

# Epizootiological Studies of Hantavirus Infection among Urban Rats in Hokkaido, Japan: Evidences for the Persistent Infection from the Sero-Epizootiological Surveys and Antigenic Characterizations of Hantavirus Isolates

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**ABSTRACT.** Epizootiological studies of hantavirus infection among urban rats were carried out through the surveys repeated 11 times at the same dumping ground area in 1983 to 1988. A total of 279 rats (*Rattus norvegicus*) were captured during the surveys. Sero-positive animals to hantavirus strain SR-11 were detected in all the surveys. Overall positive rate of rats 6 months old or more (94/128, 73.4%) was significantly higher than that of younger rats (23/151, 15.2%,  $\chi^2 = 96.4$ ,  $P < 0.001$ ). Therefore, age dependent acquisition of hantavirus infection among rats was confirmed. Seven hantavirus strains, KI-83-262 (August, in 1983, designated as strain KI-262 in our previous report (2)), KI-85-1 and 85-2 (July in 1985), KI-88-4, 88-11, 88-15 and 88-24 (October, 1988) were isolated from lung tissues of adult rats which have high titers of neutralizing antibody. Although the serum specimens of virus carrier rats neutralized the infectivity of all the KI isolates, no apparent antigenic change in the isolates was detected by indirect immunofluorescent antibody (IFA) assay using polyclonal and monoclonal antibodies (MAbs) regardless of isolation years. However, neutralization test showed slight difference of antigenicity among KI strains. These results epizootiologically confirmed that hantavirus infected persistently among urban rats in a presence of neutralizing antibody.—**KEY WORDS:** age-dependency, epizootiology, hantavirus, hemorrhagic fever, persistent infection.

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Hemorrhagic fever with renal syndrome (HFRS) is a collective term for a rodent-borne viral zoonosis characterized by fever, renal disorder and hemorrhagic manifestations [24, 27]. Causative agent of HFRS is classified into *Hantavirus* genus of the *Bunyaviridae* family. Genus *Hantavirus* comprised of at least six distinct serotypes; Hantaan, Leaky, Seoul, Prospect Hill, Puumala and Thottapalayam virus. Hantaan, Seoul and Puumala viruses are now being accepted as the causative agent of HFRS [5].

Hantaviruses have been isolated from various species of rodents which were captured at the endemic or non-endemic area [7, 11, 13, 15, 29, 30]. In addition, animal experiments using a variety of rodent species such as *Apodemus* [12], *Clethrionomys* [28], *Rattus* [14, 22, 31] and mice [8, 18, 21], indicated that all the rodent species were highly susceptible to hantavirus infection but no apparent symptoms were found. Moreover, some of the animal experiments developed persistent infection. Thus, hantaviruses were considered to be maintained among the clonically infected rodents in nature. In our previous surveys among urban rats inhabiting in a small area indicated the age-dependent acquisition of infection and virus isolation from seropositive animals [2]. To get more information concerning to the ecology of hantavirus in nature, we continued the epizootiological studies at the same area for an interval spanning six years and antigenic characteristics of the isolates during that period were studied.

## MATERIALS AND METHODS

**Collection of urban rats and sera:** Urban rats (*Rattus norvegicus*) were captured by live traps at a dumping ground area of Kami-iso (KI) town of southern part of Hokkaido, Japan, where Seoul type virus has been isolated among the rats [2]. Blood samples were collected from rats by cardiac puncture under the ether anesthesia or by filter paper method from dead rats as previously described [2].

**Age estimation of *Rattus norvegicus*:** Age of the rats was estimated by the eye lens weight method as previously described [25]. Briefly, the eye lens were fixed with formalin and dry up with oven (80°C) for overnight and then weighted to 0.1 mg level on a chemical balance. Age of the rats was calculated according to the formula described in the above report.

**Cell culture and viruses:** The E6 clone of Vero cells (ATCC C1008, CRL 1586) [19] was grown in Eagle's minimum essential medium (Eagle's MEM, Nissui Co., Tokyo, Japan) supplemented with 5% fetal calf serum and 2 mM/l of L-glutamine. Hantavirus strains Hantaan 76-118 and SR-11 were used as representatives of Hantaan type virus and Seoul type virus, respectively [9, 13]. Seven hantavirus strains, KI-83-262 [2], KI-85-1, 85-2, 88-4, 88-11, 88-15 and 88-24 were isolated from urban rats during our surveys (see text). All the hantaviruses were propagated in the Vero E6 cell culture as described previously [2].

**Virus isolation:** Virus isolation was carried out according to the procedure described before [2]. Briefly, 10%

(w/w) homogenate of lung tissues in MEM was inoculated onto the Vero E6 cell monolayer and incubated at 37°C in a CO<sub>2</sub> incubator. The cells were subcultured every two weeks and the part of the cells was examined by IFA test for the appearance of viral antigen.

**Serodiagnostic procedure:** Indirect immunofluorescent antibody (IFA) test was utilized throughout for the seroepizootiological survey using the procedure described previously [2]. Vero E6 cells infected with Seoul virus strain SR-11 were employed as an antigen. Fluorescein isothiocyanate (FITC)-conjugated anti rat Ig (G+M+A) goat IgG (Cappel Laboratories, Cochranville, Pa, U.S.A.) was used as second antibody. IFA titers of 1:32 or more were regarded as sero-positive. Focus reduction neutralization test (FRNT) was carried out in accordance with the procedure of Tanishita *et al.* [23]. FRNT titers were expressed as the reciprocals of the highest serum dilution resulted in greater than 80% reduction in the number of infected cell foci.

**Immune sera and monoclonal antibodies:** Immune sera and mouse ascitic fluid containing monoclonal antibodies (MAbs), were used for the antigenic characterization of isolates. Immune sera to strain Hantaan 76-118 and strain SR-11 were prepared by injecting the virus to rats as previously described [31]. Two MAb clones EB06 and GD05 were kindly provided from Dr. J. McCormick, Centers for Disease Control, Atlanta, GA, U.S.A. and rest of the clones were prepared by us [3]. All the MAb clones employed were directed to envelope protein G1 or G2.

**Antigenic characterizations of isolates:** IFA test was carried out according to the procedure described above. Cross neutralization test was performed by the 80% reduction method as described previously [3].

## RESULTS

**Sero-epidemiological study of hantavirus infection among urban rats:** A total of 279 urban rats (*Rattus norvegicus*) were captured at a same dumping ground area through the surveys conducted 11 times during a period from 1983 to 1989. One hundred and seventeen (41.9%) of them showed IFA titers of 1:32 or more to strain SR-11. The seropositive rats were detected in all the surveys, although the positive rates varied from 11.1% to 83.3% (Table 1). Relation between antibody positive rate and age for the 279 rats was shown in Fig. 1A. The positive rates were apparently higher in rats of six-months or more older than that of younger one. Overall positive rate of rats aged older than 6 months (94/128, 73.4%) was significantly higher than that of younger rats (23/151, 15.2%,  $\chi^2 = 96.4$ ,  $p < 0.001$ ). Geometric mean IFA titers of positive rats in each age group as plotted in Fig. 1B increased rapidly among rats aged between 3 to 4 months and then reached plateau at older age groups.

**Virus isolation:** A total of seven strains of hantaviruses were isolated from 3.9 months or more old rats captured at the same dumping ground one in 1983 (strain KI-

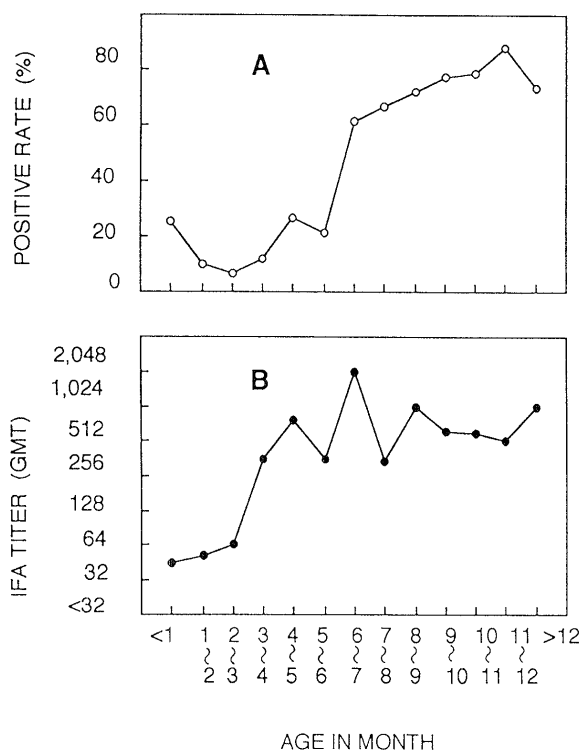


Fig. 1. Relation between age for the rats and antibody positive (IFA titers of more than 1:32) rate (A), and geometric mean IFA titers (GMT) among antibody positive rats (B). A total of 279 urban rats were captured at a same dumping ground area during a period from 1983 to 1989. Age for the rats were calculated by eye lens weight method as described in the materials and methods.

Table 1. Sero-epizootiological study of hantavirus infection among rats captured in Kami-iso town in 1983 to 1989

Date of Survey	No. of Tested	No. of Seropositive	Positive ratio (%)
Feb. 1983	6	5	(83.3)
Aug. 1983	37	13	(35.1)
Oct. 1983	27	3	(11.1)
May. 1984	3	2	(66.7)
Jul. 1984	40	11	(27.5)
Jul. 1985	41	19	(46.3)
Jun. 1986	54	32	(59.2)
Oct. 1986	9	2	(22.2)
Jun. 1987	3	1	(33.3)
Oct. 1988	24	5	(20.8)
Aug. 1989	35	18	(51.4)
Total	279	117	(41.9)

83-262) [2], two in 1985 (strains KI-85-1, 85-2) and four in 1988 (strains KI-88-4, 88-11, 88-15, 88-24), as listed in Table 2. All the virus carrier rats showed high IFA titers and two of them were confirmed to have high neutralizing antibody to their own isolates.

**Antigenicity of the isolates:** Cross neutralization test between KI isolates and strains Hantaan 76-118 of

Hantaan type and SR-11 of Seoul type was performed using immune sera to strains Hantaan 76-118 and SR-11 or sera from carrier rats of the strains KI-85-1 and KI-88-24 (Table 3). The homologous FRNT titers of anti SR-11 serum and carrier rat sera were same to that of the heterologous reactions with SR-11 and KI isolates, but anti Hantaan immune sera showed apparently lower FRNT titers to heterologous strains. Thus, all the KI strains were considered as Seoul type virus. However, the FRNT titers of anti SR-11 serum varied among the KI isolates.

*Antigenic comparison of KI strains isolated in 1983 to 1988:* To examine if the antigenic drift of the KI isolates occurred during a 5-year period, 13 anti HTN MAb clones to antigenic sites on the G1 or G2 envelope protein of Hantaan 76-118 [3] and two anti SR-11 MAb clones to G1 or G2 protein were used. As shown in Table 4, reaction patterns to the MAbs were exactly the same between KI isolates and SR-11. No apparent change of IFA titers was detected among KI isolates regardless of the isolation year. For further characterizations, neutralizing MAb clones were selected and FRNT titers to KI strains were compared (Table 5). Three of the cross reactive MAbs between strain Hantaan 76-118 and SR-11 (HCO2, 2E8 and 4D12) were able to neutralize all the KI isolates in the

same level. However, clone HCO2 showed lower FRNT titers to KI strains isolated in 1988 than to 1983 and 1985 isolates. Two of the neutralizing clones specific to Hantaan 76-118 (2D5 and 11E10) could not neutralize the infectivity of all the KI isolates.

#### DISCUSSION

Characteristics of hantavirus infection among urban rats such as age-dependent acquisition of infection and persistency of infection in sero positive rats have been reported in our previous study [1,2] and other surveys conducted at Baltimore [6] and Houston and Philadelphia [10] in U.S.A., Kobe [20] and Shimizu [16] in Japan. The present study confirm and extend these earlier observations through the longitudinal survey repeated 11 times at the same area in 1983 to 1988, by showing the maintenance of prevalence within a small rat colony even though the overall sero-positive rate and serum antibody titers are fairly high.

Viruses were isolated from lung tissues of sero-positive rats as previously described. Moreover, we confirmed that the serum specimens from virus carrier rats contained antibody which effectively neutralized the infectivity of own isolate by *in vitro* neutralization test. These results indicated that the urban rats established persistent infection without defect of function of antibody production. This seemed to be different from that of lymphocytic choriomeningitis virus (LCMV) infection in that persistently infected mice produced only negligible antibody to LCMV [4]. Therefore, the hantavirus seemed to be existing within cells so as to avoid the contact from neutralizing antibody. Yamanishi and Kondo [26] elucidated that hantaviruses persistently infected in macrophage cells in the rats experimentally inoculated with Seoul type hantavirus. In addition, no antigenic change was detected between original and re-isolated virus. Therefore, this might be an plausible mechanism for establishing persistent infection among rats. To confirm if the hantavirus was maintained among urban rats as same mechanism as that showing by the experimental infection,

Table 2. Isolation of hantavirus from rats captured at dumping ground of Kami-iso town in 1983 to 1988

No. of isolate	Date of rat captured	Sex	Age in month	IFA titers to SR-11 strain	FRNT titers to KI strain
KI-83-262	Aug. 1983	unknown	6.0	512	NT <sup>a)</sup>
KI-85-1	Jul. 1985	female	12.3	2,048	3,200 <sup>b)</sup>
-85-2	Jul. 1985	female	13.0	2,048	NT
KI-88-4	Oct. 1988	female	4.0	1,024	NT
-88-11	Oct. 1988	male	3.9	256	NT
-88-15	Oct. 1988	male	5.8	1,024	NT
-88-24	Oct. 1988	male	4.7	2,048	6,400 <sup>c)</sup>

a) Not tested. b) FRNT titer to strain KI-85-1. c) FRNT titer to strain KI-88-24.

Table 3. Antigenic comparison between Kami-iso isolates, Hantaan 76-118 and SR-11 by cross neutralization test

Hantaviruses	Immuneserum to		Urban rat serum following KI strains were isolated	
	HTN <sup>a)</sup>	SR-11	KI-85-1	KI-88-24
HTN	<u>1,600<sup>b)</sup></u>	20	160	40
SR-11	100	<u>1,600</u>	6,400	6,400
KI-83-262	20	<u>800</u>	3,200	6,400
85-1	40	<u>1,600</u>	3,200	12,800
85-2	20	320	<u>6,400</u>	6,400
88-4	40	320	3,200	3,200
88-11	20	160	1,600	3,200
88-15	20	320	6,400	3,200
88-24	20	320	3,200	<u>6,400</u>

a) Strain Hantaan 76-118.

b) Underline indicates the FRNT titers to the homologous hantavirus.

Table 4. Antigenic characterizations of KI isolates by IFA test using monoclonal antibodies to envelope proteins

Monoclonal antibody clone			Hantaviruses								
Speci- ficity	Antigenic site	Code	HTN	SR-11	Kami-iso isolates						
					83-262	85-1	85-2	88-4	88-11	88-15	88-24
HTN G1	a-1	8B6	++++	+++	++	+++	+++	++	+++	++	++
	a-2	6D4	++++	+	+	+	+	+	+	+	+
	b	2D5	++++	+	+	+	+	+	+	+	+
HTN G2	a	HCO2	++++	+++	+++	+++	+++	+++	+++	+++	+++
	b	EBO6	++++	+++	+++	+++	+++	+++	+++	+++	++
	c	11E10	++++	+	+	+	+	+	+	+	+
	d	5B7	+++	+++	+++	+++	+++	+++	+++	+++	+++
	e	20D3	++++	+	+	+	+	+	+	+	+
	f-1	8E10	++++	+++	+++	+++	+++	+++	+++	+++	+++
	f-2	23G10-1	++++	+++	+++	+++	+++	+++	+++	+++	+++
	f-3	GDO5	+++	+	+	+	+	+	+	+	+
SR-11 G1		2E8	+	++++	++++	++++	++++	++++	++++	++++	+++
G2		4D12	++	+++	++++	++++	++++	++++	++++	++++	++++

IFA test positive at serum dilution 1:10 (+), 1:100 (++), 1:1,000 (+++), 1:10,000 (++++).

Table 5. Antigenic characterizations of KI strains by neutralization test using monoclonal antibodies to envelope protein

Monoclonal antibody clone			Hantaviruses								
Speci- ficity	Antigenic site	Code no.	HTN	SR-11	Kami-iso isolates						
					83-262	85-1	85-2	88-4	88-11	88-15	88-24
HTN G1	b	2D5	320	— <sup>a)</sup>	—	—	—	—	—	—	—
HTN G2	a	HCO2	6,400	3,200	3,200	6,400	1,600	400	800	800	400
	c	11E10	160	—	—	—	—	—	—	—	—
SR-11 G1		2E8	3,200	6,400	1,600	1,600	1,600	400	800	1,600	1,600
G2		4D12	1,600	1,600	400	1,600	1,600	400	400	1,600	400

a) Neutralizing antibody titer less than 1:10.

attempts to isolate hantavirus from macrophage and other cells in infected urban rats will be required.

No detectable change of antigenicity of envelope glycoprotein antigen of isolates was found during these 5 years interval by IFA test. Although it is unclear whether single hantavirus strain prevails within this rat colony, the antigenic stability may reflect the situation of virus existence where the virus evades the host immune pressure. However, FRNT titers of KI strain to anti SR-11 serum and cross reactive neutralizing MAb clones varied among the isolates (Table 3 and Table 5). Therefore the possibility of antigenic drift was suggested. To examine the antigenic change in more detail, the nucleotide sequence of M genome segment which encodes the envelope proteins are under study. Since the neutralizing activity of MAb clones used in this experiment confined to strain Hantaan specific or hantavirus cross reactive epitopes, further studies using the neutralizing MAbs specific for Seoul type hantavirus are required for further characterizations.

Morita *et al.* [17] reported the age-dependent transmis-

sibility of hantavirus among rats experimentally inoculated with Seoul type hantavirus. In the study, rat infected during the younger age (less than 48 hrs after birth) is able to establish the persistent infection and transmit the virus to other rats, but more older one showed only transient infection without spread of infection to other rats. Nevertheless, the present study and other epizootiological observations among urban rats indicated the existence of persistent infection among adult rats, although the primary infection may occur around 8 weeks of age when maternal antibody level decreased under the protective level [31]. In addition, role of the seronegative rat population in the maintenance of infection within this rat colony should be examined. The mechanisms underlying transmission and establishing persistent infection of hantavirus among urban rats remains to be elucidated.

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