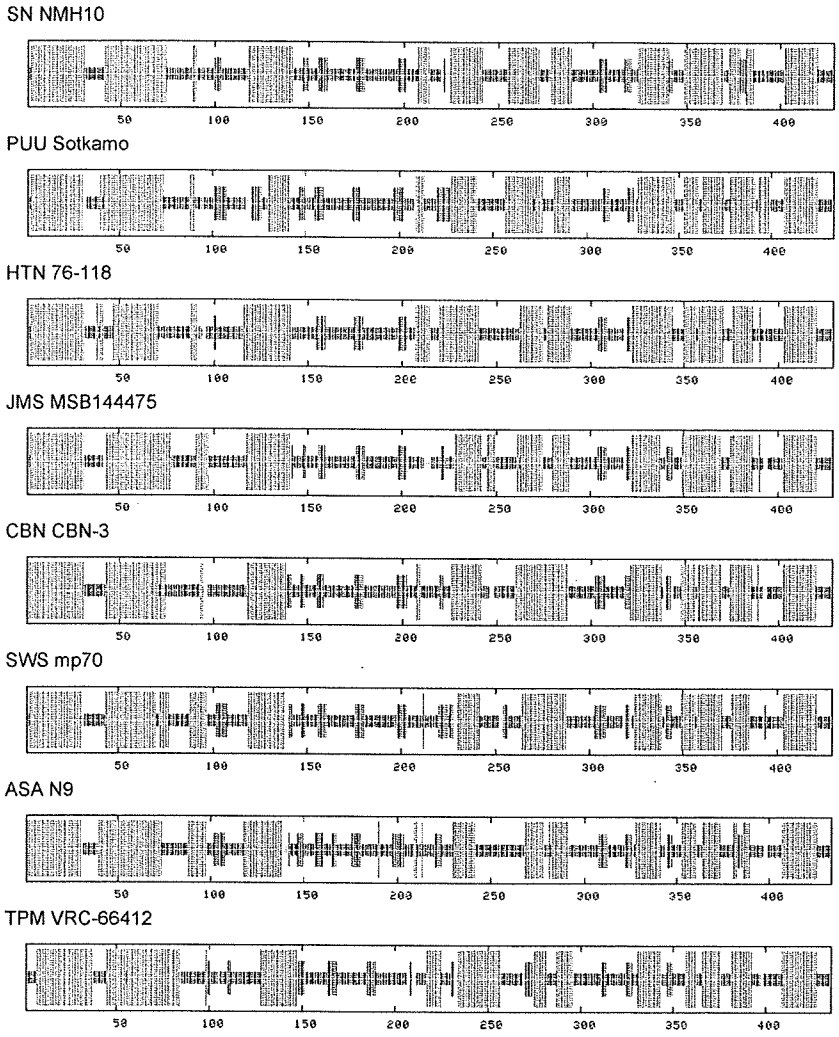

Fig. 2. Consensus secondary structure of N protein of ASAV and representative rodent- and soricid-borne hantaviruses, predicted using a high-performance method implemented on the NPS@ structure server (47). As shown, the ASAV N protein was very similar to that of other hantaviruses, characterized by the same coiled-coil helix at the amino terminal end and similar secondary structure motifs at their carboxyl terminals. The predicted structures were represented by colored bars to visualize the schematic architecture: α-helix, blue; β-sheet, red; coil, magenta; unclassified, gray. For simplicity, turns and other less frequently occurring secondary structural elements were omitted. All sequences are numbered from Met-1.

Sequence and Phylogenetic Analysis of Mole mtDNA. Molecular confirmation of the taxonomic identification of the hantavirus-infected Japanese shrew moles based on morphological features was achieved by amplification and sequencing of the 1,140-nucleotide mtDNA cytochrome *b* gene. Phylogenetic analysis showed distinct grouping of hantavirus-infected *U. talpoides* from this study with other *U. talpoides* mtDNA sequences available in GenBank, rather than with soricids or rodents (Fig. 4).

Discussion

Newfound Shrew Mole-Borne Hantavirus. Despite reports of hantavirus antigens in tissues of the Eurasian common shrew (*Sorex araneus*), alpine shrew (*Sorex alpinus*), Eurasian water shrew (*Neomys fodiens*), and common mole (*Talpa europea*) (25–28), shrews and moles have been generally dismissed as being unimportant in the transmission dynamics of hantaviruses. With the recent demonstration that TPMV and other newly identified soricid-borne hantaviruses are genetically distinct and phylogenetically distant from rodent-borne hantaviruses (19–24), the conventional view that rodents are the principal or primordial reservoir hosts of hantaviruses is being challenged. In its wake, a compelling conceptual framework, or paradigm shift, is emerging that supports an ancient origin of hantaviruses in soricomorphs (or insectivores). To this emerging concept must now be added the first molecular evidence of a newfound hantavirus, designated ASAV, in the Japanese shrew mole (family *Talpidae*, subfamily *Talpinae*). The demonstration of ASAV sequences in this endemic shrew mole species captured at different times and in two separate locations in Mie Prefecture argues strongly against this being an isolated or coincidental event. Instead, these data suggest a well established coexistence of this newfound hantavirus in the Japanese shrew mole and further solidifies the notion of a long-standing evolutionary association between soricomorphs and hantaviruses.

Shrew moles differ from typical or true moles in that they look like shrews and are much less specialized for burrowing. The greater Japanese shrew mole, which morphologically resembles semifossorial shrew moles in China (*Scaptonyx*) and North America (*Neurotrichus*), is widely distributed in the lowlands and peripheral islands of Japan, except Hokkaido, and is not found on mainland Asia (29, 30). Also endemic in Japan, the lesser Japanese shrew mole (*Dymecodon pilirostris*) is largely restricted to mountainous regions on Honshu, Shikoku, and Kyushu and is considered the more ancestral species. As determined by cytochrome *b* mtDNA and nuclear recombination activating gene-1 (RAG1) sequence analyses, the greater and lesser Japanese shrew moles are closely related, but their evolutionary origins and biogeography remain unresolved (31, 32). The existence of two distinct chromosomal races of *U. talpoides*, geographically separated by the Fuji and Kurobe rivers in central Honshu (33, 34), provides an opportunity to further clarify the evolutionary origins of shrew mole-borne hantaviruses in Japan. Studies, now underway, will examine whether ASAV is harbored by *U. talpoides* in locations east of Mie

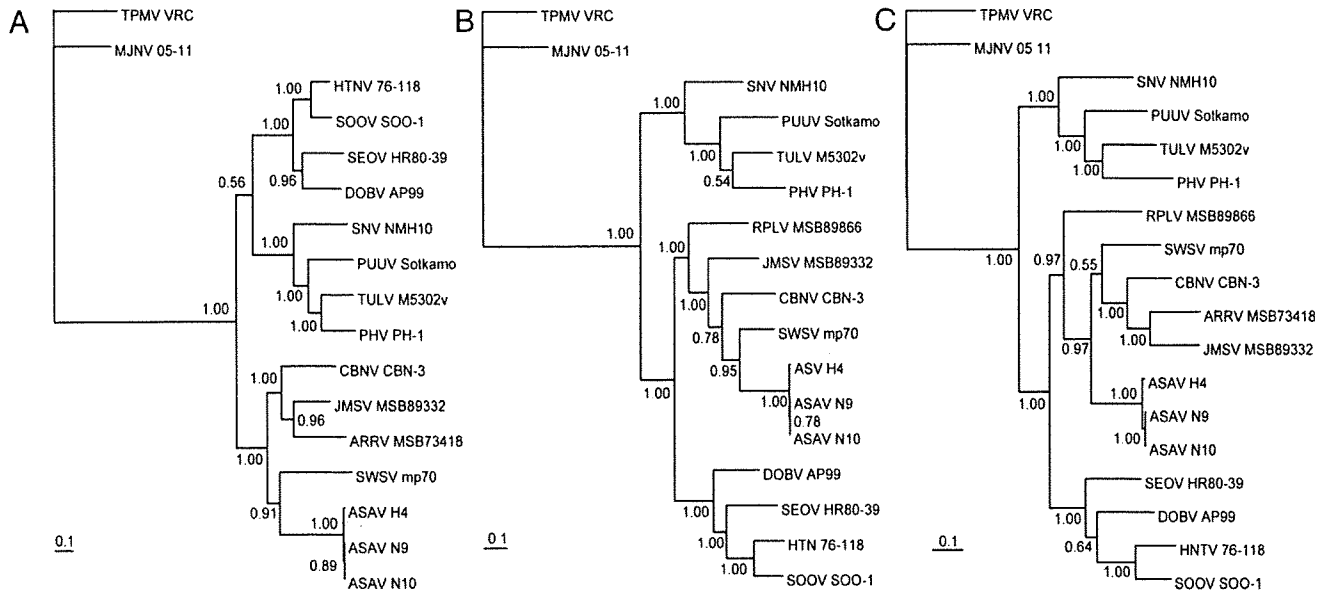

Fig. 3. Phylogenetic trees generated by the ML method, using the GTR+I+G model of evolution as estimated from the data, based on the alignment of the coding regions of the full-length (*A*) 1,302-nucleotide S and (*B*) 3,423-nucleotide M segments, and partial (*C*) 6,126-nucleotide L-genomic segment of ASAV. The phylogenetic positions of ASAV strains H4, N9, and N10 are shown in relationship to representative murinae rodent-borne hantaviruses, including Hantaan virus (HTNV 76–118, NC_005218, NC_005219, NC_005222), Soochong virus (SOOV SOO-1, AY675349, AY675353, DQ056292), Dobrava virus (DOBV AP99, NC_005233, NC_005234, NC_005235), and Seoul virus (SEOV HR80–39, NC_005236, NC_005237, NC_005238); arvicolinae rodent-borne hantaviruses, including Tula virus (TULV M5302v, NC_005227, NC_005228, NC_005226), Puumala virus (PUUV Sotkamo, NC_005224, NC_005223, NC_005225), and Prospect Hill virus (PHV PH-1, Z49098, X55129, EF646763); and a neotominae rodent-borne hantavirus, Sin Nombre virus (SNV NMH10, NC_005216, NC_005215, NC_005217). Also shown are Thottapalayam virus (TPMV VRC, AY526097, EU001329, EU001330) from the Asian house shrew (*Suncus murinus*); Imjin virus (MJNV 05–11, EF641804, EF641798, EF641806) from the Ussuri white-toothed shrew (*Crocidura lasiura*); Cao Bang virus (CBNV CBN-3, EF543524, EF543526, EF543525) from the Chinese mole shrew (*Anourosorex squamipes*); Ash River virus (ARRV MSB 73418, EF650086, EF619961) from the masked shrew (*Sorex cinereus*); Jemez Springs virus (JMSV MSB89332, EF619962, EF619960) from the dusky shrew (*Sorex monticolus*); and Seewis virus (SWSV mp70, EF636024, EF636025, EF636026) from the Eurasian common shrew (*Sorex araneus*). The numbers at each node are posterior node probabilities based on 30,000 trees: two replicate MCMC runs consisting of six chains of 3 million generations each sampled every 1,000 generations with a burn-in of 7,500 (25%). The scale bar indicates nucleotide substitutions per site. GenBank accession numbers: ASAV S segment (H4, EU929070; N9, EU929071; N10, EU929072); ASAV M segment (H4, EU929073; N9, EU929074; N10, EU929075); and ASAV L segment (H4, EU929076; N9, EU929077; N10, EU929078).

Prefecture, as well as ascertain whether *D. pilirostris* also serves as a reservoir of ASAV-related hantaviruses.

Although our RT-PCR attempts have failed to detect hantavirus sequences in other talpid species, including the long-nosed mole (*Euroscaptor longirostris*) (21) and eastern mole (*Scalopus aquaticus*) (H. J. Kang, J.-W. Song, and R. Yanagihara, unpublished observations), it may be because appropriate primers were not used. That is, based on the vast genetic diversity of soricid-borne hantaviruses, talpid-associated hantaviruses may be even more highly divergent and would require designing very different primers for amplification.

Finally, as for shrew-borne hantaviruses, the importance of this newfound shrew mole-associated hantavirus to human health warrants careful inquiry. Virus isolation attempts have been unsuccessful to date. In the meantime, an ASAV recombinant N protein is being prepared for use in enzyme immunoassays. In this regard, as evidenced by the corresponding sequence of YIEVNGIRKP in the ASAV N protein, the monoclonal antibody E5/G6, which recognizes the epitope YEDVNGIRKP (with variations) in rodent-borne hantaviruses (35), might be useful as a capturing antibody. In addition, other sensitive technologies, including nucleic acid and protein microarrays, are being developed to establish whether ASAV is pathogenic for humans.

Secondary Structure of Hantavirus N Protein. The overall N protein secondary structure of ASAV and other hantaviruses was compatible with a putative bilobed, three-dimensional protein architecture, which would allow the protein to clamp around the RNA as often observed in a variety of RNA-binding proteins. Whereas the core elements of the central β-pleated sheet appeared also to be conserved, more evolutionary variability was seen in the number of constituent strands and in the adjoining connecting elements and helices. This variability may reflect the function of this region as a flexible spacer element that can determine the relative orientation and separation of the two main α-helical domains and can accommodate the conformational changes upon RNA binding. The connecting regions could act as hinges of variable size leading to opening of the nucleocapsid. The flexible domain linkage would allow the interaction with the differently sized virus-specific RNA structures may modulate the oligomerization or assembly of the N protein in an evolutionarily and systematically changing fashion.

Phylogeny of Hantaviruses. Just as the identification of novel hantaviruses in the Therese shrew (*Crocidura theresae*) (36) and the northern short-tailed shrew (*Blarina brevicauda*) (23) heralded the discovery of other soricid-borne hantaviruses (21, 22, 24), the detection of ASAV in the Japanese shrew mole forecasts the existence of other hantaviruses in talpids. Perhaps more importantly, these findings emphasize that the evolutionary history and transmission dynamics of hantaviruses are far more rich and complex than originally imagined. That is, instead of a single progenitor virus being introduced into the rodent lineage more than 50 million years ago, mounting evidence supports a more ancient virus lineage with parallel coevolution of hantaviruses in crocidurine and soricine shrews. And given the sympatric and synchronistic coexistence of moles, shrews, and rodents, through a long continuum dating from the distant past to the present time, it seems

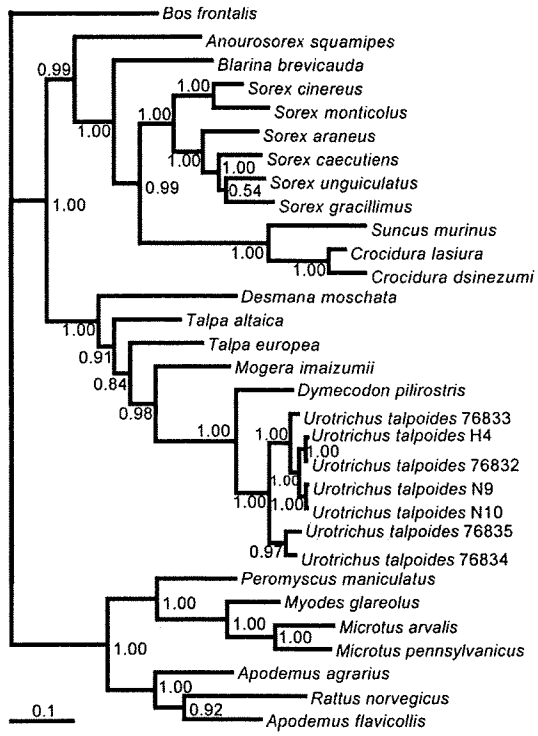

Fig. 4. Confirmation of host identification of ASAV-infected *Urotrichus talpoides* by mtDNA sequencing. Phylogenetic tree, based on the 1,140-nucleotide cytochrome *b* (cyt b) gene, was generated by the ML method. The phylogenetic positions of *Urotrichus talpoides* H4 (EU918369), N9 (EU918370), and N10 (EU918371) are shown in relationship to other *Urotrichus talpoides* cyt b sequences from GenBank (Ut76835: AB076835; Ut76834: AB076834; Ut76833: AB076833; Ut76832: AB076832), as well as other talpids, including *Desmana moschata* (AB076836), *Talpa altaica* (AB037602), *Talpa europea* (AB076829), *Mogera imaizumii* (AB037616), *Dymecodon pilirostris* (AB076830), and *Bos frontalis* (EF061237). Also shown are representative murinae rodents, including *Apodemus agrarius* (AB303226), *Apodemus flavicollis* (AB032853), and *Rattus norvegicus* (DQ439844); arvicolinae rodents, including *Microtus arvalis* (EU439459), *Myodes glareolus* (DQ090761), and *Microtus pennsylvanicus* (AF119279); and a neotominae rodent, *Peromyscus maniculatus* (AF119261), as well as crocidurinae shrews, including *Suncus murinus* (DQ630386), *Crocidura lasiura* (AB077071), and *Crocidura dsinezumi* (AB076837); and soricinae shrews, including *Anourosorex squamipes* (AB175091), *Blarina brevicauda* (DQ630416), *Sorex cinereus* (EU088305), *Sorex monticolus* (AB100273), *Sorex araneus* (DQ417719), *Sorex caecutiens* (AB028563), *Sorex unguiculatus* (AB028525), and *Sorex gracillimus* (AB175131). The numbers at each node are posterior node probabilities based on 30,000 trees: two replicate MCMC runs consisting of six chains of 3 million generations each sampled every 1,000 generations with a burn-in of 7,500 (25%). The scale bar indicates nucleotide substitutions per site.

plausible that ongoing exchanges of hantaviruses continues to drive their evolution.

In this regard, several rodent species may occasionally serve as reservoir hosts for the same hantavirus. For example, Vladivostok virus (VLAV) may be found in its natural host, the reed vole (*Microtus fortis*) (37–39), as well as an ancillary host, the tundra or root vole (*Microtus oeconomus*) (40). Similarly, the Maximowiczi vole (*Microtus maximowiczii*) is the natural reservoir of Khabarovsk virus (KHAV), which may also be harbored by *Microtus fortis* (10, 39, 40). Moreover, a KHAV-related hantavirus, named Topografov virus (TOPV), has also been found in the Siberian lemming (*Lemmus sibiricus*) (41). This is a far more extreme situation in which a hantavirus has switched from its natural rodent reservoir host and become well established in a rodent host of a different genus. Such host-switching or species-jumping events may account for the extraordinarily close phylogenetic relationship between TOPV and KHAV (41). That is, whereas *Lemmus* and *Microtus* are very distantly related, TOPV and KBRV are monophyletic.

In much the same way, as evidenced by the polyphylogenetic relationship between ASAV and other soricid-associated hantaviruses, the progenitor of ASAV may have 'jumped' from its natural soricine shrew host to establish itself in the Japanese shrew mole, or vice versa. That is, burrows and shallow tunnel systems excavated by Japanese shrew moles may be occasionally shared with sympatric species, including shrews, allowing opportunities for virus transmission through interspecies wounding or contaminated nesting materials. Such a host-switching event may have occurred in the distant past, possibly before the present-day Japanese shrew mole became endemic in Japan. Accordingly, intensive investigations of shrews in Japan and elsewhere in Far East Asia may provide further insights into the evolutionary origins of hantaviruses.

Materials and Methods

Trapping. Sherman traps (H.B. Sherman) and pit-hole traps were used to capture shrews and shrew moles in Japan between October 2006 and April 2008. Traps were set at intervals of 4 to 5 m during the evening hours of each day, over a four-day period, at sites in Hokkaido (Hamatonbetsu, Saruhutsu, and Nopporo) and Honshu (Nara and Mie), where soricomorphs had been captured. Species, gender, weight, reproductive maturity, and global positioning system (GPS) coordinates of each captured animal were recorded.

Specimen Processing. Lung tissues, dissected using separate instruments, were frozen on dry ice, and then stored at −80°C until used for testing. In some instances, portions of tissues were also placed in RNAlater RNA Stabilization Reagent (QIAGEN, Inc.) and processed for RT-PCR within 4 weeks of tissue collection.

RNA Extraction and cDNA Synthesis. Total RNA was extracted from tissues, by using the PureLink Microto-Midi total RNA purification kit (Invitrogen), in a laboratory in which hantaviruses had never been handled. cDNA was then prepared by using the SuperScript™ III RNase H- reverse transcriptase kit (Invitrogen) with a primer based on the conserved 5′-terminus of the S, M and L segments of hantaviruses (5′-TAGTAGTAGACTCC-3′).

RT-PCR. Touchdown-PCR was performed by using oligonucleotide primers designed from TPMV and other hantaviruses: S (outer: 5′-TAGTAGTAGACTCCT-TRAARAGC-3′ and 5′-AGCTCIGGATCCATITCATC-3′; inner: 5′-AGYCCIGTIATGRG-WGTIRTYGG-3′ and 5′-AIGAYTGRTARAAIGAIGAYTTYT T-3′); M (outer: 5′-GGACCAGGTGCADCTTGTGAAGC-3′ and 5′-GAACCCCADGCCCCITCYAT-3′; inner: 5′-TGTGTICCWGGITTYCATGGIT-3′ and 5′-CATGAYATCTCCAGGGTCHCC-3′); and L (outer: 5′-ATGTAYGTBAGTGCWGATGC-3′ and 5′-AACCADTCWGTYC-CRTCATC-3′; inner: 5′-TGCWGATGCHACIAARTGGTC-3′ and 5′-GCRTCRTCW-GARTGRTGDGCAA-3′).

First- and second-round PCR were performed in 20-μL reaction mixtures, containing 250 μM dNTP, 2.5 mM $MgCl_2$, 1 U of LA Taq polymerase (Takara) and 0.25 μM of each primer (24). Initial denaturation at 94°C for 2 min was followed by two cycles each of denaturation at 94°C for 30 sec, two-degree step-down annealing from 46°C to 38°C for 40 sec, and elongation at 72°C for 1 min, then 30 cycles of denaturation at 94°C for 30 sec, annealing at 42°C for 40 sec, and elongation at 72°C for 1 min, in a GeneAmp PCR 9700 thermal cycler (Perkin–Elmer). PCR products were separated by agarose gel electrophoresis and purified by using the Qiaex Gel Extraction Kit (Qiagen). Amplified DNA was sequenced directly by using an ABI Prism 3130 Avant Genetic Analyzer (Applied Biosystems).

Genetic and Phylogenetic Analyses. Sequences were processed by using the Genetyx version 9 software (Genetyx Corporation) and aligned using Clustal W and W2 (42). For phylogenetic analysis, ML consensus trees were generated by the Bayesian Metropolis–Hastings Markov Chain Monte Carlo (MCMC) tree-sampling methods as implemented by Mr. Bayes (43) using a GTR+I+G model of evolution, as selected by hierarchical likelihood-ratio test (hLRT) in MrModeltest2.3 (http://www.abc.se/~nylander/mrmodeltest2/mrmodeltest2.html) (44), partitioned by codon position.

An initial ML estimate of the model of evolutionary change among aligned viruses was generated by MrModeltest2.3. ML tree estimation in PAUP (45) was conducted starting with a neighbor-joining (NJ) tree based on this initial ML model of evolution, and proceeding with successive rounds of heuristic tree-searches to select the single most likely ML tree. Support for topologies was generated by bootstrapping for 1,000 NJ replicates (under the ML model of evolution, implemented in

PAUP) and for 100 ML replicates (data not shown). Phylogenetic relationships were further confirmed using amino acid sequences analyzed by Bayesian tree sampling, using the WAG model (46) implemented by Bayes (43).

Secondary Structure Prediction. Secondary structure prediction of the N protein was performed using the NPS@ structure server (47). To achieve 70–80% accuracy and to validate the prediction, five different methods were used jointly: DSC (48), HNN (49), PHD (50), PREDATOR (51), and MLRC (49), which in turn were based on GOR4 (52), SIMPA96 (53), and SOPMA (54). The minimum number of conformational states was set to four (helix, sheet, turn, and coil) for each analysis, and the results were combined into a consensus structure where the most prevalent predicted conformational state was reported for each residue. For convenience in visualization of the predicted structures, the NPS@ server also provided graphic outputs for the individual sequences which were subsequently combined into a multipart joint image.

PCR Amplification of Shrew Mole mtDNA. Total DNA, extracted from liver tissues using the QIAamp Tissue Kit (QIAGEN), was used to verify the identity of the hantavirus-infected shrew moles. The 1,140-nucleotide mtDNA cytochrome *b* gene was amplified by PCR, using described universal primers (5′- CGAAGCTT-GATATGAAAAACCATCGTTG-3′; 5′- AACTGCAGTCATCTCCGGTTTACAAGAC-3′) (55). PCR was performed in 50-μl reaction mixtures, containing 200 μM dNTP and 1.25 U of rTaq polymerase (Takara). Cycling conditions consisted of an initial denaturation at 95°C for 4 min followed by 40 cycles with denaturation at 94°C for 1 min, annealing at 57°C for 1 min, and elongation at 72°C for 1 min in a GeneAmp PCR9700 thermal cycler.

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Prevalence and Genetic Diversity of *Bartonella* Species Isolated from Wild Rodents in Japan[▽]

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Here, we describe for the first time the prevalence and genetic properties of *Bartonella* organisms in wild rodents in Japan. We captured 685 wild rodents throughout Japan (in 12 prefectures) and successfully isolated *Bartonella* organisms from 176 of the 685 rodents (isolation rate, 25.7%). Those *Bartonella* isolates were all obtained from the rodents captured in suburban areas (rate, 51.8%), but no organism was isolated from the animals captured in city areas. Sequence analysis of *rpoB* and *gltA* revealed that the *Bartonella* isolates obtained were classified into eight genetic groups, comprising isolates closely related to *B. grahamii* (A-I group), *B. tribocorum* and *B. elizabethae* (B-J group), *B. tribocorum* and *B. rattimassiliensis* (C-K group), *B. rattimassiliensis* (D-L group), *B. phoceensis* (F-N group), *B. taylorii* (G-O group), and probably two additional novel *Bartonella* species groups (E-M and H-P). *B. grahamii*, which is one of the potential causative agents of human neuroretinitis, was found to be predominant in Japanese rodents. In terms of the relationships between these *Bartonella* genetic groups and their rodent species, (i) the A-I, E-M, and H-P groups appear to be associated with *Apodemus speciosus* and *Apodemus argenteus*; (ii) the C-K, D-L, and F-N groups are likely implicated in *Rattus rattus*; (iii) the B-J group seems to be involved in *Apodemus* mice and *R. rattus*; and (iv) the G-O group is probably associated with *A. speciosus* and *Clethrionomys* voles. Furthermore, dual infections with two different genetic groups of bartonellae were found in *A. speciosus* and *R. rattus*. These findings suggest that the rodent in Japan might serve as a reservoir of zoonotic *Bartonella* infection.

The genus *Bartonella* is associated with aerobic, fastidious, gram-negative, slow-growing bacteria and consists of 20 species and three subspecies at the present time (19). These microorganisms infect the erythrocytes of their mammalian hosts, and some species cause a wide spectrum of illness, such as chronic bacteremia, fever, and endocarditis. In particular, *B. bacilliformis*, *B. henselae*, and *B. quintana* are known to be causative agents of Carrion's disease, cat scratch disease, and trench fever, respectively, in humans (1, 27, 32). Because of the difficulty in identifying *Bartonella* species by use of conventional biochemical tests, molecular approaches by PCR, followed by sequencing of several housekeeping genes, such as citrate synthase (*gltA*), RNA polymerase beta subunit (*rpoB*), cell division-associated protein (*ftsZ*), heat shock protein (*groEL*), riboflavin synthase alpha chain (*ribC*), and 16S rRNA genes, as targets, have been useful for identification of *Bartonella* species (26). Particularly, *rpoB* and *gltA* have been well used for differentiating *Bartonella* species because of the much lower degrees of similarity between these genes in *Bartonella* species (26). Furthermore, PCR-restriction fragment length polymorphism (PCR-RFLP) has also been applied as a simple and rapid method to identify and/or classify many microorganisms, including *Bartonella* species (30).

A number of studies have shown that *Bartonella* species are widely distributed in wild rodents in many countries, such as the United Kingdom (4, 5), the United States (10, 19, 24), Sweden (18), China (37), Greece (33), Denmark (13), France (16), Canada (20), and the Republic of South Africa (29). Ten species and two subspecies of *Bartonella*, i.e., *B. birtlesii* (3), *B. doshiae* (5), *B. elizabethae* (12), *B. grahamii* (5), *B. phoceensis* (16), *B. rattimassiliensis* (16), *B. taylorii* (5), *B. tribocorum* (17), *B. washoensis* (23), *B. vinsonii* subsp. *arupensis*, and *B. vinsonii* subsp. *vinsonii* (2, 35), have been previously isolated from wild rodent origins. Of these, four rodent-associated *Bartonella* species are thought to be implicated in human infections, with *B. elizabethae* responsible for endocarditis (11), *B. grahamii* for

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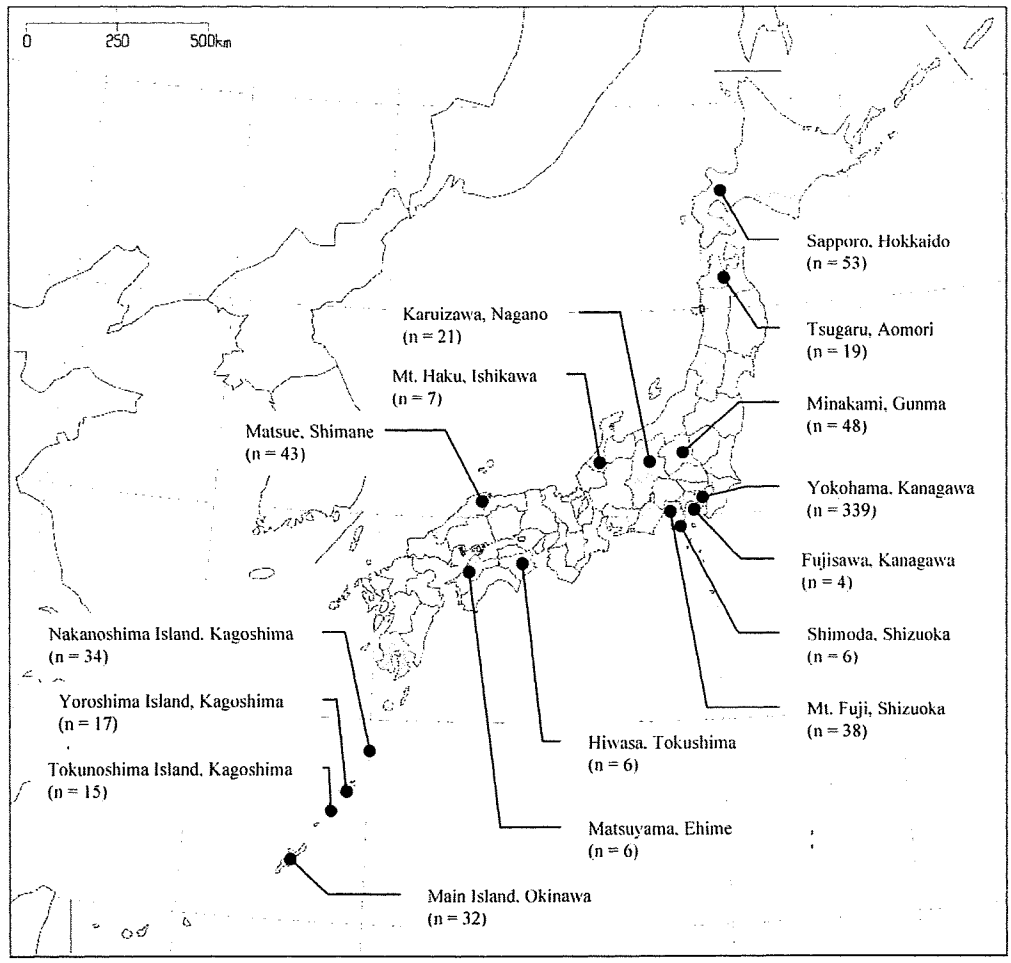

FIG. 1. Geographical representation of the locations where wild rodents were captured. Cities, areas, or islands and their prefectures (e.g., Yokohama [city], Kanagawa [Prefecture]) are shown around the map of Japan in the figure. The numbers of rodents captured are indicated in parentheses.

neuroretinitis (21), *B. vinsonii* subsp. *arupensis* for bacteremia, fever, and endocarditis (15, 35), and *B. washoensis* for cardiac disease (23). Although environmental surveillance is required for control of infectious diseases, including zoonoses, there is no information on the prevalence and genetic characteristics of *Bartonella* organisms in wild rodents in Japan so far. Therefore, the aim of this study is to clarify the distribution of *Bartonella* organisms in Japan by isolation from wild rodents collected in 16 suburban or city areas and to characterize the *Bartonella* isolates by molecular techniques.

MATERIALS AND METHODS

Blood sampling. From October 1997 to May 2006, wild rodents were captured using Sherman traps in the 12 prefectures of Hokkaido, Aomori, Gunma, Kanagawa, Shizuoka, Ishikawa, Nagano, Shimane, Tokushima, Ehime, Kagoshima, and Okinawa in Japan (Fig. 1). Blood was aseptically collected from each animal and immediately placed into sterile 1.5-ml conical plastic tubes with heparin. The blood samples were sent to the Laboratory of Veterinary Public Health, Department of Veterinary Medicine, College of Bioresource Sciences, Nihon University, under frozen conditions with dry ice and were kept at −80°C until use.

Isolation of bacteria. The frozen blood samples were thawed at room temperature, and a 100-μl sample of each was plated on heart infusion agar plates (Difco, MI) containing 5% defibrinated rabbit blood (24). The plates were incubated at 35°C under 5% CO_2 for 2 weeks. Small, rough gray colonies that required long culture periods (1 week or more) were selected. By Gram staining, we considered the microorganisms that were small, gram negative, and had bacillus shapes as *Bartonella* species. For pure culture, two or three colonies were picked from each plate, streaked out on fresh plates, and further cultured under the same conditions. The *Bartonella* isolates obtained were used for the following experiments.

PCR amplification of *rpoB* and *gltA*. Genomic DNA was extracted from each isolate of bartonellae by using an Instagene matrix (Bio-Rad, Hercules, CA). The primers used for the amplification of *rpoB* (893 bp) were 1400F (5′-CGCATT GGCTTACTTCGTATG-3′) and 2300R (5′-GTAGACTGATTAGAACGCT G-3′) (30), and the primers for *gltA* (379 bp) were BhCS.781p (5′-GGGGACC AGCTCATGGTGG-3′) and BhCS.1137n (5′-AATGCAAAAAGAACAGTAA ACA-3′) (28). The PCR was performed with 20-μl mixtures containing 20 ng of the extracted DNA, 200 μM of each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 0.5 U *Taq* DNA polymerase (Promega, Madison, WI), and 1 pmol of each primer. The PCR cycle conditions were as described previously (28, 30).

PCR-RFLP of *rpoB*. The PCR-amplified *rpoB* genes of the *Bartonella* isolates obtained from individual rodents were purified using a commercial purification kit (Spin Column PCR product purification kit; Bio Basic, Ontario, Canada). A 10-μl sample of the purified PCR products was mixed with 2 μl 10× B buffer and 5 U AcsI (identical to ApoI) restriction endonuclease (Roche Diagnostics GmbH, Penzberg, Germany) (30), and the total volume was adjusted to 20 μl with double-distilled water. After incubation for 2 h at 50°C, the digestion products were separated on 3% agarose gels by electrophoresis and visualized by ethidium bromide staining.

TABLE 1. Prevalence of Japanese *Bartonella* organisms in wild rodents

Environment	Prefecture	Area	No. of bartonella-infected rodents/no. of rodents examined (isolation rate [%])							
			A. speciosus	*A. argenteus*	*C. rufocanus* subsp. *bedfordiae*	*M. caroli*	*R. rattus*	*R. norvegicus*	Subtotal	Total
Suburban	Hokkaido	Sapporo	29/31 (93.5)	3/5 (60.0)	4/17 (23.5)				36/53 (67.9)	
	Aomori	Tsugaru	11/17 (64.7)	1/2 (50.0)					12/19 (63.2)	
	Gunma	Minakami	26/43 (60.5)	3/5 (60.0)					29/48 (60.4)	
	Kanagawa	Fujisawa	1/1 (100)	3/3 (100)					4/4 (100)	
	Shizuoka	Mt. Fuji	13/21 (61.9)	8/17 (47.1)					21/38 (55.3)	
	Ishikawa	Mt. Haku	2/7 (28.6)						2/7 (28.6)	
	Nagano	Karuizawa	16/20 (80.0)	1/1 (100)					17/21 (81.0)	
	Shimane	Matsue	8/40 (20.0)	0/2 (0.0)				0/1 (0.0)	8/43 (18.6)	
	Tokushima	Hiwasa	2/6 (33.3)						2/6 (33.3)	
	Ehime	Matsuyama	5/6 (83.3)						5/6 (83.3)	
	Kagoshima	Nakanoshima Island	22/30 (73.3)				2/4 (50.0)		24/34 (70.6)	
	Kagoshima	Yoroshima Island					10/17 (58.8)		10/17 (58.8)	
	Kagoshima	Tokunoshima Island					4/12 (33.3)		4/12 (33.3)	
	Okinawa	Main Island				0/7 (0.0)	2/6 (33.3)	0/19 (0.0)	2/32 (6.3)	176/340 (51.8)
City	Kanagawa	Yokohama					0/255 (0.0)	0/84 (0.0)	0/339 (0.0)	
	Shizuoka	Shimoda	0/2 (0.0)				0/3 (0.0)	0/1 (0.0)	0/6 (0.0)	0/345 (0.0)
Total			135/224 (60.3)	19/35 (54.3)	4/17 (23.5)	0/7 (0.0)	18/297 (6.1)	0/105 (0.0)	176/685 (25.7)	

Sequencing and phylogenetic analysis of *rpoB* and *gltA*. The PCR products of *rpoB* and *gltA* from individual *Bartonella* isolates were sequenced with specific primers for *rpoB* (1400F and 2300R, whose sequences are given above, and 1600R [5′-GGRCAAATACGACCATAATGSG-3′], 2000R [5′-CGYGGYRCC ATRAAAACTTCWCC-3′], and 2000F [5′-GGWGAAGTTTTRATGGYRCC RCG-3′]) and for *gltA* (BhCS.781p and BhCS.1137n, whose sequences are given above), using an Applied Biosystems model 3130 genetic analyzer (Applied Biosystems, Foster City, CA). The CLUSTAL_X program (34) was used for the alignment of Japanese *Bartonella* sequences obtained in this study with those of known *Bartonella* species deposited in the GenBank/EMBL/DDBJ databases. A phylogenetic tree was drawn based on the sequences of *rpoB* (825 bp) and *gltA* (312 bp), using the neighbor-joining method with Kimura's two-parameter distance method in MEGA 3.1 (22, 25, 31). Bootstrap analysis was carried out with 1,000 resamplings (14).

RESULTS

Isolation and distribution of bartonellae in wild rodents in Japan. From 1997 to 2006, a total of 685 wild rodents were collected in 16 areas of 12 prefectures throughout Japan, and the numbers of rodents captured in each area are shown in Fig. 1. The species of rodents obtained were *Apodemus speciosus* (a large Japanese field mouse; $n = 224$), *A. argenteus* (a small Japanese field mouse; $n = 35$), *Clethrionomys rufocanus* subsp. *bedfordiae* (a gray red-backed vole; $n = 17$), *Mus caroli* (a Ryukyu mouse; $n = 7$), *Rattus rattus* (a roof rat; $n = 297$), and *R. norvegicus* (a brown rat; $n = 105$). By isolation of *Bartonella* organisms from blood samples of those rodents, we eventually obtained *Bartonella* isolates from 176 of 685 rodents (25.7%) (Table 1). The isolation rate of bartonellae in suburban areas was 51.8% (176/340), but no *Bartonella* isolate was obtained from any rodents in either of the two city areas (0/345), Yokohama, Kanagawa Prefecture, and Shimoda, Shizuoka Prefecture. The isolation rates ranged from 6.3% to 100% in the 12 prefectures of Japan, although the numbers of rodents captured in some areas were small. The areas with the highest isolation rates (>80%) were Fujisawa, Kanagawa Prefecture (100% [4/4]), Matsuyama, Ehime Prefecture (83.3% [5/6]), and Karuizawa, Nagano Prefecture (81.0% [17/21]), and those with the lowest rates (<20%) were the main island of Okinawa Prefecture (6.3% [2/32]) and Matsue, Shimane Prefecture (18.6% [8/43]). Among rodent species, the isolation rates of bartonellae were 60.3% (135/224) for *A. speciosus*, 54.3% (19/35) for *A. argenteus*, 23.5% (4/17) for *C. rufocanus* subsp. *bedfordiae*, and 6.1% (18/297) for *R. rattus*. These results suggest that *Bartonella* organisms are widely distributed in wild rodents inhabiting suburban areas throughout Japan.

Genetic characterization of Japanese *Bartonella* isolates in wild rodents. To characterize the *Bartonella* isolates, we first performed a comparative analysis of the RFLP patterns of the AcsI restriction enzyme-digested *rpoB* genes amplified. For each *Bartonella* culture obtained from the blood of a rodent, two or three bacterial colonies were tested. By PCR-RFLP analysis, for 169 out of the 176 rodents, the two or three isolates from each rodent were found to have identical PCR-RFLP patterns. However, three isolates from each rodent among the remaining seven animals had two different PCR-RFLP patterns, suggesting multiple infections as described below (RFLP data not shown). Therefore, we eventually obtained a total of 183 *Bartonella* isolates which have distinguishable PCR-RFLP patterns. To further characterize the 183 *Bartonella* isolates with different RFLP patterns, we sequenced all of the *rpoB* (825-bp) and *gltA* (312-bp) genes amplified from the 183 isolates. By this sequencing, we found that the 183 isolates have 31 and 28 different sequences of *rpoB* and *gltA*, respectively. Phylogenetic analysis based on the sequence similarities revealed that those isolates with different sequences were further classified into eight clusters, designated A to H for *rpoB* and I to P for *gltA* (Fig. 2). Each cluster for *rpoB* was correlated with one of the clusters for *gltA*. All *Bartonella* isolates seem to follow a pattern in which, e.g., the isolates in cluster A of *rpoB* also belongs to cluster I of *gltA* (designated the A-I genetic group), and idem for B-J, C-K, D-L, E-M, F-N, G-O, and H-P (Fig. 2).

The closest relatives of the respective *Bartonella* isolates and

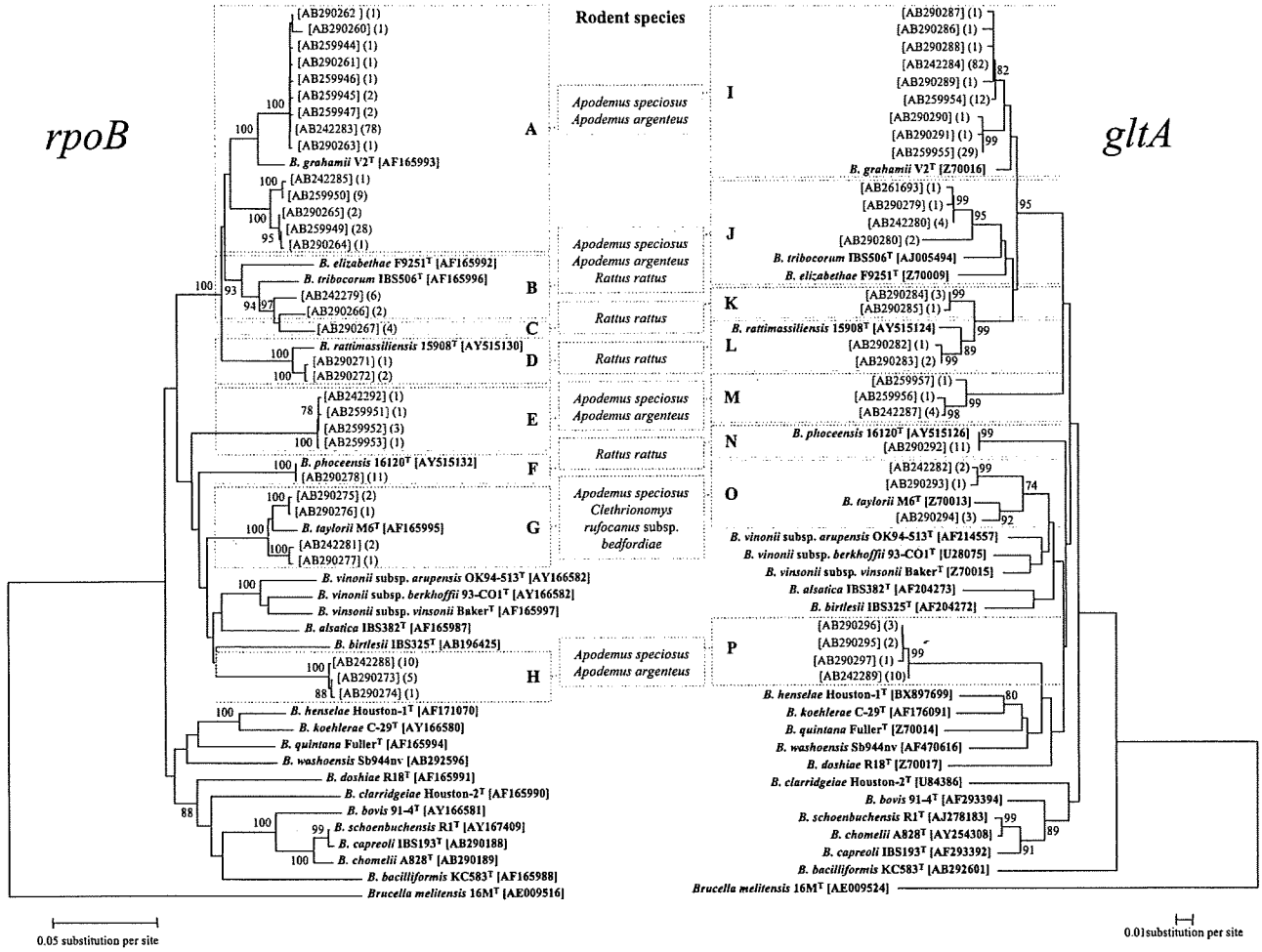

FIG. 2. Phylogenetic classification of Japanese *Bartonella* isolates based on sequences of *rpoB* (left) and *gltA* (right). The phylogenetic tree was constructed by the neighbor-joining method, and bootstrap values were obtained with 1,000 replicates. Only bootstrap replicates of >70% are noted. The 31 and 29 different sequences of *rpoB* and *gltA*, respectively, from Japanese *Bartonella* isolates were classified into eight clusters, A to H for *rpoB* and I to P for *gltA*. Based on the correlation between respective clusters in *rpoB* and *gltA*, combinations of clusters such as A-I, B-J, C-K, D-L, E-M, F-N, G-O, and H-P were assigned as *Bartonella* genetic groups. The numbers of *Bartonella* isolates with identical DNA sequences are shown in parentheses at the right and left of the respective GenBank accession numbers for the *rpoB* and *gltA* sequences, respectively. The rodent species associated with the respective *Bartonella* genetic groups are shown between the two trees. The *rpoB* and *gltA* sequences from *Brucella melitensis* 16 M^T were used as an outgroup bacterium.

the relationships between the organisms and their host species are shown in Table 2. The *Bartonella* isolates in the A-I genetic group had sequence similarities of 94.7 to 97.3% for *rpoB* and 96.8 to 98.4% for *gltA* with respect to *B. grahamii* $V2^T$ (the closest relative). The sequences also showed similarities of 98.3% for *rpoB* and 97.1 to 97.4% for *gltA* between the isolates of the D-L genetic group and *B. rattimassiliensis* 15908^T, 100% for *rpoB* and 100% for *gltA* between the isolates of the F-N group and *B. phoceensis* 16120^T, and 97.1 to 98.1% for *rpoB* and 94.1 to 97.0% for *gltA* between the isolates of the G-O group and *B. taylorii* $M6^T$. With respect to *rpoB*, the isolates of groups B and C seem to be closely related to each other, and these isolates had high similarities to *B. tribocorum* $IBS506^T$ (95.0 to 96.4% and 95.9%, respectively). However, with respect to *gltA*, the corresponding groups J and K were clearly clustered, and their closest relatives seem to be different, i.e., *B. tribocorum* $IBS506^T$ (93.6 to 96.5%) for group J and *B. rattimassiliensis* 15908^T (96.2 to 96.5%) for group K. Accordingly, in the case of genetic group C-K (for both *rpoB* and *gltA*), the isolates had the highest similarities to *B. tribocorum* $IBS506^T$ (95.9%) with respect to *rpoB*, while for *gltA*, they had higher similarities to *B. rattimassiliensis* 15908^T (96.2 to 96.5%) than to *B. tribocorum* $IBS506^T$ (93.6 to 96.5%). In the case of genetic groups E-M and H-P, the isolates from *A. speciosus* and *A. argenteus* in both groups showed the highest levels of similarity to *B. alsatica*, with only <90.1% and <91.4% for *rpoB*. With respect to *gltA*, the isolates of genetic groups E-M and H-P also showed the highest levels of similarity to *B. grahamii* and *B. vinsonii* subsp. *arupensis*, with only <91.0% and 89.1%, respectively. These low degrees of similarity of the isolates to any known *Bartonella* species suggest that the isolates of these genetic groups may be new species.

Among rodent species, (i) *A. speciosus* and *A. argenteus* mice were infected with bartonellae belonging to genetic groups A-I,

TABLE 2. Closest relatives of Japanese *Bartonella* isolates and relationships between organisms and their host species based on sequence analysis of *rpoB* and *gltA*

Genetic group	Closest relative[a]	% similarities to *rpoB* and *gltA*	No. of isolates				
			A. speciosus	*A. argenteus*	*C. rufocanus* subsp. *bedfordiae*	*R. rattus*	Total
A-I	*B. grahamii*	94.7–97.3, 96.8–98.4	116	13	0	0	129
B-J	*B. tribocorum*	95.0–96.4, 93.6–96.5	4	2	0	2	8
	B. elizabethae	93.8–94.4, 92.6–94.2					
C-K	*B. tribocorum*	95.9, 92.6–92.9	0	0	0	4	4
	B. rattimassiliensis	91.2, 96.2–96.5					
D-L	*B. rattimassiliensis*	98.3, 97.1–97.4	0	0	0	3	3
E-M	NA		5	1	0	0	6
F-N	*B. phoceensis*	100, 100	0	0	0	11	11
G-O	*B. taylorii*	97.1–98.1, 94.1–97.0	2	0	4	0	6
H-P	NA		13	3	0	0	16
Total			140	19	4	20	183

[a] NA, not applicable.

B-J, E-M, G-O, and H-P; (ii) *C. rufocanus* subsp. *bedfordiae* voles were infected only with bartonellae of the G-O group; and (iii) *R. rattus* rats were infected with bartonellae belonging to groups B-J, C-K, D-L, and F-N.

Among the genetic groups of *Bartonella* isolates, (i) the A-I, E-M, and H-P groups were obtained only from *Apodemus* mice, such as *A. speciosus* and *A. argenteus*; (ii) the C-K, D-L, and F-N groups were obtained only from *R. rattus*; (iii) the G-O group was isolated from two different host species, *A. speciosus* and *C. rufocanus* subsp. *bedfordiae*; and (iv) the B-J group was obtained from two different host genera, including three species, *A. speciosus*, *A. argenteus*, and *R. rattus* (Fig. 2). These results suggest that there is some animal host specificity among *Bartonella* species, e.g., *Apodemus* mice for genetic groups A-I, E-M, and H-P and *Rattus* rats for C-K, D-L, and F-N (Table 2).

Additionally, as described in the above section on PCR-RFLP analysis, we detected two different RFLP patterns of *rpoB* in two or three *Bartonella* isolates obtained from a single rodent out of seven wild rodents. Sequencing of the *rpoB* and *gltA* genes amplified from those isolates with different RFLP patterns revealed that five *A. speciosus* mice among the seven animals were infected with the A-I group and either the E-M, the B-J, or the H-P group and that the remaining two rodents (both *R. rattus* rats) were infected with the B-J group and either the F-N or the D-L group, showing dual infection with two different genetic groups of bartonellae (Table 3).

DISCUSSION

This study demonstrated for the first time the prevalence of *Bartonella* organisms in wild rodents in Japan and characterized the genetic properties of those *Bartonella* isolates. The overall prevalence of *Bartonella* infection in wild rodents was found to be 25.7% (176/685) in this study. Previous reports for other countries have shown that the prevalences of bartonellae in wild rodents ranged from 8.7%, in the northern part of Thailand (8), to 62.2%, in Shropshire County, United King-

TABLE 3. Dual infection with two different *Bartonella* genetic groups in wild rodents

Rodent no.	Prefecture	Area	Rodent species	Details of dual infection					
				Genetic group	Closest relative(s)	GenBank accession no. for identical *rpoB* and *gltA* sequences	Genetic group	Closest relative(s)[a]	GenBank accession no. for identical *rpoB* and *gltA* sequences
1	Aomori	Tsugaru	*A. speciosus*	A-I	*B. grahamii*	AB259944, AB242284	E-M	NA	AB259953, AB259956
2	Aomori	Tsugaru	*A. speciosus*	A-I	*B. grahamii*	AB259946, AB242284	B-J	*B. tribocorum*, *B. elizabethae*	AB242279, AB261693
3	Shimane	Matsue	*A. speciosus*	A-I	*B. grahamii*	AB242283, AB242284	E-M	NA	AB259952, AB242287
4	Kagoshima	Nakanoshima	*A. speciosus*	A-I	*B. grahamii*	AB259949, AB259955	H-P	NA	AB290273, AB290295
5	Kagoshima	Nakanoshima	*A. speciosus*	A-I	*B. grahamii*	AB259949, AB259955	H-P	NA	AB290273, AB290296
6	Kagoshima	Yoroshima	*R. rattus*	B-J	*B. tribocorum*, *B. elizabethae*	AB290270, AB290281	F-N	*B. phoceensis*	AB290278, AB290292
7	Okinawa	Main Island	*R. rattus*	B-J	*B. tribocorum*, *B. elizabethae*	AB290266, AB290280	D-L	*B. rattimassiliensis*	AB290272, AB290283

[a] NA, not applicable.

dom (4). Our result (25.7%) was similar to the percent range for Greece, 30.0% (33). The prevalences of *Bartonella* organisms isolated from the rodents captured in the three prefectures of Kanagawa, Nagano, and Ehime are considerably high (>80%), whereas the prevalences for two prefectures of Shimane and Okinawa seem to be low (<20%). Furthermore, the rodents inhabiting suburban regions appear to be predominantly infected with *Bartonella* organisms (prevalence, 51.8% [176/340]), but the rodents living in city areas are likely to be bartonella free (0/345). Among rodent species, *A. speciosus*, *A. argenteus*, *C. rufocanus* subsp. *bedfordiae*, and *R. rattus* rodents captured in suburban areas were highly infected with *Bartonella* organisms, suggesting that those rodents might be major reservoirs of those *Bartonella* species in Japan. However, no bartonellae were isolated from *M. caroli* or *R. norvegicus*, although the number of *M. caroli* mice examined was small (n = 7) in this study. The reasons why the prevalences of *Bartonella* infection varied among several locations or among different rodent species in Japan are likely to depend on the distribution of reservoirs or arthropod vectors, such as fleas, and/or to depend on host specificities due to differences in reservoirs or vector species. However, we do not know why rodents living in city areas did not harbor any *Bartonella* species, even though arthropod vectors are probably present in city areas. At least, we could not find any blood-suckling arthropod vectors in the rodents captured in the city areas in this study (data not shown).

Previously, La Scola et al. proposed sequence similarities to *rpoB* and *gltA* for validation of species, i.e., when the sequence similarities to *rpoB* and *gltA* are neither below 95.4% nor below 96.0%, respectively, those isolates can be considered members of the same species (26). According to the criteria, the genetic groups D-L and F-N in this study probably belong to the *Bartonella* species *B. rattimassiliensis* and *B. phoceensis*, respectively. All of the other genetic groups include some isolates below the cutoff value of *rpoB* or *gltA*, but some fulfill the species criteria. In this case, we used genetic groups for classification of the isolates in this study.

Bartonella isolates belonging to the A-I genetic group, which is closely related to *B. grahamii*, known as a potential causative agent of neuroretinitis in humans (21), were obtained from *Apodemus* mice, and these isolates appear to be dominant in Japanese wild rodents, suggesting the possibility of the risk of human exposures. Previously, it was reported that *B. grahamii* infects several wild rodents, such as members of the genera *Clethrionomys*, *Apodemus* (*A. speciosus* and *A. argenteus* in this study), *Microtus*, *Dryomys*, and *Mus*, in many other countries, including the United Kingdom (4), Sweden (18), Denmark (13), China (37), Canada (20), and Greece (33). This may show the wide host range and the global distribution of *B. grahamii*-like organisms. The isolates of genetic groups E-M and H-P, which were obtained only from *A. speciosus* and *A. argenteus*, had low degrees of similarity to those of all known *Bartonella* species for *rpoB* (<91.4%) and *gltA* (<91.0%). This result strongly supports the idea that groups E-M and H-P are probably new species, although further biochemical and molecular analyses, such as determination of the activity of bacterial enzymes and DNA-DNA hybridization, may be required to combine those *Bartonella* organisms as new species.

R. rattus rats captured in suburban areas were found to be infected with several bartonellae, such as genetic groups C-K, D-L, and F-N, which were closely related to *B. tribocorum*, *B. rattimassiliensis*, and *B. phoceensis*, respectively. In contrast, no bartonellae were isolated from *R. norvegicus* rats living in city or suburban areas. This suggests that *R. rattus* rodents living in suburban areas may serve as a main reservoir for several *Bartonella* species in Japan. Previous studies in other countries have shown that wild rats, including the species *R. norvegicus* as well as *R. rattus*, are known to be reservoirs for *B. phoceensis*, *B. rattimassiliensis*, *B. tribocorum*, and *B. elizabethae* in France (16, 17), the United States (12), Portugal (12), and Indonesia (36); *B. elizabethae*, a causative agent of human endocarditis and neuroretinitis, is of particular public health significance (6, 11, 12). In this study, however, we did not isolate *B. elizabethae* from any wild rats in Japan, even though this *Bartonella* species has been commonly isolated from several rat species in the world (9, 12). The reason why *B. elizabethae* was not isolated in Japan is unknown. The organisms might not yet be distributed among wild rodents in Japan. To confirm whether *B. elizabethae* is absent in wild rats of Japan, further epidemiological and ecological studies will be needed.

The *Bartonella* isolates in the G-O genetic group, which is closely related to *B. taylorii*, were obtained from both *A. speciosus* and *C. rufocanus* subsp. *bedfordiae* in this study. *B. taylorii* had previously been isolated from several *Apodemus* spp. (*A. speciosus* in this study) and from *C. glareorus* in other countries, such as the United Kingdom (5, 7), Sweden (18), and Greece (33). In Japan, *C. rufocanus* subsp. *bedfordiae* seems to harbor only *B. taylorii*-like organisms, suggesting specificity between host species and some *Bartonella* species. However, *Bartonella* isolates in the B-J group, which is closely related to *B. tribocorum* and *B. elizabethae*, were obtained from several different species of wild rodents, such as *A. speciosus*, *A. argenteus*, and *R. rattus*, suggesting the wide host range of the bartonellae in Japan. In *Bartonella*-infected rodents, dual infection with two different genetic groups of bartonellae (including two possible new species) was observed only in *A. speciosus* and *R. rattus*, suggesting that these rodent species might be potential reservoirs harboring multiple *Bartonella* species. As mentioned above, our findings in this study may become a matter of public health significance with respect to *Bartonella* infection in Japan.

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Bartonella tamiae sp. nov., a Newly Recognized Pathogen Isolated from Three Human Patients from Thailand▽

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Three strains of a novel *Bartonella* species (*Bartonella tamiae*) were isolated from human patients from Thailand. Sequence analysis of six chromosomal regions (16S rRNA, *gltA*, *groEL*, *ftsZ*, *rpoB*, and the intergenic spacer region) and phenotypical analysis supported the similarity of the three strains and placed them within the genus *Bartonella* separately from previously described species.

Several species of the genus *Bartonella* cause numerous disorders, and most are thought to be zoonoses (1, 3). These *Bartonella*-associated illnesses occur worldwide, including in Asia, and they encompass a broad clinical spectrum, including fever, skin lesions, lymphadenopathy, endocarditis, and abnormalities of the central nervous system, liver, eye, and bone tissues (5). There has been a report indicating that humans are being exposed to *Bartonella* species in Thailand, though the investigators did not isolate the agents (7).

We report the characterization of three *Bartonella* strains isolated from blood samples of patients from Khon Kaen Province, Thailand, that belong to a novel *Bartonella* species. These strains were identified during the screening of blood clot specimens from a prospective study performed to determine the etiology of febrile illnesses in Thailand. It is the first report of culture-confirmed *Bartonella* infection in humans in Thailand.

Blood clots were separated from sera, stored at −70°C, and shipped to the U.S. CDC (Fort Collins, CO) for testing. Two approaches were used for the isolation of *Bartonella* from human clots: (i) blood clots were cocultivated with Vero E6 cells at 35°C with 5% carbon dioxide for 7 days and then subcultured onto rabbit blood-enriched agar and (ii) blood clots were inoculated into a preenrichment liquid, the *Bartonella-Alphaproteobacteria* growth medium (BAPGM) developed by Maggi et al. (6), and after 7 days of incubation at 35°C with 5% carbon dioxide were plated onto rabbit blood agar. The agar plates were incubated at 35°C with an aerobic atmosphere of 5% carbon dioxide for up to 30 days. The cultured bacteria were visualized with Gram stain by using standard light microscopy with an oil immersion objective at a magnification of 1,000×.

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For negative staining, a drop of suspension was placed on a copper grid coated with Formvar-carbon film and allowed to adhere for 10 min. The grids with adherent bacteria were stained by placing them on a drop of 2% potassium-phosphotungstic acid and air dried. The grids were examined with a Phillips 201 electron microscope. For ultrathin sectioning, the bacterial suspension was fixed in a mixture of 2.5% formaldehyde, 0.1% glutaraldehyde, 0.03% trinitrophenol, and 0.03% $CaCl_2$ in 0.05 M cacodylate buffer (pH 7.2). Ultrathin sections were cut with a Leica-Reichert Ultracut S ultramicrotome, stained with 2% aqueous uranyl acetate and lead citrate, and examined with a Phillips 201 electron microscope.

The MicroScan rapid anaerobe identification panel (Dade Behring, Inc., West Sacramento, CA) was used to test the activities of preformed bacterial enzymes in accordance with the manufacturer's instructions on the preparation, incubation, and interpretation of the test results. For the study of antibiotic susceptibility, microbial suspensions were prepared according to a 0.5 McFarland standard from 5-day-old agar cultures and diluted 10-fold. Eight antibiotics (penicillin, cefotaxime, gentamicin, erythromycin, clindamycin, doxycycline, ciprofloxacin, and rifampin) diluted in Columbian agar supplemented with 5% sheep blood were tested. Results were read at the third and fifth days postinoculation.

Bacterial DNA was heat extracted at 95°C for 10 min from whole bacterial cells. Oligonucleotide primers were used for the amplification of single regions of the *Bartonella* citrate synthase (*gltA*), the cell division protein (*ftsZ*), the RNA polymerase beta-subunit (*rpoB*), the heat shock protein (*groEL*), and 16S rRNA genes, as well as the 16S-to-23S rRNA intergenic spacer (ITS) region. Positive and negative controls were included in each PCR to evaluate the presence of appropriately sized amplicons and possible contamination. Each PCR was conducted with a PTC-200 Peltier automated thermal cycler (MJ Research, Waltham, MA). PCR products were analyzed for the presence of amplicons of the correct size by

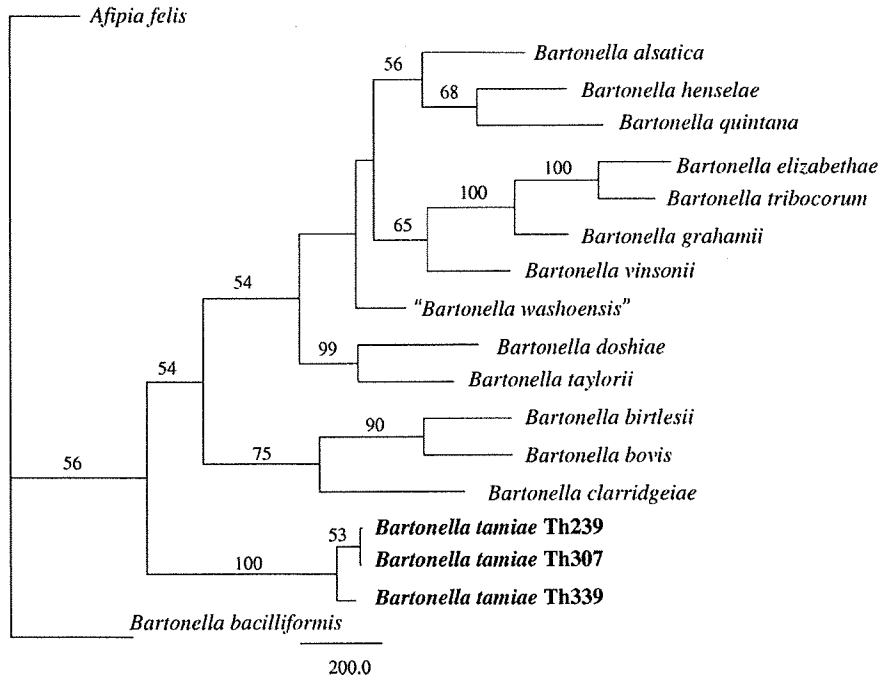

FIG. 1. Phylogenetic tree showing the positions of strains Th239, Th307, and Th339 among members of the genus *Bartonella* based on comparisons of concatenated sequences of the following six genes: the 16S rRNA gene, the citrate synthase gene *gltA*, the RNA polymerase beta-subunit gene *rpoB*, the cell division gene *ftsZ*, the heat shock protein genes *groEL*, and the 16S-to-23S rRNA ITS region sequences. The 16S rRNA gene from *Afipia felis* was included for outgroup comparison. Bootstrap values strongly support the position of these strains in a novel clade within the genus *Bartonella*; however, the values do not support separating the three strains into distinct species. Trees were constructed using a maximum likelihood-based distance algorithm and a GTR+I+G DNA substitution model using PAUP software. Numbers on branches indicate the bootstrap values derived from 500 replications. The bar indicates the number of nucleotide changes.

electrophoresis of 5 μl of the product in 1.5% agarose gels containing ethidium bromide. Amplicons of the expected size were identified by size comparison to the positive control, and the resultant PCR products were purified using the QIAquick PCR purification kit (Qiagen, Germantown, MD) and sequenced in both directions using the same primers that were used for the PCR assay. Sequencing reactions were carried out with a PTC-200 Peltier thermal cycler, using the Quick Start dye terminator cycle sequencing kit (Beckman Coulter, Fullerton, CA).

Sequences were analyzed using Lasergene sequence analysis software (DNAStar, Madison, WI). The SeqMan program (DNAStar) was used to determine consensus sequences for the amplified region of the target genes, and the Clustal V program within Megalign (DNAStar) was used to align and compare homologous sequences. The sequences were then analyzed using PAUP 4.0 (Center for Biodiversity, Illinois Natural History Survey, Champaign, IL), and phylogenies were constructed using the maximum parsimony algorithm. To improve the statistical support of the phylogeny of the *Bartonella* genus, partial nucleotide sequences of six chromosomal regions (16S rRNA, 1,216 bp [final sequence length]; *ftsZ*, 788 bp; the ITS region, 1,076 bp; *gltA*, 323 bp; *groEL*, 825 bp; and *rpoB*, 1,209 bp) from 17 *Bartonella* strains were aligned and trimmed individually using MEGA version 3.1 and then concatenated in a multilocus sequence typing approach (Fig. 1). Multiple-sequence alignments were analyzed using the PAUP 4.0 software program, and phylogenies were constructed by using a distance-based likelihood method with the GTR+I+G DNA substitution model, as chosen by Modeltest v3.1. Trees were rooted using *Afipia felis* as the outgroup; because it was intended to be used solely for rooting the tree and not for phylogenetic comparison, only the 16S rRNA gene of *A. felis* was used. Missing sequences were treated as missing data.

Strain Th239 was isolated from a 38-year-old male patient who was admitted to the hospital with fatigue, myalgia, a headache, a maculopapular rash that had lasted for 22 days, and a fever that had lasted for 6 days. Strain Th239 was obtained by the cocultivation of blood with Vero E6 cells. After 7 days, the suspension was found to be positive by PCR amplification of a *Bartonella*-specific fragment of the *gltA* gene. Subsequently, the inoculated Vero E6 cells were subcultured on rabbit blood agar, and very small colonies appeared on the agar 17 days postinoculation. The growth rate increased after several sequential subculturing passages of the collected bacterial suspension. After each passage, the suspensions of the Vero E6 cells were PCR positive for amplification of the targeted piece of the *gltA* gene.

Strain Th307 was obtained from a 41-year-old female admitted to the hospital with an initial diagnosis of a pterygium in each eye. The strain was isolated after the inoculation of the patient's blood clot into preenrichment BAPGM. After a 7-day incubation, the suspension was found to be positive for *Bartonella* organisms by PCR using the *gltA* gene as a target. The inoculated medium was then placed on rabbit blood agar and incubated at 35°C with 5% CO_2. *Bartonella*-like colonies were

observed after 5 days of cultivation and confirmed to be positive by PCR.

Strain Th339 was obtained from a 12-year-old male patient admitted to the hospital with fever, fatigue, myalgia, a headache, and a petechial rash on his arms and legs that had lasted for 2 days. The strain was isolated after the inoculation of the patient's blood clot into preenrichment BAPGM and a 10-day incubation. Inoculated medium was then placed on rabbit blood agar and incubated at 35°C with 5% CO_2. *Bartonella*-like colonies were observed on the agar plate after an additional 12 days.

BLAST searches indicated that all sequences of the 16S rRNA, *gltA*, *groEL*, *ftsZ*, *rpoB*, and the ITS region of strains Th239, Th307, and Th339 are closely related to the homologous sequences of various *Bartonella* species and unnamed *Bartonella* strains. Phylogenetic analyses based on the parsimony method (heuristic search), neighbor joining, and MegAlign alignments of all sequences (16S rRNA, *gltA*, *groEL*, *ftsZ*, *rpoB*, and the ITS region) supported the novelty of the new isolate and suggested a distant phylogenetic lineage in the genus *Bartonella*. Phylogenetic analysis of a concatenated, multiple-sequence alignment of the 16S rRNA, *gltA*, *ftsZ*, *groEL*, *rpoB*, and ITS region nucleotide sequences using a distance-based maximum likelihood algorithm supported the placement of the new isolates within a distinct phylogenetic lineage of the *Bartonella* genus (Fig. 1).

The structures of phylogenetic trees and the percentages of divergence between the sequences of the 16S ribosomal genes of strains Th239, Th307, and Th339 and sequences in the GenBank nucleic acid database demonstrated that these strains were close to representatives of the genus *Bartonella*. The genetic distances between the isolates and representatives of the genus *Brucella*, which is the taxonomic group closest to *Bartonella*, were evidently greater than the distances between these isolates and other *Bartonella* species. The 16S rRNA gene sequence analysis indicated that these three strains represent a distant phylogenetic lineage in the genus *Bartonella*. The 16S rRNA sequences of strains Th239, Th307, and Th339 exhibited the closest phylogenetic relationship with three sequences of three uncultured *Bartonella* species clones (pAJ203 [AY370185], pAJ208 [AY370186], and pAJ210 [AY370187]) obtained from the honeybee *Apis mellifera* (4).

Because of the genetic similarity among the strains, only one strain, Th239, was used for further phenotypical characterization. Gram staining of isolate Th239 revealed rod-shaped, gram-negative bacilli that were small, straight, and slightly curved. Transmission electron microscopy of negatively stained bacteria showed small, rounded rods ranging from 0.7 to 1.2 μm in length and 0.5 to 0.6 μm in width. Some bacteria had bundles of fibrils approximately 15 nm thick and 30 nm in length with a periodicity ranging from 30 to 120 nm and with polar distribution (Fig. 2A). Examination of ultrathin sections revealed typical gram-negative morphology with a wavy cell wall. The bacteria appeared to be approximately 0.3 μm by 0.8 μm (Fig. 2B). Therefore, the sizes of the organisms, taking into account measurements from both ultrathin sections and negative staining, appear to range from 0.3 to 0.6 μm in width and from 0.7 to 1.2 μm in length.

Most of the biochemical properties of strain Th239 were typical for bacteria of the *Bartonella* genus. Specifically, enzymatic hydrolysis results were negative for *p*-nitrophenyl-beta-D-galactopyranoside, *p*-nitrophenyl-alpha-D-galactopyranoside, *p*-nitrophenyl-*N*-acetyl-beta-D-glucosaminide, *p*-nitrophenyl-alpha-D-glucopyranoside, *o*-nitrophenyl-beta-D-glucopyranoside, *p*-nitrophenyl-alpha-L-fucopyranoside, *p*-nitrophenyl-alpha-D-mannopyranoside, L-proline-beta-naphthylamide, L-pyrrolidonyl-beta-naphthylamide, trehalose, urea, indole, and nitrate. Positive reactions were observed in the enzymatic hydrolysis of bis-*p*-nitrophenyl-phosphate, *p*-nitrophenyl-phosphate, L-leucine-beta-naphthylamide, L-lysine-beta-naphthylamide (acid and alkaline), DL-methionine-beta-naphthylamide, glycylglycine-beta-naphthylamide, glycine-beta-naphthylamide, L-arginine-beta-naphthylamide, L-tryptophane-beta-naphthylamide, and 3-indoxyl-phosphate. The unique property of strain Th239 in contrast to other *Bartonella* strains was its 3-indoxylphosphate activity.

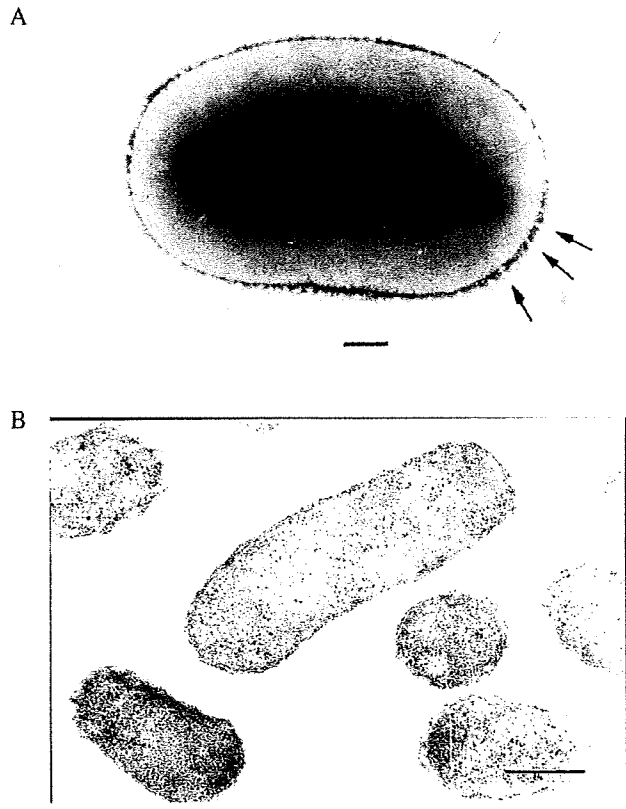

FIG. 2. Ultrastructure of *Bartonella tamiae* (strain Th239). (A) Strain Th239 after gram-negative staining showing bundles of fibrils closer to its pole (arrows). Bar = 250 nm. (B) Ultrathin section of bacterial suspension showing typical gram-negative cell walls of *Bartonella tamiae* organisms. Bar = 250 nm.

The following MICs of antibiotics were observed with strain Th239: penicillin, 4 μg/ml; cefotaxime, >4 μg/ml; gentamicin, 0.5 μg/ml; erythromycin, >4 μg/ml; clindamycin, 32 μg/ml; doxycycline, 4 μg/ml; ciprofloxacin, 8 μg/ml; and rifampin, 4 μg/ml.

Our findings support the possibility of *Bartonella* strains being causes of human disease in Thailand. Two of the three patients were febrile, and all three had clinical and laboratory findings similar to those found for patients infected with other forms of bartonellosis (5). Mild anemia observed in all patients is presumed to have resulted from the infection of red blood cells, and headache, myalgia, and abnormalities in liver func-

tion, consistent features in these three patients, are commonly identified in other *Bartonella* infections.

All three patients reported trapping or killing rats in their houses, and two of them reported recent rat exposures within the 2 weeks prior to the onset of illness. Further investigation is needed to determine the animal reservoir and any possible vectors for *B. tamiae*. Previous investigations of rodents of northern Thailand demonstrated that 9% of the tested animals were *Bartonella* culture positive (2), and phylogenetic analysis indicated a high diversity of the *Bartonella* strains obtained from Thai rodents. Also, *Bartonella* species have been identified in cats and fleas from Thailand (8, 9). However, to date, no homologous mammalian or flea *Bartonella* sequences phylogenetically similar to *B. tamiae* have been identified.

Based on a combination of genetic and phenotypic characteristics, we consider these described strains to be representatives of a novel *Bartonella* species. *Bartonella tamiae* (tam.i'ae. N.L. fem. gen. n. *tamiae*, of Tami) is the name proposed to honor the late Tamara (Tami) Fisk, who organized the febrile illness study in Thailand from which this bacterial species originated. The prototype strain (Th239) isolated from the blood of a Thai patient has been deposited in the American Type Culture Collection (ATCC BAA-1343), the National Collection of Type Cultures (United Kingdom, NCTC 13398), and the Japan Collection of Microorganisms (JCM 14580).

Nucleotide sequence accession numbers. Sixteen unique nucleotide sequences were identified among the obtained three isolates. The three *gltA* sequences were assigned the GenBank accession numbers DQ395177 (strain TH239), EF605279 (strain TH307), and EF605280 (strain TH339). The *ftsZ* sequences were assigned the accession numbers DQ395178 (TH239), EF605281 (TH307), and EF605282 (TH339). The 16S-to-23S ribosomal ITS region sequences were assigned the accession numbers DQ395180 (TH239), EF605283 (TH307), and EF605284 (TH339). The *groEL* sequence was assigned the GenBank accession number DQ395179 (TH239). The *rpoB* sequences were assigned the accession numbers EF091855 (TH239), EF605285 (TH307), and EF672730 (TH339). The novel 16S ribosomal gene sequences were assigned the accession numbers DQ395176 (TH239), EF672728 (TH307), and EF672729 (TH339).

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FULL PAPER *Public Health*

Antimicrobial Susceptibilities of *Salmonella* from Domestic Animals, Food and Human in the Mekong Delta, Vietnam

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ABSTRACT. A total of 230 *Salmonella* isolates representing 33 serotypes originated from food (pork, beef, chicken meat, duck meat, and shrimp), domestic animals (pig, chicken, and duck), and human (children with diarrhea) in the Mekong Delta, Vietnam were examined for the antimicrobial resistance to 10 antibiotics. Of the 230 *Salmonella* isolates examined, 49 (21.3%) showed antimicrobial resistance. Thirty-eight isolates (16.5%) were resistant to oxytetracycline, 26 (11.3%) to chloramphenicol, 17 (7.4%) to nalidixic acid, 16 (7.0%) to streptomycin, 5 (2.2%) to kanamycin, and 4 (1.7%) to ampicillin. No isolate showed resistance to gentamicin, cefazolin, ceftriaxone, and ciprofloxacin. Among the resistant isolates, nineteen isolates were resistant to one antimicrobial agent, 10 to two, 15 to three, 3 to four, and 2 to five antimicrobial agents. The resistance rate of *Salmonella* isolates from the Mekong Delta, Vietnam to these antimicrobial agents seems to be relatively lower than the results of developed countries and even those of the neighboring countries.
KEY WORDS: antimicrobial susceptibilities, *Salmonella*, Vietnam.

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Salmonella is one of the most common pathogens causing enteritis, and it has been a major cause of foodborne disease in many countries. In recent years, there have been significant increases in the occurrence of antimicrobial resistance in *Salmonella* in both developed and developing countries [26, 28]. In developed countries, antimicrobial resistant *Salmonella* results from the use of antimicrobial agents in food animals, and these antimicrobial resistant *Salmonella* are subsequently transmitted to humans, usually through the food supply [1]. On the other hand, increase of resistant *Salmonella* in developing countries has been associated with inappropriate use of antimicrobial agents in human medicine [26].

Because of public health concerns, antimicrobial resistance surveillance networks have been created in veterinary and human medicine [1, 9, 18, 19, 35]. Thus, a lot of information about prevalence and antimicrobial resistance of *Salmonella* are available in developed countries, notably in North America and Europe. Nevertheless, few data, especially of non-human *Salmonella* isolates, are available in developing countries. In Vietnam, the few reports dealing with antimicrobial resistance of *Salmonella* isolates are of human isolates [14, 24], and there are a few information about prevalence and antimicrobial resistance of *Salmonella* of other sources [32,33]. However, Heinitz *et al.* [12] reported that 30% of *Salmonella* isolates originated from imported seafoods from Vietnam to United States from 1990 to 1998 were resistant to some antimicrobial agents. Kiessling *et al.* [15] also reported the prevalence of antimicrobial resistant *Salmonella* in food samples imported from Vietnam to United States from 1999 to 2000, and among those isolates, one *Salmonella* Derby isolated from frozen eel showed resistance to seven antimicrobial agents. Thus, it is important to know the prevalence of resistant *Salmonella* in Vietnam, not only for public health in Vietnam, but also in view of food exporting country. The Mekong Delta consisting of 12 provinces and 1 city is located in the southern area of Vietnam, and 3 millions pigs and 44 million poultry were raised in this area in 2000. However, no reports have been published regarding the antimicrobial susceptibility of *Salmonella* spp. originated from the Mekong Delta. Therefore, in this study, the antimicrobial susceptibility of *Salmonella* originated from various sources in the Mekong Delta in Vietnam was examined.

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MATERIALS AND METHODS

Bacterial isolates: A total of 230 *Salmonella* isolates representing 33 serotypes originated from food (pork, beef, chicken meat, duck meat, and shrimp), domestic animals (pig, chicken, and duck), and human (children with diarrhea) were examined (Table 1). These were isolated from July 1999 to September 2001 in 8 provinces in the Mekong Delta, Vietnam [29,30]. Food (pork, beef, chicken meat, duck meat, and shrimp) were originated from wet markets in the Mekong Delta. Sample of domestic animals (pig, chicken, and duck) were originated from farm. Human samples were originated from the patient which visited hospitals in the Mekong Delta, Vietnam.

Table 1. Serotype distribution of 230 *Salmonella* isolates from Vietnam

Serotypes	Food					Animal			Human	Total
	Pork	Beef	Chicken meat	Duck meat	Shrimp	Pig	Chicken	Duck		
S. Aberdeen						1				1
S. Anatum	5		2	1			1			9
S. Blockley			1	1						2
S. Bovismorbificans	1	1		1	1			2	2	8
S. Braenderup								1		1
S. Derby	11	1		1	3	4		1		21
S. Dessau	2	5	2	2	4					15
S. Dublin				2				1		3
S. Emek			2				8			10
S. Enteritidis							1			1
S. Hadar			2	1						3
S. Javiana			1			8	4	3		16
S. Lexington	2		2	2	1			1		8
S. Lome								1		1
S. London	4	6	1	2		1			1	15
S. Mbandaka	1									1
S. Newport	2		1					1		4
S. Norwich	1									1
S. Ohio									3	3
S. Schleissheim	1				1					2
S. Senftenberg								2		2
S. Singapore							1			1
S. Southampton							1			1
S. Stanley	1			1		1				3
S. Tennessee	2		1		7	1				11
S. Thompson					1					1
S. Typhimurium			2	1		3	1	6	1	14
S. Virchow					1	1				2
S. Wagenia								1		1
S. Weltevreden	13	17		1	8	3	3	4		49
S. Westhampton	2									2
S. Worthington					1					1
S. II heilbron						1				1
UT[a)]		5	3	4	1	1		1	1	16
Total	48	35	20	20	29	26	19	25	8	230

a) Untypable.

Antimicrobial susceptibility test: *Salmonella* isolates were examined for susceptibility to 10 different antimicrobial agents by agar dilution method according to the National Committee for Clinical Laboratory Standards (NCCLS) procedure M7-A5 [21]. The antimicrobial agents used were ampicillin (ABPC), streptomycin (SM), kanamycin (KM), gentamicin (GM), oxytetracycline (OTC), chloramphenicol (CP), cefazolin (CEZ), ceftriaxone (CTRX), nalidixic acid (NA), and ciprofloxacin (CPFX). Antimicrobial susceptibility was assessed following the NCCLS procedure, but isolates showing intermediate susceptibility were classified as susceptible. *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 were used as a control strain according to the NCCLS.

Statistical analysis: The Chi-square test and Fisher's exact test were used for statistical analysis of the significant difference of resistant rates.

RESULTS

Of the 230 *Salmonella* isolates examined, 49 (21.3%) showed antimicrobial resistance. Thirty-eight isolates (16.5%) were resistant to oxytetracycline, 26 (11.3%) to chloramphenicol, 17 (7.4%) to nalidixic acid, 16 (7.0%) to streptomycin, 5 (2.2%) to kanamycin, and 4 (1.7%) to ampicillin. None of the isolates showed resistance to gentamicin, cefazolin, ceftriaxone, and ciprofloxacin (Table 2).

Of the 123 isolates from retail meat, a total of 33 (26.8%) isolates composed of 18 (37.5%) isolates from pork, 2 (5.7%) from beef, 9 (45%) from chicken meat, and 4

Table 2. Antimicrobial resistance of *Salmonella* isolates by food, domestic animals, and humans in Vietnam

Source	No. of examined	No. of resistant isolates[a] (%)										No. of Resistance[b] (%)
		ABPC	SM	KM	GM	OTC	CP	CEZ	CTRX	NA	CPFX	
Food												
Pork	48	3 (6.3)	7(14.6)			10 (20.8)	6 (12.5)					18 (37.5)[c]
Beef	35		2 (5.7)			1 (2.9)	1 (2.9)					2 (5.7)[c,d]
Chicken meat	20	1 (5.0)	4(20.0)	4(20.0)		9 (45.0)	5 (25.0)			7 (35.0)		9 (45.0)[d]
Duck meat	20		3(15.0)	1 (5.0)		3 (15.0)	1 (5.0)			1 (5.0)		4 (20.0)
Shrimp	29					2 (6.9)	1 (3.4)					2 (6.9)
Animal												
Chicken	19					8 (42.1)	8 (42.1)			9 (47.4)		9 (47.4)[e,f]
Duck	25					1 (4.0)	1 (4.0)					1 (4.0)[e]
Pig	26					3 (11.5)	3 (11.5)					3 (11.5)[f]
Human	8					1 (12.5)						1 (12.5)
Total	230	4 (1.7)	16 (7.0)	5 (2.2)	0	38 (16.5)	26 (11.3)	0	0	17 (7.4)	0	49 (21.3)
MIC_{50} ($\mu g/ml$)		1	8	2	0.5	2	8	1	<0.125	4	<0.125	
MIC_{90} ($\mu g/ml$)		2	8	2	0.5	128	32	1	<0.125	32	<0.125	

a) ABPC: Ampicillin, SM: Streptomycin, KM: Kanamycin, GM: Gentamicin, OTC: Oxytetracycline, CP: Chloramphenicol, CEZ: Cefazolin, CTRX: Ceftriaxone, NA: Nalidixic acid, CPFX: Ciprofloxacin.
b) Isolates resistant to at least one antimicrobial agent.
c, d, e, f) Difference is statistically significant, $P<0.01$.

Table 3. Antimicrobial resistance patterns of *Salmonella* isolates from Vietnam

No. of antimicrobial agents	Resistance pattern[a]	No. of resistant isolates	Serotypes	Source
1	SM	9	*S.* Derby	Pork (7), Beef (1), Duck meat (1)
	OTC	8	*S.* Derby	Shrimp (1)
			S. Anatum	Pork (2), Duck meat (1)
			S. London	Pork (1), Chicken meat (1), Human (1)
			S. Norwich	Pork (1)
	CP	1	*S.* Derby	Pork (1)
	NA	1	*S.* Enteritidis	Chicken (1)
2	ABPC+OTC	1	*S.* Anatum	Pork (1)
	SM+OTC	1	*S.* Hadar	Duck meat (1)
	OTC+CP	8	*S.* Derby	Pork (3), Shrimp (1), Pig (3), Duck (1)
3	ABPC+OTC+CP	3	*S.* Anatum	Pork (2)
			S. Javiana	Chicken meat (1)
	SM+OTC+CP	1	*S.* Weltevreden	Beef (1)
	OTC+CP+NA	11	*S.* Hadar	Chicken meat (1)
			S. Emek	Chicken meat (2), Chicken (8)
4	SM+KM+OTC+NA	3	*S.* Blockley	Chicken meat (1)
			UT	Chicken meat (2)
5	SM+KM+OTC+CP+NA	2	*S.* Blockley	Duck meat (1)
			S. Hadar	Chicken meat (1)
Total		49		

a) ABPC: Ampicillin, SM: Streptomycin, KM: Kanamycin, OTC: Oxytetracycline, CP: Chloramphenicol, NA: Nalidixic acid.

(20.0%) from duck meat, of the 70 isolates from domestic animals, a total of 13 (18.6%) isolates composed by 9 (47.4%) from chicken, 1 (4.0%) from duck, and 3 (11.5%) from pig, and of the 29 isolates from retail shrimp, two (6.9%) isolates, and of the 8 isolates from human, 1 (12.5%) isolate showed resistance to some antimicrobial agents. The resistance rates of *Salmonella* isolates from pork and chicken meat were significantly higher than that from beef ($P<0.01$), and among the isolates from domestic animals, resistant rates from chicken were significantly higher than those from ducks and pigs ($P<0.01$) (Table 2).

Among the resistant isolates, nineteen isolates showed resistance to one antimicrobial agent, 10 to two, 15 to three, 3 to four, and 2 to five antimicrobial agents (Table 3).

All of 17 NA resistant isolates were less susceptible to CPFX (MIC=0.25–2 $\mu g/ml$) when compared to the other

isolates.

DISCUSSION

Of the 10 antimicrobial agents analyzed in this study, *Salmonella* isolated in the Mekong Delta, Vietnam showed resistance to 6 agents. In developed countries, high resistance rates have generally been observed against those antimicrobial agents used since early times. In Japan, Miwa *et al.* [20] reported that 182 (63.4%), 184 (64.8%), and 20 (7.0%) among 287 *Salmonella* isolates, originated from chicken carcasses from 1996 to 1999, were resistant to OTC, SM, and ABPC, respectively, and Takahashi *et al.* [25] reported that 100 (80.6%), 95 (76.6%), and 11 (8.9%) among 124 isolates from feces of healthy domestic animals, mainly broiler chicken, in 1999 were resistant to OTC, dehydrostreptomycin (DSM), and ABPC, respectively. White *et al.* [36] reported that in the United States, 36 (80%), 33 (73%), and 12 (27%) among 45 of *Salmonella* isolates from retail ground meat in 1998 were resistant to tetracycline(TC), SM, and ABPC, respectively; and Poppe *et al.* [22] reported that in Canada, 341 (25.5%), 354 (26.5%), and 212 (15.9%) of 1336 isolates from animals, animal food products, and the environment of animal production were resistant to the same respective antimicrobial agents as in the United States. Similarly, resistance to those antimicrobial agents has been reported in developing countries in Southeast Asia. Rasrinaul *et al.* [23] reported that in Thailand, 40 (46%), 12 (14%), and 5 (6%) of 87 *Salmonella* isolates from food samples in 1986 were resistant to TC, SM, and ABPC, respectively. Van *et al.* [32] reported that 91 *Salmonella* isolates from retail raw food samples obtained in Ho Chi Mihn city, Vietnam were resistant to ABPC (22%), amoxicillin (22%), TC (40.7%), KM (2.2%), GM (2.2%), SM (14.3%), sulfafurazole (16.5%), enrofloxacin (8.8%), CP (2.2%), trimethoprim (3.3%) and NA (18.4%). The *Salmonella* isolates from the Mekong Delta, Vietnam also showed resistance to OTC, CP, NA, SM, KM, and ABPC. However, when compared with the results of developed countries and even with the results of neighboring countries, it seems that the resistance rate of *Salmonella* isolates from the Mekong Delta to these antimicrobial agents was relatively low. Furthermore, in the United States, besides resistance to those antimicrobial agents used since early times, resistance to CTRX, a third generation cephalosporin used to treat children with *Salmonella* infection, was detected in 7 (16%) of 45 *Salmonella* isolates in retail ground meat in 1998 [36]. Likewise, Boonmer *et al.* [2] reported that 28 (14.1%) of 199 *Salmonella* isolates from frozen chicken meat in Thailand were also resistant to CTRX. However, CTRX resistant *Salmonella* isolates as well as isolates resistant to relatively new antimicrobial agents were not detected in the Mekong Delta in Vietnam. Van *et al.* [32] also reported that no *Salmonella* isolates originated from retail raw food samples obtained in Ho Chi Mihn city, Vietnam showed resistance to cephalothin, a third generation cephalosporin. This would be partly because intensive livestock production, where antimicrobial agents are used as growth promoter or feed additives, is rare in Vietnam, and most of domestic animals are raised in small farm or farmer yard and fed agricultural by-products.

Moreover, in developed countries, the spread of particular serotypes and phage types that acquired multi-drug resistance has become an increasing public health problem. In the United States, the three most common *Salmonella* serotypes (Typhimurium, Enteritidis, and Newport) accounted for 50% of clinical isolates from human and 44% of clinical animals in 2001, and among them, *S.* Typhimurium and S. Newport have emerged as major multi-drug resistant pathogens [4]. An increase in the incidence of food-borne infections caused by *S.* Enteritidis, and human and animal infections by multi-drug resistant strains of *S.* Typhimurium has been also observed in European countries [3, 6, 31]. Among the multi-drug resistant *Salmonella*, *S.* Typhimurium definitive type DT104 has emerged as a global health problem in human and animal medicine during the last decade because of its resistance to up to nine antimicrobial agents commonly used [8, 13, 26, 27]. Van *et al.* [32] reported that 20.9% of 91 *Salmonella* isolates from retail raw food samples showed multi-drug resistance. In the Mekong Delta, many of the above serotypes have also been isolated, including *S.* Typhimurium, but no resistant strain of this serotype was detected in this study. On the other hand, *S.* Blockley, *S.* Hadar, and *S.* Emek showed a higher rate of multi-drug resistance. Those multi-drug resistant isolates were mainly originated from chicken, chicken meat and duck meat. It is not clear why mainly chicken related isolates showed a tendency to multi-drug resistance, but it might be associated with recent introduction of some commercial chicken farms using feed sold by the United States or European feed companies, in which antimicrobial agents are including in the feed.

This is the first report of quinolone resistance in nontyphoidal *Salmonella* isolated in the Mekong Delta in Vietnam, although Van *et al.* reported that *Salmonella* showing the resistance against enrofloxacin were isolated from retail raw food samples in Ho Chi Minh city in 2004 [32]. In addition, all the NA resistant isolates in the present study showed reduced susceptibility to CPFX. Hakanen *et al.* [11] suggested much lower MIC breakpoints values (MIC 0.25 μg/m*l*) for the fluoroquinolones than those recommended by the NCCLS (MIC 4 μg/m*l*) because of clinical importance of low-level CPFX resistance. If we adopt that breakpoint for the *Salmonella* isolates from the Mekong Delta in Vietnam, 4.8% (11 per 230) of the isolates should be considered resistant to ciprofloxacin. It is generally said that resistance to NA is a first-step of resistance to fluoroquinolones. Moreover, fluoroquinolones have been considered to be efficient antimicrobial agents against *Salmonella* infections and have been widely used. Therefore, as the emergence of quinolone resistance in *Salmonella* can be a serious public health problem, introduction of fluoroquinolones as food additives in food-producing animals, which would be a cause of inducing fluoroquinolone resistance to *Salmonella*,

is a cause for particular concern [10, 17, 35, 37, 38]. The results of this study, where quinolone resistant strains were detected in *Salmonella* isolates from animals and foods in Vietnam, suggested that fluoroquinolones are used in animal production as food additives or for treatment. Indeed, fluoroquinolones, such as norfloxacin and enrofloxacin, are sold at retail pharmacy for treatment of animal salmonelosis, but the detailed relationship among use of those antimicrobial agents and acquisition of quinolone resistance in the Mekong Delta is unclear due to the absence of reliable data on antimicrobial agents supplied to animals. However, the development of quinolone resistance, like in other countries, should also be considered.

Prudent use of antimicrobials in animal production system has been agreed worldwide to prevent development of antimicrobial resistance in pathogenic bacteria [5]. However, self-medication through retail pharmacies, which is a common practice in developing countries, is recognized as one of causes of inducing antimicrobial resistance in pathogenic bacteria. Similar to other developing countries, in Vietnam, antimicrobial agents can be bought easily in pharmacies without a prescription [5, 7]. Larsson *et al.* [16] also reported that ABPC, penicillin, amoxicillin, erythromycin, TC, and SM, respectively, are the most commonly used antimicrobial agents for treatment to acute respiratory tract infection in children in Bavi, Vietnam, and among the pathogens isolated from children, 88% of *Streptococcus pneumoniae* isolates and 32% of *Haemophilus influenzae* were resistant to TC, and 18% of *H. influenzae* and 19% of *Moraxella catarrhal* were resistant to ABPC. Considering that many antimicrobial agents for human and animals can be bought without control and therefore used inappropriately, increase of the antimicrobial resistance among *Salmonella* in Vietnam in future should be considered. Therefore, further investigation of *Salmonella* isolates from more extensive sources and continuous monitoring of antimicrobial resistance in Vietnam must be of great concern.

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