ORIGINAL ARTICLE

Epidemiology of *Bartonella* Infection in Rodents and Shrews in Taiwan

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Impacts

- This is the first study conducted in Taiwan to determine the epidemiology of *Bartonella* infection in small mammals, and a high prevalence of 41.3% was identified.
- The isolated *Bartonella* species were diverse and included human pathogenic *Bartonella elizabethae* and *Bartonella grahamii*. Our findings also indicated that prevalence of the infection was associated with different ecological environments and animal species.
- Future studies need to be conducted to determine whether these *Bartonella* species would be responsible for human cases with unknown fever or febrile illness in Taiwan, especially zoonotic *B. elizabethae* and *B. grahamii*.

Keywords:
Bartonella species; small mammals; epidemiology; *Rattus losea*

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Summary

During the period of August 2002 and November 2004, an epidemiological investigation for *Bartonella* infection was conducted in small mammals in Taiwan. Using whole blood culture on chocolate agar plates, *Bartonella* species were successfully isolated from 41.3% of the 310 animals tested. The isolation rate of *Bartonella* species varied among different animal species, including 52.7% of the 169 *Rattus norvegicus*, 28.6% of the 126 *Sucus murinus*, 10% of the 10 *Rattus rattus* and 66.7% of the three *Rattus losea*. Bacteremia prevalence also varied with the origin of the animals, as 56.2% of the animals captured on farms, 38.6% of the ones captured at harbour sites and 11.8% of the animals captured from urban areas were bacteremic. Through molecular analysis of the *gltA* gene and 16S/23S intergenic spacer region, genetic diversity of *Bartonella* organisms was identified, including strains closely related to *Bartonella tribocorum*, *Bartonella grahamii*, *Bartonella elizabethae*, *Bartonella phoceensis* and *Bartonella rattimassiliensis*. Moreover, this is the first report of zoonotic *B. elizabethae* and *B. grahamii* identified in *R. losea*, the lesser rice-field rat. Various *Bartonella* species were identified in *R. norvegicus*, compared to 97.2% of *Suncus murinus* with unique *Bartonella* species. By indirect immunofluorescence antibody test, using various rodent *Bartonella* species as antigens, consistently low percentage of seropositivity implied that small mammals may play a role as competent reservoirs of *Bartonella* species in Taiwan. Future studies need to be conducted to determine whether these *Bartonella* species would be responsible for human cases of unknown fever or febrile illness in Taiwan, especially zoonotic *B. elizabethae* and *B. grahamii*.

Introduction

Bartonella infections are mainly vector-borne zoonoses. Although various vertebrate animals have been documented as reservoirs of *Bartonella*, small mammals have been shown to harbour most of the known *Bartonella* species. *Bartonella talpae* was identified in a mole captured near Cambridge in UK in 1905 (Maurin et al., 1997). *Bartonella vinsonii* subsp. *visonii* was isolated only once from the blood of a Canadian vole (*Microtus pennsylvanicus*) in the 1940s (Baker, 1946). In 1995, three species isolated from wild rodents were added to the genera *Bartonella*, including *Bartonella grahamii* from *Clethrionomys glareolus*, *Bartonella taylorii* from *Apodemus* sp. and *Bartonella doshiae* from *Microtus arestis* (Birtles et al., 1995). In Peru, several *Bartonella* species were isolated from *Phyllotis* spp, and *Rattus norvegicus* was found to carry *Bartonella elizabethae* (Birtles et al., 1999). In the United States and Portugal, 18% of the rodents was *Bartonella* bacteremic, and the isolates from *R. norvegicus* were closely related to *B. elizabethae* (Ellis et al., 1999). *Bartonella tribocorum*, *Bartonella rattimassiliensis* and *Bartonella phoceensis* have been initially isolated from *R. norvegicus* rats in France (Heller et al., 1998; Gundi et al., 2004).

Of major importance, several rodent *Bartonella* species have been shown to be human pathogens. *B. elizabethae* and *B. washoensis* were found in patients with endocarditis (Daly et al., 1993; Kosoy et al., 2003). Human cases with neuroretinitis or bilateral retinal artery branch occlusions were shown to be infected with *B. grahamii* (Kerkhoff et al., 1999; Serratrice et al., 2003). In Baltimore, USA, a sero-survey showed that 33% of the inner-city intravenous drug users was seropositive for *B. elizabethae* (Comer et al., 1996). A cattle rancher with neurological disorders was found to be infected with *B. vinsonii* subsp. *arupensis*, possibly from a rodent origin (Welch et al., 1999). These findings clearly demonstrated that small mammals are important reservoirs for various zoonotic *Bartonella* species.

Prevalence of *Bartonella* infection varies in small mammals in different geographical areas. Previous studies have shown that the prevalence was 17–62% in Europe (Birtles et al., 1994; Bown et al., 2002; Holmberg et al., 2003; Engbaek and Lawson, 2004; Tea et al., 2004), 37.2% in South Africa (Pretorius et al., 2004), 6–43.5% in Asia (Bai et al., 2002; Castle et al., 2004; Li et al., 2004; Bai et al., 2005; Kim et al., 2005; Winoto et al., 2005; Inoue et al., 2008) and 42.2–57% in North America (Kosoy et al., 1997; Jardine et al., 2005). Although the discrepancy of prevalence of *Bartonella* infection in various studies has not been well explained, it is reasonable to hypothesize that different ecological environments may favour different rodent populations; and thus diverse *Bartonella* species could be observed in different geographical areas and in different species of small mammals.

The major objectives of this study were to determine the prevalence of *Bartonella* infections in small mammals in Taiwan, and to characterize *Bartonella* isolates obtained from these animals at the species level by molecular methods. The information in this study will be useful to develop control strategies and diagnostic procedures for *Bartonella* infections in humans in Taiwan.

Materials and Methods

Sample collection

During the period of August 2002 and November 2004, a total of 310 rodents and shrews (169 *R. norvegicus*, 126 *Suncus murinus*, 10 *Rattus rattus*, 3 *Rattus losea*, and 2 *Mus musculus*,) were captured in the Taichung area in central Taiwan. The Taichung area comprises Taichung County (coastal and sub-urban areas) and Taichung City, which is completely surrounded by the County. The animals were trapped from several sites with three different environmental characteristics, including harbour areas in coastal region, dairy cattle farms in the sub-urban area and traditional markets in the urban area. After being humanely anesthetized with zoletil 50 (Virbac Laboratories, 06516 Carros, France), the animals were bled to death via cardiac puncture by using 3-ml syringes fitted with 22-gauge, 1.5-inch needles. The blood was then collected in an EDTA tube and stored at −80°C for further isolation and molecular identification of *Bartonella* species.

Blood culture for *Bartonella* species

A total of 310 whole blood specimens collected from rodents and shrews were used for *Bartonella* isolation. For bacterial culture, 200 μl of thawed whole blood sample were plated onto chocolate agar. The plates were incubated at 35°C in 5% CO_2 for at least 1 month and checked for growth of *Bartonella* species on a weekly basis. For the suspected plates, at least two colonies were randomly selected and separately sub-cultured onto different fresh agar plates for further molecular identification of *Bartonella* species by PCR/RFLP (polymerase chain reaction/restriction fragment length polymorphism) and sequence analysis.

DNA extraction and PCR/RFLP procedures for *Bartonella* species

After culturing from a single colony pick from the original plate, DNA of the cultured isolates was extracted

using Viogene DNA/RNA Extraction Kit (Viogene Biotek Corp., Taipei, Taiwan) following the manufacturer's instructions. The primers BhCS.781p and BhCS.1137n were used first for amplifying a fragment ranging from 380-bp to 400-bp of the partial *gltA* gene of *Bartonella* species following the procedures developed by Norman et al. (1995). Reaction mixtures of PCR were set up as follows: 5 μl of DNA template, 0.5 μl of 100 μM each primer, 4 μl of 2.5 mM dNTPs, 5 μl of 10× PCR buffer, 3 μl of 25 mM $MgCl_2$, 31.75 μl of sterile distilled H_2O and DNA polymerase 0.25 μl (Ampli Gold, Applied Biosystems, Foster City, CA, USA). Primers bartsppA and bartsppB amplified a fragment of the 16S/23S rRNA intergenic spacer region (ITS) by single-step PCR (Jensen et al., 2000); the different molecular size of the PCR products allowed for a quick differentiation of various *Bartonella* species. Restriction fragment length polymorphism analysis (RFLP) of the *gltA* gene was also performed for quick identification of *Bartonella* organisms at species level with restriction enzymes *Taq*I (New England BioLabs., Beverly, MA, USA), *Hha*I (New England BioLabs) and *MseI* (New England BioLabs) following the manufacturer's instructions.

Sequencing and phylogenetic analyses of *Bartonella* species

The confirmed PCR products were sent for automated sequencing (Mission Biotech, Taipei, Taiwan). By phylogenetic analysis, the sequences obtained from the isolates were compared to the sequences of *Bartonella* type strains, including *Bartonella alsatica* (IBS382T) CIP 105477, *Bartonella birtlesii* (IBS 325T) CIP 106294, *Bartonella bovis* (91-4T) CIP 106692, *Bartonella clarridgeiae* (Houston-2T) ATCC 51734, *B. doshiae* (R18T) NCTC 12862, *B. elizabethae* (F9251T) ATCC 49927, *B. grahamii* (V2T) NCTC 12860 b, *Bartonella henselae* (Houston-1T) ATCC 49882, *Bartonella koehlerae* (C-29T) ATCC 700693, *Bartonella quintana* (FullerT) ATCC VR-358, *B. taylorii* (M6T) NCTC 12861, *B. tribocorum* (IBS 506T) CIP 104576, *B. vinsonii* subsp. *arupensis* (OK 94-513T) ATCC 700727, *B. vinsonii* subsp. *berkhoffii* (93-CO1T) ATCC 51672, *B. vinsonii* subsp. *vinsonii* (BakerT) ATCC VR-152. The sequences of the *Bartonella* isolates were analysed first using the BLASTN program of the NCBI website to identify the closest *Bartonella* species in the Entrez Nucleotide database. Then, after the sequences were aligned by the Clustal W method from the BioEdit program (Tom Hall, Ibis Biosciences, Isis Pharmaceuticals, Carlsbad, CA, USA), phylogenetic analysis was performed on the aligned DNA sequences using maximum parsimony as implemented in PHYLIP version 3.6 (Joseph Felsenstein, Department of Genome Sciences and Department of Biology, WA, USA). Bootstrap support was calculated by using 1000 bootstrap data replicates as implemented by Seqboot in the PHYLIP program version 3.6. The sequences of the *gltA* gene of the strains used in this study have been submitted to the Genbank. The obtained accession numbers are serial numbers from FJ179374 to FJ179394.

Indirect immunofluorescence antibody test

The bacterial antigens for indirect immunofluorescence antibody test (IFA) to determine antibody titres included *B. tribocorum*, *B. grahamii*, *B. elizabethae* and a unique *Bartonella* strain MLE053 (closely related to *B. tribocorum*) isolated from *S. munrinus* in Taiwan. Each type strain was cultured on chocolate agar plate at 35°C in 5% CO_2 for 4 days to grow up a confluent plate of bacteria. Then the IFA slides were made as described previously (Chang et al., 2006). The slides were kept at −70°C for storage.

For IFA testing, the frozen sera were thawed at room temperature and treated at 56°C for 30 min for heat inactivation. The serum samples were serially diluted from 1 : 32 to 1 : 512 by twofold dilutions using PBS (with 10% skim milk). Thirty microlitres of the diluted serum samples were dropped onto each well of slides prepared earlier. The slides were incubated at 37°C for 40 min and then washed with PBS for 10 min. The secondary antibodies used for serological testing were fluorescein-labelled goat anti-rat immunoglobulin G (Kirkegaard® Perry Laboratories Inc., Gaithersburg, MD, USA). They were diluted at 1 : 400 in PBS with 5% milk containing 0.001% Evan's blue, and the mixture was applied to each well. The slides were incubated at 37°C for 40 min, then washed with PBS for 10 min, and washed again with double distilled water for 10 min prior to reading with a fluorescent microscope (magnification, ×400). The intensity of the bacillus-specific fluorescence was scored subjectively from 1 to 4. The previous study (Kosoy et al., 1997) has shown that only 1.5% of the 394 tested rodents were with an antibody titre ≥1 : 32 by IFA test; therefore, in this study, a seropositive animal was defined as the fluorescence score of ≥2 at dilution of 1 : 32. Milk in PBS without serum was always applied to each slide as a negative control.

Statistical analysis

The data were managed using MICROSOFT OFFICE EXCEL 2003 and analysed with EpiInfo version 3.3.2 (Center for Disease Control and Prevention, Atlanta, GA, USA). The chi-square test for homogeneity was used to evaluate if the prevalence of bacteremia is significantly different among the groups. A *P*-value less than 0.05 is considered to be statistically significant.

Results

Isolation of *Bartonella* species

Among the 310 small mammals tested, *Bartonella* organisms were isolated from 128 (41.3%) animals using whole blood culture. Despite the limited sample sizes for *R. losea*, *M. musculus* and *R. rattus*, prevalence of *Bartonella* bactermia varied among the different animal species, as shown in Table 1. *Rattus norvegicus* were the most frequently trapped animals, and 52.7% of them were *Bartonella* bacteremic. It was determined that 28.6% of the shrews (*Suncus murinus*) was *Bartonella* bacteremic. Although only three *R. losea* were tested, *bartonellae* were isolated from two of them. *Bartonella* organism was also isolated from a *R. rattus*. The prevalence of *Bartonella* bacteremia was significantly different in three sampling environments, ranging from 56.2% in farms, 38.6% in harbour area and 11.8% in urban environment ($P < 0.05$). Such an observation was also identified in the population of *R. norvegicus* rats.

Molecular characterization of *Bartonella* isolates

As shown in Fig. 1, the isolates were characterized by the molecular patterns of the combined results of PCR of the 16S/23S ITS region (three patterns: A, B, and C) and PCR/RFLP of the *gltA* gene with *HhaI* (three patterns: A, B, and C), *TaqI* (one pattern: A) and *MseI* (four patterns: A, B, C, and D) digestion. It was identified a total of seven molecular patterns, named as AAAA, AAAB, AAAC, ACAA, ABAA, BAAA and CAAD. Several isolates for each molecular pattern were selected and sequenced for comparison. After sequencing and phylogenetic analysis of the *gltA* gene (Table 2 and Fig. 2), the isolate was found to be close to five *Bartonella* species. The sequences of the *gltA* gene of the strains used in this study have been submitted to the Genbank. The obtained accession numbers are serial numbers from FJ179374 to FJ179394. The isolates with molecular patterns ABAA and BAAA, which showed as two separate linages in the phylogenetic relationship, were closest to *B. tribocorum* with 96.3–99.6% of DNA similarity. Two molecular patterns AAAB and

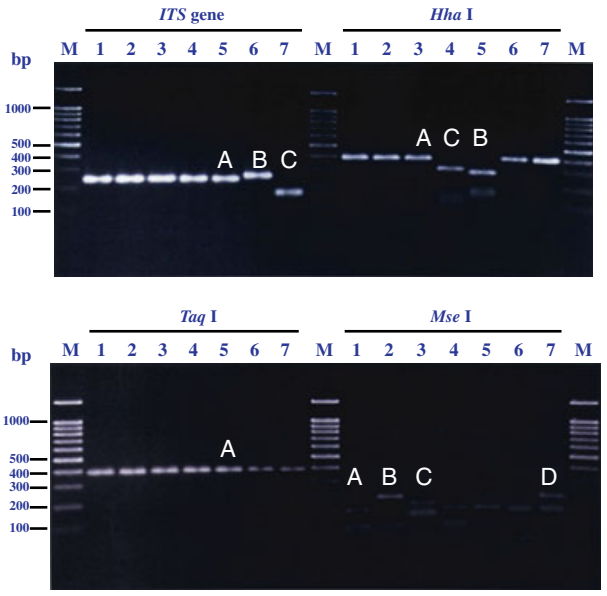

Fig. 1. Combined PCR molecular patterns for the16S-23S intergenic spacer region and PCR/RFLP analysis by digestion of the *gltA* gene with *Hha*I, *Taq*I and *Mse*I for the *Bartonella* isolates in Taiwan. Lane M, 100-bp DNA ladder; lanes 1, 2, 3, 4, 5, 5, 6, 7 are the selected isolates to show seven molecular patterns of AAAA, AAAB, AAAC, ACAA, ABAA, BAAA and CAAD, respectively.

AAAC were closest to *B. grahamii* (DNA similarity value: 93.6%-95.4%). The isolates with AAAA pattern and ACAA pattern were closest to *B. elizabethae* (96.6–96.9% of DNA similarity) and *B. rattimassiliensis* (98.4–99.6% of DNA similarity), respectively. Although the isolate with CAAD pattern was shown to be close to *B. taylorii* (92.1% of DNA similarity) by phylogenetic analysis, the sequence of the isolate was much closer to *B. phoceensis* with 94.0% similarity after BLASTN analysis. The isolates close to *B. rattimassilensis*, *B, tribocorum*, *B. grahamii*, *B. elizabethae* and *B. phoceensis* were isolated from *R. norvegicus*, and the ones close to *B. tribocorum* and *B. elizabethae* were the most prevalent. On the contrary, most (97.2%) of *S. murinus* were infected with isolates close to *B. tribocorum*. Several *R. norvegicus* rats were identified to

Table 1. Prevalence (%) of *Bartonella* bacteremia by whole blood culture in small mammals from three environments in Taichung, Taiwan

	Sampling environments			
Animal species	Harbour	Farm	Urban	Total
Rattus norvegicus	51.6 (47/91)	60.6 (40/66)	16.7 (2/12)	52.7 (89/169)
Suncus murinus	28.8 (36/125)	0.0 (0/1)	–	28.6 (36/126)
Rattus losea	66.7 (2/3)	–	–	66.7 (2/3)
Mus musculus	0.0 (0/1)	0.0 (0/1)	–	0.0 (0/2)
Rattus rattus	–	20.0 (1/5)	0.0 (0/5)	10.0 (1/10)
Total	38.6 (85/220)	56.2 (41/73)	11.8 (2/17)	41.3 (128/310)

Table 2. Prevalence (%) of *Bartonella* bacteremia by whole blood culture according to animal and *Bartonella* species, Taichung, Taiwan

	Percentage of *Bartonella* species				
Animal species	*B. rattimassiliensis*	*B. tribocorum*	*B. grahamii*	*B. elizabethae*	*B. phoceensis*
Rattus norvegicus	1.1 (1/89)	64.0 (57/89)	9.0 (8/89)	34.8 (31/89)	3.4 (3/89)
Suncus murinus	2.8 (1/36)	97.2 (35/36)	0 (0/36)	0 (0/36)	0 (0/36)
Rattus rattus	0 (0/1)	100.0 (1/1)	0 (0/1)	0 (0/1)	0 (0/1)
Rattus losea	0 (0/2)	0 (0/2)	50.0 (1/2)	50.0 (1/2)	0 (0/2)

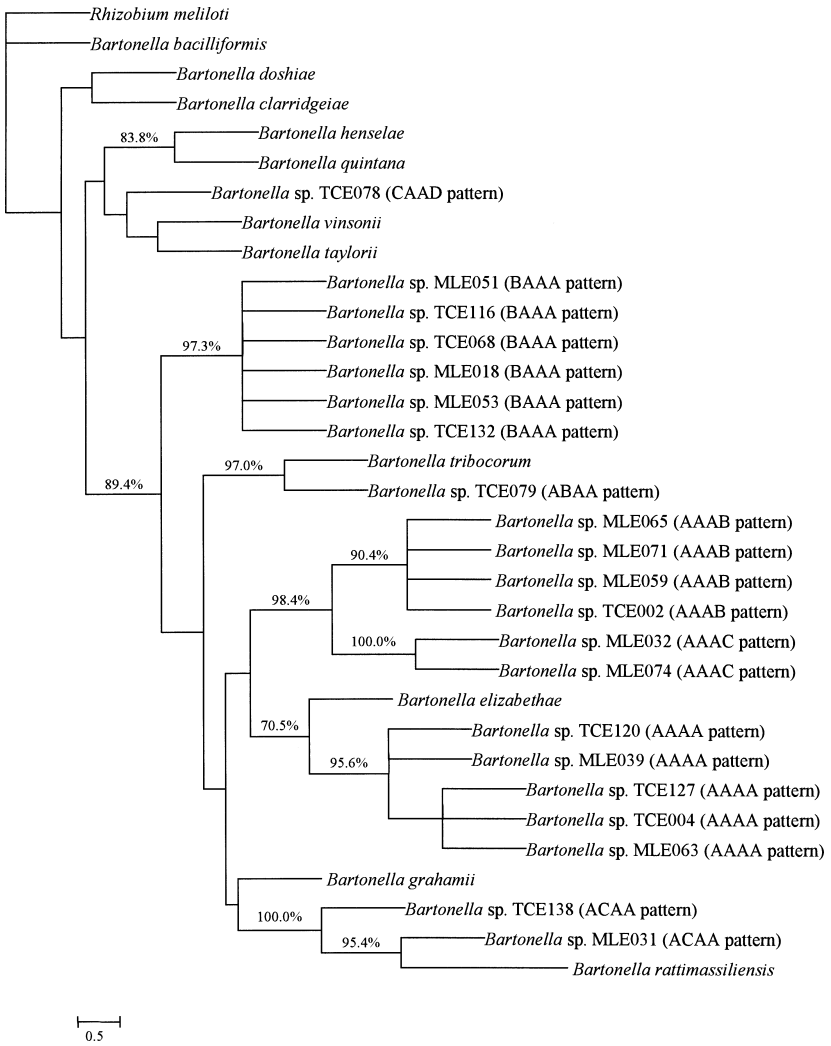

Fig. 2. Phylogenetic analysis of *Bartonella* type strains and *Bartonella* spp. isolated from small mammals in Taiwan on the basis of partial DNA sequences of *gltA* gene. The phylogenetic relationship was constructed using the maximum parsimony method of phylip version 3.6 program, and bootstrap analysis was performed with 1000 trials of bootstrap data (confidence of bootstrap values not shown if lower than 70%). The tree was rooted using *Rhizobium meliloti* as an outgroup reference. The closest *Bartonella* species for the molecular patterns were *B. tribocorum* for patterns of ABAA and BAAA, *B. grahamii* for patterns of AAAB and AAAC, *B. elizabethae* for the pattern of AAAA, *B. rattimassiliensis* for the pattern ACAA and *B. phoceensis* for the pattern of CAAD.

be co-infected with different *Bartonella* species, including 10 animals with *B. elizabethae*-like and *B. tribocorum*-like organisms, and one animal with *B. phoceensis*-like and *B. tribocorum*-like organisms.

For the isolates with zoonotic potential, *B. grahamii*-like organisms were only isolated from animals in harbour and farm areas. *Bartonella elizabethae* was identified in the three different sampling environments, yet only one *R. norvegicus* rat from the urban environment was infected with *B. elizabethae*.

Serological test by indirect immunofluorescence antibody test

For serological testing, a total of four different *Bartonella* strains were used as antigens, including *B. elizabethae*,

B. grahamii, *B. tribocorum* and the *Bartonella* strain MLE 053. The strain of MLE 053 was isolated from *S. murinus* and closest to *B. tribocorum*, but was shown to have an independent linage according to the result of phylogenetic analysis (Fig. 2). The result showed that percentages of presence of antibodies against *B. elizabethae*, *B. grahamii*, *B. tribocorum* and *Bartonella* strain MLE 053 were 0%, 3.2%, 5.2% and 3.2%, respectively.

Discussion

This is the first study conducted in Taiwan to understand the epidemiology of *Bartonella* infections in small mammals. Geographically, the study area is located North of 24° latitude North and about 120.5° longitude East, with The Central Mountain Range at its East and the Taiwan Strait at its west. Although Taiwan is located in the subtropical areas in Asia, Taichung has a milder climate than any other parts in Taiwan, with an annual average temperature of 23°C and humidity of 80%. The results indicated that the prevalence of *Bartonella* bacteremia animals was as high as 41.3%. Moreover, isolates close to *B. elizabethae* and *B. grahamii*, which have been recognized as human pathogens, were identified in these small mammals in central Taiwan. Of major importance, 34.8% of the 89 *R. norvegicus* rats with *Bartonella* bacteremia was found to be *B. elizabethae* carriers. These findings strongly suggest that rodents and shrews in Taiwan may serve as important reservoirs of *Bartonella* species; therefore humans living in this area should be aware of possible transmission from these animals. To the best of our knowledge, this is the first report to demonstrate the presence of the zoonotic *B. elizabethae* and *B. grahamii* in lesser rice-field rats, *R. losea*. This rodent species is also found in Vietnam, Thailand, Laos and southeastern coastal region of China.

In Asia, several studies have shown that small mammals, mainly rodents, are important reservoirs for various *Bartonella* species (Bai et al., 2002; Winoto et al., 2005; Bai et al., 2007; Inoue et al., 2008). Our results further indicated that the prevalence of *Bartonella* infection was significantly different in small mammals from various sampling sites in Taiwan. Overall, the lowest prevalence was identified in the urban environment; a result similar to the previous findings in Japan (Inoue et al., 2008). It is still unclear why animals from the farm areas were with the highest probability of acquiring *Bartonella* organisms. We reasonably hypothesized that the ecological diversity in farm areas may favour the survival of vectors for *Bartonella* transmission. Further studies still need to be conducted to validate this hypothesis.

Taiwan is located in East Asia and is an island with marine tropical climate. However, the Tropic of Cancer (23.5′N) running across its middle section divides the island into two regions, one with the tropical monsoon climate in the South and the other with subtropical monsoon climate in the North. The diverse climate and the Central Mountain along the island result in unique ecological environment that sustain various living creatures. *Bartonella* species in small mammals were the closest to at least five different *Bartonella* species, including *B. rattimassiliensis*, *B. tribocorum*, *B. grahamii*, *B. elizabethae* and *B. phoceensis*. A similar result is found in Japan before by Inoue et al. (2008). Although the vectors responsible for some of these *Bartonella* species have been identified in recent studies, including fleas and mites (Bown et al., 2004; Kim et al., 2005; Loftis et al., 2006; Marié et al., 2006; Abbot et al., 2007), it will be of major interest to elucidate which vectors are responsible for *Bartonella* transmission in Taiwan. Furthermore, the result of this study implied that *Bartonella* organisms may have host preference, which is similar to results reported from other parts of the world (Birtles et al., 1994; Kosoy et al., 1997, 2000). In Taiwan, *R. norvegicus* seems to be capable reservoirs for various *Bartonella* species. On the contrary, the majority of the *S. murinus* was infected with only one *Bartonella* species, closest to *B. tribocorum*. One possible reason to explain this discrepancy is that these two animals belong to two different families (*R. norvegicus* in the Muridae family and *S. murinus* in the Soricidae family), and the other possible reason is that the infested vectors could also be different for these two animal species.

A previous study has demonstrated that most of the rodents with *Bartonella* bacteremia had low antibody titres against various *Bartonella* antigens (Kosoy et al., 1997), suggesting that these animals may serve as competent reservoirs for *Bartonella* species in nature. In our study, four different *Bartonella* strains that were isolated from animals in most areas were used as antigens for serological testing. Comparing to the high prevalences of *Bartonella* bacteremia, percentages of seropositivity were fairly low in the tested animals. Of major importance, none of the animals was seropositive for *B. elizabethae*. As a result, our data not only strongly suggested that small mammals are competent reservoirs for these *Bartonella* species, but also indicated that serological testing may not be an appropriate tool for epidemiological investigation in small mammals, especially for *B. elizabethae*.

In conclusion, people should be aware that small mammals in Taiwan are reservoirs for *Bartonella* species, especially for the zoonotic *B. elizabethae* and *B. grahamii*. Further studies need to be conducted to determine whether these agents would be responsible for human cases with unknown fever or febrile illness in Taiwan.

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