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BRIEF REPORT

Lack of vertical transmission of Hantaan virus from persistently infected dam to progeny in laboratory mice

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Abstract It is unclear how the hantaviruses are transferred from infected to uninfected rodents. We studied the status of persistently infected laboratory mice and examined the frequency of viral transmission to their offspring. Expression of Hantaan virus nucleocapsid protein was detected in the lungs of persistently infected dams. None of the progeny displayed viral antigen, although they were strongly positive for IgG antibodies against hantavirus. There was neither hantavirus RNA nor virus-specific IgM antibodies or virus-specific $CD8^+$ T cells in the progeny. These results did not show any indication for a vertical transmission of hantaviruses, at least in the laboratory mouse model studied.

Hantaviruses comprise the genus *Hantavirus* in the family *Bunyaviridae*. Although hantaviruses cause two serious and often fatal human diseases, viz. hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS), their natural rodent hosts present no obvious clinical signs of infection. Instead, they carry the virus for long periods as reservoir animals and shed the virus into excreta such as urine, feces, and saliva [16]. An age-dependent increase in the infection rate was recently identified by epizootiological surveillance in a rodent colony [4, 6], suggesting that these viruses are maintained by horizontal transmission through close contact between adult rodents and not by vertical transmission to neonates from dams.

Several studies have demonstrated the protective effect of hantavirus-specific antibodies in rat fetuses from or neonates born to immune mothers. Neonates that received a lethal dose of Seoul virus (SEOV) strains B-1 or SR-11 intraperitoneally within 24 h or at 2 days after birth to immune dams survived [10, 23]. These results imply that the neonates were protected against infection by transfer of maternal anti-hantavirus antibodies. However, these reports did not address whether the mother rats were persistently infected during pregnancy and the nursing period. In addition, given the difficulty of analyzing young animals in nature, the existence of vertical transmission from persistently infected animals to their offspring remains unclear.

There are several animal models of persistent infection involving hantaviruses and their natural reservoir species of rodents, including SEOV-infected rats [7, 19], Hantaan virus (HTNV)-infected *Apodemus* mice [15], Black Creek Canal virus-infected cotton rats [12], and Sin Nombre virus-infected deer mice [5]. These model rodents harbor virus antigen for a long time without signs of disease, as wild rodents do. However, since there is little genetic information on natural rodent species, it has often been difficult to analyze the mechanism of persistent infection genetically. Previously, we established a persistent infection model in laboratory mice. Viral antigen was detected in the lungs of the animals until 90 days after infection without signs of disease [2], and the retention time of the viral antigen was dependent on their age at inoculation [3]. A weak antigen-specific cytotoxic T lymphocyte (CTL) response was detected in these laboratory mice following infection [2, 20]. Although laboratory mice are not the natural reservoir, the persistently infected mice mimic the natural reservoir hosts in important aspects: (1) they have

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large amounts of virus antigen in their lungs [2], (2) they do not show obvious signs of disease in the persistent infection phase [20], and (3) the virus is not eliminated, although persistently infected mice strongly express neutralizing antibodies (Table 1). Furthermore, an abundance of background information is available on experimental laboratory mice. Therefore, the experimental mouse model is useful for detailed analyses, particularly immunological characterization. In this study, we followed the status of persistently infected mice and analyzed their offspring for potential vertical transmission of HTNV infection.

Pregnant BALB/c/Slc mice and Slc:ICR mice were obtained from SLC (Hamamatsu, Japan). All animal experiments in this study were carried out under the guidance of the Hokkaido University Animal Research Committee and in accordance with their guidelines, performed in a BSL3 facility. For the production of persistently infected mice, BALB/c mice were subcutaneously inoculated with 1.3 focus-forming units (FFU) of HTNV strain 76-118 within 24 h of birth [1.3 FFU = 0.1 $NMLD_{50}$ (50% newborn mouse lethal dose)]. A total of 17 mice were examined at 1–12 weeks after infection. The antibody response in these animals was evaluated, as shown in Table 1. High HTNV-specific IgG antibody titers (titers $\geq$ 5,120) were observed at 4, 8, and 12 weeks after infection. The IgG antibodies were detected even at 23 weeks after infection (data not shown). Similarly, IgM antibodies (titer: 80–320) were continuously observed at least until 12 weeks post-infection. Neutralizing antibodies (titer: 320–640) were also observed in all animals at all investigated time points. In addition, virus was isolated from the brains or lungs of the mice until 12 weeks post-infection, although the isolation rate decreased slightly. Viral antigen in the lung started to disappear gradually from 8 to 15 weeks post-infection and completely disappeared 20 weeks post-infection (data not shown). The disappearance of viral antigen seemed to be correlated with a reduced viral load that is reflected in an increasing number of negatives in the bioassay used (Table 1). In contrast, adult mice that were intraperitoneally inoculated with 10^5 FFU of HTNV for the production of transiently infected mice showed HTNV-specific IgM antibodies only 1 week after infection (titer: 160–320, $n = 6$ mice), and the titer quickly dropped within 3 weeks after infection (titer: <40, $n = 6$). The titers of HTNV-specific IgG antibodies increased until 3 weeks after infection in these transiently infected mice (Table 1) and, usually, neutralizing antibodies also increased until 3 weeks after infection [2, 21].

Recently, we reported that in the persistent infection model, mice had a reduced number of virus-specific CTLs in their spleen, so that viral elimination was delayed [20]. It has been reported that CTLs are regulated by other immune cells, for example dendritic cells, or inflammatory

Table 1 Analysis of mice immunized with a sublethal dose of HTNV by IFA and FRNT, and virus isolation

Weeks after infection	No.	IFA titers[a]		FRNT titers[b]	Bioassay[c]	
		IgG	IgM		Brain	Lung
New born 1 week	1	<40	80	NT	NT	NT
	2	<40	160	NT	NT	NT
New born 4 weeks	1	10,240	160	640	+	+
	2	5,120	160	640	+	+
	3	5,120	80	320	−	+
	4	5,120	160	640	+	+
	5	5,120	160	320	−	−
	6	5,120	160	640	−	+
New born 8 weeks	1	10,240	160	320	+	+
	2	10,240	160	320	−	+
	3	5,120	320	640	−	−
	4	10,240	320	640	−	+
	5	20,480	320	320	+	+
New born 12 weeks	1	10,240	160	640	−	−
	2	10,240	320	320	−	−
	3	10,240	160	640	+	+
	4	10,240	160	320	−	+
Adult 1 week	1	80	320			
	2	80	160			
	3	80	160			
	4	160	320			
	5	80	160			
	6	80	160			
Adult 3 weeks	1	640	<40			
	2	640	<40			
	3	5,120	<40			
	4	640	<40			
	5	640	<40			
	6	320	<40			

Seventeen newborn mice (within 24 h after birth) and twelve 5-week-old mice were inoculated with HTNV. They were sacrificed at various time points

NT not tested

[a] Indirect immunofluorescent antibody (IFA) tests were performed using acetone-fixed smears of Vero E6 cells infected with HTNV 76-118 as antigen. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG [IgA + IgG + IgM(H + L)] or anti-mouse IgM (Zymed Laboratories, San Francisco, CA, USA) was used as a secondary antibody. The IFA titers are expressed as the reciprocal of the highest dilution of antiserum that resulted in specific fluorescence

[b] FRNT was performed as described previously [1]. The neutralizing antibody titer was defined as the highest serum dilution that resulted in greater than 80% reduction in the number of infected cell foci

[c] The brains and lungs were removed from HTNV-infected mice. Groups of two or three newborn ICR mice were inoculated subcutaneously with 50 µl of the homogenates. The presence of the virus was assessed by seroconversion of the mice at 4 weeks after inoculation

+, all newborn ICR mouse sera were positive for HTNV. −, all newborn ICR mouse sera were negative for HTNV

cytokines after infection [11]. We suspected such a reduction in CTLs in persistent hantavirus infection in mice was also involved in these immune cells. Therefore, we have examined the infectivity to such as immune cells in spleen by histological study. Mice were anesthetized and perfused intracardially with 4% paraformaldehyde (PFA) in phosphate buffer (PB, pH 7.4), followed by brief perfusions with sucrose [13]. The spleens and lungs were stained with the Alexa Fluor 488-labeled monoclonal antibody (MAb) E5/G6 [18]. Nuclear staining was achieved using TOTO3 (Molecular Probes). Images were obtained by confocal microscopy (FLUOVIEW FV1000; Olympus, Japan). We detected N antigen in lung and spleen tissue at 2 weeks after infection in this persistent infection model. We detected high levels of N antigen in the intra-alveolar septum region (Fig. 1a), alveolar macrophages (Fig. 1b), and the elastic fiber region (Fig. 1c) of the lungs at 2 weeks after infection. Even though most of the antigen had disappeared at 8 weeks after infection, antigen remained in the intra-alveolar septum region of the lungs (data not shown). We detected N-antigen-positive cells in the marginal zone of each spleen (Fig. 1d). Generally, immune cells are distributed in the spleen marginal zone after pathogen invasion. Therefore, we have to identify N-positive cells to know whether these cells were involved in the regulation of CTLs in persistently infected mice.

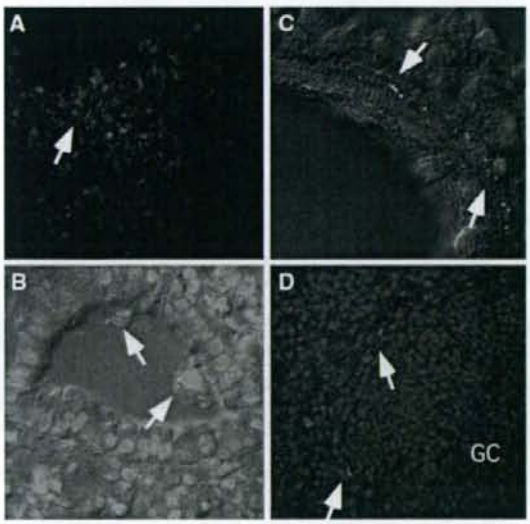

Fig. 1 Immunohistochemical analysis of persistently infected mice. Persistently infected mice were sacrificed 2 weeks after infection. The lungs and spleen were removed, fixed, sectioned, and stained with E5G6 antibody (*green*) and TOTO3 (*red*). **a** N-positive cells are observed in the intra-alveolar septum region of the lung. **b** Infected alveolar macrophages are observed in the lung. **c** Clustered N antigen is detected in the elastic fibers of the bronchiole. **d** N-positive cells are shown localized in the marginal zone of the spleen. Magnification: **a** ×20, **b** ×60, **c** ×180, **d** ×60

Finally, to examine the possibility of vertical transmission from persistently infected mice to their progeny, three pairs of these mice were mated, the offspring and dams were sacrificed at various time points (Fig. 2a), and tissues were examined by Western blotting and RT-PCR. We detected hantavirus N antigen and RNA in the dams' lungs (12 weeks old, $n = 2$); however, no antigen or RNA was detected in their progeny (10 days old; Fig. 2b, c, 4 weeks old; data not shown, $n = 6$). Although a high level of IgG antibody [immunofluorescent antibody (IFA) titer: 5,120] was observed until 28 days after birth and the mice maintained low IgG antibody levels until 90 days (data not shown), we could not observe IgM antibody in the infants (10 days old, 4 weeks old) even though their dams (12 weeks old, $n = 2$) had IgM antibody (Fig. 2d, data not shown). This indicates that the HTNV-specific IgG antibodies were maternally derived. To confirm a lack of infection in the infants derived from and nursed by the persistently infected dams, we used FACS analysis to prove the presence of HTNV-specific $CD8^+$ T cells. We used flow cytometry to assay the cytokines produced by $CD8^+$ T cells incubated with HTNV-infected antigen-presenting cells [20]. Antigen-negative 3-week-old (progeny 1,2) and 20-week-old (progeny 3,4) offspring from a 17-week-old HTNV-antigen-positive persistently infected dam were analyzed. No HTNV-specific IFN-γ^+ $CD8^+$ T cells were found in any of the progeny (Fig. 2e), suggesting that none of the offspring had ever been infected with HTNV. These data are in agreement with those from previous rodent studies [8, 14, 17].

The transfer of maternal antibodies from mother to progeny is well known in avian and mammalian species and is believed to protect the newborn against pathogens in the environment. Several reports have described this phenomenon for hantavirus infection [8, 14, 23]; however, there is a scarcity of studies examining the vertical transmission of hantaviruses in wild-living rodents that are persistently infected [15, 17]. To examine vertical transmission from persistently infected dams to their progeny, we studied the progeny of persistently HTNV-infected mice by Western blotting, RT-PCR, FACS analysis, and IFA. The progeny had no HTNV-antigen in their lungs, no virus-specific CTLs, and no anti-HTNV IgM antibodies, although they maintained anti-HTNV IgG antibodies until 90 days after birth. These results indicate that the progeny of persistently infected laboratory mice are immune to HTNV and that vertical transmission of HTNV from persistently infected dams to their offspring does not occur. Thus, these results suggest that the virus is transmitted horizontally in nature, from rodent to rodent, and several reports have suggested that a high dose is required for

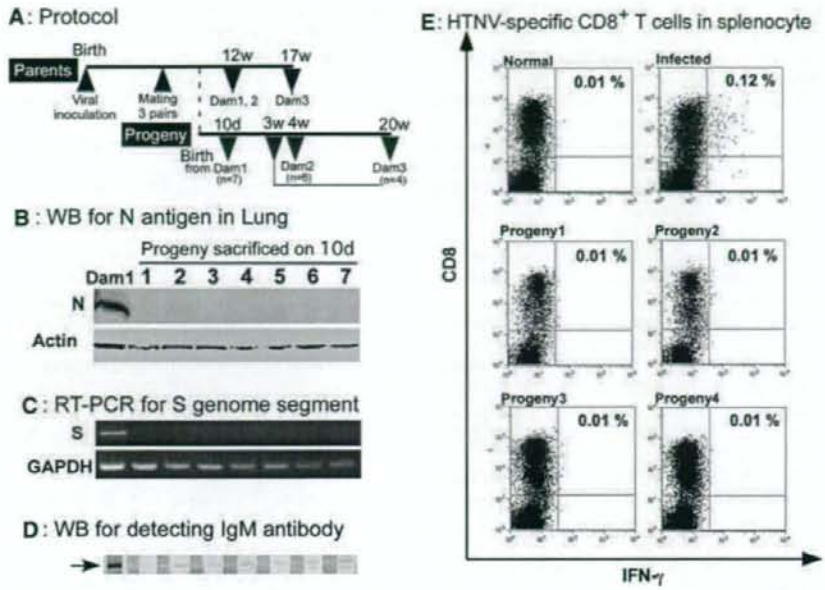

Fig. 2 Analysis of progeny from persistently infected mice. Dam1 possessed both N antigen and viral genomic RNA in lung, and its seven progenies lacked both viral antigen and RNA in lung. The same results were obtained from *dam2* and *dam3* and their six or four progenies (data not shown). **a** Experimental protocol. Three pairs of BALB/c parents were mated and analyzed at the time point indicated in *lane 1*; their newborns were analyzed at the time point indicated in *lane 2*. **b** Western blot (*WB*) analysis of lung lysates from persistently HNTV-infected mice and their newborn offspring [3]. The MAbs E5/G6 and ECO2 were used to detect the membrane-bound antigens [22]. Horseradish peroxidase (*HRP*)-conjugated goat anti-mouse IgG (Zymed Laboratories) was used as a secondary antibody. Actin, the control protein, was detected using mouse anti-β actin antibody (Sigma). **c** RT-PCR analysis of lungs from HNTV-infected mice and their newborn offspring to detect trace amounts of virus RNA. Lungs were homogenized and isolated by ISOGEN (NIPPON GENE). The isolated RNA was reverse transcribed using random hexamer and SuperScript II (Invitrogen). Each cDNA was amplified with hantavirus-specific primers MurS110F [5′CAGAAGGTIAIGGATG CAGA3′] and MurS1160R [5′TGGTCCAGTTGTATRCCCAT3′] or control GAPDH primers [5′TGCACCACCAACTGCTTAG3′, 5′GG ATGCAGGGATGATGTTC3′]. **d** Detection of IgM antibodies by WB. Recombinant HTNV N derived from high five cells was used [1]. Sera from the progeny and their persistently infected dam were used at a 1:10 dilution. Secondary antibody is HRP-anti-mouse IgM (Zymed Laboratories, San Francisco, CA, USA). **e** Immune responses of IFN-γ-producing HTNV-specific CD8$^+$ T cells in progeny (from *dam3*). Spleen cells were cultured with HTNV-infected P388D1 cells. The cells were stained with ethidium monoazide bromide (*EMA*) (Invitrogen), anti-CD8a PE (Ly-2) antibodies (eBioscience, San Diego, CA, USA), and FITC-conjugated rat anti-mouse gamma interferon (IFN-γ) antibody (Caltag Laboratories, San Francisco, CA). IFN-γ^+ cells were analyzed by flow cytometry using a FACS Calibur (Becton Dickinson, Franklin Lakes, NJ, USA) with the gates set for EMA-negative cells. The data were analyzed using FlowJo software (Tree Star, San Carlos, CA, USA). "*Infected*" indicates that the adult BALB/c mouse inoculated with HTNV recovered (positive control). "*Normal*" denotes a normal BALB/c mouse (negative control)

horizontal transmission between rodents in experimentally persistently infected animals [9, 15]. Efficient transmission may depend on the immune status influenced by infections with other pathogens of a different nature. Here, we found that vertical transmission from persistently infected laboratory mice does not occur. To prove the absence of vertical transmission of hantaviruses, further studies should be dedicated to the influence of the load and tissue distribution of hantavirus in persistently infected dams of laboratory mice and natural rodent hosts.

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Molecular phylogeny of a newfound hantavirus in the Japanese shrew mole (*Urotrichus talpoides*)

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Recent molecular evidence of genetically distinct hantaviruses in shrews, captured in widely separated geographical regions, corroborates decades-old reports of hantavirus antigens in shrew tissues. Apart from challenging the conventional view that rodents are the principal reservoir hosts, the recently identified soricid-borne hantaviruses raise the possibility that other soricomorphs, notably talpids, similarly harbor hantaviruses. In analyzing RNA extracts from lung tissues of the Japanese shrew mole (*Urotrichus talpoides*), captured in Japan between February and April 2008, a hantavirus genome, designated Asama virus (ASAV), was detected by RT-PCR. Pairwise alignment and comparison of the S-, M-, and L-segment nucleotide and amino acid sequences indicated that ASAV was genetically more similar to hantaviruses harbored by shrews than by rodents. However, the predicted secondary structure of the ASAV nucleocapsid protein was similar to that of rodent- and shrew-borne hantaviruses, exhibiting the same coiled-coil helix at the amino terminus. Phylogenetic analyses, using the maximum-likelihood method and other algorithms, consistently placed ASAV with recently identified soricine shrew-borne hantaviruses, suggesting a possible host-switching event in the distant past. The discovery of a mole-borne hantavirus enlarges our concepts about the complex evolutionary history of hantaviruses.

host switching | talpid | evolution | Japan

Dating from investigations conducted independently by Japanese and Russian medical scientists along opposite sides of the Amur River in the 1930s and 1940s, rodents have been suspected to harbor the etiological agent(s) of hemorrhagic fever with renal syndrome (HFRS) (1, 2). After a several decades-long impasse, the striped field mouse (*Apodemus agrarius*) was identified as the reservoir host of Hantaan virus (3), the prototype virus of HFRS (4). This seminal discovery made possible the identification of genetically distinct hantaviruses in other murinae and arvicolinae rodent species (5–12). Also, a previously unrecognized, frequently fatal respiratory disease, called hantavirus pulmonary syndrome (HPS) (13), is now known to be caused by hantaviruses harbored by neotominae and sigmodontinae rodents in the Americas, the prototype being Sin Nombre virus (SNV) in the deer mouse (*Peromyscus maniculatus*) (14). Remarkably, each of these hantaviruses appears to share a long coevolutionary history with a specific rodent host species. That is, based on phylogenetic analyses of full-length viral genomic and rodent mitochondrial DNA (mtDNA) sequences, these hantaviruses segregate into clades, which parallel the evolution of rodents in the murinae, arvicolinae, neotominae, and sigmodontinae subfamilies (15, 16).

Until recently, the single exception to the strict rodent association of hantaviruses was Thottapalayam virus (TPMV), a long-unclassified virus originally isolated from the Asian house shrew (*Suncus murinus*) (17, 18). Analysis of the recently acquired full genome of TPMV strongly supports an ancient non-rodent host origin and an early evolutionary divergence from rodent-borne hantaviruses (19, 20). Employing RT-PCR and oligonucleotide primers based on the TPMV genome, we have targeted the discovery of hantaviruses in shrew species from widely separated geographical regions, including the Chinese mole shrew (*Anourosorex squamipes*) from Vietnam (21), Eurasian common shrew (*Sorex araneus*) from Switzerland (22), northern short-tailed shrew (*Blarina brevicauda*), masked shrew (*Sorex cinereus*), and dusky shrew (*Sorex monticolus*) from the United States (23, 24) and Ussuri white-toothed shrew (*Crocidura lasiura*) from Korea (J.-W. Song and R. Yanagihara, unpublished observations). Many more shrew-hantavirus associations undoubtedly exist, as evidenced by preliminary studies of *Sorex caecutiens* and *Sorex roboratus* from Russia (H. J. Kang, S. Arai and R. Yanagihara, unpublished observations) and *Sorex palustris*, *Sorex trowbridgii*, and *Sorex vagrans* from North America (H. J. Kang and R. Yanagihara, unpublished observations).

In addition to challenging the view that rodents are the sole or principal reservoirs of hantaviruses, the discovery of soricid-borne hantaviruses predicts that other soricomorphs, notably talpids, might also harbor genetically distinct hantaviruses. In this regard, hantavirus antigens have been detected by enzyme immunoassay and fluorescence techniques in tissues of the European common mole (*Talpa europea*) captured in Russia (25) and Belgium (26), but no reports are available about hantavirus infection in shrew moles. Relying on oligonucleotide primers designed from our expanding sequence database of shrew-borne hantaviruses, we have identified a hantavirus genome, designated Asama virus (ASAV), in the Japanese shrew mole (*Urotrichus talpoides*). Genetic and phylogenetic analyses indicate that ASAV is distinct but related to hantaviruses harbored by Old World soricine shrews, suggesting a very ancient evolutionary history, probably involving multiple host-switching events in the distant past.

Results

RT-PCR Detection of Hantavirus Sequences. In using RT-PCR to analyze RNA extracts, from lung tissues of three Laxmann's shrew (*Sorex caecutiens*), five slender shrew (*Sorex gracillimus*), six long-clawed shrew (*Sorex unguiculatus*), one dsinezumi shrew (*Crocidura dsinezumi*), and six Japanese shrew mole (*Urotrichus talpoides*), hantavirus sequences were not detected in shrew tissues, but were found in one of two and in two of three Japanese shrew moles (Fig. 1), captured in Ohtani (34 28′14.0° N; 136 45′46.2° E) and near

Author contributions: S.A. and R.Y. designed research; S.A., S.D.O., M.A., J.A., N.O., and R.Y. performed research; S.A. and H.J.K. contributed new reagents/analytic tools; S.A., S.D.O., H.J.K., G.M., and R.Y. analyzed data; and S.A., G.M., J.A., and R.Y. wrote the paper.

The authors declare no conflict of interest.

Freely available online through the PNAS open access option.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession numbers: ASAV S segment (EU929070, EU929071, EU929072); ASAV M segment (EU929073, EU929074, EU929075); and ASAV L segment (EU929076, EU929077, EU929078].

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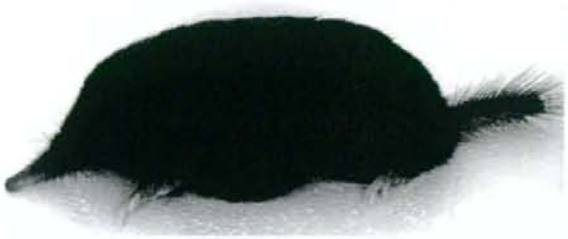

Fig. 1. Japanese shrew mole (*Urotrichus talpoides*) (family *Talpidae*, subfamily Talpinae), one of two endemic shrew mole species found only in Japan.

Asama River (34 28′12.79° N; 136 45′45.81° E), respectively, located approximately 1 km apart at an elevation of 50 m in Mie Prefecture, during February and April 2008. After the initial detection of hantavirus sequences, amplification of the S-, M-, and L-genomic segments was accomplished by using oligonucleotide primers based on conserved regions.

Nucleotide and Amino Acid Sequence Analysis. The S, M, and L segments of ASAV, as amplified from tissues of three wild-caught Japanese shrew moles, indicated an overall genomic structure similar to that of other rodent- and soricid-borne hantaviruses. The nucleotide and deduced-amino acid sequences of each ASAV genomic segment were highly divergent from that of rodent-borne hantaviruses, differing by approximately 30–40% (Table 1).

The S-genomic segment of ASAV (1,801 nucleotides for strains H4 and N9 and 1,756-nucleotides for strain N10) encoded a predicted nucleocapsid (N) protein of 434 amino acids, starting at nucleotide position 39, and a 3′-noncoding region (NCR) of approximately 465 nucleotides. The hypothetical NSs opening reading frame, typically found in the S segment of arvicolinae, neotominae, and sigmodontinae rodent-borne hantaviruses, was not found in ASAV. The interstrain variation of the S segment among the ASAV strains was negligible (1.1% at the nucleotide and 0% at the amino acid levels). In the hypervariable region of the N protein, between amino acid residues 244 and 269, ASAV differed by 18–20 and 20–22 amino acid from soricine shrew- and rodent-borne hantaviruses, respectively. Sequence similarity of the entire S-genomic segment of ASAV strains H4, N9, and N10 was higher with soricine shrew-borne hantaviruses than with hantaviruses harbored by rodents (Table 1).

Table 1. Nucleotide and amino acid sequence similarity (%) between ASAV strain N10 and representative rodent- and shrew-borne hantaviruses

	S segment		M segment		L segment	
Virus strain	1710 nt	434 aa	3604 nt	1141 aa	6126 nt	2041aa
HTNV 76–118	58.5	62.7	62.7	59.4	70.3	74.6
SEOV 80–39	63.1	62.0	62.8	59.1	70.4	74.7
SOOV SOO-1	62.8	62.9	63.3	59.7	70.2	74.3
DOBV Greece	62.2	62.2	63.0	59.3	70.2	75.7
PUUV Sotkamo	59.3	59.3	59.6	52.2	68.1	68.0
TULV 5302v	61.5	59.4	60.5	52.6	68.3	67.9
PHV PH-1	60.7	59.3	59.3	51.9	66.4	67.1
SNV NMH10	60.9	58.9	59.0	54.1	68.2	68.8
RPLV MSB89866	—	—	68.8	63.5	75.2	83.2
CBNV CBN-3	67.7	70.4	68.2	71.0	76.0	84.7
ARRV MSB73418	65.7	66.6	70.9	77.0	73.8	83.5
JMSV MSB89332	66.2	66.9	—	—	74.3	82.6
SWSV mp70	63.8	69.9	75.2	79.5	75.0	83.2
ASAV H4	98.9	100	99.3	99.6	98.2	99.6
ASAV N9	100	100	99.9	100	100	100
MJNV 05–11	57.2	46.0	56.1	44.4	65.8	61.5
TPMV VRC	58.0	45.8	57.7	43.0	64.3	62.0

Abbreviations: ARRV, Ash River virus; ASAV, Asama virus; CBNV, Cao Bang virus; DOBV, Dobrava virus; HTNV, Hantaan virus; JMSV, Jemez Spring virus; MJNV, Imjin virus; PHV, Prospect Hill virus; PUUV, Puumala virus; RPLV, Camp Ripley virus; SEOV, Seoul virus; SNV, Sin Nombre virus; SOOV, Soochong virus; SWSV, Seewis virus; TPMV, Thottapalayam virus; TULV, Tula virus. nt, nucleotides; aa, amino acids.

The 3,646-nucleotide full-length M-genomic segment of ASAV encoded a predicted glycoprotein of 1,141 amino acids, starting at nucleotide position 41, and a 183-nucleotide 3′-NCR. Four potential N-linked glycosylation sites (three in Gn at amino acid positions 138, 352, 404, and one in Gc at position 933) were found in ASAV. In addition, the highly conserved WAASA amino acid motif, which in ASAV was WAVSA (amino acid positions 649–653), was present. An interstrain variation of 0.1–0.7% and 0–0.4% at the nucleotide and amino acid levels, respectively, was found among ASAV strains H4, N9, and N10. The full-length Gn/Gc amino acid sequence of ASAV exhibited the highest similarity with Seewis virus (79.5%) from the Eurasian common shrew (Table 1).

Analysis of the nearly full-length 6,126-nucleotide (2,041-amino acid) L segment of ASAV revealed the five conserved motifs (A–E), identified among all hantavirus RNA polymerases. The overall high sequence similarity of the L segment among ASAV and rodent- and soricid-borne hantaviruses was consistent with the functional constraints on the RNA-dependent RNA polymerase (Table 1).

Secondary Structure of N Protein. Secondary structure analysis revealed striking similarities, as well as marked differences, among the N protein sequences of ASAV and 13 representative rodent- and soricid-hantaviruses. Each sequence appeared to adopt a two-domain, predominantly α-helical structure joined by a central β-pleated sheet. Whereas the length of the N-terminal domain was mostly invariant, the length of the central β-pleated sheet and of the adjoining C-terminal α-helical domain showed systematic reciprocal structural changes according to the genetic relationship and evolutionary descent of the individual sequences.

The N-terminal α-helical domain, from residues 1 to approximately 140, was composed of four helices connected by large loops (representative viruses shown in Fig. 2). The C-terminal α-helical domain, from residues 210/230 to 430, contained seven to nine helices that were connected by tighter loops (Fig. 2). And the central β-pleated region, from residues 140 to 210/230, was composed of three to five possible anti-parallel strands. Interestingly, an increasing number of strands in this section were observed when the hantaviral sequences were arranged according to their positions in the phylogenetic tree. This resulted in a widening of the central β-pleated region with a concomitant shortening of the C-terminal α-helical domain while preserving the total length of the protein. The helix adjoining the central β-sheet progressively shortened in this architectural change. These structural alterations were reversed in TPMV, which was evolutionarily more distant from the other sequences (Fig. 2).

Phylogenetic Analysis. Exhaustive phylogenetic analyses based on nucleotide and deduced amino acid sequences of the S-, M-, and L-genomic segments, generated by the maximum-likelihood (ML) method, indicated that ASAV was distinct from rodent-borne hantaviruses (with high posterior node probabilities based on 30,000 trees) (Fig. 3). Nearly identical topologies were consistently derived, by using various algorithms and different taxa and combinations of taxa, suggesting an ancient evolutionary origin. The most strikingly consistent feature was the phylogenetic position of ASAV with soricine shrew-borne hantaviruses, rather than being placed as an outgroup beyond TPMV, the prototype crocidurine shrew-borne hantavirus. That is, the prediction that a shrew mole-associated hantavirus would be phylogenetically distant from hantaviruses harbored by shrews was not validated.

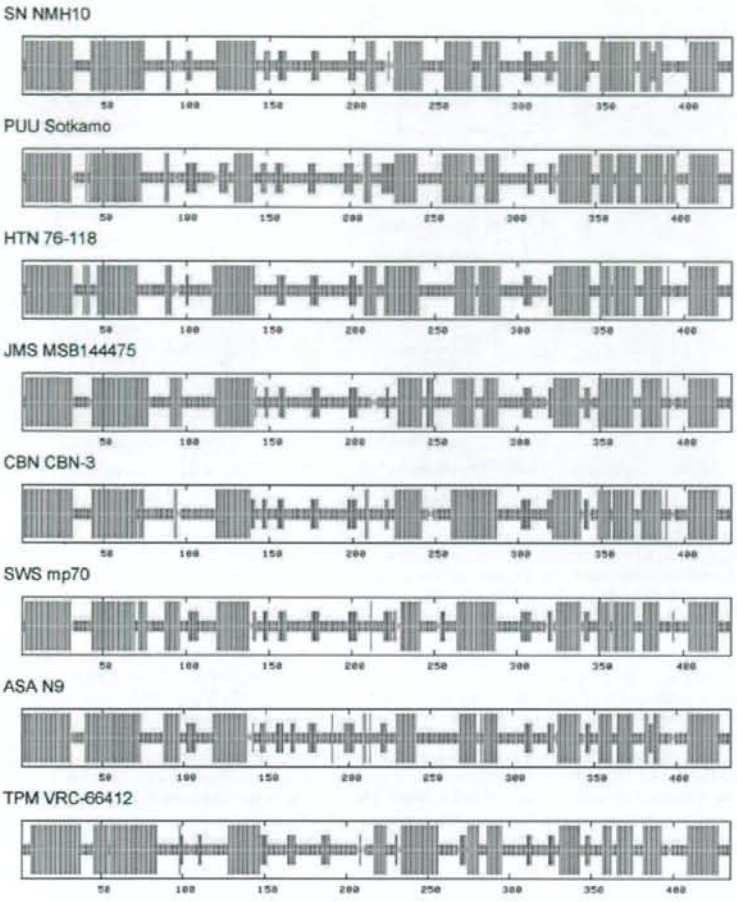

Fig. 2. Consensus secondary structure of N protein of ASAV and representative rodent- and soricid-borne hantaviruses, predicted using a high-performance method implemented on the NPS@ structure server (47). As shown, the ASAV N protein was very similar to that of other hantaviruses, characterized by 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

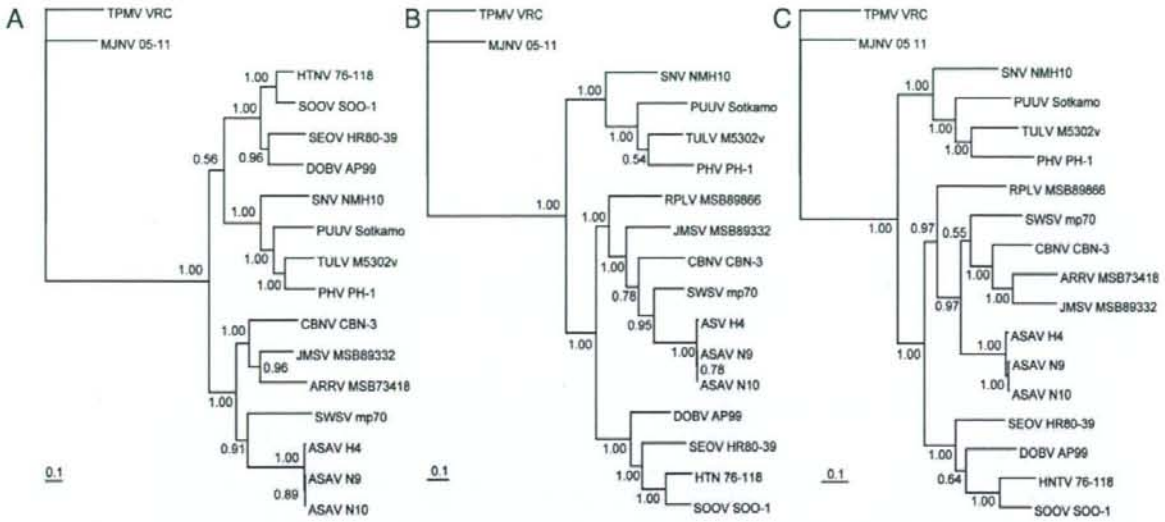

Fig. 3. Phylogenetic trees generated by the ML method, using the GTR+I+G model of evolution as estimated from the data, based on the alignment of the coding regions of the full-length (*A*) 1,302-nucleotide S and (*B*) 3,423-nucleotide M segments, and partial (*C*) 6,126-nucleotide L-genomic segment 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

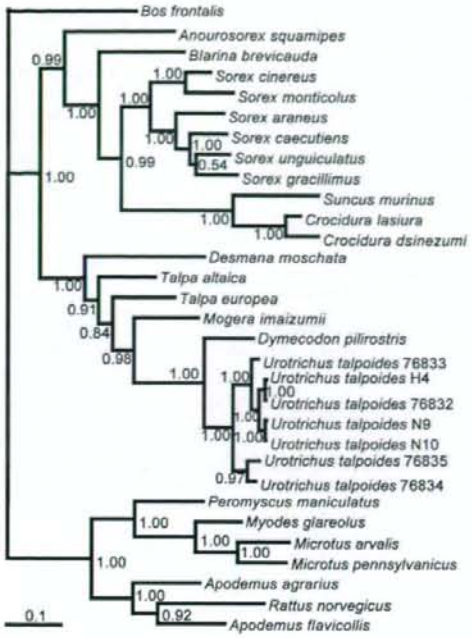

Fig. 4. Confirmation of host identification of ASAV-infected *Urotrichus talpoides* by mtDNA sequencing. Phylogenetic tree, based on the 1,140-nucleotide cytochrome *b* (cyt b) gene, was generated by the ML method. The phylogenetic positions of *Urotrichus talpoides* H4 (EU918369), N9 (EU918370), and 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.

ACKNOWLEDGMENTS. We thank Dr. Akio Shinohara (Frontier Science Research Center, University of Miyazaki) for kindly providing the photo of the Japanese shrew mole (Fig. 1). This work was supported by a Grant-in-Aid for Scientific Research (B) from the Ministry of Education, Science, and Culture of Japan (18300136), Gakujyutsu-Frontier Cooperative Research at Rakuno Gakuen University, and National Institute of Allergy and Infectious Diseases Grant R01AI075057, Centers of Biomedical Research Excellence Grant P20RR018727, and Research Centers in Minority Institutions Grant G12RR003061 from the National Center for Research Resources, National Institutes of Health.

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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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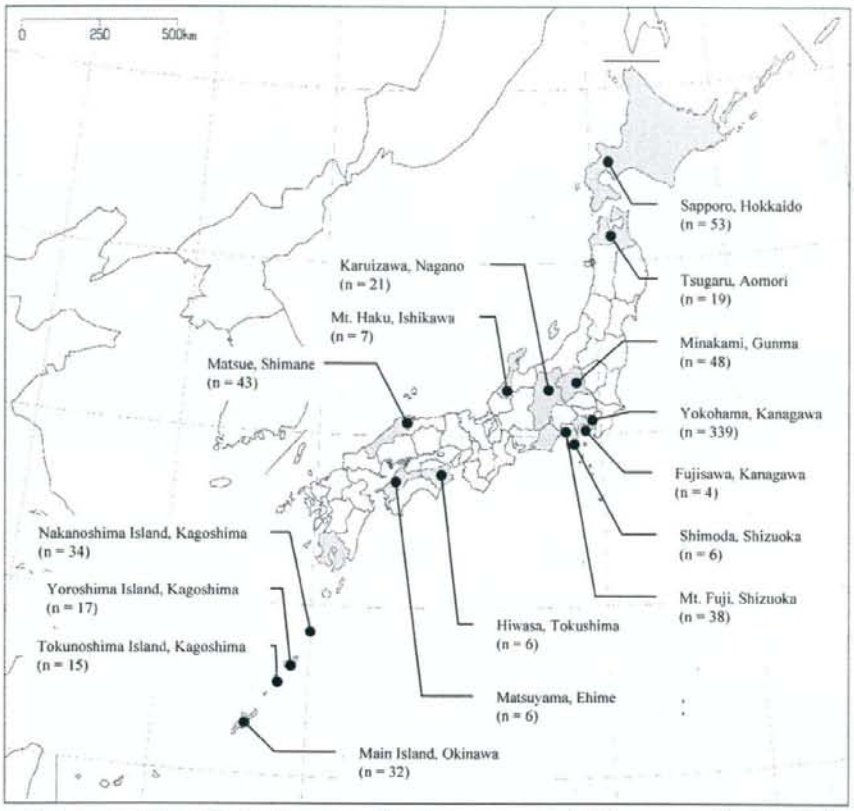

FIG. 1. Geographical representation of the locations where wild rodents were captured. Cities, areas, or islands and their prefectures (e.g., Yokohama [city], Kanagawa [Prefecture]) are shown around the map of Japan in the figure. The numbers of rodents captured are indicated in parentheses.

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′-CGCATTGGCTTACTTCGTATG-3′) and 2300R (5′-GTAGACTGATTAGAACGCTG-3′) (30), and the primers for *gltA* (379 bp) were BhCS.781p (5′-GGGGACCAGCTCATGGTGG-3′) and BhCS.1137n (5′-AATGCAAAAAGAACAGTAAACA-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'-CGYGGYRCCATRAAAACTTCWCC-3'], and 2000F [5'-GGWGAAGTTTTRATGGYRCCRCG-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

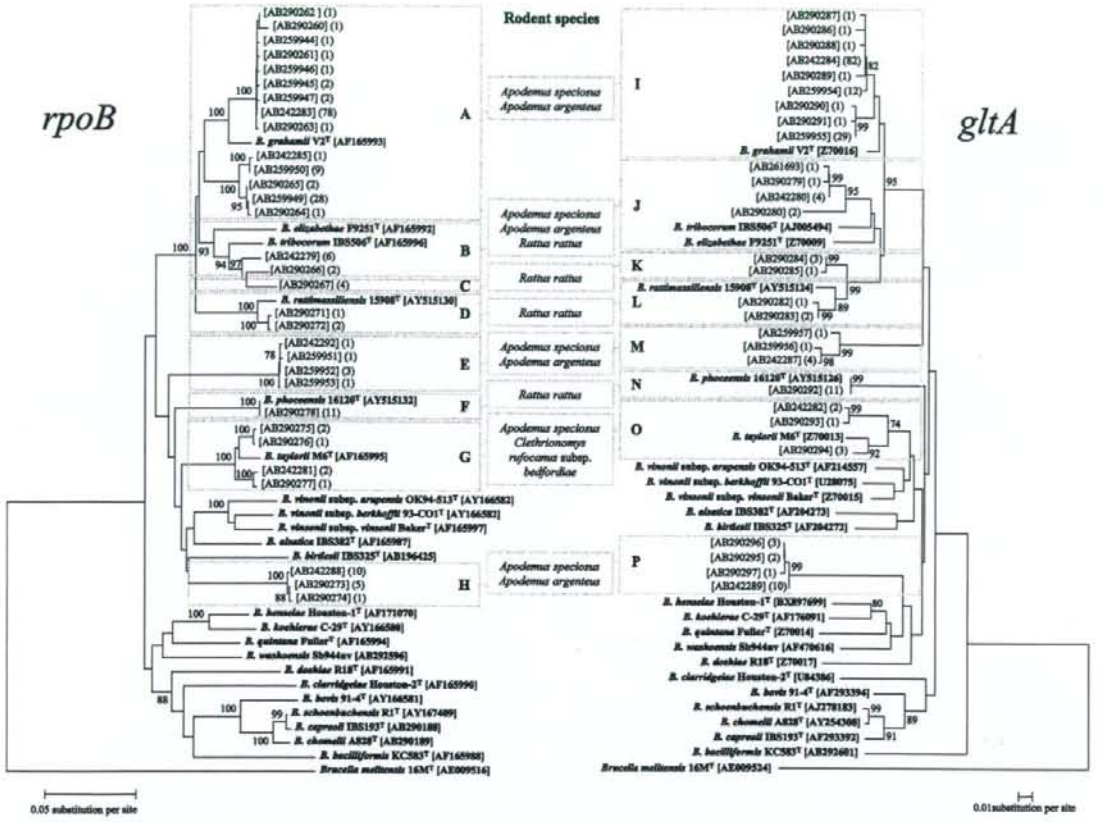

FIG. 2. Phylogenetic classification of Japanese *Bartonella* isolates based on sequences of *rpoB* (left) and *gltA* (right). The phylogenetic tree was constructed by the neighbor-joining method, and bootstrap values were obtained with 1,000 replicates. Only bootstrap replicates of >70% are noted. The 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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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