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Molecular detection of *Candidatus* Anaplasma camelii in camels (*Camelus dromedarius*) from Asir Province, Saudi Arabia

Alshahrani, M.Y.[1], Alanazi, A.D.[2*], Alouffi, A.S.[3], Abdullah, H.H.A.M.[4], Allam, A.M.[4], Mahmoud, M.S.[4], Abdel-Shafy, S.[4], Alfaifi, M.H.[1] and Alkhathami, A.G.[1]
[1]Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, King Khalid University, P.O. Box 61413, Abha, 9088, Saudi Arabia
[2]Department of Biological Sciences, Faculty of Science and Humanities, Shaqra University, P.O. Box 1040, Ad-Dawadimi 11911, Saudi Arabia
[3]King Abdulaziz City for Science and Technology, Riyadh, Saudi Arabia
[4]Department of Parasitology and Animal Diseases, Veterinary Research Division, National Research Centre, 33 Bohouth St., Dokki, 12622 Giza, Egypt
[*]Corresponding author e-mail: aalanazi@su.edu.sa
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Abstract. Knowledge of molecular identification of tick-borne pathogens in camels in Saudi Arabia is very limited; few molecular epidemiological studies have been under taken. This study was to detect *Anaplasma* spp. and *Piroplasma* spp. in camels from Asir Province, Saudi Arabia. A total of 150 blood samples were collected from camels in Asir Province and investigated by polymerase chain reaction (PCR) that targeted 18S rRNA and 23S rRNA to detect the DNA of *Piroplasma* spp. and *Anaplasma* spp., respectively. The positive samples for 23S rRNA were assayed again by PCR targeting the 16S rRNA. All the blood samples were free from *Piroplasma* spp. infection. Three camels (2%) were found to be positive for *Anaplasma* infection through use of PCR that targeted the 23S rRNA gene. There were no significant differences between ages or sexes in the camels that tested positive for *Anaplasma*. All positive *Anaplasma* infections were recorded in camels that were infested by ticks. Two *Anaplasma* sequences for the16S rRNA gene were deposited in GenBank with accession numbers MN882724 and MN882725. They recorded 99.16% and 99.34% similarities (respectively) with KF843825.1 (*Candidatus* Anaplasma camelii reported in Unizah, Saudi Arabia). Phylogenetic analyses revealed that the two sequences recorded in this study were close to each other; both were located in one cluster with *Candidatus* Anaplasma camelii isolates that were recorded before in the adjacent areas of Unizah in Saudi Arabia and Iran. In conclusion: two new *Anaplasma* genotypes close to *Candidatus* Anaplasma camelii were found in camels in Asir Province, Saudi Arabia for the first time. The camels in this province were found to be free of *Piroplasma* infection.

INTRODUCTION

Arthropods such as ticks, fleas and mosquitoes are globally important vectors of a wide range of viral, bacterial and protozoal pathogens that result in a wide range of vector-borne diseases (Otranto and Dantas-Torres, 2010). Vector-borne diseases are both emerging and re-emerging, and they pose a direct threat to human health and animal welfare (Yuasa *et al.*, 2012; de La Fuente *et al.*, 2017). In addition to acting as vectors, ticks also affect the well being of their hosts directly through irritating bites, blood loss, damage to the skin and anorexia that leads to reduced growth (Jabbar *et al.*, 2015).

Piroplasmosis and Anaplasmosis are tick-borne diseases that are globally distributed and negatively affect animal production (de La Fuente *et al.*, 2017). These diseases infect a wide range of animals,

especially ruminants, which include camels. The diseases cause fever, anaemia, weakness, lethargy and death in acute cases (Otranto and Dantas-Torres, 2010).

In Saudi Arabia, the livestock sector in 2015 contributed 2.7% to the country's Gross Domestic Product (GDP) (General Authority for Statistics, 2015). Saudi Arabia is listed among the countries that show strong recent growth in the camel population; in 2016, the country had a population of approximately one million camels, with the highest percentage in Riyadh Province (General Authority for Statistics, 2016). In addition, thousands of live camels are imported annually from neighbouring countries such as Somalia, Egypt, Sudan and Arabian Gulf countries (General Authority for Statistics, 2016). Many rural communities are dependent on these animal species for their livelihoods, since camels and cattle are a good source of meat, milk and leather (Elzaki *et al.*, 2018; Redwan *et al.*, 2018).

Camel production is severely affected by various diseases and in adequate veterinary services (Bekele, 2010). Several endo-and ectoparasites affect their health, productivity and performance, including ticks (Bekele, 2010). The feeding activity of ticks causes blood loss and anaemia, and the ticks transmit various disease pathogens to humans (Orkun *et al.*, 2014). Ticks can cause irritation, inflammation, hyper-sensitivity and damage to the animal hides, leading to production losses (Wall and Shearer, 2001).

Previous studies on ticks in Saudi Arabia have shown that there are more than 20 ixodid species and subspecies that infest domestic animals including camels (Hoogstraal *et al.*, 1981; Al-Khalifa and Diab, 1986, Alanazi *et al.*, 2019). However, knowledge of molecular identification of tick-borne pathogens in domestic animals including camels in Saudi Arabia is very limited, since few molecular epidemiological studies have been undertaken (Alanazi *et al.*, 2014; Ismael *et al.*, 2014; Mohammed *et al.*, 2017; Alanazi *et al.*, 2018; Salim *et al.*, 2019; Alanazi *et al.*, 2020). Therefore, the aim of this study was to detect *Anaplasma* spp. and *Piroplasma* spp. in camels from Asir Province, Saudi Arabia.

MATERIALS AND METHODS

Ethical approval

This study was revised and approved by the Ethical Research Committee, Deanship of Scientific Research, King Khalid University, according to the ethical principles of human and animal research (Approval no. (ECM#2019-74)–(HAPO-06-B-001).

Study site

Asir Province has an area of 76,690 km^2 and is located in the southwestern part of Saudi Arabia between 19°02N and 43°02E (Figure 1). The province is situated on a high plateau that receives more rainfall than the rest of the country and contains the country's highest peaks, which rise to almost 3,000 m. Asir has a tropical and subtropical climate and the average annual rainfall in the highlands ranges from 300 ml to 500 ml. It has two rainy seasons. As a result, there is much more natural vegetation and forests than the rest of Saudi Arabia (http://www.pme.gov.sa).

Collection of camel blood samples and DNA isolations

The cross-sectional study was conducted from August 2019 to Junary 2020 in Asir Province of Saudi Arabia. Blood samples were collected from 150 camels of both sexes and age groups; camels were selected at random. The camels ranged in age from <1 year to >15 years. Blood samples were collected from each animal (2–8 ml) from the cephalic vein into vacutainer tubes with EDTA (BD Vacutainer® Tube, Gribbles Pathology, VIC, Australia) and transported in an ice box to the parasitology laboratory at the Department of Biological Sciences, Faculty of Science and Humanities, Shaqra University, for DNA extraction. Total genomic DNA (gDNA) was isolated from the blood samples using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) and eluted in 50 µl or 100 µl of elution buffer

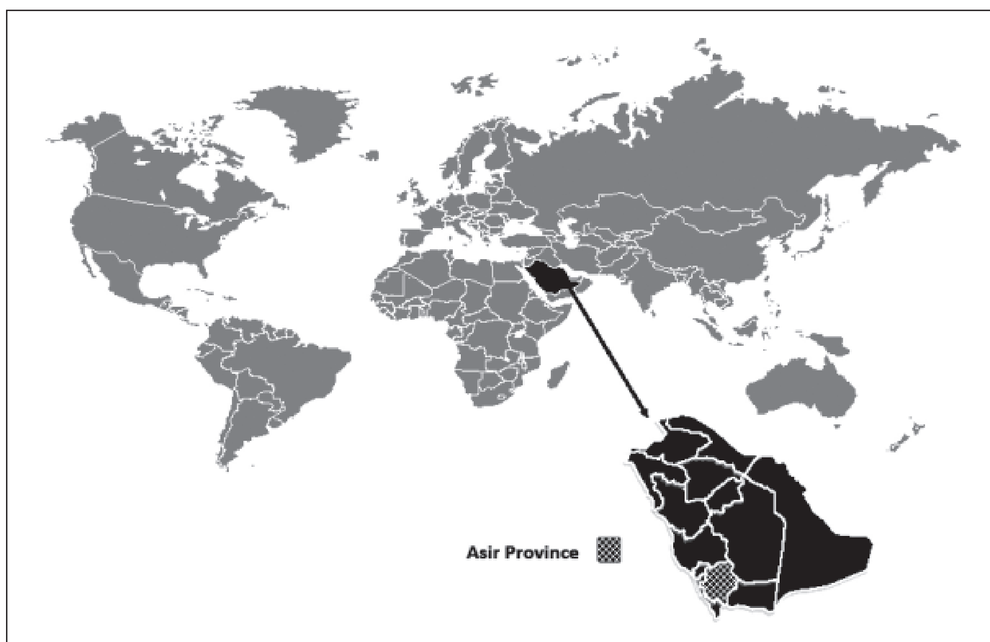

Figure 1. Map showing the location of the study site in Saudi Arabia.

Table 1. Oligonucleotide sequences of primers used for PCR and sequencing in this study

Parasite	Targeted gene	Name	Primers	Fragment length (bp)	Reference
Piroplasma	18S RNA	piro18S-F1	GCGAATGGCTCATTAIAACA	1100	Dahmana *et al.*, 2019
		piro18S-F4	CACATCTAAGGAAGGCAGCA		
Anaplasmataceae	23S RNA	Ana23S-212f	ATAAGCTGCGGGGAGTTGTC	500	Dahmani *et al.*, 2017
		Ana23S-753r	TGCAAAAGGTACGCTGTCAC		
	16S rRNA	ECB	CGTATTACCGCGGCTGCTGGCA[a]	500	Rufino *et al.*, 2013
		ECC	AGAACGAACGCTGGCGGCAAGC[a]		

as per the manufacturer's instruction. An aliquot of between 50 µl and 100 µl of gDNA from each sample was stored at –20°C prior to molecular analysis.

PCR protocol

The primers were designed to targeta 969 bp section of the conserved region that encoded for the18S rRNA gene and a 485 bp section that encoded for the 23S rRNA gene to detect the DNA of *Piroplasma* (Dahmana *et al.*, 2019) and *Anaplasma* (Dahmani *et al.*, 2017) respectively (Table 1). The positive samples for *Anaplasma* were assayed again by polymerase chain reaction (PCR), targeting the 16S rRNA gene (Rufino *et al.*, 2013). PCR assays were performed in automated BIO-RAD Thermal Cycler (BIO-RAD, Singapore) using One PCR master mix™ (GeneDireX, Taiwan). The PCR conditions for the *Piroplasma* amplification were: one incubation step at 95°C for 15 min; 35 cycles of 1 min at 95°C; 30 s of annealing at 58°C and 1 min at 72°C; followed by a final

extension for 5 min at 72°C. Positive and negative controls were included. The PCR conditions for *Anaplasma* amplification that targeted the 23S rRNA gene were: an initial denaturation step at 95°C for 15 min; followed by 40 cycles that consisted of 1 min denaturation at 95°C, 1 min annealing at 55°C, a 1 min extension at 72°C and a final extension cycle at 72°C for 7 min; and the reactions were cooled at 15°C. The PCR conditions of *Anaplasma* amplification targeting the 16S rRNA gene were: initial denaturation at 95°C for 5 min; followed by 35 cycles of denaturation at 95°C for 30 s; annealing and extension at 65°C for 30 s; 10 cycles of 62°C for 30 s; and final extension at 72°C for 30 s. After the last cycle, the extension step was continued for a further 5 min. Distilled water was used as the negative control. Positive controls were the DNA of *Theileria annulata* extracted from cattle and *Anaplasma marginale* extracted from cattle for PCR assays of *Piroplasma* and *Anaplasma*, respectively. Then, the amplifications were confirmed by electrophoresis on a 1.5% agarose gel, stained with Red Safe and examined by UV transillumination. A DNA molecular-weight marker (100bp DNA Ladder H3 RTU, GeneDireX, Taiwan) was used to estimate the size of the products. The PCR products of the positive samples were purified by using PCR Clean-Up & Gel Extraction Kit (GeneDireX, Taiwan) according to the manufacturer's instructions.

Sequencing and phylogenetic analyses

The purified PCR products were sequenced at Macrogene Lab Technology, Korea. The obtained sequences were assembled using ChromasPro software (ChromasPro 1.7, Technelysium Pty Ltd., Tewantin, Australia). The obtained sequences were submitted to GenBank and then compared with those available in the GenBank database by the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences that were obtained from positive samples were aligned with sequences of validated species already available in GenBank using Bioedit software version 7.0.5.3 (Clustal W multiple alignment) (Hall, 2011). For taxonomic analyses, the maximum-likelihood phylogenetic trees were constructed using MEGA software version X (Kumar *et al.*, 2018) with 500 bootstrap replications.

Statistical analysis

The significant differences between genders and ages of camels infected with *Anaplasma* were calculated by χ^2 test using the SPSS program (version 20.0) at $P< 0.05$.

RESULTS

***Anaplasma* infection**

Three out of 150 camels (2%) in Asir Province were positive for *Anaplasma* infection as shown by PCR that targeted the 23S rRNA (Table 2). The risk factors that are associated with *Anaplasma* infections were detected by PCR in camels in the same place (Table 3). There were no significant differences between ages or sexes of the camels that tested positive for *Anaplasma*. Two camels that were more than one year old and one that was one year old were infected with *Anaplasma*. Of these, two were female and one male. Furthermore, all positive *Anaplasma* infections were recorded in camels infested by ticks, while all animals uninfested by ticks were free of any *Anaplasma* infection.

The samples that were positive for *Anaplasma* infection showed the 520 bp band in the amplification of the 23S rRNA. Two out of three of the *Anaplasma* samples that were positive in the 23S rRNA amplification revealed a strong 500 bp band for the 16S rRNA (Figure 2). These two positive samples for *Anaplasma* infection for the 16S rRNA gene were sequenced

Table 2. Prevalence of *Piroplasma* and *Anaplasma* detected by PCR in camels in Asir Province, Saudi Arabia

Examined camels	*Piroplasma* spp.		*Anaplasma* spp.	
	Positive	%	Positive	%
150	0	0	3	2.00

Table 3. Risk factors associated with *Anaplasma* infections detected by PCR in camels in Asir Province, Saudi Arabia

Factor		Total number of camels	*Anaplasma* spp.	
			Positive	%
Age (year)	≤1	28	1	3.57
	>1	122	2	1.64
	χ^2		0.333	
	Sig.		NS	
Gender	Male	52	1	1.92
	Female	98	2	2.04
	χ^2		0.333	
	Sig.		NS	
Ticks	Yes	127	3	2.36
	No	23	0	0

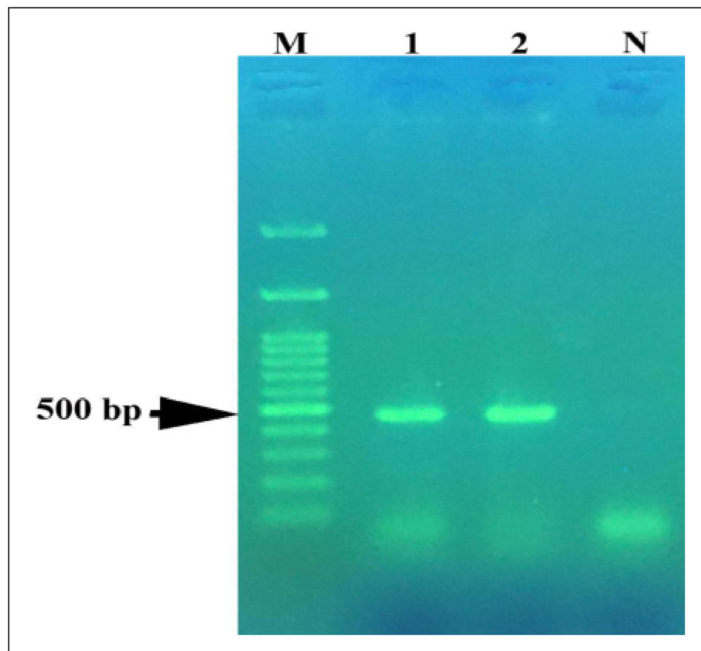

Figure 2. Image of agarose gel electrophoresis (1.5%) showing 500bp band for the amplification of 16S rRNA gene of *Anaplasma*. Lane M: DL 2000 marker; Lane N: negative control; Lanes 1-2: positive samples.

and recorded in GenBank with accession numbers MN882724 and MN882725. The accession number MN882724 has been named as *Anaplasma camelii* AC119 camel blood Saudi Arabia and recorded similarities of 99.16%, 98.74% and 98.32% with the previous accession numbers KF843825.1 (*Candidatus* Anaplasma camelii in Unizah,

Saudi Arabia covering 100%), MF289478.1 (*Anaplasma platys* isolate in China covering 100%) and MK814419.1 (*Anaplasma platys* isolate in South Africa covering 100%), respectively. The accession number MN882725 has been named as *Anaplasma camelii* AC103 camel blood Saudi Arabia and recorded similarities of 99.34%, 98.89% and 98.4% with the previous accession numbers KF843825.1 (*Candidatus* Anaplasma camelii in Unizah, Saudi Arabia covering 100%), MF289478.1 (*Anaplasma platys* isolate in China covering 100%) and MK814419.1 (*Anaplasma platys* isolate in South Africa covering 100%), respectively. A phylogenetic tree revealed that the two genotypes recorded in this study were close to each other and that both were located in one cluster with *Candidatus* Anaplasma camelii isolates that had been previously recorded in adjacent areas such as Unizah, Saudi Arabia and Iran (Figure 3).

Piroplasma infection

A total of 150 camels were screened by PCR that targeted the18S RNA gene for the presence of *Piroplasma* infection in Asir Province, Saudi Arabia. All camel blood samples were found to be negative for *Piroplasma* infection.

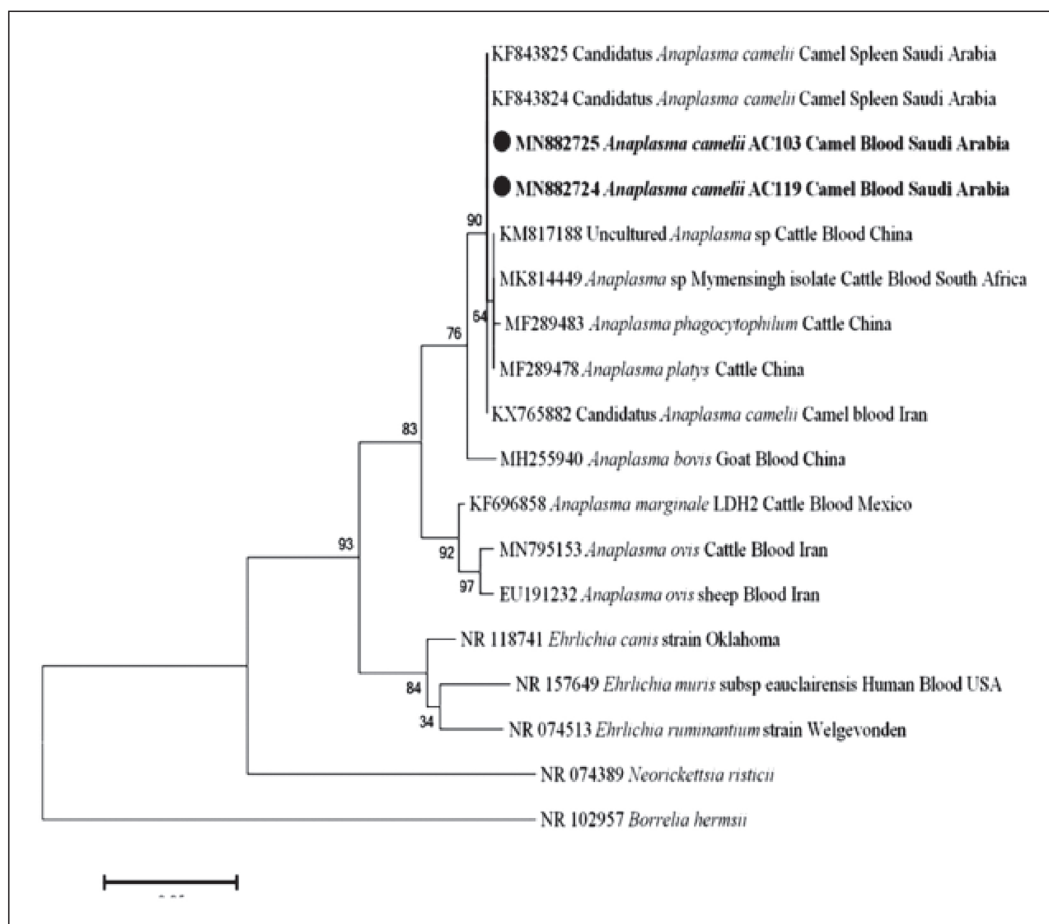

Figure 3. 16S rRNA-based phylogenetic analysis of genotypes identified in this study. Phylogenetic tree highlighting the position of *Anaplasma* sp. in the present study (bold) related to other *Anaplasma* sp. available in GenBank. The sequences of 16S rRNA were aligned using CLUSTAL W and phylogenetic inferences were constructed in MEGA X using Maximum Likelihood based on Tamura-Neimodel for nucleotide sequences with 500 bootstrap replicates. There was a total of 509 positions in the final dataset. The scale bar represents a 5% nucleotide sequence divergence.

DISCUSSION

Tick-borne pathogens such as *Piroplasma* (*Babesia* and *Theileria*) and *Anaplasma* (*Anaplasma* and *Ehrlichia*) have an economic importance regarding ruminants such as camels. Recently, many studies have used molecular tools to screen and identify these pathogens worldwide in camel hosts and their tick vectors (Mazyad and Khalaf, 2002; Sloboda *et al.*, 2011; Qablan *et al.*, 2012; Belkahia *et al.*, 2015; Khamesipour *et al.*, 2015; Li *et al.*, 2015; Lorusso *et al.*, 2016; Ait Lbacha *et al.*, 2017; Alsarraf *et al.*, 2017; Sharifiyazdi *et al.*, 2017; Azmat *et al.*, 2018; Bahrami *et al.*, 2018; Noaman, 2018; Azeem *et al.*, 2019; Sazmand *et al.*, 2019; Selmi *et al.*, 2019; Selmi *et al.*, 2020). In Saudi Arabia, very few studies concerned with these pathogens in camels have been performed (Al-Khalifa *et al.*, 2009; Ghafar and Shobrak, 2014; Mostafa and Dajem, 2014; Bastos *et al.*, 2015; Alanazi *et al.*, 2018; Alanazi *et al.*, 2020). Al-Khalifa *et al.* (2009) found that camels in the Asir region were free from blood parasites. Mostafa and Dajem (2014) found *Babesia bovis* in camels. In the Taif region, Ghafar and Shobrak (2014) showed that camels were free from *Anaplasma phagocytophilum*. Bastos *et al.* (2015) recorded novel genotypes most closely related to *Anaplasma platys* in the Unizah region. Alanazi *et al.* (2018) found that one *Hyalomma dromedarii* tick collected from a camel in Riyadh Province tested positive for *Theileria* sp. DNA. Alanazi *et al.* (2020) reported several pathogens (e.g. *Anaplasma platys*, *Anaplasma phagocytophilum*, *Anaplasma* sp., *Ehrlichia canis* and *Hepatozoon canis*) in camels from Riyadh Province.

In spite of a unique study that was conducted in the Asir region by Al-Khalifa *et al.* (2009), who indicated that the camels in this region were free of blood parasites, we believed that tick-borne pathogens might be found because most animals were found to be infested by ticks (127/150). Therefore, the goal of this study was to perform a trial to discover the presence of *Piroplasma* and *Anaplasma* in blood samples of camels in the Asir region, using two pairs of primers that belonged to the 18S and 23S rRNA genes, respectively, in addition to a pair of primers that belonged to the 16S rRNA gene. The latter was used in sequencing of *Anaplasma* that were positive for 23S rRNA due to a failure to obtain sequencing from PCR products that resulted from 23S rRNA.

The present study revealed that all camels tested were found to be free from *Piroplasma* infection which is in agreement with finding of Alanazi *et al.* (2020). This finding might be attribute to isolation of the locality from *Piroplasma* infection or to the animals having been treated against *Piroplasma*. In disagreement with the results of the present study, Selmi *et al.* (2019) found *Babesia* spp. infection (1.0%) for the first time in Tunisia, in one adult female camel that under went abortion and was anaemic. In Pakistan, Azeem *et al.* (2019) found *Babesia* and *Anaplasma* in 3% of camels, but this percentage was not accurate because the researchers used a staining procedure in their investigation. Li *et al.* (2019) obtained 18S rRNA and major piroplasm surface protein (MPSP) gene sequences of *Theileria sinensis* from Bactrian camels and ticks in Xinjiang, China. In Iran, Bahrami *et al.* (2017) investigated 248 camels for *Piroplasma* infection by 18S rRNA sequencing; they found that three and seven camels were infected with *Babesia caballi* and *Theileria equi*, respectively. These infections may be attributed to the presence of equines that lived with the camels; the same equine *Piroplasma* infection was found in camels in Jordan by Qablan *et al.* (2012). In Nigeria, Lorusso *et al.* (2016) detected *A. platys* and *T. ovis* in camels.

The present study is considered to be the first report that has recorded *Anaplasma* in Asir Province, Saudi Arabia, by use of PCR targeting the 23S rRNA. Moreover, this study has recorded two new genotypes related to *Canditatus* Anaplasma camelii with similarity of 99% for the first time. The sequences of these two genotypes were obtained from PCR products of the 16S rRNA gene and deposited in GenBank

with accession numbers MN882724 and MN882725. Three of 150 camels (2%) were found to be positive for Anaplasmosis in Asir Provonce. This level of infectionis lower than that recorded in the Taif and Unizah regions of Saudi Arabia by Ghafar and Shobrak (2014) and Bastos *et al.* (2015), who recorded more than 30% infection in camels. The high infection rates reported in these two studies may be due to the small sample sizes or the geographical locations.

Recently, Anaplasmosis was studied by molecular detection in camels of areas that surround Saudi Arabia in countries such as Iran, Tunisia, Morocco, Pakistan and China. All these studies recorded *Anaplasma* infection in camels at levels that were higher than those recorded in the current study, except for a unique study conducted in Tunisia by Selmi *et al.* (2020). This study found *A. ovis* in 1.2% of camels when the researchers investigated 412 camels for Anaplasmosis using PCR and sequencing that targeted the *groEL* and *msp4* genes. However, in Tunisia, Selmi *et al.* (2019) found *A. platys*-like strains in 5.6% (23/412) of camels by use of a PCR/restriction fragment length polymorphism assay, and Belkahia *et al.* (2015) found *Anaplasma* spp. DNA of the 16S rRNA gene in 17.7% of 226 camels. In studies of camels in Iran, Sharifiyazdi *et al.* (2017), Bahrami *et al.* (2018) and Sazmand *et al.* (2019) recorded 6% (6/100) infection by *A. platys*, 34.2% (71/207) infection by *A. phagocytophilum* and 15% (30/200) infection by *Candidatus* Anaplasmac amelii, respectively. In Pakistan, Azmat *et al.* (2018) found an overall 13.33% prevalence of Anaplasmosis in camels. In Morocco, Ait Lbacha *et al.* (2017) recorded that 39.62% of their camel sample (42/106) were positive for *Anaplasmataceae* spp. In China, Li *et al.* (2015) found that 7.2% (20/279) of the camels harboured *A. platys* DNA.

The phylogenetic tree of the genotypes obtained in the present study indicated that our genotypes were located in one cluster with *Candidatus* Anaplasma camelii isolates that had been previously recorded in Unizah, Saudi Arabia, (Bastos *et al.*, 2015) and in Iran (Sazmand *et al.*, 2019). This finding suggests that these new genotypes of *Anaplasma* circulate in Saudi Arabia and adjacent countries. The *Anaplasma* spp. in camels in other countries were discovered using different genes from those used in this study. For example, in Tunisia, Selmi *et al.* (2020) used *groEL* and *msp4* genes to identify *Anaplasma ovis* in camels; and in Morocco, Ait Lbacha *et al.* (2017) used the *groEL* gene in detection of *Candidatus* Anaplasma camelii in camels. Therefore, further extensive research that involves a large number of camels is needed in Saudi Arabian provinces to confirm the identification of *Anaplasma* spp. in camels in Saudi Arabia.

CONCLUSION

Two new *Anaplasma* genotypes close to *Candidatus* Anaplasma camelii were reported for the first time in camels from Asir Province, Saudi Arabia. The camels in this province were found to be free from Piroplasmosis. Therefore, it is recommended that a large number of animals in different provinces should be investigated through use of different genes to confirm *Piroplasma* infection in camels in Saudi Arabia.

DISCLOSURE

The authors declare that they have no competing interests.

FUNDING

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AUTHORS' CONTRIBUTIONS

All authors shared in the design of the plan of this study. MYA, ADA, ASA, MA and AGA collected blood samples from camels and extracted DNA from the blood samples. ADA, HHAMA, AMA and SA participated in the conduction of PCR protocols, purification of PCR products, sequence analyses, construction of the phylogenetic tree and analysis of the data. ADA, SA, HHAMA and AMA shared in writing the manuscript. All authors revised and approved the final version of the manuscript.

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