Molecular detection of spotted fever group rickettsiae in ticks parasitizing pet dogs in Shihezi City, northwestern China

Wurelihazi Hazihan1 · Zhihui Dong2 · Liping Guo3 · Kadyken Rizabek4 · Dzhunysov Askar4 · Kulmanova Gulzhan4 · Mahanov Kudaibergen4 · Akishev Nurlan Kenjebaevich4 · Tolegen Talgat4 · Kenesbay Kairullayev4 · Yuanzhi Wang2

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Abstract
A total of 178 adult ticks were collected from 32 pet dogs from five veterinary clinics in Shihezi City, Xinjiang Uygur Autonomous Region (XUAR), northwestern China. All the ticks were identified by comprehensive morphological and genetic analyses, and rickettsiae were detected by seven Rickettsia-specific genetic markers in the ticks. The ticks collected were identified as Rhipicephalus sanguineus sensu lato. Twenty-one of the 178 samples (11.8%) were positive for rickettsiae. Among these, in 13 (61.9%) samples Candidatus R. barbariae were identified, in five (23.8%) samples R. massiliae, and in three (14.3%) samples R. conorii. This study indicates that more attention should be paid to rickettsial infection in pet dogs and their ticks, because the latter may pose an epidemiological risk for tick-borne transmission of rickettsiae to human beings.

Keywords Rhipicephalus sanguineus sensu lato · Spotted fever group rickettsiae · Pet dogs · Northwestern China

Introduction
Ticks are among the most common ectoparasites of dogs, also involved in the transmission of a number of major diseases in both dogs and humans (Chomel 2011; Dantas-Torres and Otranto 2016). Tick-borne rickettsioses are caused by the spotted fever group rickettsiae (SFGR) of the genus Rickettsia, which contains approximately 20 species, and many of which are established or emerging human pathogens (Wood et al. 2012). Besides, more and
more new SFGR species have been found across the world, as a result of range expansion of tick populations, changes in landscape and climate, and more accurate diagnostic testing (Trotta et al. 2012; Yunik et al. 2015).

Due to the emerging and re-emerging nature of tick-borne diseases in humans, increasing focus has been placed on research of ticks parasitizing domestic animals (Hiraoka et al. 2005). As in many other countries, in China the dog has become a bonded family member. Regardless the benefits of having pet dogs, pathogens carried by ticks are potentially transmissible to humans, which may represent a health risk, especially to children, elderly people and immunocompromised individuals (Dantas-Torres and Otranto 2014). To date, at least three protozoan (Theileria, Babesia and Hepatozoon) and five bacterial (Anaplasma, Ehrlichia, Rickettsia, Coxiella and Bartonella) tick-borne genera have been reported in domestic dogs around the globe (Beck et al. 2009; Brown et al. 2006; Buharimwalla et al. 1996; Camacho et al. 2001; Conrad et al. 1991; Kaewkong et al. 2014; Kamani et al. 2013; Levin et al. 2012; Mokhtar et al. 2013; Yabsley et al. 2008). In Jiangxi Province, mid-eastern China, Babesia canis vogeli and Babesia gibsoni were molecularly detected in 780 dog ticks (749 Rhipicephalus sanguineus, 16 Haemaphysalis campaullata and 15 Haemaphysalis verticalis), while all sampled dog ticks were negative for rickettsial agents (Zheng et al. 2017). In Xinjiang Uygur Autonomous Region (XUAR), northwestern China, rickettsial agents were prevalent in ticks infesting both domestic animals and wildlife (Guo et al. 2015, 2016). However, there is limited knowledge on the species of ticks infesting dogs. Here a molecular investigation was carried out for rickettsial agents in pet dog ticks.

Materials and methods

Collection and identification of ticks

In 2016–2017, ticks were sampled from 32 pet dogs presented at five veterinary clinics with symptoms of depression, weight loss and anorexia in Shihezi City (483 m above sea level, at 44°26′21.2″N 86°06′27.4″E), the northwestern China. The ticks were placed in tubes with 75% ethanol and stored at −80 °C. All of the ticks were identified morphologically according to previous reports (Filippova 1997; Dantas-Torres et al. 2013a, b). Twenty-nine representative ticks, with 4–6 ticks at each veterinary clinic, were used to analyze tick species and genetic diversity based on partial mitochondrial 16S rRNA (460 bp), 12S rRNA (400 bp) and coxI (889 bp) gene sequences (Szabó et al. 2005; Chen et al. 2014).

DNA extraction and molecular detection

After detailed morphological analysis, genomic DNA was extracted from each individual tick using the TIANamp Genomic DNA Kit (TianGen, Beijing, China). The ticks were mechanically crushed twice in sterile water for 15 min and then dried on sterile paper, suspended in 200 µl tissue lysis buffer and 40 µl proteinase K (100 µg/ml), and incubated overnight at 56 °C. The final elution volume was 60 µl. Subsequently, the polymerase chain reaction (PCR) technology was used to detect rickettsial agents with seven genetic markers for DNA fragments [434-, 1332-, 1060-, 488-, 920-, 491-, and 812-bp products of the genes encoding the 17 kilodalton antigen (17-kDa), 16S rRNA(rrs), citrate synthase (gltA), surface cell antigen 1 (scat), PS120-protein-encoding gene (gene D), and outer membrane proteins A and B (ompA and ompB)] (Anstead and Chilton 2013; Chilton 2013; Sekeyova
et al. 2001; Wei et al. 2015). (Table 1). Rickettsia aeschlimannii from Rh. turanicus and double-distilled water were used, respectively, as positive and negative controls (Wei et al. 2015). The PCR products were purified using the TIANgel Midi Purification Kit (TIANGEN, Beijing, China), and then subjected to sequencing (BGI, Shenzhen, China). Phylogenetic analyses were conducted used MEGA version 6.0 based on the 17 kDa-rrs-gltA-ompA-ompB-gene D concatenated sequence data of the rickettsiae by Maximum Likelihood (ML) and Neighbor-Joining (NJ) methods (Tamura et al. 2013).

Results

A total of 178 adult ticks (76 males and 102 females) were collected and morphologically identified as Rh. sanguineus sensu lato. (Fig. 1). The sequencing data from the 29 representative ticks confirmed the morphological results based on Basic Local Alignment Search Tool (BLAST) analyses of 16S rRNA, 12S rRNA and cox1. Rhipicephalus sanguineus s.l. in this study had 93.3–93.8% pairwise nucleotide sequence identity to genome sequences of the reference strains Rh. sanguineus (GenBank: JX416325) for three genes analyzed. Our data were deposited in the GenBank database (16SrRNA: KY069269, 12S rRNA: KY069270, and cox1: KY069271).

Twenty-one of the 178 samples (11.8%) were positive for SFG rickettsiae. Of which, thirteen (61.9%) were identified as Candidatus R. barbariae, five (23.8%) as R. massiliae, and three (14.3%) as R. conorii subsp. indica. (Additional Table 2; Fig. 2). Rickettsia massiliae and R. conorii subsp. indica had 99.8–100% and 99.3–100% pairwise nucleotide sequence identities to the corresponding sequences of the reference strains R. massiliae (GenBank: CP000683) and R. conorii str. Malish 7 (GenBank: AE006914) for seven genetic markers, respectively. Candidatus R. barbariae in dog ticks showed 100% pairwise nucleotide sequence identity to the corresponding sequences of Candidatus R. barbariae in the flea Vermipssylla alakurt (according to the seven genetic markers, in GenBank: KT284715, KU645283, KT284716, KU645284, KT284717, KT284718, KU645286, respectively). Detailed similarities of the sequences in this study are shown in Additional Table 1. All the sequences of Rickettsia spp. obtained in this study were deposited in GenBank [17 kDa: KY069262–KY069264; rrs: KY069266–KY069268; gltA: KY069259–KY069261; scal: KY069254–KY069255, KY069265; ompA: KY069256–KY069258; ompB: KY069248–KY069250; gene D: KY069251–KY069253].

Discussion

In the present study, ticks collected from pet dogs were used to identify rickettsial agents in Shihezi City, northwestern China. Candidatus R. barbariae, R. conorii subsp. indica and R. massiliae were molecularly detected. Importantly, these rickettsial agents were shown to be present both in pet dog ticks (reported here) and in sheep ticks (Guo et al 2016), which data raise both veterinary and public health concerns in northwestern China.

Candidatus R. barbariae was originally reported from Rhipicephalus bursa ticks in Portugal (de Sousa et al. 2006), and later confirmed and characterized by five genetic markers (gltA, ompA, ompB, sca4 and rrs) from Rh. turanicus in Italy (Mura et al. 2008). Subsequently, Candidatus R. barbariae was also detected in Rh. turanicus from Cyprus and in Rh. sanguineus from Israel (Chochlakis et al. 2012; Waner et al. 2014). In 2016, our
| Target       | Gen     | Primer (reference) | Sequences (5′–3′)                   | Fragment length (bp) |
|--------------|---------|--------------------|-------------------------------------|----------------------|
| Tick         | 16S rRNA| T-16S(F)           | CTGCTCAATGATTTTTTTAAATTGCTGTGG     | 460                  |
|              |         | T-16S(R) (Chen et al. 2014) | CCGGCTCTGAACCTCAGATCAAGT      |                      |
|              | 12S rRNA| 12S(F)             | AAACCTAGGATGATCCTATATTAG        | 400                  |
|              |         | 12S(R) (Szabó et al. 2005) | CTATGCTACGACTTATACATTAAGAAGTG   |                      |
|              | coxI    | T/J-1,449          | AAATTACAGTTATGCCT               | 889                  |
|              |         | C1-N-2,312 (Chen et al. 2014) | CATAAAAAAGCCCTAATA             |                      |
| Rickettsia   | rrs     | R-16S(F)           | ATCAGTACGCCAATAACTTTTA          | 1284                 |
|              |         | R-16S(R) (Anstead and Chilton 2013) | TGGCCTCTTGGTTAGCCTAC        |                      |
|              | 17-kDa  | 17-kDa(5F)         | GCTTTAACAAAATTCTAAAACCATATA     | 434                  |
|              |         | 17-kDa(3R)         | TGTCTATCAACCACACTGGCCGT        |                      |
|              |         | 17-kDa(1F)         | GCTTTGCAACTCTATGTT             |                      |
|              |         | 17-kDa(2R) (Anstead and Chilton 2013) | CATTGTCTGCTAGGGTGCG           |                      |
|              | gltA    | gltA(F)            | ATGACCAAAGGAAAATAAAT            | 1078                 |
|              |         | gltA(R) (Anstead and Chilton 2013) | ATTCGAAAAAGTACAGTGCAACA    |                      |
|              | sca1    | sca1(F)            | GGTGATGAAAGAAGACTCTC           | 657                  |
|              |         | sca1(R) (Anstead and Chilton 2013) | CTCTTTAAAATATGGCTTAC         |                      |
|              | gene D  | gene D(F)          | CGTGAACCCTAGTACAGTGTA          | 920                  |
|              |         | gene D(R) (Sekeyova et al. 2001; Wei et al. 2015) | TATAAGCTTGGCCTCATCTC   |                      |
|              | ompA    | ompA(F)            | ATGCGGAATTTTCTCCAAA           | 530                  |
|              |         | ompA(R) (Anstead and Chilton 2013) | AGTGCAAGCTTGGCTCCTCCCT       |                      |
|              | ompB    | ompB(F)            | TACTTCCGGTACAGCAAGT            | 812                  |
|              |         | OmpB(R) (Anstead and Chilton 2013) | AAAACATAATCAAGGTACTGT       |                      |
investigation revealed that Candidatus R. barbariae is present in Vermipsylla alakurt fleas and Rh. turanicus ticks from grazing sheep (Guo et al. 2016; Zhao et al. 2016). Here, molecular evidence of Candidatus R. barbariae is provided in pet dog ticks (Rh. sanguineus s.l.).

The other two Rickettsia species, R. conorii subsp. indica and R. massiliae, had lower rates of positivity [1.7% (3/178) and 2.8% (5/178), respectively] compared to the data from grazing sheep (Wei et al. 2015; Guo et al. 2016), which might be explained by differences in tick numbers per host, as well as by varying susceptibility to rickettsiae among host species. To the best of our knowledge, however, the clinical cases were caused by R. conorii subsp. indica and R. massiliae (Cavagnaro et al. 2008; Vitale et al. 2006). Although there is no documented clinical case of rickettsia infection from pet dog ticks in China to date, more measures should be carried out to prevent its risk to dog owners, taking into account the synanthropic nature of Rh. sanguineus s.l. A diversity of tick-borne pathogens, including Anaplasma, Babesia, Borrelia, Ehrlichia and Theileriai spp. has recently been molecularly detected in Russia (Livanova et al. 2018). This, together with the present findings, draw the attention to not-yet known risks associated with tick-borne rickettsiae in several regions of Asia.
Conclusions

Three SFGR members, the *R. conorii* subsp. *indica*, *Candidatus* *R. barbaraiae* and *R. massiliiae*, were molecularly detected in *R. sanguineus* s.l. ticks from pet dogs in Shihezi City, northwestern China. The study expands the range of tick-borne pathogens in pet dog ticks in Central Asia. Effective measures should be taken into consideration to prevent tick-borne transmission of rickettsiae to human beings.

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Author contributions

YZW conceived and designed the study. LPG and KR critically revised the manuscript. HZ and ZHD analyzed the data and drafted the manuscript. DA and KG conducted the morphological test of dog ticks. MK, ANK, TT and KK conducted molecular analyses. All authors read and approved the final manuscript.
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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval This study was approved by the Animal Ethics Committee of Shihezi University (Approval No. AECSU2015-22).

Informed consent Informed consent was obtained from all the owners.

Availability of data and material The datasets supporting the conclusions of this article are included within the article and the newly-generated sequences were deposited in the GenBank database.

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Affiliations

Wurelihazi Hazihan1 · Zhihui Dong2 · Liping Guo3 · Kadyken Rizabek4 · Dzhunysov Askar4 · Kulmanova Gulzhan4 · Mahanov Kudaibergen4 · Akishev Nurlan Kenjebaevich4 · Tolegen Talgat4 · Kenesbay Kairullayev4 · Yuanzhi Wang2

Wurelihazi Hazihan
wurelihazi@shu.edu.cn

Zhihui Dong
751453835@qq.com

Liping Guo
lipingguo229@126.com

Kadyken Rizabek
agajai@mail.ru

Dzhunysov Askar
kayarat_ishan@mail.ru

Kulmanova Gulzhan
gulzhan_62@mail.ru

Mahanov Kudaibergen
makhanov_k@mail.ru

Akishev Nurlan Kenjebaevich
akishev_n@mail.ru

Tolegen Talgat
talgat_29-12-95@mail.ru

Kenesbay Kairullayev
kenes53@mail.ru

1 School of Animal Science and Technology, Shihezi University, Shihezi 832000, Xinjiang, China
2 School of Medicine, Shihezi University, Xinjiang Uygur Autonomous Region, Shihezi 832002, China
3 School of Medicine, Sun yat-sen university, Guangzhou 510080, China
4 Department of Food Engineering, Kazakh National Agrarian University, Almaty 050010, Kazakhstan