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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 Baol4 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 pathogens³. 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 excreta²⁰, 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 procedures¹⁵. 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 China²². In our previous study, we indicated that viruses that are related to the AMR and FE genotypes also exist in China and Korea⁷. 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 lineage⁷. 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 body-weights 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°C, 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°C 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°C for 1h 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 modification⁹. 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 1h incubation at 37°C in a CO₂ incubator, and the slides were overlaid with 1.5% carboxymethyl cellulose (CMC) in MEM. The slides were then incubated for 5days at 37°C in a CO₂ 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

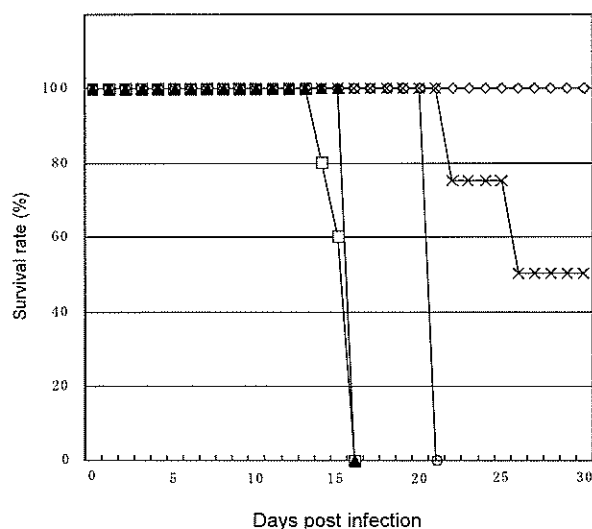


Fig. 1. Survival rates of mice that were inoculated with the AMR, FE genotype viruses and the HTN virus. Newborn BALB/c mice were inoculated sc with 1 FFU of each virus strain. The mice that were inoculated with different virus strains are indicated as follows: Control, open diamond; H5, open square;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

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 $1.1 - 5.3 \times 10^5$ 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).

The body-weight of the each inoculated animal was recorded from 0 to 30dpi (Fig. 2 A 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

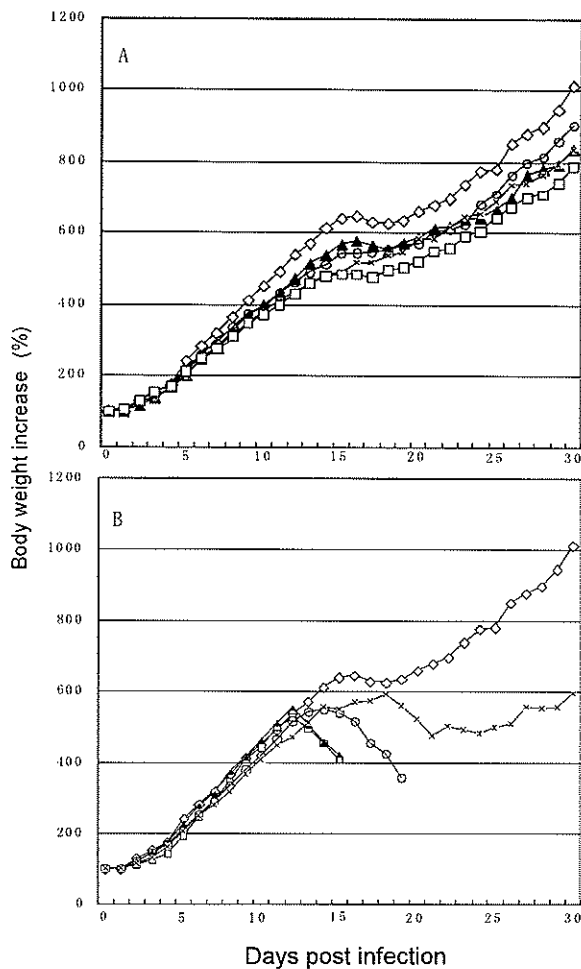


Fig. 2. Growth rates of newborn BALB/c mice that were inoculated sc with 0.001 or 1 FFU of the AMR, FE genotype viruses and the HTN virus. The infected animals were fed *ad libitum* in an animal room with biosafety level 3 containments. The body-weight of each animal was measured until 30 dpi. A : 0.001FFU 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 from 2dpi 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 1.2×10^9 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

HFRS-related viruses, except for the rhesus monkey and Syrian golden hamster models^{4,12}. Although hantavirus pathogenesis in the mouse model differs from that in humans, we found that all of the hantaviruses that are associated with severe HFRS, such as AMR, FE, and HTN viruses, are potentially virulent for newborn mice. To date, there is no adult mouse model of lethality due to HFRS-related hantaviruses. Since the adult mouse model is extremely useful for vaccine evaluation, virulence and pathological analyses in adult mice are underway using the AMR genotype virus in adult mice.

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