

Bartonella Species in Small Mammals and Their Ectoparasites in Taiwan

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Abstract. *Bartonella* spp. prevalence in small mammals and their ectoparasites was investigated in Taiwan. Blood samples were obtained from 66 rats, 20 shrews, 276 mites (*Laelaps* spp.), 74 fleas (*Xenopsylla cheopis*), 81 lice (*Polyplax* spp.), and 47 ticks (41 *Dermacentor* spp. and 6 *Ixodes* spp.). Bartonellae were isolated or detected in 27 (31.4%) animals. *Bartonella* DNA was detected in 48 (64.9%) fleas and 11 (64.7%) pooled lice samples, but not in mite and tick samples. *Bartonella phoceensis*, *B. queenslandensis*, *B. tribocorum*, *B. elizabethae*, and *B. rattimassiliensis* were isolated or detected in bacteremic mammals. For the first time in Taiwan, *B. tribocorum*, *B. elizabethae*, *B. queenslandensis*, and a *B. rochalimae*-like strain were detected in fleas, and *B. tribocorum*, *B. phoceensis*, and *B. rattimassiliensis* were detected in lice obtained from small mammals. A broader range of *Bartonella* species was identified in the ectoparasites than in the small mammals.

INTRODUCTION

Numerous *Bartonella* species have been isolated from rodents, including *B. birtlesii*,¹ *B. doshiae*,² *B. elizabethae*,³ *B. grahamii*,² *B. phoceensis*,⁴ *B. rattimassiliensis*,⁴ *B. taylorii*,² *B. tribocorum*,⁵ *B. washoensis*,⁶ *B. vinsonii* subsp. *arupensis*, *B. vinsonii* subsp. *vinsonii*,^{7,8} *B. rochalimae*,⁹ *B. rattaaustraliani*, *B. queenslandensis*, *B. cooperplainsensis*⁷ and many more unnamed species. In Thailand, two rodents (*Rattus surifer*) were infected with a new genotype of *Bartonella* (*Candidatus Bartonella thailandensis*).⁸ Several rodent-associated *Bartonella* species have been linked to human diseases. *Bartonella elizabethae* has been detected in a human with endocarditis;¹⁰ *B. grahamii* DNA was detected in ocular fluids of a patient with neuroretinitis¹¹ and antibodies to *B. grahamii* were detected in a patient with bilateral retinal artery branch occlusions;¹² *B. vinsonii* subsp. *arupensis* was isolated from a cattle rancher with bacteremia and fever¹³ and was associated with two patients with endocarditis;^{14,15} *B. washoensis* was isolated from a human case of fever and myocarditis;⁶ and *B. rochalimae* caused bacteremia, fever, and splenomegaly in a human patient.¹⁶

In Asia, many *Bartonella* species have been isolated from a wide range of small mammals. *Bartonella* isolates were obtained from seven rodent species of four genera in China: *Apodemus* (*A. chevrieri*, *A. draco*, and *A. latronum*), *Eothenomys* (*E. miletus*), *Rattus* (*R. norvegicus* and *R. flavipectus*), and *Mus* (*M. pahari*). Cultures obtained from *Rattus* rats were genetically related to *B. elizabethae*, a recognized human pathogen.^{17,18}

In Japan, *B. grahamii* has been isolated from *Apodemus speciosus* and *A. argenteus*; *B. tribocorum* and *B. elizabethae* from *Apodemus* mice and *R. rattus*; *B. rattimassiliensis* and *B. phoceensis* from *R. rattus*; *B. taylorii* from *A. speciosus* and *Myodes* (formerly *Clethrionomys*) voles. Two novel *Bartonella* species groups were isolated from *A. speciosus* and *A. argenteus*.¹⁹ In Russia, *B. grahamii* and/or *B. taylorii* were isolated from six species of small mammals (*A. flavicollis*, *A. uralensis*, *M. glareolus*, *M. musculus*, *Microtus arvalis*, and *Sorex araneus*).²⁰ In Thailand, most of the isolates from rodents *Bandicota indica*,

R. losea, and *R. rattus* were closely related to *B. grahamii* and *B. elizabethae*. Two additional isolates from *B. indica* clustered together and were nearly identical to an isolate from *A. draco* collected in southern China.²¹ In a recent study from Thailand, one isolate from a *Berylmys berdmorei* was close to *B. tribocorum*, and several *Bartonella* species were detected in rodents.²² *Bartonella cooperplainsensis*, *B. elizabethae*, *B. phoceensis*, *B. rattimassiliensis*, *B. tribocorum*, and an unknown genogroup were detected from *Rattus* spp., and *Bartonella* species isolated from *Bandicota* spp. included *B. cooperplainsensis*, *B. rattimassiliensis*, and *B. tribocorum*.²² *Bartonella* from *Mus* spp. were clustered with *B. cooperplainsensis* or *B. rattimassiliensis*.²² In Taiwan, a recent study in small mammals reported isolation of *Bartonella* species from 41.3% of 310 small mammals. *Bartonella rattimassiliensis* was isolated from *R. norvegicus* and *Suncus murinus*, *B. tribocorum* from *R. norvegicus*, *S. murinus* and *R. rattus*, *B. grahamii*, and *B. elizabethae* from *R. norvegicus* and *R. losea*, and *B. phoceensis* from *R. norvegicus*.²³

In vectors, several *Bartonella* species were detected from hematophagous arthropods feeding on rodents. *Ctenophthalmus nobilis*, a rodent flea, has been demonstrated as a competent vector for transmission of *B. grahamii* and *B. taylorii* in rodents.²⁴ However, *Bartonella* DNA has also been detected in lice,^{25,26} fleas,²⁵ *Mesostigmatid* mites,²⁷ and ticks²⁷ collected on small mammals; these ectoparasites are considered potential vectors for various *Bartonella* species. The role of these potential vectors in *Bartonella* transmission among rodent hosts needs to be confirmed.

The objectives of this study were to investigate the prevalence of *Bartonella* infection in small mammals in Taiwan and in the ectoparasites infesting these animals, especially in rodent populations close to domestic animal farms; and to determine *Bartonella* infections with respect to animal sex and age and presence or absence of ectoparasites. This investigation could help in better understanding the epidemiology of *Bartonella* infection in small mammals in Taiwan, and thus would help provide better strategies for disease control.

MATERIALS AND METHODS

Collection of blood from small mammals. Blood samples and ectoparasites were obtained from 88 trapped small mammals, 62 *R. norvegicus*, 21 *S. murinus*, and 5 *R. rattus*, during July

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2008–April 2009. The small mammals were anesthetized with zoletil 50 (Virbac Laboratories, Carros, France) by intramuscular injection. Approximately 2 mL of whole blood from each small mammal was obtained by cardiac puncture into plastic tubes containing EDTA and frozen at -80°C before testing. Animals were then euthanized as described.²³ Animal species, sex, estimated age, and presence or absence of ectoparasites were recorded for each animal at the time of sampling.

Collection and morphologic identification of ectoparasites.

Ectoparasites attached to the small mammals, such as ticks, mites, fleas, and lice, were carefully pulled out with tweezers or a flea comb and then put in individual vials (all parasites from the same animal were put into the same vial). In the laboratory, each parasite was given an identification number and then stored in separate microtubes containing 70% ethanol. The parasite species, stage, and sex were determined by using an optical microscope (Stemi 2000-C; Zeiss, Oberkochen, Germany) and morphologic keys,^{28–31} and the results were recorded in a spreadsheet.

Isolation of *Bartonella* spp. in small mammals. Two culture methods, direct culture and pre-enrichment culture, were used to isolate *Bartonella* spp. from each small mammal blood sample. For direct culture, 100 μL of blood were cultured on chocolate agar plates and incubated in an atmosphere of 5% CO_2 at 35°C and examined weekly for at least four weeks.³² For pre-enrichment culture, blood samples were incubated in liquid *Bartonella* alpha-Proteobacteria growth medium and incubated in a water-saturated atmosphere of 5% CO_2 at 35°C as described.³³ After a seven-day incubation period, 100 μL of the sample from the liquid medium were then sub-cultured on chocolate agar plates as described above and examined weekly for at least four weeks. The rest of the blood was stored at -80°C until DNA extraction.

DNA extraction from isolated colony, small mammals, and ectoparasites. For each chocolate plate that yielded suspected *Bartonella* isolates, two colonies were randomly selected for DNA extraction. Each of the two colonies picked from the original plate was heated at 100°C for 10 minutes in 50 μL of sterile water. After centrifugation for 8 minutes at $18,000 \times g$, supernatants were kept at -20°C until tested by polymerase chain reaction (PCR) as a DNA template. Two hundred microliters of each whole blood sample was used to extract DNA by using the DNeasy Tissue Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions.

When the number of ectoparasites was ≤ 5 for each developmental stage on a given animal, every ectoparasite on this animal was collected and subjected to DNA extraction. If the number of ectoparasites was > 5 on the sampled animal, 5 nymph ticks, 5 adult mites, and 5 adult fleas attached to that animal were randomly selected for molecular detection.

Because larval and nymph ticks, adult and nymph mites, adult fleas, and nymph lice were small, whole ectoparasites or pooled tick larvae (≤ 10), pooled mite nymphs (≤ 10), and pooled louse nymphs (≤ 10) were used for DNA extraction. When > 50 ectoparasites were collected from an animal, only 5 pools of 10 larvae or 10 nymphs were selected for molecular detection. DNA extraction was performed as described above.

Polymerase chain reaction and sequencing for *Bartonella* detection in isolated colonies and small mammal blood, and DNA of ectoparasites. A PCR of each DNA sample from isolated colonies, animal blood, and ectoparasites were

performed with *Bartonella*-specific primers of the citrate synthase (*gltA*) gene as described,³⁴ followed by direct sequencing of purified PCR products. The PCR amplification was performed as follows, after minor modifications: 1 cycle for 10 minutes at 95°C ; 45 cycles for 30 seconds at 95°C , 1 minute at 57°C , and 2 minutes at 72°C ; and 1 cycle for 5 minutes at 72°C for a final extension. The PCR amplification was carried out in a 25- μL reaction volume that contained 2.5 μL of extracted DNA, 2.5 μL of $10\times$ reaction buffer containing 15 mM MgCl_2 , 2 μL of dNTP master mix (1.25 mM of each of the four dNTPs), 0.25 μL (10 μM) of each primer, 0.125 μL of Gold *Taq* DNA polymerase, and 17.375 μL of sterile water. *Bartonella henselae* Houston-1 (ATCC no. 49882) and double-distilled water were used as positive and negative controls for *Bartonella* detection. After electrophoresis in 2% agarose gel for 2 hours at 100 volts, the gels were stained with ethidium bromide and PCR products were visualized by ultraviolet fluorescence.

Sequences of positive *Bartonella* PCR products from isolated colonies, animal blood, and ectoparasites were determined by using an ABI 3730 XL DNA Analyzer (Mission Biotech Co. Ltd., Taipei, Taiwan).

Phylogenetic and statistical analyses. Phylogenetic and molecular evolutionary analyses were conducted by using MEGA version 4 (<http://www.megasoftware.net/>). After sequence alignment, DNA samples having the same sequence were assigned a group number to build the phylogenetic tree. Determination of the *Bartonella* species with the highest DNA similarity value was performed by comparing the sequence of each group with corresponding fragments deposited in GenBank. Phylogenetic analysis was performed on the aligned DNA sequences using neighbor-joining method by Kimura 2-parameter model, and bootstrap support was calculated by using 1,000 bootstraps.^{35,36}

Data were analyzed by using StatXact version 8.0 (Cytel Inc., Cambridge, MA), and exact chi-square tests were used to evaluate the association between 1) the infection status, species, sex, age, and location of an animal; 2) either the presence of *Bartonella* DNA in ticks, mites, fleas, or lice and the species, sex, and age of an animal, the sex of the flea or mite, and stage of the tick or mite; 3) *Bartonella* infection in animals and ectoparasite infestation status for these animals; and 4) *Bartonella* infection in animals and the presence of *Bartonella* DNA-positive ectoparasites. A two-tailed Fisher's exact test was used when expected numbers of observations were < 5 . The Mann-Whitney test was performed to evaluate differences in ectoparasite burden between farm types conditional on rodent species (*Rattus* spp. versus *Suncus* spp.) and between rodent species conditional on farm type. A *P* value < 0.05 was considered to be statistically significant.

Additional PCR and sequencing for *Bartonella* detection.

After phylogenetic and molecular evolutionary analyses, sequences identified to have a sequence similarity value $< 95\%$ with known *Bartonella* species were tested with other genes for further molecular identification. PCR analyses of the two 16S–23S internal transcribed spacer (ITS) regions, RNA polymerase (*rpoB*), cell division (*ftsZ*), and 16S rDNA genes were performed using five pairs of *Bartonella*-specific primers as described.^{37–40} For the ITS region, two bands from the ITS pair 1 region and two bands from the ITS pair 2 region after gel expression were excised. DNA was then extracted

TABLE 1
Bartonella spp. detection in small mammals, Taiwan*

Animal species	No. animals (no. PCR/culture+, %)	Sex		Age		Location						
		F	M	A	J	CF	SF	S	AS	H	GF	DF
<i>Rattus norvegicus</i>	61 (22, 36)	35 (11)†	26 (11)	40 (18)	21 (4)	28 (19)	22 (1)	2 (1)	5	2	1 (1)	1
<i>Suncus murinus</i>	20 (4, 20)	13 (2)	7 (2)	3 (1)	17 (3)	10 (3)	5 (1)	4	1	0	0	0
<i>Rattus rattus</i>	5 (1, 20)	0	5 (1)	3 (1)	2	3 (1)	1	1	0	0	0	0
Total	86 (27, 31.4)	48 (13)	38 (14)	46 (20)	40 (7)	41 (23)	28 (2)	7 (1)	6 (0)	2 (0)	1 (1)	1 (0)

* Values in parenthesis are number of positive animals; last number in second column is a percentage. A = adult; J = juvenile; CF = cattle farm; SF = swine farm; S = university campus; AS = animal shelter; H = housing unit; GF = goose farm; DF = duck farm.

† Two small mammals, 1 *R. norvegicus*, and 1 *Suncus murinus* could not be bled but were examined for ectoparasites.

from the gel and sequenced. The PCR reaction mixtures, gel expression, and sequence performance were the same as described above.

RESULTS

Collection of blood from small mammals. Overall, 88 small mammals were trapped in 3 counties in central Taiwan (Table 1). Of these 88 mammals, blood samples could not be obtained from 2 animals (one *R. norvegicus* at one swine farm and one *S. murinus* at one cattle farm), but ectoparasites were obtained when present from all 88 animals. Among these samples, 42 (31 *Rattus* and 11 *Suncus*) were collected from 7 cattle farms (median = 6 small mammals/farm, range = 1–18 small mammals/farm) and 29 animals (24 *Rattus* sp. and 5 *Suncus*) from 6 swine farms (median = 5 animals/farm, range = 1–9 animals/farm). Overall, a higher ectoparasite burden for the small mammals, mainly rats, captured on cattle farms (rats: median = 7 ectoparasites/animal, range = 0–107 ectoparasites/animal; shrews: median = 0 ectoparasite/animal, range = 0–5 ectoparasites/animal) was observed compared with the animals captured on swine farms (rats: median = 0 ectoparasite/animal; shrews: median = 0 ectoparasite/animal, range = 0–8 ectoparasites/animal). Interestingly, among *Rattus* spp., there was a significant difference between farm types ($P = 0.004$) but not among *Suncus* spp. ($P = 0.64$) and between small mammals species on the cattle farms ($P = 0.0002$) but not on the swine farms ($P = 0.97$).

Collection and morphologic identification of ectoparasites. Ectoparasites were obtained from 54 (61.4%) of the 88 small mammals (Table 2). Of the 54 animals, 43 (79.6%) were *R. norvegicus*, 9 (16.7%) were *S. murinus*, and 2 (3.7%) were *R. rattus*. Seventeen (31.5%) animals were adult females, 12 (22.2%) were adult males, 15 (27.8%) were juvenile females, and 10 (18.5%) were juvenile males. From these ectoparasites, 276 mites, 74 adult fleas, 81 nymph lice, and 47 ticks were selected for identification and DNA extraction (Table 2). All mites belonged to the genus *Laelaps*. All fleas and lice were identified as *Xenopsylla cheopis* and *Polyplax* spp., respectively.

Most of the ticks belonged to the genus *Dermacentor*, and only six nymphs were identified as *Ixodes* spp.

Isolation of *Bartonella* spp. in small mammals and DNA extraction from isolated colonies, small mammal blood, and ectoparasites. The blood of 23 (26.7%) animals yielded suspected *Bartonella* colonies on chocolate agar plates, including 5 (5.8%) animals that also yielded suspected *Bartonella* colonies when using the pre-enrichment culture technique.

DNA was extracted from 44 colonies obtained from 23 animal blood samples (2 colonies for 21 animals and 1 colony for 2 animals) by direct culture and from 10 colonies obtained from 5 animals (2 colonies for each animal) by pre-enrichment culture. DNA was extracted from all 86 animal blood samples, 38 selected adult mites, 74 adult fleas, and 12 nymph ticks. The 238 nymph mites, 81 nymph lice, and 35 tick larvae were combined in 41, 17, and 5 pools, respectively (range = 1–10 nymphs or larvae/pool), and DNA was extracted from these pools.

Polymerase chain reaction for *Bartonella* detection from DNA of isolated colonies, small mammal blood, and ectoparasites. The 54 DNA samples (44 from direct culture and 10 from pre-enrichment culture) from the 23 animal blood culture-positive samples were confirmed to be positive for *Bartonella* by using the *gltA* primers. Of the 86 DNA samples from the small mammals, *Bartonella* DNA was detected in 18 (21%) animals by using the same primers. *Bartonella* spp. were detected in 23 (56.1%) of the 41 animals for which blood was obtained on the cattle farms (19 *R. norvegicus*, 3 *S. murinus*, and 1 *R. rattus*) (Table 1). One *R. norvegicus* and one *S. murinus* (7.1%) for the 28 animals for which blood was obtained on the swine farms were bacteremic for *Bartonella* spp. One (14.3%) of the seven small mammals from the university campus and the *R. norvegicus* from a farm raising geese were also detected as positive for *Bartonella* spp. by PCR (Table 1). No *Bartonella* species were detected in animals from the animal shelter, housing units, and the duck farm.

Of the 74 adult fleas and 17 nymph lice pooled DNA samples, the *Bartonella gltA* gene was detected in 48 (64.9%) adult fleas and 11 (64.7%) lice pooled DNA samples, but was not

TABLE 2
Bartonella spp. detection in ectoparasites, Taiwan

Ectoparasite	Species	Stage	No. ectoparasites (PCR+)*	<i>Rattus norvegicus</i> (PCR+)	<i>Suncus murinus</i> (PCR+)	<i>Rattus rattus</i> (PCR+)
Flea	<i>Xenopsylla cheopis</i>	Adult	74 (48; 64.8%)	60 (46) [F: 37 (31) and M: 23 (15)]	14 (2) [F: 7 (1) and M: 7(1)]	0
Louse	<i>Polyplax spinulosa</i>	Nymph	81 in 17 pools (11; 64.7%)	16 (11)	1	0
Mite	<i>Laelaps echianinus</i>	Adult	38 (0)	0	33 [F 32 and M 1]	5 [F 5]
	<i>Laelaps</i> spp.	Nymph	238 in 41 pools (0)	39	1	1
Tick	<i>Ixodes</i> spp.	Nymph	6 (0)	0	6	0
	<i>Dermacentor</i> spp.	Nymph	6 (0)	5	1	0
	<i>Dermacentor</i> spp.	Larvae	35 in 5 pools (0)	4	1	0

* PCR = polymerase chain reaction.

detected in mite and tick DNA samples (Table 2). Of the 48 *Bartonella* PCR-positive adult fleas, 46 (95.8%) were obtained from *R. norvegicus* and 2 (4.2%) were obtained from *S. murinus*. The 11 *Bartonella* PCR-positive louse pooled DNA samples were obtained from *R. norvegicus*.

Sequencing and phylogenetic analyses. For the *gltA* gene, complete sequences could be interpreted for all 54 animal blood isolate DNA samples, but only for 13 (72%) of the 18 PCR-positive samples from animal blood DNA extracts. For ectoparasites, complete sequences could be interpreted for 47 (97.9%) of the 48 PCR-positive samples for the flea DNA samples and for all 11 PCR-positive samples for the lice pooled DNA samples. Strains used as references for comparison of the *gltA* gene are shown in Figure 1. Most of the sequences were identical or nearly identical to *B. rattimassiliensis* (99–100%), *B. queenslandensis* (99%), *B. tribocorum* (96–100%), *B. elizabethae* (95–96%), *B. phoceensis* (94–100%) and *B. rochalimae*-like [strain Bart. Sp 1-1C from Taiwan] (96–100%) (Table 3).

By direct culture, two *Bartonella* species (*B. elizabethae* and *B. tribocorum*) were isolated from the blood of the same rodent (R85). However, different *Bartonella* species were isolated from the same rodent when using the direct culture and the pre-enrichment methods. For instance, rodent R30 and rodent R35 were respectively co-infected with *B. elizabethae* or *B. tribocorum* and *B. queenslandensis*.

When combining the sequencing results from the isolates obtained by the two culture methods and the PCR segments of *Bartonella*-positive animal blood, there were 27 (31.4%) bacteremic small mammals. Six *R. norvegicus* captured on three cattle farms were co-infected by two or three *Bartonella* species. Rodents R4, R6, and R72 were infected with *B. rattimassiliensis* and *B. phoceensis*, rodent R30 was infected with *B. queenslandensis* and *B. elizabethae*, and rodent R85 was infected with *B. elizabethae* and *B. tribocorum*. Rodent R35 was infected with *B. queenslandensis*, *B. tribocorum*, and *B. phoceensis*. Overall, *B. phoceensis*, *B. queenslandensis*, *B. tribocorum*, *B. elizabethae*, and *B. rattimassiliensis* were detected in 22 *R. norvegicus*, *B. tribocorum* from 4 *S. murinus*, and *B. queenslandensis* from 1 *R. rattus* (Supplementary Table 1, available at www.ajtmh.org).

Fleas obtained from each animal could harbor several *Bartonella* species. The *Bartonella* species detected in fleas or lice could be identical or different from the *Bartonella* species isolated or detected from animals from which fleas or lice were obtained. *Bartonella phoceensis* was detected in eight *Polyplax* spp. lice obtained from five *Bartonella*-positive *R. norvegicus*, and two of these five rats were also bacteremic for *B. phoceensis*. Isolates from a Norway rat (*R. norvegicus*) and a shrew (*S. murinus*) captured on the swine farms and the two Norway rats captured on the university campus and the goose farm, respectively, were identified as *B. tribocorum* (Supplementary Table 2, available at www.ajtmh.org).

The phylogenetic tree derived from *gltA* data set also showed the sequences to be closely related to *B. tribocorum*, *B. rattimassiliensis*, *B. queenslandensis*, *B. elizabethae*, *B. phoceensis*, and *B. rochalimae* (Figure 1).

Risk factors associated with *Bartonella* spp. infection.
Animal blood. Among the animal risk factors analyzed (species, sex, age and location) and possible association with bacteremia, only age and location were significant risk factors. The percentage of *Bartonella*-positive small mammals was

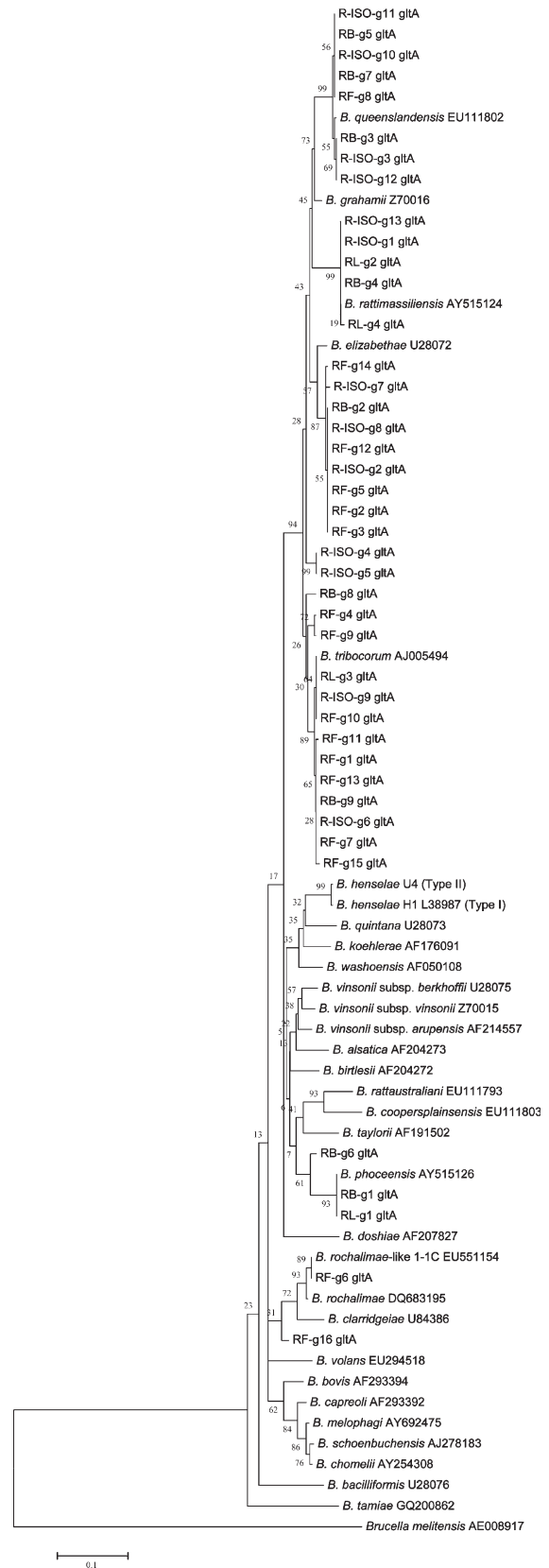


FIGURE 1. Phylogenetic tree based on 224 basepairs of the citrate synthase (*gltA*) of *Bartonella* strains, Taiwan, and constructed by using MEGA version 4 software (<http://www.megasoftware.net/>). The tree was obtained by using the neighbor-joining method and the Kimura-2 parameter model. Bootstrap support was calculated by using 1,000 bootstrap values.^{35,36}

TABLE 3
Sequence homology of *Bartonella* spp., Taiwan*

Specimen	<i>B. rattimassiliensis</i> , No. (%)	<i>B. queenslandensis</i> , No. (%)	<i>B. tribocorum</i> , No. (%)	<i>B. elizabethae</i> , No. (%)	<i>B. phoceensis</i> , No. (%)	<i>B. rochalimae</i> -like,† No. (%)
Blood culture						
Direct plating	8 (100)	8 (99)	21 (96–99)	7 (95–96)	0	0
Pre-enrichment	2 (100)	8 (99)	0	0	0	0
Blood DNA	1 (100)	4 (99)	3 (97–99)	1 (96)	4 (94–100)	0
Fleas	0	2 (99)	24 (97–99)	18 (96)	0	3 (96–100)
Lice	2 (99–100)	0	1 (100)	0	8 (100)	0

* Values are number of sequences and % similarity by source of samples

† Strain 1-1C closely related to *B. rochalimae* detected in Taiwan (9).

significantly higher on cattle farms than on swine farms (cattle farm = 56.1% [23 of 41] versus swine farm = 7.1% [2 of 28]; $P < 0.0001$) and in adult animals than in juvenile animals (adults = 43.5% [20 of 46] versus juveniles = 17.5% [7 of 40]; $P = 0.011$).

Fleas. *Bartonella* prevalence of fleas obtained from *R. norvegicus* (76.7% [46 of 60]) was significantly higher than in fleas obtained from *S. murinus* (14.3% [2 of 14]; $P < 0.0001$). Fleas obtained from male animals (81.6% [31 of 38]) were more likely to harbor *Bartonella* spp. than those obtained from female animals (47.2% [17 of 36]; $P = 0.0032$). Similarly, fleas obtained from adult animals (77.1% [37 of 48]) were more likely to harbor *Bartonella* spp. than fleas obtained from juveniles (42.3% [11 of 26]; $P = 0.0046$). Sex of the flea was not identified as a risk factor.

Lice. Percentages of *Bartonella* PCR-positive lice obtained from male or female mammals and from juvenile or adult small mammals were not statistically different.

Small mammals and fleas. Bacteremic small mammals were significantly more likely to be infested by fleas than non-bacteremic mammals (bacteremic animals = 40.7% [11 of 27] versus non-bacteremic animals = 18.6% [11 of 59]; prevalence odds ratio [POR] = 3.0, 95% confidence interval [CI] = 0.96–9.2, $P = 0.036$). In addition, fleas collected from *Bartonella*-positive animals were more likely to be *Bartonella* PCR positive than fleas obtained from *Bartonella*-negative animals (positive animals = 87.2% [34 of 39] versus negative animals = 37.9% [11 of 29]; POR = 11.1, 95% CI = 3.0–45.9, $P < 0.0001$).

Small mammals and lice. Bacteremic animals were more likely than non-bacteremic animals to be infested with lice (bacteremic animals = 33.3% [9 of 27] versus non-bacteremic animals = 6.8% [4 of 59]; POR = 6.9, 95% CI = 1.6–33.5, $P = 0.0028$). Similarly, lice obtained from *Bartonella* PCR-positive animals were more likely to be *Bartonella* PCR positive than lice obtained from *Bartonella* PCR-negative animals (positive animals = 83.3% [10 of 12] versus negative animals = 20% [1 of 5]) ($P = 0.028$, by Fisher exact test).

Additional PCR and sequencing for *Bartonella* spp. detection. One animal blood DNA sample (R35) and one flea DNA sample (RF85-3) were further analyzed with four other *Bartonella* genes. Sample RB35 and RF85-3 were PCR positive by using primers specific for the ITS pair 1 and pair 2 regions and *ftsZ* gene, but not with the other two pairs of primers. For sample RB35, two sequences of the ITS pair 1 region and two sequences of the ITS pair 2 region were obtained. One sequence of ITS pair 1 region and 1 sequence of ITS pair 2 region had 100% similarity with *B. tribocorum*. The other sequence of the ITS pair 1 region and the other sequence of the ITS pair 2 region had 96% similarity with *B. phoceensis*. Sequence of the *ftsZ* gene had 99% similarity with *B. phoceensis*. When tested by using primers for the *gltA* gene,

rodent R35 was found to be co-infected with three *Bartonella* species: *B. queenslandensis* (99% homology), *B. tribocorum* (99% homology), and *B. phoceensis* (94% homology). For sample RF85-3, sequences of ITS pair 1 and pair 2 regions and the *ftsZ* gene had 100% similarity with the *B. rochalimae*-like strain detected in Taiwan; it had 96% homology with the same strain for the *gltA* gene.

DISCUSSION

In the present study, several *Bartonella* species were isolated or detected by PCR in rodents and shrews from Taiwan. Norway rats (*R. norvegicus*) harbored the largest number of *Bartonella* species (*B. phoceensis*, *B. queenslandensis*, *B. tribocorum*, *B. elizabethae*, and *B. rattimassiliensis*). However, *B. tribocorum* and *B. queenslandensis* were also detected in *S. murinus* and *R. rattus*, respectively. Our study reports the first isolation of *B. queenslandensis* from *R. norvegicus* and *B. queenslandensis* from *R. rattus* in Taiwan. These data also confirm the previous report of isolation of strains closely related to *B. tribocorum*, *B. grahamii*, *B. elizabethae*, *B. phoceensis*, and *B. rattimassiliensis* in rodents and shrews from central Taiwan.²³

An important finding of this study is the wide diversity of *Bartonella* species identified in the ectoparasites compared with the species isolated from the blood of the small mammals from which these ectoparasites were obtained. One hypothesis could be a selective pressure or competition between *Bartonella* species, with some species more adapted to their mammalian reservoir in comparison to some species, which are more adapted to the ectoparasite vectors that are possible reservoirs for these *Bartonella* species. Such an observation was recently reported on a limited number of rodents and their ectoparasites tested.⁴¹

A surprising result was the limited value of the pre-enrichment method in detecting *Bartonella* species from small mammal blood samples. Such results may be caused by our limited experience with this new technique or a lower sensibility of this technique for isolation of rodent *Bartonella* species compared with a better yield with larger mammals, such as dogs, horses, or humans and/or for different *Bartonella* species such as *B. henselae* and *B. vinsonii* subsp. *berkhoffii*.^{33,42–44} Detection of *Bartonella* species by using PCR to amplify DNA directly from animal blood is often not successful possibly because of the inhibitory effect of hemin.¹⁵ Based on the present results, blood culture on chocolate agar seems to be the most sensitive technique for isolation of *Bartonella* species from small mammals.

Sex of the animals was not identified as a specific risk factor for *Bartonella* bacteremia in *R. norvegicus* in a study conducted in southern France.⁴ Similarly, in our study, sex of the animals was not identified as a risk factor although *Bartonella*

spp. prevalence in males was higher than that in females. Adults were also more likely to be *Bartonella* PCR positive than juveniles, as reported by Gundi and others,⁴ in which *Bartonella* spp. were isolated more often from rats with a weight of 200 grams (considered adults). In contrast, a study of *Bartonella* infection in squirrels (*Spermophilus richardsonii*) reported that juvenile squirrels were significantly more likely to be infected with *Bartonella* spp. than adults.⁴⁵ In cotton rats (*Sigmodon hispidus*), there were no differences among the proportion of cotton rats in different age classes infected by different *Bartonella* genogroups.⁴⁶ Such differences may be related to the vector infestation rate among the different rodent populations and vector transmission efficiency. Future studies on large-scale rodent and shrew populations and their ectoparasites will be necessary to demonstrate a correlation between *Bartonella* infection and animal age.

The percentage of *Bartonella*-positive small mammals was significantly higher on cattle farms than on swine farms, with more than half of the animals trapped on cattle farms being bacteremic for *Bartonella* spp. Such a difference could result either from the small number of farms tested and the limited number of small mammals trapped on swine and cattle farms or from the level of infestation by ectoparasites. There was no significant difference in the level of ectoparasite infestation of shrews on both types of farms. However, the infestation rate was significantly higher in rats from the cattle farms compared with pig farms. Zoonotic *B. elizabethae* was only detected from 5 *R. norvegicus* on cattle farms, which may have contact with domestic animals and humans because the isolates from all other locations were *B. tribocorum*.

Bartonella tribocorum, *B. elizabethae*, *B. queenslandensis*, and *Bartonella* sp. 1-1C were detected in *X. cheopis* fleas collected from small mammals. Moreover, *B. tribocorum*, *B. phoceensis*, and *B. rattimassiliensis* were detected in *Polyplax* spp. lice. Our study is the first report of detection of *Bartonella* spp. in fleas and lice obtained from small mammals in Taiwan. None of the 274 mites obtained from 37 small mammals and the 47 ticks obtained from 4 small mammals were positive by PCR for *Bartonella* spp. Interestingly, several rodents or shrews infested with various ectoparasites, as well as the fleas or lice obtained from them, were positive for *Bartonella* spp. However, the mites or ticks obtained were not positive for *Bartonella* spp. Such findings raise the possibility that in rodents and shrews in Taiwan, *X. cheopis* fleas and *Polyplax* spp. lice could have a greater potential as vectors for *Bartonella* spp. than *Laelaps* spp. mites and *Dermacentor* spp. and *Ixodes* spp. ticks. However, different tick species, including *Haemaphysalis longicornis*, *H. flava*, *I. nipponensis*, *I. turdus*, *I. persulcatus*, and *Ixodes* spp., and *Mesostigmatid* mites, have been found to be positive for *Bartonella* spp. by PCR in South Korea.²⁷ Future studies of larger small mammal populations and ectoparasites infesting them would be useful to better understand the role of the different species of ectoparasites in transmission of *Bartonella* spp. between animals and possibly to humans.

Small mammals bacteremic for *Bartonella* spp. were more likely to be infested with fleas and/or lice than non-bacteremic mammals, and fleas or lice obtained from bacteremic animals were more likely to be *Bartonella* PCR positive than those obtained from non-bacteremic animals. In this study, *B. phoceensis*, *B. queenslandensis*, *B. tribocorum*, *B. elizabethae*, and *B. rattimassiliensis* were detected in small mammals and fleas or lice. *Bartonella* sp. 1-1C, which was previously isolated from

R. norvegicus in Taiwan,⁹ was detected in fleas in the present study. In Taiwan, fleas and lice may be efficient vectors for infection of small mammal hosts with several *Bartonella* species. However, vector competency of these various flea and lice species has not been determined.

Reeves and others²⁶ hypothesized that *B. phoceensis* might be associated with or transmitted by *Hoplopleura pacifica* lice but not *P. spinulosa* because *B. phoceensis* was detected only in *H. pacifica* collected from a *B. rattimassiliensis*-infected rat. In the present study, *B. phoceensis* was detected in 8 *Polyplax* spp. lice obtained from five *Bartonella*-positive *R. norvegicus*, and two of these five animals were bacteremic for *B. phoceensis*. Our findings support the possibility that *Polyplax* spp. may also be associated with transmission of *B. phoceensis*.

Small mammals are usually hosts of ectoparasites, such as ticks, in their early stage of life. To decrease the risk of spreading zoonotic *Bartonella* spp. to humans, it will be important to determine the role of ectoparasites in transmission of these pathogens between animals and possibly to humans and to control the numbers of small mammals surrounding human residential areas and ectoparasites that parasitize them.

Received February 8, 2010. Accepted for publication June 1, 2010.

Note: Supplemental tables are available at www.ajtmh.org.

Financial support: This study was supported by grant 95AS-13.2.1-BQ-B1 from Council of Agriculture, Executive Yuan, Taiwan and grant NSC95-2313-B-005-028-MY2 from the National Science Council, Executive Yuan, Taiwan.

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