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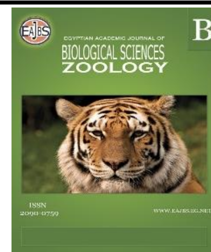

Molecular Identifications of *Hyalomma dromedarii* Infecting Camels and *Theileria annulata* from Ticks by 18S rRNA Gene in Al Disah valley, Tabuk, Saudi Arabia

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ABSTRACT

In many places of the world, ticks and tick-borne diseases constitute a severe obstacle to camel productivity. *Theileria*, an obligate intracellular protozoon, is transmitted by hard ticks and induces moderate to severe diseases in vertebrate hosts. The hard ticks *Hyalomma dromedarii* and *Hyalomma anatolicum* were collected from a camel in Saudi Arabia in October 2021. A total of 140 dromedary camels were selected by a simple random selection procedure. A total of 310 two different ixodid tick species, such as *H. dromedarii* and *H. anatolicum* were identified in camels. The infection rate of *H. dromedarii* was 78 % (242/310) and that of *H. anatolicum* was 22% (68/310). To identify *H. dromedarii* and *Theileria sp.*, polymerase chain reaction (PCR) using the *COI* gene and the 18S rRNA gene was done. The PCR-amplified 660-bp-long *H. dromedarii* and 400-bp-long *Theileria sp.* genes were analyzed with phylogenetic trees. *H. dromedarii* had a single genotype that was completely the same as that registered for *H. dromedarii*. The genotype of *Theileria sp.* found in *H. dromedarii* was highly similar to *T. annulata* by (99.9) %. This study may contribute to understanding the epidemiology of tick-borne diseases, thus, enhancing the detection and control of the diseases in Saudi Arabia.

INTRODUCTION

Ticks are hematophagous ectoparasites that feed on the blood of a variety of vertebrate hosts and play an important role in the transmission of zoonotic diseases caused by viruses, bacteria, and protozoans (Pfäffle *et al*., 2013; de la Fuente *et al*., 2017). They attack a wide range of animals including humans. Ticks are the second most important vector for transmitting diseases in domestic and wild animals (Jongejan *et al*., 2007). Tick-borne disease agents may cause a variety of dangerous diseases in humans and animals in different parts of the world, including Lyme disease, relapsing fever, Rocky Mountain spotted fever, east coast fever, tropical theileriosis, and others (Brites-Neto, Duarte, and Martins 2015; Kalume, Losson, and Saegerman 2011). The camel population on the Arabian Peninsula is reported to be 1.6 million, of which 53% are raised in Saudi Arabia (Abdallah and Faye 2012).

Camels were chosen for this study because of their widespread distribution in Asia and Africa, as well as their economic relevance in food products. The camel is the

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primary host of several types of ixodid ticks (El-Bahnsawy *et al*., 2012). The harm caused by ixodid ticks to camels is not limited to blood-sucking but includes the most fatal tick-borne theileriosis (S. A. Omer, Alsuwaid, and Mohammed 2021).

Theileriosis is a widespread tick-borne disease that has been documented in a variety of animals, including cattle, sheep, and goats. A few studies of theileriosis of camels were reported (El Kammah *et al*., 2001; Youssef *et al*., 2015). An important parasitic disease of the dairy sector in different countries including Saudi Arabia is caused by the protozoan *T. annulata* (an apicomplexan). It is transmitted by ticks of the *Hyalomma* genus (Hussein *et al*., 1991; Florin-Christensen and Schnittger 2009).

The present study used the mitochondrial cytochrome oxidase subunit I (COI) gene to molecularly identify *H. dromedarii* infecting Dromedary camels (*Camelus dromedarius*) and determine the ability of *H. dromedarii* that infect camels to serve as carriers for new genotypes of *T. annulata* in Saudi Arabia.

MATERIALS AND METHODS

Tick Collection:

A total of 140 camels were designated for the study. All camels were apparently healthy at the time of sampling. The hard tick *Hyalomma* sp. was collected from the whole body of camels which included head, tail, shoulder, belly, and perineal regions between April and October 2021 in Al Disah valley, Tabuk region, Saudi Arabia (27°36'42.1"N 36°26'23.1"E). All visible adult ticks attached were collected carefully by forceps and gently removed exerting a horizontal pull to the body surface by rotating the tick not to damage the host by the tick's mouth parts and placed into labelled vials that contained 70% ethyl alcohol. Ticks were identified at the species level by use of morphological keys and descriptions (Pegram, Hoogstraal, and Wassef 1981; Walker 2003) and stored at –20°C for molecular biology procedures.

DNA Extraction from Ticks*:*

DNA was extracted from frozen ticks using the Pure Link Genomic DNA Kits (Invitrogen, Waltham, Massachusetts, USA). Briefly, each sample was soaked with the tissue lysis buffer (between 180 µl and 540 µl depending on the size of the tick), treated with proteinase K (20 µl for each 180 µl of tissue lysis buffer), and incubated for 48h at 56°C and centrifuged for 1 min at 10000 xg.

Subsequently, according to the manufacturer's instructions (Invitrogen, Waltham, Massachusetts, USA), the supernatant was transferred to a new tube. 200 µl of ethanol and 200 µl Lysis/Binding Buffer were added to the lysate and again vortexed. The mixture was then added to the spin column and centrifuged at 10000 xg for 1 min. Thereafter, two washings were given with wash buffers and DNA was eluted in 50 µl of elution buffer and stored at -20 C till further use.

Polymerase Chain Reaction (PCR):

To identify tick species, specific primers amplifying the cytochrome oxidase CO subunit I (COI) of the mitochondrial DNA of tick LCO1490: 5′-GGTCAA CAAATCATAAAGATATTGG-3′ as a forward primer and HCO2198: 5′-TAAA CTTCAGGGTGACCAAAAAATCA-3′ as a reverse primer were used (Folmer *et al*. 1994). For the detection of the *theileria* sp. set of primers was used to amplify a 400 bp fragment of the 18S rRNA gene was used and the primers include the forward primer 5′ GTCTTGTAATTGGAATGATGG-3′ and the reverse primer 5′ CCAAAGACTTT GATTTCTCTC-3′ (Li *et al*., 2014).

PCR amplification was performed in a final reaction volume 2X (50 µl) containing 25 µl of 2X master mix solution (*i*-Taq, *iNt*RON, Seongnam, Korea), 0.2 µM

(2 µl) of each primer, 4 µl of template DNA, and 0.2 mg/ml of BSA, and 14.5 µl of nuclease-free water.

The thermal cycling program of ticks consisted of an initial denaturation at 94 °C for 10 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 42 °C for 1 min and extension at 72 °C for 1 min. A final extension was carried out for 10 min at 72 °C.

The PCR conditions of *Theileria* were an initial denaturation at 94°C for 10 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and at 72°C for 1 min, followed by a final extension at 72°C for 10 min. PCR amplicon was run on a 2 % agarose gel to check the quality and yield of the PCR product using the transilluminator (UV transilluminator, Spectroline, Westbury, USA) and digital images were documented using a digital camera.

Sequence Analysis:

PCR products were purified using a Macrogen reagent (Seoul, Korea). Single-strand DNA sequencing was performed, after which nucleotide sequences of tick *COI* and *Theileria* 18S rRNA were aligned.

Phylogenetic Analysis:

The obtained sequences were assembled using Chromas Pro 1.5 beta (Technelysium Pty., Tewantin, QLD, Australia). The resulting sequences were subjected to Basic Local Alignment Search Tool (BLAST) analysis to determine similarities with those sequences available in the NCBI GenBank database (https://www.ncbi.nlm.nih.gov). MEGA11 was used to establish the phylogenetic relationships. Phylogenetic trees were created using the maximum likelihood (ML) and neighbour joining (NJ) methods (Saitou and Nei 1987). In both ML and NJ tree construction, bootstrap support was evaluated by 1,000 replicates (Joseph FelsensteinSource: 1985).

Organisms that were used to infer the phylogenetic relationship for *Hyalomma dromedari* and *Theileria* recovered in the present study are given in Table 1.

Table 1: DNA sequences of COI from *H. dromedarii* and 18S rDNA region from *T. annulata* which were used in the phylogenetic analysis with their GenBank acc. No.

GenBank accession	Species	Country	GenBank accession	Species	Country
OM275809	*Hyalomma dromedarii*	Saudi Arabia	OM273908	*Theileria annulata*	Saudi Arabia
OK576092.1	*H. dromedarii*	Cameroon	MT341858	*T. annulata*	Italy
MZ348821.1	*H. dromedarii*	Saudi Arabia	MT341857	*T. annulata*	Italy
MT093513.1	*H. dromedarii*	Tunisia	MT318158	*T. annulata*	Pakistan
MZ348743.1	*H. dromedarii*	Saudi Arabia	MN227667	*T. annulata*	Egypt
MK305817.1	*H. dromedarii*	Saudi Arabia	MN223734	*T. annulata*	Egypt
MT107481.1	*H. dromedarii*	Tunisia	MN223729	*T. annulata*	Egypt
MG188799.1	*H. dromedarii*	Egypt	MK918607	*T. annulata*	Turkey
MK305817.1	*H. dromedarii*	Saudi Arabia	MK183002	*T. annulata*	Iran
MH590881.1	*H. dromedarii*	Saudi Arabia	KT367878	*T. annulata*	India
KU323789.1	*H. dromedarii*	Egypt	OM066186	*T. annulata*	Turkey
MT093512.1	*H. dromedarii*	Tunisia	MK300062	*T. annulata*	Saudi Arabia
AJ437061.1	*H. dromedarii*	Ethiopia	MH327773	*T. annulata*	France
AF132822.1	*H. dromedarii*	Australia	MF287932	*T. annulata*	India
KM235696.1	*H. dromedarii*	Iraq	KF429794	*T. annulata*	Iran
MK287892.1	*Argas persicus*	USA	MF287941	*T. annulata*	India
			MN223733	*T. annulata*	Egypt
			MF084761	*Borrelia theileri*	Egypt

RESULTS

Recovery of Ticks from Camel:

Out of 141 camels examined, 100 camels (71.4%) were infected by 310 ticks. These camels had both immature and adult ticks, but we collected only adult ticks for identification. We collected 165 (53.2%) male and 145 (46.8%) female ticks. Ticks were identified as 2 different species, such as *H. dromedarii* and *H. anatolicum*, in which *H. dromedarii* constituted 78% and *H. anatolicum* constituted 22%. The body parts of camel's udder/scrotum, between the groin were the most frequently infected by *H. dromedarii* and *H. anatolicum*.

Molecular Characterization of Ticks:

PCR amplification of DNA from ticks revealed positive amplification using primers which amplify COI region. The amplicon size was 660 pb (Fig. 1A). Representative samples of ticks were taken for DNA sequencing from the camel. DNA sequences of COI region (OM275809) obtained from amplification of *H. dromedarii* from camel showed close identity to sequences to some available in GenBank related to the same species (*H. dromedarii*) (**Table 2**).

Table 2: Distribution of ticks in different attachment sites.

Tick species	Predilection site					Total
	Ear	Udder/ scrotum	Between groin	Tail	Vulvar regions	
H. dromedarii	20	109	89	9	15	242
H. anatolicum	-	39	17	5	7	68
Total	20	148	106	14	22	310
Infection rate (%)	6.5	48.0	34.0	4.5	7.0	

Phylogenetic Analysis of the COI Genes of *H. dromedarii:*

The nucleotide sequence of the COI gene of *H. dromedarii* was compared to those from other organisms retrieved from GenBank. The sequences showed 100% sequence identity with that from Cameroon, Egypt, and Tunisia (OK576092, KU323789, and KU323789, respectively), and 99.5 % identity with that from Saudi Arabia (MZ348821.1, MK305817.1, and MH590881.1) (Fig. 2A)

PCR Detection and Molecular Characterization of *Theileria:*

Amplification of disease agents (*Theileria* sp.) from the DNA extracted from ticks collected from camels revealed positive results from 20 samples indicating a prevalence rate of 6.5%.

Positive samples were only from *H. dromedarii* which was collected from camels. No amplification was detected from *H. anatolicum* which was collected from camels indicating the absence of *Theileria* sp. DNA in the extracted samples.

A single 400 bp fragment of *Theileria* sp. was amplified (Fig. 2B). Sequencing of the amplified product showed DNA that is identical to *Theileria annulata*. A representative DNA sequence obtained from sequencing of the 18S rDNA resulted from the amplification of DNA from ticks using primers that amplify Theileria DNA was deposited at the GenBank database with the accession number: OM273908 .The sequence obtained was identical to several other *T. annulata* deposited in GenBank (Table 1).

The 18S rDNA sequences of *T. annulata* from Saudi Arabia (MT341858, MN223734, and MK918607) were found to have 100% sequence identity with *T. annulata* from Italy, Egypt, and Turkey (MT341858, MN223734, and MK918607), respectively.

Saudi Arabia, India, and Pakistan (MK300062, KT367878, and MT318158) have a 99.5 % phylogenetic relationship. (Fig. 2B).

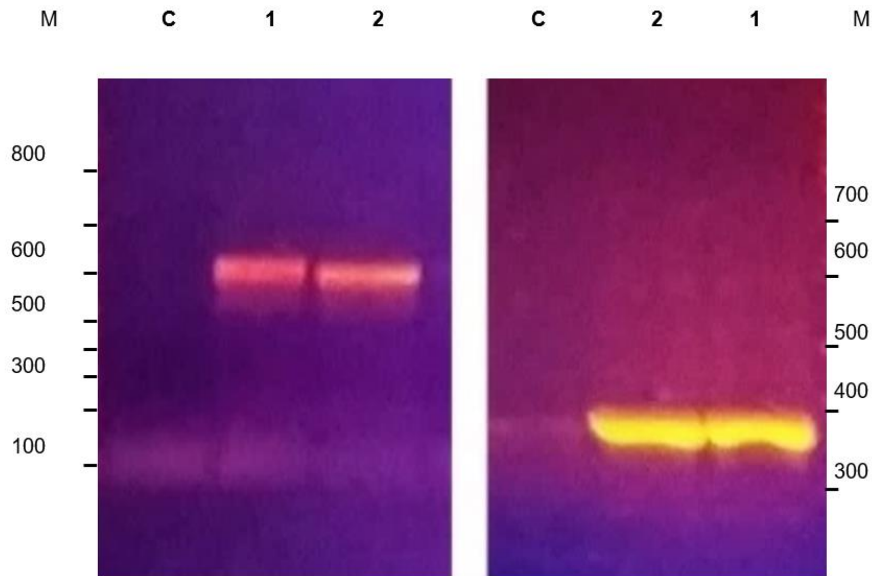

Fig. 1: Agarose gel electrophoresis (2% agarose, stained with ethidium bromide): analysis of polymerase chain reaction amplification for (A) identification of *H. dromedarii* M: 100 bp DNA marker; C: negative control; 1 & 2) positive tick sample with *H. dromedarii* at 660 bp; (B) detection of *T. annulata* M: 100 bp DNA marker; C: negative control; 1 & 2) positive ticksample with *T. annulata* from *H. dromedarii* at 400 bp. *Borrelia theileri* serving as an outgroup.

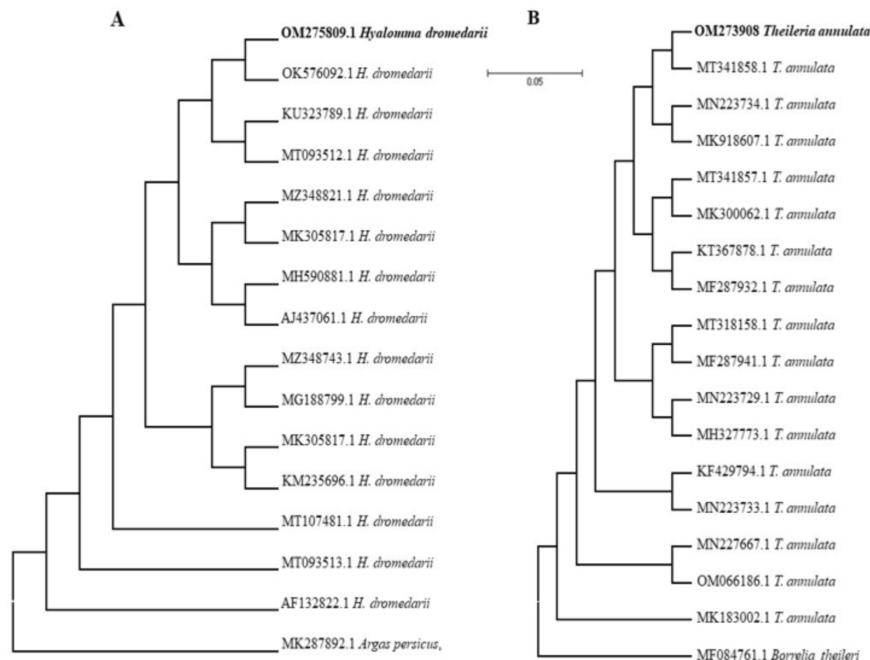

Fig. 2: (**A**) Neighbor-joining phylogenetic tree based on 660 bp of COI gene sequence of *H. dromedarii*, the tree was rooted using *Argas persicus* as an outgroup. (**B**) Neighbor-joining phylogenetic tree based on 400 bp of 18S rDNA gene sequence of T. annulata. The tree was rooted using *Borrelia theileri* as an outgroup. New sequences derived from this study are bolded. The phylogenetic tree was constructed using the maximum likelihood method, with bootstrap analysis with 1,000 replicates. Numbers on branches indicate support for each clade ≥ 80%. The evolutionary episode was analyzed by MEGA11.

DISCUSSION

A total of 140 camels were investigated for ixodid tick infestation in this study. Based on morphological characteristics, we identified *H. dromedarii* and *H.anatolicum* in 310 collected ticks from camels (Pegram, Hoogstraal, and Wassef 1981). Recently, another report showed ticks classified as *H. dromedarii* and *H. anatolicum* from camels in the eastern province by Omer (S. A. Omer, Alsuwaid, and Mohammed 2021). The camel tick *H. dromedarii*, which is the most common parasitic species on dromedary camels, has the greatest prevalence rate (78%) (Elghali and Hassan 2009; Hassan *et al*., 2017). *H. dromedarii* is a thermophilic tick that lives in the desert and hyper-arid environments. It has been detected in camels in Sudan, Iran, Egypt, Saudi Arabia, and Tunisia, with infection rates ranging from 49 to 89% (Hussein *et al*., 1991; Moshaverinia and Moghaddas 2015; Ghashghaei *et al*., 2016; El-Azazy, El-Metenawy, and Wassef 2001). The tick species identified during this investigation have also been recognized as vectors of *Theileria spp.* in Saudi Arabian domestic and wild ungulates (El-Azazy, El-Metenawy, and Wassef 2001; Al-Khalifa *et al*. 2009; Alanazi *et al*. 2020). In this study, the camels were highly infested with ticks (71.4%, 100/140), however low infectivity of *Theileria* in ticks (6.5%) was observed. COI sequences from *H. dromedarii* obtained in this work were determined to be identical to *H. dromedarii* sequences in the GenBank.

In this investigation of tick predilection sites on the host body, different tick species were discovered to have varied preferences for predilection sites. As a result, between the groins, *H. dromedarii* and *H. anatolocum* displayed a high predilection for udder/scrotum. This finding was consistent with Musa and Tesgera (Musa *et al*., 2014; Tesgera *et al*., 2017) found that lengthy hypostome ticks such as *Hyalomma* attach to lower areas of the animal body, which was also the case in this study. Tick attachment sites are determined by a range of characteristics such as host density, the interaction between tick species, time of the season, and inaccessibility for grooming (Hassan *et al*., 2017).

In the current investigation, *T. annulata* DNA was found in 6.5 % of the ticks examined. *T. annulata* DNA was amplified from *H. dromedarii* ticks collected from camels, but no amplification was detected from *H. anatolicum* ticks collected from camels, indicating that the extracted samples did not include *Theileria* DNA. Previous studies have found evidence of tropical theileriosis caused by *T. annulata* in Saudi Arabia (Al-Khalifa *et al*., 2009; Al-Atiya, Elbihari, and Gameel 1991).

The current study's prevalence of *T. annulata* (6.5%) was lower than the 40 % reported previously by Omer (S. A. Omer, Alsuwaid, and Mohammed 2021) in other Saudi Arabian regions. Variances in husbandry, tick distribution, tick species, detecting methods, and sample period may all contribute to regional differences in prevalence. *T. annulata* was isolated from *H. dromedarii* ticks collected from camels in the Al Disah valley, which matches (Hassan *et al*., 2017) found in Giza, Egypt. This similarity could be attributable to the density of *H. dromedarii* and the similarity of the study areas and environmental conditions.

Contrary to the present findings (S. A. Omer, Alsuwaid, and Mohammed 2021; Al-Khalifa *et al*., 2009; O. H. Omer *et al*., 2003) indicated that the *T. annulata* has been reported from both *H. anatolicum* and *H. dromedarii* ticks collected from cattle in Saudi Arabia. In the Tabuk region's Al Disah valley, no molecular research on *T. annulata* has been done. This discovery represents the first molecular characterization of *T. annulata* in the Kingdom of Saudi Arabia's Tabuk region. Furthermore, the role of *H. dromedarii* as a primary vector for the parasite and transmission of infection in camels has been documented.

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Conflict of Interest:

The authors declare that there is no conflict of interest.

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