Table 1 (*continued*)

Virus species	Strain	Abbreviation	Principal species	Original isolation in	Geographical distribution of reservoir	Disease
	Sin Nombre virus-NMH10 virus	SNV	*Pelomyscus maniculatus*	US		
Arvicolinae subfamily-associated viruses						
Prospect Hill virus	Bloodland Lake virus	BLLV	*Microtus ochrogaster*	USA, Canada		?
	Prospect Hill virus	PHV	*Microtus pennsylvanicus*	USA, Canada		
Isla Vista virus		ISLAV	*Microtus californicus*	Western USA		?

interstitial infiltrates is also common. After the prodromal period, the clinical course of patients with severe disease can be divided into five phases: febrile, hypotensive, oliguric, diuretic and convalescent [29]. A more mild type of hantavirus infection, nephropathia epidemica (NE), is caused by PUUV and occurs in northern Europe [30]. In NE patients, although renal manifestations are common, haemorrhage is rare and the five phases typical of severe HFRS are absent. The mortality of NE patients is 0.1–0.3% and is thus much lower than the 5–10% of HFRS patients infected with HTN, SEO or DOB viruses [9].

HPS is characterised by bilateral interstitial pulmonary infiltrates, respiratory compromise usually requiring the administration of supplemental oxygen and clinical symptoms resembling those of ARDS. HPS can be divided into two phases: a prodromal phase, which usually lasts 3–5 days, and a cardiopulmonary stage marked by diffuse pulmonary oedema and hypotension within 2–5 days after the onset of pulmonary symptoms. The rapid progression of interstitial pulmonary oedema to alveolar oedema, with severe bilateral involvement and the accumulation of pleural effusion, accounts for the 30–40% mortality associated with HPS [29].

Although the characteristic symptoms of HFRS and HPS differ, increased capillary permeability is considered to be the common underlying factor of the two diseases [31,32]. Since hantavirus is usually non-cytopathogenic in cultured cells, cell-mediated immune responses, such as activation of virus-specific CD8+ T cells and increased levels of tumour necrosis factor receptor (TNF-r), interleukin (IL-6 and IL-10) are most likely responsible for the symptoms observed in HFRS and HPS [33].

3.2. Disease in animals

All hantavirus reservoir animals are rodents, with the exception of TPMV [11], whose reservoir is *S. murinus*, a member of the order Insectivore. As is the case for

many other zoonoses, all natural reservoirs of hantavirus are asymptomatic following infection, and in many of these animals, a persistent infection is established, although the animals possess high titres of neutralising antibody [18]. The suppression of cellular immunity has been considered as the most plausible mechanism behind the maintenance of persistent infection [34] but this has yet to be determined conclusively due to the technical difficulties in immunologically characterising wild rodents. However, hamsters infected with the Andes virus, which belongs to the genus *Hantavirus*, manifest symptoms similar to those associated with HPS, such as pulmonary oedema, accumulation of pleural exudates and high mortality [35]. Therefore, the hamster model has provided a useful tool for studying the mechanisms of HPS pathogenesis.

Although few reports exist on hantavirus infection of *Mus musculus* in nature, laboratory mice are highly susceptible [36]. In particular, newborn mice [37] and immunologically defective animals, such as nude mice [38] and SCID mice [39,40], were found to be vulnerable to systemic infection with a fatal outcome, whilst surviving mice became persistently infected, with suppression of the virus-specific CD8 T-cell response [34]. This observation supports the suppression of cellular immunity as the mechanism establishing persistent infection of hantavirus reservoir animals.

4. Epidemiology and epizootiology of HFRS in Asian countries

Since more than 90% of HFRS cases have been reported in eastern Asia, including China, the far-eastern parts of Russia, and Korea, epidemiological and epizootiological studies have mainly been conducted in those countries. Accordingly, this section will first review the results of those studies and then summarise what is known about hantavirus infection in other East Asian countries, particularly in Southeast Asia.

4.1. East Asia

The majority of reported cases of hantavirus infection in East Asia have come from China [41]. HFRS was first recognised in northeastern China in 1931 as a febrile disease with haemorrhagic manifestation and renal dysfunction. Beginning in 1955, the areas in which the disease was endemic expanded to many other parts of the country. The number of cases of HFRS in China from 1950 to 1997 was 1,258,402. From 1990 to 1997, the number of cases, morbidity (per 100,000) and number of deaths (fatality %) were 391,046 (4.21%) and 6310 (1.61%), respectively. More recent reports examined the distribution of HFRS cases for all of mainland China [42]. From 1994 to 1998, 257,127 cases of HFRS were reported; these were documented in 28 of 31 provinces and in 1397 of the country's 2359 counties. However, spatial analysis indicated hot spots, i.e., large ($>10{,}000\,km^2$) areas where the risk of HFRS risk was higher, in Shandon, Hebei, Heilongjiang, Hunan, Zhejiang, Jiangxi and Guangxi provinces. Furthermore, in several smaller areas ($<10{,}000\,km^2$) the risk of HFRS was also found to be higher. These areas were

located in provinces of central, eastern and northeastern China. Therefore, as an infectious disease, HFRS is an important public health concern throughout China.

A nationwide study of the epidemiology of HFRS conducted between 1984 and 1986 indicated that 67 species of vertebrates were infected with hantaviruses. Most of those animals were rodents, but several species of domestic animals, including cats, pigs, rabbits and dogs were reported [41]. Antigenic and genetic characterisations of the hantaviruses isolated from HFRS patients and from rodents in China suggested that two different hantaviruses, HTNV and SEOV, and viruses related to them, were co-circulating in endemic areas [43]. Phylogenetic analysis based on the nucleotide sequences of the S and M genome segments of 46 hantaviruses from China, 13 from patients, 23 from rodents and 10 from unknown hosts, were compared with hantaviruses from Korea, Japan, the US and Europe [44]. The HTNV type was divided into nine distinct genetic subtypes, one consisting of isolates from Korea. The Da Bie Shan virus strain NC167 of HTNV, isolated from a Chinese white-bellied rat (*Niviventer confucianus*) captured in a mountainous area of Anhui Province, was the most distant from the remaining HTNV strains. A comparison of the M segment of HTNV with those of eight other genotypes revealed 24.1–25.0% nucleotide and 15.3–16.2% amino-acid sequence differences. By contrast, the nucleotide and deduced amino-acid sequence differences in the M segments of the remaining HTNV strains were 5.4–15.4% and 1.8–4.6%, respectively. Strain Da Bie Shan NC167 and HTNV strain 76118, which is a prototype of HTNV, also showed more than fourfold two-way differences in cross-neutralisation tests. Based on these findings, strain Da Bie Shan NC167 was classified as a novel type of hantavirus [44] (Fig. 1).

A correlation between the subtype and province of origin was established for seven of the nine genetic subtypes of HTNV, which suggests immigration of the reservoir rodent of ancestral HTNV from the southern province of Guizho to three northern provinces (Helongjian, Shandong and Zhejiang) and to Korea. SEOV is also divided into five genetic subtypes, but the variability amongst them is smaller than that between HTNV subtypes. Of those five subtypes, the Gou3 virus, isolated from *Rattus rattus*, showed the greatest divergence from the other four SEOV subtypes, which were isolated from *Rattus norvegicus*. The diversity in the M segment nucleotide sequence and the Gn/Gc amino-acid sequence between Gou3 and the other SEOVs was 15.4–16.0% and 3.1–3.6%, respectively, compared to 3.5–1.9% and 0.5–1.1%, amongst the other SEOVs [44].

Far-eastern Russia is also an area where hantaviruses are endemic. The first case of HFRS in Asian Russia was reported in 1934, in the Khabarovsk region [45]. Since then, many other cases of HFRS have been reported throughout Russia. Although the majority of cases (84,687) reported between 1978 and 1995 occurred in the European part of the country, several different hantaviruses are known to be present in far-eastern Russia and to cause HFRS. In fact, from 1978 to 1995, 3145 cases were registered in 15 of the 29 regions of Asian Russia [46].

In northeastern China, HTNV carried by *Apodemus agrarius* caused several severe cases of HFRS. PUUV infection has been found amongst *Clethrionomys rufocanus* in Asian Russia and may be the cause of a milder form of HFRS. Recently, Amur virus

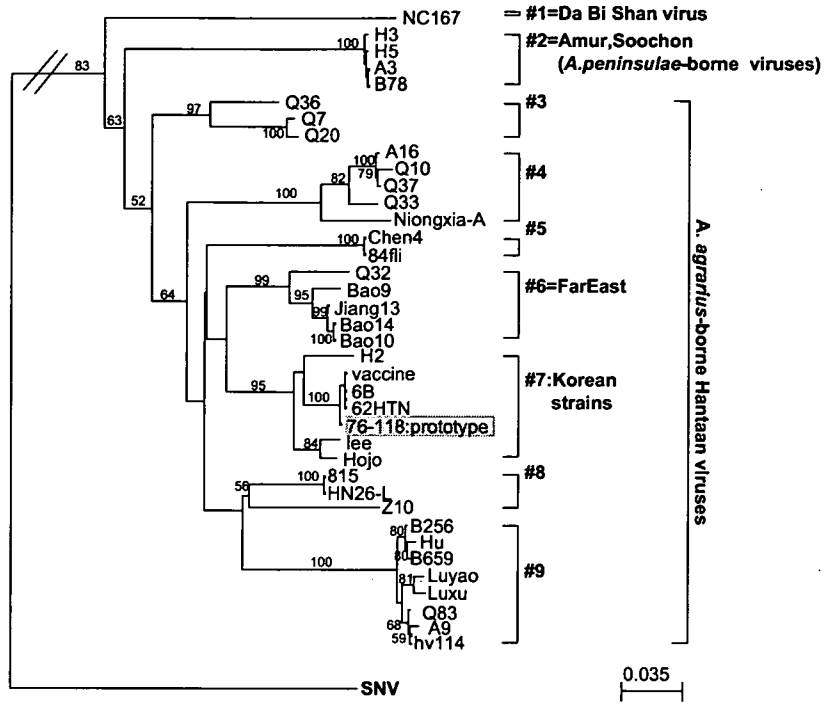

Fig. 1. Phylogenetic tree for *Apodemus*-borne hantaviruses. The neighbour-joining phylogenetic analysis was performed based on the partial sequences of the M (nucleotides 2001–2301) segment. The numbers at the nodes are bootstrap confidence levels for 100 replicates. Only bootstrap confidence levels more than 50% are shown [44].

and Far East virus, which are antigenically and genetically related but distinct from HTNV, were isolated from a patient with severe HFRS. An epizootiologic study indicated that the reservoir animals of the two viruses were *Apodemus peninsulae* and *A. agrarius*, respectively [47,48]. The deduced amino-acid sequences of the S and M segments of Amur virus showed 96.7% and 92.0–92.2% similarities with HTNV, respectively. For the Far East virus, the similarities were 99.1% and 97.9% in the S and M segments, respectively.

As noted above, HTNV isolated in China and Korea can be divided into nine distinct genetic subtypes [44]. Phylogenetic analysis of Amur virus and Far East virus together with analyses of other HTNVs indicated that both Amur virus and Far East virus belong to genetic subtypes that were previously identified amongst Chinese HTNVs. Two of the Chinese isolates with the same genetic subtype as Amur virus and the prototype HTNV strain 76118 showed more than a fourfold difference in cross-neutralisation tests to immune sera and to *A. peninsulae* sera obtained from an animal infected with Amur virus [49]. Therefore, the Amur virus and related viruses isolated in China may constitute a distinct serotype within the genus *Hantavirus* (Fig. 1). Recently, a new hantavirus, Khabarovsk virus, was isolated in reed voles

(*Microtus fortis*) in far-eastern Russia. Although the Khabarovsk virus is genetically most closely related to Puumala virus, its relationship to HFRS is still unclear [50].

In Korea, HTNV was first isolated in 1976 from the lung tissues of *A. agrarius*, captured along the Hantaan River [14]. With the eventual development of a hantavirus vaccine, the number of patients has decreased [51–53]; however, approximately 100–300 cases of HFRS, with an overall mortality of 4.5%, are reported annually [54]. Amongst all reported cases, approximately 70% and 20% are considered to be caused by HTNV and SEOV, respectively. In the remaining 10% of cases, the causative hantavirus has not been identified. Recently, a new hantavirus, designated Soochon virus, was isolated from *A. peninsulae* captured in the mountains of east-central Korea [54]. Phylogenetic analysis based on the almost entire sequences of the S and M segments indicated that Soochon virus is distinct from the HTNV virus of *A. agrarius*. Soochon virus is a member of the same clade as Amur virus and related viruses isolated in China, indicating that they shared a common ancestor virus. The results of cross-neutralisation tests with Soochon virus were similar to those obtained with the other viruses, suggesting that *A. peninsulae* HTNVs constitute a new hantavirus serotype.

An epidemiologic and epizootiologic study conducted in Russia, China and Korea indicated that *Apodemus*-borne HTNVs are the main causative agents of severe HFRS, although antigenic and genetic variability exist with respect to geographical distribution [48].

Although Japan borders those countries where HFRS is endemic, no large epidemics of the disease have been reported [55,56]. Both the urban rat (*R. norvegicus*) and laboratory rats were associated with cases of HFRS in the 1960s [57] and in the 1970s until 1984 [58], respectively, and during both episodes, the Seoul-type hantavirus was the causative agent. In the urban rat-mediated infections, 119 cases, two of which were fatal, were reported in an area with poor sanitary conditions. The endemic disappeared after conditions were improved. Infections associated with laboratory rats were reported by 21 institutions throughout Japan, and involved 126 cases, including one death. Since 1984, no cases of HFRS have been reported. However, SEOV-infected brown rats have been detected throughout Japan, mainly at port areas, and PUUV-infected grey red-backed voles (*C. rufocanus*) were identified in Hokkaido, northern Japan [59]. The limited number of seroepidemiological studies of human sera found that seropositive sera had been obtained from workers at a dumping-ground area (Tokyo), members of the Japanese self-defence force (Hokkaido) [55] and patients with hepatitis and renal failure of unknown etiology [60]. The virus was not isolated in any of these cases, but animals that were positive for the virus were suspected to be the sources of the infections. The PUUV genome amplified from the infected *C. rufocanus* in Hokkaido was most related to those obtained from the same species of voles captured in far-eastern Russia [61].

4.2. East Asian countries other than China, Korea and Japan

Seroepidemiological surveys confirmed hantavirus infections of humans and rodents in Taiwan [62,63], Hong Kong [64], Fiji [64], Malaysia [65], India [66],

Indonesia [67], Singapore [68], Sri Lanka [69], Thailand [70–72] and Vietnam [73]. In Myanmar and Australia [64], positive sera were obtained only from humans, whereas in Cambodia only positive rodents were detected [74]. Nonetheless, taken together, it is clear that hantavirus infections affect humans and rodents throughout Asia. Since the sizes of the epidemiologic studies varied depending on the country, the following section will be limited to a brief summary of the results from each country.

In Taiwan, the results of an extensive seroepizootiologic study of hantavirus infection amongst rodents were reported in 2000 [62]. From 1994 to 1995, 5461 rodents belonging to 16 species were collected throughout Taiwan and examined in an ELISA aimed at detecting anti-hantavirus antibodies. The most common host species belonged to the genus *Rattus*. A much higher antibody prevalence was found in urban regions (20%) than in rural areas (5%). Phylogenetic characterisation indicated SEOV infection. Unlike the situation in mainland China, no *A. agrarius* individuals positive for the virus were found (0/67). By May 2006, a total of seven cases of HFRS had been reported. However, the overall prevalence of hantavirus in Taiwan has yet to be examined.

In Thailand, *R. norvegicus* obtained from the port area of Bangkok was reported to be infected with hantavirus, probably SEOV, transported from abroad by ship [70]. In addition, various species of inland rodents are infected with hantavirus. Amongst them, the greater bandicoot rat (*Bandicota indica*) is a main reservoir of the virus, whilst several species of rice-field rats, such as *R. rattus*, *Rattus exulans* and *Rattus losea*, are also natural reservoirs albeit to a lesser extent. The Thailand virus (THAIV), one of the distinct species of virus within the genus *Hantavirus*, was isolated from *B. indica* captured in a village near the western province of Kanchanaburi in 1985 [70]. Therefore, THAIV or related viruses appear to be distributed throughout Thailand. Ten of the 30 sera obtained from residents of the village where virus-infected *Bandicota* was captured showed antibody to hantavirus, which demonstrated that Thailand virus is able to infect humans. However, the virulency of THAIV towards humans has not been determined. Hantavirus has been suspected as one of the pathogens in fevers of unknown origin (FUOs) in Thailand. In the period 1999–2000, 115 cases of FUO were reported in patients admitted to Bangkok Hospital who were examined for antibodies to hantavirus. Paired sera from one patient showed high antibody titres to HTNV by IgG ELISA, IgM ELISA and IFA test. Between 2002 and 2003, 260 paired sera from patients with FUO were collected in Surin Province. One of the sera showed a neutralising antibody titre to THAIV of 1:160, whilst the titres to HTNV and SEOV were less than 1:40. Furthermore, convalescent-phase serum did not contain hantavirus IgM antibody. Since the symptoms of the patient were comparable to those typical for HFRS, THAIV might be an additional causative agent of HFRS [72].

In Cambodia, a recent epidemiological study found that black rats, brown rats, and an unidentified *Rattus* species were infected with SEOV-like virus [74]. Furthermore, the viral genome amplified from seropositive black rats (*R. rattus*) was most related to that of THAIV [72]. Therefore, THAIV or a THAIV-like virus

circulates throughout Indochina and may also represent an additional causative agent of HFRS.

In Malaysia, positive sera were obtained both from renal patients and rats. Amongst the 119 sera from renal patients, 3 were positive for antibodies to HTNV, SEOV or Sin Nombre virus. In 14 of 87 rats, serum antibody titres were highest against SEOV [64].

In Indonesia, of the 94 patients from central Java with FUO, 5 were positive for hantavirus-specific IgM and IgG and another 5 were positive for HTNV- or SEOV-specific IgM, as determined by ELISA [75]. A more recent investigation of rodents, conducted on an island near Jakarta, showed that 43 of 185 *Rattus* sera were positive for SEOV (Dr. Ima, personal communication).

Positive sera, mostly to SEOV, from humans and from rodents were also reported in Vietnam [73]. Eight of 308 sera obtained from healthy people residing in the Haiphong port area and in HaNam Province, in northern Vietnam, were positive for SEOV. Four of 204 serum samples obtained from FOU patients living in the northern provinces of HaNam and ThanhHoa were likewise positive. Positive sera were also detected in *Rattus* individuals captured at the Haniphong port area and in HaNam and ThanhHoa provinces (Dr. Truong Uyen Ninh, National Institute of Hygiene and Epidemiology, Hanoi, Vietnam, personal communication).

In India, a high rate of positivity for hantavirus infection was reported. Of 152 sera obtained from patients with FOU, 23 were positive for IgM to either HTNV, SEOV or PUUV, as shown by ELISA, and 18 were positive by IFA. In sera from 87 healthy donors, two were shown to be positive by IFA [66].

More recently, sera positive for TPMV antibodies were found in 2 of 478 FOU patients in Thailand and in 2 of 14 *Suncus* sera obtained from animals captured on an island near Jakarta. Although these finding provide mainly serological evidence, the data indicate that TPMV is able to infect humans and can be maintained in the musk shrew as its natural host [76].

5. Conclusions

Epidemiological studies have shown that hantaviruses are widely distributed in Asia, both in humans and in rodents. Unlike the situation in Far East Asia, the number of hantavirus antibody-positive sera has so far been quite small, even amongst FOU patients. Therefore, the significance of hantavirus infection as the causative agents for FOU in East Asia remains unclear and further serological surveys amongst healthy people are needed. Nevertheless, these observations indicate that unidentified pathogens that cause FOU are prevalent in this region.

Epizootiologic studies, particularly amongst rodents, have confirmed the close relationship between hantavirus and rodents, which act as reservoirs for the virus. Since it thought that hantaviruses co-evolved with their rodent hosts, an understanding of the virus' ecology may provide unique and important information about other rodent-borne pathogens as causative agents of emerging infectious diseases.

References

[1] Nichol ST, Beaty BJ, Elliott RM, Goldbach R, Plyusnin A, Schmaliohn CS, et al. Bunyaviridae. In: Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA, editors. Virus taxonomy: classification and nomenclature of viruses: eighth report of the international committee on the taxonomy of viruses. 8th ed. San Diego, London, Tokyo: Elsevier Academic Press; 2005. p. 695–716.

[2] Schmaljohn CS, Hasty SE, Dalrymple JM, LeDuc JW, Lee HW, von Bonsdorff CH, et al. Antigenic and genetic properties of viruses linked to hemorrhagic fever with renal syndrome. Science 1985;227:1041–4.

[3] Nichol ST, Spiropoulou CF, Morzunov S, Rollin PE, Ksiazek TG, Feldmann H, et al. Genetic identification of a hantavirus associated with an outbreak of acute respiratory illness. Science 1993;262:914–7.

[4] Xu ZY, Guo CS, Wu YL, Zhang XW, Liu K. Epidemiological studies of hemorrhagic fever with renal syndrome: analysis of risk factors and mode of transmission. J Infect Dis 1985;152:137–44.

[5] Armstrong LR, Zaki SR, Goldoft MJ, Todd RL, Khan AS, Khabbaz RF, et al. Hantavirus pulmonary syndrome associated with entering or cleaning rarely used, rodent-infested structures. J Infect Dis 1995;172:1166.

[6] Tsai TF. Hemorrhagic fever with renal syndrome: mode of transmission to humans. Lab Anim Sci 1987;37:428–30.

[7] Douron E, Moriniere B, Matheron S, Girard PM, Gonzalez JP, Hirsch F, et al. HFRS after a wild rodent bite in the haute-savoie-and risk of exposure to Hantaan-like virus in a Paris laboratory. Lancet 1984;1:676–7.

[8] Elliott RM. The Bunyaviridae. New York: Plenum Press; 1996.

[9] Schmaljohn C, Hjelle B. Hantaviruses—a global disease problem. Emerg Infect Dis 1997;3:95–104.

[10] Plyusnin A, Morzunov SP. Virus evolution and genetic diversity of hantaviruses and their rodent hosts. Curr Top Microbiol Immunol 2001;256:47–75.

[11] Carey DE, Reuben R, Panicker KN, Shope RE, Myers RM. Thottapalayam virus: a presumptive arbovirus isolated from a shrew in India. Indian J Med Res 1971;59:1758–60.

[12] Meyer BJ, Schmaljohn CS. Persistent hantavirus infections: characteristics and mechanisms. Trends Microbiol 2000;8:61–7.

[13] Plyusnin A, Vapalahti O, Lundkvist A. Hantaviruses: genome structure, expression and evolution. J Gen Virol 1996;77:2677–87.

[14] Lee HW, Baek LJ, Johnson KM. Isolation of Hantaan virus, the etiologic agent of Korean hemorrhagic fever, from wild urban rats. J Infect Dis 1982;146:638–44.

[15] Taller AM, Xiao SY, Godec MS, Gligic A, Avsic-Zupanc T, Goldfarb LG, et al. Belgrade virus, a cause of hemorrhagic fever with renal syndrome in the Balkans, is closely related to Dobrava virus of field mice. J Infect Dis 1993;168:750–3.

[16] Avsic-Zupanc T, Toney A, Anderson K, Chu YK, Schmaljohn C. Genetic and antigenic properties of Dobrava virus: a unique member of the *Hantavirus* genus, family *Bunyaviridae*. J Gen Virol 1995;76:2801–8.

[17] Brummer-Korvenkontio M, Manni T, Ukkonen S, Vaheri A. Detection of hemagglutination-inhibiting antibodies in patients with nephropathia epidemica and Korean hemorrhagic fever by using Puumala virus cell culture antigen [letter]. J Infect Dis 1986;153:997–8.

[18] Kitamura T, Morita C, Komatsu T, Sugiyama K, Arikawa J, Shiga S, et al. Isolation of virus causing hemorrhagic fever with renal syndrome (HFRS) through a cell culture system. Jpn J Med Sci Biol 1983;36:17–25.

[19] Plyusnin A, Kruger DH, Lundkvist A. Hantavirus infections in Europe. Adv Virus Res 2001;57:105–36.

[20] Lee HW. Epidemiology and pathogenesis of hemorrhagic fever with renal syndrome. In: Elliott RM, editor. The Bunyaviridae. New York: Plenum Press; 1996. p. 253–67.

[21] CDC home page: all about hantavirus, 2006, 〈http://www.cdc.gov/ncidod/diseases/hanta/hps/no-frames/caseinfo.htm〉.

[22] Hantavirus—frequently asked questions, 2006, 〈http://www.cbc.ca/news/background/health/hantavirus.html〉.

[23] Pan American Health Organization Homepage, 2006. Number of cases and deaths from hantavirus pulmonary syndrome (HPS), Region of Americas, 1993–2004.
[24] Schmaljohn CS. Molecular biology of hantaviruses. In: Elliott RM, editor. The Bunyaviridae. New York: Plenum Press; 1996. p. 63–90.
[25] Elliot RM. Molecular biology of the Bunyaviridae. J Gen Virol 1990;71:501–22.
[26] Tao H, Xia SM, Chan ZY, Song G, Yanagihara R. Morphology and morphogenesis of viruses of hemorrhagic fever with renal syndrome. II. Inclusion bodies-ultrastructural markers of hantavirus-infected cells. Intervirology 1987;27:45–52.
[27] Lee J-S. Clinical manifestations and treatment of HFRS and HPS. In: Lee HW, Calisher C, Schmaljohn CS, editors. Manual of hemorrhagic fever with renal syndrome and hantavirus pulmonary syndrome. Seoul: WHO Collaborating Center for Virus Reference and Research; 1999. p. 18–27.
[28] Frederick K, Levy H. Clinical manifestations and treatment of HPS. In: Lee HW, Calisher C, Schmaljohn CS, editors. Manual of hemorrhagic fever with renal syndrome and hantavirus pulmonary syndrome. Seoul: WHO Collaborating Center for Virus Reference and Research; 1999. p. 33–8.
[29] Peters CJ, Simpson GL, Levy H. Spectrum of hantavirus infection: hemorrhagic fever with renal syndrome and hantavirus pulmonary syndrome. Annu Rev Med 1999;50:531–45.
[30] Lahdevitra J. Clinical manifestations and treatment of HFRS (Puumala virus). In: Lee HW, Calisher C, Schmaljohn CS, editors. Manual of hemorrhagic fever with renal syndrome and hantavirus pulmonary syndrome. Seoul: WHO Collaborating Center for Virus Reference and Research; 1999. p. 28–32.
[31] Cosgriff TM. Mechanisms of disease in hantavirus infection: pathophysiology of hemorrhagic fever with renal syndrome. Rev Infect Dis 1991;13:97–107.
[32] Kanerva M, Mustonen J, Vaheri A. Pathogenisis of Puumala and other hantavirus infection. Rev Med Virol 1998;8:67–86.
[33] Zaki SR, Greer PW, Coffield LM, Goldsmith CS, Nolte KB, Foucar K, et al. Hantavirus pulmonary syndrome. Pathogenesis of an emerging infectious disease. Am J Pathol 1995;146:552–79.
[34] Araki K, Yoshimatsu K, Lee B-H, Kariwa H, Takashima I, Arikawa J. Hantavirus-specific CD8+ T cell responses in newborn mice persistently infected with Hantaan virus. J Virol 2003;77: 8408–17.
[35] Hooper JW, Larsen T, Custer DM, Schmaljohn CS. A lethal disease model for hantavirus pulmonary syndrome. Virology 2001;289:6–14.
[36] Kawamura K, Zhang XK, Arikawa J, Takashima I, Dempo K, Hashimoto N. Susceptibility of laboratory and wild rodents to *Rattus* or *Apodemus*-type hantaviruses. Acta Virol 1991;35:54–63.
[37] Nakamura T, Yanagihara R, Gibbs Jr CJ, Gajdusek DC. Immune spleen cell-mediated protection against fatal Hantaan virus infection in infant mice. J Infect Dis 1985;151:691–7.
[38] Nakamura T, Yanagihara R, Gibbs CJ, Amyx HL, Gajdusek DC. Differential susceptibility and resistance of immunocompetent and immunodeficient mice to fatal Hantaan virus infection. Arch Virol 1985;86:109–20.
[39] Yoshimatsu K, Arikawa J, Ohbora S, Itakura C. Hantavirus infection in SCID mice. J Vet Med Sci 1997;59:863–8.
[40] Araki K, Yoshimatsu K, Lee BH, Kariwa H, Takashima I, Arikawa J. A new model of Hantaan virus persistence in mice: the balance between HTNV infection and CD8(+) T-cell responses. Virology 2004;322:318–27.
[41] Song G. Epidemiological progresses of hemorrhagic fever with renal syndrome in China. Chin Med J 1999;112:472–7.
[42] Fang L. Spatial analysis of hemorrhagic fever with renal syndrome in China. BMC Infect Dis 2006;6:77.
[43] Liang M, Li D, Xiao SY, Hang C, Rossi CA, Schmaljohn CS. Antigenic and molecular characterization of hantavirus isolates from China. Virus Res 1994;31:219–33.
[44] Wang H, Yoshimatsu K, Ebihara H, Ogino M, Araki K, Kariwa H, et al. Genetic diversity of hantaviruses isolated in China and characterization of novel hantaviruses isolated from *Niviventer confucianus* and *Rattus rattus*. Virology 2000;278:332–45.

[45] Targanskaia V. Clinical course of acute nephritis. Mater Far Eastern Med Inst (Khabarovsk) 1935;2:156–61.
[46] Tkachenko E, Ivnidze E, Zagidullin I, Dekonenko A. HFRS in Eurasia: Russia and Republics of the former USSR. In: Lee HW, Calisher C, Schmaljohn CS, editors. Manual of hemorrhagic fever with renal syndrome and hantavirus pulmonary syndrome. Seoul: WHO Collaborating Center for Virus Reference and Research; 1999. p. 49–57.
[47] Yashina L, Mishin V, Zdanovskaya N, Schmaljohn C, Ivanov L. A newly discovered variant of a hantavirus in *Apodemus peninsulae*, far eastern Russia. Emerg Infect Dis 2001;7:912–3.
[48] Lokugamage K, Kariwa H, Hayasaka D, Cui BZ, Iwasaki T, Lokugamage N, et al. Genetic characterization of hantaviruses transmitted by the Korean field mouse (*Apodemus peninsulae*), far east Russia. Emerg Infect Dis 2002;8:768–76.
[49] Lokugamage K, Kariwa H, Lokugamage N, Miyamoto H, Iwasa M, Hagiya T, et al. Genetic and antigenic characterization of the Amur virus associated with hemorrhagic fever with renal syndrome. Virus Res 2004;101:127–34.
[50] Horling J, Chizhikov V, Lundkvist A, Jonsson M, Ivanov L, Dekonenko A, et al. Khabarovsk virus: a phylogenetically and serologically distinct hantavirus isolated from *Microtus fortis* trapped in far-east Russia. J Gen Virol 1996;77:687–94.
[51] Lee HW, Ahn CN, Song JW, Baek LJ, Seo TJ, Park SC. Field trial of an inactivated vaccine against hemorrhagic fever with renal syndrome in humans. In: Calisher CH, editor. Archives of virology supplementum 1, hemorrhagic fever with renal syndrome, tick- and mosquito-borne viruses. Wien, New York: Springer; 1990. p. 35–47.
[52] Park K, Kim C, Moon K-T. Protective effectiveness of hantavirus vaccine. Emerg Infect Dis 2004;2004.
[53] Cho HW, Howard CR, Lee HW. Review of an inactivated vaccine against hantaviruses. Intervirology 2002;45:328–33.
[54] Baek LJ, Kariwa H, Lokugamage K, Yoshimatsu K, Arikawa J, Takashima I, et al. Soochong virus: an antigenically and genetically distinct hantavirus isolated from *Apodemus peninsulae* in Korea. J Med Virol 2006;78:290–7.
[55] Arikawa J, Yoshimatsu K, Kariwa H. Epidemiology and epizootiology of hantavirus infection in Japan. Jpn J Infect Dis 2001;54:95–102.
[56] Lokugamage N, Kariwa H, Lokugamage K, Iwasa MA, Hagiya T, Yoshii K, et al. Epizootiological and epidemiological study of hantavirus infection in Japan. Microbiol Immunol 2004;48:843–51.
[57] Tamura M. Occurrence of epidemic hemorrhagic fever in Osaka city: first cases found in Japan with characteristic feature of marked proteinurea. Biken J 1964;7:79–94.
[58] Kawamata J, Yamanouchi T, Dohmae K, Miyamoto H, Takahaski M, Yamanishi K, et al. Control of laboratory acquired hemorrhagic fever with renal syndrome (HFRS) in Japan. Lab Anim Sci 1987;37:431–6.
[59] Kariwa H, Yoshizumi S, Arikawa J, Yoshimatsu K, Takahashi K, Takashima I, et al. Evidence for the existence of Puumala-related virus *Clethrionomys rufocanus* in Hokkido, Japan. Am J Trop Med Hyg 1995;53:222–7.
[60] Kariwa H, Yoshimatsu K, Araki K, Chayama K, Kumada H, Ogino M, et al. Detection of hantaviral antibodies among patients with hepatitis of unknown etiology in Japan. Microbiol Immunol 2000;44:357–62.
[61] Kariwa H, Yoshimatsu K, Sawabe J, Yokota E, Arikawa J, Takashima I, et al. Genetic diversities of hantaviruses among rodents in Hokkaido, Japan and far east Russia. Virus Res 1999;59:219–28.
[62] Chin C, Chiueh TS, Yang WC, Yang TH, Shih CM, Lin HT, et al. Hantavirus infection in Taiwan: the experience of a geographically unique area. J Med Virol 2000;60:237–47.
[63] Liu YH, Huang JH, Hsueh PR, Luh KT. Hantavirus infection with marked sinus bradycardia, Taiwan. Emerg Infect Dis 2002;8:644–5.
[64] Lee HW. Epidemiology and epizootiology. In: Lee HW, Calisher C, Schmaljohn CS, editors. Manual of hemorrhagic fever with renal syndrome and hantavirus pulmonary syndrome. Seoul: WHO Collaborating Center for Virus Reference and Research; 1999. p. 40–8.
[65] Lam SK, Chua KB, Myshrall T, Devi S, Zainal D, Afifi SA, et al. Serological evidence of hantavirus infections in Malaysia. Southeast Asian J Trop Med Public Health 2001;32:809–13.

[66] Chandy S, Mitra S, Sathish N, Vijayakumar TS, Abraham OC, Jesudason MV, et al. A pilot study for serological evidence of hantavirus infection in human population in south India. Indian J Med Res 2005;122:211–5.
[67] Plyusnina A, Ibrahim IN, Winoto I, Porter KR, Gotama IB, Lundkvist A, et al. Identification of Seoul hantavirus in *Rattus norvegicus* in Indonesia. Scand J Infect Dis 2004;36:356–9.
[68] Chan KP, Chan YC, Doraisingham S. A severe case of hemorrhagic fever with renal syndrome in Singapore. Southeast Asian J Trop Med Public Health 1996;27:408–10.
[69] Vitarana T, Colombage G, Bandaranayake V, Lee HW. Hantavirus disease in Sri Lanka. Lancet 1988;2.
[70] Elwell MR, Ward GS, Tingpalapong M, LeDuc JW. Serologic evidence of Hantaan-like virus in rodents and man in Thailand. Southeast Asian J Trop Med Public Health 1985;16:349–54.
[71] Suputthamongkol Y, Nitatpattana N, Chayakulkeeree M, Palabodeewat S, Yoksan S, Gonzalez JP. Hantavirus infection in Thailand: first clinical case report. Southeast Asian J Trop Med Public Health 2005;36:217–20.
[72] Pattamadilok S, Lee BH, Kumperasart S, Yoshimatsu K, Okumura M, Nakamura I, et al. Geographical distribution of hantaviruses in Thailand and potential human health significance of Thailand virus. Am J Trop Med Hyg 2006;75:994–1002.
[73] Rollin PE, Nawrocka E, Rodhain F, Sureau P, McCormick JB. Serological data on hemorrhagic fever with renal syndrome in Southeast Asia. Bull Soc Pathol Exot Ses Filiales 1986;79:473–5.
[74] Reynes JM, Soares JL, Hue T, Bouloy M, Sun S, Kruy SL, et al. Evidence of the presence of Seoul virus in Cambodia. Microbes Infect 2003;5:769–73.
[75] Groen J, Suharti C, Koraka P, van Gorp EC, Sutaryo J, Lundkvist A, et al. Serological evidence of human hantavirus infections in Indonesia. Infection 2002;30:326–7.
[76] Okumura M, Yoshimatsu K, Kumperasart S, Nakamura I, Ogino M, Taruishi M, et al. Development of serological assays for Thottapalayam virus, an insectivore-borne hantavirus. Clin Vac Immunol 2007;14:173–81.

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Mode of Infection of Hokkaido Virus (Genus *Hantavirus*) among Grey Red-Backed Voles, *Myodes rufocanus*, in Hokkaido, Japan

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***Abstract*: Hokkaido virus (HOKV) is a member of the genus *Hantavirus*, in the family *Bunyaviridae*. To investigate HOKV infection in the host *Myodes rufocanus*, the grey red-backed vole, 199 animals were captured at Tobetsu (October 2004 and July 2005) and Nakagawa (October 2004) in Hokkaido, Japan, for detection of antibody, antigen, and viral RNA. In the surveys in Tobetsu (2004) and Nakagawa (2004), seropositive animals were detected at a frequency of 6.0% (5/84) and 10.4% (5/48), respectively. No seropositive animals were detected in Tobetsu in 2005. Seroprevalence in males in Tobetsu and Nakagawa in 2004 was 25% (1/4) and 45.5% (5/11), respectively, which was higher than in females, at 5.0% (4/80) and 0% (0/37), respectively ($P<0.01$). These results suggest that male animals play an important role in the maintenance of HOKV in *M. rufocanus*. Two females were seronegative but viral RNA-positive, indicating that these animals had acute infections before antibody was produced. Another five infected animals in Nakagawa were all male and had high levels of antibodies and viral RNA, suggesting that they had persistent infections. Viral RNA copies in organs of infected animals in Nakagawa were quantified by real-time polymerase chain reaction. Two acutely infected animals had ≥10 times the number of RNA copies in their lungs compared to those of persistently infected animals. In most cases, lungs or spleen had the highest RNA copy number, regardless of infection status.**

***Key words*: Hantavirus, Rodent, Epidemiology**

Hantaviruses are the causative agents of rodent-borne zoonotic diseases called hemorrhagic fever with renal syndrome (HFRS) in Eurasian countries, and hantavirus pulmonary syndrome (HPS) in American countries (12, 41). Viruses in the genus *Hantavirus* within the family *Bunyaviridae* are maintained in rodents and are transmitted to humans via excreta of infected rodents (48, 56). The virus genome is tripartite, single, and negative-stranded RNA consisting of small (S), medium (M), and large (L) segments, which encode nucleocapsid protein (NP), glycoproteins (G1 and G2), and viral RNA polymerase, respectively (2, 52). Humans are considered a "dead-end" host for hantaviruses, but there is the one exceptional case of Andes virus (ANDV) infection that is transmitted from human to human (13, 47). Hantavirus infection among rodents occurs by

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Abbreviations: Ab, antibody; Ag, antigen; ANDV, Andes virus; BAYV, Bayou virus; BCCV, Black Creek Canal virus; cDNA, complementary deoxyribonucleic acid; DDW, deionized distilled water; dNTP, deoxyribonucleotide triphosphate; DOBV, Dobrava virus; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetate acid; ELISA, enzyme-linked immunosorbent assay; FAM, carboxyfluorescein; FITC, fluorescein isothiocyanate; G, glycoprotein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HFRS, hemorrhagic fever with renal syndrome; HOKV, Hokkaido virus; HPS, hantavirus pulmonary syndrome; HTNV, Hantaan virus; IFA, immunofluorescent antibody assay; KCl, potassium chloride; L, large; LECV, Lechiguanas virus; LNV, Laguna Negra virus; M, medium; MGB, minor groove binder; NaCl, sodium chloride; NP, nucleocapsid protein; NYV, New York virus; OD, optical density; OPD, *o*-phenylenediamine; PBS, phosphate buffered saline; PBST, PBS with Tween 20; PCR, polymerase chain reaction; PMSF, phenylmethylsulphonyl fluoride; PO, peroxidase; PUUV, Puumala virus; RMV, Rio Mamore virus; RNA, ribonucleic acid; rNP, recombinant NP; RT, reverse transcription; S, small; SD, standard deviation; SEOV, Seoul virus; SNV, Sin Nombre virus; Tris-HCl, Tris (hydroxymethyl) aminomethane hydrochloride.

direct physical contact (46) or through saliva, feces, and urine (59).

Each hantavirus is associated predominantly with one host rodent, and more than 30 different viruses have been identified in the genus *Hantavirus* (61). The host-virus relationships related to HFRS are *Apodemus agrarius*-Hantaan virus (HTNV) (32), *Rattus norvegicus*-Seoul virus (SEOV) (10), *Apodemus flavicollis*-Dobrava virus (DOBV) (4), and *Myodes glareolus*-Puumala virus (PUUV) (44, 60). The host-virus relationships in HPS are *Peromyscus maniculatus*-Sin Nombre virus (SNV) (43), *Peromyscus leucopus*-New York virus (NYV) (53), *Sigmondon hispidus*-Black Creek Canal virus (BCCV) (50, 51), *Oryzomys palustris*-Bayou virus (BAYV) (29, 55), *Oligoryzomys longicaudatus*-ANDV (8), *Oligoryzomys flavescens*-Lechiguanas virus (LECV) (8), *Oligoryzomys microtis*-Rio Mamore virus (RMV) (6), and *Calomys laucha*-Laguna Negra virus (LNV) (21).

In Japan, all HFRS cases have been reported in relation to urban rats (33, 54) and laboratory rats (27, 57). However, no HFRS cases have been officially reported in the past 20 years (3, 24), although antibody to SEOV was detected in one person from the Japan Ground Self-defense Forces in Hokkaido (35), and seropositive individuals have been reported among patients with unknown hepatic disorders (24).

Seroepizootiological surveys on Hokkaido Island, the northern-most major island of Japan, have revealed that *M. rufocanus* is a predominant host for Hokkaido virus (HOKV), which is serologically related to PUUV, but has a genetically distinct lineage from the European PUUV (25). Although HOKV is widely distributed in Hokkaido (26, 35), little is understood about the mode of infection of HOKV in *M. rufocanus*. Recently, Iwasa et al. (20) reported that horizontal infection may be the main means of maintaining HOKV in the *M. rufocanus* population, as shown by microsatellite analysis. In this study, we analyzed HOKV infection in *M. rufocanus* populations from various areas, by detection of anti-HOKV antibody, viral antigen and viral RNA, to reveal the mode of HOKV infection in *M. rufocanus*. This should enable us to understand the mechanism of hantavirus maintenance in nature.

Materials and Methods

Epizootiological surveys. In 2004 and 2005, a total of 199 indigenous wild voles (*M. rufocanus*) were captured in the forests of Tobetsu, Ishikari District, and Nakagawa, Rumoi District, Hokkaido, Japan, by using live traps (Fig. 1). The traps were baited with oatmeal and left overnight. Blood samples were collected from

Fig. 1. Geographic location of survey points in Hokkaido at which *M. rufocanus* were captured. Filled circles indicate survey points in 2004 and 2005.

live animals via cardiac puncture under anesthesia, followed by collection of lungs, kidney, and spleen. Filter paper was used to collect blood samples from dead animals.

Sample preparation. Serum samples were heat-inactivated at 56 C for 30 min and stored at −40 C until use. Lungs, kidney, spleen, and blood clots were mixed with ISOGEN (Nippon Gene, Tokyo) and homogenated by shaking with a zirconium bead at 3 frequency/sec for 3 min, using Qiagen M300 (Retsch, Haan, Germany) for total RNA extraction. After being kept at room temperature for 5 min, the homogenate samples were stored at −80 C until used for RNA extraction.

Lungs were also mixed with a lysis buffer [0.01 M Tris-HCl pH 7.8 (Kanto Chemical, Tokyo), 2% Triton X-100 (Nacalai Tesque, Kyoto, Japan); 0.15 M NaCl, 0.6 M KCl, 5 mM EDTA (Kanto Chemical); Aprotinin 2 μg/ml, Pepstatin 2 μg/ml, Leupeptin 5 μg/ml (Wako, Osaka, Japan), and 1 mM PMSF (Sigma, St. Louis, Mo., U.S.A.)](37) for viral NP detection. The homogenates were kept on ice for 30 min and centrifuged at 6,000–7,000 rpm for 10 min. Supernatants were collected and stored at −80 C until used for viral-antigen detection. Sample preparation was performed in a biosafety level 3 containment room, except for sample collections from animals that were carried out in the field.

Enzyme-linked immunosorbent assay (ELISA). (i) ELISA for antibody detection (Ab-ELISA): Recombi-

nant NP (rNP) of HOKV was expressed as a fusion protein with N-utilization substance A (NusA). The rNP was diluted to 1.6 μg/ml with 0.05 M carbonate/bicarbonate buffer (pH 9.8; Sigma). *Escherichia coli*-expressed NusA was also diluted to 1.6 μg/ml. These recombinant proteins were added to 96-well plates (100 μl/well) and kept at 4 C overnight. The plates were washed six times with phosphate-buffered saline containing 0.05% Tween 20 (PBST), blocked with Block Ace (Dai Nippon Pharmaceutical, Osaka, Japan) at a dilution of 1:5 in deionized distilled water (DDW), and incubated at 37 C for 1 hr. After washing, 1:100 dilutions of wild-rodent sera were applied to the plates (50 μl/well) and incubated at 37 C for 1 hr. The plates were washed and incubated with protein G/peroxidase (PO) conjugate (Zymed, San Francisco, Calif., U.S.A.; 50 μl/well) at 37 C for 1 hr. After washing, 200 μl *o*-phenylenediamine tablets (OPD; Sigma) with hydrogen peroxide were added to each well, and the plate was left at room temperature for 30 min. Finally, optical density (OD) values were measured at 450 nm using a Labsystem Multiskan MS (Labsystem, Helsinki, Finland). The OD value of the rNP well minus that of the NusA well with the same serum was calculated and regarded as the ELISA value.

(ii) ELISA for antigen detection (Ag-ELISA): Ninety-six-well flat-bottom microtiter plates (Coaster, Corning, N.Y., U.S.A.) were coated with anti-rNP rabbit IgG, diluted 1:100 with carbonate/bicarbonate buffer (Sigma), at 4 C overnight. The well coated with anti-NusA rabbit IgG at the same dilution was used as a control and kept at 4 C overnight. After washing, diluted Block Ace (1:5) was added to the plate and incubated at 37 C for 1 hr. Lung homogenates were diluted with PBST (1:40) and added to the plates, followed by incubation and washing. Diluted anti-PUUV mouse serum with PBST (dilution 1:1,000) was added to the plates, and incubated as described above. After washing, anti-mouse IgG PO (1:10,000 dilution) was added to the plates, which were further incubated. After washing, 200 μl OPD (Sigma) with hydrogen peroxide were added to each well, and the plate was left at room temperature for 30 min. The OD value of the anti-rNP rabbit IgG well minus that of the anti-NusA rabbit IgG well with the same serum was calculated and regarded as the ELISA value.

Indirect immunofluorescent antibody assay (IFA). Vero E6 cells were infected with PUUV Sotkamo strain and cultured for 21 days in a CO_2 incubator. The infected cells were collected by trypsinization, seeded onto 24-well slides, and incubated for 4 hr in a CO_2 incubator. The cells were washed twice with PBS and fixed with cold acetone at −20 C for 20 min. After fixation, the slides with the fixed cells were washed with distilled water, air dried, and stored at −30 C until use as the antigen slide. The sera from wild rodents were tested for anti-hantavirus antibodies by using the antigen slide and FITC-conjugated protein G (Zymed) (34, 39).

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR). (i) RNA extraction: RNA was isolated from tissues (lungs, kidneys, spleen, and blood clots) using ISOGEN (Nippon Gene), according to the manufacturer's protocol.

(ii) Reverse transcription: Preparation for the first-strand synthesis of complementary DNA (cDNA) was done by mixing 11 μl extracted RNA (5 μg), 1 μl Random Primer (3 μg/μl) (Invitrogen, Carlsbad, Calif., U.S.A.), and 1 μl 10 mM dNTP (TaKaRa, Otsu, Japan). The RNA mixtures were heated at 70 C for 10 min, cooled to 25 C for 10 min, and chilled on ice for 3 min. The samples were further mixed with 4 μl 5× first-strand buffer (Invitrogen), 2 μl 0.1 mM DTT, and 1 μl SuperScript II (200 U/μl) (Invitrogen). cDNA synthesis was carried out at 42 C for 50 min and heated at 70 C for 15 min.

(iii) PCR: The reaction mixture for PCR was prepared by mixing with 1 μl cDNA sample, 18.75 μl DDW, 2.5 μl 10× HiFi buffer (Invitrogen), 1 μl 50 mM $MgSO_4$ (Invitrogen), 0.5 μl 10 mM dNTP (TaKaRa), 0.5 μl forward primer (10 μM), Hokkaido S172Fw (5′-CTGCAAGCACGGCAACAAACAGTGTCAGCA-3′), 0.5 μl reverse primer (10 μM), Hokkaido S894Rv (5′-GTCGGGGACATGATTCTTATCAAGCACATC-3′), and 0.25 μl Platinum *Taq* DNA polymerase High Fidelity 5 U/μl (Invitrogen). Partial S segment was amplified according to a thermal cycling program with 40 cycles of denaturation at 94 C for 30 sec, annealing at 60 C for 30 sec, and extension at 68 C for 2 min. The PCR product was further amplified by a nested PCR with inner primer pairs, PUUV S269Fw (5′-CTAAGC-CTGCTGACCCGACTGG-3′) and PUUV S707Rv (5′-ACCCCCATGACAGGACTCAT-3′). The PCR program consisted of 40 cycles of denaturing at 94 C for 30 sec, annealing at 57.5 C for 30 sec, and extension at 68 C for 2 min.

Real-time PCR. (i) DNase treatment: The mixture of total RNA for DNase treatment was prepared in a tube by adding 15 μg sample RNA, 5 μl 10× DNase buffer (TaKaRa), 2 μl DNase I (5 U/μl, RNase free; TaKaRa), 0.5 μl RNase Out Ribonuclease Inhibitor (40 U/μl; Invitrogen), and DDW to make 50 μl. The sample was incubated at 37 C for 30 min, precipitated with Lithium Chloride Precipitation Solution (Ambion, Austin, Tex., U.S.A.), and dissolved in 30 μl DDW. The DNase-treated RNA was used for cDNA synthesis as described above.

(ii) Real-time PCR: The primers and minor groove binder (MGB) probes targeting HOKV S segment were designed using the software package Primer Express version 2.0 (Applied Biosystems, Foster City, Calif., U.S.A.). Probe was labeled with the 5'-reporter dye FAM and a 3'-MGB/non-fluorescent quencher, respectively. After optimization of primer and probe concentrations, samples were assayed in quadruplicate in a 25-µl reaction mixture. Each well of the sample plate for real-time PCR consisted of 2.25 µl cDNA, 12.5 µl 2× *Taq*Man Universal PCR Master Mix (Applied Biosystems), 0.225 µl forward primer (100 µM), Hokkaido 91Fw (5'-ATGGACCCAGATGACGTTAACAA-3'), 0.225 µl reverse primer (100 µM), Hokkaido 231Rv (5'-TCAGCAGGCTTAGTATCCATCTT-3'), 0.46 µl fluorescent probe (10.9 µM), Hokkaido S (5'-ACAGTGTCAGCATTGG-3'), and 9.34 µl DDW. The sample was kept at 50 C for 2 min and 95 C for 10 min, followed by 60 thermal cycles at 95 C for 15 sec and 60 C for 1 min, with real-time data collection using the 7000 Sequence Detection System (Applied Biosystems). For standardization, the same amount of cDNA was applied for rodent GADPH control with 12.5 µl 2× *Taq*Man Universal PCR Master Mix, 0.25 µl 10 µM Rodent GADPH Forward Primer, 0.25 µl 10 µM Rodent GADPH Reverse Primer, and 0.25 µl 20 µM Rodent GADPH Probe (VIC Probe). All primers and probes for real-time PCR were purchased from Applied Biosystems.

Statistical analyses. Viral RNA and antibody prevalence (i.e., the number of animals with detectable viral RNA or antibody, respectively) were compared between males and females using Chi-square analyses. The different was considered statistically significant if $P<0.01$.

Results

Evaluation of Ab-ELISA by Comparison with IFA and RT-PCR in M. rufocanus *Samples*

To efficiently detect antibodies to HOKV in *M. rufocanus* for analyzing the mode of infection in a rodent population, we expressed rNP in bacterial cells and tried to establish an Ab-ELISA by using rNP as the antigen. A total of 199 serum samples collected in three different surveys (Tobetsu, 2004 and 2005, and Nakagawa, 2005) were tested by IFA and Ab-ELISA. In IFA, eight sera had 1:32 or higher titers, ranging from 1:32 to 1:512, and were determined as positive (Fig. 2). In Ab-ELISA, the OD values ranged from −0.229 to 1.273. All IFA positive sera were also positive by Ab-ELISA (Fig. 2, Group A), if the cutoff value was set at OD 0.3. Above this value, two IFA negative sera (Tobetsu, 2004, #79 and #85) were considered positive by ELISA (Fig. 2, Group B). Since viral RNA was detected by RT-PCR from lung samples of these two voles in Group B and seven of eight voles in Group A (Fig. 2), ten rodents in Groups A and B were infected with HOKV. Ab-ELISA gave a sensitivity of 100% (8/8) and a specificity of 99.0% (189/191) to IFA in 199 *M. rufocanus* sera (Table 1). In the same way, Ab-

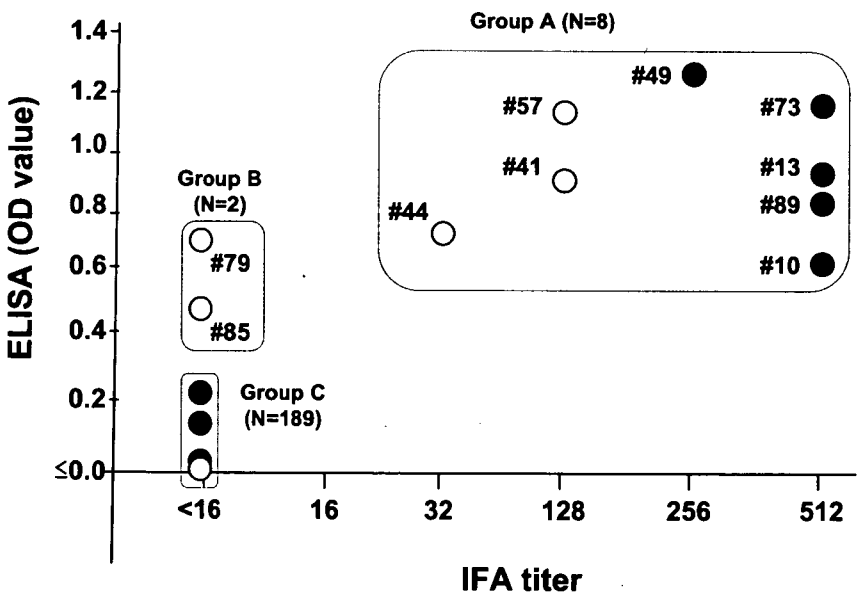

Fig. 2. Correlation of IFA titers and ELISA. The filled and open circles indicate samples from Nakagawa and Tobetsu, respectively. Group A, eight samples positive for ELISA and IFA; Group B, two samples positive for ELISA but negative for IFA; Group C, 189 samples negative for both tests. Cutoff points were 0.3 and ≥16 for ELISA and IFA, respectively.

ELISA gave a sensitivity of 81.8% (9/11) and 99.5% (187/188) specificity to RT-PCR.

Table 1. Ab-ELISA evaluation by comparison of IFA and RT-PCR

			ELISA		
			Positive (≥0.3)	Negative (<0.3)	Total
IFA	Positive	(≥16)	8	8	0
	Negative	(<16)	2	189	191
	Total		10	189	199
RTP-CR	Positive		9	2	11
	Negative		1	187	188
	Total		10	189	199

Detection of HOKV NP by Ag-ELISA

Ag-ELISA was used for detection of HOKV NP in *M. rufocanus*. OD values ranged from −0.092 to 0.566, with a mean of 0.005 and a standard deviation (SD) of 0.056. The cutoff value was set as the mean+5 SD, which was 0.286. Only two out of 199 (1.0%) *M. rufocanus* had detectable OD values but were negative for antibody. These two samples, #40 (OD 0.566) and #55 (OD 0.455) captured in Nakagawa (Fig. 3), were also positive for viral RNA, and the OD values of Ag-ELISA were significantly higher than the cutoff point. All other samples were negative for NP by Ag-ELISA.

Prevalence of HOKV Infection in M. rufocanus *Populations*

In a survey conducted at Tobetsu in October 2004,

Table 2. Prevalence of Puumala-related virus infection in *M. rufocanus* captured in Hokkaido

Place of survey	Date of survey	Prevalence (%)					
		Antibody[a]			Viral RNA[b]		
		Male	Female	Total	Male	Female	Total
Tobetsu	Oct, 2004	1/4 (25.0)	4/80 (5.0)	5/84 (6.0)	1/4 (25.0)	3/80 (3.8)	4/84 (4.8)
Tobetsu	July, 2005	0/37 (0.0)	0/30 (0.0)	0/67 (0.0)	0/37 (0.0)	0/30 (0.0)	0/67 (0.0)
Nakagawa	Oct, 2004	5/11 (45.5)	0/37 (0.0)	5/48 (10.4)	5/11 (45.5)	2/37 (5.4)	7/48 (14.6)
Total		6/52 (11.5)	4/147 (2.7)	10/199 (5.0)	6/52 (11.5)	5/147 (3.4)	11/199 (5.5)

[a] Antibody to HOKV was tested by Ab-ELISA.
[b] Viral RNA was detected by RT-PCR targeting for S RNA.

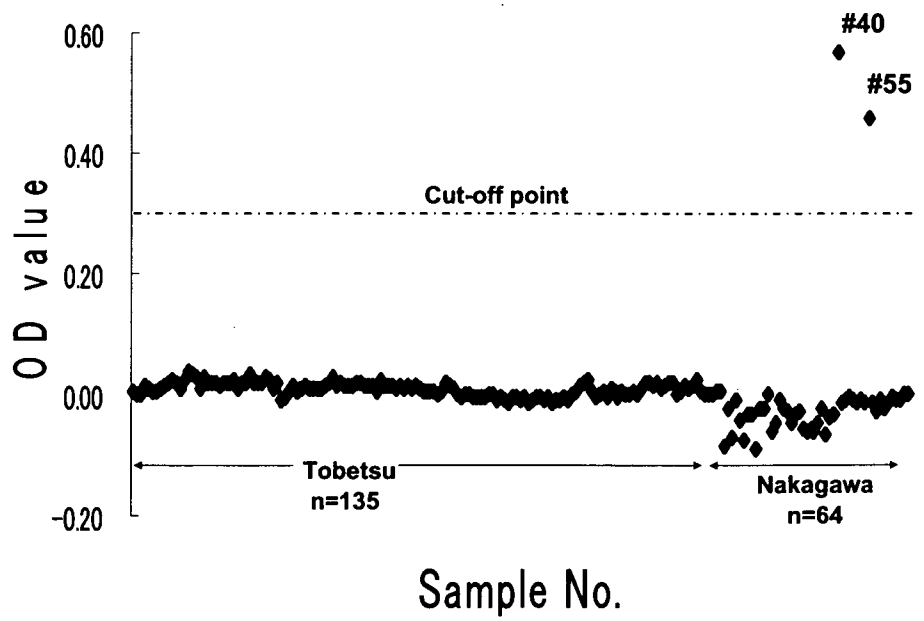

Fig. 3. Detection of NP of HOKV in *M. rufocanus* by Ag-ELISA (n=199). Cutoff point at 0.3 was set at the mean+5 SD.

five of 84 animals (6%) were positive by Ab-ELISA, with a seroprevalence of 25.0% (1/4) in male and 5.0% (4/80) in females (Table 2). Prevalence of viral RNA in males and females was 25% (1/4) and 3.8% (3/80), respectively. In a survey at Tobetsu in July 2005, no antibodies or RNA were detected in 67 voles captured. In a survey at Nakagawa in October 2004, 10.4% (5/48) and 14.6% (7/48) of animals were positive for antibodies and RNA, respectively. All seropositive animals were male and had viral RNA with a high prevalence of 45.5% (5/11).

Quantitative Detection of Viral RNA in Infected M. rufocanus *by Real-Time PCR*

Viral RNA-positive samples from Nakagawa were further tested by real-time PCR (Fig. 4). All seven rodents positive by RT-PCR also had positive results by real-time PCR, with varying RNA copy numbers in different voles and organs. Five (#13, #40, #55, #49, and #73) out of seven animals had the highest viral RNA copy number in the lungs or spleen, and a lower number in the kidneys or blood clots. Voles #40 and #55, which were antibody negative in both IFA and ELISA, had higher RNA copies than did the other animals. The RNA copies in lungs of voles #40 and #55 were 123,856 and 17,534, respectively, which were at least 10 times higher than those in other infected animals. Vole #89 had a unique pattern of viral RNA distribution, with lower RNA copy numbers in the lungs and higher numbers in the spleen and kidneys. No viral RNA was detected in any of the organs and blood clots of vole #1, which was an antibody- and viral-RNA-negative animal.

Discussion

Hantaviruses are maintained in a variety of rodent species throughout the world. It is widely believed that hantaviruses and rodents have been co-evolving on a geological time scale, according to phylogenetic analyses of hantaviruses and rodents (40, 49). The infection dynamics among rodent populations may greatly influence the evolutionary process of hantaviruses. Therefore, studies on the transmission and maintenance of viruses in the rodent population are important for understanding the evolution of hantaviruses. Our repeated surveys in Kamiiso and Tobetsu revealed that SEOV and HOKV have been maintained stably for years in *R. norvegicus* and *M. rufocanus*, respectively (22, 26). In both SEOV and HOKV, transmission appears to occur mainly by horizontal infection. However, it is still unclear how the virus is maintained and transmitted in the rodent population, due to the lack of useful tools for monitoring infections among rodents.

To elucidate the mechanism of hantavirus maintenance in more detail, we tried to establish Ab-ELISA, Ag-ELISA, and real-time PCR for HOKV infection. Compared to IFA, Ab-ELISA had high sensitivity and

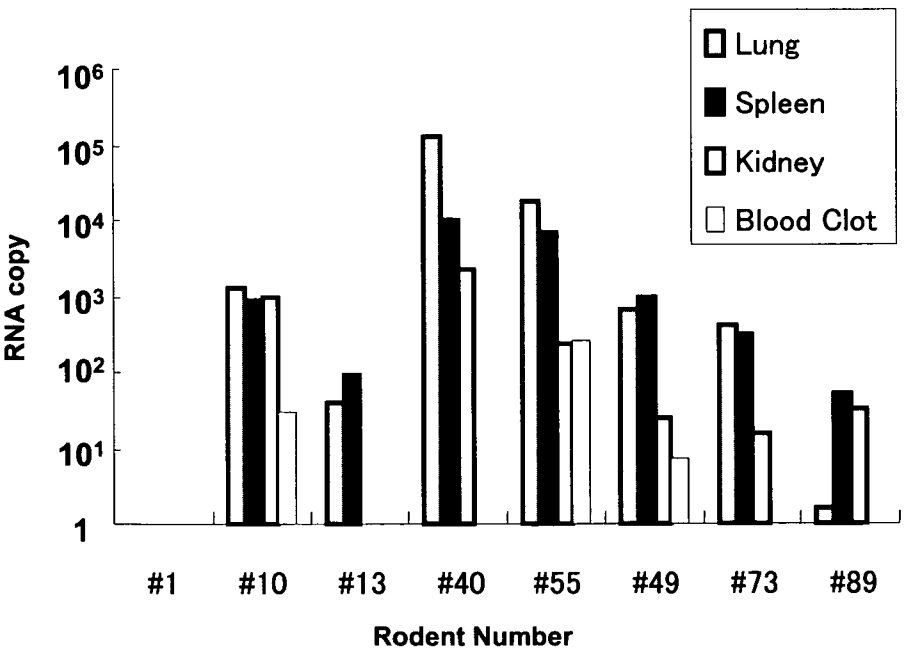

Fig. 4. Quantitative detection of HOKV RNA by real-time PCR in *M. rufocanus* captured at Nakagawa. Bars show RNA copy numbers. Ab-ELISA and viral-RNA-negative rodent #1; Ab-ELISA and RT-PCR-positive rodents #10, #13, #49, #73, #89; and Ag-ELISA and RT-PCR positive rodents #40 and #55.

Table 3. Comparison of antibody and antigen ELISAs, IFA and RT-PCR results in *M. rufocanus* (1994–1995)

Place (Year)	Total sample	Sex	Sample number	IFA (Titer)	Ab-ELISA (OD value)	Ag-ELISA (OD value)	RT-PCR
Tobetsu (2004)	5	F	41	128 [a)]	0.91	0.018	+
		F	44	32	0.76	0.007	−
		F	57	128	1.17	0.018	+
		M	79	<16	0.71	0.005	+
		F	85	<16	0.48	0.018	+
	79	Negative by all tests					
Nakagawa (2004)	7	M	10	512	0.62	−0.032	+
		M	13	512	0.93	−0.025	+
		F	40	<16	0.01	0.566	+
		M	49	256	1.27	−0.015	+
		F	55	<16	−0.01	0.455	+
		M	73	512	1.17	−0.010	+
		M	89	512	0.85	−0.010	+
	41	Negative by all tests					
Tobetsu (2005)	67	Negative by all tests					

[a)] Positive samples for IFA, Ab-ELISA, Ag-ELISA and RT-PCR were in shaded color.

specificity for detection of anti-HOKV antibodies and was useful for rapid antibody detection. Although sensitivity was relatively low, Ag-ELISA could detect virus antigen in seronegative voles. In the group that was positive by either Ab-ELISA or Ag-ELISA, 11 out of 12 animals had viral RNA (Table 3). In contrast, no viral RNA was detected in virus-negative animals (187 cases) with both Ab- and Ag-ELISA (Table 3). Therefore, combined application of Ab- and Ag-ELISA can detect infected *M. rufocanus* efficiently.

Among host reservoir species, including *M. glareolus* and *R. norvegicus*, adult males are more likely to be infected with hantavirus than are females (5, 18). In our study, male *M. rufocanus* showed higher prevalence than females at two different survey points ($P<0.01$). Our results were also in agreement with studies on New World rodents, in which male *Peromyscus* spp. were more frequently infected with SNV or SNV-related viruses than were females (1, 7, 9). The higher antibody prevalence in males may be due to a wider range of activity, aggressive behavior toward other males during breeding periods, and longer survival (1, 38). During breeding, male *M. glareolus* frequently distribute small amounts of excreta and urine to mark their boundaries, and consequently, the overlap in habitat areas for males may result in a greater risk of infection than in females (14, 45). Another possible means of transmission in male *M. rufocanus* is related to fighting and wounding, as has been suggested for *R. norvegicus* (17, 18). The prevalence of infected males may be influenced by hormone and immune status. Klein et al. (28) reported that SEOV-infected male rats produce a higher level of antibodies and shed the virus for longer periods than females do. These observations suggest that male rats play an important role in transmitting the virus, especially during the mating season, and maintaining the virus for longer periods.

All infected males captured at Nakagawa in October 2004 had a high level of antibody and viral RNA, suggesting that these animals had a persistent infection (11, 23). Wild and laboratory-colonized bank voles (*M. glareolus*) acquire persistent PUUV infection, with antibodies, viral antigen, and infectious virus throughout their life time (59). HTNV, SEOV, BCCV, and SNV can also persist in their hosts (18, 19, 23, 31). During the summer mating season, *M. rufocanus* males may be more aggressive and have more opportunity to fight and become infected. Infected male *M. rufocanus* may be able to harbor the virus during winter.

We found two female voles in Nakagawa that had viral antigen and RNA without antibodies (Table 3). This indicated that these animals had become infected, probably within 2 weeks before the capture, because more than 10 days are required for hantavirus-infected animals to produce a detectable level of antibody (16, 31, 59). These newly infected animals (#40 and #55) had a higher number of copies of viral RNA in several organs than other persistently infected animals (Fig. 4). Therefore, animals in the acute phase of infection may also play an important role as a source of virus transmission, regardless of sex. In studies on deer mice (*P. maniculatus*) during an HPS outbreak, 55% of seronegative animals captured were PCR positive (11). In the cases of Tobetsu and Nakagawa in 2004, 0.0% (0/79)

and 4.7% (2/43) of seronegative animals were viral-RNA-positive, respectively. This may mean that HOKV in our study was transmitted among *M. rufocanus* with lower activity. In *M. rufocanus* at Tobetsu, 6.0% and 0% of animals were seropositive, respectively. We have experienced this fluctuation of seroprevalence at this survey point over 10 years (data not shown). We do not have a clear answer to this phenomenon at this moment. However, it may be related to seasonal and/or annual fluctuations. Similar phenomena are observed in different hantaviruses. For example, *Apodemus agrarius* infected with HTNV in Korea had higher prevalence in autumn than that in summer (36). In addition, *M. glareolus* had strong fluctuation of PUUV prevelance in 2–5 year interval (15, 58).

We found that HOKV RNA can be maintained in several organs of *M. rufocanus*, such as lung, spleen, kidney, and blood clots, as seen in other hantaviruses (30, 31, 42). In particular, HOKV appears to replicate well in lung and spleen. To understand how the virus is transmitted, excreta such as saliva and urine should be tested for the source of infection.

In conclusion, the mode of infection of Hokkaido virus among *M. rufocanus* was analyzed. We suggest that male voles may play a more important role than females in natural infection among *M. rufocanus* population.

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References

1) Abbott, K.D., Ksiazek, T.G., and Mills, J.N. 1999. Long-term hantavirus persistence in rodent populations in central Arizona. Emerg. Infect. Dis. **5**: 102–112.
2) Arikawa, J., Schmaljohn, A.L., Dalrymple, J.M., and Schmaljohn, C.S. 1989. Characterization of Hantaan virus envelope glycoprotein antigenic determinants defined by monoclonal antibodies. J. Gen. Virol. **70**: 615–624.
3) Arikawa, J., Yoshimatsu, K., and Kariwa, H. 2001. Epidemiology and epizootiology of hantavirus infection in Japan. Jpn. J. Infect. Dis. **54**: 95–102.
4) Avsic-Zupanc, T., Xiao, S.Y., Stojanovic, R., Gligic, A., van der Groen, G., and LeDuc, J.W. 1992. Characterization of Dobrava virus: a Hantavirus from Slovenia, Yugoslavia. J. Med. Virol. **38**: 132–137.
5) Bernshtein, A.D., Apekina, N.S., Mikhailova, T.V., Myasnikov, Y.A., Khlyap, L.A., Korotkov, Y.S., and Gavrilovskaya, I.N. 1999. Dynamics of Puumala hantavirus infection in naturally infected bank voles (*Clethrionomys glareolus*). Arch. Virol. **144**: 2415–2428.
6) Bharadwaj, M., Botten, J., Torrez-Martinez, N., and Hjelle, B. 1997. Rio Mamore virus: genetic characterization of a newly recognized hantavirus of the pygmy rice rat, *Oligoryzomys microtis*, from Bolivia. Am. J. Trop. Med. Hyg. **57**: 368–374.
7) Boone, J.D., Otteson, E.W., McGwire, K.C., Villard, P., Rowe, J.E., and St. Jeor, S.C. 1998. Ecology and demographics of hantavirus infections in rodent populations in the Walker River Basin of Nevada and California. Am. J. Trop. Med. Hyg. **59**: 445–451.
8) Calderon, G., Pini, N., Bolpe, J., Levis, S., Mills, J., Segura, E., Guthmann, N., Cantoni, G., Becker, J., Fonollat, A., Ripoll, C., Bortman, M., Benedetti, R., Sabattini, M., and Enria, D. 1999. Hantavirus reservoir hosts associated with peridomestic habitats in Argentina. Emerg. Infect. Dis. **5**: 792–797.
9) Calisher, C.H., Root, J.J., Mills, J.N., Rowe, J.E., Reeder, S.A., Jentes, E.S., Wagoner, K., and Beaty, B.J. 2005. Epizootiology of Sin Nombre and El Moro Canyon hantaviruses, southeastern Colorado, 1995–2000. J. Wildl. Dis. **41**: 1–11.
10) Childs, J.E., Glass, G.E., Korch, G.W., and Leduc, J.W. 1989. Effects of hantaviral infection on survival, growth and fertility in wild rat (*Rattus norvegicus*) populations of Baltimore, Maryland. J. Wildl. Dis. **25**: 469–476.
11) Childs, J.E., Ksiazek, T.G., Spiropoulou, C.F., Krebs, J.W., Morzunov, S., Maupin, G.O., Gage, K.L., Rollin, P.E., Sarisky, J., Enscore, R.E., Frey, J.K., Peters, C.J., and Nichol, S.T. 1994. Serologic and genetic identification of *Peromyscus maniculatus* as the primary rodent reservoir for a new hantavirus in the southwestern United States. J. Infect. Dis. **169**: 1271–1280.
12) Duchin, J.S., Koster, F.T., Peters, C.J., Simpson, G.L., Tempest, B., Zaki, S.R., Ksiazek, T.G., Rollin, P.E., Nichol, S., Umland, E.T., Moolenaar, R.L., Reef, S.E., Nolte, K.B., Gallaher, M.M., Butler, J.C., and Breimen, R.F. 1994. Hantavirus pulmonary syndrome: a clinical description of 17 patients with a newly recognized disease. N. Engl. J. Med. **330**: 949–955.
13) Enria, D., Padula, P., Segura, E.L., Pini, N., Edelstein, A., Posse, C.R., and Weissenbacher, M.C. 1996. Hantavirus pulmonary syndrome in Argentina. Possibility of person to person transmission. Medicina (B Aires) **56**: 709–711.
14) Escutenaire, S., Chalon, P., De Jaegere, F., Karelle-Bui, L., Mees, G., Brochier, B., Rozenfeld, F., and Pastoret, P.P. 2002. Behavioral, physiologic, and habitat influences on the dynamics of Puumala virus infection in bank voles (*Clethrionomys glareolus*). Emerg. Infect. Dis. **8**: 930–936.
15) Escutenaire, S., Chalon, P., Verhagen, R., Heyman, P., Thomas, I., Karelle-Bui, L., Avsic-Zupanc, T., Lundkvist, A., Plyusnin, A., and Pastoret, P.P. 2000. Spatial and temporal dynamics of Puumala hantavirus infection in red bank vole (*Clethrionomys glareolus*) population in Belgium. Virus Res. **67**: 91–107.
16) Gavrilovskaya, I., Apekina, N., Okulova, N., Demina, V., Bernshtein, A., and Myasnikov, Y. 1993. IgG avidity assay for estimation of the time after onset of hantavirus infection

in colonized and wild bank voles. Arch. Virol. **132**: 359–367.

17) Glass, G.E., Childs, J.E., Korch, G.W., and LeDuc, J.W. 1988. Association of intraspecific wounding with hantaviral infection in wild rats (*Rattus norvegicus*). Epidemiol. Infect. **101**: 459–472.
18) Hinson, E.R., Shone, S.M., Zink, M.C., Glass, G.E., and Klein, S.L. 2004. Wounding: the primary mode of Seoul virus transmission among male Norway rats. Am. J. Trop. Med. Hyg. **70**: 310–317.
19) Hutchinson, K.L., Rollin, P.E., and Peters, C.J. 1998. Pathogenesis of a North American hantavirus, Black Creek Canal virus, in experimentally infected *Sigmodon hispidus*. Am. J. Trop. Med. Hyg. **59**: 58–65.
20) Iwasa, M.A., Kariwa, H., Cui, B.Z., Lokugamage, K., Lokugamage, N., Hagiya, T., Mizutani, T., and Takashima, I. 2004. Modes of hantavirus transmission in a population of *Clethrionomys rufocanus bedfordiae* inferred from mitochondrial and microsatellite DNA analyses. Arch. Virol. **149**: 929–941.
21) Johnson, A.M., Bowen, M.D., Ksiazek, T.G., Williams, R.J., Bryan, R.T., Mills, J.N., Peters, C.J., and Nichol, S.T. 1997. Laguna Negra virus associated with HPS in western Paraguay and Bolivia. Virology **238**: 115–127.
22) Kariwa, H., Isegawa, Y., Arikawa, J., Takashima, I., Ueda, S., Yamanishi, K., and Hashimoto, N. 1994. Comparison of nucleotide sequences of M genome segments among Seoul virus strains isolated from eastern Asia. Virus Res. **33**: 27–38.
23) Kariwa, H., Kimura, M., Yoshizumi, S., Arikawa, J., Yoshimatsu, K., Takashima, I., and Hashimoto, N. 1996. Modes of Seoul virus infections: persistency in newborn rats and transiency in adult rats. Arch. Virol. **141**: 2327–2338.
24) Kariwa, H., Yoshimatsu, K., Araki, K., Chayama, K., Kumada, H., Ogino, M., Ebihara, H., Murphy, M.E., Mizutani, T., Takashima, I., and Arikawa, J. 2000. Detection of hantaviral antibodies among patients with hepatitis of unknown etiology in Japan. Microbiol. Immunol. **44**: 357–362.
25) Kariwa, H., Yoshimatsu, K., Sawabe, J., Yokota, E., Arikawa, J., Takashima, I., Fukushima, H., Lundkvist, A., Shubin, F.N., Isachkova, L.M., Slonova, R.A., Leonova, G.N., and Hashimoto, N. 1999. Genetic diversities of hantaviruses among rodents in Hokkaido, Japan and Far East Russia. Virus Res. **59**: 219–228.
26) Kariwa, H., Yoshizumi, S., Arikawa, J., Yoshimatsu, K., Takahashi, K., Takashima, I., and Hashimoto, N. 1995. Evidence for the existence of Puumula-related virus among *Clethrionomys rufocanus* in Hokkaido, Japan. Am. J. Trop. Med. Hyg. **53**: 222–227.
27) Kawamata, J., Yamanouchi, T., Dohmae, K., Miyamoto, H., Takahaski, M., Yamanishi, K., Kurata, T., and Lee, H.W. 1987. Control of laboratory acquired hemorrhagic fever with renal syndrome (HFRS) in Japan. Lab. Anim. Sci. **37**: 431–436.
28) Klein, S.L., Bird, B.H., and Glass, G.E. 2000. Sex differences in Seoul virus infection are not related to adult sex steroid concentrations in Norway rats. J. Virol. **74**: 8213–8217.
29) Ksiazek, T.G., Nichol, S.T., Mills, J.N., Groves, M.G., Wozniak, A., McAdams, S., Monroe, M.C., Johnson, A.M., Martin, M.L., Peters, C.J., and Rollin, P.E. 1997. Isolation, genetic diversity, and geographic distribution of Bayou virus (Bunyaviridae: hantavirus). Am. J. Trop. Med. Hyg. **57**: 445–448.
30) Lee, H.W., Baek, L.J., and Johnson, K.M. 1982. Isolation of Hantaan virus, the etiologic agent of Korean hemorrhagic fever, from wild urban rats. J. Infect. Dis. **146**: 638–644.
31) Lee, H.W., Lee, P.W., Baek, L.J., Song, C.K., and Seong, I.W. 1981. Intraspecific transmission of Hantaan virus, etiologic agent of Korean hemorrhagic fever, in the rodent *Apodemus agrarius*. Am. J. Trop. Med. Hyg. **30**: 1106–1112.
32) Lee, H.W., Lee, P.W., and Johnson, K.M. 1978. Isolation of the etiologic agent of Korean hemorrhagic fever. J. Infect. Dis. **137**: 298–308.
33) Lee, H.W., Lee, P.W., Tamura, M., Tamura, T., and Okuno, Y. 1979. Etiological relation between Korean hemorrhagic fever and epidemic hemorrhagic fever in Japan. Biken J. **22**: 41–45.
34) Lokugamage, N., Kariwa, H., Lokugamage, K., Hagiya, T., Miyamoto, H., Iwasa, M.A., Araki, K., Yoshimatsu, K., Arikawa, J., Mizutani, T., and Takashima, I. 2003. Development of an efficient method for recovery of Puumala and Puumala-related viruses by inoculation of Mongolian gerbils. J. Vet. Med. Sci. **65**: 1189–1194.
35) Lokugamage, N., Kariwa, H., Lokugamage, K., Iwasa, M.A., Hagiya, T., Yoshii, K., Tachi, A., Ando, S., Fukushima, H., Tsuchiya, K., Iwasaki, T., Araki, K., Yoshimatsu, K., Arikawa, J., Mizutani, T., Osawa, K., Sato, H., and Takashima, I. 2004. Epizootiological and epidemiological study of hantavirus infection in Japan. Microbiol. Immunol. **48**: 843–851.
36) LeDuc, J.W. 1987. Epidemiology of Hantaan and related viruses. Lab. Anim. Sci. **37**: 413–418.
37) Lundkvist, A., Fatouros, A., and Niklasson, B. 1991. Antigenic variation of European haemorrhagic fever with renal syndrome virus strains characterized using bank vole monoclonal antibodies. J. Gen. Virol. **72**: 2097–2103.
38) Mills, J.N., Ksiazek, T.G., Ellis, B.A., Rollin, P.E., Nichol, S.T., Yates, T.L., Gannon, W.L., Levy, C.E., Engelthaler, D.M., Davis, T., Tanda, D.T., Frampton, J.W., Nichols, C.R., Peters, C.J., and Childs, J.E. 1997. Patterns of association with host and habitat: antibody reactive with Sin Nombre virus in small mammals in the major biotic communities of the southwestern United States. Am. J. Trop. Med. Hyg. **56**: 273–284.
39) Miyamoto, H., Kariwa, H., Araki, K., Lokugamage, K., Hayasaka, D., Cui, B.Z., Lokugamage, N., Ivanov, L.I., Mizutani, T., Iwasa, M.A., Yoshimatsu, K., Arikawa, J., and Takashima, I. 2003. Serological analysis of hemorrhagic fever with renal syndrome (HFRS) patients in Far Eastern Russia and identification of the causative hantavirus genotype. Arch. Virol. **148**: 1543–1556.
40) Morzunov, S.P., Rowe, J.E., Ksiazek, T.G., Peters, C.J., St. Jeor, S.C., and Nichol, S.T. 1998. Genetic analysis of the diversity and origin of hantaviruses in *Peromyscus leucopus* mice in North America. J. Virol. **72**: 57–64.