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...
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; Paștiu 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. aesculimanni* 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 Qatars’ 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, & Guglielmone, 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 bidirectional 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, Filipski, & Kumar, 2013). The *gltA* gene and *ompA* gene sequences identified in this study and representative sequences for the *R. africae* and *R. aesculimanni* 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 replications 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*...
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. africae in the ompA region (GenBank accession no. JQ691730), 100% nucleotide identity with R. africae in the 381 bp partial gltA region (GenBank accession no. HQ335126) and 99.9% nucleotide identity with R. africae 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. aeschilimanii in the ompA region (GenBank accession no. MH500083) and 99%–100% nucleotide identity with R. aeschilimanii 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. aeschilimanii ticks.

R. aeschilimanii-positive ticks were found in the same T. graeca, while the R. africae-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 to 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. aeschilimanii), MN025497 (ompA gene fragment of R. africae), MN025499 and MN025500 (partial gltA gene fragments of R. aeschilimanii), and MN306555 (near complete gltA gene fragment of R. africae).

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. africae and R. aeschilimanii. Analysis of the near complete gltA gene was only possible on the previously determined R. africae and confirmed the classification as R. africae.

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, Petrzclkova, & Kamler, 2011). Since the pre-adult stages of H. aegyptium also feed on humans (Vatanscver, Gargili, Aysul, Sengoz, & Estrada-Pena, 2008), this tortoise tick may play a role in the transmission of R. aeschilimanii and R. africae. H. aegyptium geographic distribution is the same of their tick 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 tortoises 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. africae, 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. africae tick vectors with these reptiles.

Interestingly, we have also detected the pathogenic R. aeschilimanii in H. aegyptium ticks infesting tortoise. R. aeschilimanii 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. aeschilimanii is on the rise (Mokrani et al., 2008).

Our study reports for the first time the detection of R. africae and R. aeschilimanii 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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