Characterization of Imjin Virus, a Newly Isolated Hantavirus from the Ussuri White-Toothed Shrew (*Crocidura lasiura*)[▽]

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Until recently, the single known exception to the rodent-hantavirus association was Thottapalayam virus (TPMV), a long-unclassified virus isolated from the Asian house shrew (*Suncus murinus*). Robust gene amplification techniques have now uncovered several genetically distinct hantaviruses from shrews in widely separated geographic regions. Here, we report the characterization of a newly identified hantavirus, designated Imjin virus (MJNV), isolated from the lung tissues of Ussuri white-toothed shrews of the species *Crocidura lasiura* (order Soricomorpha, family Soricidae, subfamily Crocidurinae) captured near the demilitarized zone in the Republic of Korea during 2004 and 2005. Seasonal trapping revealed the highest prevalence of MJNV infection during the autumn, with evidence of infected shrews' clustering in distinct foci. Also, marked male predominance among anti-MJNV immunoglobulin G antibody-positive Ussuri shrews was found, whereas the male-to-female ratio among seronegative Ussuri shrews was near 1. Plaque reduction neutralization tests showed no cross neutralization for MJNV and rodent-borne hantaviruses but one-way cross neutralization for MJNV and TPMV. The nucleotide and deduced amino acid sequences for the different MJNV genomic segments revealed nearly the same calculated distances from hantaviruses harbored by rodents in the subfamilies Murinae, Arvicolinae, Neotominae, and Sigmodontinae. Phylogenetic analyses of full-length S, M, and L segment sequences demonstrated that MJNV shared a common ancestry with TPMV and remained in a distinct out-group, suggesting early evolutionary divergence. Studies are in progress to determine if MJNV is pathogenic for humans.

Hantaviruses (family *Bunyaviridae*, genus *Hantavirus*) are medically important rodent-borne pathogens, causing hemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome (HCPS). The belief in long-standing coevolutionary relationships between hantaviruses and their reservoir rodent host species is supported by virus and rodent gene phylogenies. That is, phylogenetic analyses, based on full-length viral genomic sequences and rodent mitochondrial DNA (mtDNA) or nuclear gene sequences, indicate that antigenically distinct hantaviruses segregate into clades, which parallel the evolution of rodents in the subfamilies Murinae, Arvicolinae, Neotominae, and Sigmodontinae (23, 25, 26, 28, 39, 54). Previously, this phylogenetic insight has been successfully employed to direct the discovery of new hantaviruses, such as those found in the Korean field mouse (*Apodemus peninsulae*) (5) and the royal vole (*Myodes regulus*) (47).

Renewed interest in the role of nonrodent reservoirs in the evolution of hantaviruses has been spurred by recent analysis of the entire genome of Thottapalayam virus (TPMV), a hantavirus isolated from the Asian house shrew (*Suncus murinus*) (10, 61), which revealed a separate phylogenetic clade, suggest-

ing early evolutionary divergence from rodent-borne hantaviruses (44, 56). Armed with oligonucleotide primers designed on the basis of conserved regions of the TPMV genome and guided by long-ignored reports of serologic and antigenic evidence of hantavirus infection in shrews (20, 33, 52), we have previously detected genetically distinct hantaviruses in the Eurasian common shrew (*Sorex araneus*) from Switzerland (45); the Chinese mole shrew (*Anourosorex squamipes*) from Vietnam (46); and the northern short-tailed shrew (*Blarina brevicauda*), masked shrew (*Sorex cinereus*), and dusky shrew (*Sorex monticolus*) from the United States (1, 2) by reverse transcription-PCR (RT-PCR). Novel hantavirus genomes in Therese's shrew (*Crocidura theresae*) from Guinea (29); the vagrant shrew (*Sorex vagrans*), Trowbridge's shrew (*Sorex trowbridgii*), and the American water shrew (*Sorex palustris*) from the United States (H. J. Kang and R. Yanagihara, unpublished data); and the flat-skulled shrew (*Sorex roboratus*) and Laxmann's shrew (*Sorex caecutiens*) from Russia (Kang and Yanagihara, unpublished) have also been detected.

Here, we report the antigenic, genetic, and phylogenetic characterization of a newly identified hantavirus, designated Imjin virus (MJNV), isolated from Ussuri white-toothed shrews of the species *Crocidura lasiura* (order Soricomorpha, family Soricidae, subfamily Crocidurinae) captured near the demilitarized zone (DMZ) in the Republic of Korea. The discovery of MJNV and other soricid-borne hantaviruses from widely separated geographic regions, spanning four continents,

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FIG. 1. Map of Paju City, Yeoncheon County, and Pocheon City near the DMZ, showing the locations of the 13 trap sites on U.S. Army installations. MJNV RT-PCR-positive Ussuri shrews (red boxes) were trapped at six sites (designated DN, F1, L1, L3, MP, and SR).

challenges the conventional view that rodents are the principal and primordial reservoir hosts. Moreover, viewed within the emerging context that soricid-borne hantaviruses are far more genetically diverse than those harbored by rodents, this gateway investigation on a newfound shrew-borne hantavirus heralds a paradigm-shifting conceptual framework for the evolutionary history of hantaviruses.

MATERIALS AND METHODS

Trapping. *Crocodylus lasiura* shrews were captured near the DMZ along the Imjin River (38°N, 126°40' to 127°20'E) in the Republic of Korea during the winter, spring, summer, and autumn of 2004 and 2005 by using Sherman traps (8 by 9 by 23 cm; H. B. Sherman, Tallahassee, FL) baited with peanut butter placed between two saltine crackers. A total of 50 traps were set at intervals of approximately 4 to 5 m at each of six sites on the outskirts of Paju City, situated 20 km northeast of Seoul and directly south of the Imjin River, during the daylight hours of each day over a 4-day period. In addition, traps were set at six sites in Yeoncheon County and one site in Pocheon City, which lie north and east of Paju City, respectively (Fig. 1).

Specimen processing. All specimen-processing procedures were performed in the biosafety level 3 animal facility at Korea University. Shrews were sacrificed by cervical dislocation and exsanguinated by cardiac puncture. Serum was separated by centrifugation within 24 h of blood collection. Lung, liver, kidney, and spleen tissues were dissected using separate instruments and were stored at -80°C until the samples were used for virus isolation and RT-PCR and mtDNA analyses. Except for U.S. Army personnel, all staff engaged in the trapping of shrews and rodents and the processing of tissue samples had been vaccinated with a hantavirus vaccine (Hantavax) licensed by the Korean Food and Drug Administration (11) or possessed preexisting immunity to hantaviruses as a result of natural infection.

Virus isolation. Virus isolation in Vero E6 cells (CRL 1586; American Type Culture Collection) obtained from the lung tissues of wild-caught Ussuri shrews was attempted using previously described methods (58). Briefly, subconfluent monolayers of Vero E6 cells, grown in 25-cm² flasks, were inoculated with 5% suspensions of lung or spleen tissue homogenates from Ussuri shrews with RT-PCR evidence of hantavirus infection. Cells were subcultured at 10- to 14-day intervals, at which time an aliquot of cells was examined for hantaviral antigens by the indirect immunofluorescent-antibody (IFA) technique using sera from MJNV-infected Ussuri shrews. Supernatants from IFA antigen-positive cell cultures were then examined for hantavirus sequences by RT-PCR.

IFA test. With the isolation of MJNV, the seroprevalence of infection in Ussuri white-toothed shrews was assessed. Sera, diluted 1:16, were placed into duplicate wells of acetone-fixed Vero E6 cells infected with MJNV, and the wells were incubated for 30 min at 37°C (34). After the wells were washed three times with phosphate-buffered saline, fluorescein isothiocyanate-conjugated goat anti-body to rat and mouse immunoglobulin G (IgG) antibodies (ICN Pharmaceuti-

cal, Inc., Aurora, OH) was added and the wells were incubated at 37°C for 30 min and then washed three more times with phosphate-buffered saline. The detection of virus-specific fluorescence was considered to be evidence of MJNV infection.

Thin-section electron microscopy. MJNV-infected Vero E6 cells were collected 72 h postinfection, fixed with 2.5% glutaraldehyde (Electron Microscopy Sciences, PA) in Sorensen's Na-K phosphate buffer (29% 0.066 M KH₂PO₄, 71% 0.066 M Na₂HPO₄, pH 7.2; Electron Microscopy Sciences), and treated with 2% osmium tetroxide (Electron Microscopy Sciences) as a secondary fixative. Infected cells were then dehydrated in a series of ethanol washes (48). Thin sections were placed onto 400-mesh square copper electron microscopy grids (Electron Microscopy Sciences) and viewed with a transmission electron microscope (model H-7500; Hitachi, Japan).

Antigenic characterization of MJNV by PRNT. With the advent of gene amplification, the ease of DNA sequencing, and the difficulty of culturing hantaviruses, members of the *Hantavirus* genus are now typically grouped by genotyping (41, 55). However, serotypic classification by plaque reduction neutralization tests (PRNT), which closely recapitulate genotyping results, is still considered the conventional "gold standard" (34). Briefly, rat immune sera prepared against MJNV strain 05-11, TPMV strain VRC66412, Hantaan virus (HTNV) strain 76-118, Puumala virus (PUUV) strain Sotkamo, and New York virus (NYV) strain NY-1, as well as sera from anti-MJNV antibody-positive Ussuri shrews, were used to analyze the antigenic relationships among MJNV, TPMV, and representative rodent-borne hantaviruses. Serial twofold dilutions of immune sera were incubated with 50 to 100 PFU of each hantavirus at 4°C overnight. Thereafter, virus-serum mixtures were inoculated onto confluent monolayers of Vero E6 cells grown in 6-well flat-bottomed tissue culture plates, adsorbed at 37°C for 1 h, and then overlaid with Dulbecco's modified Eagle's medium containing agarose (0.33 g/100 ml). After incubation (6 days for HTNV, 9 days for MJNV, TPMV and PUUV, or 11 days for NYV), monolayers were overlaid with agarose containing 5% neutral red (0.167 mg/ml) and plaques were enumerated. PRNT titers were expressed as the reciprocal of the highest level of serum dilution giving 80% or greater reduction in plaque formation.

Genetic characterization of MJNV. Total RNAs extracted from MJNV-infected Vero E6 cells and from the lung tissues of wild-caught Ussuri shrews were reverse transcribed using the SuperScript II RNase H⁻ reverse transcriptase kit (GIBCO/BRL) with a primer based on the conserved 5' ends of the S, M, and L segments of hantaviruses (5'-TAGTAGTAGACTCC-3') and with random hexamers. For subsequent amplification, touchdown PCR was performed using oligonucleotide primers based on TPMV sequences, and amplified products were cloned using the TOPO-TA cloning system (Invitrogen Corp., San Diego, CA). DNA sequencing in both directions for at least three clones of each PCR product was performed using a dye termination cycle sequencing ready reaction kit (Applied Biosystems Inc., Foster City, CA) and an automated sequencer (model 377; Perkin Elmer Co.). Full-length S, M, and L genomic segment sequences from MJNV were aligned and compared with sequences from TPMV and previously published sequences from rodent-borne hantaviruses by using the Clustal W method with the Lasergene program, version 5 (DNASTAR, Inc., Madison, WI) (51).

Phylogenetic characterization of MJNV. Phylogenetic analysis to assess the evolutionary relationships between MJNV and other hantaviruses was based on maximum likelihood (ML) methods, which although computationally expensive, provide robust statistical methods for analyzing diverse genetic information. An initial ML estimate of the model of evolutionary change among aligned viruses was generated by Modeltest 3.7 (40). ML tree estimation in PAUP* (50) was conducted by starting with a neighbor-joining 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 the topology was generated by bootstrapping for 1,000 neighbor-joining replicates (under the ML model of evolution, implemented by PAUP) and by bootstrapping for 100 ML replicates, implemented by the RAXML Web server prototype using a novel rapid bootstrapping algorithm (<http://phylobench.vital-it.ch/raxml-bb/>) (49). Phylogenetic relationships were further confirmed using amino acid sequences analyzed by quartet puzzling using 10,000 puzzling steps, implemented by TREE-PUZZLE (42).

PCR amplification of shrew mtDNA. Total DNA, extracted from liver tissues using the QIAamp tissue kit (Qiagen), was used to verify the identity of the MJNV-infected Ussuri shrews. The cytochrome *b* region of mtDNA was amplified by PCR using previously described universal primers (+L14115, 5'-CGA AGC TTG ATA TGA AAA ACC ATC GTT G-3', and -L14532, 5'-GCA GCC CCT CAG AAT GAT ATT TGT CCA C-3') that permit the amplification of a 482-bp product (6, 43). PCR was performed in 50-μl reaction mixtures containing 200 μM deoxynucleoside triphosphate and 1.25 U of recombinant *Taq* poly-

FIG. 2. Detection of new hantavirus. (A) *Crocidura lasiura* (Ussuri white-toothed shrew) inhabits forests and fields, occasionally near human habitation. (B) The geographic range of *Crocidura lasiura* extends throughout Korea, southeastern Siberia, and northeastern China (shaded area). (C) Representative agarose gel showing 696-bp product (circled) amplified by RT-PCR from RNA extracted from the lung tissue of an Ussuri shrew. M, molecular size markers.

merase (Takara, Shiga, Japan). Cycling conditions consisted of initial denaturation at 95°C for 4 min, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 1 min, in a PTC-200 DNA Engine Peltier thermal cycler (MJ Research, Inc., Watertown, MA). PCR products were cloned and sequenced, as indicated above.

Nucleotide sequence accession numbers. The nucleotide sequences determined in this study were deposited in GenBank with the following accession numbers: MJNV S segment, EF641804 and EF641805; MJNV M segment, EF641797, EF641798, and EF641799; MJNV L segment, EF641806 and EF641807.

RESULTS

RT-PCR detection of hantavirus sequences in shrews. As part of a U.S. Army surveillance program aimed at monitoring the prevalence of HTNV infection in striped field mouse (*Apodemus agrarius*) populations, 115 *Crocidura lasiura* shrews (Fig. 2A and B) were unintentionally captured along the Imjin River near the DMZ in the Republic of Korea during 2004 and 2005 (Fig. 1). Rather than employing the time-honored approach of first screening shrew sera for antibodies against TPMV and other hantaviruses, we resorted to a more brute-force strategy of analyzing RNAs from lung tissues for hantaviral sequences by RT-PCR using oligonucleotide primers based on the TPMV S and M genomic segments. In so doing, we detected novel hantaviral sequences in lung (Fig. 2C), liver, and spleen tissues from seven Ussuri shrews (five males and two females) (Table 1).

Isolation of a new shrew-borne hantavirus. Characteristic intracytoplasmic granular fluorescence in Vero E6 cells was initially detected 14 days after inoculation with lung tissue homogenates from two RT-PCR-positive Ussuri shrews (designated 05-11 and 04-55). The new hantavirus was spherical and measured 80 to 120 nm in diameter, as visualized by thin-section transmission electron microscopy (Fig. 3). MJNV virions appeared to bud from the plasma membranes of in-

TABLE 1. Prevalence of MJNV infection among Ussuri white-toothed shrews captured near the DMZ in Korea in 2004 and 2005^a

Location	Trap site	No. of shrews tested	No. positive by IFA test	No. positive by RT-PCR
Paju City	DN	12	3	2
	SR	3	1	1
	M7	4	0	0
	NC	1	0	0
	TB	1	0	0
	WB	1	0	0
Yeoncheon County	CH	8	1	0
	L1	2	2	1
	L2	3	1	0
	L3	3	2	1
	F1	6	1	1
	F2	43	4	0
Pocheon City	MP	28	3	1

^a The locations of the trap sites are shown in Fig. 1. Seropositivity, as determined by the IFA test, was defined as the detection of virus-specific granular fluorescence in MJNV-infected Vero E6 cells at a serum dilution of 1:16 or greater.

fected Vero E6 cells, and filamentous intracytoplasmic viral inclusions were occasionally observed.

Prevalence of MJNV infection in Ussuri shrews. The availability of MJNV-infected Vero E6 cells made possible the screening of Ussuri shrew sera for IgG antibodies against the newfound hantavirus by using the IFA technique. Of 55 male and 60 female Ussuri shrews, 13 males (23.6%) and 5 females (8.3%) were found to have anti-MJNV IgG antibodies in their sera, indicating a marked male predominance of 2.6:1.0. Among seronegative Ussuri shrews, the male-to-female ratio was near 1 (1.0:1.3). None of the 115 shrews examined had IgG antibodies against HTNV, and conversely, anti-MJNV IgG antibodies were not detected in 59 *Apodemus agrarius* mice captured at the trap sites during the same period as the shrews.

FIG. 3. Thin-section electron micrograph of MJNV virion in Vero E6 cells.

FIG. 4. Comparative seasonal prevalences of MJNV infection, as determined by IFA and RT-PCR tests, in Ussuri white-toothed shrews captured near the DMZ during the spring, summer, autumn, and winter of 2004 and 2005.

Although not all shrews with anti-MJNV IgG antibodies detectable by the IFA test had MJNV RNA detectable by RT-PCR, all shrews with detectable MJNV RNA had IFA test-determined virus titers of 1:16 or higher. Also, antibody- and MJNV RNA-positive shrews tended to be heavier than uninfected shrews (mean weight, 11.4 versus 10.0 g) and were presumably older. However, two subadult male shrews (weighing 8.5 and 9.5 g) had detectable anti-MJNV IgG antibodies and MJNV RNA. The male Ussuri shrew (05-11) from which MJNV was isolated weighed 10.5 g and had an IFA test virus titer of 1:256.

Anti-MJNV IgG antibody-positive Ussuri shrews were captured at 9 of the 13 trap sites (Table 1). However, the number of shrews from each site was generally low, with only three sites (designated DN, F2, and MP) yielding more than 10 shrews over the 2-year study period. In fact, at three of the sites, only one shrew was captured. Nevertheless, the high prevalence of MJNV infection at some sites, such as L1 and L3, was suggestive of microfocal distribution (Table 1).

The prevalence of MJNV infection among shrews, as determined by the IFA test and RT-PCR, was highest during the autumn (Fig. 4). Very few shrews were captured during the summer (one in June 2004 and five in June 2005), and none of these shrews had evidence of MJNV infection. By far, the largest numbers of shrews were captured during the winter (30 in December 2004 and 43 in December 2005), possibly because of diminished food sources.

Antigenic analysis of MJNV. Antisera prepared against representative HFRS- and HCPS-causing rodent-borne hantaviruses did not react with MJNV-infected cells in the IFA test (data not shown). Moreover, as determined by PRNT, no cross-neutralizing antibodies for MJNV and HTNV, PUUV, or NYV were detected. However, partial one-way cross neutralization between MJNV and TPMV, as well as between HTNV and PUUV and between HTNV and NYV, was found (Table 2).

Sequence analysis of MJNV. The full-length S, M, and L genomic segments of MJNV, as amplified from the Vero E6 cell isolates (strains 05-11 and 04-55) and from isolates obtained from the tissues of three other wild-caught Ussuri white-toothed shrews from different trap sites, were sequenced. The overall genomic structure of MJNV was similar

to those of TPMV and other hantaviruses. However, the nucleotide and deduced amino acid sequences for each genomic segment of MJNV were significantly divergent from those of rodent-borne hantaviruses, suggesting early evolutionary divergence. That is, the amino acid sequences of the nucleocapsid and Gn and Gc glycoproteins of MJNV and rodent-associated hantaviruses differed by more than 50% (Table 3).

The full-length S genomic segments of MJNV strains 05-11 and 04-55 were 1,577 nucleotides, starting at nucleotide position 68, with a 199-nucleotide 3' noncoding region and a region encoding a predicted nucleocapsid protein of 436 amino acids. The hypothetical NSs opening reading frame, typically found in the S genomic segments of hantaviruses harbored by arvicoline, neotomine, and sigmodontine rodents, was not found in MJNV. The degrees of variation among MJNV strains based on the entire S genomic segment were 90.7 and 99.3% at the nucleotide and amino acid levels, respectively. In the hyper-variable region of the nucleocapsid protein, between amino acid residues 244 and 269, MJNV diverged by 11 amino acids from rodent-borne hantaviruses, but the functional significance of the substitutions involved is unknown. Sequence analysis of the entire S genomic segment showed that MJNV strain 05-11 differed from TPMV by 68.7 and 69.9% at the nucleotide and amino acid levels, respectively (Table 3).

The full-length M genomic segment of MJNV was 3,619 nucleotides, starting at nucleotide position 41, with a 216-nucleotide 3' noncoding region and a region encoding a predicted glycoprotein of 1,120 amino acids. Like the TPMV glycoprotein, the MJNV glycoprotein had five potential N-linked glycosylation sites (three in Gn at amino acid positions 134, 289, and 388 and two in Gc at positions 916 and 1079), as well as the highly conserved WAASA amino acid motif (amino acid positions 632 to 636), found in all rodent-borne hantaviruses. Sequence analysis of the entire M segment revealed degrees of variation among MJNV strains 04-3, 04-55, and 05-11 of 97.6 to 99.4% and 99.3 to 99.8% at the nucleotide and amino acid levels, respectively.

Nucleotide and amino acid pairwise analyses of the full-length M segment of MJNV showed it to have approximately 70% sequence similarity to the corresponding segment of TPMV (Table 3). The deduced amino acid sequence of the MJNV M segment, like that of the TPMV segment, was less than 50% similar to the Gn/Gc sequences of all rodent-borne

TABLE 2. Cross PRNT-measured titers of HTNV, PUUV, NYV, TPMV, and MJNV^a

Antiserum target	PRNT titer of:				
	HTNV	PUUV	NYV	TPMV	MJNV
HTNV	320	20	20	<20	<20
PUUV	80	320	20	<20	<20
NYV	80	<20	320	20	<20
TPMV	<20	<20	20	320	20
MJNV	<20	<20	<20	80	320

^a Antisera against HTNV, PUUV, NYV, TPMV, and MJNV were prepared by intramuscular inoculation of 4-week-old Sprague-Dawley rats with 10^4 to 10^5 PFU of virus. Rats were bled 4 weeks postinoculation. Serial twofold dilutions of rat antisera (1:20 to 1:320) were tested. PRNT-determined titers were expressed as the reciprocal of the highest level of serum dilution giving 80% or greater reduction in plaque number. Maximum detected neutralizing antibody titers to homologous viruses are shown in boldface.

TABLE 3. Degrees of nucleotide and amino acid sequence similarity between MJNV strain 05-11 and other representative hantaviruses^a

Virus strain	% Similarity of:					
	S segment nucleotides (1,577)	S segment amino acids (436)	M segment nucleotides (3,619)	M segment amino acids (1,120)	L segment nucleotides (6,570)	L segment amino acids (2,149)
HTNV 76-118	53.7	46.8	51.9	43.1	62.6	62.3
SEOV 80-39	53.8	45.6	52.1	42.8	62.0	61.5
SOOV SC-1	54.1	47.1	51.7	43.1	63.5	62.1
DOBV Greece	52.6	46.1	52.6	42.6	62.8	61.7
PUUV Sotkamo	52.7	45.9	52.2	42.7	63.2	62.1
TULV 5302v	52.5	47.7	53.4	43.5	62.4	61.7
PHV PH-1	54.0	46.6	52.7	42.2	62.2	61.1
SNV NMH10	52.5	47.3	51.9	42.6	62.4	62.0
ANDV Chile	53.4	47.8	52.5	44.3	62.8	61.6
TPMV VRC66412	68.7	69.9	68.8	71.8	74.2	81.0

^a Numbers in parentheses are numbers of nucleotides and amino acids corresponding to the indicated segments in MJNV strain 05-11. Abbreviations: ANDV, Andes virus; DOBV, Dobrava virus; PHV, Prospect Hill virus; SNV, Sin Nombre virus; SOOV, Soochong virus; and TULV, Tula virus.

hantaviruses (Table 3). This finding would predict the absence of cross neutralization between MJNV and hantaviruses harbored by rodents, as verified by cross PRNT (Table 2).

For the L segment, the five conserved motifs (A, B, C, D, and E) previously identified in all hantavirus RNA polymerases were found in MJNV. The level of sequence similarity of approximately 60% between the L segments of MJNV and rodent-borne hantaviruses, much higher than the levels of similarity between other segments, probably signifies the func-

tional constraints of the RNA-dependent RNA polymerase (Table 3).

Phylogenetic analysis of MJNV. Unlike all other hantaviruses, which segregate into clades that parallel the evolution of rodents belonging to the subfamilies Murinae, Arvicolinae, Neotominae, and Sigmodontinae, MJNV and TPMV remained in a distinct and divergent group in phylogenetic analyses of the full-length S, M, and L genomic segments by the ML method (Fig. 5). Similar topologies for deduced amino

FIG. 5. Phylogenetic trees generated by the ML method using the GTR+I+ Γ model of evolution as estimated from the data based on the alignment of the entire coding regions of the 1,353-nucleotide S, 3,498-nucleotide M, and 6,480-nucleotide L genomic segments of MJNV. The phylogenetic positions of MJNV strains 05-11 and 04-55 are shown in relationship to those of representative murine rodent-borne hantaviruses, including HTNV strain 76-118 (GenBank accession no. NC_005218, NC_005219, and NC_005222), Sangassou virus strain SA14 (SANV; GenBank accession no. DQ268650, DQ268651, and DQ268652), Dobrava virus strain AP99 (DOBV; GenBank accession no. NC_005233, NC_005234, and NC_005235), and SEOV strain 80-39 (GenBank accession no. NC_005236, NC_005237, and NC_005238); arvicoline rodent-borne hantaviruses, including Tula virus strain M5302v (TULV; GenBank accession no. NC_005227, NC_005228, and NC_005226) and PUUV strain Sotkamo (GenBank accession no. NC_005224, NC_005223, and NC_005225); and sigmodontine and neotomine rodent-borne hantaviruses, including Andes virus strain Chile 9717869 (ANDV; GenBank accession no. NC_003466, NC_003467, and NC_003468) and Sin Nombre virus strain NMH10 (SNV; GenBank accession no. NC_005216, NC_005215, and NC_005217). The position of TPMV strain VRC66412 (GenBank accession no. AY526097, EU001329, and EU001330), from the Asian house shrew (*Suncus murinus*), is also shown. Host identification of *Crociodura lasiura* was confirmed by mtDNA sequencing (data not shown). The numbers at each node are bootstrap support values (expressed as the percentage of replicates in which the node was recovered), as determined for 100 ML iterations under the same model of evolution by the RAXML Web server (39). The scale bars indicate the numbers of nucleotide substitutions per site.

acid sequences of the encoded proteins were found by using quartet puzzling (with TREE-PUZZLE) (data not shown). Collectively, these data strongly supported the hypothesis of an ancient nonrodent host origin for both MJNV and TPMV. That is, MJNV and TPMV appear not to represent spillover from as-yet-unidentified rodent reservoir species but rather to represent phylogenetically distinct hantaviruses that have co-evolved with their soricid hosts.

Phylogenetic analysis of host mtDNA. Phylogenetic analysis of the mtDNA cytochrome *b* gene, amplified from liver tissues of anti-MJNV IgG antibody-positive *Crocidura lasiura* shrews, validated the taxonomic identification based on morphological features. Importantly, the shrews from which MJNV strains 05-11 and 04-55 were isolated in Vero E6 cells were identified as *Crocidura lasiura* by mtDNA sequence analysis.

DISCUSSION

Search for novel hantaviruses. New hantaviruses have previously been targeted for discovery by focusing on rodent species which are evolutionarily related to known rodent reservoir hosts (5, 47). In much the same way, we posited that shrew species belonging to the subfamily Crocidurinae, particularly species that were phylogenetically related to *Suncus murinus*, the reservoir of TPMV, would similarly harbor hantaviruses. Fortuitously, we were provided access to tissues and sera of *Crocidura lasiura* shrews that had been captured coincidentally as part of a surveillance program for HTNV infection in *Apodemus agrarius* in Korea. Although the trapping method was not ideally suited for capturing shrews, the trapping expeditions resulted in the collection of adequate materials to search for a hantavirus in the Ussuri white-toothed shrew.

By designing oligonucleotide primers based on the recently acquired full genome sequence of TPMV, we successfully amplified and subsequently isolated a new hantavirus, designated MJNV, from *Crocidura lasiura*, the first mammal recorded scientifically in Korea (21). MJNV was genetically distinct from but clearly related to TPMV, as evidenced by the results of phylogenetic analysis, which are consistent with the evolution of crocidurine shrews (15). In this regard, hantaviruses are likely to be harbored by shrew species closely related to *Crocidura lasiura*, including the dsinezumi shrew (*Crocidura dsinezumi*) and the Asian lesser white-toothed shrew (*Crocidura shantungensis*) (37). A recent analysis of cytochrome *b* mtDNA sequences has shown that *Crocidura dsinezumi* and *Crocidura lasiura* form a well-supported monophyletic group, with two distinct clusters of *Crocidura dsinezumi* shrews corresponding to western and eastern Japan. Based on the low level of genetic divergence, *Crocidura dsinezumi* shrews on Jeju Island and in Hokkaido, Japan, appear to have resulted from recent introductions from western and northeastern Japan, respectively (37). Thus, studies of *Crocidura dsinezumi* shrews on Jeju Island will provide a window into the existence of crocidurine shrew-borne hantaviruses in Japan, just as further studies of *Crocidura lasiura* shrews captured along the DMZ will provide information about MJNV and MJNV-like hantaviruses in North Korea.

The isolation of MJNV from the Ussuri white-toothed shrew and the recent identification of soricid-borne hantaviruses, which are more genetically diverse than rodent-borne hanta-

viruses, from four continents (1, 2, 29, 45, 46) would predict that many more shrew-hantavirus associations undoubtedly exist. Also, these findings raise the possibility that other soricomorphs, notably moles (family Talpidae), harbor hantaviruses. In support of this possibility, novel hantavirus genomes in the Japanese shrew mole (*Urotrichus talpoides*) (3), as well as in the American shrew mole (*Neurotrichus gibbsii*) (27) and the European common mole (*Talpa europaea*) (Kang and Yanagihara, unpublished), have recently been identified, suggesting a far more ancient and complex evolutionary history, characterized by codivergence and cross-species transmission, than was recognized previously. Although unthinkable just a few years ago, a reasonable next step now includes studies to ascertain if other soricomorphs, such as hedgehogs and gymnures (family Erinaceidae), are involved in the evolutionary dynamics of hantaviruses.

Antigenic relationships between MJNV and other hantaviruses. As noted, no antigenic cross-reactivity between MJNV and representative rodent-borne hantaviruses was found by the IFA test or PRNT. However, partial one-way cross neutralization between MJNV and TPMV was found. The closer sequence homology between the Gn and Gc envelope glycoproteins of MJNV and TPMV than between these glycoproteins and those of other hantaviruses presumably accounts for the observed cross neutralization, and future investigations to dissect the precise epitopes are warranted. Past studies have demonstrated a similar pattern of cross neutralization between HTNV and PUUV, as well as HTNV and Prospect Hill virus (12), but the epitopes have not been mapped.

In the epitope region recognized by the monoclonal antibody E5/G6, with the consensus sequence FEDVNGIRKP (4), the corresponding TPMV and MJNV N proteins had sequences of MEDRNGIKQH and METVNGIQRH, respectively, more comparable to each other than to the consensus sequence. Thus, this epitope region, at amino acid positions 176 to 185 in MJNV, would presumably need to be modified by site-directed mutagenesis to allow binding by monoclonal antibody E5/G6, as has been demonstrated for TPMV (38). This approach would provide a common enzyme immunoassay-based platform for the detection of IgG antibodies to MJNV.

Transmission dynamics of hantaviruses. In the reservoir rodent host, hantavirus infection is subclinical, with short-lived viremia and the dissemination of virus in multiple tissues, particularly those of the lungs, salivary glands, and kidneys (31, 32, 59). The demonstration of virus antigen in brown fat of overwintering bank voles (*Myodes glareolus*) suggests a possible mechanism of virus maintenance (20). Virus excretion in urine and feces from infected rodents persists for months or possibly for life, despite high-titered neutralizing antibodies in sera (31, 59). However, vertical transmission of hantavirus in rodents does not occur (7, 8, 32), and arthropods and insects do not appear to be involved in the maintenance of the enzootic cycle or the transmission of infection to humans (32, 53).

Unfortunately, appropriate samples, including ectoparasites, from Ussuri shrews were not collected in this study to answer fundamental questions about virus shedding and transmission dynamics. However, especially noteworthy was the overrepresentation of MJNV infection among adult male Ussuri shrews, a pattern which has been reported previously for Seoul virus (SEOV) infection in the Norway rat (*Rattus norvegicus*) (13, 22,

24), Sin Nombre virus infection in the deer mouse (*Peromyscus maniculatus*) (9, 14), and Bayou virus infection in the marsh rice rat (*Oryzomys palustris*) (35). Whether the male predominance among MJNV-infected shrews results from prolonged shedding of high-titered infectious virus in secretions and excretions or reflects intraspecific transmission through wounding, as reported for SEOV-infected rats (24), is unclear. Also, studies are warranted to ascertain if the persistence of MJNV in Ussuri shrews results from heightened regulatory T-cell responses, as described previously for SEOV-infected male Norway rats (17, 18).

Chronic hantavirus infection in rodents is usually characterized by alternating positivity and negativity for hantavirus RNA, instead of strict concordance between the presence of detectable anti-hantavirus antibody and that of hantavirus RNA (30). Similarly, in this study, the detection of anti-MJNV IgG antibodies in the absence of detectable MJNV RNA may be attributed either to the insensitivity of the RT-PCR analysis or fluctuating viral loads in tissues. Another possibility is that antibodies detected in some of the shrews may represent passively acquired maternal IgG antibodies or may have been falsely identified, since the commercially available secondary antibodies employed were prepared with specificity for mouse and rat, rather than for crocidurine shrew. The future development of improved reagents for detecting shrew immunoglobulins would provide greater sensitivity and specificity.

Evolutionary history of hantaviruses. Whereas not all “orphan viruses” warrant intensive study at the time of their discovery, selected zoonotic viruses, particularly those related to viruses known to cause life-threatening diseases, such as HFRS and HCPS, are worthy of high research priority. That is, by investing early in studies of such newly identified viruses, we may be better equipped to more rapidly respond to and diagnose future outbreaks caused by emerging hantaviruses. In this regard, prospective in-depth studies on neotomine and sigmodontine rodent-borne hantaviruses in the early 1980s might have provided important clues about their pathogenicity long before the recognition of HCPS in 1993. In much the same way, the identification of MJNV in the Ussuri white-toothed shrew provides an opportunity to investigate its genetics, transmission dynamics, and disease potential before the next generation of health care workers is faced with a newly recognized hantavirus disease caused by hantaviruses carried by shrews.

That is not all. Rarely do seemingly tangential findings challenge the very tenets of a well-established scientific field and/or stimulate serious inquiry about the fundamental dogma for a virus genus. That shrews harbor hantaviruses which are far more genetically diverse than hantaviruses harbored by rodents heralds a compelling paradigm-shifting conceptual framework for reconstructing the evolutionary origins of hantaviruses. For example, since all other members of the *Bunyaviridae* family involve insect or arthropod vectors, the long coevolutionary history of hantaviruses may have entailed the emergence of a primordial virus through species jumping from an insect or arthropod host into an early soricomorph ancestor, with subsequent host switching many millions of years before the present. New knowledge from these gateway investigations will help to rewrite the textbook chapters on hantaviruses.

Pathogenicity of newfound soricid-borne hantaviruses. Like their counterparts in Korea 40 years earlier who were faced

with HFRS, a disease then unknown to American medicine (19, 57, 60), health care workers in the Four Corners region of the southwestern United States were confronted by a terrifying outbreak of a rapidly progressive, frequently fatal respiratory disease, now called HCPS, in the spring of 1993 (16, 36). No one had the prescience to predict that a once-obscure group of rodent-borne viruses, previously known to cause HFRS, would also cause HCPS. The realization that rodent-borne hantaviruses are capable of causing diseases as clinically disparate as HFRS and HCPS raises the possibility that MJNV may be similarly pathogenic for humans. That is, MJNV may cause a disease or syndrome that is clinically distinct from HFRS and HCPS. Scant evidence to date suggests that TPMV or TPMV-related viruses may cause infection in humans (38, 44), but a definitive demonstration of disease association in humans is lacking. Intensive investigations are warranted to ascertain if MJNV and/or other newly identified genetically distinct soricid- and talpid-borne hantaviruses are pathogenic.

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