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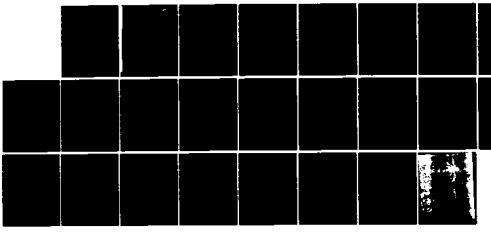

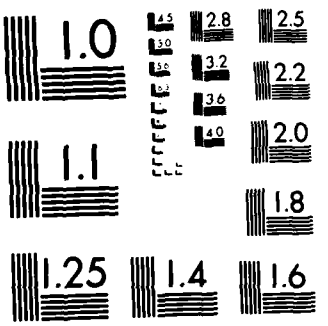

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KOREAN HEMORRHAGIC FEVER

Final Report

HO WANG LEE, M. D.

May 1982

Supported by
U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701

Grant No. DAMD 17-81-G-9482

Korea University College of Medicine
Seoul, Korea

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Korean hemorrhagic fever (KHF) is a disease transmitted to man by field mice. Although predominantly associated with rural areas, it is now being recognized as an urban problem in some countries and a particular hazard to laboratory stuff using

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SUMMARY

Urban rats captured in Seoul and four nearby Korean cities were found to have immunofluorescent (IF) antibodies reactive with Hantaan virus, the etiologic agent of Korean hemorrhagic fever (KHF). Antibodies were detected in 13% of sera from 477 Rattus norvegicus and 11% of 47 Rattus rattus. Hantaan viral antigen was found in pulmonary tissues of 42 animals and Hantaan virus strains were recovered from 23 rats, all but two of which were R. norvegicus. Wistar rats were qualitatively much more sensitive than Apodemus agrarius rodents for isolation of virus strains from wild rat tissues. Wistar rats inoculated with one of these strains had virus in lung and spleen for at least 75 days. These results document the existence of an urban cycle for Hantaan virus, previously suspected on the basis of the occurrence of sporadic urban human cases of KHF, and suggest that Rattus-borne Hantaan virus may be widely distributed in urban centers of Asia and elsewhere.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1976).

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INTRODUCTION

Korean hemorrhagic fever (KHF) and nosologically related syndromes variously termed epidemic hemorrhagic fever, Songo fever, hemorrhagic fever with renal syndrome, hemorrhagic nephroso-nephritis, and nephropathia epidemica are predominantly rural diseases transmitted to man by field mice [1-6]. The etiologic agent of KHF, Hantaan virus, shares biochemical and morphological properties with the family Bunyaviridae and immunofluorescent (IF) antigens with agents causing similar clinical syndromes in Euro-Asia [7-15]. Apodemus agrarius is the principal reservoir for Hantaan virus in Korea and chronic infection of this mouse with persistent viruria is thought to be the important mechanism leading to human infections [16]. A similar role for the field vole, Clethrionomys glareolus, has been proposed for nephropathia epidemica in Finland [17].

Several recent events, however, suggest that Hantaan virus or a closely related agent, may have additional epidemiological features. When an IF serological method became available, it was found that persons who had experienced clinically compatible KHF-like illness in urban Osaka, Japan during the 1960s had persistent IF antibodies against the Hantaan agent in their sera [12]. In addition, sporadic cases of KHF have been documented in the city of Seoul [18]. Outbreaks of serologically confirmed KHF among laboratory personnel in medical centers in Japan were circumstantially linked to Wistar albino rats which were found to have antibodies against Hantaan antigen [19,20].

Finally, the occurrence of clinical KHF in four persons exposed for less than 4 months to Wistar rats inoculated with Hantaan virus provided strong evidence that these animals were capable of producing highly infectious virus aerosols [21].

We report here initial results of a search for Hantaan virus infection among urban wild rats in Seoul and nearby Korean cities, together with data concerning characteristics of the agents which we isolated.

MATERIALS AND METHODS

Survey areas:

Survey areas for collection of urban house rats were primarily Mapo-ku, Chongro-ku, and Seungbuk-ku districts of Seoul where cases of KHF occurred in 1977-1979. During the coll ∧ of rats the survey area was expanded to nearby districts and towns which are located within 40 miles from Seoul (Figure 1).

Capture and processing of rodents:

Rats were purchased from householders who used live traps supplied by us. Live animals were identified upon receipt at the laboratory. They were bled by cardiac puncture under chloroform anesthesia; samples centrifuged to obtain sera were stored at -20° C for antibody studies. The rats were then sacrificed to provide lung, liver, spleen, kidney and parotid gland tissues. Portions of lung tissue were saved for immediate cryostat sectioning to search for Hantaan virus antigens and the remaining samples were stored at -70° C.

Measurement of Hantaan IF antibodies:

Two Hantaan virus antigen preparations were used (a) frozen sections of Apodemus lung infected with the 76/118 strain, 12th Apodemus lung passage [7] and (b) spot slides of A549 cells using 76/118 virus after three Apodemus lung and seven A549 passages. Both of these preparations were free of IF reovirus antigens when tested with polyvalent anti-reovirus antiserum (Reference Reagents Branch, Centers for Disease Control, Atlanta Ga.). The indirect IF method [7] was used for detection and titration of Hantaan virus antibodies. Fluorescein isothiocyanate-conjugated goat anti-rat and anti-mouse IgG reagents were obtained from Cappel Laboratories; slides were examined with an epi-illumination ultraviolet microscope (Leitz SM-Lux).

Detection of viral antigens:

Frozen cryostat sections (4 mu) of lung and other tissues were examined for viral antigens by indirect IF using serum from a patient convalescent from KHF. Details of this technique have been described [7].

Isolation and identification of virus strains:

Ten percent (w/v) suspensions for virus isolation were made by tituration of lung tissue in diluent consisting of 1% bovine albumin in pH 7.4 phosphate saline containing gentamycin 50 ug/ml and Fungizone^{R} 2 ug/ml, and supernatants were harvested after centrifugation at 2,600 g for 20 min at 4° C. We injected 0.3 ml of this material by the im route into each of four A. agrarius captured on Cheju island where no KHF, Hantaan virus, or antigen

has been found; 0.5 ml was injected into each of four Wistar rats previously shown to be negative for IF Hantaan antibodies. Lungs of these animals were examined by IF at 20 and 30 days as an index of virus replication. Reisolation of virus from original lung tissue was made in several instances using both species of rodent. Paired sera from KHF patient 78/63 were used for identification of rat strains. These sera had titers to the 76/118 virus strain of 1:128 and 1:16,384, respectively, and did not react with reovirus antigens at a 1:20 dilution. The 80/39 strain isolated from a wild Rattus norvegicus was compared to the prototype 76/118 virus by serum neutralization tests. The sera tested were no. 81/73 from a convalescent KHF patient and no. 76/118 prepared by single injection of a rabbit with a lung suspension from the 12th Apodemus passage. Equal volumes of 10-fold dilutions of viruses and sera were incubated at 37° C for 1 hr after which 0.3 ml of each mixture was inoculated im into each of four Apodemus (76/118) or Wistar rats (80/39). Normal species serum controls were employed; animals were sacrificed 20 days later and detection of pulmonary IF antigen was taken as evidence of Hantaan virus infection. Neutralization was calculated on a $\log_{10}$ index basis. The replication of Hantaan virus strains from urban rats and the associated pattern of antigen distribution in organs of different rodents were determined by im inoculation of virus strains into serologically negative Wistar rats from Korea and Japan, wild

R. norvegicus and Rattus rattus, and albino mice. Virus content in organs of Wistar rats at certain intervals after virus inoculation was determined by titration in Wistar rats.

RESULTS

Five cases of KHF were recognized in urban Seoul; three during 1976 and two in 1979 [18]. All of these persons were adult males, and became ill during November and December. None had left the city during the month preceeding onset of illness, and all survived infection, although two patients were moderately to severely ill. All of these persons worked within a short distance of their residences and all reported seeing rats in their houses or work places shortly before becoming ill. Two of the five patients had killed rats in their offices several days before onset of fever. The houses of these people are shown on a map of Seoul which is divided into districts (Figure 1).

Detection of Hantaan IF antibodies and viral antigen in urban rats

We examined 335 R. norvegicus and 47 R. rattus captured in 10 of the 13 political districts of Seoul. Most of these rats were obtained from only six districts. In addition, 142 R. norvegicus were obtained from four other Korean cities (Figure 1). The number of animals tested and those positive for antigen and antibody are given in Table 1 and Figure 1. In general, we detected antigen in lungs of rats which also had Hantaan virus antibodies.

Only six animals had antigen but no antibody, whereas 38 rats with antibody were negative for Hantaan antigen. Evidence for infection of rats was found in each district where the sample size was at least 16. Infection of urban rats with a Hantaan-like agent was not limited to Seoul; each of four other cities yielded animals positive for antigen or antibodies.

Isolation of virus strains from wild rats

Lung suspensions from 39 animals with detectable pulmonary Hantaan IF antigen were inoculated into Apodemus and Wistar rats. Lungs of these animals were in turn examined 20 and 30 days later for IF antigen as an index of virus replication. Twenty-three isolates were obtained, all but two of these from R. norvegicus. As shown in Table 2, intensity of pulmonary IF antigen was directly related to subsequent recovery of virus from wild rats. Wistar rats were much more sensitive than Apodemus for detection of wild rat virus (Table 3). Sixteen isolates were detected only in Wistar rats, whereas three strains were detected only by Apodemus, and reisolation was not successful for two of these strains. Sera from Apodemus and Wistar rats inoculated with lung suspensions also were tested for Hantaan IF antibodies. All antigen-negative rodents had no antibodies, an indication that antigen was a reliable measure of infectivity for these hosts. Reisolation was achieved for each of eight strains originally detected by Wistar rats or by both host systems. Also shown in Table 3 are data indicating that the amount of IF serum antibody did not influence recovery

of virus strains. As set out in Table 3 and Figure 1, isolates were made in five districts of Seoul and in two other Korean cities. It was noteworthy that only four animals had pulmonary IF antigen not correlated with presence of virus, antibody, or both.

Table 4 summarizes data on distribution of IF antigen in tissues of 18 R. norvegicus, 11 of which yielded viral isolates. Although the greatest amount of antigen was usually observed in lungs, IF foci were detected in kidney and spleen of a majority of the animals. Antigen was detected in liver and lacrimal glands of five of the virus-positive rats, but not in liver and in only a single lacrimal gland from the virus-negative animals. Titration of infectivity in four tissues from one rat yielded values of 1.6-4.3 $\log_{10}$/ml with the highest concentration in lung.

Characteristics of virus recovered from wild rats

Serial passages of rat strain HR/80/39 were made in Wistar rats and were screened for infectivity in both rats and Apodemus (Table 5). By the 5th passage all Wistar rats exhibited pulmonary IF antigen whereas Apodemus continued to respond irregularly. At the 6th passage ID_{50} values were 7.6 and 4.0 $\log_{10}$/ml respectively, for Wistar rats and Apodemus. This strain was identified as Hantaan virus by IF and neutralization tests shown in Table 6.

Preliminary observations concerning virus content of tissues of Wistar rats inoculated with 3rd passage HR/80/39 strain are depicted in Table 7. The data show that viremia occurred, that

lung was a sits of infection persistent for at least 75 days as was spleen, and that kidney and liver were transiently virus-positive. This pattern correlated quite well with results of IF antigen distribution in Wistar rats 30 days after inoculation with four different wild rats isolates (Table 8). Lung and spleen were the predominant sites of antigen deposit.

A variety of wild and colonized rodent species was tested as detection hosts for Hantaan virus strains of different origin. As depicted in Table 9, none of four strains tested produced pulmonary IF antigen in laboratory mice. Two strains isolated and passaged in Apodemus failed to induce IF antigen in any other rodent examined. Both R. norvegicus isolates replicated in all Rattus rodents tested, but only HR/80/39 was capable of producing infection resulting in formation of detectable IF antigen in Apodemus.

DISCUSSION

Detection of Hantaan virus, viral antigen and antibodies in urban Korean Rattus confirms the existence of a natural reservoir for this agent which significantly expands the epidemiologic horizon for transmission of the virus to man. Although much remains to be learned concerning the biology of Hantaan virus in Rattus, the recovery of virus from tissues of Wistar rats 75 days after experimental inoculation and the isolation of virus from

more than half of the wild Rattus positive for IF antigen suggest that chronic infection similar to that previously documented for the field mouse, A. agrarius [16] occurs in Rattus. If accompanied by chronic viruria, such a pattern has significant global implications. Like Mus musculus in the case of lymphocytic choriomeningitis virus, wild rats may have carried Hantaan virus far beyoud its presumptive Euro-Asian origins. Surveys of urban rats worldwide are urgently needed and port cities represent the most inviting targets.

The general inability of Hantaan virus isolates from Rattus to produce IF-positive infection in Apodemus suggests that these strains have been circulating among rats for some time, an observation which correlates well with the complete absence of this field mouse in long established, highly urban centers where the Rattus viruses were encountered. A reciprocal study of replication of low passage Apodemus and Rattus strains is required to substantiate this assumption.

Attempts to recover virus strains from the 29 Rattus with IF antibodies but no pulmonary antigen are in progress. Pendigg the outcome of this study it is important to note that virus was recovered from 30% of animals which had circulating Hantaan IF antibodies. In the case of Apodemus rodents the antibody-pulmonary antigen correlation exceeded 85% and antigen-positive animals were virtually the only ones to yield Hantaan viral isolates (Lee, H. W. et al, unpublished observations).

Thus a practical approach to the search for Hantaan virus among these wild rodents is to test sera for antibodies and to inoculate visceral tissues of positive individuals into susceptible rodent hosts.

The isolation of Hantaan virus from Rattus using Wistar rats provides a laboratory system in which to assess the biology of this agent, especially in terms of possible vertical transmission. At the same time these new findings reemphasize the need for further work to devise simpler and more sensitive host systems for production and measurement of infectious virus, viral antigens and specific antiviral antibodies. We were unable, for example, to detect the HR/80/39 strain, when the original lung suspension was inoculated into the A549 human adenocarcinoma cell line previously employed to adapt an Apodemus isolate for production of IF antigen [22]. Until these fundamental problems are solved, work with Hantaan virus, and related agents, must be done with wild or laboratory rodents, a laborious and rather hazardous technique, both in terms of potential laboratory infection in man [19-21] and of the constant possibility of recovery of unwanted agents indigenous to the hosts employed.

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Legends for Figures

Figure 1. Map of Seoul City, South Korea and metropolitan area showing locations of urban Korean hemorrhagic fever cases, and *Rattus* positive for antigen and/or antibody to Hantaan virus.

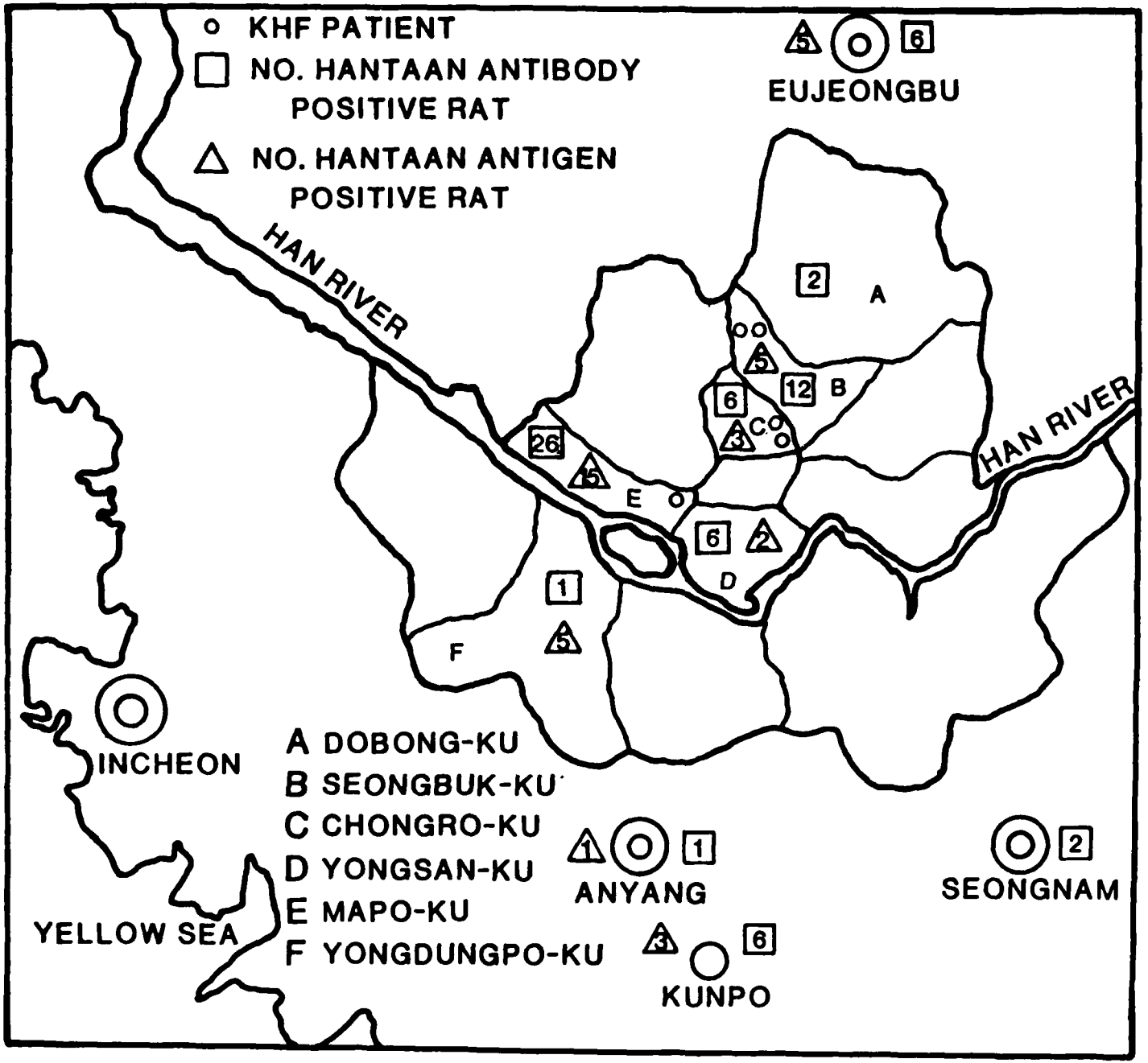

Figure 1. Map of Seoul City, and metropolitan area showing locations of urban KHF cases, and Rattus positive for antigen and/or antibody to Hantaan virus.

Table 1
Hantaan viral IF antibodies and pulmonary antigen in wild rattus, urban Korean cities, 1980.

Locality	IF antibody and pulmonary antigen: R. norvegicus			R. rattus			Total	% Positive	
	No.	antibody	antigen	No.	antibody	antigen		Antibody	Antigen
Seoul-District									
Chongno-Ku	31	5	3	11	1	0	42	14.3	7.1
Mapo-Ku	99	23	14	9	3	1	108	24.1	13.9
Seongbuk-ku	102	11	4	19	1	1	121	9.9	4.1
Yongsan-Ku	40	6	2	6	0	0	46	13.0	4.3
Dongdaemun-Ku	4	0	0				4		
Kwanak-Ku	4	0	0				4		
Dobong-Ku	17	2	0				17	11.8	
Seodaemun-Ku	2	0	0				2		
Yongdungpo-Ku	35	1	5	1	0	0	36	2.8	13.9
Seongdong-Ku				1	0	0	1		
Other cities									
Eujeongbu	58	6	5				58	10.3	8.6
Anyang	16	1	1				16	6.3	6.3
Kunpo	66	6	3				66	9.1	4.5
Seongnam	3	2	0				3	66.7	
Total	477	63	37	47	5	2	524	13.0	7.4
% Positive		13.2	7.8		10.6	4.3			

Table 2
Isolation of Hantaan virus from antigen-positive wild house rats, Korea, 1980.

	Isolation of virus from rats having IF antigen in lungs; by intensity		
	+	++	+++
Positive	9	9	5
Tested (% positive)	22 (41%)	12 (75%)	5 (100%)

Table 3
Differential sensitivity of *Apodemus* and Wistar rat for detection of Hantaan virus in lungs of wild house rats, Korea, 1980.

Code no.	Date of collection	Locality	Sex	Weight (g)	IF serum titer (Hantaan)	Lung IF antigen	No. isolations/total	
							Apodemus	Wistar
1 HR/42	Apr 15	Mapo-ku	F	109	512	+++	1/9	0/4
2 HR/95[1]	May 13	"	F	197	1,024	+	1/10	0/4
3 HR/237	Jun 18	Eujeongbu	F	114	16	+	3/4	0/4
4 HR/4	Mar 29	Mapo-ku	F	180	4,096	++	0/17	2/4
5 HR/11	Mar 31	"	M	192	1,024	++	0/17	1/4
6 HR/15	Apr 4	"	M	232	1,024	+	0/4	1/4
7 HR/21	Apr 7	Seongbuk-ku	F	105	1,024	+	0/4	1/4
8 HR/23	Apr 8	Mapo-ku	F	178	1,024	+	0/4	2/4
9 HR/26	Apr 9	"	F	175	2,048	+	0/8	4/7 (2/4,2/3[2])
10 HR/200	Jun 13	Yongsan-ku	M	125	2,048	++	0/4	6/8 (3/4,3/4[2])
11 HR/256	Jun 19	Mapo-ku	M	157	32	++	0/4	2/4
12 HR/273	Jun 21	Eujeongbu	M	145	4,096	+++	0/4	3/7 (1/3.2/4[2])
13 HR/337	Jun 28	Mapo-ku	F	140	32	++	0/4	1/4
14 HR/348	Jun 30	Eujeongbu	M	160	32	+	0/4	1/3
15 HR/397	Jul 4	Yongdungpo	M	175	16	++	0/4	2/4
16 HR/399	Jul 4	"	M	140	16	+++	0/4	1/4
17 HR/404	Jul 4	Kunpo	M	135	1,024	+	0/4	2/4
18 HR/482	Jul 15	Eujeongbu	F	120	512	+++	0/4	1/4
19 HR/502[1]	Jul 16	Seongbuk-ku	M	130	512	+	0/4	2/8 (1/4,1/4[2])
20 HR/39	Apr 15	Mapo-ku	F	183	512	+++	13/24 (4/8,9/16[2])	8/9 (2/3,6/6[2])

21 HR/115	May 26	Mapo-ku	F	133	1,024	++	4/7 (2/3,1/4[2])	4/8 (2/4,2/4[2])
22 HR/120	May 28	Chongno-ku	M	146	2,048	++	1/6	6/8 (3/4,3/4[2])
23 HR/254	Jun 19	Mapo-ku	F	125	2,048	++	1/4	4/7 (2/4,2/3[2])
24 - 39	April-July	Korea	M-F	75-252	16-2,048	+ - ++	0/64	0/64

1: R. rattus. All other strains from R. norvegicus.

2: Number positive/number tested on primary and re-isolation attempt.

Table 4
Distribution of Hantaan virus IF antigen in the organs of wild Rattus norvegicus collected in urban areas of Korea, 1980.

Code no. of R. norvegicus	Weight (g)	Sex	IF antibody titer against Hantaan virus	Presence of Hantaan virus antigen in tissue of						
				Lung	Kidney	Liver	Spleen	Parotid gland	Lacrimal gland	Virus isolation
HR/80/4	180	F	256	++	-	-	-	-	-	+
HR/80/21	105	F	256	+	-	-	-	-	-	+
HR/80/23	178	F	128	+	-	-	-	-	-	+
HR/80/26	175	F	128	+	-	-	-	-	-	+
HR/80/39	183	F	512	+++[1]	+[2]	+[3]	+[4]	+	+	+
HR/80/42	109	F	1,024	+++	+	+	++	-	+	+
HR/80/115	133	F	1,024	++	+	+	+	-	+	+
HR/80/120	146	M	512	++	+	+	+	-	+	+
HR/80/237	114	F	64	+	-	-	-	-	-	+
HR/80/254	125	F	512	++	++	+	+	+	+	+
HR/80/273	145	M	2,048	+++	-	-	-	-	-	+
HR/80/24	132	M	32	+	-	-	-	-	-	-
HR/80/71	174	F	1,024	+	+	-	+	-	+	-
HR/80/85	195	M	512	+	+	-	+	-	-	-
HR/80/257	252	F	128	+	-	-	+	-	-	-
HR/80/276	170	M	1,024	++	+	-	+	-	-	-
HR/80/290	115	F	16	+	-	-	-	-	-	-
HR/80/321	160	F	128	++	+	-	+	-	-	-

1: ID_{50} in Wistar rat = $10^{4.3}$/ml.
2: ID_{50} = $10^{1.6}$/ml.
3: ID_{50} = $10^{2.6}$/ml.
4: Not detectable.

Table 5
Passage and growth of Hantaan virus, HR/80/39 strain, isolated from urban wild R. norvegicus, in various rodents.

Passage no.	Total days	No. positive/no. inoculated	
		Wistar rat	A. agrarius
1	30	4/5	12/28
2	60	4/4	2/4
3	90	8/9	not tested
4	120	9/11	3/4
5	149	12/12	3/4
6	179	12/12[1]	4/7[2]
7	201	4/4	not tested

1 : ID_{50} rat = $10^{7.6}$/ml; neutralization index of KHF patient serum, 81-73 = 800,000.

2 : ID_{50} in Apodemus = $10^{4.0}$/ml.

Table 6
Identity of Hantaan virus strains isolated from R. norvegicus and A. agrarius.

Virus	Titer in indicated serum			
	IF, Human 78-63		LNI[1]	
	Acute (d-5)	Convalescent (d-16)	Human (81-73)	Rabbit (76-118)
76/118 (AP/12)				
Apodemus	128	16,384	5.1	4.3
HR/80/39 (WRP6)				
R. norvegicus	32	4,096	4.9	4.1

1 : LNI + Log_{10} neutralizing index with indicated serum vs. homologous normal serum.

Table 7
Growth and distribution of Hantaan virus strain HR/80/39/WRP3 isolated from wild R. norvegicus in the tissue of Wistar rats.

	No. positive/no. inoculated by days						
Tissue	10	14	23	31	49	61	75
Blood	0/4	4/4	not tested				
Lung		0/4	2/4	4/4	4/4	6/6	4/4
Kidney		0/4	0/4	2/4	0/4	0/6	0/4
Liver		0/4	0/4	1/4	0/4	0/6	0/4
Spleen		0/4	2/4	2/3	1/3	2/6	2/4
Parotid glands		0/4	0/4	1/4	0/4	0/6	0/4
Lacrimal glands		0/4	0/4	0/4	0/4	0/6	0/4

NOTE: All rats inoculated im with 0.5 ml of 10% lung suspension.

Table 8
Distribution of Hantaan IF antigen in Wistar rats inoculated by im route with 4 strains of viurs recovered from wild R. norvegicus, Korea, 1980.

Strain	Rat no.	Presence of virus antigen in tissues at 30 days					
		Lung	Kidney	Liver	Spleen	Parotid gland	Lacrimal gland
HR/80/39 WRP4	1	++++*	-	+	+	-	-
	2	+++	+	+	++	+	-
	3	+	-	-	+	-	-
	4	++	-	-	+	-	-
HR/80/115	1	++	-	-	+	-	-
	2	++	-	-	+	-	-
	3	+++	-	-	-	-	-
	4	++	-	-	+	-	-
HR/80/120	1	+++	-	-	++	+	-
	2	+++	-	-	+	-	-
	3	+++	-	-	++	-	+
	4	++	-	-	+	-	-
HR/80/200	1	+++	-	-	++	-	-
	2	++	-	-	+	-	-
	3	++	-	-	+	-	-
	4	+	-	-	-	-	-

* Distribution of fluorescent antigen of Hantaan virus in tissue from Wistar rat was graded as - or + (from + to +++)

NOTE: All rats inoculated im with 0.5 ml of 10% lung suspension.

Table 9
Infection (IF antigen) patterns in different rodent hosts of Hantaan virus strains from distinct sources.

Animal	No. positive/ni. inoculated by strains			
	Han/AP3[1]	76/118/AP24[2]	HR/80/39/WRP4[3]	HR/80/26 lung suspension[4]
A. agrarius	10/10	5/5	3/4	0/8
Wistar rat	0/4	0/5	8/9	4/7
R. norvegicus	0/4	0/16	3/5	4/6
R. rattus (korea)	0/4	0/4	3/7	5/7
R. rattus (Japan)	0/4	0/16	2/4	2/4
Albino mouse	0/6	0/10	0/10	0/7

1: 3rd passage in Apodemus lung. Isolated from KHF patient in Apodemus.

2: 24th passage in Apodemus lung. Isolated from wild Apodemus.

3: 4th passage in Wistar lung. Isolated from wild R. norvegicus.

4: Original lung material. Isolated from wild R. norvegicus.

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