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A new natural reservoir of hantavirus: isolation of hantaviruses from lung tissues of bats

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Summary. Two species of bats were confirmed as new natural reservoirs of hantavirus. Antibodies to Hantaan virus were detected in 3.40% (23 of 677) of bats captured from 1989 to 1992 in Korea by the IFA technique. Areal distribution of immunofluorescent antibody were different, and seropositive rates were much high in sera of bats captured in summer (3.82%) and winter (5.82%). Viral antigens were observed in the lungs (3 of 16) and kindney (1 of 7). Two hantaviruses were isolated from lung tissues of *E. serotinus* and *R. ferrum-equinum* through a cell culture system, designated CUMC-92B8 and -92B48, respectively. Using Rous associated virus-2 reverse transcriptase-directed PCR and 2 oligonucleotide primer pairs, genomic sequences of the isolates were amplified. Amplified products of the isolates and reactivities to monoclonal antibodies very closely resembled those of Hantaan virus. These data suggest that the serotype of the isolates is closely related to Hantaan virus, and bats serve as reservoirs of hantavirus.

Introduction

Hemorrhagic fever with renal syndrome (HFRS) is a viral zoonosis caused by the hantavirus genus of the *Bunyaviridae* family [5, 15, 16, 21]. The prototype virus, Hantaan virus (strain 76–118), was initially isolated from the lung tissue of *Apodemus agrarius coreae* [8]. Infection with hantavirus may occur throughout year, but biphasic occurrence of HFRS has been observed in endemic regions in Far East Asia including Khabarovsk, Amur, and Korea [13, 20]. The breeding behavior of *Apodemus* mice coincides with the planting and harvesting activites of farmers in these areas [8]. Incidence rates of HFRS vary considerably different between endemic regions and form one year to the next in the same region. In Korea, approximately 600–1 000 cases of Korean hemorrhagic fever occur annually.

The importance of wild rodents including *Apodemus agrarius, Microtus fortis,* and *Clethrionomys glareolus* as natural reservoirs of the etiologic agents of HFRS was proposed by the early investigations of Soviet and Japanese scientists. *Apodemus agrarius, Clethrionomys glareolus, Microtus pennsylvanicus,* and *Rattus norvegicus* were shown to be the primary reservoirs of Hantaan, Puumala, Prospect Hill, and Seoul viruses, respectively [2, 10, 13, 14]. Hantaviruses have been isolated from many other wild rodent species [1, 4, 17], house rats [10, 19] and laboratory rats [12] in widely divergent geographical areas. These rats serve as the reservoirs of Seoul virus and Hantaan virus in urban centers around the world. Furthermore, serological evidence of hantavirus infection has been demonstrated in species of arvicolid, cricetid, and sciurid rodents [24]. Until now 4 families of the *Rodentia* order (*Muridae, Arvicolidae, Cricetidae,* and *Sciuridae*), 2 families of the *Insectivora* order (*Soricidae* and *Talpidae*), and 1 family of the *Canivora* order (*Felidae*) have been known as natural reservoirs of hantaviruses [24]. Detection of antibodies to Hantaan virus in the sera of birds in Korea has been reported [11]. In addition, antibodies to hantavirus have been found in domestic animals such as chickens and pigs [24]. It is increasingly likely that a new reservoir of hantavirus will be found.

In this paper, we report new natural reservoirs of hantavirus. We demonstrated antigens of hantavirus in the lung and kidney of bats captured in our country and isolated hantaviruses from lung tissues of bats.

Materials and methods

Capturing and processing of bats

Using butterfly nets, we captured bats in caves and under eaves of roofs from January 1989 to December 1992. The five capture regions were located in four Provinces in Korea. Details are designated on the map (Fig. 1). We selected the regions on the basis of previous sero-epidemiological surveys of hantavirus. All bats captured alive were transported to the laboratory as early as possible, then anesthetized with ether, bled by cardiac puncture, and samples of lung, spleen and kidney were removed aseptically. Specimens were stored at $-70\,^{\circ}C$ until use.

Antibody detection

Bat sera were made in two dilutions of 1:16 and 1:32 in 0.01M phosphate buffered saline (PBS, pH 7.2), and then tested for antibody to Hantaan virus (strain 76–118) by the IFA technique [6]. Fluorescein isothiocyanate (FITC)-conjugated goat antibody (IgG $F(ab)'_2$) to mouse (Cappel Laboratories, Cochranville, PA, U.S.A.) was employed for the IFA staining. Eight antiglobulin units were employed. Sera exhibiting specific immunofluorescence in two dilutions were considered seropositive, and titrated in two-fold increments. IFA titers were expressed as the reciprocal of the highest dilution displaying specific immunofluorescence.

Antigen detection

Specimens of lung, spleen kidney were embedded in a polyethylene compound (Tissue-Tek II, Miles Laboratories, Napierville, IL, U.S.A.), then frozen at $-70\,^{\circ}C$ until examination.

Fig. 1. Bat capture regions in Korea. Bats were captured annually at five regions, using butterfly nets from 1989 to 1992

4 μm sections were cut in a Cryocut microtome (2800 Frigocut, Reichert-Jung, Federal Republic of Germany) at −25 °C, and fixed in cold acetone, then stained by the IFA technique [6]. Immunofluorescence was examined with a fluorescence microscope (Axiophot, Carl Zeiss, Federal Republic of Germany). Random sections were screened for the presence of reovirus and lymphocytic choriomeningitis (LCM) virus.

Isolation of hantavirus

20% tissue emulsions of lung and kidney from antigen-bearing bats were prepared in a maintenance medium consisting of Eagle's minimum essential medium with 10% fetal calf serum, 4% L-glutamine (200 mM, 100 X; Gibco, Grand Island, NY, U.S.A.), and 1% non-essential amino acids (10 mM of each amino acid : M.A. Bioproducts Walkersville, MD, U.S.A.) [6]. Monolayers of Vero-E6 cells grown in 25-cm^2 culture bottles were inoculated with 0.3 ml of the 20% emulsions, absorbed for 90 min at 37 °C, then maintained with maintenance medium. Serial blind passages were performed at 14 day intervals [13]. Identification of the isolate was performed by the usual procedures [14]. Antigenic determinants of the isolates were compared using 5 monoclonal antibodies produced in our laboratory. 5A3, 7B1, and 8E7 were reactive with the N protein (M.W.50 kD) of Hantaan and Seoul viruses, and 6E7 and SV2C2 reactive with the G2 glycoprotein (M.W. 55 kD).

RNA extraction

RNA from virus infected Vero-E6 cells was extracted by the guanidinium thiocyanate (GT)/cesium chloride method [3]. Briefly, $2 \sim 4 \times 10^7$ cells were washed with PBS, then completely lysed in 3 ml of GT (Fluka Biochemica, Switzerland) buffer consisting of 2.8 ml GT solution and 0.2 ml 2-mercaptoethanol. The lysate was layered onto 0.8 ml of 5.7 M cesium chloride (Grade I, Sigma Chemical Co., St. Louis, MO, U.S.A.) in the Beckman Ultra-clear centrifuge tube, and centrifuged at 35 000 rpm for 15 h at 20 °C using SW 50.1 Ti rotor (L8-70M, Beckman Instruments, Cedar Grove, NJ, U.S.A.). The RNA pellet was reconstituted with 0.2 ml of 1 × TE buffer (pH 7.2) and transferred to a polyethylene microtube. RNA was precipitated with 100% cold ethanol. The RNA pellet was resuspended in 20 µl of 1 × TE buffer. The RNA concentration was determined at 260 nm spectrophotometrically. To avoid false-positive results by product caryover [7], all samples were handled in a biosafety hood.

Reverse transcriptase-directed PCR

RNA was initially reverse-transcribed using random hexomer (hexadeoxynucleotide mixture: dp(N) 6 : Takara Biochemicals, Kyoto, Japan) and Rous associated virus-2 reverse transcriptase (RAV-2; Takara Biochemicals, Kyoto, Japan) [22, 23]. The resulting cDNA was used as a template to amplify hantavirus sequences, using 2 oligonucleotide primer pairs (Takara Biochemicals, Kyoto, Japan) (Table 1). After covering with 20 µl mineral oil (heavy white, Sigma, Chemical Co., St. Louis, MO, U.S.A.), 30 cycles of 1-min denaturation (94 °C), 2-min annealing (55 °C) and 3-min primer extension (72 °C) steps were performed in the thermal cycler (Perkin-Elmer-Cetus). Size fractionation of PCR products was performed by electrophoresis in 1.6% agarose gel (Ultra Pure, Bethesda Research Laboratories, Bethesda, MD, U.S.A.) prepared in 0.5 × TBE buffer containing 0.5 µg/ml of ethidium bromide.

Results

Detection of antibody to hantavirus in bats

A total of 677 bats caught alive over four years were classified into eight species. Antibodies to Hantaan virus were detected in 3.78% (16 of 423) of *R. ferrum-equinum* and in 3.70% (7 of 189) of *E. serotinus* (Table 2). *R. ferrum-equinum* was 62.5% (423 of 677) and *E. serotinus* was 27.9% (189 of 677). Reciprocal IFA titers ranged from 16 to 128. The genometric mean titers of anti-hantaviral antibodies

Table 1. Oligonucleotide primer pairs for hantavirus detection

Primer	Sequences (5′ → 3′)	Position	Product length (bp)
[a]HVSp 1	d(GCAGACTGGCTGAGCATCATCGTC)	1289–1312	405
HVSp 2	d(TGCTGTATCTGGAAGAAGCTTGCA)	908–931	
[b]BVMp 1	d(AGACTCCGCAAGAAACAGCAGTTA)	148–171	171
BVMp 2	d(CAGTTCTGIATAGCCTGACACACT)	1–24	

[a] *HVS* S segment region of Hantaan virus (76–118 strain)
[b] *BVM* M segment region of Seoul virus (B-1 strain)

Table 2. Immunofluorescent antibody against Hantaan virus in different species of bats (1989–1992)

Species	No. of positive/no. of tested	(%)[a]
Rhinolophus ferrum-equinum	16/423	(3.78)
Miniopterus schreibersii	0/47	(0.00)
Vespertilio superans	0/6	(0.00)
Myotis mystatinus	0/8	(0.00)
Murina leucogaster	0/2	(0.00)
Eptesicus serotinus	7/189	(3.70)
Myotis formosus	0/1	(0.00)
Myotis macrodactylus	0/1	(0.00)
Total	23/677	(3.40)

[a] Sera with reciprocal titers $\leqslant 16$ by IFA were considered seronegative

Table 3. Provincial distribution of immunofluorescent antibody against Hantaan virus in sera of *E. serotinus* and *R. ferrum-equinum* (1989–1992)

Province	No. of positive/no. of tested	(%)
Kangwon-do (region 1)	1/66	(1.52)
Chungchongnam-do (region 2, 3)	13/335	(3.88)
Kyongsangbuk-do (region 4)	2/88	(2.27)
Chunlabuk-do (region 5)	7/188	(3.72)
Total	23/677	(3.40)

Table 4. Seasonal distribution of immunofluorescent antibody against Hantaan virus in sera of *E. serotinus* and *R. ferrum-equinum* (1989–1992)

Season	No. of positive/No. of tested	(%)
Spring	1/24	(4.17)
Summer	15/393	(3.82)
Autumn	1/157	(0.64)
Winter	6/103	(5.82)
Total	23/677	(3.40)

were 1:29 in seropositive *R. ferrum-equinum*, and 1:26 *in E. serotinus*. Prevalence rates were high in sera of bats captured in the region 2 (3.88%) and region 5 (3.72%) (Fig. 1, Table 3). 87.0% (20 of 23) of seropositive bats were captured from region 2, 3 and 5. High incidence rates were observed in winter (5.82%) and

summer (3.82%) (Table 4). Antibodies to Hantaan virus were detected in 91.3% (21 of 23) of bats caught during two seasons.

Hantaviral antigens in tissues

Frozen sections of lung, spleen and kidney dissected from 16 *R. ferrum-equinum* and 7 *E. serotinus* were examined for the presence of hantavirus antigen by the IFA technique using convalescent-phase sera from patients with Korean hemorrhagic fever. Sixteen units of serum antibody to Hantaan virus were used for detection of hantaviral antigens in tissues. Fine granular immunofluorescence was detected in two lungs and one kidney section of *R. ferrum-equinum*, and one lung section of *E. serotinus* (Table 5, Fig. 2). Block titrations were carried out with convalescent-phase sera of KHF patients and antisera from ICR mice immunized with Hantaan virus with use of the IFA technique. Antigens of reovirus and/or LCM virus were not observed in random sections.

Isolation of hantavirus

Through the Vero-E6 cell culture system, two isolates were isolated from the lung emulsions (one from *E. serotinus* and the other from *R. Ferrum-equinum*),

Table 5. Detection of hantaviral antigen in the tissues of seropositive bats

Tissue	Species (No. of positive/no. of tested)	
	R. ferrum-equinum	*E. serotinus*
Lung	2/16	1/7
Spleen	0/16	0/7
Kidney	1/16	0/7

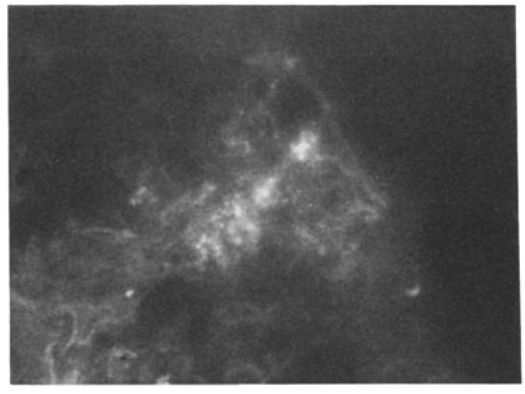

Fig. 2. Hantavirus-specific immunofluorescence in a bat lung section. Monoclonal antibody (7B1 in Table 6) was used for the IFA staining (400 ×)

and designated CUMC-92B8 and CUMC-92B48, respectively. Hantavirus specific immunofluorescence was initially detected 45–75 days after inoculation of Vero E6 cells with tissue emulsions. 0.1–0.3 percent of cells was infected at the time of first detection. Cytopathic effect of infected Vero-E6 cells was not observed. Virus-specific immunofluorescence was not detected in negative controls including uninfected Vero-E6 cells, conjugate control, and antisera to mycoplasmal species. Blind tests conducted with the coded sera of patients with Korean hemorrhagic fever and normal human sera produced 100% agreement of negative and positive results. When inoculated with 10^3 $TCID_{50}$/ml, CUMC-92B8 grew rapidly, but CUMC-92B48 grew slowly. 100% of the cells infected

Table 6. Reactivities of five monoclonal antibodies to two isolates

Hantavirus	Monoclonal antibody[a]				
	5A3	6F7	7B1	8E7	SV2C2
CUMC-92B8	++[b]	−	+++	++	−
CUMC-92B48	+++	−	+++	++	+
Hantaan (76–118)	++	−	+++	++	+
Seoul (80–39)	−[c]	+	+++	−	+++

[a] SV2C2 was produced to Seoul virus (strain 80–39), the others to Hantaan virus (strain 76–118) in our laboratory. 5A3, 7B1, and 8E7 were reactive with the N protein (M.W. 50 kD) and 6E7 and SV2C2 were reactive with the G2 glycoprotein (M.W. 55 kD)

[b] Reaction with antibody (+: < 30%, ++: 30%–70%, +++: 70% ≤)

[c] No reaction with antibody

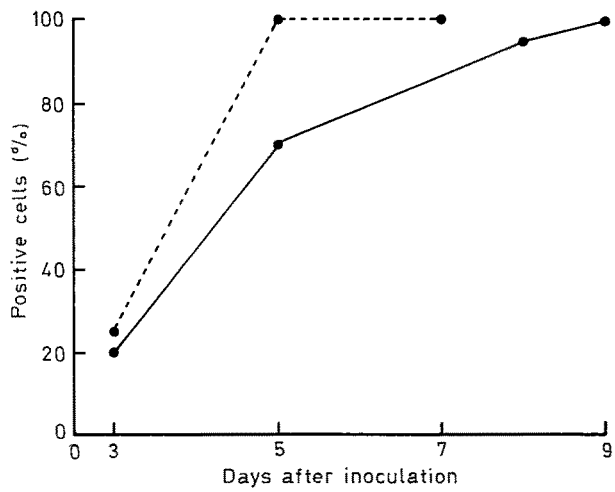

Fig. 3. Propagation of CUMC-92B8 and CUMC-92B48 isolates in Vero-E6 cells. ●---● CUMC-92B8; ●—● CUMC-92B48

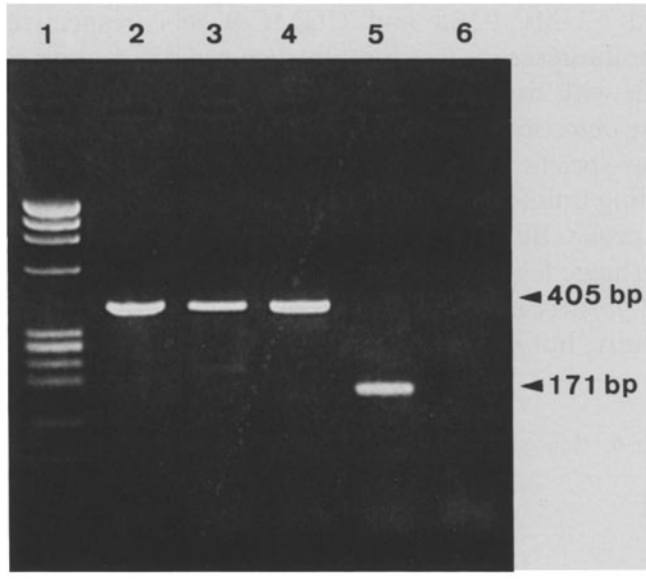

Fig. 4. PCR products amplified from RNA from extracted from infected Vero-E6 cells. *1* Phage 174 Hae III lysate; *2* S segment of Hantaan virus strain 76–118; *3,4* CUMC-92B8 and CUMC-92B48, respectively; *5* M segment of Seoul virus strain 80–39; *6* uninfected normal Vero-E6 cells

with the CUMC-92B8 displayed specific immunofluorescence by day 5, while CUMC-92B48 was delayed (Fig. 3). Peak titer of 3.0×10^7 $TCID_{50}$/ml was observed on day 7 in the culture supernatant of CUMC-92B8, and 1.0×10^8 $TCID_{50}$/ml on day 11 in that of CUMC-92B48. There were no differences between the isolates except one antigenic determinant (SV2C2). The two isolates shared identical antigenic determinants with Hantaan virus (Table 6).

Amplification of genomic sequences

The time of RNA extraction was monitored by the IFA technique. RNAs of two isolates were extracted on day 7 (CUMC-92B8) and 11 (CUMC-92B48) postinoculation. Amplified products of 405-bp and 171-bp were obtained for four hantavirus strains, using 2 primer pairs each specific for S segment of Hantaan and M segment of Seoul viruses (Fig. 4). The S segment of two isolates were amplified by the Hantaan virus S segment-specific primer pair (HVSpl:HVSp2), but not by the Seoul virus M segment-specific primer (BVMpl:BVMp2). The PCR products of the isolates and Hantaan virus (strain 76–118) were found to be the same size.

Discussion

The clinical severity of HFRS can be predicted by the geographical distribution of the predominant reservoir. The mild form without hemorrhagic symptoms is seen in Europe where Puumala virus-infected *C. glareolus* abound [17]. The

moderate form is recognized in Asia associated with *Rattus*-derived Seoul virus [12]. The severe form with typical hemorrhagic symptoms is common in Asia and in the eastern part of Europe where *A. agrarius* are the principal reservoirs [8]. Both the mild and severe forms are expected in eastern Europe and European U.S.S.R. where *Apodemus* and *Clethrionomys* species serve as reservoirs. The respiratory droplet or airborne route of infection is efficient and probably constitutes the mode of transmission of hantavirus. Particularly during periods of virus shedding, excretions and secretions from the infectious reservoir play a pivotal role in the maintenance of the enzootic cycle [24].

E. serotinus and *R. ferrum-equinum* were confirmed as new reservoirs of hantavirus by the demonstration of viral antigens and isolation of the virus. High prevalence rates of antibodies to Hantaan virus were found in sera of bats captured in domestic area (region 2) and those in caves (region 5). CUMC-92B8 was isolated from *E. serotinus* captured in region 2, and CUMC-92B48 from *R. ferrum-equinum* captured in region 5. Sporadic cases of HFRS occurred annually in the above regions, but these regions are not endemic regions. The habitat of *E. serotinus* is similar to that of house rats. We have caught them under the eaves of roofs (region 2). Therefore, *E. serotinus* has many chances of frequent contact with the infectious excretions and secretions of house rats. In contrast, *R. ferrum-equinum* inhabits caves in mountains far from human dwelling places. Serological evidence of infection with hantavirus was reported in two species of bats, *R. ferrum-equinum* and *Vespertilo abramus*, captured from different regions in Korea [11]. However, they neither demonstrated antigens in tissue nor isolated the virus from bats. *V. abramus* was not caught during our trapping. *R. ferrum-equinum* and *E. serotinus* were evenly distributed, but *V. abramus* and other species of bats seemed to be localized in Korea. Bats are classified into the order *Chiropera*. *E. serotinus* belongs to the *Vespertilionidae* family, and *R. ferrum-equinum* to the *Rhinolophidae* family.

Incidence rates of hantavirus infection in bats were much low in autumn (Table 4). Most of seropositive bats was caught during summer and winter. There were no differences of incidence rates between the two seasons. These findings are very important because seasonal incidence rates of bats were much different from those of *Apodemus* mice. In *Apodemus* mice, the minor peak of incidence is seen in spring and the major peak in autumn, and about one month after each peak, HFRS occurrences follow [8, 13, 20]. We have found the peak incidence rates between bats and *Apodemus* mice alternate in Korea. We can now explain more exactly how hantavirus infection occurs throughout the year. In Bulgaria and Hungary, most HFRS cases have occurred during the summer [24]. It is possible that some species of bats may serve as reservoirs in these countries. From now on, it will be necessary to look through the ecology of bats in relation to other reservoirs of hantavirus.

Although an inactivated vaccine has been inoculated into the high risk groups such as soldiers and farmers since 1990 [10], there are no actual decrements of HFRS patients in Korea as yet. Emerging new serotypes of hantavirus [1, 4] and increasing of new reservoirs [18] can partially explain this, but further

studies will be necessary for complete understanding. Preliminary data of PCR-amplified products and reactivities to monoclonal antibodies suggest that the bat isolates come close to Hantaan virus. However, genomic analysis of these isolates will be needed for further clarification.

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