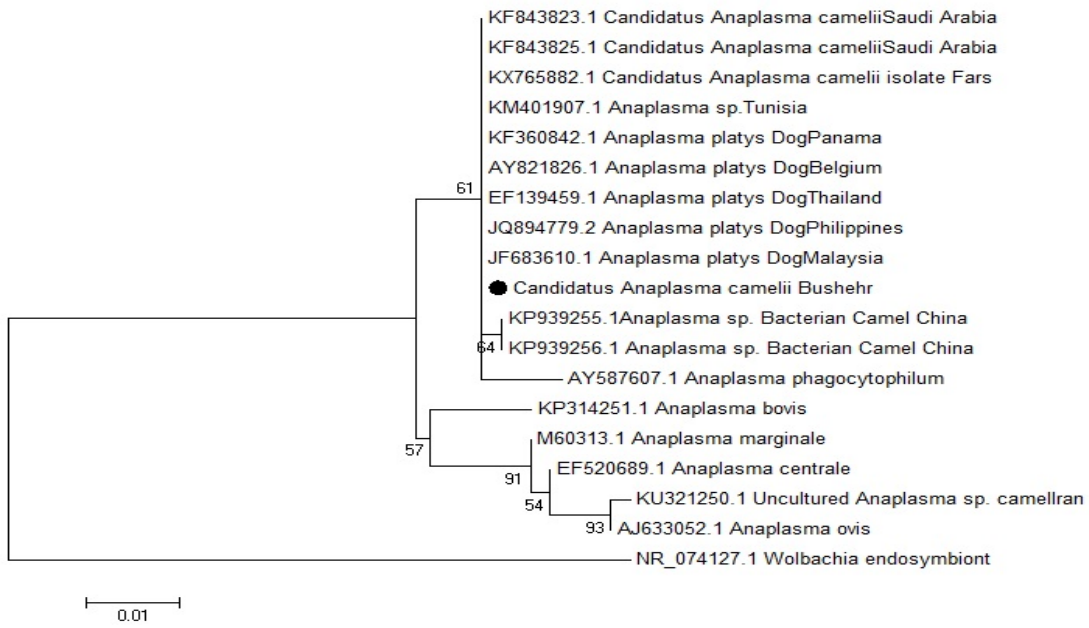

Figure 2. Molecular Phylogenetic analysis based on the 16S rDNA gene of *Anaplasma* obtained from dromedary camel in Bushehr province (showed with ●) by using Maximum Likelihood method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates)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

Acknowledgments

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Conflict of Interest

There is no conflict of interest.

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بررسی مورفولوژیک و مولکولی عفونت آناپلاسما در شتر یک‌کوهانه استان بوشهر، ایران

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چکیده

زمینه مطالعه: آناپلاسموزیس توسط یک جرم داخل سلولی اجباری گرم منفی متعلق به خانواده آناپلاسماتاسهآ ایجاد می‌شود که می‌تواند توسط کنه‌ها و سایر بندپایان منتقل شود.

هدف: هدف مطالعه حاضرتعیین وضعیت آلودگی آناپلاسما در شترهای یک‌کوهانه استان بوشهر با روش مولکولی و میکرسکوپی است.

روش کار: در تحقیق حاضر ۱۳۹ نمونه خون از شترهای استان بوشهر جمع‌آوری شد. رنگ‌آمیزی گیمسا و nested-PCR به‌منظور بررسی حضور عفونت آناپلاسما در شترهای یک‌کوهانه استفاده شد. همچنین نسبت به شناسایی ملکولی گونه انا‌پلاسما اقدام گردید.

نتایج: در این بررسی در۲۷ (۱۹/۴٪) نمونه خون از مجموع ۱۳۹ گسترش خون با روش میکروسکوپی اجرام آناپلاسمایی مشاهده گردید. نتایج *PCR* وجود آلودگی آناپلاسما را در ۱۱۱(۸۰٪) نمونه و نتایج nested-PCR وجود آلودگی را در ۱۳۴ (۹۶٪) نمونه تایید کرد. نتایج تعیین توالی و آنالیز آن نشان‌دهنده شباهت ۱۰۰٪ با نمونه های تعیین توالی‌شده *کاندیداتوس* آناپلاسما کاملی بود.

نتیجه‌گیری نهایی: نتایج مولکولی نشان داد فراوانی کاندیداتوس آناپلاسما کاملی در شترهای یک‌کوهانه در شهرستان بوشهر بالا بود.

واژه‌های کلیدی: آناپلاسما، آناپلاسموزیس، کاندیداتوس *آناپلاسما کملی*، شتر یک‌کوهانه، مطالعه مولکولی

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