Letter to the Editor

Bartonella Species Detected in the Plateau Pikas (*Ochotona curzoiae*) from Qinghai Plateau in China

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***Bartonella* species can infect a variety of mammalian hosts and cause a broad spectrum of diseases in humans, but there have been no reports of *Bartonella* infection in Ochotonidae. This is the first study to detect *Bartonella* in plateau pikas in the Qinghai plateau, providing baseline data for the risk assessment of human *Bartonella* infection in this area. We obtained 15 *Bartonella* strains from 79 pikas in Binggou and Maixiu areas of Qinghai with a positive rate of 18.99%. Based on the phylogenetic analysis of the *Bartonella* citrate synthase (*gltA*) gene sequences, most strains were closely related to *B. taylorii* (3/15) and *B. grahamii* (12/15). The latter is a pathogenic strain in humans. Our results suggest that a corresponding prevention and control strategy should be taken into consideration in the Qinghai province.**

Bartonella spp. are vector-borne, facultative, gram-negative intracellular, and slow-growing bacteria belonging to the family *Bartonellaceae* and can infect erythrocytes and endothelial cells in their hosts[1]. Since the first *Bartonella* sp., *B. bacilliformis*, was discovered in 1905 by Alberto Leonardo Barton Thompson, more than 30 species or subspecies of *Bartonella* have been identified[2]. Twelve species were recognized as human pathogens responsible for a large spectrum of diseases. For example, *B. henselae* can cause cat scratch disease (CSD), bacillary angiomatosis-bacillary peliosis, and endocarditis; *B. quintana* can cause trench fever, endocarditis, and bacillary angiomatosis; *B. bacilliformis* is responsible for verruga peruana and Oroya fever[3]. In China, *Bartonella* infections among humans have mainly been reported in the central plain area, such as Jiangsu, Zhejiang, Anhui, and Hubei province. However, no cases or suspected cases have been reported in the Qinghai-Tibetan plateau. Since specific laboratory diagnostic tools, including serology, culture, and PCR detection for routine clinical identification and detection of individual *Bartonella* agents are not widely available, clinical reports mostly describe presumptive cases of CSD. Few cases were detected in paraffin-embedded lymph node tissues using immunochemical staining with monoclonal antibodies to *B. henselae*[4].

Bartonella species have been found in a variety of mammals (rodents, carnivores, and ungulates) and arthropods (sandfly, human body louse, fleas, ticks, and blood sucking flies, etc.)[5]. A particularly high infection rate of *Bartonella* has been reported in rodents[6-7], mainly detected in *Rattus flavipectus*, *R. norvegicus*, *R. losea*, *Apodmus chevrieri*, *A. speciosus*, *A. agrarius*, *Eothenomys melanogaster*, *Suncus murinus*, and *Microtus fortis*[8]. Four *Bartonella* spp, *B. elizabethae*, *B. grahamii*, *B. washoensis*, and *B. vinsonii subsp. arupensis* isolated from rodents are suspected to cause human diseases[5]. *Bartonella* infection has been mostly reported in Rodentia, but few cases have been reported in Lagomorpha, and there have been no reports of *Bartonella* infection in Ochotonidae.

The plateau pika (*Ochotona curzoniae*) belongs to Mammalia, Lagomorpha, Ochotonidae, *Ochotona* and is a small non-hibernating rodent mammal that resides throughout the Qinghai-Tibetan plateau of China. Previous studies showed that plateau pika can carry a variety of pathogens, such as *Echinococcus granulosus* and *Yersinia pestis*, which are both transmissible to humans[9]. The objective of the present study was to detect the *Bartonella* infection

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in plateau pikas to provide a scientific basis for the control and prevention of *Bartonella* infection in humans in the Qinghai plateau. This study was approved by the Ethics Committee of Chinese Center for Disease Control and Prevention (No: ICDC-2015002).

Plateau pikas were captured using mouse snap traps in August 2013. The Binggou scenic area and Maixiu forest were selected as our study sites. The Binggou scenic area (longitude 100.22°E, latitude 38.2°N) is located in Qilian county, Haibei Tibetan autonomous prefecture, in the northeast of Qinghai province. Maixiu forest (longitude 101.5°E, latitude 35.03°N) is located in Zeku county, Huangnan Tibetan autonomous prefecture, in the southeast of Qinghai province (Figure 1). After capturing the animals, we identified the gender, collected the livers and spleens samples of plateau pikas under sterile conditions, and stored the samples in liquid nitrogen until use.

In the laboratory, 30 mg tissue of each sample was homogenized by adding 200 μL sterilized trypsin soy broth (BD Biosciences, Franklin Lakes, NJ, USA), plated onto two trypsin soy agars containing 5% (vol/vol) defiber sheep blood (BD Biosciences), incubated at 37 °C and 5% CO_2, and checked for growth of *Bartonella* species on alternate days for up to 30 d. Suspected colonies were randomly selected and separately subcultured on fresh agar plates 1-3 times to obtain pure colonies[10]. The purified suspected colonies of each sample were added into 100 μL sterile deionized water (pH 8.0), boiled at 100 °C for 10 min, and centrifuged at 6080 ×g for 5 min; the supernatant was used as the crude DNA template[10]. Next, PCR was performed to detect the *Bartonella* citrate synthase (*gltA*) gene. The primers used to amplify the 379-base pair fragment were forward BhCS781.p (5′-GGGGACCAGCTCATGGTGG-3′) and reverse BhCS1137.n (5′-AATGCAAAAAGAACAGT AAACA-3′)[1,7]. PCR was performed on Labcycler (SensoQuest, Göttingen, Germany) in 50 μL mixtures containing 25 μL 2×TransTaq-T PCR SuperMix (Beijing TransGen Biotech Co., Ltd., Beijing, China), 22 μL double-distilled H_2O, 1 μL (10 μmol/L) of each

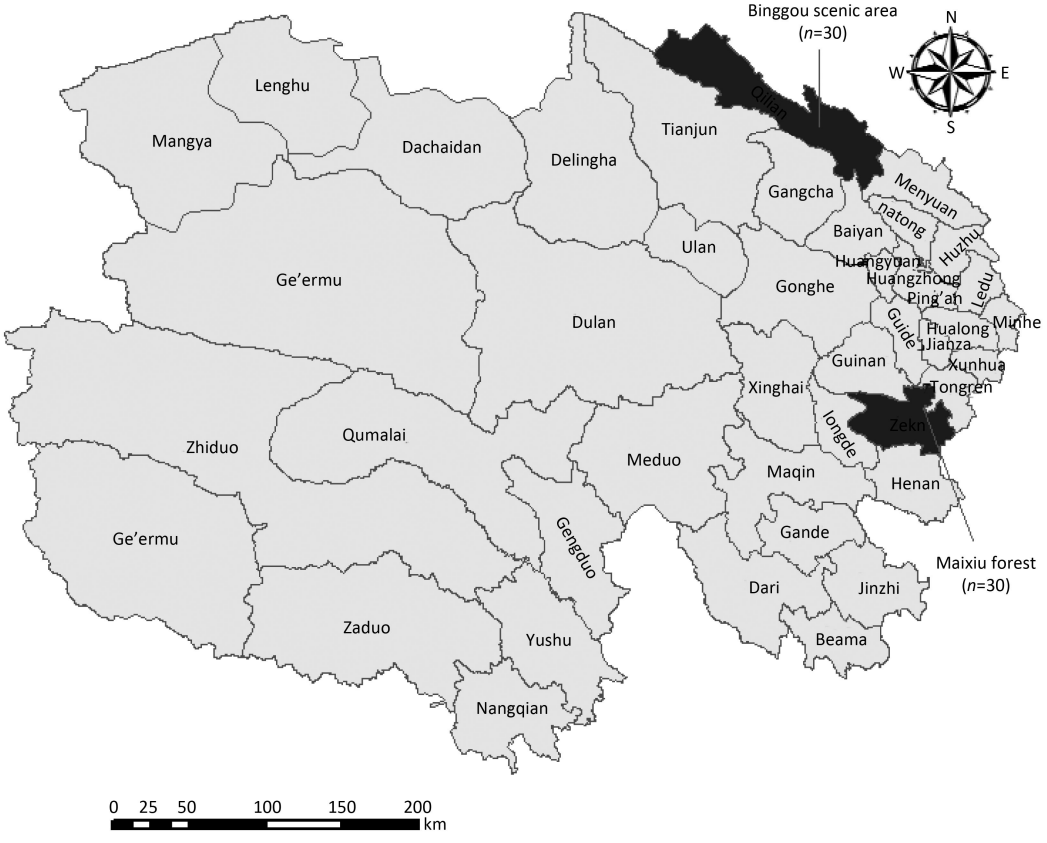

Figure 1. Geographical distribution of the trapped pikas and the study areas consist of the sampling sites in two counties of Qinghai province, China.

primer, and 1 μL of DNA template. Amplification was performed under the following conditions: 1 cycle for 5 min at 94 °C; 33 cycles for 30 s at 94 °C, 30 s at 53 °C, and 20 s at 72 °C; final extension for 7 min at 72 °C. Next, 5 μL of each PCR product was run on a 1% agarose gels, stained with ethidium bromide, and visualized using a gel imaging system (Bio-Rad, Hercules, CA, USA).

The *gltA* PCR-positive products were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocols, then sequenced using specific primers for *gltA* with an Applied Biosystems 3730xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The nucleic acid sequence homology was blasted against reported *Bartonella* species sequences in GenBank using the BLAST program at the National Center for Biotechnology Information Website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The phylogenetic analysis with type strains of Bartonella were performed by using ClustalW (1.6) and MEGA 6 software, and Brucella abortus was used as the outlier group. Bootstrap calculations were carried out for 1000 replicates[6,10].

The colony formation time of suspected colonies in the liver and spleen group were described as the median (quartile range), and the Wilcoxon rank sum test was used to determine statistical significance. The positive rates of *Bartonella* in different areas and genders were analyzed using the Chi-square test. All data were analyzed using SPSS 13.0 (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to be statistical significant.

In our study, a total of 79 plateau pikas were captured, 49 in the Binggou scenic area (OC 1-49 QH) and 30 in the Maixiu forest (OC 50-79 QH), including 21 males, 37 females, and 21 without gender information. Fifteen isolates (18.99%) were identified. The prevalence of *Bartonella* DNA found in *Ochotonidae* was higher than the prevalence rates of 14.57%, 13.81%, and 9.23% reported in rodents from Fujian, Heilongjiang, and Hainan, respectively, which were detected by PCR after culture, but lower than the values of 62.20%, 57.50%, and 25.99% reported from England, the US, and West Africa, respectively, which were only detected by PCR[1,4,7]. The high prevalence of detection of *Bartonella* DNA in the *Ochotonidae* suggests their role as vectors of *Bartonella*.

Colony formation was visible after culture for 1-2 weeks, which is consistent with the results of previous studies[4]. The suspected colonies of *Bartonella* spp. were tiny circular bulges (ϕ<1.0 mm), gray-white in color, slightly transparent, had smooth or rough edges, and had colony numbers of 10-1000 colony-forming units. The colonies were generally pitted on the agar and became rough after incubation. Small round holes remained after removing the colonies with inoculation loops, which were caused by the growth of *Bartonella* spp. into the agar. Furthermore, the subculture colonies grew faster and were larger than the primary generation (Figure 2).

The results showed that 10 and 5 positive strains were detected in samples from Binggou and Maixiu; 2, 8, and 5 positive strains were detected for male, female, and non-identified genders, respectively, but the difference between the rate of detection based on the regions and gender were not statistically significant (20.41% of Binggou *vs.* 16.67% of Maixiu, $\chi^2=0.17$, $P=0.68$; 9.52% of males *vs.* 21.62% of females, adjusted $\chi^2=0.66$, $P=0.42$). In addition, 8 and 4 strains

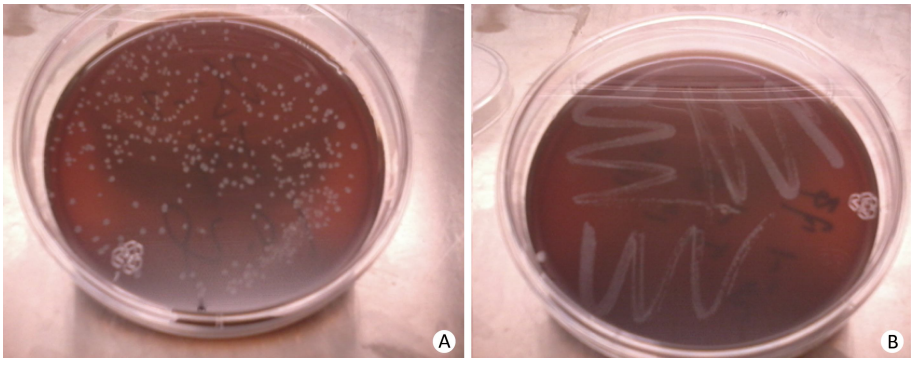

Figure 2. Suspected colonies of *Bartonella* isolated from *Ochotona curzonize* (OC19QH) were cultured in trypsin soy agars containing 5% (V/V) defiber sheep blood. A: Primary culture colony on the 13th day; B: Subculture colony on the 5th day.

were detected in the liver and spleen, respectively, while 3 strains were detected in both tissues, and the difference between the rate of detection based on the organs was also not statistically significant (13.92% in livers *vs* 8.86% in spleens, paired χ^2=0.75, *P*=0.39). The colony formation time was 6-13 days, with an average time of 12 (7) d in the liver and 10-18 d, with an average time of 12 (6) d in the spleen, and the difference between the colony formation times based on the organs was also not statistically significant (*u*=1.36, *P*=0.17). The positive rate of *Bartonella* in different regions and gender as well as colony formation times in different organs were not statistically significant; these results are supported by those of previous studies[7].

After PCR, all *gltA* sequences of the isolates from the plateau pikas were compared with *Bartonella* sequences deposited in GenBank using the BLAST tool. The homology of 12 strains was greater than 97% with *B. grahamii* (CP001562, AY584857, AY584856, AY584855, AB426654), and 3 strains were above 97% with *B. taylorii* (AF191502). The novel *gltA* genotypes were submitted to the nucleotide databases of GenBank with the following accession numbers: KT445915 to KT445929. We used the maximum likelihood (ML), neighbor-joining (NJ), minimum-evolution (ME), and unweighted pair-group method with arithmetic mean to construct phylogenetic trees and obtained the same results; thus, the ML method was used for further analyses (Figure 3). Two distinct clusters A and B were formed, including 12 and 3 strains respectively.

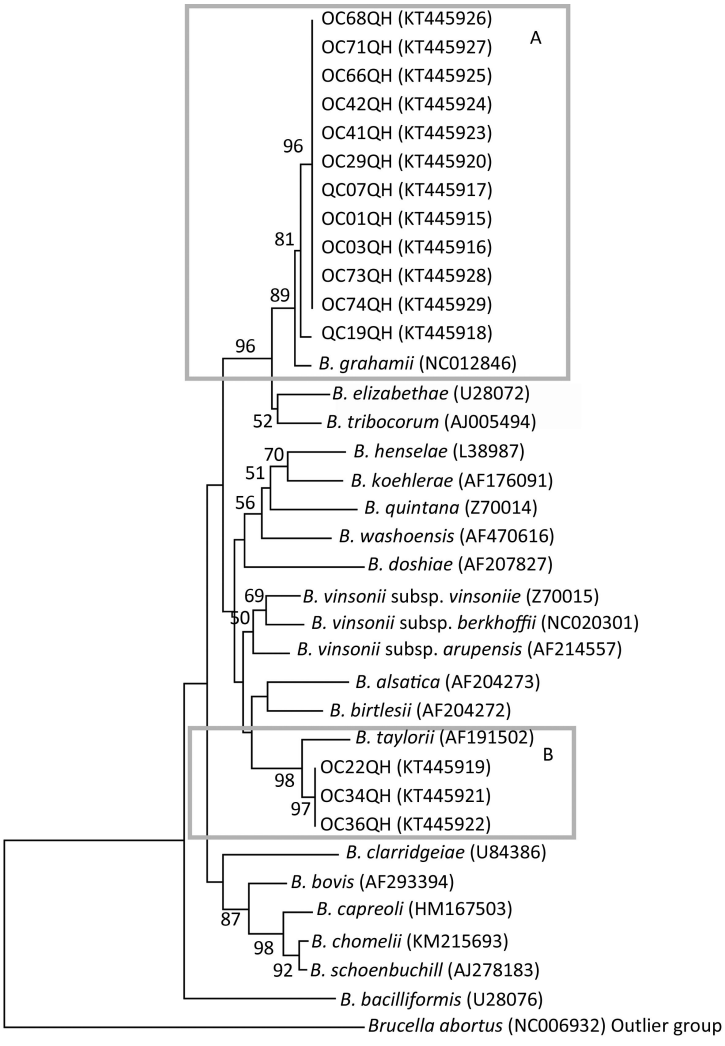

Figure 3. Phylogenic analysis of *gltA* sequences derived from the *Bartonella* isolated from Plateau pikas using ML method.

The strains belonging to cluster A were closely related to *B. grahamii*, and strains belonging to cluster B were closely related to *B. taylorii*. Previous studies confirmed that *B. grahamii* was associated with neuroretinitis and CSD[4,10], suggesting that *Bartonella* species detected in *Ochotonidae* may have the ability to cause human disease.

In Qinghai, plateau pikas are very common in pastoral areas. In these areas, more than 90% of the total population is the Tibetan nationality. They have strong wildlife protection consciousness, and may have close contact with wild animals during for work or living conditions, which may increase the possibility of infection, resulting in a higher prevalence of zoonoses. In addition, travelers to the Binggou scenic area and Maixiu forest are susceptible to many kinds of pathogens. If these visitors contact the plateau pikas, they may become infected with *Bartonella*. Therefore, the infection risk of *Bartonella* species should be assessed.

In conclusion, our study identified two genotypes of *Bartonella* in Ochotonidae from Qinghai province, *B. grahamii* and *B. taylorii*. However, further studies are required to determine the following: 1) Except for *gltA*, other related genes such as *ribC* and *rpoB* were not detected; 2) The epidemiological significance of *B. taylorii* was not explored; 3) Some fleas and ticks have been found on the plateau pikas, but their roles in the transmission of *Bartonella* species have not been examined.

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