Molecular Epidemiological and Serological Studies of Hantavirus Infection in Northern Vietnam

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ABSTRACT. The distribution of anti-hantavirus antibodies in humans and rodents in northern Vietnam was examined. In total, 837 serum samples from healthy humans (617) and patients with fever (220), living in six different areas were screened for IgG antibodies against Hantaan or Seoul virus (SEOV) by ELISA, IFA, and Western blot analysis. Antibody-positive sera were identified in 7/617 (1.1%) healthy donors, 5/150 port workers in the port of Hai Phong, and 2/185 residents of Ha Nam Province. In comparison, positive sera were detected in 5/220 (2.3%) fever patients in the provinces of Ha Nam (1/58) and Thanh Hoa (4/146). Antibody-positive *Rattus norvegicus* were found in the provinces of Ha Nam (7/52) and Thanh Hoa (1/67), in Haibatrung District (7/43) in Hanoi, and in Hai Phong Port (21/62), while antibody-positive *R. rattus* (2/17) were found in Hai Phong Port. Part of the Gc region from the viral genome was amplified by RT-PCR using lung tissue samples from *R. norvegicus* in Haibatrung (2/7) and Hai Phong Port (7/9), but not from *R. rattus* (0/2). Viral sequences were located in the SEOV clade and formed a single lineage with Indonesian SEOV, suggesting that Vietnamese SEOV is part of a distinct lineage among Asian SEOVs.

KEY WORDS: HFRS, *Rattus*, rodent, Seoul virus, zoonosis.

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Hantaviruses are enveloped RNA viruses that belong to the *Hantavirus* genus in the *Bunyaviridae* family. The hantavirus virion contains three negative-sense RNAs, designated S, M, and L, based on their relative sizes; they encode a nucleocapsid protein (N), enveloped glycoproteins (Gn and Gc), and an RNA-dependent RNA polymerase (L protein), respectively [8].

Hantaviruses are found primarily in rodents and are the causative agents of two severe viral zoonoses, hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS). A close relationship exists between each viral species and its particular rodent reservoir species; thus, endemic areas of HFRS and HPS are confined to areas inhabited by the reservoir rodents. Consequently, HFRS exists throughout Eurasia, while HPS is found in North and South America [23].

The hantaviral species that have been causally associated with HFRS, Hantaan virus (HTNV), Seoul virus (SEOV), and Dobrava/Belgrade virus (DOBV), are carried by rodents in the subfamily Murinae (Old World rats and mice), while Puumala virus (PUUV) is carried by rodents in the subfamily Arvicolinae (voles and lemmings) of the family Muridae [20].

The total number of HFRS cases per year is about 60,000–150,000, but more than 90% of these cases occur in East Asian countries, including China, Russia, and Korea [14]. HFRS is caused by HTNV, which is carried by striped

field mice (*Apodemus agrarius*), SEOV, which is carried by brown rats (*Rattus norvegicus*), and PUUV, which is carried by voles (*Myodes* spp.) [23]. Epidemiological and epizootiological studies have shown that hantavirus infections spread in both humans and rodents in South and Southeast Asia [6, 9, 22]. Because most of the affected animals are *Rattus* spp., SEOV infections introduced by infected brown rats through international freight transportation is suspected. However, the existence of different hantaviruses has also been reported. For example, Thailand virus (THAIV) is the only hantavirus species carried by *Bandicota indica*, of the subfamily Murinae, in Thailand [9], while Thottapalayam virus (TPMV), which was isolated from insectivore mammals in India [5], is antigenically and genetically quite distant from other rodent-derived hantaviruses. Several studies have reported antibody-positive human sera against THAIV [19, 27] and TPMV [18] in Thailand. Further, Cao Bang virus was isolated from a Chinese mole shrew (*Anourosorex squamipes*) captured in Cao Bang Province in Vietnam [26]. These data indicate that various hantavirus species are circulating throughout South and Southeast Asia. Because the clinical symptoms of leptospirosis and other febrile illnesses are similar to those of HFRS, undiagnosed cases of HFRS may exist in patients with febrile illnesses of unknown origin (FUO). However, limited data are available regarding human and rodent infections with hantaviruses in South and Southeast Asia.

In this study, human and animal sera derived from various districts of Vietnam were screened for evidence of hantavirus infection. Additionally, hantavirus genomes were amplified from lung tissue collected from rodents captured

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in hantavirus-positive areas. Finally, the relationship between Vietnamese hantaviruses and other hantaviruses found in Asia was analyzed phylogenetically.

MATERIALS AND METHODS

Viral strains and cells: HTNV strain 76–118 [15], SEOV strain SR-11 [11], THAIV strain Thai749 [9], TPMV strain VRC-66412 [5], and PUUV strain Sotkamo [4] were used as representative strains of HTNV, SEOV, THAIV, TPMV, and PUUV respectively. All of the viruses were propagated in Vero cells (clone E6; ATCC C1008) prior to their molecular and antigenic characterization or use in a focus reduction neutralization test (FRNT).

Human sera and methods for antibody detection: In total, 837 serum samples were obtained from healthy individuals or patients. The sera were identified by numbers to prevent subject identification. The use of the sera for the investigation of fever of unknown origin (FUO) in Vietnam was explained to the blood donors orally. All blood donors provided informed consent. The FUO were initially suspected to be dengue fever; however, they were seronegative for dengue virus. Sera were collected in the Vietnamese provinces of Bac Giang, Hai Phong, Ha Nam, Ha Noi, Hoa Binh, and Thanh Hoa (Table 1, Fig. 1). Screening for anti-hantavirus IgG was performed by ELISA at a serum dilution of 1:200. Recombinant N proteins of HTNV, PUUV, and TPMV were expressed by a recombinant baculovirus system and designated rNP-HTNV, rNP-PUUV and rNP-TPMV, respectively. The three different rNPs were used as ELISA antigens, as described previously [18, 19]. Baculovirus-expressed bornavirus P24 antigen was used as a negative control. All positive sera were subjected to Western blotting using recombinant SEOV antigen and indirect immunofluorescent antibody (IFA) testing, using SEOV-infected Vero E6 cell antigen, as previously described [31]. Those sera confirmed as positive by ELISA, Western blotting, and IFA were subjected to serotyping ELISAs to determine the type of hantavirus [3, 17]. Briefly, the serum samples were diluted 1:200 and then applied to microtiter plate wells coated with truncated N antigen lacking 49 amino acids from the N-terminal end. The truncated N antigens used for serotyping ELISA were prepared from HTNV, SEOV, and THAIV by a recombinant baculovirus system and were designated HTNV50, SEOV50, and THAIV50, respectively. Screening for virus-reactive IgM was performed by μ -capture ELISA using recombinant N protein from HTNV, as described previously [16]. Sera from four HFRS patients who had been previously diagnosed with HTNV, SEOV, THAIV, and PUUV, by FRNT, were used as positive controls, while negative human control sera (NHS), which were confirmed to contain no antibodies against hantaviruses, were used as negative controls.

Animal sera and antibody detection: In total, 442 serum samples were obtained from *Rattus* spp. or shrews (*Suncus murinus*) captured at six locations in Vietnam between 1998 and 2006 (Table 2, Fig. 1). The sera were screened by indi-

Table 1. Results of serological screening for hantaviruses in human sera

Location	Healthy		Patient	
	Tested	Positive	Tested	Positive
Bac Giang	20	0	—	—
Hai Phong*	150	5	16	0
Ha Nam	185	2	58	1
Ha Noi	73	0	—	—
Hoa Binh	14	0	—	—
Thanh Hoa	175	0	146	4
Total (%)	617	7 (1.1)	220	5 (2.3)

* Healthy port workers of Hai Phong port belonged to the “healthy” group of Hai Phong province. The patient group of Hai Phong province did not contain patients from Hai Phong port workers. Numbers in parentheses indicate the percentage positive.

Fig. 1. Map showing the collection sites for the human serum samples and trapping sites for the rodents in Vietnam (•).

rect IgG ELISA using *Escherichia coli*-expressed His- and NUS-tagged partial N protein (103 amino acids from the N-terminus) from HTNV strain 76–118 or full-length N protein from TPMV, expressed using a pET43.1 vector system (Novagen) [18]. The sera were then diluted 1:200 and screened by ELISA. The presence of bound antibodies was detected using a horseradish peroxidase-conjugated goat

Table 2. Trapping sites, collected rodent species, and seropositivity for hantavirus

Location	Species	No. Positive (PCR positive/tested)	No. Tested (% positive)
Ha Nam	<i>Rattus norvegicus</i>	7	52 (13.5)
Thanh Hoa	<i>R. norvegicus</i>	1	67 (1.5)
Tay Nguyen	<i>R. norvegicus</i>	0	38
	<i>R. exulans</i>	0	86
	<i>Suncus murinus</i>	0	22
	<i>R. hosaensis</i>	0	5
	<i>R. rattus</i>	0	1
Vin Phuc	<i>R. norvegicus</i>	0	2
	<i>R. rattus</i>	0	21
Ha Noi	<i>R. norvegicus</i>	7 (2/7)	43 (16.3)
Haibatrung District	<i>R. rattus</i>	0	7
	<i>S. murinus</i>	0	19
Hai Phong Port	<i>R. norvegicus</i>	21 (7/9)	62 (33.9)
	<i>R. rattus</i>	2 (0/2)	17 (11.8)
Total		38	442

anti-rat IgG antibody (Zymed Laboratories Inc.) or Protein A (Prozyme). The antibody-positive sera were then subjected to Western blotting using recombinant hantavirus N proteins from HTNV strain 76–118 and baculovirus-expressed TPMV (strain VRC-66412), as described previously [18, 32], and IFA using HTNV strain -76–118- and TPMV strain VRC-66412-infected Vero E6 cells as antigens. As positive controls, three serum samples from Wistar rats that had been experimentally infected with SEOV strain SR-11 were used. As negative controls, sera from five wild-trapped, uninfected rats from Japan were used [29]. For the shrew sera, experimentally infected and uninfected shrew serum samples were used as positive and negative controls [18].

IFA assay: The IFA assay was performed as described previously [31]. Briefly, acetone-fixed monolayers of Vero E6 cells infected with hantavirus were used as antigens. To detect the antibodies bound to antigen, FITC conjugated anti-rat, anti-human, and Protein A were used, as described previously [13]. These serum samples (1:100 dilution) showing characteristic fluorescence in infected Vero cells, but that were negative with uninfected Vero cells, were regarded as positive.

PCR, nucleotide sequencing, and phylogenetic analysis: Total RNA isolated from rat lung tissue was used to produce hantavirus-specific cDNA, as described previously [30]. Nucleotides (nt) 2000–2300 of the M genome segment were amplified using the primers SEOMF1936, (5'-gtgactcttcttctattatt-3') and SEOMR2353 (5'-tgggcaatctgggggtgcatg-3'). Similarly, nt 2000–3101 were amplified from the M genome segment using the primers SEOMF1936 and M12 (5'-f-AACCACTATGGCCACCTTTC-3'). All products of the expected size were purified using a PCR purification kit (Qiagen) and sequenced using the original PCR primers. Sequencing was performed using a BigDye Termini-

nator Cycle Sequencing Kit (ver. 3.1; Perkin Elmer) with a model 3100 DNA Sequencing System (Perkin Elmer) by Hokkaido System Science Co., Ltd. The sequences were aligned using CLUSTALW and Genetyx Mac (ver. 13.0.6) with default parameters (gap insert penalty, -12; gap extend penalty, -4). In subsequent phylogenetic analyses, neighbor-joining (NJ) phylogenetic trees and bootstrap analyses were calculated by Genetyx Mac (ver. 13.0.6) and/or Phylip (ver. 3.65).

Species specification by sequencing of mitochondrial cytochrome b: Total DNA, isolated from rat lung tissue using DNAzol Reagent (Invitrogen), was subjected to PCR to amplify the entire coding region of cytochrome *b* (*cytb*; 1140 bp). The products were subsequently sequenced, as previously described [28].

RESULTS

Twelve of 837 human serum samples were positive by all three tests, ELISA, IFA, and Western blot, and were regarded as hantavirus-infected cases (Table 1). None of the samples were positive for PUUV or TPMV by ELISA (data not shown). Additionally, none of the samples contained IgM antibodies against HTNV (data not shown). The positive samples were collected in the provinces of Ha Nam, Thanh Hoa, and Hai Phong (Fig. 1). The positive rate among the febrile patients (2.3%) was roughly twice that among the healthy donors (1.1%).

ELISA was used to estimate the serotypes of the Vietnamese hantaviruses in the positive cases (Table 3). Two of the four positive samples from Thanh Hoa Province also showed a clear SEOV infection pattern. These results indicate the presence of an SEOV, whose rodent host belonged to the genus *Rattus* in the Hai Phong Port area and Thanh Hoa Province. However, the other two samples, 40B and

Table 3. Serotyping of antibody-positive human sera found in Vietnam by ELISA

Location	Serum ID	Status	ELISA OD				Determination
			rN	HTNV50	SEOV50	THAIV50	
Hai Phong	192	H	1.343	0.252	0.636	0.286	SEOV
Hai Phong	198	H	0.745	0.372	0.806	0.392	SEOV
Hai Phong	258	H	0.572	0.002	0.413	0.026	SEOV
Hai Phong	322	H	0.572	0.017	0.123	0.024	SEOV
Hai Phong	366	H	1.066	-0.002	0.413	0.026	SEOV
Ha Nam	75	P	0.370	-0.007	-0.017	-0.024	NI
Ha Nam	12	H	0.800	0.005	-0.028	-0.038	NI
Ha Nam	33	H	0.424	0.046	0.061	-0.041	NI
Thanh Hoa	63	P	0.343	0.035	0.240	0.032	SEOV
Thanh Hoa	38	P	0.317	0.106	0.188	0.044	SEOV
Thanh Hoa	40B	P	0.325	0.098	0.104	-0.002	NI
Thanh Hoa	149	P	0.298	0.089	0.113	-0.021	NI
HTNV antibody-positive			1.166	1.177	0.204	0.130	HTNV
SEOV antibody-positive			1.373	0.476	1.086	0.569	SEOV
THAIV antibody-positive			1.022	0.343	0.316	0.853	THAIV
Negative human sera-1			-0.008	-0.019	-0.013	-0.013	Negative
Negative human sera-3			-0.018	-0.019	-0.023	-0.009	Negative

Status H, healthy; status P, patient; NI, not identified.

149, showed virtually identical OD values, based on ELISA, when HTNV50, SEOV50, and THAIV50 were used as the serotyping antigens. Additionally, three of the positive samples from Ha Nam Province showed low OD values with three of the serotyping antigens, although they reacted strongly to full-length rN antigen from HTNV.

As shown in Table 2, 38 (8.6%) of the animal samples were antibody-positive in the three tests, ELISA, IFA, and Western blot. All but two of the positive samples were from *R. norvegicus*. The highest positive rate (33.9%) was observed among *R. norvegicus* captured at Hai Phong Port, in a warehouse. Two *R. rattus* captured in the warehouse were also antibody-positive. All of the sera were antibody-negative for PUUV, based on ELISA (data not shown). These results suggest that in Vietnam, hantavirus exists primarily in the brown rat (*R. norvegicus*).

The two antibody-positive *R. rattus* captured in the Hai Phong Port warehouse were classified as *R. rattus flavipectus*, based on morphological characteristics. To determine their classification genetically, the *cytb* gene from the mitochondrial DNA of the rats was sequenced and compared to representative sequences from other *Rattus* spp. [28]. As shown in Fig. 2A, the sequences obtained from three rats [all *R. rattus flavipectus*: #42 and #82 (antibody-positive) and #72 (antibody-negative)] were of the same lineage as those obtained from *R. tanezumi*, captured in Japan and China.

Among the seropositive brown rats (*R. norvegicus*) captured in Haibatrung and Hai Phong Port, 2 of 7 and 7 of 9 lung specimens, respectively, from the seropositive rats were positive for virus RNA, based on PCR. However, no part of the hantavirus genome was amplified from lung tissue collected from seropositive *R. rattus flavipectus* (*R. tanezumi*) captured at Hai Phong Port, although the two species of *Rattus* were found in the same warehouse as the positive

R. norvegicus.

Among the PCR-positive specimens obtained from *R. norvegicus* captured in Hanoi Haibatrung District and the Hai Phong Port area, two and four specimens, respectively, were selected and the partial nucleotide sequences of their M genomic segments were compared. First, the Vietnamese hantaviruses were compared, based on short sequences from part of the Gc coding region, to various hantavirus strains. As shown in the phylogenetic tree drawn using 271nt of the M genomic segment (Fig. 2B), the Vietnamese hantaviruses were all found to belong to the SEOV clade. The Vietnamese viruses and Indonesian SEOV (Jakarta) [21] formed one group within the SEOV clade; thus, SEOV strains originating from Southeast Asia form a distinct lineage among SEOVs. To confirm the relationship among the Vietnamese and other SEOVs, a phylogenetic tree was drawn based on a longer sequence. As shown in Fig. 2C, both of the Vietnamese SEOVs obtained from Hai Phong and Hanoi Haibatrung District belong to the same SEOV cluster. In this tree, strain B1, which was isolated in Osaka, Japan, was located outside of the Vietnamese virus, alongside other Japanese strains obtained from Hokkaido in northern Japan, and it made a distinct cluster with the Korean SEOV. Another distinct cluster consisted of the Chinese SEOV.

DISCUSSION

Through serological examination of human sera for hantavirus infection, 12 antibody-positive sera were found from northern Vietnam. These results indicated that hantaviruses related to HTNV/SEOV are currently circulating in Vietnam. Roughly, a two-fold higher antibody positive rate was obtained among FUO patient sera compared with those of healthy donors. However, no statistically significant differ-

Fig. 2. Phylogenetic analysis of the hantaviruses derived from Haibatrung District and the Hai Phong Port area. A: NJ analysis of the mitochondrial *cytb* gene (7). Sequences of *Rattus argentiventer* (AB033701), *R. norvegicus* (AB033713), *R. rattus* [Oceanian-type black rat captured in Otaru, Japan (AB211039), and Indonesia (AB033702)], Asian-type black rats (*R. rattus tanezumil*) captured in Japan (AB211040 and AB211041), and black rats captured in Thailand (unpublished data) and the Philippines (DQ191488) were used as reference sequences. *Mus musculus* (AB125774) was used as an outgroup. The accession numbers for *cytb* from the black rats captured in Hai Phong Port (#42, #72, and #82) were AB355899–AB355901, while those of the brown rats (#16 and #20) were AB355902 and AB355903, respectively. B: Phylogenetic analysis of hantavirus based on 271 nt from the Gc region. Sequences of SEOV strains Gou3 (AB027521), Beijing-Rn (AB027087), Guang199 (AB027086), HN71 (AB027084), HB55 (AF035832), NM39 (AB027080), L99 (AF035833), J12 (AB027082), Wan (AB027081), Shanxi (AB027085), KI83 (D17592), SR-11 (M34882), Girard Point (U00464), Jakarta (AJ620583), B1 (X53861), C3 (AB027088), and Hebei4 (AB027089). SD10 (AB027092), SD227 (AB027091), Hebei1 (S72343), Seoul 8039 (S47716), and Tchoupitoulas (U00473) were used. Sequences from representative hantaviruses, HTN (M14626), DOBV (AJ009773), and THAIV (AB186420), were used. C: Phylogenetic analysis of hantavirus based on 1101 nt from the Gc region. Sequences of SEOV strains Gou3 (AB027521), L99 (AF035833), Z37 (AF190119), Seoul 8039 (S47716), KI83 (D17592), SR11 (M34882), B1 (X53861) and THAIV (L08756), DOBV (AJ009744), and HTNV (M14627) were used. Gou3 was used as an outgroup. The sequences of Vietnamese SEOV derived from brown rats captured in the Hai Phong Port area (#7, #16, #20, and #28) and in Hanoi (#9 and #25) correspond to AB355728–31, and AB355732–3, respectively.

ence was shown between healthy and patient groups in a χ^2 test or Fisher test. Additionally, because no hantavirus-IgM antibody-positive serum was detected from IgG-positive sera, the relationship between FUO and hantavirus infection remains unclear. Positive sera from Ha Nam and two from

Thanh Hoa could not be serotyped using the three serotyping antigens, HTNV50, SEOV50, and THAIV50. These positive human sera strongly reacted with rNP-HTNV, but were negative with PUUV (data not shown). Thus, these cases were considered to be infected with a novel hantavi-

rus, which might be related to Murinae-rodent associated hantaviruses, such as HTNV, SEOV, and THAIV. Previous studies have shown that hantaviruses belonging to three Murinae-associated species (HTNV, SEOV, THAIV) exist in East and Southeast Asia [10]; thus, serotyping antigens capable of distinguishing these three hantavirus species were used. All of the antibody-positive samples from the Hai Phong Port area showed higher OD values to the SEOV50 antigen than the other serotyping antigens (HTNV50 and THAIV50; Table 3). Thus, they were considered to be infected with SEOV. Generally in the port area, the brown rat (*R. norvegicus*) was found as the host rodent for SEOV. Also, in the Hai Phong Port area, antibody-positive brown rats and black rats were found (Table 2). To identify the host-virus relationship, the mitochondrial *cytb* gene and hantavirus genome from lung tissues of rats were examined. As shown in Table 2, the hantavirus genome was amplified from antibody-positive brown rats, but not from lung tissue of black rats. Further, the sequences were identified as brown rat-borne SEOV, different to the strain Gou3 sequence, which was a black rat-borne SEOV in China (30) (Fig. 2B, 2C). These results suggested that in the Hai Phong Port area, SEOV has spread from brown rats to black rats. These brown rats may also be the source of SEOV infection in human port workers in Hai Phong port. Also, the SEOVs circulating in Hai Phong port belonged to a distinct Vietnamese SEOV clade, with the SEOV derived from the Hanoi Haibatrung district.

Phylogenetic analysis of the *cytb* gene of rats also revealed at least three varieties among the black rats (*R. rattus*) in Asia: *R. tanezumi* (Oceanian black rat), *R. rattus* in Indonesia and Japan (European-type black rat), and *R. rattus* in the Philippines and Thailand. The black rats, classified to *R. rattus flavipectus* morphologically, that were captured in Hai Phong port, were classified as *R. tanezumi* by the *cytb* gene system. Novel hantaviruses were recently detected in black rats from China [30] and Cambodia [22]. Thus, to examine the relationship between hantaviruses and their reservoir rodents, rodent classification using the *cytb* gene system should be adopted.

None of the *R. rattus (tanezumi)* captured in Hai Phong Port warehouse were positive by PCR, although the *R. norvegicus* captured in the same warehouse were highly PCR-positive. This different PCR positivity between *R. norvegicus* and *R. rattus (R. tanezumi)* suggested that the principal host for Vietnamese hantavirus is *R. norvegicus*. *R. rattus (R. tanezumi)* was probably transiently infected, via a spill-over event from *R. norvegicus*. Primers used in this study were also compatible with the *R. tanezumi*-borne virus, strain Gou3, from a previous report [30]. However, the possibility that this primer set was not able to amplify the *R. tanezumi*-borne virus gene found in Hai Phong port still remains.

As shown in Table 3, three of the positive samples from Ha Nam Province showed low OD values with the three serotyping antigens, although they reacted strongly to full-length rN antigen from HTNV. The low reactivity of the

three Ha Nam cases, as well as the 2 unserotyped cases from Thanh Hoa, suggests the possibility of the existence of a novel species of hantavirus. As all of the positive sera were antibody-negative for PUUV, the novel species may be a Murinae-associated hantavirus.

Throughout the serological surveillance of small animals, numerous *S. murinus*, the natural host for TPMV [5], were captured. The presence of anti-TPMV antibodies was also examined by ELISA; however, all of the samples from the rats and *S. murinus* were negative. Recently, genetically distinct rodent-borne hantaviruses were detected in small mammals belonging to various species of Soricomorpha worldwide [1, 2, 12, 24–26]. Because these hantaviruses are phylogenetically distinct from TPMV, the antigenicity of TPMV may also differ from that in other soricomorph-borne hantaviruses. Thus, further serological surveillance for TPMV and other hantaviruses, including soricomorph-borne hantaviruses, is necessary.

In conclusion, we found that SEOV is circulating in northern Vietnam, in both humans and rodents; however, the consequence of SEOV infection as a cause of HFRS remains unclear. The Vietnamese SEOV is phylogenetically distinct from SEOVs originating in other regions, suggesting that Southeast Asian SEOVs form a separate cluster. As the existence of novel hantaviruses was also suggested, additional epidemiological and epizootiological studies are required to clarify the variation in, and distribution of, hantaviruses in East and Southeast Asia.

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