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DETECTION OF *BARTONELLA* SPECIES IN SMALL MAMMALS FROM ZHEJIANG PROVINCE, CHINA

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ABSTRACT: To estimate the prevalence of *Bartonella* in small mammals of different species, during different seasons, and at different study sites, and to provide baseline data for the risk assessment of human *Bartonella* infection, we captured small mammals using snap traps in Zhejiang Province, China. *Bartonella* species were detected in small-mammal samples by polymerase chain reaction and positive amplicons were sequenced. *Bartonella* DNA was detected in 47% (90/192) of *Apodemus agrarius*, 31% (14/45) of *Rattus losea*, 16% (7/43) of *Rattus norvegicus*, 24% (9/37) of *Eothenomys melanogaster*, 4% (1/28) of *Niviventer confucianus*, 30% (7/23) of *Suncus murinus*, 22% (2/9) of *Microtus fortis*, 27% (2/7) of *Rattus tanezumi*, and 29% (2/7) of *Apodemus peninsulae*. No *Bartonella* DNA was detected in 27 unidentified Soricidae or nine *Mus musculus*. This is the first report of *Bartonella* DNA detected in *E. melanogaster* and *N. confucianus*. The prevalence of *Bartonella* DNA varied among small-mammal species, study sites, and seasons; the prevalence of *Bartonella* DNA between genders did not vary significantly within a species. The sequences we report were most similar to *Bartonella grahamii.*

Key words: *Bartonella*, China, PCR, small mammals.

INTRODUCTION

Bartonella spp. are fastidious gram-negative bacteria that infect the erythrocytes and endothelial cells of their hosts (Boulouis et al., 2005). More than 20 species or subspecies of *Bartonella* have been described; 12 are recognized as human pathogens (Henn et al., 2007) responsible for a large spectrum of diseases. *Bartonella quintana* is the etiologic agent of trench fever, endocarditis, bacillary angiomatosis (Maurin and Raoult, 1996), and chronic bacteremia in homeless patients (Brouqui et al., 1999). *Bartonella henselae* can cause bacillary angiomatosis (Weisburg et al., 1991; Koehler et al., 1992) and endocarditis (Hadfield et al., 1993; Drancourt et al., 1996), but is most commonly associated with cat scratch disease (Regnery et al., 1992; Dolan et al., 1993), an illness which also has been attributed to *Bartonella clarridgeiae* (Kordick et al., 1997). One isolate of *Bartonella elizabethae* has been obtained from the heart valve of a patient with endocarditis (Daly et al., 1993). *Bartonella bacilliformis* is responsible for verruga peruana and Oroya fever (Keier and Ristic, 1981). Several other pathogenic bartonellae including *Bartonella vinsonii arupensis* (Welch et al., 1999) and *Bartonella tamiae* (Kosoy et al., 2008) have been described recently.

Bartonella spp. also infect a variety of mammalian hosts and cause a broad spectrum of disease. In addition to animal hosts (e.g., cats, dogs, and cattle) in close contact with humans, small mammals are one of the most important reservoirs of these agents. Many *Bartonella* spp. have been identified in small mammals and an increasing number of human and canine infections have been reported to be caused by rodent-borne *Bartonella*. *Bartonella* infection prevalences in small mammals from England and the southeastern United States were 62% (n=37) and 42.2% (n=279), respectively (Birtles et al., 1994; Kosoy et al., 1997). Small

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TABLE 1. Study site locations and prevalences of *Bartonella* infection as determined by polymerase chain reaction in small mammals, Zhejiang Province, China.

Location (no. of trapping sites)	Latitude	Longitude	Habitat	No. tested	No. PCR positive	Prevalence (%)
Tiantai (4)	29.14°N	121.03°E	Mountain	229	85	37.1
Longquan (2)	28.09°N	119.12°E	Mountain	76	29	38
Jindong (2)	29.05°N	119.41°E	Mountain	41	0	0
Linhai (2)	28.86°N	121.12°E	Suburb	16	9	56
Chun'an (2)	29.61°N	118.84°E	Residential	14	0	0
Shangyu (2)	30.02°N	120.88°E	Farm	12	1	8
Jiangshan (2)	28.73°N	118.61°E	Farm	12	0	0
Longyou (2)	29.04°N	119.19°E	Mountain	10	4	40
Ouhai (2)	27.58°N	120.32°E	Residential	10	4	40
Jiande (2)	29.48°N	119.27°E	Suburb	7	2	29
Total				427	134	31.4

mammals in which *Bartonella* spp. have been detected include *Rattus norvegicus*, *Rattus tanezumi*, *Rattus rattus, Mus musculus*, *Apodemus speciosus*, *Apodemus argenteus, Apodemus flavicollis, Apodemus sylvaticus, Apodemus agrarius, Myodes (Clethrionomys) glareolus, Myodes* spp., *Eothenomys* spp., *Microtus* spp., *Peromyscus maniculatus*, *Tamias minimus*, *Spermophilus lateralis*, *Spermophilus beecheyi, Neotoma micropus, Neotoma albigula, Sorex vulgaris,* and *Sorex araneus* (Holmberg et al., 2003; Kosoy et al., 2003; Engbaek and Lawson, 2004; Li et al., 2004; Knap et al., 2007; Inoue et al., 2008; Morway et al., 2008). In areas with a high prevalence of *Bartonella* infection, small-mammal–associated *Bartonella* are highly diverse. *Bartonella* spp. isolated from small mammals include *Bartonella tribocorum*, *B. elizabethae*, *Bartonella grahamii*, *Bartonella taylorii*, *Bartonella vinsonii vinsonii*, *Bartonella washoensis*, *Bartonella doshiae*, *Bartonella birtlesii*, *B. vinsonii arupensis*, *Bartonella rattimassiliensis*, and *Bartonella phoceensis* (Gundi et al., 2004).

According to clinical reports, the number of cat scratch disease cases in Zhejiang Province is the second largest in China. Zhejiang Province is a developed region of great economic importance for industry and tourism, suggesting a high potential for zoonotic exposure to *Bartonella* spp. Although investigators have reported *Bartonella* infections in several provinces in China (Ying et al., 2002, 2007; Li et al., 2004; Ye et al., 2006), no one has investigated *Bartonella* among small mammals in Zhejiang Province. The objective of this study was to investigate the epidemiology of *Bartonella* spp. associated with small mammals in Zhejiang Province.

MATERIALS AND METHODS

Small-mammal samples

Small mammals were captured using snap traps in a diversity of habitats in Zhehiang Province during December (Tiantai, Jindong, Longyou, and Jiangshan) and April 2005 (Tiantai, Chun'an, Jiande, and Longyou) and August (Tiantai, Longquan, and Ouhai) and November (Tiantai, Linhai, and Shangyu) 2006. Three hundred snap traps were set for one night at each site during each sampling period. Ten cities, representing different habitats and geographic locations, were selected for trapping (Table 1). Small mammals were identified by species and gender and capture locations and collection dates were recorded. Spleens of captured small mammals were collected using a sterile technique and stored at −20 C before analyses. In order to compare *Bartonella* prevalence among seasons, one site (Tiantai) was designated to capture small mammals in four seasons.

Polymerase chain reaction

Twenty-five milligrams of each spleen collected was placed into a sterilized mortar and crushed with a sterilized pestle in liquid

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TABLE 2. Sequences, target gene, product size, and references of the three primer pairs used to detect *Bartonella* in small mammals, Zhejiang Province, China.

Name	Sequence	Target gene	Product size[a]	Reference
BhCS.781p-BhCS.1137n	5′-GGGGACCAGCTCATGGTGG-3′, 5′-AATGCAAAAAGAACAGTAAACA-3′	*gltA*	379	Norman et al., 1995
Bh.311p-Bh.452n	5′-CTCTTTCTTCAGATGATGATCC-3′, 5′-AACCAACTGAGCTACAAGCCCT-3′	16S/23S rRNA ITS	200	Jensen et al., 2000
TIle.455p-TAla.885	5′-GCTTGTAGCTCAGTTGGTTAG-3′, 5′-TGCTTGCAAAGCAGGTGC TCT-3′	16S/23S rRNA ITS	200–400	Li et al., 2004

[a] Number of base pairs.

nitrogen. DNA was extracted from the crushed spleen using a QIAamp Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

Polymerase chain reaction (PCR) amplification was done using a MyCycler™ thermal cycler (BIO-RAD, Hercules, California, USA). Three pairs of primers synthesized by SBS Genetech (Beijing, China) were used for amplification of *Bartonella* DNA (Table 2).

Conditions for PCR amplification have been described (Norman et al., 1995; Jensen et al., 2000; Li et al., 2004). DNA amplification was performed in a 25-μl volume. Each reaction mixture contained 1μl (10 pmol/μl) of each primer, 1.5 U *Taq* DNA polymerase (Sino-American Biotechnology Co., Henan, China), 2 μl 2.5 mM deoxynucleosides triphosphate mixture, 2.5 μl 10× PCR buffer, 1.5 μl 20 mM $MgCl_2$, and 5 μl of DNA template. Each PCR performed had a negative control (molecular-grade water) and positive control (DNA from *B. henselae* strain Houston-1, [ATCC49882]).

Polymerase chain reaction products underwent electrophoresis on a 1.5% agarose gel, stained with ethidium bromide and visualized under ultraviolet light. DNA extractions, reaction mixture preparation, and sample addition were performed in different rooms to minimize cross-contamination.

Following electrophoresis, eight PCR amplicons of *gltA* gene fragments from *A. agrarius*, which were amplified using BhCS.781p-BhCS.1137n primers, were purified with the Promega Wizard PCR Preps Kit (Promega, Madison, Wisconsin, USA) and then cloned into the pGEM-T Easy vector system (Promega) following the manufacturer's protocol. The white-colored recombinant clones were selected for sequencing. The amplicons sequenced were 379 bp.

Nucleotide sequence accession numbers

Partial sequences of the *gltA* gene generated during this study were submitted to GenBank with accession numbers EU179229 (R21ZJ from Longquan), EU179230 (R29ZJ from Tiantai), EU179231 (R60ZJ from Tiantai), EU179232 (R1ZJB from Tiantai), EU179233 (R17ZJB from Tiantai), EU179234 (R66ZJB from Linhai), EU179235 (R2ZJJD from Jiande), and EU179236 (R3ZJJD from Longyou).

Data analysis

To detect and identify *Bartonella* DNA, PCR amplicons derived from the *gltA* gene of *Bartonella* species were sequenced after cloning. The sequences were then compared to

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TABLE 3. Study site, number of small mammals collected, and prevalence of *Bartonella* infection as determined by polymerase chain reaction in Zhejiang Province, China.

Species	Location										Total tested	No. PCR positive	Prevalence (%)
	Tiantai	Longquan	Jindong	Linhai	Chunan	Shangyu	Jiangshan	Longyou	Ouhai	Jiande			
Apodemus agrarius	100	58	0	9	0	0	12	10	2	1	192	90	46.9
Rattus losea	23	8	11	3	0	0	0	0	0	0	45	14	31
Rattus norvegicus	21	1	3	1	13	1	0	0	2	1	43	7	16
Eothenomys melanogaster	37	0	0	0	0	0	0	0	0	0	37	9	24
Niviventer confucianus	7	0	21	0	0	0	0	0	0	0	28	1	4
Soricidae	16	0	6	0	0	0	0	0	0	5	27	0	0
Suncus murinus	11	0	0	2	0	4	0	0	6	0	23	7	30
Microtus fortis	0	9	0	0	0	0	0	0	0	0	9	2	22
Mus musculus	0	0	0	1	1	7	0	0	0	0	9	0	0
Rattus tanezumi	7	0	0	0	0	0	0	0	0	0	7	2	28.6
Apodemus peninsulae	7	0	0	0	0	0	0	0	0	0	7	2	28.6
Total	229	76	41	16	14	12	12	10	10	7	427	134	31.4

those detected in other small mammals with published sequences using the BLAST program in the National Center for Biotechnology Information Website (http://blast.ncbi.nlm.nih.gov/blast.gov).

Chi-square tests and logistic regression analyses of *Bartonella* prevalence among species, sites, gender, and seasons were conducted using the Statistical Package for the Social Sciences (SPSS 11.0, Chicago, Illinois). Data from all sites except Tiantai were used for logistic regression. The dependent variable in the logistic regression was infection status and the independent variables were location, species, gender, location×species, location×gender, and species×gender. The method of logistic regression was forward-conditional. The stepwise probability for entry was 0.05 and for removal was 0.10. The classification cutoff was 0.5 and the maximum number of iterations was 20. Validation analysis, regression diagnostics, and goodness-of-fit of the logistic regression model were also conducted.

RESULTS

Bartonella infection prevalence

A total of 427 small mammals of 11 species were captured (Table 3). Of these, 230 were female and 197 were male.

Amplified PCR products of the expected size from all primer sets were considered positive. Instances when only one of three or two of three primers amplified PCR products were considered negative. Overall, 31.4% (134/427) of the small mammals were PCR-positive. Negative controls failed to yield detectable PCR products; positive controls gave consistent PCR products of the appropriate size. The prevalence of *Bartonella* DNA varied significantly among species (0% in *M. musculus* and the unidentified Soricidae to 46.9% in *A. agrarius* [χ^2=53.747, P<0.001]) and locations (0% to 56.3% among locations [χ^2 = 44.032, P<0.01], Table 1).

Bartonella prevalence varied significantly among seasons at Titantai (χ^2 = 24.219, P<0.01). Prevalences in spring, summer, autumn, and winter were 25% (17/67), 21% (11/53), 39% (12/31), 58% (45/78) respectively. Prevalence of *Bar-*

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tonella DNA was similar among male (32.0%; n=197) and female (30.9%; n=230) small mammals (χ^2, P>0.05).

Logistic regression

The χ^2 for goodness-of-fit of the logistic regression model was 85.55 (P<0.01). The overall correct percentage was 81.8%. Variables in the most parsimonious model were location, species, and location×species.

Characterization of *gltA* amplicons

A pair-wise comparison was performed using reference *Bartonella* retrieved from GenBank. There was only one nucleotide mutation, T→C, at position 131 between the DNA sequences of R21ZJ and R66ZJB, and the DNA sequence of R1ZJB had only one nucleotide difference when compared to R21ZJ (position 338, C→T). These three sequences were most similar (>95%) to *Bartonella* strain OL11554ks (DQ357612), which was detected in blood of *Onychomys leucogaster* in the western United States (Ying et al., 2007). They had the most similarity (96%) to *B. grahamii* (AY584855 and AY584856 from *A. agrarius*).

DISCUSSION

The overall *Bartonella* prevalence in our study (31.4%) was similar to that found by Li et al. (2004). These authors found that most positive rodents were *R. tanezumi*. We report *Bartonella* DNA in nine species of small mammals (Table 3) including the first records of *Bartonella* DNA from *E. melanogaster* and *N. confucianus*. Prevalences varied among small mammal species. *Apodemus agrarius*, *Rattus losea*, and *Suncus murinus* had the highest prevalences. *Bartonella* DNA was not detected in *M. musculus*.

The finding of *Bartonella* DNA in seven of 10 study sites indicates that bartonellae are broadly present in Zhejiang Province. However, the prevalence in small mammals varied among sites within the province. Tiantai, Longquan, Linhai, Jiande, and Longyou had the highest prevalences, and *Bartonella* was not detected at Jindong, Chun'an, and Jiangshan. Other factors that may have influenced our results include the presence of discontinuous populations of hosts that undergo local extinctions of *Bartonella*, the transmission of *Bartonella* from mammal species that may not occur in the sites where *Bartonella* was not detected, effects of temporal differences among sites, and small sample sizes.

The difference in prevalence of *Bartonella* among seasons was unexpected. We speculate that the differences may correlate with population growth periods and variations in the species of small mammals captured. We captured many juvenile rodents in spring, but all captured rodents were adults during the winter. The results of this study suggest the need to determine whether *Bartonella* infection varies with the life stages of the host.

The citrate synthase gene sequence (*gltA*) is acknowledged as the phylogenetic marker for *Bartonella* (Birtles and Raoult, 1996; Lascola et al., 2003). The *gltA* sequences of R29ZJ, R60ZJ, R17ZJB, R2ZJJD, and R3ZJJD were identical and those of R21ZJ, R66ZJB, and R1ZJB were very similar. Both clusters were genetically related to the recognized human pathogen *B. grahamii* (Kerkhoff et al., 1999; Serratrice et al., 2003). Because we did not sequence all PCR-positive amplicons, there could be other potentially zoonotic *Bartonella* species in our collections. However, these results demonstrated that *A. agrarius* was the host of one species of *Bartonella* that was similar to *B. grahamii* (a human pathogen). These *Bartonella* associations in small mammals may represent a public health risk.

The similarity of *Bartonella* from *A. agrarius* from China to a *Bartonella* isolated from an *Onychomys leucogaster* from the western United States is of interest. Bown et al. (2002) reported that isolates from the United States and the United Kingdom were identical and most

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similar to *B. vinsonii*, a species associated with New World rodents. However, there are fundamental deference between those results and ours. *Apodemus agrarius* and *O. leucogaster* are from different subfamilies, but *Sciurus carolinensis* and *Sciurus vulgarisgrey* belong to the same genus.

In summary, to our knowledge, this is the first report on *Bartonella* from the eastern coastal region of China. The prevalence of *Bartonella* was high in small mammals from Zhejiang Province and varied among species, sites, and seasons. In addition, a *Bartonella* sp., similar to *B. grahamii*, exists in association with *A. agrarius* in Zhejiang Province. Prevention disease of due to Bartonella infection in humans in Zheijiang Province should include control programs directed at *A. agrarius*, *R. losea*, and *S. murinus*.

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