Journal of Wildlife Diseases, 46(1), 2010, pp. 173–178

BARTONELLA SP. INFECTION OF VOLES TRAPPED FROM AN INTERIOR ALASKAN SITE WHERE TICKS ARE ABSENT

Kotaro Matsumoto,[1,2] Joseph A. Cook,[3] Heidi K. Goethert,[1] and Sam R. Telford, III[1,4]

[1] Cummings School of Veterinary Medicine, Tufts University, 200 Westboro Road, North Grafton, Massachusetts 01536, USA
[2] Obihiro University of Agriculture & Veterinary Medicine, Inada-cho, Obihiro 080-8555, Japan
[3] Museum of Southwestern Biology and Department of Biology, University of New Mexico, Albuquerque, New Mexico 87131, USA
[4] Correspondence author (email: sam.telford@tufts.edu)

ABSTRACT: To determine whether *Bartonella* species may perpetuate in sites where ticks are absent, we analyzed blood and spleen samples from small mammals trapped near Cantwell, Alaska during September 1999. Blood smears from seven of 48 *Myodes* (*Clethrionomys*) *rutilus* suggested a *Bartonella* species infection; five of the seven yielded cultures consistent with *Bartonella* species; PCR of *rrs*, *gltA*, and *rpoB* genes confirmed *Bartonella* infection in four of these culture samples. Phylogenetic analysis of *rrs*, *gltA*, and *rpoB* genes demonstrated that the sequences clustered with the *Bartonella vinsonii* clade. Based on PCR, two additional blood samples and four of 22 spleen samples also contained DNA of this *Bartonella* species for an overall prevalence of 14% (10/70) among the 70 *M. rutilus* sampled in this study. We conclude that *Bartonella* species may commonly infect *M. rutilus* in a site where ticks are absent.

Key words: Alaska, *Bartonella*, *Myodes* (*Clethrionomys*) *rutilus*, ticks.

INTRODUCTION

The genus *Bartonella*, gram negative, hemotropic alpha-proteobacteria, currently contains 18 valid species and three subspecies. *Bartonella* spp. (some previously referred to as *Grahamella* spp.) are among the most common blood parasites detected in rodents, second only to trypanosomes (Kreier and Ristic, 1968), and have been identified in diverse mammals ranging from rodents to ungulates to phocids (Billeter et al., 2008). Ten *Bartonella* spp. have been implicated as the causative agents of human diseases, namely, *Bartonella bacilliformis* (Carrion's disease); *Bartonella clarridgeiae*, *Bartonella alsatica*, and *Bartonella henselae* (cat-scratch or similar disease); *Bartonella elizabethae*, *Bartonella koehlerae*, *Bartonella vinsonii* subsp. *arupensis*, and *Bartonella vinsonii* subsp. *berkhoffii* (endocarditis); *Bartonella grahamii* (neuroretinitis); and *Bartonella quintana* (trench fever). These bacteria are transmitted by a variety of hematophagous arthropods including *B. bacilliformis* by sand flies (Hertig, 1942), *B. quintana* by human body lice (Maurin and Raoult, 1996), *B. henselae* by cat fleas (Chomel et al., 1996), and uncharacterized rodent *Bartonella* sp. (Grahamella) by fleas (Krampitz and Kleinschmidt, 1960).

The DNA of *Bartonella* species has also been detected in ticks, including *Ixodes pacificus* (Chang et al., 2001; Holden et al., 2006) and *Ixodes ricinus* (Schouls et al., 1999), which are the main vectors of *Borrelia burgdorferi*, the agent of Lyme disease. To date, tick vectorial capacity for *Bartonella* spp. remains undescribed (Billeter et al., 2008), although elements of vector competence have been demonstrated for *B. henselae* and *I. ricinus* (Cotte et al., 2008). The role of ticks in the perpetuation of *Bartonella* species should be carefully defined because of the implied clinical consequences of co-infection, particularly in Lyme disease-associated sequelae.

If ticks are critical to the perpetuation of rodent-infecting *Bartonella* spp., the prevalence of infection within rodents from sites where ticks are absent should approach zero. Accordingly, we determined whether rodent-infecting *Bartonella* spp. were maintained in a central Alaskan site where ticks are absent; we corroborated the absence of vector ticks by searching for evidence of infection by *Babesia microti*,

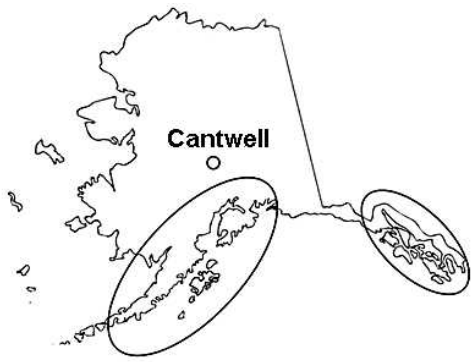

FIGURE 1. Map of Alaska. *Myodes rutilus* voles were captured in September 1999 near Cantwell. Open circles show the area where *Babesia microti* commonly infects voles and where *Ixodes angustus* ticks have been reported.

which is not found in rodents from Alaskan sites where ticks are absent (Fay and Rausch, 1969; Goethert et al., 2006).

MATERIALS AND METHODS

Collection of small mammals

Small mammals were snap-trapped or live-trapped in the vicinity of Cantwell in central Alaska (Denali Borough, 63°23′17″N, 148°54′1″W, elevation 670 m) during September 1999 as part of the Beringian Coevolution Project (Goethert et al., 2006; Fig. 1). Dead or euthanized animals were carefully inspected for ectoparasites by visual examination as well as by combing the fur with a toothbrush, particularly focusing on ears, face, and nape of neck. Blood and spleen were removed and stored in Alsevers solution for parasitologic analyses; other tissue vouchers were frozen in liquid nitrogen for deposition with traditional skin, skull, and postcranial specimens in the University of Alaska Museum mammal collection. Blood smears made at the time were subsequently fixed in absolute methanol, stained in Giemsa pH 7.0, and examined for hematozoa by brightfield microscopy at 400×; *Bartonella* spp. were presumptively identified based on characteristic Romanowsky-stained, rod-like clusters on the surface of, or within, red cells (Tyzzer, 1941).

Screening for Babesia microti

The absence of *B. microti* from blood smears was confirmed by PCR, as described and previously reported for these samples (Goethert et al., 2006). Briefly, a 238-base pair (bp) portion of the 18S rDNA was targeted using primers Bab1/Bab4; this assay has an analytic sensitivity of less than one parasite/μl of blood (Persing et al., 1992).

Isolation of *Bartonella* spp. from blood samples

Whole blood samples, which matched blood smears containing erythrocytic inclusions suggestive of *Bartonella* infection, were cultivated on brain heart infusion (BHI)-5% rabbit blood agar slants (modified from media and suggestions of Tyzzer, 1941) at room temperature for as long 1 mo.

DNA extraction and screening PCR on blood and spleen samples

The DNA was extracted from archived (frozen at −20 C) blood samples using ammonium hydroxide as described elsewhere (Schouls et al., 1999) or archived spleens of the animals using the QIAamp DNA Mini Kit (Qiagen, Valencia, California, USA) according to the manufacturer's recommendations. The extracted DNA samples were screened by PCR, using primers BhCS.781p and BhCS.1137n, which amplify a 379-bp fragment of the *gltA* gene of the genus *Bartonella* (Norman et al., 1995). Samples which were positive for screening PCR were confirmed by PCR targeting a different gene as well as a larger portion of the *gltA*, using primer pairs 1400F and 2300R, and BvCS.205p and BhCS.1137n, which amplify a 893-bp fragment of the *rpoB* gene and a 958-bp fragment of the *gltA* gene of *Bartonella* spp., respectively (Renesto et al., 2001; Winoto et al., 2005). Negative controls for PCR consisted of distilled water and a blank DNA extraction added to the PCR mix instead of template DNA. One sample of DNA from *B. vinsonii* subsp. *arupensis* (strain 2198) was included as a positive control in each PCR.

Screening PCR for *Bartonella* spp. on colonies

The colonies grown on the blood agar slants were suspended in 100 μl of PBS, boiled for 10 min, and used as the template DNA for screening by PCR using primers BhCS.781p and BhCS.1137n. Larger portions of the *gltA* and the *rpoB* gene target were amplified as confirmation.

Sequencing of *rrs*, *gltA*, and *rpoB* genes of *Bartonella* spp.

The *rrs* gene of positive samples was amplified using primers fD1, and Rp2, which amplify about a 1,450-bp fragment of *rrs* gene of eubacteria (Weisburg et al., 1991; Inokuma et al., 2001). PCR products of *rrs*, *gltA*, and

rpoB genes were purified by spin columns (Qiagen, GmbH, Germany) and sent to the University of Maine sequencing facility (Orono, Maine, USA) for sequence analysis. The fragment of *rrs* gene was sequenced using the primers fD1, Rp2, and Bh16S426F (5′-CGG CTA ACT TCG TGC CAG CA-3′), Bh16S538R (5′-CCT GGG ATT TCA CCT CTG AC-3′), Bh16S954F (5′-AGG CTG GAT CGG AGA CAG GT-3′), and Bh16S1048R (5′-CTA AGG GCG AGG GTT GCG CT-3′). A 958-bp fragment of *gltA* was sequenced using the primers BvCS.205p, BhCS.781p, and Bv322R (5′-GAG ATG AGG CGA ACA GAA GC-3′) and BhCS.1137n (Norman et al., 1995; Winoto et al., 2005) and an 893-bp fragment of *rpoB* using primers 1400F, 2028F, 1596R, and 2300R (Renesto et al., 2001).

Phylogenetic analysis

To identify the detected agents, phylogenetic analysis was performed. The obtained sequences were used in a BLAST search (Altschul et al., 1997) and aligned with sequences of other *Bartonella* species registered in GenBank using the program CLUSTAL W (Thompson et al., 1994). A simple neighbor-joining analysis was performed using the alignments and the program MEGA3 (Kumar et al., 2004). An unrooted star phylogeny was constructed based on concatenated sequences of *gltA*, *rpoB*, and *rrs* gene sequences of *Bartonella* spp.

GenBank accession numbers of the *gltA*, *rpoB*, and *rrs* gene sequences used to construct the tree are shown below: *B. bacilliformis*, NC_008783, AF165988, CP000524; *Bartonella doshiae*, Z70017, AF165991, Z31351; *B. elizabethae*, Z70009, AF165992, L01260; *B. grahamii*, Z70016, AF165993, Z31349; *B. henselae*, BX897699, AF171071, M73229; *B. koehlerae*, AF176091, AY166580, AF076237; *Bartonella phoceensis*, AY515126, AY515132, AY515119; *B. quintana*, NC_005955, AF165994, M11927; *Bartonella rattimassiliensis*, AY515124, AY515130, AY515118; *Bartonella schoenbuchensis*, AJ278183, AY167409, AJ278187; *Bartonella taylorii*, AY584852, AF165995, Z31350; *B. vinsonii* subsp. *arupensis*, AF214557, AY166582, AF214558; and *B. vinsonii* subsp. *berkhoffii*, AF143445, AF165989, U26258.

RESULTS

Collection of small mammals and isolation of *Bartonella* spp.

Seventy *Myodes* (*Clethrionomys*) *rutilus* (northern red-backed vole, hereafter "vole") were captured, and 48 blood and 22 spleen samples were collected specifically for parasitologic analysis. Voles were selected based upon their apparent age (nonjuveniles) and randomly with respect to sex. Ticks were not found on any vole or other animals (shrews, *Sorex* spp.) that were concurrently captured. Blood smears from captured voles were examined by microscopy, and seven samples had evidence of *Bartonella* infection. Of these seven, five yielded blood agar cultures with colonies demonstrating typical "grahamella" morphology (Tyzzer, 1941); the other two became contaminated with a fungus and were discarded.

PCR on blood and spleen samples

DNA was extracted from 48 archived blood and 22 spleen samples and tested by screening PCR. Six blood samples (13%), including the four yielding cultures that were confirmed by PCR and the two that were discovered solely by PCR, and four spleen samples (18%) were PCR positive, demonstrating that a total of 10 of 70 voles (14%; 95% confidence interval 6.1–22.5%) contained DNA of *Bartonella* spp.

PCR and sequencing on colonies

The colonies from each presumptive culture were suspended in 100 μl of PBS, boiled for 10 min, and tested by screening PCR, and four samples were positive; an additional culture had colonies with typical morphology, although PCR failed to confirm their identity. The four positive (screening PCR) samples also tested PCR-positive for *gltA*, *rpoB*, and *rrs* genes. All positive and negative controls were consistently positive and negative in each PCR, respectively. The PCR products from the four cultures were sequenced; 915-bp fragments of *gltA* gene, 852-bp fragments of *rpoB* gene, and 1,409- and 1,410-bp fragments of *rrs* gene were obtained from three samples (samples 28647, 28649, and 28667).

Phylogenetic analysis

Sequences of *gltA*, *rpoB*, and *rrs* (1,409-bp) genes from AF28647 (AF = field sample number; University of Alaska Museum accession number UAM 70686 for other tissue vouchers) had 93.8% (839/894) similarity to those of *Bartonella* sp. C4phy (GenBank Z70019), 93.0% (792/852) to *Bartonella washoensis* (DQ825688), and 99.5% (1,403/1,410) to *B. henselae* (DQ645426), respectively. The sequences of *gltA*, *rpoB*, and *rrs* (1,410-bp) from AF28649 (UAM 70688) were 92.8% (850/916) identical to those of *B. vinsonii* subsp. *arupensis* (Genbank AF214557), 92.6% (790/853) to *B. vinsonii* subsp. *arupensis* (AY166582), and 99.4% (1,403/1,411) to *Bartonella queenslandensis* (EU111758), respectively. The obtained sequences from AF28667 were identical to those of AF28647. A neighbor-joining analysis based on concatenated sequences of *rrs*, *gltA*, and *rpoB* genes demonstrated that the obtained sequences from AF28647 and AF28649 were in same clade with *B. vinsonii* (Fig. 2). The Alaskan sequences were deposited in GenBank with accession numbers EU979531, EU979533, and EU979532 for *gltA*, *rpoB*, and *rrs* genes of *Bartonella* strain 28647, and EU979534, EU979536 and EU979535 for *gltA*, *rpoB*, and *rrs* genes of *Bartonella* strain 28649.

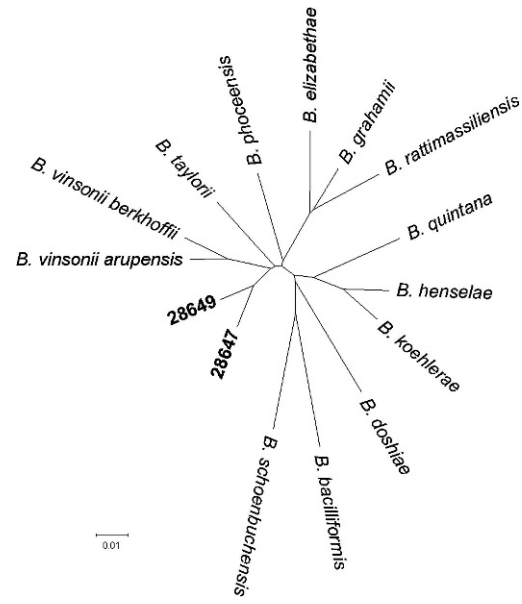

FIGURE 2. Unrooted star phylogeny (neighbor-joining algorithm) based on concatenated sequences of *gltA*, *rpoB*, and *rrs* gene sequences of *Bartonella* spp. demonstrating clustering of Alaskan vole *Bartonella* sequences (28647, 28649) with the *B. vinsonii* clade.

DISCUSSION

Bartonella spp., or their DNA, were detected in blood and spleen samples of 14% of voles from a site where ticks are absent. *Ixodes angustus* ticks infest rodents and commonly maintain *Babesia microti* in coastal Alaska, but Cantwell is too far inland for stable infestations of any tick (Fig. 1) (Fay and Rausch, 1969; Goethert et al., 2006). We did not detect *B. microti* by microscopy or by PCR, even though sampling was done during September, which would represent the end of the tick transmission season in coastal sites, thereby ensuring that many voles would have been exposed to infection. The absence of *B. microti* argues that ticks are rare or absent in this site. If we assume that voles are infested at an average rate of 11%, as they are in southern Alaska (Murrell et al., 2003), our sample size of 70 animals would have yielded seven animals with ticks; but if infestation rates are on the order of 1%, 70 animals would be an inadequate sample. Thus, although examination of more animals might have uncovered a low-level infestation, it is likely that ticks are very rare, at best, in this site and such an infestation rate is consistent with our failure to detect *B. microti.*

Phylogenetic analysis using concatenated sequences of *gltA*, *rpoB*, and *rrs* genes was performed, which clarified some of the ambiguity in the nodes of the resulting trees that was seen when sequences from individual genes were used. The *Bartonella* spp. that were detected in this study are most similar to *B. vinsonii* (Fig. 2).

However, the *rpoB* and *gltA* sequences of Alaskan samples showed only 92.6–93.0% and 92.8–93.8% similarity to those of the closest *Bartonella* strains, respectively. La Scola et al. (2003) proposed that newly encountered *Bartonella* isolates should be considered a new species, if a 327-bp *gltA* fragment shares 96.0% sequence similarity with those of the validated species, and if an 825-bp *rpoB* fragment shares 95.4% sequence similarity with those of the validated species; this suggests that the bacteria detected in our study represent new *Bartonella* species. The "*Grahamella*-like organism" reported by Fay and Rausch (1969) as having been transmitted to voles by inoculation of flea (*Megabothris abantis)* homogenates is likely to represent the agent that we detected (Fay and Rausch, 1969).

We conclude that a bacterium that may represent a new member of the *B. vinsonii* clade may commonly infect voles in a site where ticks are absent. It has been suggested that *B. vinsonii arupensis* and *B. vinsonii berkhoffii* are transmitted by ticks (Hofmeister et al., 1998; Kordick et al., 1999), but the evidence is circumstantial. Ticks are clearly not required to perpetuate the bartonellae that we detected in central Alaskan voles, and it is possible that this is true for others within the *B. vinsonii* clade. As experimentally demonstrated by Fay and Rausch in their seminal 1969 paper, the agent that we detected is undoubtedly maintained by fleas. The role of fleas as the main vector should remain the null hypothesis for epizootiologic studies on the perpetuation of the rodent-infecting *Bartonella* spp.

ACKNOWLEDGMENTS

Aspects of the project were supported through the Beringian Coevolution Project (NSF 0196905, 0415668), as well as through NIH RO1 AI 064218 and AI 067159.

LITERATURE CITED

Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Research 25: 3389–3402.

Billeter, S. A., M. G. Levy, B. B. Chomel, and E. B. Breitschwerdt. 2008. Vector transmission of *Bartonella* species with emphasis on the potential for tick transmission. Medical and Veterinary Entomology 22: 1–15.

Chang, C. C., B. B. Chomel, R. W. Kasten, V. Romano, and N. Tietze. 2001. Molecular evidence of *Bartonella* spp. in questing adult *Ixodes pacificus* ticks in California. Journal of Clinical Microbiology 39: 1221–1226.

Chomel, B. B., R. W. Kasten, K. Floyd-Hawkins, B. Chi, K. Yamamoto, J. Roberts-Wilson, A. N. Gurfield, R. C. Abbott, N. C. Pedersen, and J. E. Koehler. 1996. Experimental transmission of *Bartonella henselae* by the cat flea. Journal of Clinical Microbiology 34: 1952–1956.

Cotte, V., S. Bonnel, D. Le Rhun, E. Le Naour, A. Chauvin, H. Boulouis, B. Lecuelle, T. Lilin, and M. Vayssier-Taussat. 2008. Transmission of *Bartonella henselae* by *Ixodes ricinus*. Emerging Infectious Diseases 14: 1074–1080.

Fay, F. H., and R. L. Rausch. 1969. Parasitic organisms in the blood of arvicoline rodents in Alaska. The Journal of Parasitology 55: 1258–1265.

Goethert, H. K., J. A. Cook, E. W. Lance, and S. R. Telford. 2006. Fay and Rausch 1969 revisited: *Babesia microti* in Alaskan small mammals. The Journal of Parasitology 92: 826–831.

Hertig, M. 1942. Phlebotomus and Carrion's disease. American Journal of Tropical Medicine 22 (Suppl.): 1–81.

Hofmeister, E. K., C. P. Kolbert, A. S. Abdulkarim, J. M. Magera, M. K. Hopkins, J. R. Uhl, A. Ambyaye, S. R. Telford, III, F. R. Cockerill, III, and D. H. Persing. 1998. Cosegregation of a novel *Bartonella* species with *Borrelia burgdorferi* and *Babesia microti* in *Peromyscus leucopus*. The Journal of Infectious Diseases 177: 409–416.

Holden, K., J. T. Boothby, R. W. Kasten, and B. B. Chomel. 2006. Co-detection of *Bartonella henselae*, *Borrelia burgdorferi*, and *Anaplasma phagocytophilum* in *Ixodes pacificus* ticks from California, USA. Vector-Borne and Zoonotic Diseases 6: 99–102.

Inokuma, H., Y. Terada, T. Kamio, D. Raoult, and P. Brouqui. 2001. Analysis of the 16S rRNA gene sequence of *Anaplasma centrale* and its phylogenetic relatedness to other ehrlichiae. Clinical and Diagnostic Laboratory Immunology 8: 241–244.

Kordick, S. K., E. B. Breitschwerdt, B. C. Hegarty, K. L. Southwick, C. M. Colitz, S. I. Hancock, J. M. Bradley, R. Rumbough, J. T. McPherson, and J. N. Maccormack. 1999. Coinfection with multiple tick-borne pathogens in a Walker hound kennel in North Carolina. Journal of Clinical Microbiology 37: 2631–2638.

KRAMPITZ, H. E., AND A. KLEINSCHMIDT. 1960. [Grahamella Brumpt 1911. Biological and morphological research.]. Zeitschrift für Tropenmedizin und Parasitologie 11: 336–352.

KREIER, J. P., AND M. RISTIC. 1968. Haemobartonellosis, eperythrozoonosis, grahamellosis, and ehrlichiosis. *In* Infectious blood diseases of man and animals, Vol. II, D. Weinman and M. Ristic (eds.). Academic Press, New York, USA, pp. 386–472.

KUMAR, S., K. TAMURA, AND M. NEI. 2004. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. Briefings in Bioinformatics 5: 150–163.

LA SCOLA, B., Z. ZEAITER, A. KHAMIS, AND D. RAOULT. 2003. Gene-sequence-based criteria for species definition in bacteriology: The *Bartonella* paradigm. Trends in Microbiology 11: 318–321.

MAURIN, M., AND D. RAOULT. 1996. *Bartonella* (*Rochalimaea*) *quintana* infections. Clinical Microbiology Reviews 9: 273–292.

MURRELL, B. P., L. A. DURDEN, AND J. A. COOK. 2003. Host associations of the tick, *Ixodes angustus* (Acari: Ixodidae), on Alaskan mammals. Journal of Medical Entomology 40: 682–685.

NORMAN, A. F., R. REGNERY, P. JAMESON, C. GREENE, AND D. C. KRAUSE. 1995. Differentiation of *Bartonella*-like isolates at the species level by PCR-restriction fragment length polymorphism in the citrate synthase gene. Journal of Clinical Microbiology 33: 1797–1803.

PERSING, D. H., D. MATHIESON, W. F. MARSHALL, S. R. TELFORD, III, A. SPIELMAN, J. THOMFORD, AND P. A. CONRAD. 1992. Detection of *Babesia microti* by polymerase chain reaction. Journal of Clinical Microbiology 30: 2097–2103.

RENESTO, P., J. GOUVERNET, M. DRANCOURT, V. ROUX, AND D. RAOULT. 2001. Use of *rpoB* gene analysis for detection and identification of *Bartonella* species. Journal of Clinical Microbiology 39: 430–437.

SCHOULS, L. M., I. VAN DE POL, S. G. RIJPKEMA, AND C. S. SCHOT. 1999. Detection and identification of *Ehrlichia*, *Borrelia burgdorferi* sensu lato, and *Bartonella* species in Dutch *Ixodes ricinus* ticks. Journal of Clinical Microbiology 37: 2215–2222.

THOMPSON, J. D., D. G. HIGGINS, AND T. J. GIBSON. 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research 22: 4673–4680.

TYZZER, E. E. 1941. The isolation in culture of grahamellae from various species of small rodents. Proceedings of the National Academy of Sciences, USA 27: 158–162.

WEISBURG, W. G., S. M. BARNS, D. A. PELLETIER, AND D. J. LANE. 1991. 16S ribosomal DNA amplification for phylogenetic study. Journal of Bacteriology 173: 697–703.

WINOTO, I. L., H. GOETHERT, I. N. IBRAHIM, I. YUNIHERLINA, C. STOOPS, I. SUSANTI, W. KANIA, J. D. MAGUIRE, M. J. BANGS, S. R. TELFORD, III, AND C. WONGSRICHANALAI. 2005. *Bartonella* species in rodents and shrews in the greater Jakarta area. The Southeast Asian Journal of Tropical Medicine and Public Health 36: 1523–1529.

Received for publication 6 October 2008.