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Genetic Diversity of Hantaviruses Isolated in China and Characterization of Novel Hantaviruses Isolated from *Niviventer confucianus* and *Rattus rattus*

Hua Wang,*,† Kumiko Yoshimatsu,† Hideki Ebihara,† Michiko Ogino,† Koichi Araki,‡ Hiroaki Kariwa,‡ Zhaoxiao Wang,§ Zhaozhuang Luo,¶ Dexin Li,* Changshou Hang,* and Jiro Arikawa†,[1]

*Institute of Virology, Chinese Academy of Preventive Medicine, Beijing 100052, China; †Institute for Animal Experimentation, Hokkaido University School of Medicine, Sapporo 060-8638, Japan; ‡Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan; §Anti-Epidemic Station, Anhui, Hefei, China; and ¶Anti-Epidemic Station, Guizhou, Guiyang, China

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The antigenic and genetic properties of 46 hantaviruses from China, 13 from patients, 23 from rodents, and 10 from unknown hosts, were compared with those of other hantaviruses. The viruses were classified as either Hantaan (HTN) or Seoul (SEO) viruses. A phylogenetic analysis of the partial M (300 bp) and S (around 485 bp) genomes of HTN viruses identified nine distinct genetic subtypes, one consisting of isolates from Korea. The SEO viruses were divided into five genetic subtypes, although they had less variability than the HTN subtypes. There was a correlation between the subtype and province of origin for four subtypes of HTN viruses, confirming geographical clustering. Hantaan virus NC167 isolated from *Niviventer confucianus* and SEO virus Gou3 isolated from *Rattus rattus* were the basal clades in each virus. The phylogenetic trees constructed from the entire S and M segments suggested that NC167 was introduced to *N. confucianus* in a host-switching event. The reactivity of a panel of 35 monoclonal antibodies was almost exactly the same in NC167 and a representative HTN virus and in Gou3 and a representative SEO virus. However, there was a one-way cross-neutralization between them. These results confirm the varied nature of Murinae-associated hantaviruses in China.

INTRODUCTION

Hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS) are rodent-borne viral zoonoses caused by viruses in the genus Hantavirus, family *Bunyaviridae* (Schmaljohn and Dalrymple, 1983; Nichol *et al.,* 1993). Like other viruses in the family *Bunyaviridae,* hantaviruses possess a negative-sense RNA genome consisting of three segments: the large (L), medium (M), and small (S) segments. The S RNA segment encodes the nucleocapsid protein (NP), the M RNA segment encodes a glycoprotein precursor that is cleaved into the envelope glycoproteins G1 and G2 cotranslationally, and the L segment is believed to encode RNA polymerase (Elliot, 1990). At least 14 virus species and 10 serotypes have been identified by genetic and antigenic characterizations, respectively (Schmaljohn and Hjelle, 1997). Four of the hantavirus species, Hantaan (HTN), Seoul (SEO), Dobrava/Belgrade (DOB), and Puumala (PUU), which are all different serotypes, are known to cause HFRS and are called the "Old World hantaviruses." At least four virus species are causative agents of HPS in North and South America (Young *et al.,* 1998). Their primary rodent hosts belong to the subfamily Sigmodontinae, which inhabits the Americas. Therefore, these are called the "New World hantaviruses."

Each hantavirus appears to have a single rodent species that serves as a natural reservoir (Plyusnin *et al.,* 1996; Schmaljohn and Hjelle, 1997). In Asia, striped field mice, (*Apodemus agrarius*) carry HTN virus (Lee *et al.,* 1978), while in Europe DOB viruses are reported in both yellow-necked mice (*A. flavicollis*) (Taller *et al.,* 1993) and striped field mice (*A. agrarius*) (Nemirov *et al.,* 1999). Seoul viruses are carried by the urban rat (*Rattus norvegicus*) and roof rat (*R. rattus*) in Asia, North America, South America, and Europe (Chin *et al.,* 2000; Kitamura *et al.,* 1983; Xiao *et al.,* 1994). Another hantavirus serotype, Thailand, was originally isolated from rodents in the genus *Bandicota* in Thailand, and its pathogenicity in humans is unknown (Plyusnin *et al.,* 1996). The rodent species associated with HTN, SEO, and DOB viruses belong to the subfamily Murinae. The rodents associated with PUU and PUU-related hantaviruses belong to the Arvicolinae, while in North and South America Sin Nombre virus (SNV) and related viruses are carried by New World rodents in the genera *Peromyscus* and *Oligoryzomys* of the subfamily Sigmodontinae. The close association between each hantavirus and a particular rodent species and the phylogenetic analysis of hantaviruses suggest that hantaviruses and their rodent hosts have coevolved (Plyusnin *et al.,* 1996).

[1] To whom correspondence and reprint requests should be addressed at Institute for Animal Experimentation, Hokkaido University School of Medicine, Kita-15, Nishi-7, Kita-ku, Sapporo, 060-8638, Japan. Fax: +81-11-706-7879. E-mail: j_arika@med.hokudai.ac.jp.

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HFRS caused by HTN and SEO viruses is a significant public health problem in Asia, especially in China. It has been reported that nearly 100,000 patients are hospitalized annually (Lee, 1996; Song, 1999). Recently, many hantaviruses have been isolated from various rodent species and patients throughout China. Serologic and antigenic studies showed that these isolates were related to either HTN or SEO, which are associated with the rodent subfamily Murinae (Tang *et al.,* 1991; Liang *et al.,* 1994).

Shi *et al.* (1998) compared the nucleotide sequence of the M genome segment of Chinese HTN and SEO viruses to those of Korean and Japanese isolates and showed that Chinese HTN viruses form lineages distinct from other Asian isolates. Liang *et al.* (1994) also showed that at least three subtypes of HTN viruses are present in China.

It has been reported that HTN and SEO viruses, which showed different fatality rates (HTN for 5–10%, SEO 1–2%) and inapparent infection rates (HTN for 1–4%, SEO 8–20%), coexist in China (Song, 1999). Furthermore, epidemiological investigations showed that morbidity and mortality rates vary considerably from village to village where HTN viruses are spreading in both of the villages (unpublished observation by Dr. Wang Hua). This suggests that hantaviruses with different degrees of virulence coexist in China. Therefore, to obtain basic information on the variability of hantaviruses in China, we antigenically and genetically characterized 46 Chinese hantavirus isolates from rodents and humans, along with isolates from other countries.

RESULTS

Antigenic characterization of Chinese isolates

A total of 46 isolates from Chinese patients or rodents were grouped into HTN or SEO by their reactivities to serotype-specific monoclonal antibodies, C24B4 and DCO3, in an indirect immunofluorescence antibody (IFA) assay (Table 1).

TABLE 1

Screening of Chinese Hantavirus Isolates by Reactivities against Monoclonal Antibodies in IFA Assay

Origin	Viruses		Total
	HTN	SEO	
Human	9	4	13
Rodents	16	7	23
Apodemus agrarius	15	0	15
Niviventer confucianus	1	0	1
Rattus norvegicus	0	5	5
Rattus rattus	0	1	1
Cricetulus barabensis	0	1	1
Unknown	7	3	10
Total	32	14	46

Note. A total of 46 Chinese hantavirus isolates were divided into HTN and SEO viruses according to the reactivities to HTN-specific monoclonal antibody C24B4 (Yoshimatsu *et al.,* 1996) or SEO-specific monoclonal antibody DCO3 (Ruo *et al.,* 1991).

Genetic detection and phylogenetic analysis

The nucleotide sequences of the 300-bp fragment of the G2 encoding region of virus M segments amplified by reverse transcriptase-PCR (RT-PCR) from 43 isolates were used for phylogenetic analysis. Xiao *et al.* (1993) published a phylogenetic analysis for this region that is suitable for typing hantaviruses. Chen4 and HV114 were included in the 43 isolates; these sequences were the same as published sequences. Additional published strains (10 HTN viruses, 9 SEO viruses, Thailand, DOB/Slovenia, and SNV) were included in this analysis (Fig. 1a). An approximately 485-bp nucleotide sequence containing the noncoding region of the S segment of the virus was amplified by RT-PCR from 21 HTN and 3 SEO viruses. These sequences and the published sequences listed in Table 2 were used (Fig. 1b). The phylogenetic trees based on the M (Fig. 1a) and S (Fig. 1b) sequences both had two branches, one consisting of the HTN viruses and the other consisting of the SEO viruses.

Genetic diversity of HTN viruses

The branches of the phylogenetic tree for the partial M segment formed nine HTN clades (designated subtypes HTN 1 to 9) and five SEO clades (designated subtypes SEO 1 to 5). Of the HTN viruses, NC167, which was isolated from *Niviventer confucianus,* formed a separate branch. The viruses in subtype HTN 2 were quite similar to one another, but it is not known where they were collected. Subtype HTN 3 consisted of isolates from Guizhou Province. Subtype HTN 4 consisted of isolates from Niongxia and Guizhou Provinces and A16. Subtype HTN 5 contained two isolates, Chen4 and 84fli, which had identical sequences. The viruses were isolated in Anhui and Sanxi Provinces, respectively. With the exception of Q32, the isolates belonging to HTN subtype 6 were from Helongjian Province. HTN subtype 7 consisted of Korean viruses. HTN subtype 8 consisted of an isolate from Hinan Island (HN26-L) and one from Zhejiang Province (815). Since these two sequences are quite different, they might be classified in different subtypes after additional isolates are collected. HTN subtype 9 consisted mainly of Shandon and Hubei isolates.

Nine subtypes of the 22 HTN viruses were also found in the phylogenetic trees based on the partial S segment (Fig. 1b). The composition of the subtypes corresponded to that in the M segment analysis (Fig. 1a), except for Q32, which was placed in subtype HTN 3 in the S segment analysis and in subtype HTN 6 in the M segment analysis. The branching order within the lineages

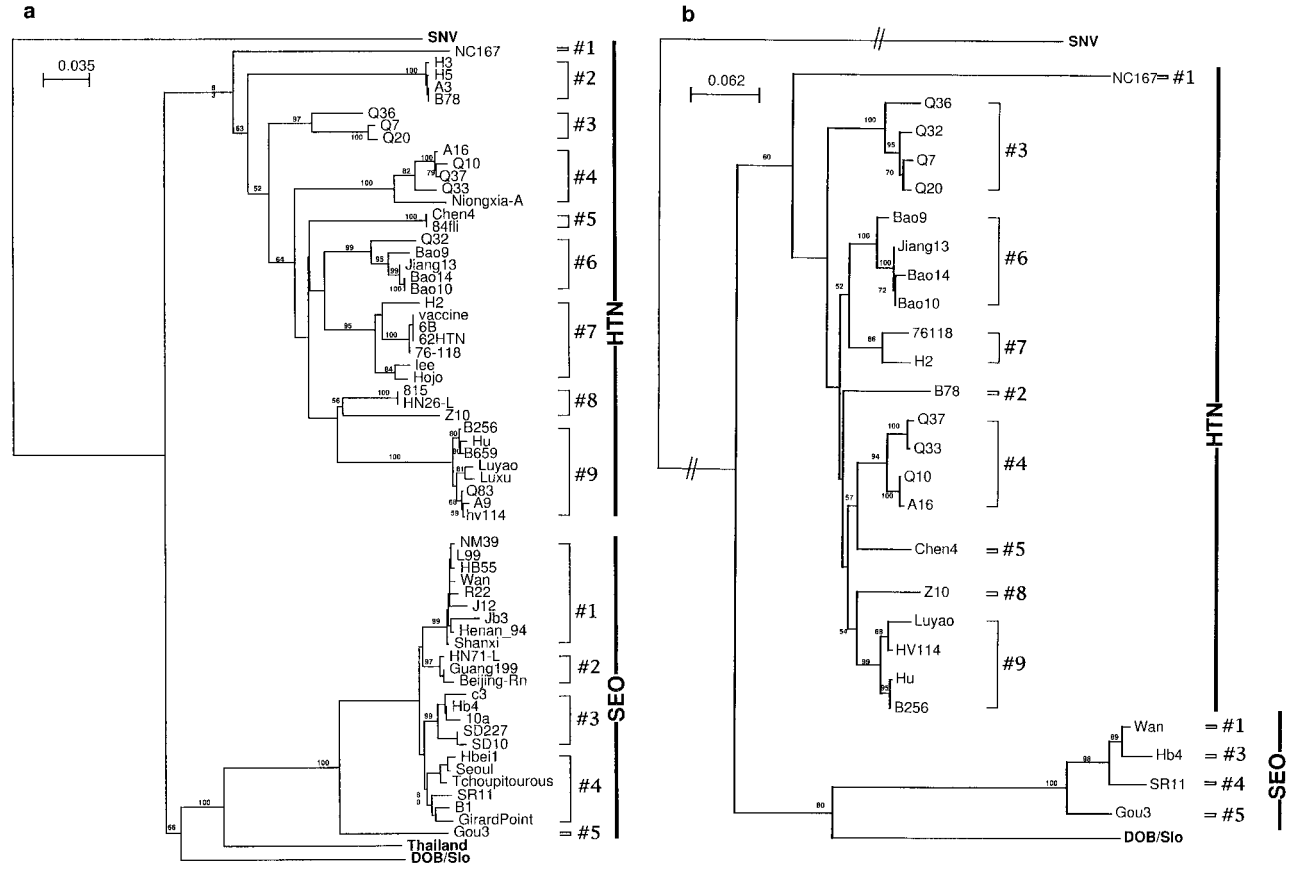

FIG. 1. Phylogenetic trees for hantaviruses based on the partial sequences of the (a) M (nucleotides 2001–2301) and (b) S (nucleotides 1211–terminal) segments, excluding the primer sequences. The numbers at the nodes are bootstrap confidence levels for 100 replicates. The numbers on the right side of the figure indicate the subgroup number of HTN viruses and SEO viruses. The reference viruses, Sin Nombre virus, Thailand virus, and Dobrava/Belgrade virus, are indicated in boldface type.

differed in the S and M trees. However, bootstrap values for the nodes separating the subtypes in the partial S tree were lower than those of the partial M tree, indicating that confidence in the partial S tree is lower. Furthermore, there was a high level of sequence conservation at the amino acid level among isolates with a significant level of diversity at the nucleotide level. The contribution of reassortant events to the diversity of HTN viruses remains unclear.

The entire S segments of Chinese HTN isolates Q32, Chen4, and Hu were sequenced and the sequences excluding the terminal primer regions were compared (Table 3). The nucleotide homologies between the Chinese isolates were approximately 88%, while the homologies of the Chinese isolates with Korean HTN virus strain 76118, which is the prototype virus, were approximately 85%. However, the amino acid sequences of the Chinese and Korean isolates were conserved (97%). The entire M segment of Chinese HTN isolates A9 and HV114 also formed a distinct cluster from the Korean HTN isolates, HTN 76118, Hojo, and Lee (Table 4). As in the S segment, the amino acid sequences of the Chinese and Korean isolates were conserved (95%). Although the Korean and Chinese HTN viruses are essentially identical at the protein level, they are distinct at the nucleotide level.

Genetic diversity of SEO viruses

The genetic diversity of the SEO viruses was lower than that of the HTN viruses, in both the M and the S trees (Figs. 1a and 1b). Although there were five subtypes in both phylogenetic trees, subtypes 1 to 4 appeared to be closely related to one another. Subtypes 1 to 3 consisted of Chinese isolates. All but one isolate in subtype 4 were isolated outside China (Korea, Japan, and the United States). Subtype 5 consisted of Gou3, isolated from *R. rattus* in Zhejiang Province, and it seemed distinct from the other SEO viruses in both trees.

Antigenic characterization of NC167 and Gou3 by MAbs and cross-focus reduction neutralizing test (FRNT)

Thirty-five monoclonal antibodies (MAbs) including 21 MAbs to glycoproteins G1 or G2 and 14 MAbs to NP, were used for the antigenic characterization of NC167

TABLE 2

Hantavirus Strains Used in This Study

Group	Strain	Source	Location	Accession No. M	Accession No. S	References
HTN viruses						
1	NC167	N.c.	Anhui	AB027115	AB027523	
2	H3	Human	Hubei	—	—	Liang *et al.* (1994)
	H5	Human	Helongjiang	—	—	Liang *et al.* (1994)
	A3	A.a.	Zhejiang	AB027055	—	
	B78	Human	Shandong	AB027056	AB027093	
3	Q36	A.a.	Guizhou	AB027057	AB027094	
	Q7	A.a.	Guizhou	AB027058	AB027095	
	Q20	A.a.	Guizhou	AB027059	AB027096	
4	Niongxia-A	A.a.	Niongxia	AB027060	—	
	Q32	A.a.	Guizhou	AB027061	AB027097	
	Q10	A.a.	Guizhou	AB027062	AB027098	
	A16	A.a.	Sanxi	AB027063	AB027099	
	Q37	A.a.	Guizhou	AB027064	AB027100	
5	Chen4	Human	Anhui	—	AB027101	Liang *et al.* (1994)
	84fli	Human	Sanxi	—	—	Liang *et al.* (1994)
6	Q33	A.a.	Guizhou	AB027065	AB027102	
	Bao9	A.a.	Helongjiang	AB027066	AB027103	
	Jiang13	A.a.	Helongjiang	AB027067	AB027104	
	Bao14	A.a.	Helongjiang	AB027068	AB027105	
	Bao10	A.a.	Helongjiang	AB027069	AB027106	
7	HTN76118	A.a.	South Korea	**M14627**	**M14626**	Schmaljohn *et al.* (1986, 1987)
	Lee	Human	South Korea	**D00376**	—	Schmaljohn *et al.* (1988)
	HoJo	Human	South Korea	**D00377**	—	Schmaljohn *et al.* (1988)
	62HTN	—	—	AB027070	—	
	6B	—	—	AB027071	—	
	Vaccine	—	—	AB027072	—	
	H2	—	North Korea	AB027073	AB027107	
8	HN26-L	A.a.	Hainan	AB027074	—	
	815	—	—	AB027075	—	
	Z10	Human	Zhejiang	AB027076	AB027108	
9	Luyao	Human	Shandong	—	AB027109	Liang *et al.* (1994)
	Luxu	Human	Shandong	—	—	Liang *et al.* (1994)
	B659	Human	Shandong	**S72339**	—	Liang *et al.* (1994)
	A9	A.a.	Jiangsu	**AF035831**	—	Shi *et al.* (1998)
	HV114	A.a.	Hubei	**L08753**	AB027110	Xiao *et al.* (1993)
	Hu	Human	Hubei	AB027077	AB027111	
	Q83	—	Guizhou	AB027078	—	
	B256	—	—	AB027079	AB027112	
SEO viruses						
1	NM39	R.n.	Neimeng	AB027080		
	L99	R.losea	Jiangxi	**AF035833**	—	Liu *et al.* (1984); Shi *et al.* (1998)
	HB55	Human	Henan	**AF035832**	—	Shi *et al.* (1998)
	R22	R.n.	Henan	**AF035834**	—	Shi *et al.* (1998)
	Wan	Human	Jiangsu	AB027081	AB027113	
	J12	Human	Jieling	AB027082	—	
	Jb3	Human	Jiangsu	—	—	Liang *et al.* (1994)
	Henan94	R.n.	Henan	AB027083	—	
	Shanxi	—	—	AB027084	—	
2	HN71-L	R.n.	Hainan	AB027085	—	
	Guang199	—	—	AB027086	—	
	Beijing-Rn	R.n.	Beijing	AB027087	—	
3	c3	Human	Hebei	AB027088	—	
	Hebei4	Cr.b.	Hebei	AB027089	AB027114	
	10a	R.n.	Hebei	AB027090	—	
	SD227		Shangdong	AB027091	—	
	SD10	R.n.	Shangdong	AB027092	—	

TABLE 2—*Continued*

Group	Strain	Source	Location	Accession No. M	Accession No. S	References
4	Hbei1	Human	Hubei	**S72343**	—	Liang *et al.* (1994)
	Seoul	R.n.	South Korea	**S47716**	—	Antic *et al.* (1992)
	Tchoupitoulas	R.n.	North America	**U00473**	—	Xiao *et al.* (1993)
	SR11	R.n.	Japan	**M34882**	**M34881**	Arikawa *et al.* (1990)
	B-1	R.n.	Japan	**X53861**	—	Isegawa *et al.* (1990)
	Girard Point	R.n.	North America	**U00464**	—	Xiao *et al.* (1993)
5	Gou3	R.r.	Zhejiang	AB027521	AB027522	
Reference strain						
	SNV	P.m.	North America	**L25783**	**L25784**	Spiropoulou *et al.* (1994)
	DOB/Slo	A.f.	Europe	**L33685**	**L41916**	Avsic-Zupanc *et al.* (1995)
	DOB/Saa	A.a.	Estonia	**AJ009774**	**AJ009773**	
	Thailand		Thailand	**L08756**	—	Xiao *et al.* (1993)
	Tula	M.a.	Russia	**Z69993**	**Z30941**	Vapalahti *et al.* (1996); Plyusnin *et al.* (1994)
	KBR	M.f.	Russia	—	**U35255**	Horling *et al.* (1996a)
	PRH	M.p.	North America	**X55129**	**M34011**	Plyusnin *et al.* (1996)
	Prair_vole	M.o.	North America	—	**U19303**	
	PUU/Sotkamo	Cl.g.	Finland	**X61034**	**X61035**	Vapalahti *et al.* (1992)
	PUU/Tobetsu	Cl.r.	Japan	—	**AB010731**	Kariwa *et al.* (1999)

Note. —, not reported. Host animals are abbreviated as follows: R.n., *Rattus norvegicus;* R.r., *Rattus rattus;* A.a., *Apodemus agrarius;* N.c., *Niviventer confucianus;* P.m., *Peromyscus maniculatus;* Cr.b., *Cricetulus barabensis;* A.f., *A. flavicollis;* M.a., *Microtus arvalis;* M.f., *M. fortis;* M.p., *M. pennsylvanicus;* M.o., *M. ochrogaster;* Cl.g., *Clethrionomys glareolus;* Cl.r., *Clethrionomys rufocanus;* HTN, Hantaan 76-118; SNV, Sim Nombre virus strain NM H10; DOB/Slo, Dobrava/Belgrade virus strain Slovenia; DOB/Saa, Dobrava virus strain Saaremaa/160V; PRH, Prospect Hill virus 1; Tula, Tula/5302 (Z69993), Tula/76Ma (Z30941); Prair_vole, prairie vole hantavirus, Sotkamo, Puumala virus strain Sotkamo. Accession numbers of published sequences used phylogenetic analysis or homology search are indicated in boldface type.

and Gou3 in the IFA tests (Table 5). Except for MAb 20D3, the reactive pattern of the MAbs to NC167 was the same as to the prototype HTN strain 76118. Although there were a few SEO-specific MAbs, Gou3 had exactly the same pattern as strain SR11. These results showed that NC167 and Gou3 are antigenically closely related to HTN and SEO viruses, respectively.

The FRNT measures antibodies against neutralization-related epitopes on the G1 and G2 envelope proteins. The cross-neutralization test was performed to evaluate

TABLE 3

Comparison of Nucleotide and Encoded Amino Acid Sequences of the S Segment of NC167, Gou3, and Hantaviruses

Viruses	Homology % (nucleotide) DOB/Slo	DOB/Saa	NC167	HTN	Q32	Chen4	Hu	Gou3	SR11	SNV	PRH	Tula	PUU
DOB/Slo	—	88.0	68.5	72.2	71.2	70.6	71.1	66.4	67.9	60.3	61.1	60.9	61.0
DOB/Saa	92.1	—	70.1	71.5	71.5	70.8	71.4	66.5	72.5	61.6	61.0	61.4	60.6
NC167	77.6	81.6	—	75.9	76.9	75.5	76.1	67.8	70.3	60.9	60.7	60.8	60.7
HTN/76118	78.1	82.3	92.1	—	85.3	85.5	85.4	67.4	68.7	60.3	61.3	59.6	59.1
Q32	77.6	81.4	91.1	96.7	—	89.4	87.9	67.5	67.5	61.3	60.4	59.2	58.7
Chen4	78.6	82.3	91.4	97.4	98.1	—	88.9	68.6	66.6	60.8	61.3	58.8	60.1
Hu	78.6	82.3	91.1	97.2	97.2	97.9	—	68.2	67.5	60.8	61.5	60.2	60.9
Gou3	76.0	80.0	83.2	83.0	81.6	82.3	82.1	—	87.9	59.0	59.7	59.6	60.6
SR11	76.0	80.0	82.3	82.3	80.9	81.6	81.4	97.2	—	60.7	61.2	60.0	59.2
SNV	59.2	62.5	60.6	62.7	61.8	62.5	62.5	62.0	61.3	—	65.9	63.7	63.1
PRH	58.9	62.1	60.3	61.9	61.2	61.7	61.2	62.4	62.4	73.4	—	70.2	68.5
Tula/65Ma	59.3	63.3	62.1	63.5	62.6	63.0	62.6	62.3	62.8	73.7	81.8	—	69.1
PUU/Sotkamo	57.5	60.7	60.7	60.7	59.8	60.3	59.8	61.2	61.7	70.7	78.5	79.9	—

Homology % (amino acid)

Note. ▭, subfamily of host rodents; ▒, serotype; ▓, subtype; ▒, intermediate virus. Sequences used in this study are listed in Table 2.

TABLE 4

Comparison of Nucleotide and Encoded Amino Acid Sequences of the M Segment of NC167, Gou3, and Hantaviruses

Viruses	Homology % (nucleotide) DOB/Slo.	DOB/Saa.	NC167	HTN	Hojo	Lee	A9	HV114	Thai.	Gou3	KI83	SR-11	B-1	Seoul	SNV	PRH	Tula	PUU
DOB/Slo	—	81.6	70.6	70.1	70.0	70.2	70.4	70.4	71.2	70.0	70.4	70.5	70.7	70.6	59.9	58.9	59.5	59.6
DOB/Saa	93.7	—	69.8	70.7	70.2	70.4	69.9	70.0	70.7	70.2	70.2	70.1	69.9	70.1	60.0	58.4	59.5	60.1
NC167	75.7	75.1	—	75.4	75.0	75.1	75.8	75.9	71.4	71.5	70.9	71.3	71.1	71.1	59.8	59.3	59.3	58.8
HTN/76-118	77.4	76.7	84.7	—	94.6	95.0	84.6	84.7	71.3	71.3	71.2	71.5	71.1	71.0	59.1	59.9	59.7	59.8
Hojo	76.5	75.9	83.8	97.4	—	98.3	83.6	83.6	70.7	70.6	71.0	71.0	70.8	70.7	58.6	58.9	59.4	58.9
Lee	77.0	76.5	84.2	98.2	98.5	—	84.1	84.1	70.8	70.9	71.0	71.0	70.7	70.8	58.8	59.3	59.4	59.3
A9	76.5	75.9	84.1	95.4	94.4	95.1	—	99.5	70.4	70.5	70.9	70.9	70.9	70.8	58.7	59.7	60.2	59.9
HV114	76.6	76.2	84.1	95.4	94.4	95.1	98.7	—	70.4	70.6	70.9	70.9	70.9	70.8	58.8	59.7	60.2	60.1
Thailand	76.9	76.4	74.9	77.1	76.3	76.7	76.1	76.1	—	73.2	73.2	73.3	73.4	73.5	60.5	59.3	59.5	59.2
Gou3	76.6	76.1	76.0	77.0	76.6	77.0	76.8	76.8	81.6	—	84.5	84.2	84.1	84.0	59.1	58.9	59.7	59.6
KI83	77.7	76.3	76.1	77.1	76.1	76.0	76.5	76.5	82.2	96.9	—	98.1	96.5	96.4	59.1	59.1	59.9	60.5
SR11	77.5	76.1	75.8	77.0	75.9	76.3	76.3	76.3	82.0	96.6	99.5	—	96.5	96.1	59.0	59.3	59.9	60.5
B-1	77.1	75.7	75.6	76.6	75.6	76.0	75.9	75.9	81.7	96.5	98.9	98.8	—	96.7	59.7	59.2	59.7	60.3
Seoul 80-39	77.4	76.1	75.8	77.1	76.1	76.5	76.3	76.3	82.0	96.4	99.2	98.9	98.3	—	58.8	59.1	59.8	60.5
SNV	54.2	54.3	54.4	54.7	54.8	54.8	54.7	54.3	53.5	52.8	53.2	53.1	52.6	53.0	—	66.3	67.1	65.9
PRH	53.3	53.2	53.2	54.4	54.0	54.3	54.1	54.1	54.0	53.5	54.2	54.0	53.7	53.8	67.6	—	72.3	70.2
Tula	55.1	54.5	53.9	55.6	54.9	55.2	55.7	55.6	54.3	53.9	54.8	54.5	54.2	54.5	69.2	80.5	—	71.7
PUU/Sotkamo	52.9	52.8	52.6	53.8	53.4	53.8	53.6	53.5	53.8	53.5	54.1	53.8	53.5	53.8	66.8	75.9	79.5	—

Homology % (amino acid)

Note. ▭, subfamily of host rodents; ▭, serotype; ▭, subtype; ▭, intermediate virus. Sequences used in this study are listed in Table 2.

the serological relationships between the representative HTN virus strain 76118 and NC167 and the representative SEO virus strain SR11 and Gou3. As shown in Table 6, the FRNT titer of anti-HTN immune serum to homologous HTN virus strain 76118 was 2 times higher than to NC167, while the anti-NC167 serum titer to NC167 was at least 32 times higher than to strain 76118. The anti-SR11 serum titer to the homologous virus was 2 times higher than to Gou3, but the anti-Gou3 serum titer to the homologous virus was 16 times higher than to strain SR11. These results showed that there was one-way cross-reactivity between HTN and NC167 and between SR-11 and Gou3.

Genetic characterization of NC167 and Gou3

To examine the divergence of NC167 and Gou3 in more detail, the entire S and M genome segments were sequenced and compared with other published sequences of selected strains. The nucleotide and amino acid sequence homologies of the S segment between NC167 and the rest of the HTN viruses (Table 3) were 75 and 92%, respectively; those among the rest of the HTN viruses were 85 and 97%. Comparison of the M genome sequence (Table 4) demonstrated the same tendency. Therefore, NC167 is distinct from the rest of the HTN viruses. Since most of the nucleotide sequence data for SEO viruses are for the M segment, the M segment of Gou3 was compared with those of five other SEO viruses. As shown in Table 4, the nucleotide and amino acid sequence homologies between Gou3 and the other SEO viruses (nucleotide, 84%; amino acid, 97%) were apparently lower than those between the other SEO viruses (nucleotide, 96%; amino acid, 99%). Therefore, Gou3 is also distinct from previously isolated SEO viruses.

To determine the phylogenetic relationships of NC167 and Gou3 to other hantaviruses, a phylogenetic analysis was carried out using complete S segment sequences. A clear relationship between the type of hantavirus and the classification of the host rodent species has been reported. The results in Fig. 2 confirm the previous findings that NC167 and Gou3 clustered with the lineages of *Apodemus*-derived and *Rattus*-derived viruses, respectively. Although NC167 clustered with the HTN viruses originating from Korea (HTN 76118) and China (Chen4, Q32, and Hu), the phylogenetic tree showed that NC167 and DOB viruses, which are also *Apodemus*-associated viruses, diverged from a common ancestral virus before the Korean and Chinese HTN viruses diverged. The distance between Gou3 and SR11 is much less than the distances among viruses derived from *Clethrionomys* or *Microtus* in the Arvicolinae rodents. This may reflect the recent differentiation of *R. rattus* and *R. norvegicus,* from which Gou3 and SR11 were isolated, respectively.

Subfamily Murinae-associated hantaviruses were further characterized phylogenetically using the entire M segment sequences (Fig. 3). As in the S segment anal-

TABLE 5

Antigenic Characterization of NC167 and Gou3 by Using Monoclonal Antibodies in an IFA Assay

Virus	Antigenic sites	MAb	Viruses			
			HTN76118	NC167	SR11	Gou3
HTN	G1-a[a]	6D4/10F11	++	++	−	−
	G1-b[a]	2D5/3D5	++	++	−	−
		16D2	++	++	−	+
	G2-a[a]	HCO2/16E6	++	++	++	++
	G2-b[a]	EBO6	++	++	++	++
	G2-c[a]	11E10	++	++	−	−
	G2-d[a]	17G6/3D7/5B7	++	++	++	++
	G2-e[a]	20D3	++	−	+	+
	G2-f[a]	1G8/1C6/8E10/23G10-1-1/3B6/7G6/18F5	++	++	++	++
		GDO5	+	+	−	−
	NP-I[b]	ECO2/ECO1/GBO4	++	++	++	++
	NP-II[b]	E5G6	++	++	++	++
	NP-III[b]	C16D11/F23A1	++	++	++	++
		C24B4	++	++	−	−
		BDO1	++	+	−	−
SEO	NP	DCO3[c]	−	−	++	++
		BCO2/JDO5/2E8	++	++	++	++

Note. −, <100; +, 100; ++, >1000.
[a] Arikawa *et al.* (1989).
[b] Yoshimatsu *et al.* (1996).
[c] Ruo *et al.* (1991).

ysis, NC167 and Gou3 were placed in the basal clusters of HTN and SEO viruses, respectively. However, the distance between NC167 and the rest of the HTN viruses was almost equal to that between Thailand and the other representative strains of SEO virus. The homology of the nucleotide and amino acid sequences of the M segment of NC167 to other HTN viruses (nucleotide, 75%; amino acid, 84%) was almost equal to that of Thailand viruses from *Rattus*-born SEO viruses (nucleotide, 73%; amino acid 82%) (Table 4). Since Thailand is a different type of hantavirus, these results suggest that NC167 might also be defined as a new type of hantavirus within the Murinae-associated hantaviruses. In the phylogenetic analysis of the M segment (Fig. 3), the distance between Gou3 and the rest of the SEO viruses was the same as that between the clusters of HTN viruses isolated in China (HV114 and A9) and in Korea (Lee, HoJo, and HTN76118). The homology of Gou3 to other SEO viruses (nucleotide, 84%; amino acid, 96%) was similar to that between HTN viruses isolated in China and Korea (nucleotide, 84%; amino acid, 95%) (Table 4). Since a proposal was made to distinguish these two clusters of viruses as subtypes, Gou3 might be defined as the representative strain of a new subtype of SEO virus.

TABLE 6

Antigenic Characterization of NC167 and Gou3 by Cross-FRNT

Antiserum	FRNT titers to virus			
	HTN 76-118	NC167	SR11	Gou3
Anti-HTN 76-118[a]	160	80	20	40
Anti-NC167[a]	<20	640	<20	80
Anti-SR11[b]	20	80	320	160
Anti-Gou3[b]	20	40	40	640

Note. Underlines indicated homologous reactions.
[a] Mouse immune sera.
[b] Rat immune sera.

DISCUSSION

HFRS outbreaks have been reported in the People's Republic of China since the 1930s, and control of this disease is urgently required there. This study examined the variability of hantaviruses in China in more detail. The antigenic and genetic properties of 46 hantaviruses from rodents and humans were compared. Our results were consistent with previous findings in several ways. First, HTN and SEO viruses are found in both humans and rodents in China (Chen *et al.,* 1986; Yan *et al.,* 1986). Second, the primary rodent reservoirs of HTN and SEO viruses in China are *A. agrarius* and *R. norvegicus,* respectively. Third, the genetic variability among HTN viruses is higher than that among SEO viruses (Shi *et al.,* 1998). In a previous report, the HTN viruses in China were divided into at least three subtypes, which were distinct from the prototype HTN virus isolated in Korea

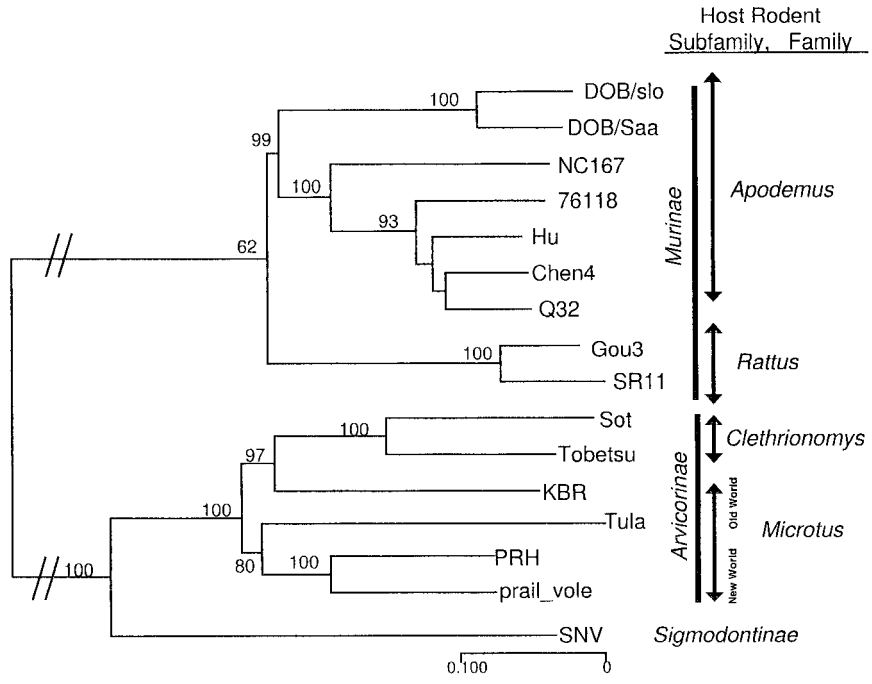

FIG. 2. Phylogenetic analysis using the entire S segment. The numbers at the nodes are bootstrap confidence levels for 100 replicates. The sequences used in this study are listed in Table 2. Sin Nombre virus, which belongs to the subfamily Sigmodontinae-associated hantavirus, was used as the outgroup.

(Liang *et al.,* 1994; Shi *et al.,* 1998). Our studies confirmed and extended these earlier findings by showing that at least eight subtypes of Chinese HTN viruses exist, based on a phylogenetic analysis of the partial sequence of the M genome.

Although the exact location of virus isolation was not specified, there seemed to be a relationship between subtype and geographic origin. In particular, most or all of the isolates in subtypes HTN 3, 4, 6, and 7 originated from Guizhou, Guizhou, and Helongjian Provinces and

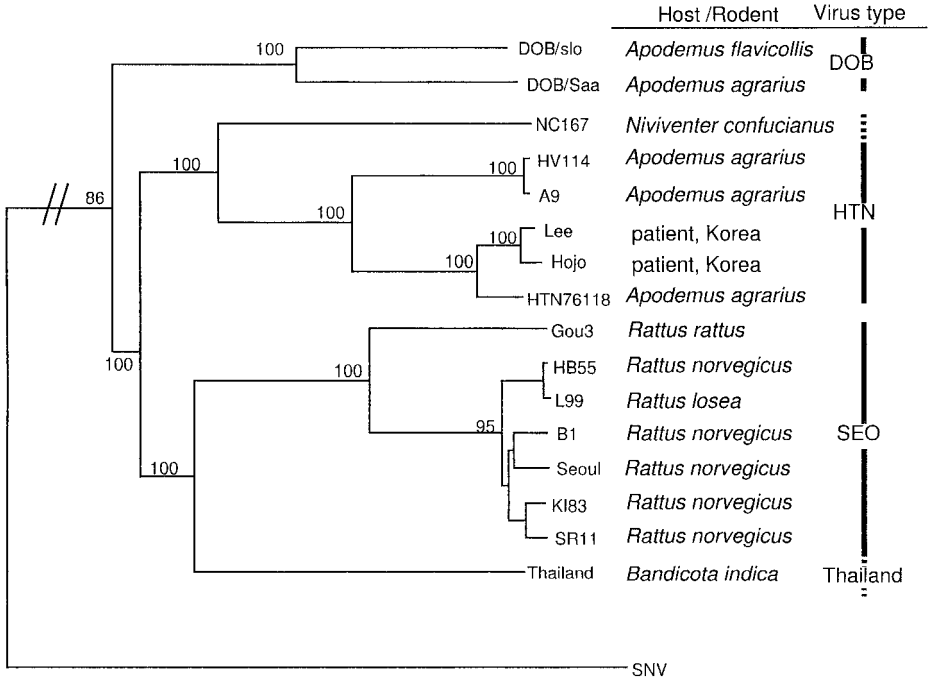

FIG. 3. Phylogenetic analysis of subfamily Murinae-associated hantaviruses using the entire M segment. The numbers at the nodes are bootstrap confidence levels for 100 replicates. The sequences used in this study are listed in Table 2. Sin Nombre virus, which belongs to the subfamily Sigmodontinae-associated hantavirus, was used as the outgroup.

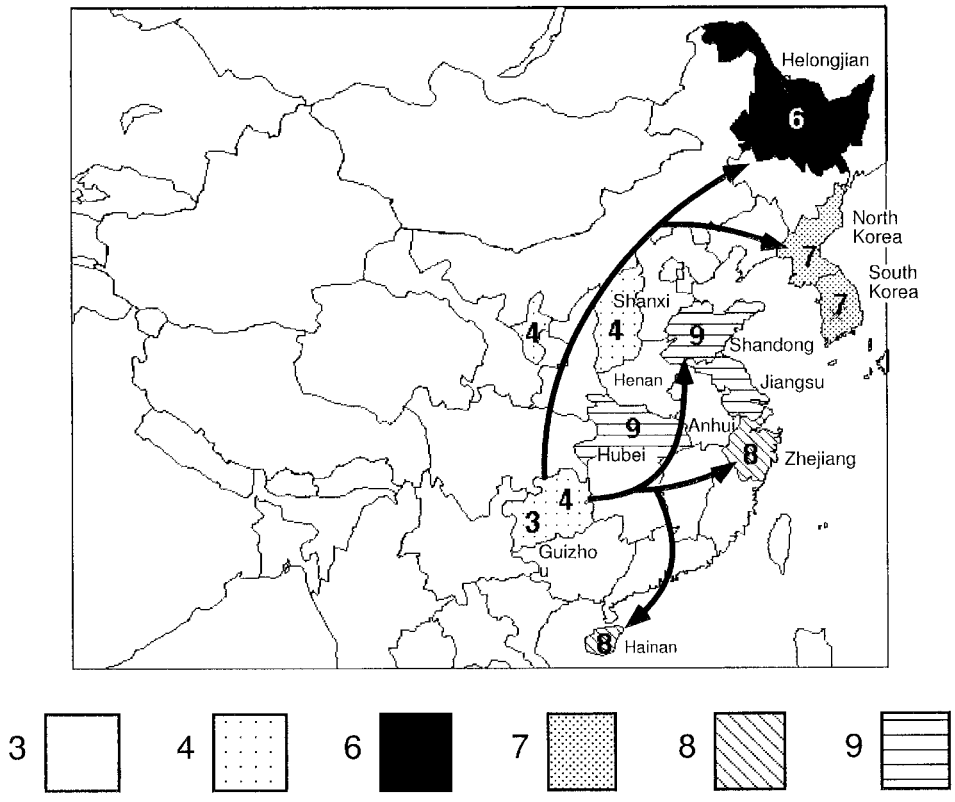

FIG. 4. Immigration route of HTN virus. Subtype 3, 4, 7, 8, and 9, which are clearly associated with a geographical region, were plotted on a map of China, North Korea, and South Korea. The immigration route was deduced from the order of divergence of the genotypes and is indicated by arrows. This map also shows where hantavirus strains NC167 and Gou3 were isolated. NC167 was isolated from a Chinese white-bellied rat captured in Anhui Province. Gou3 was isolated from a roof rat captured in Zhejiang Province.

Korea, respectively. In a study of Puumala and Tula hantaviruses in Europe, genetic variants that formed subtypes in the phylogenetic tree were isolated in distinct regions. This pattern of virus spread is called geographic clustering. Since our results are quite similar to those for Puumala and Tula viruses (Horling *et al.,* 1996b; Plyusnin *et al.,* 1996), the HTN viruses also exist in geographic clusters.

In this study, we found that NC167 is a distinct virus that is antigenically and genetically related to HTN viruses from *N. confucianus,* Chinese white-bellied rat, which belongs to the Subfamily Murinae. In initial studies in China, a Chinese epidemiologist and virologist discovered that *N. confucianus* (previously known as *R. confucianus*) carried both hantavirus and anti-hantavirus antibodies. Chen *et al.* (1986) reported capturing antibody-positive *N. confucianus* in Shaanxi and Gansu provinces, a mountainous region of China. NC167 was isolated from *N. confucianus* captured in the mountainous region of Anhui Province. These results indicate that the *Niviventer*-associated hantavirus might be endemic throughout the mountainous region of central China.

Although the genetic variability of the partial M genome segment of SEO viruses seemed smaller than that of HTN viruses, at least five subtypes were identified (Fig. 1). Interestingly, subtype 4 grouped SEO viruses isolated in the United States (Tchoupitourous and Girard Point), Japan (SR11 and B1), and Korea (Seoul). The relatively smaller genetic variability among SEO viruses has been explained by the recent invasion of various countries by infected *Rattus* transported by ship from a common source. However, the reason for the grouping of these diverse viruses in one subtype needs to be clarified. In the same phylogenetic tree, Gou3 was the basal clade of the SEO branch. In the monoclonal antibody study, Gou3 had the same reactive pattern as SEO strain SR11, confirming the finding that Gou3 belongs to the SEO virus.

Although the MAb profile confirmed that NC167 is an HTN virus and Gou3 is an SEO virus (Table 5), clear one-way cross-neutralization was observed between NC167 and HTN virus strain 76118 and between Gou3 and SEO virus strain SR11 (Table 6). The different neutralizing patterns indicate that NC167 and Gou3 possess strain-specific epitopes on their envelope proteins. Phylogenetic analysis and comparison of the nucleotide and amino acid identities based on the whole S and M genomes (Figs. 2 and 3 and Tables 3 and 4) clearly showed that NC167 and Gou3 are unique from other previously isolated HTN or SEO viruses. NC167 and Gou3 may form a new type and subtype, respectively. The discovery of distinct viruses in rodent species other

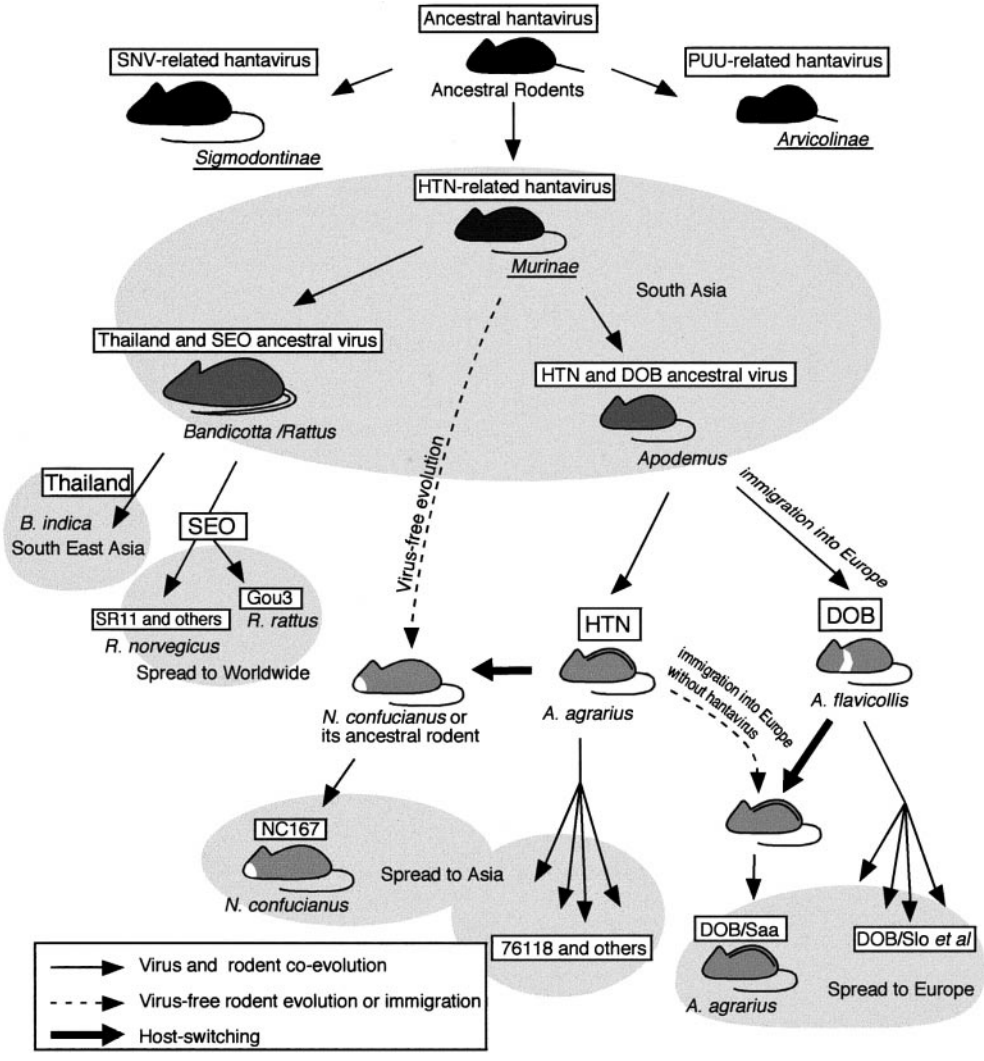

FIG. 5. Schematic diagram for proposed mechanisms of diversity of Murinae-related hantaviruses based on hypotheses of virus–host coevolution and host-switching events.

than *A. agrarius* and *R. norvegicus* confirmed the hypothesis of hantavirus coevolution with their primary rodent hosts. So far, information on the evolutionary relationships among Murinae-derived hantaviruses is limited compared to the Arvicolinae-derived viruses, such as PUU, Tula, and PRH viruses. Therefore, the characterization of the new viruses is useful in understanding the relationships among Murinae-associated hantaviruses, as well as showing the varied nature of hantaviruses in China (summarized in Fig. 5).

Vapalahti *et al.* (1999) showed that Khabaravsk virus isolated from *Microtus fortis* was derived from a *Lemmus*-associated virus in a comparison of two phylogenetic trees, one for mitochondrial cytochrome b nucleotide sequences of various rodent species and the other for hantavirus nucleotide sequences. They considered this the result of a host-switching event. Morzunov *et al.* (1998) and Monroe *et al.* (1999) also reported another likely host-switching event in the Sigmodontinae-associated viruses and proposed a host-switching event within the genus *Peromyscus,* between *P. maniculatus* and *P. leucopus.* As shown in the phylogenetic trees in Figs. 2 and 3, the NC167 branch is separated from the other *Apodemus*-borne viruses. This suggests that a host-switching event occurred after DOB and that the HTN diverged from a common ancestor (Fig. 5). *Niviventer* rodents are considered to have originated in Southeast Asia and migrated north. *N. confucianus* inhabits the mountainous region of central China (Bobrov and Neronov, 1995). We speculate that after ancestral HTN infected *A. agrarius* and *N. confucianus,* the two species of rodents dispersed into different regions and the ancestral virus subsequently evolved in the two rodents independently.

From the nodes in the partial M segment phylogenetic tree (Fig. 1) and the geographic distribution of these isolates, we speculated on the migration route of HTN viruses in Asia (Fig. 4). Subtypes 3 and 4 diverged from

the ancestral virus earlier than other HTN subtypes. Since most of the isolates in the two subtypes originated in Guizhou Province, this suggests that the ancestral HTN virus migrated into this area and subsequently spread north and east in China, as shown in Fig. 4. The genetic characterization of rodent mitochondrial DNA indicates that *Apodemus* spp. originated in south Asia 6 to 7 million years ago. It was speculated that *Apodemus* followed two different migration routes, one to northeast Asia, which gave rise to *A. agrarius,* and the other to Europe, which produced *A. flavicollis* (Chelomina *et al.,* 1998). This indicates that HTN virus migrated from Southeast Asia and spread throughout China at the same time as *Apodemus* mice. However, several nodes showed low probabilities demonstrated by bootstrap confidence value among 100 replicates. To obtain a more reliable phylogenetic tree for the investigation of the immigration root of HTN, we must collect and analyze more HTN isolates derived from various provinces of China.

In Europe, *A. flavicollis* is reported to be the primary rodent reservoir of DOB virus. Recently, it was discovered that *A. agrarius,* which is the primary host rodent for HTN virus in Asia, is also the rodent reservoir for DOB virus on Saaremaa Island, in Estonia. The sequence of strain DOB/Saaremaa (DOB/Saa) is distinct from that of the representative strain for DOB virus, DOB/SIovenia (DOB/SIo), and there are also slight differences in their antigenicity in FRNT (Nemirov *et al.,* 1999).

In our study, Gou3 from *R. rattus* diverged from a common ancestor to other *R. norvegicus*-derived SEO viruses following a *Bandicota*-derived virus in the phylogenetic tree (Fig. 3). The genus *Rattus* originated in Southeast Asia, and SEO viruses evolved with their host rodents with the radiation of *Rattus* spp. (Fig. 5).

In China, HFRS first appeared in northeast China in the 1930s and spread south into the rest of China over half a century (Song, 1999). However, the severity of reported HFRS varies with endemic foci. The relationship between the HTN and SEO subtypes and the severity of HFRS should be studied in the future. The pathogenicity of *Niviventer*-borne and *R. rattus*-borne hantaviruses in humans is still unclear. Further studies of the epidemiological and epizootiological features of hantaviruses are required.

MATERIALS AND METHODS

Viruses and cells

A total of 46 isolates of hantavirus isolated from humans and animals in China in the 1980s and 1990s were grown in Vero E6 cells using a previously described procedure (Schmaljohn and Dalrymple, 1983). The serotypes of the isolates were determined by indirect IFA assay, as described below. The viruses were divided into HTN and SEO viruses according to the binding of HTN-specific monoclonal antibody C24B4 (Yoshimatsu *et al.,* 1996) or SEO-specific monoclonal antibody DCO3 (Ruo *et al.,* 1991).

Indirect immunofluorescence antibody assay

An IFA assay was carried out as previously described (Yoshimatsu *et al.,* 1993). Briefly, acetone-fixed smears of Vero E6 cells infected with hantaviruses were used as antigens. Fluorescent isothiocyanate-conjugated anti-mouse immunoglobulin (H + L) goat IgG (Zymed, San Francisco, CA) was used as the second antibody. The IFA reactivity was determined by the appearance of characteristic fluorescence in the cytoplasm.

Reverse transcriptase-PCR

Total RNA was extracted from Vero E6 cells inoculated with hantaviruses using Isogen reagent (Nippon Gene, Tokyo, Japan) according to the manufacturer's directions. This procedure is based on the acid guanidine isothiocyanate–phenol–chloroform method. First, cDNA was synthesized from total RNA using SuperScript II (Gibco BRL) and oligonucleotide primers, which consisted of 14 nucleotides complementary to the 3′ end of virus sense S RNA genome segments (designated 14-PRIMER by Schmaljohn *et al.* (1986); TAGTAGTAGACTTC), according to the manufacturer's directions. The nucleotide sequences of the 300-bp fragment of the virus M genome segment, which encodes the G2 protein of HTN or SEO viruses, were amplified using oligonucleotide primer pairs HMF1958 (GAATCCATACTGTGGGCTGCAAGTGC)/HMR2340 (GGATTACAACCCCAGCTCGTCTC) and SEOMF1936 (GTGGACTCTTCTTCTCATTATT)/SEOMR2353 (TGGGCAATCTGGGGGGTTGCATG), respectively. An approximately 485-bp nucleotide sequence that contains the noncoding region of the virus S genome segment of both HTN and SEO viruses was amplified using oligonucleotide primer pair GS4 (GAIIGITGTCCACCAACATG)/CS8 (TAGTAGTAGGCTCCCTAAAAAGACAA). Primers GS4 and GS6 were described in a previous report (Arthur *et al.,* 1992). The entire S segment of isolates Hu, Chen4, and Q32 was amplified as two overlapping fragments using oligonucleotide primer pairs GS4/CS8 and CS1 (TAGTAGTAGACTCCCTAAAGAGCTAC)/GS6 (AGCTCIGGATCCATITCATC). Dr. Isegawa, of the Osaka University Medical School, Osaka, Japan, supplied primers CS1 and CS8, which were synthesized for a sequencing study of the HTN virus strain clone-1 (Isegawa *et al.,* 1994). The entire S segment of NC167 and Gou3 was amplified with primer HTV-MTF (TAGTAGTAGACTCCGCAARAAAIAS). The entire M segment of NC167 was amplified with HTV-MTF2 (TAGTAGTAGACWCCGCAAIAIAIAGCAGT) and HTV-MTR (TAGTAGTAKICTCCGCAIGATGTYAAG) and that of Gou3 was amplified with primer pair HTV-MTF and HTV-MTR. To sequence the terminal region of the M genome segments, RNA was extracted from 1 ml of cultured supernatant of Vero cells inoculated with each virus using Isogen-LS reagent (Nippon Gene). The two ends of the RNA were li-

gated to each other using T4 RNA ligase (TaKaRa, Tokyo, Japan) according to the manufacturer's directions, and the ligated RNA was extracted again. First, cDNA was synthesized from the treated RNA fraction using SuperScript II (Gibco) and random hexamer oligonucleotide primers. An approximately 400-bp nucleotide sequence that contains the junction of the 3′- and 5′-terminals of the M genome segments was amplified with the appropriate primer pair. These PCR products were cloned into PCR2.1 plasmid vector using The Original TA Cloning Kit (Invitrogen, Groningen, The Netherlands) according to the manufacturer's directions. Three clones of each were sequenced and their consensus sequences were determined. To sequence the PCR products completely, we synthesized several oligonucleotide primers based on the product sequences.

Nucleotide sequence determination and analysis

PCR products of the correct size were purified using a Gene Clean III Kit (Bio 101, Vista, CA) and sequenced with the same primers used for PCR amplification. The sequencing reaction was performed with dye terminator reactions using an ABI PRISM Dye Terminator Cycle Sequencing Kit FS or a BigDye Terminator Cycle Sequencing Kit (Perkin–Elmer, Applied Biosystems Division, Foster City, CA). The samples were sequenced on a Model 373A or 377 DNA Sequence System (Perkin–Elmer, Applied Biosystems Division). Alignment and comparative nucleotide sequence analysis were carried out using Clustal W, Version 1.6 (Thompson *et al.,* 1994).

Phylogenetic analysis

A neighbor-joining phylogenetic analysis was performed using the programs DNADIST, PROTDIST, NEIGHBOR, SEQBOOT, and CONSENSE from PHYLIP Version 3.57c (Felsenstein, 1995) on a SUN Spark Station 2. One hundred bootstrap replicates were performed. The accession numbers of the sequences used in the phylogenetic analysis are listed in Table 2.

Immune rat and mouse sera and monoclonal antibodies

Each of 10 female 7-week-old SIc/ICR mice was inoculated intraperitoneally with 10^3 focus-forming units (FFU) of either HTN or NC167. Each of 10 female 8-week-old Hkm/Wistar rats was inoculated intraperitoneally with 10^4 FFU of SEO virus strain SR11 or Gou3. Serum specimens were obtained 4 weeks after the inoculation. All the animals were treated according to the Laboratory Animal Control Guidelines of the Hokkaido University School of Medicine, Institute for Animal Experimentation. A total of 35 monoclonal antibodies to envelope glycoprotein and nucleocapsid protein (Arikawa *et al.,* 1989; Ruo *et al.,* 1991; Yoshimatsu *et al.,* 1996) were used to characterize the antigenicity of Gou3 and NC167 in the IFA test.

Focus reduction neutralization test

The FRNT was carried out using a previously described procedure (Kariwa *et al.,* 1995). Briefly, 25 μl of serial twofold dilutions of serum were mixed with an equal volume of virus suspension, which contained 50 FFU of virus, at 37°C for 60 min. They were then inoculated onto Vero E6 cell monolayers in 8-well glass slides and incubated at 37°C for 60 min in a CO_2 incubator. After the inoculum was removed, the cells were overlaid with medium containing 1.5% carboxymethyl cellulose. After incubation for 2 days, the residual virus infectivity was determined by indirect immunofluorescence staining, as described above. The FRNT titer was expressed as the reciprocal of the highest serum dilution resulting in a reduction in the number of infected cell foci greater than 80% inhibition.

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