Molecular detection of Bartonella in ixodid ticks and plateau pika (Ochotona curzoniae) in Shiqu county, China

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Abstract

Background Bartonella bacteria have been associated with an increasingly wide range of human and animal diseases and were also recognized to be globally dispersed as emergent pathogens. Ticks and small rodents are known vectors of human and animal bartonellosis and play important roles in maintenance and circulation of bartonellae in nature. In China, Shiqu county is located on the eastern Qinghai-Tibetan plateau and about 26 thousands square kilometers with an average altitude of above 4,200 meters and vast area of pastureland. In present study, the occurrence of Bartonella spp. in ticks and plateau pika was firstly investigated in Shiqu county.

Results A total of 818 ticks (Dermacentor everestianus, 79.0%, 646/818; Haemphysalis qinghaiensis, 21.0%, 172/818), were collected in 4 villages in Shiqu county. Only Bartonella melophagi was detected in tick samples with a total prevalence of 30.1% (246/818). Significant difference was observed (P<0.05) between D.everestianus (17.0%) and H.qinghaiensis (79.1%). The infection rates of Bartonella spp. in ticks from Arizha, Maga, Derongma and Changxgma villages were 4.8%, 76.8%, 12.5% and 18.0%, respectively. Compared with other villages, the infection rate of Bartonella spp. in Maga was higher (P<0.01). As for plateau pika, total infection rate of Bartonella spp was 24.1%, with 20.8% (15/72), 30.9% (25/81), 13.8% (9/65) and 29.4% (20/68) in Arizha, Maga, Derongma and Changxgma, respectively. Totally, B. queenslandensis, B.grahamii and two unvalidated Bartonella species were detected. No significant difference in infection rates was observed (P>0.05) between theses study sites.

Conclusion At present, only D.everestianus and H.qinghaiensis were found in Shiqu county with high infection of Bartonella spp. in theses ticks and plateau pika. The threats to public health by these Bartonella species should be monitored.

Background

The Bartonella genus, currently includes 36 named and 17 Candidatus species [1], which can be found in a wide range of mammalian and arthropod hosts and some of them are zoonoses, including: B.alsatica, B.bacilliformis, B.elizabethae, B.henselae, B.koehlerae, B.melophagi, B.quintana, B.rochalimae, B.tamiae, B.vinsonii subsp.berkhoffii, B.vinsonii subsp.arupensis and B.washoensis [2-8]. Ticks and small rodents are known vectors of human bartonellosis and play important roles in maintenance and circulation of bartonellae in nature within arthropod-mammal systems. Shiqu county is about 26 thousands square kilometers with an average altitude of above 4,200 meters and vast area of pastureland on the eastern Qinghai-Tibetan plateau, where lives a population of estimated 97 thousands with low education level and poor public health. Yak is the largest population of local livestock (about 600 thousand) and severe tick infestation is often observed. Except yak, plateau pika (Ochotona curzoniae) is the largest population of local small rodents with close interaction with local people and livestock. The significance of ticks (Acari: Ixodida) has long been recognized due to their ability to feed on a large range of host species and to transmit Bartonella pathogens that can infect a variety of vertebrate hosts, including humans. However, little information exists on bartonellae and their vectors in Shiqu county. The objective of this
study was to provide evidence of the presence of *Bartonella* spp. in plateau pika and ticks and preliminary results to establish prevention and control measures for this tick-borne disease.

**Results**

A total of 818 ticks were collected at 4 villages in Shiqu county and morphological and molecular identification using the 16S rRNA gene, confirmed the presence of two different tick species, belonging to *Dermacentor everestianus* (79.0%, 646/818) and *Haemaphysalis qinghaiensis* (21.0%, 172/818). Pictures of ticks and sequences information of 16S rRNA are included in Supplementary file 3-6. Ticks were first screened using the *Bartonella* spp. *gltA* gene and *gltA*-positive samples were then screened with *rpoB*, showing a total prevalence of 30.1% (246/818). Significant difference was observed (*P*<0.05) between *D. everestianus* (17.0%) and *H. qinghaiensis* (79.1%). The infection rates of *Bartonella* spp. in Arizha, Maga, Derongma and Changxgma were 4.8%, 76.8%, 12.5% and 18.0%, respectively (Table 2). Compared with other villages, the infection rate of *Bartonella* spp. in Maga (marked with “**” in Table 2) was higher (*P*<0.01). In Maga, no significant difference was observed (*P* 0.05), although the infection rate of *Bartonella* in *H. qinghaiensis* (79.1%) was higher compared with *D. everestianus* (69.2%). As for plateau pika, total infection rate of *Bartonella* spp was 24.1%, with 20.8% (15/72), 30.9% (25/81), 13.8% (9/65) and 29.4% (20/68) in Arizha, Maga, Derongma and Changxgma, respectively. No significant difference in infection rates was observed (*P* 0.05) between these study sites.

In this study, all amplicons of *gltA* and *rpoB* were sequenced and compared to each other. Totally, seven sequences of *gltA* and nine sequences of *rpoB* were obtained and deposited in GenBank with ID numbers (*gltA*: MN056882- MN056888; *rpoB*: MN296286- MN296294). For *gltA* gene, sequence (MN056882) from ticks was 100% identical to *B. melophagi* (AY724768) with 100% coverage; Sequences (MN056883 and MN056888) from plateau pika showed 97.03%-100% identity with *B. queenslandensis* (MH748120) with 99%-100% coverage; Sequences (MN056884, MN05686 and MN05687) from plateau pika showed 100%, 97.61% and 96.73% identity with *B. grahamii* (KT445918 and CP001562) with 100% coverage; Sequence (MN056885) from plateau pika was 98.81% homologous to *B. rochalimae* (KU292571) with 100% coverage. For *rpoB* gene, sequences (MN296287- MN296291) from ticks were 99.12-99.71% identical to *B. melophagi* (EF605288) with 99-100% coverage; Sequences (MN296286 and MN296294) from plateau pika showed 95.65-97.86% identity with *B. grahamii* (AB426697 and JN810811) with 100% coverage; Sequence (MN296292) from plateau pika was 99.69% homologous to *B. queenslandensis* (MH748136) with 100% coverage. However, Sequence (MN296293) from plateau pika was only 92.28 and 92.58% similar to *Bartonella* sp. (AB529489) and *B. grahamii* (AB426696) with 100% coverage, respectively.

According to criteria (*Bartonella* spp. species thresholds: *gltA* ≥ 96.0% and *rpoB* ≥ 95.4%) proposed by La Scola, et al [13], for tick samples, only *B. melophagi* was detected (Table 2); For plateau pika, as shown in Table 3, *B. grahamii* was predominantly identified species found in four villages with *B. queenslandensis* detected only in Maga and 2 unvalidated *Bartonella* species (*Bartonella*.sp* and *Bartonella*.sp**) found in Ariza and Changxgma, respectively. Furthermore, *gltA* and *rpoB* based phylogenetic analysis supported the classification of *Bartonella* spp. detected in the present work (Fig.2 and Fig.3).
Discussion

In this study, two tick species were found: *H. qinghaiensis* (only in Maga) and *D. everestianus* (in all of four sites). *D. everestianus* was only reported in Northwestern China and Nepal [15] with an altitude of 2600-4700m [16]. Larvae and nymphs of this tick species often infest lagomorphs and rodents, while adult ticks usually utilize medium-large sized, modest and wild mammals as hosts, including hares, sheep, yaks, and horses [15, 16]. However, *H. qinghaiensis*, a typical three-host tick, is only recorded in China [17-21], particularly prevalent in the western plateau, including the provinces of Qinghai, Gansu, Sichuan and Tibet [21]. Its natural hosts include sheep, goat, yak, cattle and hare (*Lepus oiostolus*). It is known that all stages of the tick could develop in sheep, goat, yak and cattle [21-27]. Comparing to ticks at high altitude, the activity of *H. qinghaiensis* is more frequently at low altitude. In this study, Arizha, Changxgma and Derongma villages belong to sub-frigid zone, with an altitude of 4300-4600m. Maga village is located in the cold temperate zone, with an altitude of 3799m. There was a significant difference in altitude between Maga and the other three villages, which was probably why *H. qinghaiensis* was only found in Maga village.

All types of ticks were found to contain *Bartonella* DNA, although in varying percentages and locations. A survey of ticks from 16 states in the U.S revealed that the overall prevalence of *B. henselae* in *Ixodes* ticks was 2.5% [28]. In Austria, *Bartonella* spp. (*B. henselae, B. doshiae, and B. grahamii*) were detected in 2.1% of *I. ricinus* with the highest rate in ticks derived from Vienna (with a 7.5% infection rate), and that adult ticks had a higher prevalence than other stages [29]. Furthermore, a recent One Health perspective review on *Bartonella* indicated that the overall presence of *Bartonella* in ticks (combining evidence from multiple surveillance studies) was about 15% [30]. In our results, a total prevalence of 30.1% in ticks (especially in Maga, 76.8%) indicated the severity in Shiqu county.

*B. melophagi*, a human bacterial pathogen, was firstly isolated from sheep blood in 2007 [31] and the same bacteria were then isolated from the blood of two female patients with pericarditis and skin lesions in the USA [32]. In this study, this is the first report of DNA of *B. melophagi* detected in *D. everestianus* and *H. qinghaiensis* and it was the first molecular evidence of *B. melophagi* found in Shiqu county. However, currently there is no evidence supporting the ability of these ticks to transmit *B. melophagi* to livestock or human. To address this issue, experiments need to be performed to assess vector competency of *D. everestianus* and *H. qinghaiensis* to transmit *B. melophagi* in the future.

*Bartonella* infection has been mostly reported in Rodentia [33-42], with few cases reported in Lagomorpha. Until now, there has only one report of *Bartonella* infection in plateau pika with a positive rate of 18.99% [43]. Totally, 15 *Bartonella* strains were obtained and most of them were closely related to *B. taylorii* and *B. grahamii* [43]. In our results, *B. grahamii*, a pathogenic strain in humans, was detected in all of four villages while *B. queenslandensis* was detected only in Maga. Nevertheless, as *B. coopersplainsensis*, the zoonotic potential of *B. queenslandensis* has not been reported. Additionally, for two unvalidated *Bartonella* species (*Bartonella. sp* and *Bartonella. sp**) found in Ariza and Changxgma, respectively, sequences analysis showed: 1) based on *gltA* gene, they were clustered with
*B.rochalimae* and *B. queenslandensis*, respectively; 2) based on *rpoB*, however, they were clustered with *B. melophagi*. The causes of this conflicting result can be classified as follows: 1) potential presence of multiple *Bartonella* species in the sample although it is not common based on culturing; 2) different primer sets may have amplification bias towards particular species based on the annealing affinity. These complications may cause the observed *Bartonella* diversity to differ depending on which marker was used for amplification; 3) homologous recombination, a specific form of LGT (lateral gene transfer) among *Bartonella* spp. This problem has been documented in several studies of *Bartonella* strains from cats, rodents and bats based on sequencing multiple protein-coding loci [12, 44-48]. However, culturing, sequencing multiple loci (including 16S rRNA, *ftsZ*, *gltA*, *groEL*, *ribC* and *rpoB* and ITS), cloning sequences into vectors before sequencing or deep sequencing approaches can be sufficient to describe a potentially novel *Bartonella* specie or subspecies and may differentiate these possible scenarios.

In Shiqu, plateau pika, the largest population of local small rodents, has close contact with local people and livestock and can be infested with fleas and ticks, implicating them in transmission cycles of *Bartonella* spp. In China, *Bartonella* infections among humans have mainly been reported in the central plain area, such as Jiangsu, Zhejiang, Anhui, and Hubei province. No cases or suspected cases have been reported in the Qinghai-Tibetan plateau. So, the relationship of plateau pika and the transmission of *Bartonella* should be studied closer and more thoroughly with controlled experiments to determine the exact routes of transmission between plateau pika, the transmission between plateau pika and their vectors, as well as the transmission between plateau pika to humans and livestock.

**Conclusion**

At present, only *D.everestianus* and *H.qinghaiensis* were found in Shiqu county with high infection of *Bartonella* spp. in these ticks and plateau pika and further research need to be conducted to determine the risk of *Bartonella* infections to humans and livestock.

**Methods**

**Study design and samples collection**

The study was conducted in Shiqu county (N32°58′46.95″, E98°06′10.58″), Sichuan province, China. Total 818 ticks were collected by blanket dragging between June and August, 2018, among which, 168, 224,192 and 234 ticks were collected from Arizha (N32°59′49.19″, E98°31′57.54″), Maga (N32°25′15.41″, E98°08′19.14″), Derongma (N33°04′15.75″, E97°58′20.50″) and Changxgma (N32°52′41.95″,E98°37′18.91″) villages, respectively. Also, in the same period, total 286 pikas were captured, 72 in Arizha, 81 in Maga, 65 in Derongma, and 68 in Changxgma.

**Biological sampling**

Ticks were carefully removed from blanket and stored in 70% ethanol at 4°C. The specimens were morphologically identified using the guidelines for tick identification [9]. Then, molecular identification of
tick species was performed targeting the mitochondrial 16S rRNA gene [10]. Plateau pikas were captured using mouse snap traps. After capturing the animals, spleens were collected under sterile conditions, and stored in liquid nitrogen until use. The bodies of pika were deeply buried to avoid being eaten by dogs, cats and other wild carnivores.

**DNA extraction, PCR and sequencing**

Ticks were sectioned longitudinally and one half per each tick was used for DNA extraction; For all of spleen samples, an average of 30 mg of tissue was used. Total DNA of all samples were extracted using the TIANamp Genomic DNA Kit (TIANGEN Biotech Co., Ltd, Beijing, China; Cat No: DP304) for tick molecular identification and characterization of *Bartonella* spp. All samples were submitted to previously described PCR assays targeting *gltA* (379 bp) [11]. All *gltA*-positive samples were further analyzed with PCR targeting *rpoB* (379 bp) [12]. In this study, all primers were listed in Table 1. PCR amplifications were conducted in a 25μl reaction mixture consisting of 1μl of genomic DNA (2-3 ng), 1μl of each primer (10μM), 12.5μl of PCR Supermix (Transgen Co., Ltd, Beijing, China; Cat No: AS111-11) and 9.5μl of nuclease-free water. Each PCR reaction included a positive control (DNA of *B. henselae*, preserved in lab) and a negative control (nuclease-free water). Observed bands were purified using the QIAquick Gel Extraction Kit and sent for sequencing (Sangon Biotech (Shanghai) Co., Ltd). Obtained sequences were analyzed by using the Bioedit v.7.0.2 and submitted for nucleotide BLAST search through the NCBI database. Sequences with ≥95% quality cover and identity were considered to be positive for *Bartonella* spp. and compared with validated *Bartonella* species in GenBank/EMBL/DDBJ using the Clustal X program (http://www.clustal.org/clustal2/). Clones that share ≥96.0% and ≥95.4% similarity in *gltA* and *rpoB* sequences with the validated species, respectively, can be considered as the same species [13].

**Phylogenetic analysis and statistics**

For phylogenetic analysis, Neighbor-Joining phylogenetic trees were constructed based on *Bartonella gltA* and *rpoB* sequences using the Kimura two-parameter model with partial gap deletion and a cutoff of 95% site coverage, respectively. The evolutionary distance was calculated and bootstrap analysis with 1,000 iterations was carried out with the MEGA6 [14]. SPSS19.0 (One-way ANOVA) was applied to compare the difference in prevalence of *Bartonella* spp. between different sampling locations, plateau pika and tick species. A p-value of ≤ 0.05 was considered significant.

**Declarations**

**Competing interests**

The authors declare that there is no conflict of interest in this study.

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Ethics approval and consent to participate

This study was carried out in full compliance with the framework for the collection of wild species of biological diversity for purposes of non-commercial scientific research, authorized by the Sichuan's Department of Agriculture and Rural Affairs. The study received approval from the Animal Ethics Committee of Southwest Minzhu University Plateau pikas were collected during inspection by qualified veterinary officers. In this study, no experiment was conducted on live animals.

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Availability of data and materials

The sequences generated in this study were submitted to the GenBank database under the accession numbers MN056882- MN056888 and MN296286- MN296294 (see Supplementary files).

Authors' contributions

TTC and LCC performed the experiments. TTC and H LL designed the project, analyzed the data and drafted the manuscript together. YA, YD and GL collected the tick samples. HW, MX and YJ collected the pika samples. All authors read and approved the final manuscript.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1 Primer sequences used for ticks and *Bartonella* spp. identification

| Target gene | Primer sequence (5’-3’) | Product (bp) | References |
|-------------|-------------------------|--------------|------------|
| 16S rRNA    | 16S+1: CTGCTCAATGATTTTTTAAATTGCGG <br> 16S-1: CCGGTCTGACAGATCAAGT | 460          | [10]       |
| gltA        | bart781: ATGCGGAATATTTCCTTTAAAATTG | 379          | [11]       |
| rpoB        | rpoF: GACGATTGATCAATTTTTTTCTTTT <br> rpoR: GGCATTATGTGTATTGTC | 379          | [12]       |

Table 2 The prevalence of *Bartonella* spp. in ticks in Shiqu county

| Location | No of samples | Infection rates % | Total |
|----------|---------------|-------------------|-------|
|          | *H. qinghaiensis* | *D. everestianus* | *H. qinghaiensis* | *D. everestianus* |     |
| Ariza    | 0/168 | 168/168 | 4.8(8/168) | 4.8 |
| Maga     | 172/224 | 52/224 | 79.1(136/172) | 69.2 (36/52) | 76.8* |
| Derongma | 0/192 | 192/192 | 12.5 (24/192) | 12.5 |
| Changxgma | 0/234 | 234/234 | 18.0(42/234) | 18.0 |

Table 3 The prevalence of *Bartonella* spp. in plateau pika in Shiqu county

Supplementary Files

Supplementary file1 Sequences of *gltA* gene
| Location         | Bartonella.sp* | B. queenslandensis | B. grahamii | Bartonella.sp** | Total |
|------------------|----------------|--------------------|-------------|------------------|-------|
| Ariza            | 4.2 (3/72)     | 0                  | 16.7 (12/72)| 0                | 20.8  |
| Maga             | 0              | 8.6 (7/81)         | 22.2 (18/81)| 0                | 30.9  |
| Derongma         | 0              | 0                  | 13.8 (9/65) | 0                | 13.8  |
| Changxiguma      | 0              | 0                  | 23.5 (16/68)| 5.9 (4/68)       | 29.4  |

**Supplementary file2** Sequences of *rpoB* gene

**Supplementary file3** Adult specimen of *H.qinghaiensis*. A Dorsal view; B. Ventral view.

**Supplementary file4** Adult specimen of *D.everestianus*. A Dorsal view; B. Ventral view.

**Supplementary file5** Sequences of 16S rRNA (*H.qinghaiensis*)

**Supplementary file6** Sequences of 16S rRNA (*D.everestianus*)

**Figures**
Figure 1

The map of Shiqu county. A. The map of China where Sichuan province is marked as yellow; B. The map of Ganze Tibetan autonomous prefecture where Shiqu county is marked as yellow; C. The map of Shiqu where location of samples collection is represented with black triangles (1. Ariza; 2. Maga; 3. Derongma; 4. Changxgma). Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal
status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

Figure 2

Neighbor joining (NJ) phylogenetic trees based on Bartonella gltA gene; Sequences obtained in this study were marked with black triangles.
Figure 3

Neighbor joining (NJ) phylogenetic trees based on Bartonella rpoB gene; Sequences obtained in this study were marked with black triangles.

Supplementary Files
This is a list of supplementary files associated with this preprint. Click to download.

- Supplementaryfile3.jpg
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- Supplementaryfile6.txt
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