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Original article

Molecular characterization of ticks and tick-borne piroplasms from cattle and camel in Hofuf, eastern Saudi Arabia

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ABSTRACT

The aims of the present study were to characterize ticks infesting the dromedary camel and cattle in Hofuf, Eastern Saudi Arabia and to determine the piroplasms that they may harbor. DNA was extracted from ticks, collected from camels and cattle, using commercial kits and subjected to polymerase chain reaction using specific primers for the amplification of ticks and piroplasms DNA. The cytochrome oxidase subunit I mitochondrial gene (*COI*) was used for characterization of ticks whereas partial 18S rRNA gene (*18S rRNA*) was used for piroplasms characterization. Ticks were genetically identified as *Hyalomma dromedarii* and *Hyalomma anatolicum*. Both cattle and camel in Hofuf, were found to be infested with both species. Both ticks identified as *H. dromedarii* and *H. anatolicum* from camels and cows showed 100% identity to *COI* sequences from the same species available in GenBank. Only *Theileria annulata* DNA was amplified from both *H. anatolicum* and *H. dromedarii* infesting cattle. None of the ticks collected from camels revealed DNA of piroplasms. *T. annulata* DNA was reported for the first time from Hofuf and the role of both *H. anatolicum and H. dromedarii* as potential vectors for this parasite in cattle in Saudi Arabia has been documented for the first time.

1. Introduction

Ticks are known arthropod vectors and capable of transmitting several pathogenic agents (viruses, bacteria, helminthic stages and protozoa) to various hosts including vertebrates (Jongejan and Uilenberg, 2004). They feed on the blood of mammals, birds, and sometimes reptiles and amphibians. They are essentially established in tropic and subarctic areas and some abiotic factors such as temperature and humidity play a fundamental role in their growth and development.

Disease agents transmitted by ticks can cause a wide range of serious diseases in humans and animals such as Lyme disease, relapsing fever, Rocky mountain spotted fever, east coast fever, tropical theileriosis, Crimean Congo haemorrhagic fever etc. in different parts of the world (Kalume et al., 2011; Brites-Neto et al., 2015).

The camel population in the Arabian Peninsula is approximately 1.6 million, 53% of which were found in the Kingdom of Saudi Arabia (Abdallah and Faye, 2012). Camel has a great importance due to its meat and milk production (Faye and Bonnet, 2012), and this productivity increases with increasing population growth of dromedary camel (Alanazi et al., 2020). Cattle used in Saudi Arabia mainly for milk production. Ticks infesting domestic livestock including camel and cattle in Saudi Arabia have been presented in some studies based on morphological description; (Al-Khalifa and Diab, 1985; Diab et al., 1987; Al-Afaleq et al., 2018). They have reported several species infesting cattle and cattle including various *Hyalomma* spp.

The protozoan *Theileria annulata* (an apicomplexan) is the causative agent for an important parasitic disease of dairy industry in several countries including Saudi Arabia. It is transmitted by ticks of the genus *Hyalomma* (Hussein et al., 1991; De Kok et al., 1993). *Theileria* and *Babesia* species are tick-borne haemoprotozoan parasites mainly infect cattle and small ruminants, in tropical and subtropical areas that decreasing their productivity (Mehlhorn and Schein, 1984). *T. annulata* has been reported from cattle in Saudi Arabia from the eastern province as well as the Qassim area based

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on microscopic examination of Giemsa-stained blood films collected from infected animals (Al-Atiya et al.1991; Hussein et al., 1991; Omer et al., 2003).

The aims of the present study were to identify and molecularly characterize ticks infesting the Dromedary camel (*Camelus dromedarius*) and cattle (*Bos taurus*) in Hofuf, Eastern region, Saudi Arabia using the mitochondrial cytochrome oxidase subunit I (*COI*) gene. Furthermore, the role of these ticks as potentials vectors for piroplasms in both hosts was evaluated using *18S rRNA* gene.

2. Material and methods

2.1. Samples collection

Fifty ticks infesting each of cows and camels from Kilabyiah village, 10 km north west of Hofuf, Eastern province, Saudi Arabia were collected. Hofuf and related villages constitute Al-Asha oasis which a large agricultural area with tropical climate which dry with five months summer and cold winter. Animals from which ticks were collected related to local people and they are raised traditionally in small paddocks. Ticks were collected from the under the tail as well as from ears of cattle and camel. These are the areas which were infested with ticks.

Ticks were kept in 70% ethanol and frozen at −20 °C for further identification and DNA extraction. Ticks were examined under stereoscope and morphological identification was performed using the key produced by Hoogstraal et al. (1981) and Diab et al. (2006). The animals from which the ticks were collected appear clinically healthy, however, few individuals particularly cows showed clinical signs in the form of weakness, respiratory distress, pale mucous membranes and inappetence.

2.2. DNA extraction from ticks

DNA was extracted from ticks after being washed individually in 500 µL of sterile double distilled water to avoid contamination and they were transferred into liquid nitrogen. Each individual tick was manually crushed with a sterile ceramic mortar and was placed in a sterile 1.5 ml Eppendorf tube. DNA was extracted from the tick by DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), following the manufacturer's protocol. Extracted DNA was stored at −20 °C till use.

2.3. Polymerase chain reaction and DNA sequencing

For tick identification specific primers amplifying the cytochrome *oxidase c* subunit I (*COI*) of the mitochondrial DNA of ticks Fish1F: 5′-TCAACCAACCACAAAGACATTGGCAC-3′ as a forward primer and Fish1R: 5′-TAGACTTCTGGGTGGCCAAAGAATCA-3′ as a reverse primer were used (Ward et al., 2005).

For the detection of the piroplasms (*Theileria*/*Babesia* spp.) one set of primers was used to amplify a 580 bp fragment of the *18S rRNA* gene was used and the primers include the forward primer 5′-GTCTTGTAATTGGAATGATGG-3′ and the reverse primer 5′-CCA AAGACTTTGATTTCTCTC-3′ (Li et al., 2014).

The DNA was amplified in a 25 µL reaction mixture containing about 80 ng of the extracted DNA from ticks, 10 pmol of each primer pair, and 12.5 µL 2X PCR Master Mix (Promega, USA). PCR amplifications were carried out in a Multigene™ thermocycler (Labnet International, Inc., Edison, NJ, USA). The cycling conditions used to amplify the desired DNA, for both regions, were 1 cycle at 94 °C for 2 min for initial followed by 35 cycles of 92 °C for 30′, 55 °C for 30′, 72 °C for 30′ and final extension at 72 °C for 10 min.

Amplified DNA was visualized using the transillumintor and UV light and digital images were documented using a digital camera. Amplified PCR products will be subjected to DNA sequencing using Macrogen facility (Seoul, South Korea) for DNA sequencing.

2.4. Phylogenetic analysis

Sequences obtained were compared with related sequences available in GenBank database using BLAST service. The analysis program MEGA7 was used to infer the phylogenetic relationships between sequences obtained from the present study with sequences from related organisms available in GenBank (Kumar et al., 2016). Phylogenetic trees were constructed using maximum likelihood (ML) and neighbour joining (NJ) methods. In both ML and NJ trees construction, bootstrap support was evaluated by 1,000 replicates. Organisms which were used to infer phylogenetic relationship for ticks recovered in the present study are given in Table 1. Whereas related organisms to infer the phylogenetic relationship for the piroplasm recovered are given in Table 2.

2.5. DNA sequences and GenBank accession numbers

DNA sequences from *COI* region obtained from tick were deposited in GenBank and given the following accession number MK305816 and MK305817 for *H. dromedarii*, MW084955 for *H. anatolicum*. DNA sequences from 18S rDNA region for the piroplasm (*Theileria annulata*) was given as MK300062.

3. Results

3.1. Morphological identification of ticks

Of the 100 ticks collected from both hosts, 56 were identified as *H. dromedarii* and 44 as *H. anatolicum* (Table 3). Forty-six *H. dromedarii* was collected from camels and 10 was collected from cows. Forty *H. anatolicum* was collected from cows while 4 were from camels. A total of 14 ticks identified morphologically as *H. drome-*

Table 1
DNA sequences of *COI* from *Hyalomma* spp. Used in the phylogenetic analysis with their GenBank accession numbers and the outgroups.

GenBank Accession	Parasite species	Country
MK305817.1 H2	***Hyalomma dromedarii***	**This study (Saudi Arabia)**
MH590881.1	*Hyalomma dromedarii*	Saudi Arabia
MK305816.1 H1	***Hyalomma dromedarii***	**This study (Saudi Arabia)**
MT107481.1	*Hyalomma dromedarii*	Tunisia
MT040954.1	*Hyalomma dromedarii*	Tunisia
KT989619.1	*Hyalomma dromedarii*	Israel
MT093513.1	*Hyalomma dromedarii*	Tunisia
MT093506.1	*Hyalomma dromedarii*	Tunisia
MG188799.1	*Hyalomma dromedarii*	Egypt
MG188798.1	*Hyalomma dromedarii*	Egypt
GQ483461.1	*Hyalomma dromedarii*	India
MW084955.1	***H. anatolicum***	**This study (Saudi Arabia)**
MT876644.1	*H. anatolicum*	Pakistan
MK462197.1	*H. anatolicum*	Pakistan
KU130581.1	*H. anatolicum*	Pakistan
KM235704.1	*H. anatolicum*	Iraq
MN728993.1	*H. anatolicum*	Pakistan
MK462202.1	*H. anatolicum*	Pakistan
MN728991.1	*H. anatolicum*	Pakistan
MK462195.1	*H. anatolicum*	Pakistan
MK462203.1	*H. anatolicum*	Pakistan
MH459376.1	*H. anatolicum*	China
KT920180.1	*H. anatolicum*	Iran
MN268573.1	*H. excavatum*	Iran
MN264497.1	*H. excavatum*	Egypt
MN264496.1	*H. excavatum*	Egypt
MN264491.1	*H. excavatum*	Egypt
KU130571.1	*Nosomma monstrosum*	Sri Lanka
KU130569.1	*Amblyomma variegatum*	Nigeria

Table 2
DNA sequences from 18S rDNA region from *Theileria* spp. which were used in the current analysis with their GenBank accession numbers and the outgroup.

GenBank Accession	Parasite species	Country
MK300062.1	*Theileria annulata*	This study (Saudi Arabia)
MK918607.1	*Theileria annulata*	Turkey
MK415058.1	*Theileria annulata*	China
MK838106.1	*Theileria annulata*	Pakistan
MG599091.1	*Theileria annulata*	China
MK183002.1	*Theileria annulata*	Azerbaijan
MH327773.1	*Theileria annulata*	Algeria
MF287932.1	*Theileria annulata*	India
MF287930.1	*Theileria annulata*	India
EU073963.1	*Theileria annulata*	China
MK849885.1	*Theileria annulata*	India
AF081135.1	*Theileria lestoquardi*	China
LC495915.1	*Theileria* sp. Yokohama isolate	Japan
L28999.1	*Theileria parva*	Kenya
FJ426360.1	*Theileria buffeli*	Italy
L19082.1	*Theileria taurotragi*	South Africa
MN704656.1	*Theileria ovis*	Iraq
L24381.1	*Toxoplasma gondii*	Australia

Table 3
Ticks collected from Cattle and camels from Hofuf, Saudi Arabia.

Tick species	Host		Sex		
	Cattle	Camel	M	F	Total
Hyalomma dromedarii	10	46	14	42	56
Hyalomma anatolicum	40	4	7	37	44

darii were males while 42 were females. Whereas, 7 of those identified as *H. anatolicum* were males and 37 were females (Table 3). Females of *H. dromedarii* and *H. anatolicum* were identified by the characteristic shape of the posterior lip of the genital aperture which is V shaped in the first and U shaped in the latter. Males of both species were distinguished by the sub-anal plates as it was aligned outside the longitudinal axis of the adanal plates in *H. dromedarii*, whereas in *H. anatolicum* the sub-anal plates are aligned in the same axis.

Females *H. dromedarii* showed narrow genital aperture, triangular in shape (V-shaped). Scutum is yellow to reddish brown in color; pale marbling absent; nearly as broad as long. Basis capitula are slightly concave. Hypostome is club-shaped. Legs with coxae showing posteromedian and posterolateral spurs of coxa I long. Similar coloration of legs to male ticks (Fig. 1A).

Males *H. dromedarii* showed long adanal plates, markedly convex lateral margin, rounded posterior margin. Scutum is yellow to reddish brown in color; no marbling; broadly oval in shape. Basis capitula are without lateral projections; dorsal posterior margin angular, deeply concave. Hypostome is club-shaped; denticulate portion slightly longer than denticle-free portion. Legs: Coxae: posteromedian and posterolateral spurs of coxa I long and colored stripes on dorsal aspects of leg segments.

Females *H. anatolicum* showed steep scapular grooves, legs with pale rings, small porose area and large sized engorged females. They also showed long distinct internal and external spur in coxa 1, coxae 2 and 3 with indistinct external spurs whereas coxa 4 with no distinct internal spur.

Males *H. anatolicum* showed visible cervical field depression, short lateral grooves, two posterior ridges. Visible adanal and accessory shields, coxa 1 with long and distinct internal and external spur. Coxae 2 and 3 with indistinct external spur whereas coxa 4 with indistinct internal spur (Fig. 1B).

Fig. 1. A photomicrograph showing a ventral view of a female *Hyalomma dromedarii* (A) and a ventral view of a male *Hyalomma anatolicum* showing morphological features of the species.

3.2. Molecular characterization of ticks

PCR amplification of DNA from ticks revealed positive amplification using primers which amplify *COI* region. The amplicon size was ~750 pb (Fig. 2). Representative samples of ticks were taken for DNA sequencing from both camel and cows. DNA sequences of *COI* region (MK305816) obtained from the amplification of *H. dromedarii* from camel showed close identity to sequences to some available in GenBank related to the same species (*H. dromedarii* H1). However, one of the sequences (MK305817) showed a transition mutation at the position 615 of the alignment (C instead of T) (*H. dromedarii* H2). The overall mean distance between *H. dromedarii* sequences used in the analysis was 0.002. All ticks examined and sequenced showed the same morphological characters.

Ticks which were identified as *H. anatolicum* based on morphology, revealed sequences (471 bp) which showed identity to related sequences from the same region related to *H. anatolicum* in GenBank (accession number MW084955). The overall mean distance between sequences of *H. anatolicum* was 0.31 while the overall sequences distance between *H. anatolicum* sequences and the related *H. excavatum* was 0.032. Three sequences obtained from *H. anatolicum* showed DNA sequences which were found to be related to *Bos taurus*, *Camelus dromerdarius* and to the Japanese quail (*Coturnix japonica*).

Phylogenetic analysis using *COI* data from both taxa obtained in the present study revealed a phylogeny with good statistical support with bootstrap values >70% (Fig. 1). DNA sequences from

Fig. 2. Representative 1.5% agarose gel electrophoresis showing the polymerase chain reaction product resulted from amplification of Ticks' DNA. The amlicon size expected was 750 bp. M is for the 100 base pair ladder whereas samples from 1 to 5 were from *H. dromedarii* while samples from 6,7,9,10 were from *H. anatolicum*. Sample number 8 was negative control without DNA.

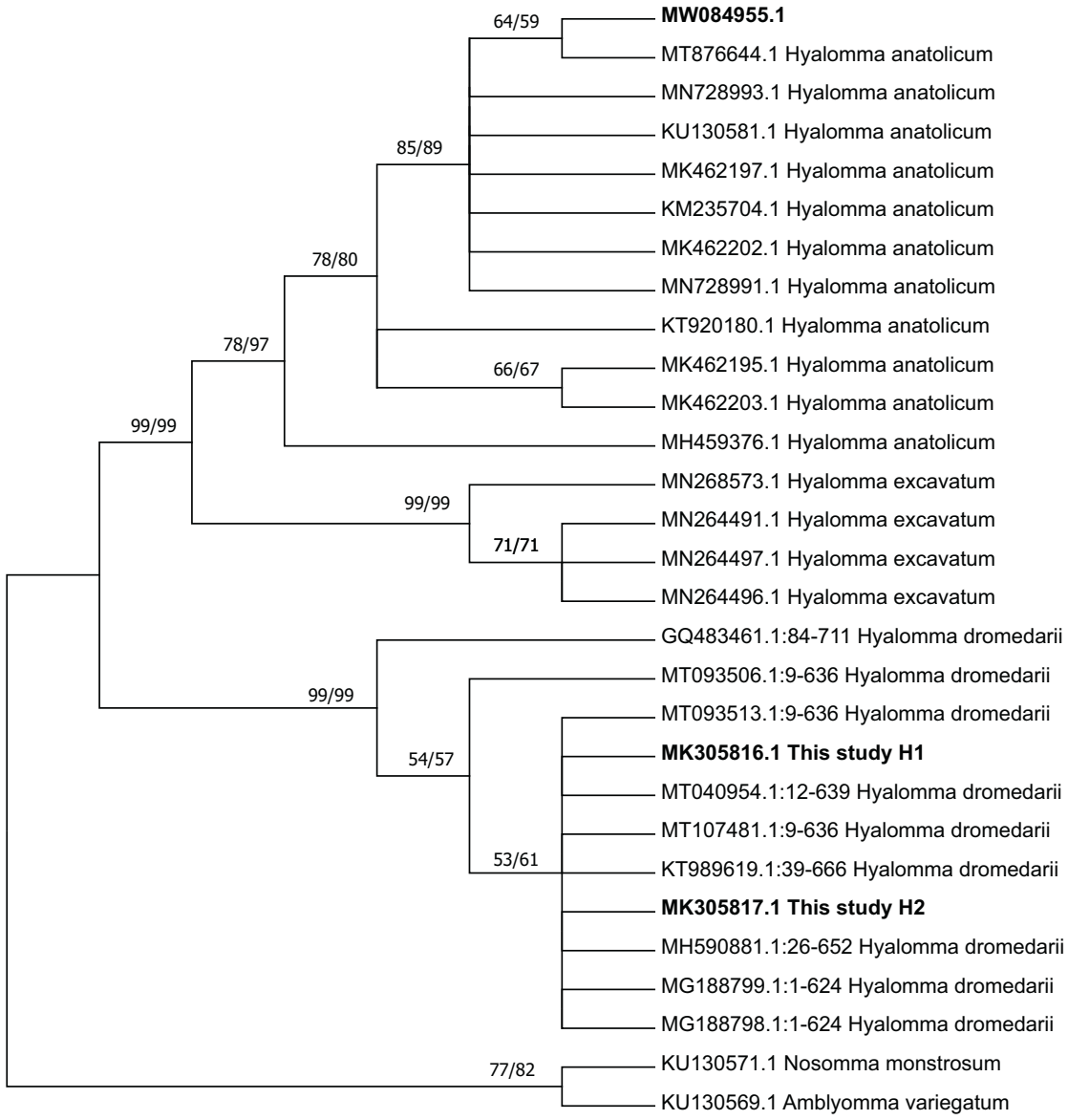

Fig. 3. A consensus phylogenetic tree constructed with maximum-likelihood (ML) and neighbor joining (NJ) methods, demonstrating phylogenetic relationships of the two tick species identified in the present study and related species of ticks, with *Nosomma monstrosum* and *Amblyomma variegatum* as outgroups inferred from *COI* gene sequence data generated from *H. dromedarii* (2 sequences) and *H. anatolicum* (1 sequence) and other taxa from GenBank. Numbers indicated at branch nodes are bootstrap values and the posterior probability (ML /NJ). Only bootstraps > 50% are shown. New sequences are shown on bold.

the GenBank together with outgroups (*Nosomma monstrosum* and *Amblyomma variegatum*) are given in Table 1. *H. dromedarii* and *H. anatolicum* were separated into two distinct clades. *H. anatolicum* formed a distinct group with its morphologically related species *H. excavatum* (Fig. 3).

3.3. PCR detection and molecular characterization of piroplasms

Amplification of disease agents (*Theileria*/*Babesia* spp.) from the DNA extracted from ticks collected from cows revealed positive results from 20 samples indicating a prevalence rate of 40%. Positive samples were only from ticks which were collected from cows. Of the positive samples, 3 were from *H. dromedarii* and 17 were from *H. anatolicum*. The prevalence in *H. dromedarii* was 5.4% (3 out of 56) of the total number examined of this species whereas it was 38.6% (17 out of 44) in *H. anatolicum*. No amplification was detected from ticks which were collected from camels indicating absence of piroplasm DNA in the extracted samples.

Sequencing of the amplified product showed DNA which is identical to *Theileria annulata*. A representative DNA sequence obtained from sequencing of the 18S rDNA resulted from amplification of DNA from ticks using primers which amplify *Theileria*/*Babesia* DNA was deposited at the GenBank database with the accession number: MK300062. The sequence obtained was identical to several other *T. annulata* deposited in GenBank (Table 2).

None of the sequences obtained as a result of the amplification of the PCR product resulted from using primers which amplify *Theileria*/*Babesia* spp. showed DNA related to *Babesia* spp.

The piroplasm detected in both *H. anatolicum* and *H. dromedarii* from cattle was found to be related to *T. annulata*. The DNA sequence obtained grouped with DNA sequences obtained from *T. annulata* from Algeria, Tajikistan, India, Turkey, Pakistan and China. However, it was not grouped with *T. lestoquardi*, *T. buffeli*, *Theileria* sp. (Yokohama isolate), *T. parva*, *T. ovis* and *T. taurotragi* which clustered separately (Fig. 4).

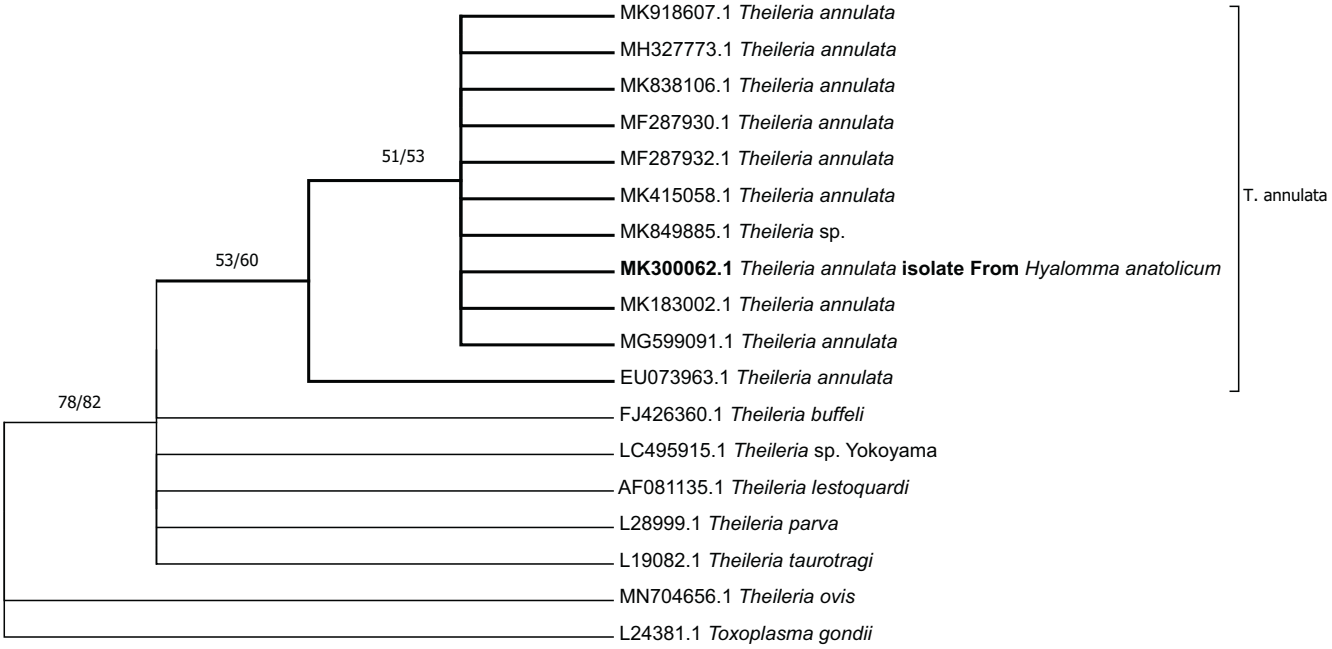

Fig. 4. Phylogeny of the partial *18S rRNA* gene. A maximum likelihood (ML) and a neighbor joining (NJ) phylogenetic trees were constructed using partial *18S rRNA* gene representative sequence determined in the present study. Bootstrap values ML/NJ were given on branches. The DNA of the organism *T. annulata* from other countries as well as other *Theileria* spp. and the out group (*Toxoplasma gondii*) were retrieved from GenBank. The sequences obtained in the present study grouped with other *T. annulata* obtained from other countries. New sequence is shown on bold.

4. Discussion

In the present study morphological identification of ticks infesting cattle and camel in Hofuf was conducted. The identification based on morphological characters was performed following the keys of indicated in the references (Hoogstraal et al., 1981; Al-Khalifa et al., 1984; Diab et al., 2006). The identification was further confirmed by DNA-based molecular method through PCR and sequencing of the cytochrome oxidase subunit I region of the mitochondrial DNA of ticks.

Ticks which infest cattle and camel were identified as *Hyalomma anatolicum* and *Hyalomma dromedarii* based on morphological features (Hoogstraal et al., 1981). Both *H. dromedarii* and *H. anatolicum* were found in both animal species studied. Al-Afaleq et al. (2018) reported ticks which they identified as *H. dromedarii* and *H. anatolicum* from camel in the eastern province. The DNA characterization confirms the identity of *H. anatolicum* and *H. dromedarii*. *H. dromedarii COI* DNA sequences obtained from ticks collected from the camels and cattle in the eastern province were found to be related to those obtained from *H. dromedarii* from other parts of the world. They have been reported from camel, sheep, goat, cattle and horse in Sudan and Saudi Arabia (El Ghali and Hassan, 2010; El Tigani and Mohammed, 2010; Alanazi et al., 2018). Finding *H. dromedarii* in cattle was not surprising as it was originally a camel parasite. On a previous study, *H. dromedarii* has been reported to be associated with cattle although it chiefly infests camel (Walker et al., 2003).

COI sequences obtained from *H. dromedarii* in the present study were found to be identical to sequences from *H. dromedarii* in the GenBank. However, one isolate showed one transition mutation at the position 615 bp and showed a nucleotide C (MK305816) instead of a T (MK305817) nucleotide. There was no obvious morphological difference in this particular tick when studied before extraction. Furthermore, it has not changed the protein sequence resulted from the translation of the DNA sequence.

On the other hand, sequences obtained from ticks collected from cattle were identical to sequences related to *H. anatolicum* from different localities for the same region of *COI*, hence confirming morphological identification. Detection of *Bos Taurus*, *Camelus dromedarius* and the Japanese quail (*Coturnix japonica*) *COI* sequences from *H. anatolicum* ticks indicated that these ticks may have fed on a cow, a camel or Japanese quail, before it landed on the cow.

DNA of *T. annulata* was detected in 40% of the ticks investigated in the present study. *T. annulata* DNA was amplified from both *H. anatolicum* and *H. dromedarii* ticks collected from cattle. Tropical theileriosis caused by *T. annulata* has been reported from Saudi Arabia on previous studies (Al-Atiya et al., 1991; Hussein et al., 1991; Al-Khalifa et al., 2009; Omer et al., 2003). The detection was based on demonstration of the organism on Giemsa-stained blood smears together with the associated clinical signs and the hematological changes in diseased animals. It has been reported from the eastern province as well as the central region of Saudi Arabia (Al-Atiya et al., 1991; Al-Khalifa et al., 2009; Omer et al., 2003). No molecular investigation on the tropical theileriosis has been conducted in Saudi Arabia. This finding is considered the first molecular characterization report on tropical theileriosis in the Kingdom of Saudi Arabia. Furthermore, it has also documented the role of *H. anatolicum* as a main vector for the parasite. It has also shown that the camel tick *H. dromedarii* is capable of transmitting the infection in cattle and can be a potential vector in transmitting tropical theileriosis in Saudi Arabia. Previously, *H. dromedarii* nymphs were able to transmit the disease experimentally in Sudan (Mustafa et al., 1983).

None of the ticks collected from camels revealed any amplifiable DNA related to *Theileria* or *Babesia* spp. confirming the finding of the inability of camels to be infected with these organisms when injected with them (Wernery and Kaaden, 2002). However, it is not possible to speculate that camels are not infected with these piroplasms in the light of the information indicating that DNA of *T. equi, T. mutans, T. ovis* and *B. caballi* are found in the blood of camels from different parts of the world (Qablan et al., 2012; Youssef et al., 2015; Lorusso et al., 2016; Hassan et al., 2017). Unlike what we have reported in the present study, Ismael et al. (2014) reported

what they identified as *T. camelensis* from blood smears prepared from 38.7% of camels they investigated in the Riyadh area. Further work and a Kingdom wide study is required in order to document that ticks in Saudi Arabia are probably suitable hosts for camel piroplasms or not?

The absence of *Babesia* spp. DNA in the current study is in congruence with the recent study by Ghafar and Amer (2019) when they were searching for the human pathogen *B. divergens* in cattle slaughtered in Taif abattoir. However, Al-Khailfa *et al.* (2009) reported detection of *B. bigemina*, based on microscopy, from cattle in Jizan area. Alanazi et al. (2020) did not detect neither *Theileria* spp. nor *Babesia* spp. from ticks collected from camels in the Riyadh province, Saudi Arabia. The inability to detect *Babesia* DNA in the current study could be explained by the fact that either both *H. dromedarii* and *H. anatolicum* are not suitable hosts to transmit *Babesia* spp. or the absence of *Babesia* infection from the eastern province of Saudi Arabia as Al-Khalifa et al. (2009) only detected it from cattle from Jizan where the conditions may be suitable for the organism and the host to survive.

5. Conclusion

Ticks infesting cattle and camel in Hofuf, eastern Saudi Arabia were identified using morphological methods and confirmed using molecular techniques. Molecular characterization confirmed the morphological description as *H. dromedarii and H. anatolicum*. The role played by both of *H. dromedarii* and *H. anatolicum* as vectors for *T. annulata* in cattle in eastern Saudi Arabia has been documented for the first time.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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