





SHORT COMMUNICATION

Pathogenic *Rickettsia* in ticks of spur-thighed tortoise (*Testudo graeca*) sold in a Qatar live animal market

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Abstract

The dissemination of vector arthropods harbouring zoonotic pathogens through the uncontrolled transboundary trade of exotic and pet animals poses an important threat to Public Health. In the present report, we describe the introduction of pathogenic *Rickettsia africae* and *R. aeschlimanni* in ticks removed from imported tortoises in Qatar. A total of 21 ticks were collected from pet spur-thighed tortoises (*Testudo graeca*) from Doha, May 2018, and studied for species identification and characterization of *Rickettsia* spp. Morphological and molecular analysis of ticks allowed their identification as *Hyalomma aegyptium*. Molecular analysis of partial *ompA* and *gltA* genes showed that *Rickettsia* sequences found on these ticks clustered with sequences classified as *R. aeschlimanii* and *R. africae*. Since pre-adult stages of *H. aegyptium* also feed on humans, this tick species may play a role in the transmission of *R. aeschlimanii* and *R. africae*. We alert for the introduction of non-native pets as vehicles for tick importation, known vectors for animal and human pathogenic agents. Importation of exotic species into non-autochthonous countries deserves strict control to enforce robust surveillance and mitigate potential exotic diseases epidemics.

KEYWORDS

Hyalomma aegyptium, *Rickettsia aeschlimanii*, *Rickettsia africae*, *Testudo* tortoise

1 | INTRODUCTION

The dissemination of vector arthropods harbouring zoonotic pathogens through the uncontrolled transboundary trade of exotic

and pet animals poses an important threat to Public Health (Fevre, Bronsvoort, Hamilton, & Cleaveland, 2006). Several epidemics have been traced back to the introduction of vector-borne agents in otherwise disease-free regions (Sergon et al., 2007; Yssouf et al., 2011), adding concerns to the potential emergence of infectious diseases in naive populations. As such, efforts in controlling

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the introduction of non-autochthonous species and in preparing regulatory systems to counter biological invasions have been made worldwide (Goka, Okabe, & Takano, 2013). Nonetheless and despite these efforts, the risk of exotic species introduction is still high due to the observed growing economic globalization, and thus, continuous surveillance programs have to be made to provide early warning on exotic pathogens introduction (Goka et al., 2013).

Rickettsia species are pathogens with zoonotic characteristics that are strictly intracellular, Gram-negative bacteria from the order Rickettsiales, comprising 30 recognized species and numerous uncharacterized sequences (Guo et al., 2019; Shpynov, Pozdnichenko, & Gumenuk, 2015).

Ticks are both vectors and reservoirs for several rickettsial agents and studies have reported the importation of tick-infested animals, which were later found to be carrying rickettsiae, many of which are pathogenic to humans (Erster, Roth, Avni, King, & Shkap, 2015; Ojeda-Chi et al., 2019; Parola et al., 2013).

A few studies have also reported the presence of *Rickettsia* spp. in ticks found in tortoises (Ehlers et al., 2016; Paştıu et al., 2012). Interestingly, a recent study has highlighted the introduction of *R. bellii* in *Amblyomma rotundatum* ticks infesting red-footed tortoise (*Chelonoides carbonaria*) (Erster et al., 2015). The infested tortoises were shipped to Ben Gurion Airport, Israel, having been legally imported from a pet trader in Florida, USA. The introduction of tick-infested tortoise, compliant with the legal requirements in the country, raises alerts on the facilitated introduction of pathogenic *Rickettsia* spp. In the present report, we describe the introduction of pathogenic *R. africae* and *R. aeschlimanni* in ticks collected from imported tortoises (*Testudo graeca*) in Qatar.

2 | MATERIALS AND METHODS

2.1 | Study area

Qatar is a small country located on the eastern side of the Arabian Peninsula. It is surrounded by the Persian Gulf with a land border at the south with Saudi Arabia. The landscape is generally sand flat with an arid desert climate with summer seasons occurring from May to September, and the hottest months having an average daytime maximum air temperature exceeding 50°C and night-time temperatures not dropping below 30°C. Rainfall is scarce (75.6 mm per year), falling with erratic patterns from October to March (Matzarakis & Frohlich, 2015).

2.2 | Ticks

A total of 21 ticks were directly removed from two pet tortoises (*T. graeca*), that had been acquired from one of Qatar's largest animal markets just before presentation at Parkview Pet Center Veterinary Clinic for a health check and ectoparasitic control in Doha, May 2018. After removal, ticks were immediately stored in 70% ethanol at room temperature until further investigation. Tick identification

was performed using the morphological criteria already described (Apanaskevich, 2003). To further characterize ticks at the species level and to ensure the successful DNA extraction for pathogen screening, tick specimens' DNA was extracted and screened using mitochondrial genes as molecular targets.

2.3 | Nucleic acid extraction

Tick individual DNA was extracted using alkaline hydrolysis according to previously described methods (Schouls, Pol, Rijpkema, & Schot, 1999). Conventional PCRs targeting partial regions of the 12S rDNA (Szabó, Mangold, João, Bechara, & Guglielmo, 2005) and 16S rDNA (Black & Piesman, 1994) were performed as previously described (Coimbra-Dores et al., 2018).

2.4 | Detection of rickettsial DNA in ticks

Tick DNA specimens were initially screened for the presence of spotted fever group *Rickettsia* using a conventional PCR targeting a broad spectrum 511 bp fragment of the outer membrane protein B (*ompB*) gene, as previously described (Choi et al., 2005). To confirm positive results and genetically characterize *Rickettsia* spp., ticks were further tested for a 532 bp fragment of the outer membrane protein A (*ompA*) gene (Regnery, Spruill, & Plikaytis, 1991) and for a partial 381 bp fragment of the citrate synthase (*gltA*) gene (Regnery et al., 1991). Amplification of the near complete (806 bp) *gltA* gene was also attempted (de Sousa et al., 2005). For PCR the KAPA HiFi HotStart ReadyMix, KAPA Biosystems (Woburn, MA, USA) was used according to the manufacturer's instructions.

2.5 | Sequencing and phylogenetic analysis

All *Rickettsia*-positive amplicons and both 12S/16S rDNA amplicons obtained were sequenced for genetic characterization. Amplicons were purified with ExoSAP-IT® (Affymetrix), and bi-directional sequencing was performed by Sanger method at genomics core facility of Institute of Molecular Pathology and Immunology of the University of Porto. Sequence editing and multiple alignments were performed with the BioEdit Sequence Alignment Editor v7.1.9 software package, version 2.1 (Ibis Biosciences), and further analysis was performed by comparison with the sequences available in the NCBI nucleotide database (<http://blast.ncbi.nlm.nih.gov/Blast>). Phylogenetic analysis was performed using MEGA version 6.0 software (Tamura, Stecher, Peterson, Filipinski, & Kumar, 2013). The *gltA* gene and *ompA* gene sequences identified in this study and representative sequences for the *R. africae* and *R. aeschlimanii* obtained from GenBank were used for the phylogenetic analysis. A maximum-likelihood (ML) (Kumar, Stecher, Li, Knyaz, & Tamura, 2018) method was applied. The ML bootstrap values were estimated using 1,000 replicates with Tamura 3-parameter as the correction model. Tamura 3-parameter model was estimated as the best substitution model by MEGA version 6.0 software.

3 | RESULTS

3.1 | Morphological and molecular identification of ticks

All 21 ticks collected from the two *T. graeca* were females, sharing identical morphological features. General morphological features included a wide arch with straight posterior margin genital orifice; a funnel-like swollen vestibular part of vagina; stick-like setae of alloscutum, tapering in the apical region; second segment of palps with proximal narrowing; and spurs of coxae I widely separated, triangular and wide. Morphological analysis of ticks allowed the

identification as *Hyalomma aegyptium*. BLAST analysis of the 16S and 12S segments obtained from both ticks showed 100% nucleotide identity with *H. aegyptium* (GenBank accession no. MG418680) and 99% with *H. aegyptium* (GenBank accession no. AF031854), respectively.

3.2 | Identification of Rickettsia in the examined ticks

Of the total ticks ($n = 21$) screened for *Rickettsia*, three (14%) showed to be positive for *ompB* gene. To confirm positive results by *ompB*

FIGURE 1 Phylogenetic analysis of *Rickettsia* spp. identified in *Hyalomma aegyptium* ticks. A maximum-likelihood method based on the Tamura 3-parameter model phylogenetic tree was constructed based on *Rickettsia ompA* DNA sequences. *Rickettsia* spp. characterized in this study are shown as Qatar/tick species/*Rickettsia* ([QtHyaegR] 1 and 16—*R. aeschlimanii*; 9—*R. africae*). Reliability of internal branches was assessed using the bootstrapping method (1,000 replicates)

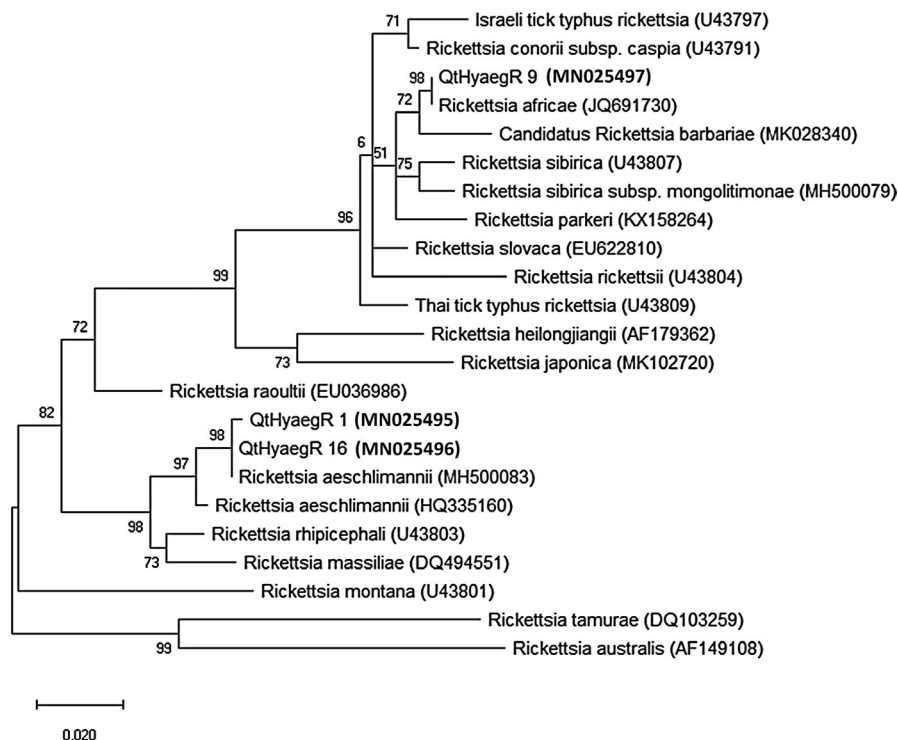
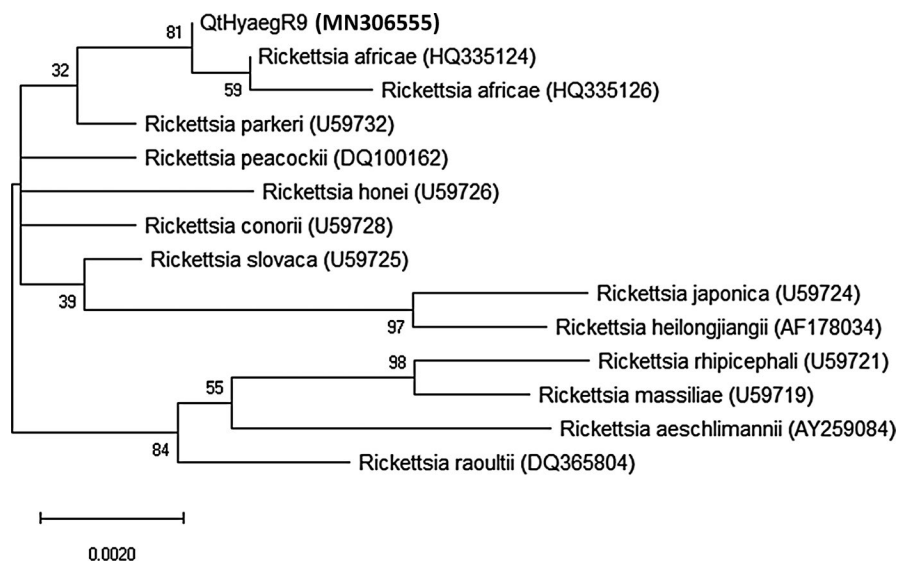


FIGURE 2 Phylogenetic analysis of *Rickettsia* spp. identified in *Hyalomma aegyptium* ticks. A maximum-likelihood method based on the Tamura 3-parameter model phylogenetic tree was constructed based on *Rickettsia gltA* DNA sequences. *Rickettsia* spp. characterized in this study are shown as Qatar/tick species/*Rickettsia* ([QtHyaegR] 9—*R. africae*). Reliability of internal branches was assessed using the bootstrapping method (1,000 replicates)



gene and to genetically characterize *Rickettsia* at the species level, ticks were further studied in the *ompA* and *gltA* gene regions. BLAST analysis of the obtained *ompA* and *gltA* sequences from one tick showed 100% nucleotide identity with *R. africanae* in the *ompA* region (GenBank accession no. JQ691730), 100% nucleotide identity with *R. africanae* in the 381bp partial *gltA* region (GenBank accession no. HQ335126) and 99.9% nucleotide identity with *R. africanae* in the near complete (806 bp) *gltA* gene (GenBank accession no. HQ335124).

Regarding the other two *ompB* positive ticks, BLAST analysis of the *ompA* and 318 bp partial *gltA* sequences showed that both shared 100% nucleotide identity with *R. aeschilimannii* in the *ompA* region (GenBank accession no. MH500083) and 99%–100% nucleotide identity with *R. aeschilimannii* in the partial *gltA* region (GenBank accession no. MH932014). No amplification was obtained with the primers for the near complete *gltA* gene in these two *R. aeschilimannii* ticks.

R. aeschilimannii-positive ticks were found in the same *T. graeca*, while the *R. africanae*-positive tick was found in another *T. graeca*. Phylogenetic analysis was performed for both partial *ompA* (Figure 1) and near complete *gltA* gene (Figure 2) sequences in order to obtain information about their genetic relatedness with other *Rickettsia* species reference sequences. The following accession numbers were assigned to the sequences obtained in this work: MN025495 and MN025496 (*ompA* gene fragments of *R. aeschilimannii*), MN025497 (*ompA* gene fragment of *R. africanae*), MN025499 and MN025500 (partial *gltA* gene fragments of *R. aeschilimannii*), and MN306555 (near complete *gltA* gene fragment of *R. africanae*).

4 | DISCUSSION

The present study shows the circulation of rickettsiae in ticks infesting pet spur-thighed tortoises (*T. graeca*) sold in a live animal market in Qatar. Molecular analysis of a 532 bp stretch of the *ompA* gene and partial 381 bp long sequence of the *gltA* gene showed that *Rickettsia* sequences found in these ticks clustered with sequences classified as *R. africanae* and *R. aeschilimannii*. Analysis of the near complete *gltA* gene was only possible on the previously determined *R. africanae* and confirmed the classification as *R. africanae*.

Morphological and molecular analysis of ticks also allowed the identification as *H. aegyptium*, known for predominantly infest tortoises (genus *Testudo*), most frequently *T. graeca* (Siroky, Erhart, Petrzalkova, & Kamler, 2011). Since the pre-adult stages of *H. aegyptium* also feed on humans (Vatansver, Gargili, Aysul, Sengoz, & Estrada-Pena, 2008), this tortoise tick may play a role in the transmission of *R. aeschilimannii* and *R. africanae*. *H. aegyptium* geographic distribution is the same of their tortoise hosts and extends from northwestern Africa, the Mediterranean region, the Balkans, Turkey, the Middle East, the Caucasus, Central Asia, Afghanistan and Pakistan (Siroky et al., 2011). To the best of our knowledge, pet spur-thighed tortoise are not native to Qatar and were hence imported.

Tick-borne rickettsioses are considered reemerging zoonoses, being increasingly recognized in many countries worldwide as a cause of significant morbidity among infected individuals (Parola et

al., 2013). *R. africanae*, the agent of African tick bite fever, is the second most frequent cause of systemic febrile illness among travellers returning from endemic areas and has been previously described in *H. aegyptium* ticks in humans and tortoises (Orkun, Karaer, Cakmak, & Nalbantoglu, 2014), thus supporting the possibility of humans sharing *R. africanae* tick vectors with these reptiles.

Interestingly, we have also detected the pathogenic *R. aeschilimannii* in *H. aegyptium* ticks infesting tortoise. *R. aeschilimannii* is known for causing the classical triad of clinical manifestations of rickettsial infection that include fever, eschar and rash (Germanakis, Chochlakis, Angelakis, Tselentis, & Psaroulaki, 2013). Noteworthy, the number of published cases of human infection by *R. aeschilimannii* is on the rise (Mokrani et al., 2008).

Our study reports for the first time the detection of *R. africanae* and *R. aeschilimannii* in *H. aegyptium* ticks collected from pet spur-thighed tortoises, in Qatar, contributing for an extent of the geographic location of these pathogenic rickettsiae.

The dissemination of the vectors of zoonotic infectious diseases through the uncontrolled transboundary trade of pet animals poses significant Public Health threats. In particular, the international trade of *Testudo* tortoises is of major importance as a vehicle for tick importation, namely *H. aegyptium*, vectors for human and animal pathogens (Mihalca, 2015). Importation of exotic species into non-autochthonous countries deserves strict control to enforce robust surveillance and mitigate potential exotic disease epidemics.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ETHICAL APPROVAL

Not applicable.

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