Rickettsiae in Red Fox (*Vulpes Vulpes*), Marbled Polecat (*Vormela Peregusna*) and Their Ticks in Northwestern China

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Abstract

Background

Previously, twelve *Rickettsia* species were found in ticks, fleas, sheep keds (*Melophagus ovinus*), bats (common pipistrelle: *Pipistrellus pipistrellus*) and a tick-bitten patient in Xinjiang Uygur Autonomous Region (XUAR), northwestern China. Here we aimed to molecularly detect rickettsial agents in red fox (*Vulpes vulpes*), marbled polecat (*Vormela peregusna*) and their ticks.

Methods

During 2018-2019, 12 red foxes, 1 marbled polecat and their ticks were sampled in two counties and a city of Xinjiang Uygur Autonomous Region (northwestern China). The heart, liver, spleen, lung and kidney of these 13 carnivores were dissected, followed by DNA extraction. Hard ticks were identified both morphologically and molecularly. All samples were examined for the presence of rickettsiae by amplifying four genetic markers.

Results

A total of 26 adult ticks and 28 nymphs (38 *Ixodes canisuga*, nine *Ixodes kaiseri*, six *Haemaphysalis erinacei* and one *Dermacentor marginatus*) were collected from red foxes, and four *H. erinacei* ticks were removed from a marbled polecat. Analysis of cytochrome *c* oxidase subunit I (*COI*) gene sequences indicated that 2-32 nucleotides differed between *I. canisuga*, *I. kaiseri* and *H. erinacei* from northwestern China and Europe. *Rickettsia raoultii* was detected in three red foxes, *Candidatus Rickettsia barbariae* in a red fox, *Rickettsia sibirica* in a red fox and a marbled polecat, and *R. raoultii* in two tick species (*I. canisuga* and *D. marginatus*).

Conclusions

To the best of our knowledge, *I. canisuga* and *I. kaiseri* have not been previously reported from red foxes in China. The DNA of *R. sibirica* and *R. raoultii* was detected for the first time in organs of red foxes, and *R. sibirica* in organs of marbled polecat. This is also the first molecular evidence for the presence of *R. raoultii* in *I. canisuga*. Our findings add to the range of tick-borne pathogens in wildlife species and associated ticks in China.

Background

The red fox (*Vulpes vulpes*) is widely distributed throughout Europe, Asia, North Africa, and North America [1]. Its habitats highly overlap with those of other wildlife species, domestic animals and even humans [2]. Previously, red foxes were reported to harbor several vector-borne pathogens of veterinary-medical importance, such as tick-borne encephalitis virus [3], *Borrelia burgdorferi* [4], *Ehrlichia canis* [5], *Leishmania infantum* [6], *Hepatozoon canis* [7] and *Babesia vulpes* [8, 9]. Serological investigation of red foxes indicated that 50.3% had antigens of SFG rickettsiae, including *Rickettsia massiliae* and *Rickettsia*

conorii in Spain [10]. In addition, immuno-fluorescence assay showed that 1.9% of red foxes had antibodies to *Rickettsia typhi*, and 6.7% of them to *Rickettsia slovaca* in Spain [4].

The geographical range of marbled polecat (*Vormela peregusna*) covers Central Asia, northwestern China and Europe [2]. Considering studies on its epidemiological role, seroconversion was detected in a marbled polecat to plague F1 antigen in Xinjiang Uygur Autonomous Region (XUAR), northwestern China. *Borrelia burgdorferi sensu lato* and *Babesia* sp. were molecularly identified in a marbled polecat in Romania and China, respectively [11, 12]. Furthermore, *Rickettsia raoultii* and *Candidatus Rickettsia barbariae* were molecularly identified in marbled polecats in XUAR [12].

In the temperate climate zone, hard ticks (Acari: Ixodidae) are regarded as the most important vectors of pathogens [13]. Among them, *Ixodes persulcatus*, *Ixodes ricinus*, *Ixodes hexagonus*, *Ixodes kaiseri*, *Ixodes canisuga*, *Dermacentor reticulatus*, *Dermacentor marginatus*, *Haemaphysalis punctata* and *Rhipicephalus sanguineus* were reported from red foxes [10, 14-16]. In Spain, *Rickettsia massiliae*, *Rickettsia aeschlimannii* and *Rickettsia slovaca* were detected in red fox ticks [10]. In addition, *Haemaphysalis erinacei* from marbled polecats contained the DNA of *Rickettsia raoultii* in China [17].

The aim of the present study was to investigate rickettsial agents in 12 red foxes, a marbled polecat and their ticks in China.

Methods

Sample collection and species identification

A total of 12 illegally hunted or road-killed red foxes and one naturally died marbled polecat were sampled in two counties and a city of XUAR during 2018-2019 (data shown in Additional file 1). The red foxes and the marbled polecat were morphologically identified by an experienced zoologist. The heart, liver, spleen, lung and kidney of all 13 carcasses were removed. Simultaneously, the entire body surface of each individual was checked for ticks, all of which were removed. The ticks were morphologically identified to the species level according to the standard taxonomic keys as previously described [18]. This was also confirmed by molecular and phylogenetic analyses based on two mitochondrial markers, the 16S rDNA and the cytochrome c oxidase subunit I (*COI*) genes [16].

Detection, sequencing and phylogenetic analysis of rickettsiae

Genomic DNA was extracted from organs (heart, liver, spleen, lung and kidney) of wild carnivores, as well as from their ticks using the TIANamp Genomic DNA Kit (TIANGEN, Beijing, China). To investigate the presence of rickettsiae in ticks, four genetic markers were targeted, including the 17 kDa antigen (*17-kDa*), the citrate synthase (*gltA*), the outer membrane protein A (*ompA*) and the surface cell antigen 1 (*sca1*) genes. Two (the *gltA* and *ompA*) genes were used to detect rickettsiae in organs of wild carnivores [19]. The primers and PCR cycling conditions of this study are shown in Additional file 2. Each PCR assay included a negative control (distilled water instead of DNA template) and a positive control (containing

sequence-verified DNA of *R. massiliae* from *Rhipicephalus turanicus* ticks collected in XUAR) [20]. Purification and sequencing of the PCR products were performed as described before [21, 22]. Sequences were manually edited, aligned and compared to reference GenBank sequences by nucleotide BLASTn program (<https://blast.ncbi.nlm.nih.gov>). A phylogenetic tree was constructed using the Maximum-Likelihood method in MEGA 7.0 software [17].

Results

Tick identification

A total of 26 adult ticks and 28 nymphs (38 *I. canisuga*, nine *I. kaiseri*, six *H. erinacei* and one *D. marginatus*) were collected from 12 red foxes, and four *H. erinacei* ticks were found on the marbled polecat. Morphological characteristics are shown in Supplementary Figure 1.

Molecular and phylogenetic analyses

Analysis of *COI* sequences revealed 2-32 nucleotide differences in case of *I. canisuga* (3-6 bp), *I. kaiseri* (2-7 bp) and *H. erinacei* (30-32 bp) between Europe and China. Phylogenetic analysis showed that i) *I. canisuga* in XUAR was in a basal position to eleven European haplotypes ("A to K") [18] (Fig. 1A); ii) *I. kaiseri* from red foxes in XUAR was also in basal position to nine European haplotypes ("L to T"), and had identical sequence with conspecific ticks from long-tailed ground squirrels and Asian badgers [16, 19, 23] (Fig. 1B); iii) *H. erinacei* from red foxes and marbled polecat had identical sequences, and formed a distinct clade from those reported in Turkey, Italy and Romania (Fig. 1C); and iv) *D. marginatus* from red fox #2 had identical sequence with the off-host tick collected formerly in Altaw City, XUAR.

Red fox #3, #5 and #11 were positive for *R. raoultii*, and red fox #8 was positive for *Candidatus R. barbariae*. At the same time, red fox #12 and the marbled polecat were positive for *Rickettsia sibirica*. In addition, *R. raoultii* was detected in *I. canisuga* from red fox #11 (Manas County) and *D. marginatus* from red fox #2 (Nilka County). Nucleotide sequences of rickettsial agents were deposited in the GenBank database (MT890502-MT890525). Phylogenetic analyses are shown in Fig. 2 and Additional Figure 2.

Discussion

Molecular studies on *I. canisuga* and *I. kaiseri* were mostly reported from Europe, where these tick species typically infest Eurasian badgers, red foxes, steppe polecats, raccoon dogs, common hedgehogs and domestic dogs. Among them, dogs and red foxes can also be co-infested with *I. canisuga* and *I. kaiseri* [16, 24]. In this study, *I. canisuga* and *I. kaiseri* were found on red foxes, as also confirmed by 16S rDNA gene sequences (GenBank: MT889694-MT889698 and MT889701-MT889705). To the best of our knowledge, *I. canisuga* and *I. kaiseri* have been discovered here for the first time on red foxes in China. Phylogenetic analysis of the *COI* gene showed that i) *I. canisuga* specimens collected from the same host species (red fox) are genetically different between China (MT890495-MT890498) and Germany (KY962044-KY962045), Croatia (KY962037-KY962040), Bosnia-Herzegovina (KY962016-KY962017),

Serbia (KY962030-KY962031), Romania (KY962025, KY962021-KY962023); ii) *I. kaiseri* from red foxes and long-tailed ground squirrels in XUAR had 100% identity, but clustered in a separate phylogenetic position compared to European ticks; and iii) *H. erinacei* infesting red fox and marble polecat in XUAR shared identical *COI* gene sequences (MT890493 and MT890494), but differed from those collected in Italy, Turkey and Romania (Fig. 1C). These findings support that the genetic diversity of *I. kaiseri*, *I. canisuga* and *H. erinacei* might reflect geographical distribution rather than host associations, consistently with Klompen et al [25].

Considering previous reports, *Rickettsia helvetica* was detected in the blood sample of a red fox in Switzerland [26]. In addition, *R. raoultii* and *Candidatus Rickettsia barbariae* were reported in a marbled polecat in Altaw City, XUAR [12]. Here, *R. raoultii* was detected for the first time in organs of red foxes, and *R. sibirica* in the lung and kidney of a marbled polecat and in the liver of a red fox. Moreover, the DNA of *R. raoultii* was shown to be present in *I. canisuga*. In our previous work, *R. raoultii* and *R. sibirica* were identified in several tick species [12, 17, 19, 27, 28], and *R. raoultii* was even found in organs/tissues of bats (*Pipistrellus pipistrellus*) and a tick-bitten patient [19, 29]. Based on the above findings, the epidemiological role of red foxes, marble polecats and their ticks in transmitting rickettsiae can be postulated. However, further studies (including transmission experiments) are necessary to eventually verify this. In addition, the scope of this work should be extended to more wildlife species from China and Central Asia.

Conclusions

To our knowledge, *I. canisuga* and *I. kaiseri* have not been previously identified from red foxes in China. The genetic diversity of *I. kaiseri*, *I. canisuga* and *H. erinacei* might be more related to geographical distribution than parasitized hosts. *Rickettsia raoultii* in *I. canisuga* and organs of red foxes, and *R. sibirica* in organs of a red fox and a marbled polecat are reported here for the first time. Our findings add to the range of tick-borne pathogens in wildlife species and associated ticks.

Abbreviations

COI: cytochrome *c* oxidase subunit I; ompA: outer membrane protein A; gltA: citrate synthase; 17-kDa: 17-kDa antigen; sca1: cell surface antigen 1; XUAR: Xinjiang Uygur Autonomous Region

Declarations

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Authors' contributions

GL, SZ, WT and YW conceived and designed the study, and wrote the manuscript. WY, LM, SW, YZ, ZL, WH and XG performed the experiments, analyzed the data. SH contributed to study design and edited the manuscript. All authors read and approved the final manuscript.

Ethical approval and consent to participate

This study was approved by the Animal Ethics Committee of Shihezi University (Approval No. AECSU2015-11).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Phylogenetic tree based on COI sequences of ticks collected from 12 red foxes and a marble polecat in northwestern China. The evolutionary history was inferred using the Maximum Likelihood method (bootstrap replicates: 1000) with MEGA 7.0. New sequences obtained in this study are indicated by black triangles. A. *Ixodes canisuga*, B. *Ixodes kaiseri*, C. *Haemaphysalis erinacei*, and D. *Dermacentor marginatus*.

Figure 2

Phylogenetic tree of the *ompA-gltA* concatenated sequences of rickettsial agents in 12 red foxes and a marble polecat.

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