

## Molecular identification of ticks infesting camels and the detection of their natural infections with *Rickettsia* and *Borrelia* in Riyadh province, Saudi Arabia

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**Abstract.** The present work aimed to identify camel ticks *Hyalomma dromedarii* and *Hyalomma marginatum* using direct sequence of the mitochondrial 16S rRNA gene and the detection of their natural infection rate with *Rickettsia* and *Borrelia* using the PCR/ hybridization method for amplification of the citrate synthase (gltA) gene. The phylogenetic analysis showed 99% similarity between *Hyalomma dromedarii* and its reference with accession # L34306.1, as well as between *Hyalomma marginatum* and its reference with accession # KT391060.1 obtained from GenBank data base. The prevalence of *H. dromedarii* and *H. marginatum* was about 99% and 1%, respectively. The intraspecific variation among *H. dromedarii* ranged between 0.2–6.6%. The interspecific variation between *H. dromedarii* and *H. marginatum* was 18.3%. PCR/hybridization of the sampled *H. dromedarii* detected about 31%, 37% and 18% natural infection with *Rickettsia*, *Borrelia* and co-infection with both pathogens, respectively. In contrast, none of *Rickettsia* or *Borrelia* was detected in *H. marginatum*. The present study emphasizes the accuracy of the identification of camel ticks based on molecular techniques. The ability of *H. dromedarii* to spread more than one disease is an important issue from the epidemiological standpoint. Future epidemiological research should be carried out in Saudi Arabia to monitor the distribution of tick species and suggest effective control strategies.

### INTRODUCTION

Ticks are vectors of *Rickettsia* and *Borrelia* (Keskin *et al.*, 2016; Koetsveld *et al.*, 2016). *Rickettsia* is the causative agent of many diseases such as Rocky Mountain spotted fever and African tick-bite fever (Sambou *et al.*, 2014), while *Borrelia* is the causative organism of Lyme disease (Tilly *et al.*, 2009). Adult ticks can pass infection to the next generation through trans-ovarian transmission, which may increase the incidence of spreading the infectious agents (Raoult & Roux, 1997). Moreover, tick infestation results in increased calf mortality (Nelson *et al.*, 1977; Schwartz & Wilson, 1983).

Desert countries, for example, Saudi Arabia pay attention to studying infestation

of camels with ticks, where camels are an important source of meat and milk. Most of the previous studies on ticks that may attack camels in different regions of Saudi Arabia were focusing on their morphological identification and epidemiology (Diab *et al.*, 2006; Al-Khalifa *et al.*, 2007; Alanazi *et al.*, 2018). Molecular identification of ticks is widely used based on several nuclear and mitochondrial markers which allow differentiation between closely related species and determination of evolutionary relationships among ticks (Lv *et al.*, 2014; Hornok *et al.*, 2015; Kaur *et al.*, 2016). Among mitochondrial genes, 16S rRNA gene has proved to be a good marker to identify different tick species (Lv *et al.*, 2014; Pesquera *et al.*, 2015; Kaur *et al.*, 2016; Li *et*

*al.*, 2017; Jiang *et al.*, 2018). Citrate synthase (*gltA*) gene has been used by several authors to detect the incidence of *Rickettsia* in ticks (Demoncheaux *et al.*, 2012; Špitalská *et al.*, 2014; Abdullah *et al.*, 2016).

The current study aimed to identify camel ticks collected from Riyadh region, Saudi Arabia using direct sequence of the mitochondrial 16S rRNA gene, and the detection of the incidence of their natural infection with *Rickettsia* and *Borrelia* using PCR/ hybridization method for the amplification of the citrate synthase (*gltA*) gene. Study of the pathogen-vector system is an important issue from the epidemiological standpoint and for developing an approach to control the vector.

## MATERIALS AND METHODS

### Ethics statement

This study does not involve endangered or protected species or any studies with human participants performed by any of the authors. Camel ticks were collected from a private farm after permission given by the owner.

### Study area

Riyadh province has an area of 404,240 km<sup>2</sup> and is located in the central part of Saudi Arabia between 24°38'N and 46°43'E. It has a very hot summer with temperatures often approaching 50°C. The average high temperature in July is 45°C. Winter is cold with windy nights. The overall climate is arid, receiving very little rainfall (around 21.4 mm as an average) and with relative humidity ranging from 10–47% throughout the year. The province is also known to have many dust storms. It has one of the largest livestock populations in Saudi Arabia, accounting for 20% of the country's camels, 33% of its cattle, 17% of its sheep and 14% of its goats (Alanazi *et al.*, 2018).

### Tick sampling

During May 2014, camel ticks (n=100; adult males and females) were randomly collected manually with a cotton piece wet with 70% alcohol from a herd (n=20) of one-humped camel (*Camelus dromedarius*) mostly

females, kept in a farm located in Riyadh province, Saudi Arabia. All collected specimens were morphologically identified according to Hoogstraal *et al.* (1981). Then different samples were separately preserved in 70% ethanol and stored at -20°C for further molecular identification according to methods of Demoncheaux *et al.* (2012).

### DNA isolation and PCR protocol

DNA was extracted from individual tick specimens (n=100) with QIAmp<sup>®</sup> Micro Kit (Qiagen, Valencia, ca USA) as described by the manufacturer. The extracted DNA was stored at -20°C for further use.

A fragment of about 460 bp of the mitochondrial 16S rRNA gene was amplified with 16S+1 (5'-CCG GTC TGA ACT CAG ATC AAG T-3') as a forward primer and 16S-1(5'-CTG CTC AAT GAT TTT TTA AAT TGC TGT GG-3') as a reverse primer (Black & Piesman, 1994). The mixture of PCR contained 4 µL of 5× PCR GoTaq<sup>®</sup> Green Master Mix (Nippon Genetics, Europe GmbH), 0.6 µL (final concentration of 10 µM/µL) of each primer, 2 µL of DNA template and 12.8 µL of nuclease free water in a total volume of 25 µL. PCR was performed in a gradient thermal cycler (Peqlab Biotechnologie GmbH, Germany) according to the following protocol: initial denaturation at 95°C for 5 min, followed by 35 cycles; each consisted of denaturation at 95°C for 45 sec, annealing at 52°C for 45 sec, extension at 72°C for 45 sec, and a final extension step for 10 min at 72°C. All applications were comparable with a negative control containing no DNA template. The concentration and purity of the extracted DNA were determined with a NanoDrop 2000 UV-Vis spectrophotometer (Thermo Fisher Scientific Inc., USA). PCR products were stained with ethidium bromide, and then analyzed on 1.5% (w/v) agarose gel with a 100 bp DNA ladder (Solis Biotdyne) for 10 v/cm for 40 min. Gels were visualized under UV light trans-illuminator gel documentation system. All extracted PCR products were stored at -20°C for further analysis.

### Sequencing and bioinformatics analysis

PCR products with the same PCR primers were sent for sequencing in the Central Lab,

King Saud University, Female Section. The chromatograms were edited with BioEdit Sequence Alignment Editor according to the method of Burland (2000) where the DNA sequences were aligned by the ClustalW tool incorporated into MEGA 6 software. All sequences obtained were identified by comparing with those available in GenBank with the NCBI BLAST tool. Based on the sequences of 16S rRNA gene, one sequence from each identified tick species was randomly selected, and then submitted and registered to GenBank.

The evolutionary history was inferred using the Maximum Likelihood method (Tamura & Nei, 1993). The tree with the highest log likelihood (-774.2360) is shown. Initial tree(s) for the heuristic search was obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log Likelihood value. The analysis involved four nucleotide sequences. Codon positions included were 1<sup>st</sup> + 2<sup>nd</sup> + 3<sup>rd</sup> + nNoncoding. All positions containing gaps and missing data were eliminated. There were a total of 399 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al.*, 2013).

#### PCR amplification/hybridization

Extracted DNA of each tick specimen was used for the detection of both *Rickettsia* and *Borellia* with two different specialized kits, based on the amplification of the citrate synthase (gltA) gene. The first kit was rapidSTRIPE *Rickettsia* Assay and the second kit was rapidSTRIPE *Borrelia* Assay, both from Biometra Analytik Jena, Germany. The protocol described by the manufacturer was followed. In each reaction both positive and negative controls were added. The PCR machine was adjusted for 42 cycles after an initial denaturation at 95°C for 2 min. Each cycle consisted of denaturation at 95°C for 30 sec, annealing at 57°C for 30 sec, and extension at 72°C for 1 min. A final denaturation step was added at 95°C for 5 min and at 45°C for 10 min to allow to the

amplified DNA to attach to the probe provided with the kit. The products were stored at -20°C.

A 10-µL aliquot of each reaction product was added to a specialized strip provided with the kit and incubated at room temperature for one minute. Finally, 150 µL of running buffer was added to each strip and incubated in specialized tubes (provided with kits) at room temperature for 20 min. The results were recorded.

## RESULTS

Analysis of PCR products revealed that most of the tick specimens gave a band approximately 470 bp length. However, five bands did not give any PCR product profile as confirmed with the agarose gel electrophoresis (Figure 1).

Phylogeny tick samples from family of Hyalomminae were inferred using the 16S rRNA mitochondrial gene sequences (Figure 2). Seven reference species were used for comparison with the query sequences including *Hyalomma dromedarii* (GenBank ID MG972372.1, L34306.1, KU130423.1, KU130425.1, MG7575400.1, KU130424.1, KY945490.1) and *Hyalomma marginatum* (GenBank ID KT391060.1, L34307.1, KT391059.1, KP776645.1, KR870973.1, KU130447.1, KU130448.1). However, four randomly chosen sequences of *H. dromedarii* (1-4) were incorporated in phylogenetic tree, whereas only one species was identified as *H. marginatum* and included in the tree. As outgroup references, two members of subfamily Haemaphysalinae were selected including the *Haemaphysalis humerosa* (Genbank ID JX573138.1) and *Haemaphysalis longicornis* (GenBank ID KX083342.1). With respect to evolutionary analysis, the bootstrap values indicated the strong support along the nodes in the ML phylogenetic tree. The two identified subspecies of the genus *Hyalomma* are not showing the divergent evolutionary relationship. Furthermore, the isolates taken from both species are closely related to each other, indicating the similarity with reference to the molecular and

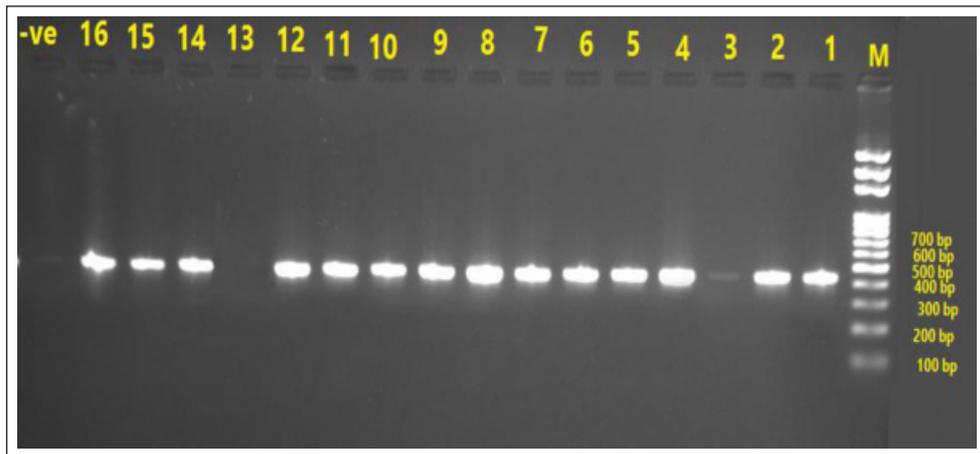


Figure 1. Analysis of PCR products on 1.5% (w/v) agarose gel of 16S rRNA gene. M: DNA marker; -ve: negative control; specimens 1, 2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15 and 16 show bands of 470 bp. Specimen 3 shows a very weak band and specimen 13 does not show any band.

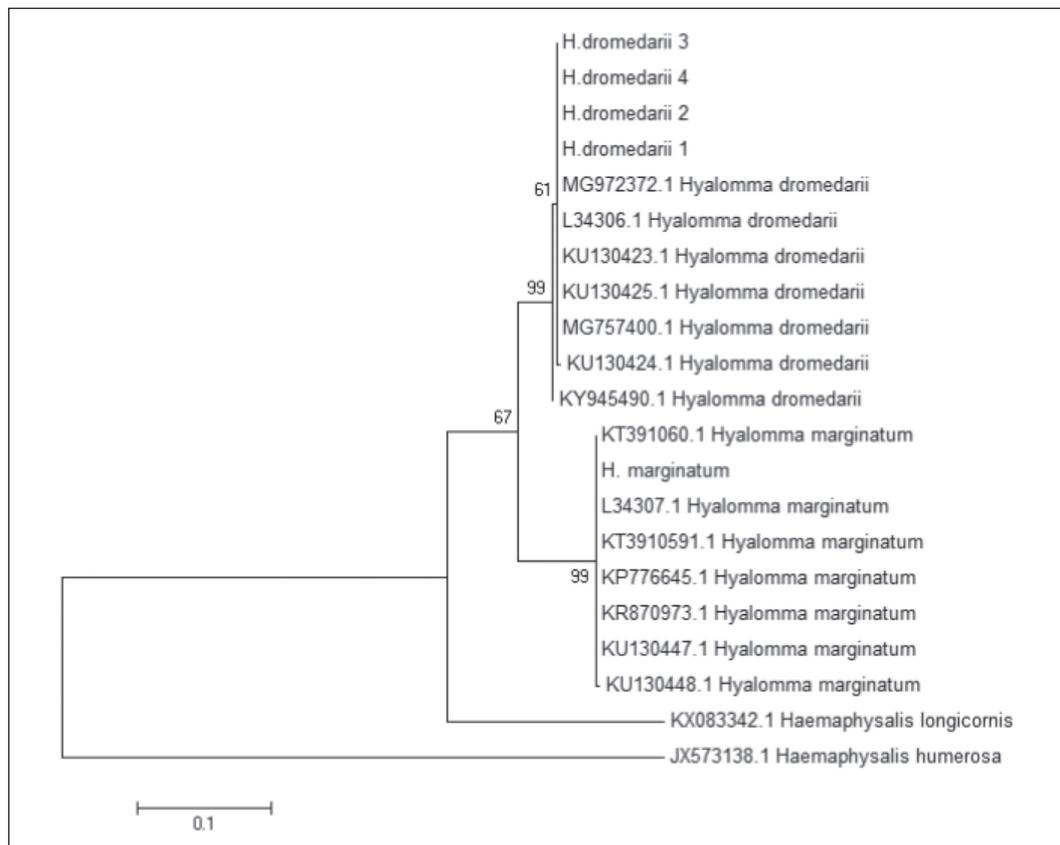


Figure 2. Maximum likelihood based phylogenetic tree of *Hyalomma dromedarii* and *Hyalomma marginatum* ticks with respect to Tamura-Nei model, using the fragment of mitochondrial 16S rRNA gene sequences. Node support representing the percentage of replicate trees in which the associated taxa clustered together in the bootstrap iterations of 1000 is indicated before each node. The analysis involved 21 nucleotide sequences. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA6.0.

Table 1. Percentage of infection of *Hyalomma dromedarii* and *Hyalomma marginatum* with *Rickettsia* and *Borrelia* using PCR /hybridization kit

Tick species	No. of identified specimens	% Infection		
		<i>Rickettsia</i>	<i>Borrelia</i>	<i>Rickettsia</i> + <i>Borrelia</i>
<i>H. dromedarii</i>	94	30.5	36.8	17.9
<i>H. marginatum</i>	1	0	0	0

morphological data of the specimens of ticks collected from camels from different regions of Saudi Arabia (Figure 2).

The results of 16S rRNA sequence showed that out of 95 molecularly identified samples, 94 specimens were found to be *H. dromedarii* with accession # 2088592 and one specimen was *H. marginatum* with accession # 2088597. The prevalence of the two species was 98.95% and 1.05%, respectively.

In the present survey, genetic relationships, based on direct sequences of 16S rRNA as a marker for molecular characterization of the two *Hyalomma* species, revealed intraspecific variation within *H. dromedarii* ranged between 0.2–6.6%. The interspecific variation between *H. dromedarii* and *H. marginatum* reached 18.3%.

Subsequently, PCR/hybridization technique for the natural infection with *Borrelia* and *Rickettsia*, based on the amplification of the citrate synthase (*gltA*) gene in surveyed *H. dromedarii* (n=94), detected 30.5% and 36.8% infection with *Rickettsia* and *Borrelia*, respectively. Additionally, 17.9% co-infection with both pathogens was detected (Table 1). In contrast, no infection with *Borrelia* or *Rickettsia* was detected in the sole specimen of *H. marginatum* (Table 1).

## DISCUSSION

The current study showed that *H. dromedarii* was the predominant identified tick species (about 99%). The molecular identification supports the previous morphological identification of this tick species which indicated that *H. dromedarii* is the predominant tick species infesting camels in Saudi Arabia (Diab *et al.*, 2006; Al-Khalifa

*et al.*, 1984, 1987, 2006, 2007). *H. dromedarii* is the predominant species in other Middle East countries, as well (Loftis *et al.*, 2006; Elghali & Hassan, 2009; Nazifi *et al.*, 2011; Demoncheaux *et al.*, 2012; Djerbouh *et al.*, 2012).

Our results indicated that *H. marginatum* is a rare species as it represents about 1% of collected and identified species. These results are in agreement with the results of several authors that showed low population of this tick species in Saudi Arabia (Diab *et al.*, 2006; Al-Khalifa *et al.*, 1984, 2007). This result could be explained based on the suggestion of Hoogstraal *et al.* (1981) that Riyadh region lacks the environmental conditions that are favorable for the development of *H. marginatum* such as temperature, humidity, precipitation, vegetation, and favorite hosts. Elghali & Hassan (2009) reported that camel is not the favorable host to *H. marginatum*; thus low population of this tick species might be expected.

The intraspecific genetic variation within *H. dromedarii* (>6%) herein is suggestive of cryptic species. In agreement with our results, several authors recently have reported the prevalence of sibling/cryptic species in different hard tick genera including *Hyalomma* (Liu *et al.*, 2013; Burger *et al.*, 2014). COI gene has been found useful for uncovering cryptic lineages in ticks. For example, the study of Low *et al.* (2015) for haplotype network and phylogenetic analyses based on COI+16S rRNA sequences has resulted in four genetically divergent groups among Malaysian *Rhipicephalus microplus*, and the significantly low genetic differentiation and high gene flow among the populations of this tick species support the occurrence of genetic admixture.

In comparison to our results, lower and higher intraspecific variations within different species of *Ixodes* have been reported by several authors (Lv *et al.*, 2014; Lah *et al.*, 2014; Pesquera *et al.*, 2015). Intraspecific variation may be due to the individual ticks having a low bacterial burden, sex- or stage-specific differences, and the presence of PCR inhibitors (Jourdain *et al.*, 2015).

In the present study, the interspecific variation between *H. dromedarii* and *H. marginatum* was 18.3%. Lah *et al.* (2014) reported interspecific variation of 7.2–23.7% between different species of *Ixodes*.

The current study showed that about 31% and 37% of *H. dromedarii* were naturally infected with *Rickettsia* and *Borrelia*, respectively. Variable prevalence of *Rickettsia* and *Borrelia* in other hard tick species has been reported by several authors (Demoncheaux *et al.*, 2012; Djerbouh *et al.*, 2012; Keskin *et al.*, 2016; Koetsveld *et al.*, 2016; Kumsa *et al.*, 2016; Jiang *et al.*, 2018). The ability of *H. dromedarii* to transmit more than one pathogen (about 18% co-infection with *Rickettsia* and *Borrelia* in the present study) is an important issue from the epidemiological standpoint.

## CONCLUSION

The present study encourages the molecular identification of the camel ticks *H. dromedarii* and *H. marginatum* using direct sequence of the mitochondrial 16S rRNA gene, as well as the detection of the pathogens transmitted by them (*Rickettsia* and *Borrelia*) using the PCR/ hybridization method for the amplification of the citrate synthase (*gltA*) gene. These genetic markers appear to be an accurate tool; thus any possible confusion which may result from morphological identification of ticks can be avoided. The relative high infection rate of tick-borne rickettsiosis and borreliosis, and co-infection with both *Rickettsia* and *Borrelia* may cause high opportunities for human infections through zoonotic factors, particularly at mass gatherings, for example, travelling to Saudi Arabia or pilgrims in it. Climatic conditions and numbers of load of bacteria transmitted

by tick bites may determine the severity of clinical manifestations in humans. In Saudi Arabia, Bedouin shepherds and farmers are in continuous exposure to ticks parasitizing their domestic animals; thus they become more liable to rickettsiosis and borreliosis infection than other people. Future epidemiological research should be carried out in Saudi Arabia to monitor the distribution of tick species and suggest effective control strategies.

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