大韓바이러스學會誌 : 第 28 卷 第 2 號 1998
J. Korean Soc. Virology, Vol. 28, No. 2, 1998

Detection of Puumala and Hantaan Viruses among Bats in Korea by Nested RT-PCR

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=Abstract=

Hantavirus is a genus of the Bunyaviridae family consisting following serotype groups: Hantaan, Seoul, Puumala, Prospect Hill, Thailand, Belgrade, Thotta palayam, Sin Nombre. Most of Hantavirus group have been associated with many clinically similar disease known collectively as hemorrhagic fever with renal syndrome (HFRS). Hantaan virus is the prototype of the genus hantavirus, originally isolated from *Apodemus agrarius*. Bat was found as a natural host for Hantaan virus in Lee's lab for the first time. Then, Hantaan-like virus was isolated Hantaan-like virus from bat. To identify hantaviruses that are present in Korea among bats, bats were collected from Jeong-Sun, Won-Joo, Chung-Ju and Hwa-Cheon area, RNA was isolated from lung and serum. RT-PCR was performed with a universal primer from M segment. Nested RT-PCR was carried out to differentiate Hantaan, Seoul and Puumala virus using serotype specific primers. As we expected, Hantaan viruses were detected in bats and Seoul virus was not detected. Interestingly, Puumala viruses were also detected in bats from Won-Ju, but not in other areas. Puumala virus is originally isolated from *Clethrinomys glareolus*, and cause light HFRS. Recently, *Paradoxomis webbiana*, a wild bird turn out to be a reservoir for Puumala virus in Korea. These data indicate that bat is a new natural reservoir of Puumala virus.

Key Words: Puumala virus, Bat, RT-PCR, Nested RT-PCR

INTRODUCTION

The Hantavirus genus of the Bunyaviridae family possesses a three-segmented, single-stranded RNA genome with negative polarity [25]. Hantavirus is consist of eight serologically distinct groups, including Hantaan [18], Seoul [19], Puumala [20], Prospect Hill [21], Belgrade [22], Thailand [23], Muerto Canyon [17], and Thottapalayam [24] virus. Hantaviruses have been associated with many clinically similar diseases known as Korean haemorrhagic fever (KHF), epidemic haemorrhagic fever, nephropathia epidemica and other pathogenic desease. Among these 8 hantaviruses, 5 serotypes have been known to be pathogenic. Hantaan virus, the prototype of hantaviruses, originally isolated from *Apodemus agrarius* cause most severe disease with syndrome in Korea and East Asia [18]. Seoul virus isolated from *Rattus norvegicus* is distributed all around the world causing haenorrhagic fever with renal syndrome (HFRS) with intermediate severity [19]. In Korea, Hantaan and Seoul virus are the major cause of HFRS. Belgrade virus isolated from

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This study was supported by Korean Ministry of Education throughBasic Sciences Research Institute (BSRI-96-4443).
This study was presented in The Fourth International Conference on HFRS and Hantaviruses. March 5-7, 1998 Holliday Inn Hotel and Conference Plaza, Atlanta, Georgia, USA.

Apodemus flavicolis also cause severe disease with syndrome in Balkan area [22]. Recently, Muereto canyon virus transmited by *Peromyscus maniculatus* was identified as the etiological agent of the hantavirus pulmonary syndromes with 76% mortality [17].

Puumala virus, the causative agent of the human disease nephropathia epidemica [20], occurs endemically in Europe and is spread mainly by the bank vole (*Clethrionomys glareolus*). Recent genotype studies have disclosed several PUU-like genotypes spread in Europe and Russia by the genera of the Arvicoline-rodent subfamily: Tula, Tobetsu, Khabarov, and Topografov [27]. Their importance for human pathogenicity is not clear yet.

Immunoflourescent antibody screening test (IFAT) is one of the most commonly used method to detect antibody against hantavirus from samples with limitations due to the relatively low sensitivity and complex procedures. Since an introduction of RT-PCR to detect hantanvirus [26], RT-PCR was emerging as a powerful technique to detect hantavirus. Furthermore, by using serotype specific primers, significant improvements were made in differentiating hantavirus serotypes and genetic variations.

The local distribution of hantavirus infection has been studied by IFA to detect antibodies against hantavirus. Detecting the viral antigen is more informative to determine whether the animals are carriers of the viruses. To detecting viral antigen in the sample, isolation of virus by cell culture, and Immunoenzyme detection are used, however, those are not popular due to the complex procedures and the low reliabilities. Recently, RT-PCR has been performed to detect viral antigen, because it can detect virus with very low titration and the nucleotide sequence can be analyzed with the amplified products. RT-PCR was carried out to study local distributions of hantavirus infection in our studies to get more reliable data.

Since bats were found as a carrier of Hantaan virus antibody by IFA in Lee's lab for the first time [30,31], Hantaan virus was isolated and sequenced [6,7]. Puumala virus was also found in bats captured in 1996-1997 by nested RT-PCR in Won-Joo [32]. In 1998, bats were also collected, and nested RT-PCR with serotype specific primers was performed. Puumala virus were identified only in Won-Joo, not in Jeong-Sun, Hwa-Cheon (Kang-Won Do) and Chung-Ju (Chung-Buk). Puumala virus was fuoud in a wild bird, Paradoxomis webbiana in Korea [4]. Here, we report bat is also a new natural reservoir of Puumala virus.

MATERIALS & METHODS

1. Samples

Bats have been collected from 1989 to 1998 as in Table 1. Bats collected from Jeong-Sun, Won-Joo, Chung-Ju and Hwa-Cheon were tested by RT-PCR to detect hantaviruses. Serum, lungs and kidneies were dissected and stored at -70℃.

2. Antibody detection

Bats 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 [26]. 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.

3. Primers

Primers for RT-PCR were listed in Table 2 [1]. Primers were derived from G1 region of M segment. Hantaan specific primers (HTNV) were used for RT-PCR to detect Hantaan viruses. Nested RT-PCR was performed to detect hantavirus in bats. A universal primer pair (MOF103, MOR204) was used for the first round of PCR, and Hantaan (HANV), Seoul (SEOV), Puumala (PUUV) specific primers were used for nested RT-PCR.

Table 1. Hantaan virus in bats captured from 1989 to 1997

Year of collection		Province	Species	No. of positive / No. of tested
1989	2	Chung Nam	*Rhinolophus ferrumequinum*	1 / 18
	3	Chung Nam	*Rhinolophus ferrumequinum*	1 / 9
	4	Kang Won	*Rhinolophus ferrumequinum*	0 / 1
		Chung Nam	*Rhinolophus ferrumequinum*	0 / 5
	5	Chung Nam	*Rhinolophus ferrumequinum*	0 / 9
	6	Chun Buk	*Rhinolophus ferrumequinum*	0 / 6
	7	Kang Won	*Rhinolophus ferrumequinum*	0 / 17
		Chun Buk	*Rhinolophus ferrumequinum*	0 / 47
	8	Kang Won	*Rhinolophus ferrumequinum*	1 / 28
		Chun Buk	*Rhinolophus ferrumequinum*	7 / 61
		Chun Buk	*Myotis mystatinus*	0 / 8
		Chun Buk	*Miniopterus schreibersii*	0 / 20
	9	Chun Buk	*Rhinolophus ferrumequinum*	0 / 20
		Chun Buk	*Miniopterus schreibersii*	0 / 26
1990	6	Gyeong Buk	*Rhinolophus ferrumequinum*	0 / 6
	7	Kang Won	*Rhinolophus ferrumequinum*	0 / 12
		Chung Nam	*Rhinolophus ferrumequinum*	0 / 5
		Chung Nam	*Murina leucogaster*	0 / 1
		Chung Nam	*Vespertilio superans*	0 / 4
	8	Chung Nam	*Rhinolophus ferrumequinum*	0 / 1
		Chung Nam	*Murina leucogaster*	0 / 1
		Chung Nam	*Miniopterus schreibersii*	0 / 1
		Gyeong Buk	*Rhinolophus ferrumequinum*	0 / 4
		Gyeong Buk	*Vespertilio superans*	0 / 1
	9	Kang Won	*Rhinolophus ferrumequinum*	0 / 8
	10	Chung Nam	*Rhinolophus ferrumequinum*	1 / 34
	11	Gyeong Buk	*Rhinolophus ferrumequinum*	0 / 50
	12	Chung Nam	*Rhinolophus ferrumequinum*	1 / 25
1991	7	Chung Nam	*Eptesicus serotinus*	7 /170
	9	Chung Nam	*Eptesicus serotinus*	0 / 19
	12	Chung Nam	*Rhinolophus ferrumequinum*	0 / 5
1992	1	Chung Nam	*Rhinolophus ferrumequinum*	2 / 25
			Vespertilio superans	0 / 1
			Myotis formosus	0 / 1
			Myotis macrodactylus	0 / 1
		Gyeong Buk	*Rhinolophus ferrumequinum*	2 / 27
1995	8	Chung Nam	*Eptesicus serotinus*	4 /102
		Che Ju	*Vespertilio superans*	0 / 22
			Plecotus auritus	0 / 1
1996*	10	Kang Won	*Rhinolophus ferrumequinum*	7 / 10
1997*	8	Kang Won	*Myotis macrodactylus*	5 / 11
1998*	2	Kang Won	*Rhinolophus ferrumequinum*	10 / 33
	3	Chung Buk	*Rhinolophus ferrumequinum*	0 / 6
		Total		49 /862 (5.7)

( ) : %. *RT-PCR

4. RNA isolation

Total RNA was isolated from serum with Viral RNA kit from QUIAGEN, and followed the acompanied protocols. RNAzol B was used to isolate RNA from lungs. 1 ml RNAzol B was added to lungs, and the tissue was homogenated with bead beater. Then, chloroform extracted twice. RNAs were precipitated with 50% isopropanol, and the RNA pellets were washed with 70% ethanol. Pellets were dried and resuspended in 50 μl DEPC ddH_2O.

5. RT-PCR

1 μl (1 pmol) primer MOF103 was added to 10 μl RNA, and heat treated at 72℃ for 10 mins to denature the secondary structure of RNA. Cool down to 40℃ to anneal the primer, then add 0.5 μl RNAsin (10 unit), 1.5 μl 50 mM dNTP mix, 3 μl 5x M-MLV Reverse transcriptase buffer, 1 μl M-MLV Reverse transcriptase (Promega). Incubate at 40℃ for 1 hr to synthsize DNA, then at 95℃ for 3 mins to inactivate M-MLV reverse transcriptase.

For nested RT-PCR, the first PCR was performed with universal primers (MOF103, MOR-204). Then, 5 μl the first RT-PCR product was mixed with 2.5 μl 10x Taq polymerase buffer (+10 mM $MgCl_2$), 1 μl (20 pmol) serotype specific primer pair (HANT or PUUVor SEOV), 0.25 μl Taq polymerase and ddH_2O to adjust volume to 30 μl. 33 PCR cycles were perforemd and each cycle is 94℃ for 30 secs, 55℃ for 45 secs, 72℃ for 45 secs. Electrophoresis was performed in 2% agarose gel PCR products.

RESULTS

Hantavirus detection among bats in Kang-Won and Chung-Buk by RT-PCR

Nested RT-PCR was performed, because virus was not detected with RT-PCR in bat's serum or lungs. Total RNA was prepared with lung and serum, then the first round of RT-PCR was performed with the genus-specific primers. Amplified PCR products were used as templates for nested RT-PCR with three serotype specific primers, HTNV, PUUV, and SEOV (Table 2). 10 *Rhinolophus ferrumequinum* in

Table 2. Neucleotide sequence of Hantavirus genus-reactive and serotype specific primers[12)]

	Name	Neucleotide position	Primer sequence	Sense	Function	Products (bps)
Genus-specific	MOF103	1190-1212	5'>GGACCAGGTGCAGCTTGTGAAGC<3'	+	CDNA synthesis and downstream primer	490 bp
	MOR204	1661-1680	5'>ACCTCACAAACCATTGAACC<3'	−	1' PCR upstream primer	
HTNV	HTN G1F	1343-1362	5'>TGCAACGGGCAGAGGAAAGT<3'	+	nested PCR downstream primer	285 bp
	HTN G1R	1604-1625	5'>GTACTGATTTTAGCCTATTCTC<3'	−	nested PCR upstream primer	
SEOV	SEO G1F	1343-1362	5'>TGTAATGGTCAGAAAAAGAC<3'	+	nested PCR downstream primer	289 bp
	SEO G1R	1607-1629	5'>CGTAGAATGGCTTTGAATCGGTT<3	−	nested PCR upstream primer	
PUUV	PUU G1F	1296-1315	5'>GTGTCCAGAGATTCCGTGGT<3'	+	nested PCR downstream primer	327 bp
	PUU G1R	1599-1620	5'>GAACATAAGTATGCGAATGCAA<3'	−	nested PCR upstream primer	

Table 3. Hantaan virus infection among bats in Won-Joo, Jeong-Sun, Hwa-Cheon and Chung-Ju

Area	Year of collection	Species	No. of Positive / No. of Tested
Won-Joo	1996	*Rhinolophus ferrumequinum*	7 / 10
	1998	*Rhinolophus ferrumequinum*	10 / 21
Jeong-Sun	1997	*Myotis macrodactylus*	5 / 11
Hwa-Cheon	1998	*Rhinolophus ferrumequinum*	0 / 12
Chung-Ju	1998	*Rhinolophus ferrumequinum*	0 / 6
Total			22 / 60 (36.7%)

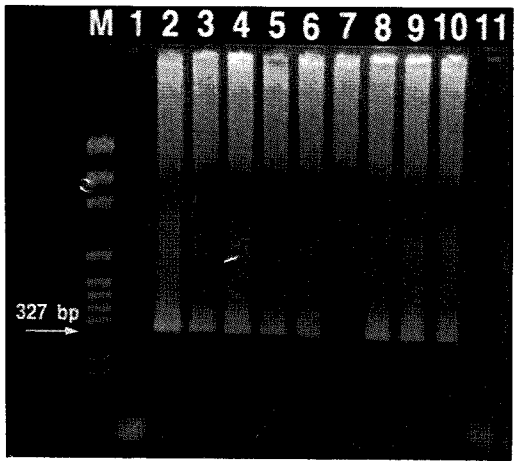

Fig. 1. Detection of Puumala virus in bats by nested RT-PCR. Nested RT-PCR was performed to detect Puumala virus in total RNA from serum. Lane 1: pGEM marker, Lane 2: negative control, Lane 3, 4, 5, 6, 7, 9, 10, 11: Puumala virus positive, Lane 8, 12: Puumala virus negative. Arrow indicates the nested RT-PCR product of Puumala virus, 327 bp.

Won-Joo and 11 *Myotis macrodactylus* in Jeong-Sun from 1996 to 1997 were tested for hantavirus infection (Table 3). 7 out of 10 *Rhinolophus ferrumequinum* in Won-Joo were carrying Hantaan virus, and the rate of infection was 70%. In 1998, 21 *Rhinolophus ferrumequinum* in Won-Joo were tested, 10 of them were carrying Hantaan viruses and the infection rate was 50%. The average rate of Hantaan virus infection was 60% in 1996-1998. 5 out of 10 *Myotis macrodactylus* captured in Jeong-Sun were carrying Hantaan virus and the infection rate was 50% (Table 3). This confirms the previous studies that bats are a natural host of Hantaan-like virus [7,30,31,32]. 6 *Rhinolophus ferrumequinum* in Chung-Ju and 11 *Rhinolophus ferrumequinum* in Hwa-Cheon were tested for hantavirus infection. Hantaan virus was not detected both in Chung-Ju and Hwa-Cheon (Table 3).

Rhinolophus ferrumequinum in Won-Joo is a putative new natural reservoir of Puumala virus

Puumala virus, which is a prevalent hantavirus strain in Europe, was detected by nested RT-PCR with Puumala virus specific primer (PUUV) in *Rhinolophus ferrumequinum* in Won-Joo city. This indicates that *Rhinolophus ferrumequinum* is a natural reservoir of Puumala virus as well as Hantaan virus. Recently, a wild bird, *Paradoxomis webbiana* turn out to be a natural host for Puumala virus in Korea [5]. Eight carried Puumala virus among ten *Rhinolophus ferrumequinum* in Won-Joo in 1996-1997 (Table 4) (Fig. 1). The infection rate of Puumala virus was 80%. 6 *Rhinolophus ferrumequinum* were carrying both Puumala and Hantaan virus. In 1998, 6 out of 23 *Rhinolophus ferrumequinum* were carrying Puumala virus in Won-Joo, and the infection rate was 26%. This confirms that *Rhinolophus ferrumequinum* a natural host of Puumala virus. 6 *Rhinolophus ferrumequinum* were carrying both Puumala and Hantaan viruses. The average

Table 4. Puumala virus infection among bats in Won-Joo, Jeong-Sun, Hwa-Cheon, Chung-Ju

Area	Year of collection	Species	No. of Positive / No. of Tested
Won-Joo	1996	*Rhinolophus ferrumequinum*	8 / 10
	1998	*Rhinolophus ferrumequinum*	6 / 23
Jeong-Sun	1997	*Myotis macrodactylus*	0 / 11
Hwa-Cheon	1998	*Rhinolophus ferrumequinum*	0 / 12
Chung-Ju	1998	*Rhinolophus ferrumequinum*	0 / 6
Total			14 / 62 (22.6%)

Puumala virus infection rate was 53% in Won-Joo 1996-1998. Puumala virus was not detected with Myotis macrodactylus and *Rhinolophus ferrumequinum* in Jeong-Sun, Chung-Ju, Hwa-Cheon. Seoul virus was not detected both with *Rhinolophus ferrumequinum* in Won-Joo and *Myotis macrodactylus* in Jeong-Sun.

Hantaan virus was detected by IFA and RT-PCR in Korea from 1989 to 1998

Since the first detection of Hantaan virus Antibodies by IFA in 1989, the local distribution has been studied in our lab (Table 1). Viral antibody was detected by IFA from 1989 to 1995, the average infection rate was 4.7%. Nested RT-PCR was performed to detect virus since 1996. The infection rate was 50%. It was high compare to the previous data with IFA (Immunoflourescent Antibody), the possession rate of IFA against Hantaan virus was average 3.4% in bats. It demonstrates that nested RT-PCR is much more sensitive compare to IFA technique.

DISCUSSION

Rodents were found as the principle natural reservoir of hantavirus as an etiological agent of hoemorragic fever with renal syndrome (HFRS) in Korea. Hantavirus exist in various serotypes with different pathogenicity for human, varying form asymptomatic infection to highly fatal disease. HFRS by Hantaan virus are common in Asia, whereas Nephropathia epidemica caused by Puumala virus are common in northern part of Europe. In Korea, 1000 HFRS cases are caused chiefly by Hantaan and Seoul viruses every year, and *Apodemus* mice and rats are the principle natural reservoirs respectively in HFRS occuring areas. Recently, patients with light HFRS carried antibody against Puumala virus or show high antibody titre in Korea, according to unpublished data. In central and northern Europe, the Puumala virus is predominent causing influenza-like symptoms and renal disfunction. However, the natural host and the infection routes of Puumala virus in Korea has not well been studied yet.

The hantavirus can be transmitted through inhalation of contaminated air or dust with excretion of the virus-infected wild animals. Antibodies against Hantaan and Seoul virus were detected from sera of bats (*Rhinolopus ferrumequinum* and *Vespertilo abramus*) [28] and wild birds (*Paradoxmis webbiana* and *Emberiza elegans*) [4,29] in Korea. Hantaan-like viruses were isolated and sequenced from bats, *Rhinolopus ferrumequinum* and *E. serotinus* [6, 7]. Hantaan virus might be transmitted from *Apodemus* mice to bats by uncertain routes. In the case of Puumala virus, it has been a prevalent serotype and causative agent for nepropathia epidemica in Europe. Light HFRS pa-

tients possessed antibody against Puumala virus were found in Korea, indicating the possibbilities of Puumala virus in Korea. The natural reservoir of Puumala virus in Europe is the rodents *Clethrionomys glareolus* [20]. Recently, antibodies against Puumala virus were also detected in the sera of wild birds, *Paradoxmis webbiana* in Korea [5]. Here, in this reports, we also found bat is also a carrier of Puumala virus in Korea for the first time. The ecological relationship between *Apodemus* mice and other reservoirs of hantaviruses is not known. The infection routes and maintenance of hantaviruses in nature needs to be studied.

To detect hantavirus from HFRS patient, IFAT (immunoflourescent antibody screening test) has been commonly used. In addition to IFA, ELISA [8] (Enzyme linked immuno-sorbent assay), HI [9] (Hemagglutination Inhibition), IAHA [10] (Immuno adherence hemagglutination), PRNT [11] (Plaque reduction netralization test), SPRIA [13] (solid phase radioimmunoassay) and western blot [14] analysis were developed and used to detect antibodies against hantavirus. Recently, RT-PCR has been performed to detect viral antigen, and to analyze the viral genome [15,16] due to the relatively high sensitivity and simplicity. Furthermore, the introduction of nested RT-PCR to detect and differentiate [12] hantavirus serotypes by using serotype-specific primers make the technique much more sensitive up to 1 PFU.

Nested RT-PCR was carried out to detect and differentiate hantavirus serotypes among bats with RNA from lungs. Bats collected from Jeong-Sun, Won-joo, Hwa-Cheon and Chung-Ju. Bats both from Hwa-Cheon and Chung-Ju did not carry hantaviruses. *M. macrodactylus* from Jeong-Sun were infected only with Hantaan virus, neither with Seoul virus nor Puumala virus. The Hantaan virus infection rate was 45% in Jeong-Sun in 1996-1997. Bats collected in Won-Joo were *Rhinolophus ferrumequinum* carrying Puumala virus as well as Hantaan virus. 7 out of 10 bats were infected with Puumala virus, 8 out of 10 bats were infected with Hantaan virus alone 1996-1997. 6 out of 10 bats were infecte both Puumala and Hantaan virus 1996-1997. This indicates that bats are a new natural reservoir of Puumala virus. In 1988, Puumala and Hantaan viruse are also detecetd from *Rhinolophus ferrumequinum* in Won-Ju. 6 out of 23 bats were infected with Puumala virus, 10 out of 21 bats were infected with Hantaan virus in 1998. 6 out of 21 bats were infected both Puumala and Hantaan virus in 1998. These data confirm that bats are a new natural reservoir of Puumala virus, although, it will be necessary to isolate and sequence the virus in bats to confirm it. The hantavirus infection rates among bats were around 50%. The infection rates were high compare to the previous data, 3～7% infection rates in bats by IFA [28]. This implies that nested RT-PCR is much more sensitive than IFA, although bats were collected at different times. The infection routes of Puumala virus to bats or wild birds has not been known yet. It is necessary to investigate the etiological relation among the natural hantavirus reservoirs and life cycle of hantavirus in nature.

CONCLUSION

We studied the local distribution and identification of hantaviruses among bats in Kang-Won and Chung-Buk areas. Nested RT-PCR was performed to detect viral antigen not antibody against virus. Detecting viral antigen is more direct and reliable to analyze the viral infection rate among natural reservoirs. Around 50% bats tested were infected to hantavirus. The infection rate by nested RT-PCR was much higher compare to that by IFA. Interestingly, Puumala virus was also detected in bats from Won-Joo by nested RT-PCR with serotype specific primers. The average Puumala virus infection rate was 53% in 1996-1998. It will be necessary to isolate and sequence Puu-

mala viruses from bats. Here, we found bat is also a good candidate for a new host for Puumala virus.

Acknowledgement

Thanks to Prof. Kim, Gum Ryoung and Dr. Jung, Yong Tae for advise and gifts of primers.

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