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Original Article

Molecular Detection of Novel Genetic Variants Associated to *Anaplasma Ovis* Among Dromedary Camels in Iran

Noaman *, V.

Department of Veterinary Group of Animal Science Research, Isfahan Agricultural and Natural Resources Research and Education Center, Agricultural Research, Education, and Extension Organization, Isfahan, Iran

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Corresponding Author: V.noaman@areeo.ac.ir

ABSTRACT

To the best of our knowledge, little information is available regarding the presence of Anaplasma species in camels in Iran. This study sought to investigate the presence of Anaplasma species by microscopy and polymerase chain reaction (PCR) assays in 100 healthy dromedaries (Camelus dromedarius) arriving for slaughter. The microscopic examination of Giemsa-stained blood films revealed that Anaplasma like structures could be identified in the erythrocytes of two blood smears. To confirm the presence of and to identify the species of Anaplasma spp., a PCR technique was performed using primers amplifying a 750 bp fragment of the 16S rRNA gene of Anaplasma and the PCR products were analyzed by sequencing. The nucleotide sequence was compared to the sequences available in GenBank using the Basic Local Alignment Search Tool (BLAST). According to the results, the sequences of two 16S rRNA PCR products clearly fit within the Anaplasma genus in the family *Anaplas mataceae*. In this study, phylogenetic analysis using the 16S rRNA gene sequences revealed that two sequences obtained from monophyletic clusters included *Anaplasma ovis* (*A. ovis*). The obtained sequences had 99.6-100% similarity with previously published 16S rRNA gene sequences. This study aimed to evaluate the presence of novel genetic variants associated to *A. ovis* in dromedaries in the world. Further studies are recommended to establish the vector(s), as well as the veterinary and medical significance of these apparently novel variants in Iran.

Keywords: *Anaplasma ovis*, one-humped camel, Molecular identification, 16S rRNA gene, Iran

Détection moléculaire de nouvelles variantes génétiques associées à *Anaplasma ovis* chez les dromadaires iraniens (*Camelus dromedarius*)

Résumé: Peu d'informations sont disponibles concernant la prévalence des infections *anaplas miques* chez les Camélidés iraniens. Une centaine de dromadaires (*Camelus dromedarius*) sains, arrivant à l'abattoir pour y être abattus, ont été examinés par microscopie et par des tests de réaction en chaîne de la polymérase (PCR) afin de détecter des contaminations anaplasmiques éventuelles. L'observation microscopique des frottis de sang périphérique colorés au Giemsa a révélé la présence de structures analogues à l'Anaplasma dans deux pourcent des échantillons sanguins. Afin de confirmer ces résultats et d'identifier les espèces d'Anaplasma impliquées, une technique de PCR a été mise au point en utilisant des amorces amplifiant un fragment de 750 pb du gène de l'ARNr 16S de l'Anaplasma. Les produits de cette PCR ont été ensuite analysés par séquençage. La séquence nucléotidique obtenue a été comparée aux séquences disponibles dans GenBank en utilisant le programme BLAST (Basic Local Alignment Search Tool). Ces analyses ont démontré que les séquences de deux produits de PCR ARNr 16S correspondent au genre Anaplasma appartenant à la famille des *Anaplas mataceaes*. L'analyse phylogénétique utilisant les séquences du gène de l'ARNr 16S classait les deux séquences obtenues

dans cette étude dans des groupes monophylétiques comprenant *A. ovis*. Les résultats ont indiqué une similitude de 100 à 99,6% avec les séquences publiées du gène de l'ARNr 16S d'*A. ovis*. La présente étude rapporte la présence de nouvelles variantes génétiques associées à *A. ovis* chez les dromadaires dans le monde. Des recherches supplémentaires sont nécessaires afin d'identifier le ou les vecteur(s) de cette maladie, ainsi que l'impact de ces nouvelles variantes identifiées en Iran aux niveaux vétérinaire, médical et sanitaire.
Mots-clés: *Anaplasma ovis*, dromadaire, identification moléculaire, gène de l'ARNr 16S, Iran

INTRODUCTION

Anaplasmosis is an arthropod-borne disease caused by Anaplasma species that are obligate intracellular parasites (Rickettsiales: *Anaplas mataceae*) (Dumler, 2001). In Iran, molecular methods detected five species of this pathogen in the blood smears of cattle and sheep including *A. marginale*, *A. centrale*, *A. phagocytophilum*, *A. bovis* and *A. ovis* (Noaman, 2013 ;Noaman, 2009; Noaman, 2010; Noaman, 2010 ;Noaman, 2009; Noaman, 2009). *A. marginale* and *A. ovis* are similar in morphology and biology, and they are transmitted by Ixodid ticks. However, they are different in terms of the ability to infect a particular host. *A. marginale* is the primary causative agent of bovine anaplasmosis and is of economic significance in the cattle industry. *A. ovis* causes mild disease in sheep, the infection can cause acute clinical symptoms associated with stressors such as long-distance transportation, animal migration, drought, poor pasture, hot weather, vaccination, deworming, and tick infestation (Renneker, 2013). Several studies reported the co-infection of *A. ovis* with other pathogens among sheep (Anaplasma spp., Theileria spp., and Babesia spp.) (Renneker, 2013; Torina, 2010). Immune deficiency in infected sheep increases the chance of co-infection and mortality. (Kocan, 2005; Renneker, 2013). Additionally, the infection is obviously of public health significance due to its infectivity for man. (Chochlakis, 2010) Reported a human infection from Cyprus confirmed by polymerase chain reaction (PCR) amplification of 16S rRNA genes of Anaplasma spp., major surface protein 4, and heat shock protein 60 (GroEL). Camel is capable of surviving in arid and semi-arid ecosystem because of its unique adaptive characteristics (Schwartz, 1992). In Iran, camel is an important multipurpose animal, which can be used for milk, meat, skin, and transport. The dromedary (*Camelus dromendarius*) with an estimated population of 162,000 is the most prominent camel species in Iran. Generally, there was a constant increase in the population of camels over the last decade. Diseases are the main factors affecting camel production in most of the Middle East countries, which lead to an increased cost of production, lower productivity, and often animal loss. In Iran, camels are usually raised under a free range nomadic management over a vast expanse of uncultivated land with low or no veterinary attention. According to the literature, anaplasmosis is a subclinical infection among dromedaries. So far, limited cases of anaplasmosis in dromedaries, llamas, and bacterians were reported to be infected by A. marginale, A. phagocytophilum, and A. platys (Bastos, 2015; Belkahia, 2015; Ben Said, 2014); Ghafar, 2014; Li, 2015; Sudan, 2014; Wernery, 2014). It is indicated that molecular techniques are sensitive and specific for detecting the Anaplasma spp. (Noaman, 2010). To the best of our knowledge, no study was conducted in Iran to investigate Anaplasma infection among dromedaries (*Camelus dromendarius*). This study aimed to evaluate the presence of Anaplasma spp. in the blood smears of domestic dromedaries in Iran using PCR and sequence analysis.

MATERIALS AND METHODS

This study was carried out in Golestan Province, north-east of Iran. This province, which covers an area

of approximately 20,367 square km, lies between latitude 38.15-36.30°N and longitude 45-56°E. The present study was conducted on 100 randomly selected adult dromedaries brought to the Gorgan abattoir for slaughter over a period of one year. Jugular vein blood samples were taken from all the animals immediately after the point of slaughter. About 5 ml of blood was withdrawn into vacutainers containing disodium-ethylenediaminetetra-acetic acid as an anticoagulant. Thereafter, thin blood smears were prepared, air dried, fixed in methanol, and stained with Giemsa. Microscopic examination was performed using a microscope (Nikon, 1000X, Japan). At least 50 fields per slide were examined for all the obtained smears, and the presence of Anaplasma spp. was recorded. Two blood smears were positive for Anaplasma like structures. DNA extraction was performed only on two blood samples with the presence of Anaplasma spp. in the marginal point of erythrocytes. DNA was extracted using a DNA isolation kit (MBST, Iran) according to the manufacturer's instructions. Briefly, 50 µl of each blood sample was lysed with 180 µl of erythrocyte lysis buffer, and the proteins were degraded by 20 µl of proteinase K for 10 min at the temperatute of 55 °C. After adding 360 µl of binding buffer and incubation for 10 min at 70°C, 270 µl of ethanol (96%) was added to the solution, and after vortexing, the complete volume was transferred to the MBST-column. The solution was first centrifuged and then washed twice with 500 µl of washing buffer. Ultimately, DNA was eluted from the carrier using 100 µl of elution buffer. The amount of extracted DNA and its purity was measured by optical density (OD) 260 and the ratio of OD260 to OD280, respectively. Prior to the study, agarose gel electrophoresis was used to separate the DNA molecules. In addition, PCR was performed using two primer sets, namely, F1 (5'-CTGGCGGCAAGCTT AACACA-3') and EHR16SR (5'-TAGCACTCATCGT TTACAGC-3') in 50 µl volume containing one time PCR buffer, 2.5 U Taq Polymerase (Cinnagen, Iran), 2 µl of each primer (F1/ EHR16SR, 20 µM, Cinnagen), 200 µM of each dATP, dTTP, dCTP, and dGTP (Cinnagen), 1.5 mM MgCl2, and 2.5 µl of extracted DNA (Parola, 2000). DNA amplification was executed in an automated thermocycler (T100 Thermal Cycler, Bio-Rad, USA) with the following PCR program: initial denaturation (five minutes at 95 °C), 40 cycles of denaturation (45 s at 94 °C), annealing (45 s at 55 °C), and extension (1.5 min at 72 °C). Finally, PCR was completed with the additional extension step for 10 min. The PCR products were analyzed on 1.5% agarose gel containing 0.5× Tris-Boric acid- Ethylenediamine tetraacetic acid buffer and visualized by UV-illuminator after staining the gel with ethidium bromide. The amplicon was purified and digested using restriction endonuclease Bst 1107I. Briefly, 10µl of purified PCR product was exposed to 0.1 µl of Bst 1107I (Roche, Germany, 10U/µl) in 2.5 µl of 10× corresponding buffer and 12.5 µl H2O for one hour at 37 °C. About 10 µl of PCR products that was treated with 2.5 µl of 10× corresponding buffer and 12.5 µl H2O was used as the control solution. The restriction endonuclease Bst 1107I recognizes the sequence GTATAC in corresponding PCR product of A. marginale and cut it in the position 68 bp. However, the used restriction enzyme cannot cut the corresponding PCR product of A. ovis (GTACGC) (Noaman, 2013; Noaman, 2010). To confirm the results, the PCR products were purified using PCR Product Purification Kit (MBST, Iran) and sent to Pishgam Biotech Company (Tehran, Iran) for direct sequence analysis. The obtained sequence was compared with available sequences in GenBank using the nucleotide Basic Local Alignment Search Tool (BLAST). Phylogenetic analysis was performed using a neighbor-joining maximum-likelihood method in the Molecular Evolutionary Genetics Analysis software, version 5.05 (Tamura, 2011). The tree stabilities were estimated by bootstrap analysis for 1000 replications.

Table 1. 16S rRNA sequence GenBank

Species	GenBank accession numbers	% 16S rRNA gene similarity	
		KT601343	KU321250
A. ovis partial sequence	AF318945	99.6	100
A. ovis isolate CGX	EF587237	99.6	100
A. ovis isolate LYG495 partial sequence	JQ917886	99.6	100
A. ovis strain Ao499 partial sequence	KJ639880	99.6	100
A. ovis isolate Zhangjiachuan	AJ633051	99.4	99.8
A. centrale from Japan	AF318944	98.9	99.3
A. centrale from South Africa	AF414869	98.9	99.3
A. centrale strain Israel vaccine	AF309869	98.9	99.3
A. centrale strain vaccine from Australia	AF414868	98.9	99.3
A. marginale 16S strain Virginia	AF311303	98.6	99
A. marginale strain F12 from Australia	AF414874	98.6	99
A. marginale strain Florida	AF309867	98.6	99
A. marginale strain South Idaho	AF309868	98.6	99
A. marginale strain Veld from South Africa	AF414873	98.6	99
A. marginale strain Virginia	AF309866	98.6	99
A. marginale from Uruguay	AF414877	98.5	98.9
A. marginale	M60313	98.5	98.9
A. centrale Amori Japan	AF283007	97	97.4
A. bovis	U03775	95.3	95.7
A. phagocytophila partial sequence	M73220	94.9	95.3
A. phagocytophila	M73224	94.9	95.3
A. platys isolate Okinawa partial sequence	AY077619	94.9	95.3
A. platys isolate A.pl.87 partial sequence	JQ396431	91.6	92
New Anaplasma spp. Iran camel	KT601343	100	99.6
New Anaplasma spp. Iran camel	KU321250	99.6	100

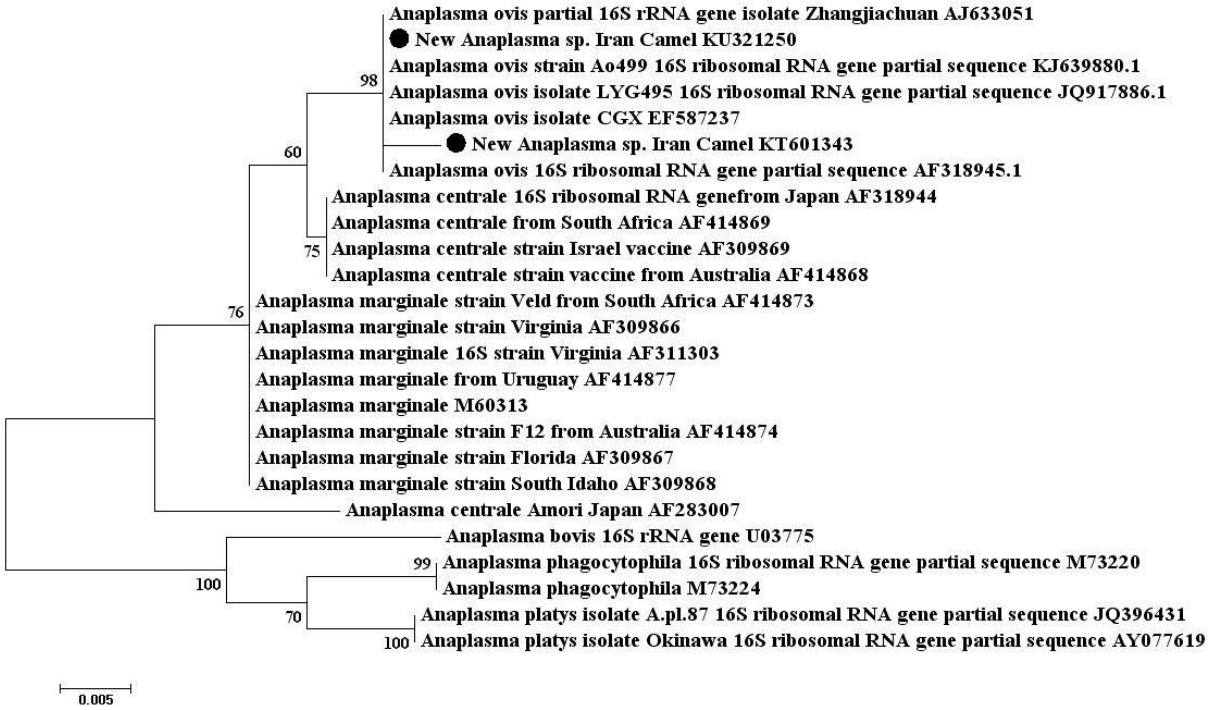

Figure 3. Neighbor-joining phylogenetic tree based on Anaplasma partial 16S rRNA nucleotide sequences. Sequences included in the analysis are indicated with species and GenBank accession number. Sequences newly identified in the present study are indicated in black circle. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. The evolutionary distances were computed using the maximum-composite-likelihood method. All positions containing gaps and missing data were eliminated. There were a total of 750 positions in the final data set.

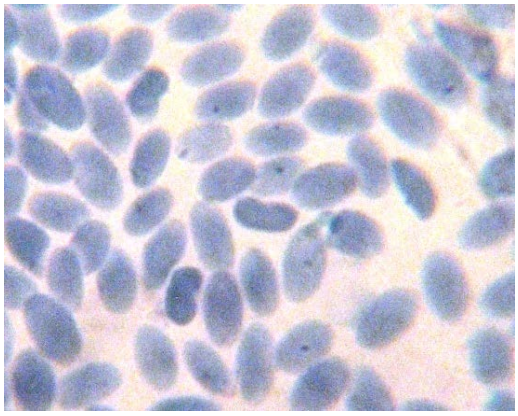

Figure 1. Giemsa-stained blood smear of camel demonstrating Anaplasma like structures in the erythrocytes.

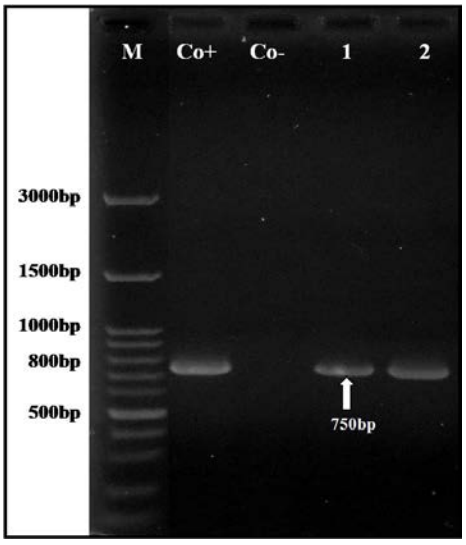

Figure 2. Agarose gel electrophoresis of polymerase chain reaction products obtained by the amplification of 16S rRNA gene. The expected size (~750 bp) is indicated (lanes 1 and 2). Co+ and Co- are positive and negative controls, respectively. M= Marker

GenBank 16S rRNA gene sequences and accession numbers were used to construct a phylogenetic tree and to calculate percent identities. These sequences can be retrieved under the accession numbers of AJ633051, KJ639880, JQ917886, EF587237, and AF318945 for *A. ovis*, AF318944, AF414869, AF309869, AF414868, and AF283007 for *A. centrale*, AF414873, AF309866, AF311303, AF414877, M60313, AF414874, AF309867, and AF309868 for *A. marginale*, U03775 for *A. bovis*, M73220 and M73224 for *A. phagocytophilum* and JQ396431 and AY077619 for *A. platys*. Pairwise/multiple sequence alignments and sequence identities were computed using Identity Matrix option available in BioEdit software.

RESULTS

In this study, it was indicated by microscopy technique that two thin blood smears were positive for Anaplasma spp. (Figure 1). Additionally, PCR method and agarose gel electrophoresis confirmed the mentioned result by demonstrating the expected 750 bp band (Figure 2). Fragment length polymorphism (RFLP) method and was employed to distinguish A. marginale by using restriction endonuclease Bst1107I. This enzyme recognizes the GTATAC sequence that is common among multiple *A. marginale* strains. Nevertheless, it could not cut the PCR products of 16S rRNA gene of Anaplasma spp. The sequences of the PCR products were submitted to GenBank database and can be retrieved under the accession numbers of KT601343 and KU321250. The two sequences obtained from two camels showed 99.6% similarity to each other. The comparison of the sequences indicated that these two sequences were 91-100% identical to various sequences of the Anaplasma spp. 16S rRNA sequence was deposited in GenBank with accession numbers of KU321250, AF318945, EF587237, JQ917886, KJ639880, and AJ633051 exhibited the greatest similarity with *A. ovis* (Table 1). The sequence similarity of KU321250 and KT601343 with *A. ovis* 16S rRNA variants that have been deposited in GenBank (AF318945, EF587237, JQ917886, KJ639880 and AJ633051) was 100%-99.8% and 99.6%-99.4%, respectively. Sequence similarities with other species of the genus were lower than 99.4 %. The results of the comparison of nucleotide sequences between 16S rRNA genes of new Anaplasma agents and other Anaplasma agents is shown in Figure 3. Phylogenetic analysis demonstrated that these two new variants are clustered in the *A. ovis* clade.

DISCUSSION

The taxonomic classification of Anaplasma spp. is difficult due to the lack of a simple in vitro bacterial culturing technique, the absence of a suitable laboratory animal model, and the inability to microscopically identify the isolates (Lew, 2003 #9). 16S rRNA gene-based PCR is an invaluable method in detecting the pathogenic microorganisms that are difficult to isolate from specimen and grow in the laboratory. This technique is widely used to identify newly discovered bacteria and evaluate the existing taxonomy (Dumler, 2001). The 16S rRNA gene is less variable compared to other genes, which lead to high similarities among closely related species (Noaman, 2010). The 16S rRNA gene is considered as a sensitive molecular tool for the discrimination of Anaplasma species in phylogenic studies (Dumler, 2001; Lew, 2003). In this study, the partial amplification of 16S rRNA gene of two blood smears was successful. According to the results of BLASTn searches, the obtained sequences were similar to the published *A. ovis* 16S rRNA gene sequences (accession numbers of AF318945, EF587237, JQ917886, KJ639880, and AJ633051), which were previously obtained from small ruminants in Mozambique and small ruminants, ticks, and cervidae in china. Regarding the literature, *A. ovis* was considered to be moderately pathogenic in small ruminants but not in camels. The observed sequence similarities were confirmed by phylogenetic analysis. The phylogenetic trees inferred from the partial 16S rRNA gene clustered into six groups representing *A. ovis*, *A. centrale*, *A. marginale*, *A. bovis*, *A. phagocytophilum*, and *A. platys*. Moreover, this systematic division was confirmed by the biological and antigenic analyses of each species (Dumler, 2001). One of the obtained sequences (KU321250) was 100% identical to the published *A. ovis* 16S rRNA sequences (AF318945, EF587237, JQ917886, and KJ639880). The document is the first report of camel anaplasmosis by *A. ovis* in the world. *A. ovis* infections in small ruminants have been reported to be an endemic infection in Europe, Africa, and Asia. In addition, this infection was observed in the small ruminants of Iran and its neighbors such as Iraq and Turkey (Noaman, 2009; Renneker, 2013). Globally, there are several reports demonstrating the infection of *A. marginale*, A. phagocytophilum, and *A. platys* in dromedary camels. However, there was no evidence of A. ovis infection in these animals (Bastos, 2015; Belkahia, 2015; Ben Said, 2014; Sudan, 2014). Infection with A. ovis is frequently subclinical, nevertheless it may cause disease, which is more severe among goats than sheep. Furthermore, this disease is more prevalent among stressed or debilitated animals (Renneker, 2013). In addition, the infection with A. ovis may predispose animals to other infectious diseases that aggravate the condition of the animal and can lead to mortality (Kocan, 2005). Consistent with the studies conducted by (Bastos, 2015; Belkahia, 2015 ; Ben Said, 2014), in this study, the selected camels appeared healthy and had no apparent clinical manifestations.

According to the results of the current study, there were novel genetic variants associated with *A. ovis* in dromedary camels in the world. However, it should be pointed out that the molecular diagnosis was performed only on the two blood samples with the presence of Anaplasma spp. in the marginal point of erythrocytes. Therefore, it was difficult to draw any conclusions about the source of the infection. Various factors such as climate, host abundance, tick hosts, diversity, and topography affect the epidemiology of A. ovis. Further studies are recommended to investigate the range, prevalence, transmission route, vector(s), and clinical impact, as well as the veterinary and medical significance of these apparently novel species.

Ethics

I hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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