cross-reactivities of the SP-based and commercial ELISAs. Whereas 3 of 10 samples gave inconclusive results by the commercial IgG and IgM ELISAs, respectively (Table 5), all 10 samples were negative by both the SP-IgG ELISA and the SP-IgM ELISA. These results indicated that the SP-IgG and SP-IgM ELISAs were specific to antibodies against TBE virus.

4. Discussion

The virus neutralization test is often used as a specific serological diagnostic test for TBE infection. However, the test is time-consuming and must be carried out in a high-level biosafety facility. ELISA tests using inactivated whole virus as antigen have also been widely used for the serological diagnosis of TBE virus infection, but the production and inactivation of live TBE virus for this application is also restricted by safety considerations. Thus, the generation of recombinant viral proteins is an important approach for the development of alternative antigens that are both less expensive and less hazardous to prepare and use.

During in vitro infection of cells with TBE virus, subviral particles with no nucleocapsids are released from cells at the same time as mature virions (Allison et al., 1995; Russell et al., 1980). Likewise, when recombinant flaviviral prM and E proteins are co-expressed in mammalian cells, SPs are secreted into the culture medium (Allison et al., 1995; Fonseca et al., 1994; Konishi et al., 1992). Similarly, it has been confirmed that transfection of the pCAGprME plasmid, which encodes the prM and E proteins of TBE virus, into 293T cells results in the release of TBE SPs into the culture medium (Yoshii et al., 2003). The E proteins in SPs are considered to have almost the same structure and function as those in complete virions (Allison et al., 1995). As SPs do not have genomic RNA, they cannot replicate even if they enter cells and can thus be handled in laboratories with no biosafety facilities. For these reasons, SPs are gradually replacing viruses for a variety of applications. ELISAs using SPs from Japanese encephalitis virus, West Nile virus, and European subtype TBE virus have been presented as useful serological diagnostic methods (sequentially, Davis et al., 2001; Hunt et al., 2001; Konishi et al., 1996; Jaaskelainen et al., 2003).

As presented in a previous report, ELISAs have been developed for the serological diagnosis of TBE infection based on recombinant viral proteins (Yoshii et al., 2003). In that study, lysates of cells transfected with pCAGprME were used as the ELISA antigen. In the present study, new ELISA tests were developed using antigens prepared from TBE virus SPs harvested from the supernatant of transfected cells and compared these ELISAs with other testing methods.

The SP-IgG ELISA was found to have a high sensitivity (82/83, 98.8%) and specificity (12/12, 100%) as compared with the neutralization test when the cut-off value for the ELISA was set at a P/N ratio of 1.155 (Table 1). In contrast, the commercial IgG ELISA had a relatively low sensitivity and generated many inconclusive test results (Table 2). These findings suggest that the SP-IgG ELISA can substitute for the neutralization test and a commercial IgG ELISA for the detection of anti-TBE virus IgG antibodies. The SPs used as antigen in the SP-IgG ELISA were concentrated from culture supernatants without further purification or fixation, and an E-protein-specific mAb was used as the capture antibody. This ELISA may have led to the high degree of sensitivity and specificity of the SP-IgG ELISA.

The distribution of the P/N ratios from the SP-IgM ELISA indicated that the serum samples were clearly separated into a low P/N group and a high P/N group, which were presumably IgM-negative and IgM-positive, respectively (Fig. 2). This distribution curve permitted a cut-off value at the mean of the maximum P/N ratio of the negative group and the minimum P/N ratio of the positive group.

The diagnosis of TBE infection using the neutralization test requires paired serum samples and the measurement of a significant (greater than four-fold) increase in the neutralizing antibody titer. We found that the SP-IgM ELISA was superior to the neutralization test for diagnostic testing using paired and single serum samples. Only one of 17 neutralization-positive patients was negative according to the SP-IgM ELISA. Of the 15 paired sera that yielded equivocal results by the neutralization test owing to the lack of significant increases in antibody titers, 11 were positive and 4 were negative using the SP-IgM ELISA. The SP-IgM ELISA could also be applied to single serum samples for the diagnosis of TBE infection. Of seven single serum samples with NT titers $\geq$1:160, six (NT titers were from 1:1280 to 1:20,480) were judged to be positive by the SP-IgM ELISA.

The SP-IgM ELISA was both more sensitive and more specific than the commercial IgM ELISA when both tests were compared (Table 4). Of 95 samples tested, 52 were positive by both tests. Eleven (21.2%) of these 52 samples were negative by the IgM ELISA using recombinant antigens (Yoshii et al., 2003). However, of 36 samples that were negative by both the commercial IgM ELISA and the IgM ELISA using recombinant antigens, 23 were negative and 13 were positive by the SP-IgM ELISA. The 13 samples that were positive by the SP-IgM ELISA also were positive by the neutralization. Seven serum samples that yielded inconclusive results with the commercial IgM ELISA were positive with the SP-IgM ELISA. The discrepancy between the two ELISA tests may be attributed to differences in the antigens used. The SP-IgM ELISA uses unfixed SPs, whereas the commercial ELISA uses formalin-fixed virions. Formalin fixation may cause a loss of antigenicity of the virion proteins (Heinz et al., 1995). In addition, the two tests use different strains of TBE virus as the antigen source; the SP-IgM ELISA uses the prM and E proteins of a Far Eastern subtype strain, whereas the commercial ELISA uses a European subtype strain.

It has been reported that infection and/or vaccination with other flaviviruses, including yellow fever virus, dengue virus, West Nile virus, and JE virus, can induce cross-reactive antibodies (Dobler et al., 1996; Holzmann et al., 1996; Niedrig et al., 2001). Both the IgG and IgM TBE-specific SP ELISAs had little cross-reaction with antibodies against the JE virus. Whereas 3 of 10 serum samples from JE patients had marginal cross-reactivity on the commercial IgG and IgM ELISAs, respectively, all 10 samples were negative on both the SP-IgG and SP-IgM ELISAs (Table 5). Again, although the reason for this difference is not

known, it may be attributed to differences in the antigens, as discussed above.

These newly developed ELISA systems based on safe and inexpensive SPs are potential alternatives to the conventional diagnostic ELISA methods based on inactivated whole virions. These new methods had high sensitivity and specificity and no cross-reactivity with anti-JE virus antibodies. Therefore, these SP-IgG and SP-IgM ELISAs can be applied to epidemiological research and the diagnosis of TBE in Japan, where TBE virus and JE virus are both endemic.

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Modes of hantavirus transmission in a population of *Clethrionomys rufocanus bedfordiae* inferred from mitochondrial and microsatellite DNA analyses

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Summary. To elucidate the mode of transmission of Puumala-related hantavirus in a population of gray red-backed voles, *Clethrionomys rufocanus bedfordiae*, in Hokkaido, Japan, we analyzed the kin structure and dispersal patterns of individual voles using microsatellite and mitochondrial DNA markers. Siblings or dam/offsprings was identified within the population based on the relatedness calculation with the microsatellite data. The pairwise relatedness values obtained could reveal kinship among all vole individuals within the population. Based on the assessment of kinship, we did not find a positive relationship between hantavirus transmission and close kinship. Males infected with the hantavirus carried a relatively uncommon mitochondrial haplotype. However, these infected males shared low relatedness values and were not considered closely related, *i.e.*, they were not siblings or parent/offspring. These observations imply that hantavirus transmission in the vole population may not be related to close kinship but by random horizontal infection.

Introduction

Epidemiological studies have shown that a variety of populations of wild rodents occasionally act as reservoirs of hantaviruses (genus *Hantavirus*, family *Bunyaviridae*). Several rodent-borne hantaviruses cause serious diseases in humans, including hemorrhagic fever with renal syndrome (HFRS) in Europe and Asia and hantavirus pulmonary syndrome (HPS) in the Americas [2, 8, 11, 36]. Ecological observations have suggested that the hantaviruses associated with HFRS are transmitted horizontally among individual rodents *via* aerosolized con-

taminated excreta [3, 4, 12, 35, 37]. A relationship between HPS-related hantavirus infections and the host rodents has also been documented in the U.S.A. [5, 27]. Based on these studies, sexually related transmission and inter-population invasions of males are considered important sources of new infections of HPS-related hantavirus in rodent populations [5]. Conversely, little is known about the relationship between the ecological interactions among individual rodents and hantavirus infections in natural rodent populations, particularly for HFRS-related viruses. Therefore, these relationships based on the vole behavioral and dispersal data in nature would help to trace hantavirus transmission in host rodent populations.

Hantaviruses are divided into several serological types. *Puumala virus* (PUUV) is maintained in the bank vole, *Clethrionomys glareolus* (Rodentia, Arvicolinae), in Europe [6, 29, 30, 34]. Hokkaido, the northernmost island of Japan, is home to the gray red-backed vole, *C. rufocanus bedfordiae*, which is especially common in forests with dense undergrowth of *Sasa* bamboo [19, 20, 28]. Previously, we detected PUUV-related hantavirus infection in *C. rufocanus bedfordiae* in epizootiological surveys [22], although *C. glareolus* and *C. rufocanus* are not closely related phylogenetically [9, 16]. This PUUV-related hantavirus was considered to be endemic to the reservoir *C. rufocanus bedfordiae* in Hokkaido, as it is genetically distinct from PUUV [25, Kariwa et al. unpublished data]. In addition, the frequency of infected *C. rufocanus bedfordiae* in natural populations was 10.4% in individuals ($n = 278$) collected from eight localities in Hokkaido [22]. However, the mechanisms maintaining this level of horizontal infection in *C. rufocanus bedfordiae* populations have not been studied ecologically, from the perspective of hantavirus transmission *via* aerosolized contaminated excrement and vole behavior [12, 13, 35, 37]. The previous investigations suggest that hantavirus infection occur by horizontal transmission not by vertical way in rodent populations [38]. However, these observations were not fully discussed by the kinship and pedigrees of rodent populations. Accordingly, we postulated two hypotheses for horizontal infection within a small population of voles from an ecological standpoint such as knowledge of vole behavior and dispersal patterns. First, there is a high probability of an infected dam transmitting its litter if the litter is continuously exposed to the dam's contaminated excrement in the nest before dispersal. To trace the mode of hantavirus transmission within a small vole population and to obtain the information about intra-populational lineages and the kinship of individual voles, including infected animals, in more detail, rodent molecular markers are invaluable [13, 14, 17, 18]. Furthermore, as a second hypothesis, hantavirus transmission may be spread by the dispersal of infected voles based on the differences in the dispersal patterns of male and female voles [13, 33].

In this study, we analyzed mitochondrial DNA sequences and PCR fragment lengths at microsatellite loci in a small *C. rufocanus bedfordiae* population containing individuals infected with PUUV-related hantavirus in a small forest in Hokkaido. As ecological studies, such as elucidation of the vole's behavior and kinship, have shown, these DNA markers have a high resolution in detecting kinship within a small vole population; mitochondrial DNA is useful for tracing maternal lineages and microsatellite DNA can also be used to detect pedigree

[13, 14, 17, 18]. The mode of hantavirus transmission within the vole population is discussed based on the relationship and the behavioral interactions between individual *C. rufocanus bedfordiae* and hantavirus infection.

Materials and methods

Vole samples and detection of hantavirus antibody and virus RNA

In all, 96 live voles were captured in a shelterbelt forest at Tobetsu, Ishikari Subprefecture, Hokkaido, Japan (43° 12′ 21″N, 141° 25′ 18″E), using live traps (100 night-traps, two nights), on 27–29 September 2000. The forest consisted mainly of ash *Fraxinus mandshurica* var. *japonica* and the ground was completely covered with the bamboo *Sasa senanensis* (Fig. 1). The collected vole species were identified based on morphological and molecular measures [17, 18, 20]. The family units of all the voles were unknown. The age and sex of the vole samples are summarized in Table 1. The age (over-wintered or young) was determined using body weight and size [1].

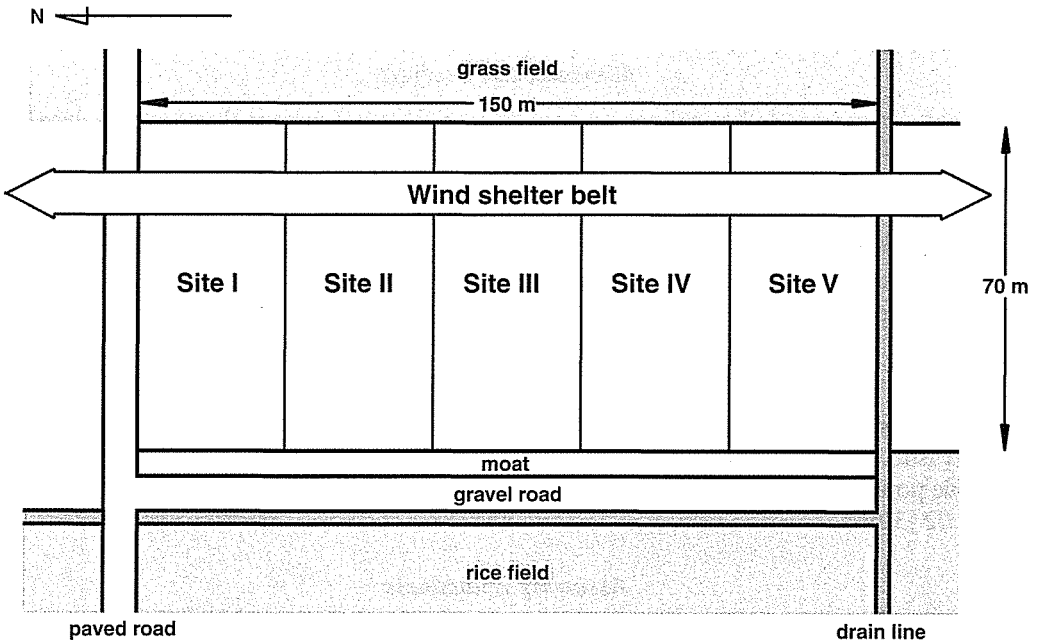

Fig. 1. Schematic of the forest surveyed in this study

Table 1. Age and sex constitutions of vole samples within the small population examined

	Site I		Site II		Site III		Site IV		Site V		Total
	F	M	F	M	F	M	F	M	F	M	
Young	11	4	20	3	13	1	9	6	12	3	82
Over-wintered	1	0	3	2	2	1	2	1	1	1	14
Total	12	4	23	5	15	2	11	7	13	4	96

F, female; M, male

Each captured vole was tested for antibodies to hantaviruses using the protein G antibody assay [21]. In addition, we performed RT-PCR reaction for an amplification of the partial length of the S segment using a primer set specific for PUUV-related hantavirus in Hokkaido [23].

Mitochondrial DNA analysis

Total DNA was extracted from the voles using the conventional phenol-chloroform method. The mitochondrial D-loop region (624 base pairs) was amplified by PCR with the primer set L15926/Hpr-1 [13, 24]. PCR was carried out for 35 cycles each consisting of 30 sec at 96 °C, 60 sec at 50 °C, and 60 sec at 72 °C. The reaction mixtures (20 μl) contained 0.1 mM dNTPs, 10 mM Tris-HCl, 50 mM KCl, 2.5 mM $MgCl_2$, 0.05 μM primers, and 0.5 units of Ampli*Taq*® polymerase (Applied Biosystems, Foster City, CA). The PCR products were sequenced using an automated sequencer (model 310, Applied Biosystems) using a Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems). All the sequences for the mitochondrial control region from the vole samples determined in this study have been deposited to the GenBank/EMBL/DDBJ nucleotide sequence databases with accession numbers AB104401–AB104411.

Microsatellite analysis

We used six microsatellite primer sets, with each forward primer fluorescently labeled: MSCRB-1, -2, -3, -4, -5, and -6 (Applied Biosystems) [14]. PCR amplification was performed in 10-μl reaction volumes as described previously under the conditions described for MSCRB-2 [14]. The PCR products were directly electrophoresed using an ABI model 373A automated sequencer (Applied Biosystems) and the lengths of the PCR products were determined using GeneScan ver. 1.02 (Applied Biosystems) based on the size marker GeneScan-500 TAMRA (Applied Biosystems).

Assessing Hardy-Weinberg equilibrium

The observed allele frequencies at six loci were compared with Hardy-Weinberg expectations using Fisher's exact test [10], to assess the equilibrium condition of the genetic structure of the vole population examined in this study. The comparison was performed with GENEPOP ver. 1.02 [32].

Assessing relatedness

The genotypes at the six microsatellite loci were determined for all individuals from PCR fragment length data. Pairwise relatedness between individuals was estimated from the genotypes and allele frequencies at the six microsatellite loci using Relatedness ver. 5.0.8 [31].

Results

Antibody assay

Three voles had a positive response for PUUV (n = 3, individual codes: 2000/9/27-29#32, #84 and #89; antibody titer: #32, 1:40; #84, 1:40; and #89, 1:80) and Hantaan virus (HTNV) (antibody titer: #32, <1:10; #84, 1:40; and #89, 1:20) [21, 22]. Furthermore, these three individuals showed positive signals on the RT-PCR reaction. Thus, we recognized that these three (#32, #84 and #89) were infected with PUUV-related hantavirus.

Mitochondrial DNA analysis

In all, 11 mitochondrial D-loop haplotypes (haplotypes A to K: Table 2, Fig. 2) were obtained from the 96 *C. rufocanus bedfordiae* individuals captured. In the vole population examined in this study, haplotype F was most major, followed by haplotypes A and C (>10%) (Fig. 2). The other eight haplotypes were relatively

Table 2. Nucleotide substitutions among 11 mitochondrial D-loop haplotypes (624 bp)

Haplotype	Site															
	1	1	1	1	1	2	2	2	2	2	2	2	2	3	3	4
	0	1	1	2	9	0	2	3	5	5	5	5	7	4	5	0
	1	5	6	1	0	3	3	3	0	2	3	5	2	7	7	1
A	G	T	T	T	T	T	A	C	T	A	G	C	T	C	A	C
B	A	T	T	C	T	T	A	C	T	C	G	T	T	A	A	C
C	A	T	T	C	C	T	A	C	C	C	A	T	T	C	A	C
D	A	C	T	C	T	T	A	C	T	C	A	T	T	C	A	C
E	A	T	T	C	T	T	A	C	T	C	A	T	T	C	A	C
F	A	T	T	C	T	T	A	C	T	C	G	T	T	C	A	C
G	A	T	T	C	T	C	A	C	T	C	G	T	C	C	A	C
H	A	T	T	T	T	T	A	T	T	C	G	T	T	C	A	T
I	A	T	C	T	T	T	A	C	T	C	G	T	T	C	A	C
J	A	T	T	C	T	T	G	C	T	A	G	T	C	C	G	C
K	A	T	T	C	T	T	A	C	T	C	G	T	T	G	A	C

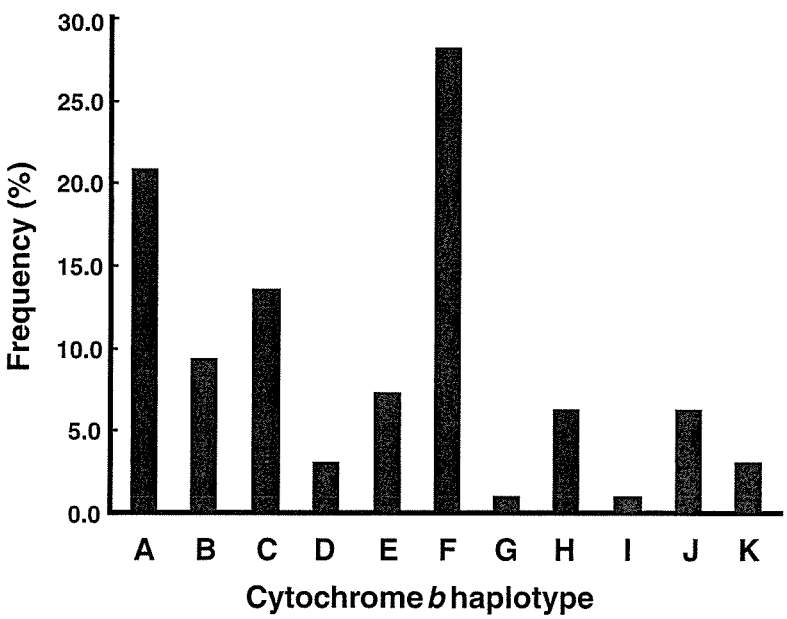

Fig. 2. Frequencies of mitochondrial D-loop haplotypes (A to K) in all individuals of *Clethrionomys rufocanus bedfordiae* examined in this study

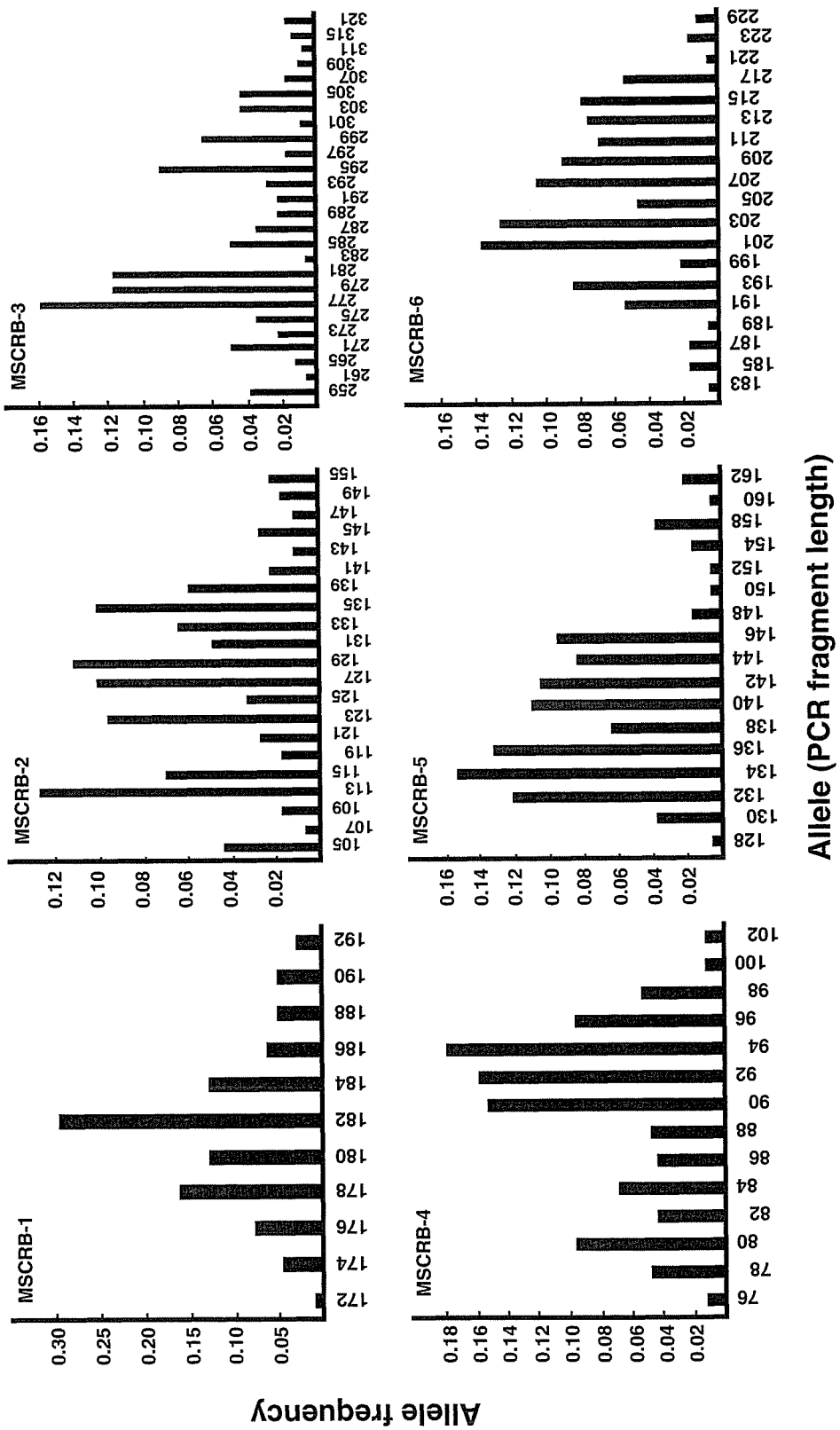

Fig. 3. Allele frequencies at six microsatellite loci (MSCRB-1 to -6) in all individuals of *Clethrionomys rufocanus bedfordiae* examined in this study

minor (<10%). Three individuals infected with hantavirus, specimens #32, #84, and #89, carried haplotypes H, C, and H, respectively. Of these, #84 was a young female, #32 was an over-wintered male, and #89 was a young male, as inferred from their body weights (data not shown). Both infected males had the same haplotype H (6.3%) which was relatively less frequent among all haplotypes within the population (Fig. 2).

Microsatellite analysis

All six loci were highly heterozygous. There were 11, 21, 26, 14, 17, and 19 alleles for MSCRB-1, -2, -3, -4, -5, and -6, respectively, and the allele frequencies at each locus are given in Fig. 3. The heterozygosities observed at MSCRB-1, -2, -3, -4, -5, and -6 were 0.8446, 0.9242, 0.9315, 0.8885, 0.8998, and 0.9154, respectively. All the observed heterozygosities agreed with Hardy-Weinberg expectations ($p < 0.01$ at MSCRB-1, -3, -4, -5, and -6; $p < 0.1$ at MSCRB-2). Kinships among all the individuals were estimated using pairwise relatedness (R) values, which were determined by symmetric assessment ($-1 < R < 1$), where higher R-values indicate close kinships among the individuals [31]. R ranged from -0.2779 to 0.7795. There was a slightly negative correlation ($r = -0.0768$, which is not significant at $p < 0.05$) between R and site distance, which was categorized into five distance classes (Fig. 4), but this relationship was not supported by Mantel's test [26]. Therefore, we concluded that the R-values were weakly correlated with geographic distance within the surveyed forest. Accordingly, we considered that the voles examined were obtained from a random-mating population ecologically and genetically based on the microsatellite analysis and that they were appropriate for examination to evaluate our hypotheses.

Of the individuals with haplotype C (n = 13), five (#5, 7, 10, 80, and 84) shared relatively high R-values and allele combinations (0.2359–0.6154, Tables 3 and 4, Fig. 4). In addition, they were thought to have been born at the same time based on their similar body weights (Table 3), the season, and the proximity of their sites of capture (Sites I and II). Therefore, they were assumed to be sibling offspring from a dam at Site I born in the early summer of 2000. Of these, #84 was infected with hantavirus, while the others were negative for hantavirus infection. Therefore, within one sibling group showing haplotype C, there were infected and uninfected individuals.

In contrast, the allele combinations of the three individuals infected with hantavirus (#32, #84, and #89) were relatively random, and their pairwise R-values ranged from -0.0542 to 0.0721 (Tables 5 and 6). These R-values were low compared with the pairwise R-values for all the individuals studied (Table 6). Although #84 had haplotype C, #32 and #89 carried the same haplotype, H, which was uncommon in the study population. Furthermore, the latter two individuals were male and they were not related according to the kinship calculation considering R-values (Table 6, Fig. 4) and their ages estimated from body weight. Therefore, males #32 and #89 may have come from other forest areas to the surveyed forest [13].

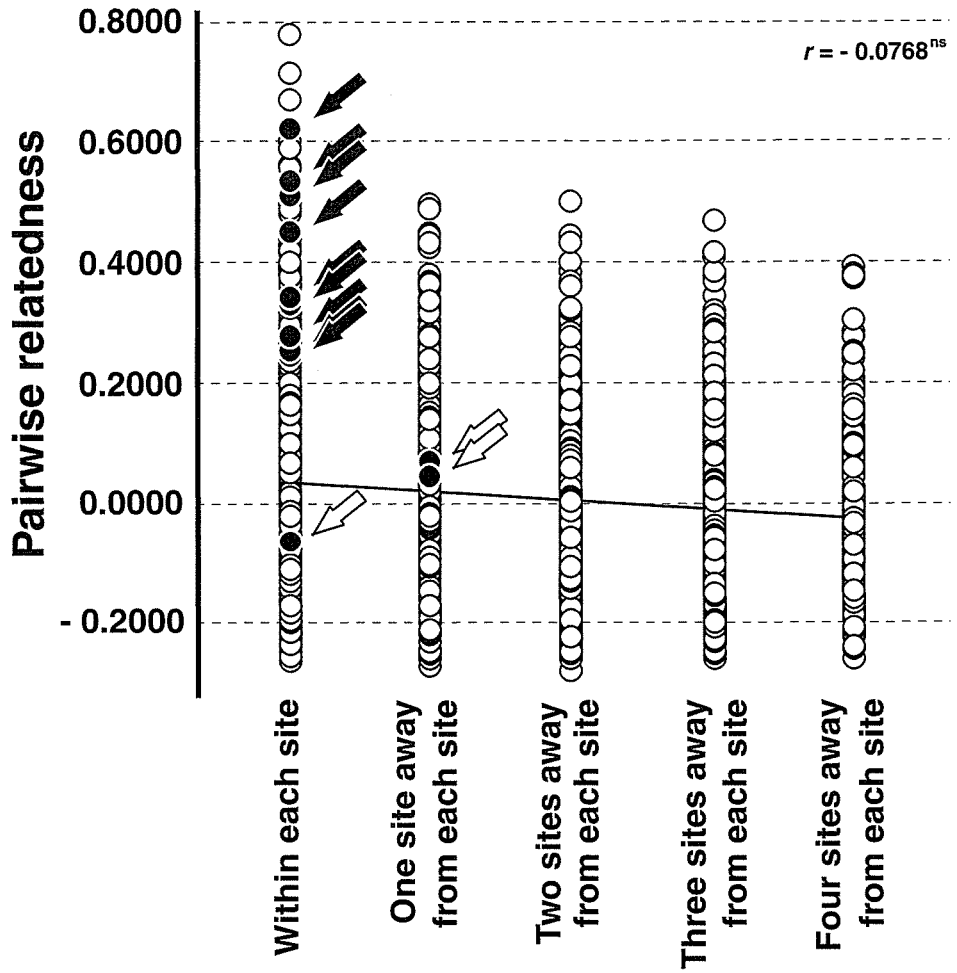

Fig. 4. Relationship between relatedness and distance among trapping sites. Solid arrows indicate pairwise relatedness in a sibling group carrying mitochondrial haplotype C (see text and Table 4). Open arrows indicate pairwise relatedness among infected individuals with PUUV-related hantavirus (see text and Table 6)

Table 3. Allele combinations of five individuals which were considered as sibling offsprings carrying D-loop haplotype-C

Individual	Sex	BW*	mtDNA	Microsatellite loci (MSCRB)					
				-1	-2	-3	-4	-5	-6
#5	f	18.0	C	182/184	105/113	277/285	78/90	134/136	203/217
#7	m	17.4	C	180/182	105/141	277/285	78/78	136/146	203/223
#10	f	16.8	C	178/184	113/119	277/287	78/80	136/146	209/217
#80	m	19.6	C	180/182	105/113	277/285	80/80	134/144	217/223
#84**	f	17.8	C	178/180	113/119	285/285	80/80	134/140	203/223

*BW, body weight

**#84 individual was infected with PUUV-related hantavirus

Table 4. Pairwise relatedness ($-1 < R < 1$) among five individuals carrying D-loop haplotype-C in all individuals examined

Individual	#5	#7	#10	#80	#84*
#5	–				
#7	0.5346	–			
#10	0.3854	0.2764	–		
#80	0.4370	0.3305	0.2707	–	
#84*	0.2369	0.2359	0.2566	0.6154	–

*#84 individual was infected with PUUV-related hantavirus

Table 5. Allele combinations of three individuals which were infected with PUUV-related hantavirus

Individual	Sex	BW* (g)	mtDNA	Microsatellite loci (MSCRB)					
				-1	-2	-3	-4	-5	-6
#32	m	33.4	H	178/182	123/135	279/279	90/92	130/140	203/215
#84	f	17.6	C	178/180	113/119	285/285	80/80	134/140	203/223
#89	m	23.6	H	178/180	113/131	271/299	90/96	134/136	191/193

*BW, body weight

Table 6. Pairwise relatedness ($-1 < R < 1$) among three individuals which were infected with PUUV-related hantavirus

Individual	#32	#84	#89
#32	–		
#84	0.0495	–	
#89	−0.0543	0.0722	–

Discussion

Random occurrence of virus transmission within the vole population

Our kin structure data for the sibling group (individuals. #5, #7, #10, #80, and #84) carrying D-loop haplotype C suggest that hantavirus transmission among closely related voles, such as parent-offspring or siblings, does not always occur in the vole population. As the antibody titer to hantavirus was relatively low in individual #84, the infection may have occurred recently [22], perhaps after dispersal, based on experimental evidence of the relationship between the time after infection and titer in rats [3, 4]. Therefore, the infection of individual #84 with the PUUV-related hantavirus might not originate from its dam or siblings (Table 4, Fig. 4). Furthermore, horizontal transmission to individual #84 might have occurred randomly through aerosolized excrement contaminated with the hantavirus or from close contact with infected individuals in the forest. Thus, our first hypothesis assuming

"close relationship between close vole kinship and hantavirus transmission" would be rejected and these data suggest that hantavirus transmission occurs randomly in the vole population regardless of kinship. Previous surveys have also supported that virus transmission within close kinships in host rodents rarely occurs [7]. In the most previous study [7], traditional and ecological methods such as the capture-mark/release-recapture method was used to trace rodent behavior and kinship within a forest. In contrast, we used microsatellite analysis to understand the detailed behavior and kinship of the rodents and their relationships were determined at a higher resolution. Considering the resolution of current analysis, we thought that our research method is invaluable epizootiologically and ecologically to trace transmission mode of hantavirus under natural conditions.

Although we obtained valuable data, we were only able to detect one set of siblings (Tables 3 and 4, Fig. 4). Considering our methods, this would be influenced by the choice of season (only autumn in this study), the locations of the area surveyed and control areas, and the total trapping period [7]. Accordingly, to confirm our data, we should expand our survey, perhaps trapping individuals twice per year, and establish a control forest. In particular, it will be necessary to examine sex-related differences in dispersal in each season to evaluate the sexual bias in the collection results in the present study.

Relationship between vole dispersal and virus transmission

Our second hypothesis considered the importance of male dispersal and the spread of virus transmission. Although our samples were biased towards females (74/96, 77.1%; Table 3), 2 of 22 males caught were infected. The reported frequencies of infection with *Sin Nombre virus* in *Peromyscus maniculatus* and with PUUV in *C. glareolus* were significantly higher in males [5, 7]. In addition, our voles were collected in September 2000, which was thought to be after the dispersal of young voles born in the spring of 2000 [13, 15]. On the basis of sexually different dispersal patterns of the voles [13, 33] and current data of mitochondrial DNA sequences and *R*-values, the two infected adult males (#32 and #89) might have probably come from other forest areas (Table 6, Fig. 4). Accordingly, considering the vole behavior outlined above and previous studies, the spread of virus transmission seems to be related to the dispersal patterns of male vole individuals [7, 13, 33].

Our results may suggest the presence of a relationship between male dispersal and the spread of virus transmission but there were a few problems remain with the ecological surveillance methods. To resolve these problems, it is necessary to address the following points. First, it is possible that small vole populations in surrounding forest areas moved into the study forest after vole collection. To consider this artificial effect on the population, it is necessary to reconfirm the kin structure of the study population and the invasion frequency in the study forest. Namely, we should investigate samples from other forest areas to confirm whether the two infected males had invaded our study forest. Second, to clarify the infection frequency in voles of both sexes and of various ages in the forest, ongoing surveillance is required in different seasons and years [7]. Third, we focused on

the kin structure of a small population of *C. rufocanus bedfordiae* in the autumn of 2000. However, other factors may affect virus transmission in nature. Hence, we should consider factors that might be related to infection, such as wounds on the voles or the condition of the forest undergrowth, as suggested in the literature [7]. These additional studies would clarify the modes of hantavirus transmission.

Elucidating hantavirus-transmission using microsatellite markers

This study was the first to apply microsatellite markers, which were usually used to study animal ecology [13], to examine the modes of hantavirus transmission in nature. The detailed relationships between hantavirus transmission and kinship and host dispersal patterns have not been studied previously. We considered two hypotheses for the mechanism of hantavirus transmission from the data on vole ecology. Thus, the results of the present study are valuable for understanding the modes of hantavirus transmission, such as the tendency to random transmission and the contribution of male dispersal to the spread of hantavirus transmission within a vole population. To clarify our conclusion, we will continue our surveillance of the level of hantavirus infection and of the modes of hantavirus transmission within *C. rufocanus bedfordiae* populations in Hokkaido.

Many serious rodent-borne viral and bacterial infectious diseases occur in nature, but their modes of transmission and infection mechanisms are currently investigated using only traditional epizootiological methods. Our method using microsatellite markers will prove valuable for tracing the mode of virus transmission in other zoonoses.

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FULL PAPER

Comparison of virulence of various hantaviruses related to hemorrhagic fever with renal syndrome in newborn mouse model.

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Abstract

The virulence of hantaviruses that are antigenically related but have different genetic characteristics from the prototype of hantavirus , Hantaan (HTN) virus, was examined in newborn mice. The H5 and B78 strains of the Amur (AMR) genotype, the Bao14 strain of the Far East (FE) genotype, and the 76-118 strain of HTN virus were inoculated subcutaneously (1 focus-forming unit ; FFU) into newborn mice. All of the AMR and FE genotype viruses inoculated mice were died by 16 days post-infection (dpi) and 21 dpi, respectively, while 50% of the HTN virus inoculated mice survived until 30 dpi. The AMR and FE genotype viruses inoculated mice had high viral titers in the lung (1.3×10^6 to 1.3×10^8 FFU/gram [g] tissue) , brain (2.1×10^7 to 1.2×10^9 FFU /g tissue), and kidney (2.5×10^5 to 1.6×10^7 FFU/g tissue), and showed a detectable level of antibodies (titers 1 : 16-1 : 32) at 14 dpi. In contrast, the HTN virus infected mice had viruses only in the lungs at low titers (1.1-5.3×10^5 FFU /g tissue). Observations of body-weight changes revealed that the AMR and FE genotype viruses inoculated mice had lower growth rates than the HTN virus inoculated mice. These data suggest that the AMR and FE genotype viruses are more virulent than the HTN virus in newborn mice.

Key words : Amur, Far East, Hantavirus, mouse, virulence

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Introduction

The genus *Hantavirus* is the only genus of the family *Bunyaviridae* that comprises rodent-borne human pathogens[3]. Although persistently infected rodents shed virus in the urine, saliva, and feces, hantaviruses do not produce any clinical sign of illness in their reservoir hosts. Generally, humans acquire infection mainly *via* inhalation of aerosolized infected rodent excreta[20], and are the dead-end host. Hantaviruses are distributed worldwide, causing two forms of human disease : hemorrhagic fever with renal syndrome (HFRS) in Eurasia, and hantavirus pulmonary syndrome (HPS) in the Americas[1,2,8,13,16]. The geographic distribution of the rodent reservoirs of pathogenic hantaviruses is the major factor that determines the occurrence of human hantavirus infection globally[17,18].

In East Asia, which includes China, Korea, and Far East Russia, two different hantaviruses have been identified., i.e., the Hantaan (HTN) and Seoul (SEO) viruses, which cause severe and moderate forms of HFRS, respectively. The HTN virus is maintained by *Apodemus* agrarius and the SEO virus is carried by *Rattus norvegicus*[5,6,19]. HFRS causes a serious public health problem, mainly in China and also in Far East Russia. The lists of hantaviruses and their rodent reservoirs have grown in recent years with the development of new diagnostic procedures[15]. New hantaviruses and their reservoirs have been identified in Far East Russia[10,20]. The Amur (AMR) genotype is one of the newly identified genotypes of hantaviruses in HFRS patients, for which *Apodemus peninsulae* is the natural reservoir of the virus[7,23]. This genotype appears to be distinct from the prototype HTN virus, based on genetic, antigenic, and ecological characteristics (unpublished data). Another virus belonging to the Far East (FE) genotype has also been identified in HFRS patients in the same region[11,23]. The FE genotype is more related to the HTN virus in terms of genetic and antigenic characteristics (unpublished data). However, the reservoir of the FE -lineage viruses remains unknown.

Recent studies have provided evidence that numerous hantaviruses persist in China[22]. In our previous study, we indicated that viruses that are related to the AMR and FE genotypes also exist in China and Korea[7]. Since newborn mice are susceptible to HTN virus and the infection is lethal for inoculated mice, hantavirus virulence was evaluated using this mouse model. In order to provide information on the virulence of the viruses that circulate in China and Far East Russia, which are distinct from the HTN virus, we inoculated strains of AMR, FE genotypes, and HTN viruses into newborn mice. The results show that the AMR and FE genotype viruses are more virulent than the HTN virus. All of the animals that were inoculated with the AMR and FE genotype viruses died within 21 days post inoculation (dpi), while 50% of HTN virus inoculated animals survived until 30 dpi.

Materials and methods

Viruses :

The hantavirus strains, H5, B78, and Bao 14, which were isolated in China, were used in this study. Phylogenetic analysis based on limited nucleotide sequences indicates that the H5 and B78 isolates belong to the AMR lineage and that Bao14 belongs to the FE lineage[7]. Thus, H5 and B78 were used to examine the virulence of the AMR genotype virus in mice, and Bao14 was used to represent the FE genotype.

The viruses were propagated in Vero E6 cells. The HTN virus strain 76-118 was passaged through Vero E6 cells eleven times, while the H5, B78, and Bao 14 strains were

passaged three times.

Animal experiments :

Specific-pathogen-free pregnant inbred (BALB/c) mice were obtained from SLC (Hamamatsu, Japan). Newborn BALB/c mice (within 24h after birth) were inoculated subcutaneously (sc) with 1 or 0.001 focus-forming units (FFU) of H5, B78, Bao14, and HTN 76-118. Each group included ten newborn mice. The mortality rate, clinical signs, and bodyweights were recorded until 45 dpi. Four animals in each group that were inoculated with 1 FFU-virus were sacrificed at 14dpi, and the sera and organs were collected, to determine antibody responses and to titrate the virus loads in the lungs, liver, kidney, spleen, and brain. The dams were allowed to suckle the neonatal mice and food pellets and water were provided ad *libitum*. All of the animal experiments were carried out under biosafety level 3 containment conditions, according to the guidelines of the Graduate School of Veterinary Medicine, Hokkaido University.

Indirect immunofluorescent antibody assay (IFA):

The sera that were collected at 14 dpi were tested by IFA for antibodies against H5, B78, Bao14, or HTN viruses. Antigen slides for each virus were prepared by spotting virus-infected Vero E6 cells onto 24-well slides. After incubation for 4h at 37℃, the cells were fixed with cold acetone and air-dried. The mouse sera were diluted in phosphate-buffered saline (PBS) and spotted onto homologous antigen slides. After incubation at 37℃ for 1h, the slides were washed three times with PBS, and fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (ICN Pharmaceuticals, Aurora, OH, USA) was applied. The slides were incubated at 37℃ for 1 h and washed with PBS three times. When observed under a fluorescence microscope, scattered granular fluorescence in the cytoplasm of infected Vero E6 cells was taken as a positive reaction.

Titration of virus in the organs of infected mice :

The virus titers (FFU) in the organs were measured using previously described method, with slight modification[9]. Briefly, 10% tissue homogenates of brain, kidney, and lung tissues of infected mice were serially diluted in minimum essential medium (MEM : Gibco, Invitrogen, NY, USA) and inoculated onto Vero E6 cells that were seeded in 8-well chamber slides. The inocula were discarded after 1 h incubation at 37℃ in a CO_2 incubator, and the slides were overlaid with 1.5% carboxymethyl cellulose (CMC) in MEM. The slides were then incubated for 5days at 37℃ in a CO_2 incubator. The virus titers were determined by averaging the IFA-visualized foci from four individual wells.

Results

Although the AMR and FE genotype viruses cause severe HFRS, virulence comparisons in the animal model with the HTN virus, which is the prototype hantavirus, are lacking. In this study, we compared the virulence attributes of the AMR, FE and HTN viruses in the newborn mouse model. The clinical symptoms, such as ruffled coats, lethargy, and paralysis, became apparent one day prior to the death (starting from 14 and 21 dpi, respectively) of mice inoculated with the AMR and FE genotype viruses. In contrast, mice inoculated with HTN virus showed only ruffled coats and excitability for 2-3 days before the death (starting from 22 dpi). Mice inoculated with the H5 and B78 strains of the AMR genotype showed mortality at 14 dpi, and all of the animals were dead by 16 dpi. All of the mice

inoculated with strain Bao14 of the FE genotype died by 21 dpi. In contrast, the mice inoculated with 76-118 strain of HTN virus started dying at 22 dpi, and 50% of the animals survived until 30 dpi (Fig. 1). Antibodies were detected in mice inoculated with the AMR and FE genotype viruses at 14 dpi, with titers that ranged from 1:16 to 1:32, while the HTN virus infected mice did not produce detectable antibodies. Furthermore, we quantitated the infectious virus loads in various organs of the infected animals at 14 dpi. In the AMR viruses inoculated mice, the virus loads in the brain, lungs, and kidney were 1.5×10^8 to 1.2×10^9, 1.3×10^6 to 1.3×10^8, and 6.5×10^5 to 3.4×10^6 FFU/g tissue, respectively. In the FE virus inoculated animals, the virus loads in the brain, lungs, and kidney were 2.1 to 4.3×10^7, 6.6 to 7.5×10^7, and 2.5×10^5 to 1.6×10^7 FFU/g tissue, respectively (Table 1). In contrast, in the HTN virus inoculated mice, virus was detected only in the lungs, at titers of 1.1-5.3x 10^5 FFU/g tissue. The order in terms of decreasing virus titer in the brain tissues was B78 = H5 > Bao14 > 76-118. Our histological analysis also indicates that strains of the AMR and FE can infect to more cells and spread to more organs than HTN virus does, especially in brains (data not shown).

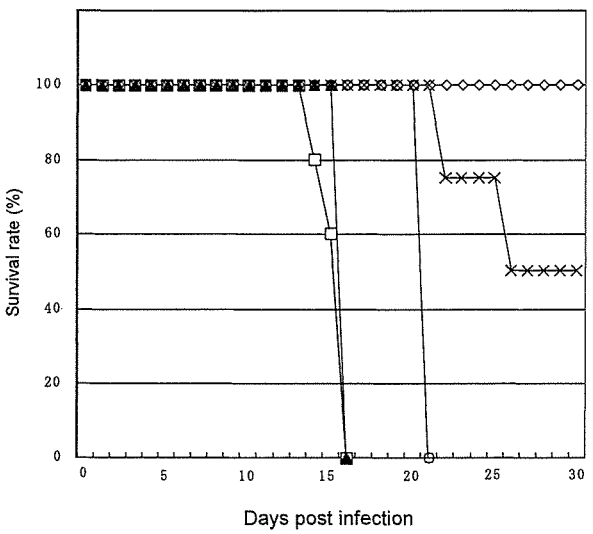

Fig. 1. Survival rates of mice that were inoculated with the AMR, FE genotype viruses and the HTN virus. Newborn BALB/c mice were inoculated sc with 1 FFU of each virus strain. The mice that were inoculated with different virus strains are indicated as follows: Control, open diamond; H5, open square; B78, closed triangle; Bao14, open circle; and HTN 76-118, cross.

The body-weight of the each inoculated animal was recorded from 0 to 30dpi (Fig. 2A and 2B). The growth of the mice infected with 1 FFU of viruses was apparently delayed compared to the control animals. The body-weights of the animals inoculated with the AMR genotype viruses were significantly lower than those of the control from 14 to 15 dpi ($p < 0.05$). The growth of the mice inoculated with the FE genotype virus was also sig-

Table 1. Virus and IFA antibody titers in BALB/C mice inoculated with H5, B78, Bao 14, and HTM viruses at 14 days post inoculation

Inoculated virus strain	Animal	Virus titer (FFU-g. tissue)			IFA antibody titer
		Brain	Lung	Kidney	
HTN	1 [a)]	$< 5 \times 10^3$	1.1×10^5	$< 5 \times 10^3$	<16
	2	$< 5 \times 10^3$	5.3×10^5	$< 5 \times 10^3$	<16
Bao14	5	2.1×10^7	6.6×10^7	2.5×10^5	32
	6	4.3×10^7	7.5×10^7	1.6×10^7	32
H5	9	1.5×10^8	1.6×10^6	6.5×10^5	32
	10	5×10^8	1.3×10^8	3.4×10^6	<16
B78	11	1.2×10^9	1.2×10^8	2.5×10^6	16
	12	2.3×10^8	1.3×10^6	1.8×10^6	16

[a)] Individual animal

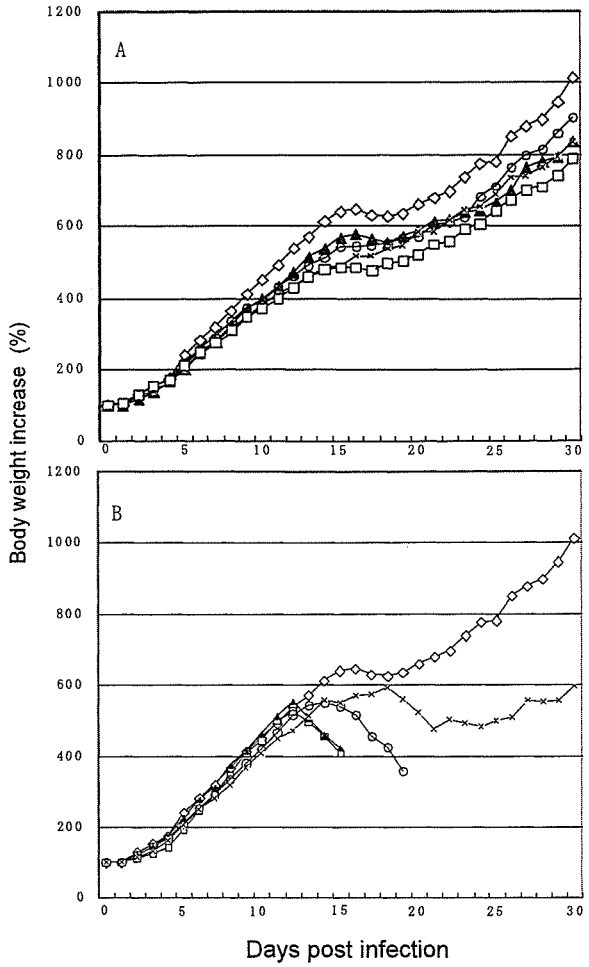

Fig. 2. Growth rates of newborn BALB/c mice that were inoculated sc with 0.001 or 1 FFU of the AMR, FE genotype viruses and the HTN virus. The infected animals were fed *ad libitum* in an animal room with biosafety level 3 containements. The body-weight of each animal was measured until 30 dpi. A : 0.001FFU inoculation ; B : 1 FFU inoculation. The mice that were inoculated with the different strains of viruses designated as follows : Control, open diamond ; H5, open square ; B78, closed triangle ; Bao 14, open circle ; and HTN 76-118, cross.

nificantly delayed from 15 to 19 dpi ($p<0.05$). However, there was no significant growth delay in the HTN virus inoculated mice. Interestingly, the growth of mice inoculated with 0.001 FFU of the AMR genotype viruses was significantly delayed from2dpi until the end of the observation period. There was a tendency towards delayed growth rates in the FE genotype virus and HTN virus inoculated mice.

Discussion

Various animal studies with different hantaviruses have reported that newborn mice are susceptible to hantavirus infection, with lethal outcome[14,24,25]. In this study, newborn mice were infected sc with the AMR, FE, and HTN viruses to evaluate the virulence of these hantaviruses, which are associated with HFRS.

The AMR, FE, and HTN viruses showed different virulence profiles in newborn mice. All of the newborn mice infected with the AMR and FE genotype viruses died between 16 to 21 dpi, while 50% of the HTN virus inoculated mice survived until 30 dpi. These results indicate that the AMR and FE genotype viruses are more virulent than the HTN virus in this mouse model. Increases in body-weight were delayed to a greater extent in the mice inoculated with the AMR and FE genotype viruses than that in the HTN virus inoculated mice. In addition, strains of the AMR and FE genotypes showed dissemination among the various organs examined, while the HTN virus was detected only in the lungs with lower titer. The virus titer in the brains of the AMR genotype viruses inoculated mice reached 1.2 x 10^9 FFU/g tissue at 14dpi. The order in terms of decreasing virus titer in the brains of infected animals was as follows : AMR>FE> HTN. The early deaths in the AMR and FE genotype viruses inoculated mice may have been related to the higher virus titers in the brains of the animals. These results indicate that replication of the AMR and FE genotype viruses is more rapid in mice than that of the HTN virus, and that the AMR and FE genotype viruses are more virulent than the HTN virus.

There are few reliable animal models for