大韓바이러스學會誌 : 第 28 卷 第 1 號 1998
J. Korean Soc. Virology, Vol. 28, No. 1, 1998

Correlation of Virologic Property and Phylogenetic Analysis of Hantaan Viruses Isolated from Patients and Reservoirs in Korea

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=Abstract=

Twenty isolates of Hantavirus were isolated from patients and reserovirs from 1988 to 1994 in Korea. Isolation rate was 1.9% (10/538) in patients, 6.2% (5/81) in *Apodemus sp.*, 2.6% (1/38) in *Rattus sp.* and 0.6% (4/677) in bats. Reciprocal mean IFA titers ranged from 27.5 to 1,024 at the specimen collection. According to the growth rate and reaching peak titier of infectivity, the isolates were grouped as rapid, intermediate, and slow growing groups. All isolates were confirmed as Hantaan type by the nested RT-PCR on the G1 region of the M segment. Comparison of nucleotide sequence (Nt: 2101 - Nt: 2280) of the G2 region revealed that the sequence homology bewteen Hantaan 76/118 virus and the isolates was more than 90%. Several nucleotide positions of the isolates showed high variation. The variation rate of patientisolates was about one-half when compared with that of rodentisolates. On the basis of phylogenetic analysis Hantaan viruses isolated were divided into two genogroups.

These results indicate that Hantaan virus is highly dominant serotype in Korea and the virologic property and genogroup are not correlated.

Key Words: Hantaan isolation, Growth rate, M segment, Nucleotide sequence, Genogroup

INTRODUCTION

Hantavirus, the etiologic agent of hemorrhagic fever with renal syndrome (HFRS), is one of rodent-born viruses. Serologic [1] and virologic [2] findings have established the presence of Hantaan-like viruses in rodents virtually worldwide. Wild rodents chronically infected with hantaviruses, natural reservoirs, play an important role in virus transmission and maintenance of the enzootic cycle. Principal reservoirs are colsely associated with serotypes of Hantavirus genus and clinical severity of HFRS [3,4]. These can be predicted by the geographical distribution of the predominant reservoir. *Apodemus* species and Rattus species are known to be primary reseroirs of Hantaan and Seoul types in the Korean peninsula [3,5]. In recent, two species of bats, *E. serotinus* and *R. ferrume-quinum*, were confirmed as new natural reservoir of Hantaan virus [6,7].

It is important to isolate hantavirus from specimens in endemic areas. However, it is really difficult to isolate hantavirus. Vero E6 clone is most commonly used for isolation and

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propagation of hantaviruses, but virus growing and infectivity titer are dependent on a serotype of hantavirus infected [8,9]. Among Hantaan virus isolates, viral growing seems to be slightly different in Vero E6 cells.

The Hantavirus genus within the Bunyaviridae family [10,11] has three single-standed negative sense RNA segments (S, M and L segments). The S genome segment encodes the nucleocapsid (N) protein (Mr: 50-53 kDa) [12], the M genome segment encodes envelpe glycoproteins G1 (Mr: 68kDa) and G2 (Mr: 55 kDa) [13], and the L segment encodes the L protein, RNA-dependent RNA polymerase [14].

The glycoprotein G1 and G2 contain serotype-specific antigens of Hantavirus genus. Since 1988, twenty hantaviruses had been isolated from patients and natural reservoirs for serveral years in our laboratory. Virologic properties and data of genome analysis were described in this paper.

MATERIALS AND METHODS

Specimen collection

Heparinized peripheral whole blood specimens of in-patients with HFRS were collected from 1988 to 1994. Peripheral blood monocyte (PBM) was separated by Ficoll/Hypaque gradient centrifugation. 1 ml suspension of PBM with growth medium was stored at -80℃ until use [15]. Wild rodent was captured from several regions in Kyungi-do province by using a Sherman trap. All rodents captured alive were transported to the laboratory as early as possible. Specimens of sera, lung and kidney were collected aseptically, and stored at -80℃ until use.

Cell, medium and virus

Vero E6 cell (ATCC C1008, CRL 1586) was purchased. Eagle's minimal essental medium containing 10% fetal bovine serum, 4% L-glutamine (Gibco, Grand Island, NJ), 1% non-essential amino acids (M.A.Bioproducts, Walkersville, MD), and 1% penicillin-streptomycin was used for propagation and isolation of hantavirus [16]. As a reference virus and antigen, Hantaan 76/118 strain, Seoul 80-39 strain and Puumala Hallnas B1 strain were supplied by H. W. Lee, WHO Collaborating Center for Hantaviruses, Seoul, Korea.

RNA extraction

The time of RNA extraction was monitored by the IFA technique [6]. RNAs were extracted from virus infected Vero E6 cells by the guanidinium thiocyanate (GT)/cesium chloride method [17]. RNA pellet by centrifugation at 35,000 rpm for 15h at 20℃ using SW 50.1 Ti Rotor (L8-70M, Beckman Instruments, Cedar Grove, NJ) was reconstituted with 1 X TE buffer. To avoid false-positive results by product carryover [18], all samples were handled in a biosafety hood.

RT-PCR

RNA was initially reverse-transcribed using random hexamer and Rous associated virus-2 reverse transcriptase (Takara Biochemicals, Kyoto, Japan) [19]. One set of genus-specific primer and four sets of type-specific primers were synthesized (Table 1). In the first step, the resulting cDNA was used as a template to amplify the G1 region of the M segment, using a genus-specific primer pairs. In the second step, the nested PCR was done with the amplified products by using type specific primer pairs [20]. PCR products from the G2 region of the M segment was done as previously described [7,21]. Thirty cycles of 1 min denaturation at 90℃, 2 min annealing at 55℃, and 3 min primer extension at 72℃ were repeated in the thermal cycler (Temp. Tronic, Thermolyne, Barnstead/Thermolyne Co., Dubuque, IW). Size fractionation of PCR products was performed by electrophoresis on 1.6% agarose gel (Ultra Pure, Bethesda Research Laboratories, Bethesda, MD) prepared in 0.5 X TBE buffer containing 0.5 μg/ml of ethidium bromide.

Table 1. Oligonucleotide primer pairs for amplification of G1 and G2 regions of M genomic segment

Region	Genus-specific primer (outer primer)	Sequence (5'→3')	Product length (bp)
G1	MOF 103	5'-GGACCAGGTGCAGCTTGTGAAGC-3'	490
	MOR 204	5'-ACCTCACAAACCATTGAACC-3'	
	Type-specific primer n(inner primer)		
	Hantaan (76/118)	5'-TGCAACGGGCAGAGGAAAGT-3'	282
		5'-GTACTGATTTTAGCCTATTCTC-3'	
	Seoul (80-30)	5'-TGTAATGGTCAGAAAAAGAC-3'	286
		5'-CGTAGAATGGCTTTGAATCGGTT-3'	
	Puumala (Hallnas B1)	5'-GTGTCCAGAGATTCCGTGGT-3'	324
		5'-GAACATAAGTATGCGAATGCAA-3'	
G2	Hantaan (76/118)	5'-ATACCCAAGTAAGTTGGAGAGG-3'	365
		3'-TTGTCCAATTCTTTAGGAAA-5'	

Table 2. Isolation of hantaviruses from specimens of patients and natural reservoirs from 1988 to 1994 in Korea

Host	Specimen	IFA antibody* titer at specimen collocted	Antigen detection rate	Virus isolation rate
Patient	serum	1:144.0 (<20 - 512)	ND+	0.7% (4/538)
	PBM	1:405.3 (<20 - 1024).	ND	1.1% (6/538)
Apodemus sp	lung	1:742.4 (64 - 1024).	14.8% (12/81)	3.7% (3/81)
	kidney		17.3% (14/81)	2.5% (2/81)
Rattus sp.	lung	1:1,024 (128 - 5120)	10.5% (4/38)	2.6% (1/38)
	kidney		13.2% (5/38)	0.0% (0/38)
Bat sp.	lung	1:27.5 (16 - 128)	1.3% (9/677)	0.6% (4/677)
	kidney		0.3% (2/677)	0.0% (0/677)

*reciprocal mean antibody titer
+ND: not done

Cloning

The PCR product was ligated to pT7 Blue T-Vector (Novagen, Madison, WI). The ligated plasmid was introduced into the competent *E. coli*, and transformants were selected based on antibiotic resistance, *LacZ* disruption and eletrophoretic analysis using a alkaline-lysis miniprep procedure. The procedure was performed as the company's instructions.

Nucleotide sequencing

The cloned plasmid DNA was sequenced by the dideoxy-chain-termination method [22] using a sequencing kit (United States Biochemical, Cleveland, OH). The tube containing template, primer, DNA polymerase, dNTPs and ddNTPs, was placed in the themal cycler. The cycling conditions were as follows: 20 cycles of 20 secs at 95℃, 20 secs at 55℃, and 20 secs at 72℃. The annealing, labelling and termination reaction were done according to the manufacturer's protocols. After completion of reactions, the sequence product was analyzed on 8% high-resolution denaturing polyacrylamide gel at 1600V constant power, and au-

Table 3. Comprasion of growth rate of hantavirus isolates

Growth	No. of isolate	Percent of antigen positive cells after inoculation				Specimen/Host
		3 day	5 day	7 day	10 day	
Rapid	3	≥80	100			serum/Human lung/*Apodemus sp.*
Intermediate	4	≤20<80		100		lung/*Rattus sp.* lung/Bat kidney/*Apodemus sp.*
Slow*	13	<20		60-80	100	lung/*Apodemus sp.* lung/Bat lung/*Rattus sp.* *serum & PBM/Human*

*Peak titer of infectivity in supernatant reached $1.2x10^{6.8}$ $TCID_{50}$/ml in 10-14 days postinoculation
Each well of 24-well plate was inoculated with 10 $TCID_{50}$/ml. All experiments were duplicated

toradiographed to visualize the DNA sequence.

Phylogenetic analysis

Based on the nucleotide sequences of a partial G2 region of M segment, the phylogenetic relationship of the hantavirus isolates relative to Hantaan 76/118 virus was determined using PAUP version 3.1 (Illinois Natural History Survey, Champaign, IL) [23].

RESULTS

Hantavirus isolation and growth

A total of twenty hantaviruses was isolated from specimens collected from various hosts (Table 2). In patients, four isolates from sera (0.7%: 4/538) and six isolates from PBMs (1.1%; 6/538) was obtained, and the mean IFA titers were 1:144.0 and 1:405.3, respectively. In *Apodemus sp.*, three hantaviruses from lung tissues (3.7%;3/81) and two from kidney tissues (2.5%;2/81) were isolated, and the mean IFA titer was 1:742.4. One strain of hantavirus was isolated from lung tissue (2.6%;1/38) of *Rattus sp.*, and the mean IFA titer was 1:1,024. Four isolates were obtained from lung tissues of bats (0.6%;4/677), and the mean IFA titer was 1:27.5. Antigen detection rate from tissues of *Apodemus* and *Rattus sp.* was about ten times higher than that of bats. Propagation pattern of the isolates in Vero E6 cells was divided into three groups according to growing and reaching peak titer of infectivity (Table 3). Two isolates from patients' sera and one isolate from lung tissue of *Apodemus sp.* were grown rapidly. On day 3 after inoculation, about 80 percent of cells displayed virus specific fluorescence, and peak titer of $1.3x10^{6.8}$ $TCID_{50}$/ml was observed on day 5. Growth of thirteen isolates was slow, and infectivity of culture supernatant reached maximal level in 10-14 days postinoculation.

Genotype of the isolates

On the basis of the isolate origin and difference of the biologic property, genotypes of fifteen isolates were analysed. Amplified product of 490-bp was obtained using a generic primer pairs, MOF 103 and MOR 204, specific for G1 region of M segment of hantavirus. Nested RT-PCR was performed using three pairs of type-specific primers. A 282-bp fragment was amplified from all isolates tested by Hantaan specific inner primers (Fig. 1). The specificity of Hantaan virus, Seoul virus, and Puumala virus specific primers was examined,

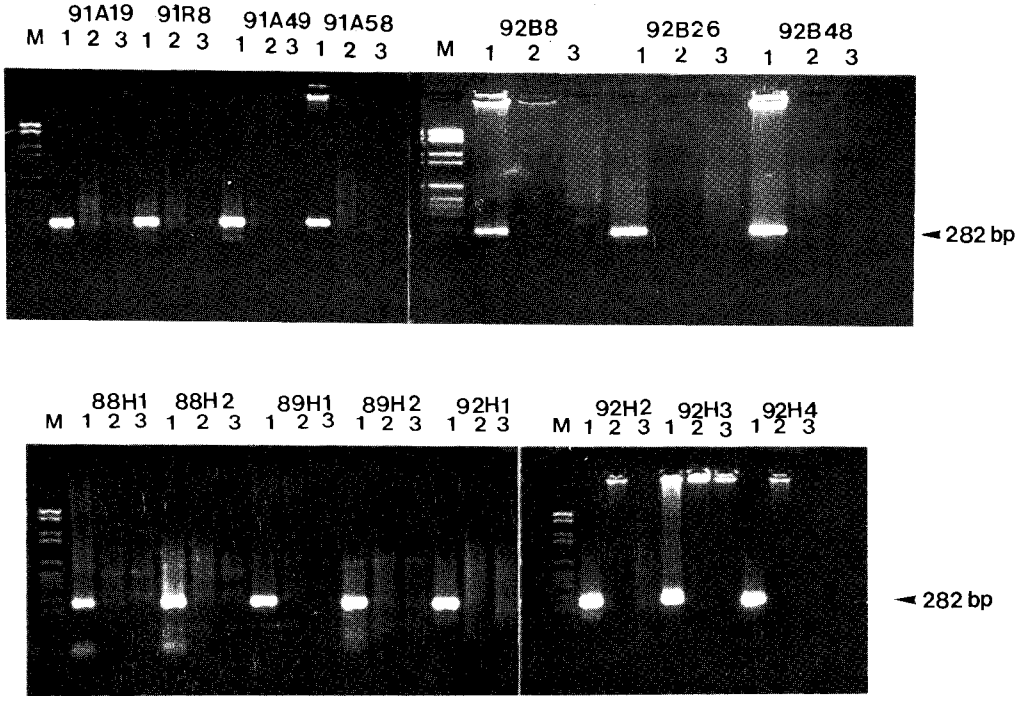

Fig. 1. Nested RT-PCR products of 15 hantavirus isolates using three sets of serotype-specific primer pairs. M: *Hae*III digested ϕ X-174 RF-DNA. Lane 1: amplified products by Hantaan-specific primers. Lane 2: Seoul-specific primers. Lane 3: Puumula-specific primers two arabic numbers before a single large alphabet indicate year isolated, a single large alphabet indicates host isolated (A: *Apodemus sp.*, R: *Rattus sp.*, B: bat, and H: patient). Arabic number after alphabet indicates random number of specimen collected.

and each virus specific primer pairs amplified only homologous virus (data not shown).

Nucleotide difference of the G2 region

The 365-bp of PCR products were amplified from the G2 region of the M RNA segments of the isolates and Hantaan 76/118 virus by using a set of Hantaan-specific primer pair, and the PCR products were cloned. A 180-bp fragment of the cloned plasmid DNA (Nt: 2101 - Nt: 2280) was sequenced and 100-nucleotide positions that showed nucleotide changes were compared (Fig. 2). Identical portions (80 nucleotides) were deleted in this figure. A nucleotide sequence comparison of the hantavirus isolates to the corresponding sequence of the Hantaan virus revealed that the sequence homology was 91.1%~100.0%. The range of sequence differences between Hantaan virus and six isolates from rodents was 1.7%~3.3%, and that between Hantaan virus and eight patient isolates was less than 2.8%. Nucleotide sequences of three bat isolates and Hantaan virus were identical. Replacement of nucleotides at four positions (Nt: 2111, 2146, 2170 and 2188) was 100.0% in six isolates from rodents, and 50.0% in eight isolates from patients (Table 4). Overall change of four positions was 71.4%. Two nucleotide positions (Nt: 2245, 2248) showed high replacement, also.

Phylogentic analysis

Genetic relatedness of the Hantaan virus isolates from 1988 to 1994 in Korea was constructed on the basis of 180 nucleotide sequence of the G2 region of the M segment by

M segment G2 region

	2111				2160
HTN(76/118)	TTAACAAACC	CACTTGAGGA	AGCACAATCC	ATTGACCTAC	ATATTGAAAT
CUMC-91A19	C---------	----------	---------T	-----T----	--G-------
-91R8	C---------	----------	---------T	----GT--C-	--G-------
-91A34	C---------	----------	---------T	----GT--C-	G---------
-91A49	C---------	----------	----------	----GT--C-	--G-------
-91A57	C---------	-------A--	----------	-----T----	----------
-91A58	C---------	-------A--	----------	-----T----	----------
-92B8	----------	----------	----------	----------	----------
-92B26	----------	----------	----------	----------	----------
-92B48	----------	----------	----------	----------	----------
-88H1	----------	----------	----------	----------	----------
-88H2	----------	------G---	----------	----------	----------
-89H1	C---------	-------A--	----------	-----T----	----------
-89H2	C---------	----------	----------	-----T----	----------
-92H1	----------	----------	----------	----------	----------
-92H2	C---------	----------	---------T	----GT----	--G-------
-92H3	----------	-------A--	----------	----------	----------
-92H4	C---------	----------	---------T	----GT----	----------

	2161				2250
HTN(76/118)	AGAAGAACAG	TTGATGTGCA	TGCTCTAGGA	ACATCCTTTC	ACTGTTATGG
CUMC-91A19	---------A	-------A--	----------	-----T----	----C--C--
-91R8	---------A	-------A--	----------	-----T----	----C--C--
-91A34	---------A	-------A--	----------	-----T----	----C--C--
-91A49	---------A	-G-----A--	----------	-----T----	----C--C--
-91A57	---------A	-G-----A--	----------	----------	----------
-91A58	---------A	----------	----------	----------	----------
-92B8	----------	----------	----------	----------	----------
-92B26	----------	----------	----------	----------	----------
-92B48	----------	----------	----------	----------	----------
-88H1	----------	----------	----------	----------	----------
-88H2	----------	----------	----------	----------	----------
-89H1	---------A	-G-----A--	C---------	----------	----------
-89H2	---------A	-G-----A--	----------	----------	----------
-92H1	----------	----------	----------	----------	----------
-92H2	---------A	-------A--	C---------	----------	----C--C--
-92H3	----------	----------	----------	----------	----------
-92H4	---------A	-------A--	C---------	----------	----C--C--

Fig. 2. The nucleotide sequence alignment of G2 region of M RNA segment of 17 isolates and Hantaan 73/118 virus. 180-nucleotides of G2 region (Nt: 2101-Nt: 2280) was sequenced, and identical portions (80 nucleotides) were deleted in this figure. Abbreviations see in Fig. 1.

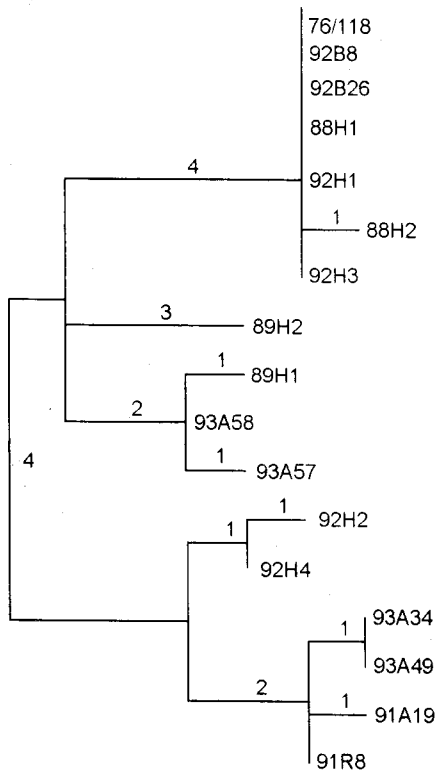

Fig. 3. Phylogenetic relationship of the G2 region (180 bases) of the M RNA segments of 17 hantavirus isolates to the equivalent segments of Hantaan 76/118 virus. Phylogenetic analysis was carried out by the maximum parsimony method using PAUP Version 3.1. Horizontal lengths are proportional to nucleotide step differences, and vertical distances are graphic representation only.

the PAUP method. Trees were caculated by the mid-portion rooting relative to sequence region of Hantaan 76/118 virus, and bootstrap confidence limits were expressed as percent on the horizontal line of each tree. Seventeen isolateds of Hantaan virus tested were divided into two genogroups, but any relatedness between genogroup and virologic property or origin of the isolate was not observed.

DISCUSSION

Hantaan 76/118 virus, the prototype virus of HFRS, was isolated from the lung tissue of *Apodemus agrarius coreae* [5] that is a principal reservoir of Hantaan virus. Thereafter, numerous hantaviruses have been isolated from various rodent species and other small mammals in widely divergent geographical areas in the world [24]. Isolation and characterization of hantavirus isolates are very significant in predicting the clinical severity of HFRS, and an unknown reservoir. Since discovery of Muerto Canyon virus (MCV) from *Peromyscus maniculatus* captured at the region in southwestern United States [25], investigations on Hantavirus genus and viral diseases have been increasing.

Despite well establishment of virus isolation methods and detection of viral antigen from tissues, hantavirus isoltation is really difficult. The isolation rate of hantaviruses from patient and bats was very low, but that from rodents was relatively high (Table 2). Antigen detection by the IFA technique was more than 10 percent in rodent specimens. IFA antibody titer at the specimen collection influcenced on the hatavirus isolation from specimens. When the antibody titer was more than 1:1,024, virus isolation was rare, especially from patient specimens. Development of more susceptible methods will be needed for an increasing of hantavirus isolation.

Two to four hantaviruses were isolated annually from various hosts for seven years. Specimens of 538 sera and PBMs were collected from patients with HFRS occurred in regions of northern Kyunggi-do and Kangwon-do Provinces. Eighty-one *Apodemus sp.* and thirty-eight *Rattus sp.* were captured from several different regions of Kyunggi-do Province.

The isolates showed different growth patterns. The isolates from patient's sera grew rapidly, but those from patient's PBMs grew slowly. To analyze the growth differences, the nested RT-PCR on the G1 region of the M genome segment was done. All isolates were confirmed as Hantaan type, regardless of specimen sources and host origins. During the time, we failed to isolate Seoul virus from patient

Table 4. Comparison of nucleotide sequence in the M segments of Hantaan 73/118 virus and the isolates Isolates from

Nucleotide postition	Hantaan 76/118	Isolates from		Total
		Apodemus sp.	Human	
2111	T	C (6/6;100.0%)	C (4/8;50.0%)	71.4% (10/14)
2146	C	T (6/6;100.0%)	T (4/8;50.0%)	71.4% (10/14)
2170	G	A (6/6;100.0%)	A (4/8;50.0%)	71.4% (10/14)
2188	G	A (6/6;100.0%)	A (4/8;50.0%)	71.4% (10/14)
2245	T	C (4/6;66.7%)	C (2/8;25.0%)	42.9% (6/14)
2248	T	C (4/6;66.7%)	C (2/8;25.0%)	42.9% (6/14)
2140	C	T (3/6;50.0%)	T (2/8;25.0%)	35.7% (5/14)
2145	A	G (3/6;50.0%)	G (2/8;25.0%)	35.7% (5/14)
2128	G	A (2/6;33.3%)	A (2/8;25.0%)	28.6% (4/14)
2153	A	G (2/6;33.3%)	G (2/8;25.0%)	28.6% (4/14)
2182	T	G (2/6;33.3%)	G (2/8;25.0%)	28.6% (4/14)
2149	A	C (3/6;50.0%)	A (0/8;0.0%)	21.4% (3/14)
2236	C	T (4/6;66.7%)	C (0/8;0.0%)	28.6% (4/14)
2191	T	T (0/6;0.0%)	C (3/8;37.5%)	21.4% (3/14)

specimens, and lung tissues of *Rattus sp.* which is a principal reservoir of Seoul type. These data support that Hantaan virus infection is predominant in Korea, and also it will be necessary to estimate the incidence of Seoul virus infection in this country.

For further analysis, nucleotide sequences of the G2 region of the M segment of Hantaan viruses were compared. The reason is major epitopes of the Hantavirus genus were encoded in this region [26]. A sequence difference of the isolates to the corresponding sequence of the prototype virus was less than 3.3%. Several nucleotide positions of Hantaan viruses isolated from *Apodemus agrarius* captured in 1991 exhibited high rates of nucleotide replacements, when compared with those of the prototype, Hantaan 76/118 virus, isolated in 1978. The nucleotide replacement rate of the patient's isolates was one-half of that of the isolates from *Apodemus sp.*, When considering the mode of transmission of the Hantavirus from reservoir to human, this suggests that variant and prototype of Hantaan viruses coexist equally in reservoirs. Interestingly, the nucleotide position of replacement observed in the G2 region of the isolates from *Apodemus sp.* is not coincident with that recognized in the identical region of HV cl-1 and cl-2 which were obtained from in vitro serial subculture of Hantaan 76/118 virus [27].

Virulence of HV cl-1 in suckling mice was much stronger than that of HV cl-2, and the molecular basis is an amino acid substitution in position 1124 of the G2 region of HV cl-1. Although a nucleotide replacement does not always accompany amino acid change, nucleotide for long period.

Among 135-nucleotides of the N protein region of the S segment, thirteen positions of rodent-isolates and only one position of human-isolates were changed (data not shown). These findings are similar to those of the G2 region described above between HV cl-1 [27] and the isolates by us. An accumulation of minor changes in the G2 region of the M genome segment could be responsible sequence analysis of hantavirus isolates should be done for emerging a new variant of hantavirus. Two genogroups of Hantaan virus were found in our

country, but any relation of genogroup and host origin or virologic property was not observed.

Acknowledgements

We would like to gratefully acknowledge technical assistance of W. B. Park in our Department, and phylogenetic analysis of K-J Song in Department of Micorbiology, College of Medicine, Korea University. We also thank H. W. Lee for suppling reference viruses.

This work was supported by a grant G015515 from the Basis Medical Research Promotion Fund of the Ministry of Education, R.O.K

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