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Partial Sequence Analysis of Puumala Virus M Segment from Bats in Korea

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=Abstract=

Hantavirus is a genus of the *Bunyaviridae* family causing two serious diseases, hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS). Puumala virus is a member of *hantavirus* originally found in Europe, and its natural reservoir is *Clethrionomys glareolus*. It is also associated with the human disease nephropathia epidemica, a milder form of HFRS. To identify the hantaviruses in bats, bats were collected from Jeong-Sun, Won-Joo, Chung-Ju and Hwa-Cheon area in Korea, and nested RT-PCR was performed with serotype specific primer from M segment. Interestingly, Puumala virus was detected in bats (*Rhinolophus ferrum-equinum*) only from Won-Joo. The 327 bp nested RT-PCR product, was sequenced. The sequence database search indicates that the sequence is homologous to the published sequence of Puumala viruses. The sequence similarities were ranged from 71% to 97%. The highest sequence similarity was 97% with Puumala virus Vranicam strain, and the lowest was 71% with Puumala virus K27 isolate. Puumala virus Vranicam strain was isolated from a bank vole (*Clethrionomys glareolus*) in Bosnia-Hercegovina. Puumala virus K27 was isolated from human in Russia. This analysis confirms that bats (*Rhinolophus ferrum-equinum*) in Korea are natural reservoir of Puumala virus.

Key Words: Puumala virus, bat, nested RT-PCR, DNA sequence, deduced amino acid sequence

INTRODUCTION

Hantaviruses, a member of *Bunyaviridae*, are enveloped viruses possessing a three-segmented, single-stranded RNA genome with negative polarity [20]. Small mammals, mainly rodents, are the natural reservoirs of hantaviruses. Viral transmission is mediated by aerosolized animal excreta. The genus *Hantaviridae* consists of nine different serological groups, including Hantaan [7], Seoul [8], Puumala [16], Prospect Hill [11], Sin Nombre [15], Thailand [3], Dobrava [1], Tula [18] and Thottapalayam [23] virus, and the number of serotype is expected to rise. Hantaviruses have been associated with two clinically distinct diseases known as hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS). Among these 9 hantaviruses, 5 serotypes have been known to be pathogenic. Hantaan virus, the pro-totype of hantavirus, ori-

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ginally isolated from *Apodemus agrarius* cause most severe disease with syndrome in Korea and East Asia [7]. Seoul virus isolated from *Rattus norvegicus* is distributed all around the world causing HFRS with intermediate severity [8]. In Korea, Hantaan and Seoul virus are the major cause of HFRS. Dobrava virus isolated from *Apodemus flavicolis* also cause severe disease with syndrome in Balkan area [1]. Puumala virus cause a milder form of HFRS or nephropathia epidemica [16], occurs endemically in Europe and is spread mainly by the bank vole (*Clethrionomys glareolus*). Recently, a new hantavirus was discovered in the United States as the etiological agent of a deadly disease, HPS with mortality over 50% [15]. Sin Nombre virus is carried mainly by deer mice (*Peromyscus maniculatus*). Serologically and genetically, Hantaan, Seoul and Dobrava virus are carried by *Murinae* rodents which are closely related each other, while Puumala, Prospect Hill, Tula and Sin Nombre viruses are carried by *Arvicolonae* and *Sigmodontinae* rodents closely related. However genetic dissection of the infectivity and pathogenicity, as well as host specificity of hantaviruses require further investigations.

We have reported that *Rhinolophus ferrumequinum* is a novel natural host of Puumala virus [14]. Puumala virus was detected in the serum and lung of bats captured in Won-Joo by nested RT-PCR with Puumala virus serotype specific primers from G1 region of M segment. Bats are also known as a carrier of Hantaan virus by studying with IFA [10, 12]. Hantaan virus from bats was isolated and sequenced [5, 6]. Puumala virus was also found in bats captured in 1996, 1997, 1998 by nested RT-PCR in Won-Joo [14]. The 327 bp nested RT-PCR product was sequenced. The nucleotide sequence of the 327 bp was homologous to that of the published Puumala virus. Homologue search found 8 Puumala virus strains mainly isolated bank vole and human. The nucleotide sequence homologies were 62~97%. Puumala virus Vranicam strain showed the highest nucleotide sequence homology of 97%. Vranicam was isolated from a bank vole in Bosnia-Hercegovina, and associated with the occurence of HFRS in human [19]. Puumala virus K27 showed the lowest similarity of 62%. K27 is a human isolate obtained in an HFRS endemic region of the former Soviet Union [22]. As far as we know, Puumala virus detected from a wild bird, *Paradoxomis webbiana*, in Korea, has not been sequenced or isolated yet [9]. Here, we report the bat is also a new natural reservoir of Puumala virus. The infection route of Puumala virus to bats or wild birds has not been known yet. It is necessary to investigate the etiological relation among the natural Puumala virus reservoirs and life cycle of Puumala virus in nature.

MATERIALS & METHODS

1. Samples

Bats have been collected from 1989 to 1998. Bats collected from Jeong-Sun, Won-Joo, Chung-Ju and Hwa-Cheon were subjected to by RT-PCR to detect hantaviruses. Sera, lungs and kidneys were dissected and stored at -70℃.

2. Primers

Primers were derived from G1 region of M segment [4]. Genus specific primers MOF103 (1190-1212: 5'>GGACCAGGTGCAGCTTGTGAAGC<3) and MOR204 (1661-1680: 5'>ACCTCACAAACCATTGAACC<3') were used to perform RT-PCR and Puumala specific primers, PUUG1F (1269-1315: 5'>GTGTCCAGAGATTCCGTGGT<3') and PUUG1R (1599-1620: 5'>GAACATAAGTATGCGAATGCAA<3') [4] were used for nested RT-PCR to detect Puumala viruses in bats.

3. RNA isolation

Total RNA was isolated from serum with Viral RNA kit from QUIAGEN, and followed the manufacturer's protocols. RNAzol B was

used to isolate RNA from lungs. One milliliter RNAzol B was added to lung, and the tissue was homogenated with a bead beater. Then, chloroform-extracted twice. The RNA was precipitated with 50% isopropanol, and the RNA pellet was washed with 70% ethanol. The pellet was dried and resuspended in 50 μl DEPC ddH_2O.

4. Nested RT-PCR

One pico mole of the primer in 1 μl primer MOF103 was added to 10 μl RNA, and heat treated at 72℃ for 10 mins to denature the secondary structure of RNA. After cooling down to 40℃ to anneal the primer, 0.5 μl RNAsin (10 unit), 1.5 μl 50 mM dNTP mix, 3 μl 5x M-MLV Reverse transcriptase buffer, and 1 μl M-MLV Reverse transcriptase (Promega) were added. The reaction mixture was incubated at 40℃ for 1 hr to synthesize DNA, then at 95℃ for 3 mins to inactivate M-MLV reverse transcriptase.

For nested RT-PCR, the first PCR was performed with the universal primers (MOF103, MOR204). 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) Puumala virus specific primer pair. Zero point two-five microliter Taq polymerase and double-distilled H_2O were added to adjust volume to 30 μl. The 33 PCR cycles were carried out and each cycle is 94℃ for 30 secs, 55℃ for 45 secs, 72℃ for 45 secs. The PCR products were analyzed by electrophoresis with 2% agarose.

5. Sequencing of the nested RT-PCR product and sequence analysis

The nested RT-PCR product was subcloned into pGem-T-Easy vector (Promega). The plasmids of the subclones were prepared by boiling method and digested with EcoRI to analyze the subclones. The subclone of the 327 bp nested RT-PCR product was sequenced by dideoxy chain termination with universal primers (5'CAGGAAACAGCTATGAC3' and 5'GTAAAACGACGGCCAGT3'). The sequenced RT-PCR product was named PUUBAT. The DNA sequence comparison was performed with GCG (Genetics Computer Group) and GeneDoc (PC), computer programs.

RESULTS

Sequence analysis of the 327 bp nested RT-PCR product of Puumala virus M segment from bats

Nested RT-PCR was performed, because virus was not detected with RT-PCR in bat's serum or lungs. Total RNA was prepared from the lung and serum, then the first round of RT-PCR was performed with the genus-specific primers, MOF103 and MOR204. The amplified PCR products were used as templates for nested RT-PCR with Puumala virus specific primers, PUUG1F and PUUG1R.

Puumala virus, which is a prevalent hantavirus strain in Europe, was detected by nested

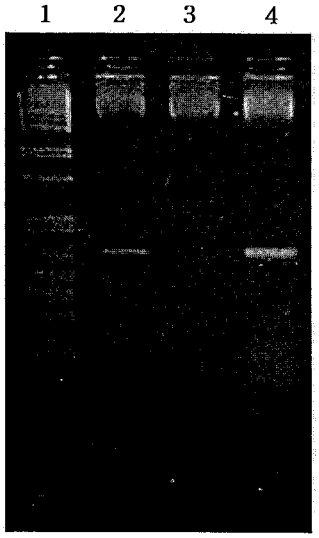

Figure 1. Electrophoresis of the 327 bp nested RT-PCR product subclones in pGem T easy vector. Plasmid was treated with EcoRI. lane 1: 100 bp DNA ladder, lane 2-4: subclones digested with EcoRI. Lane2 and lane4 are subclones containing the 327 bp nested RT-PCR product. Arrow indicates the 327 bp nested RT-PCR product.

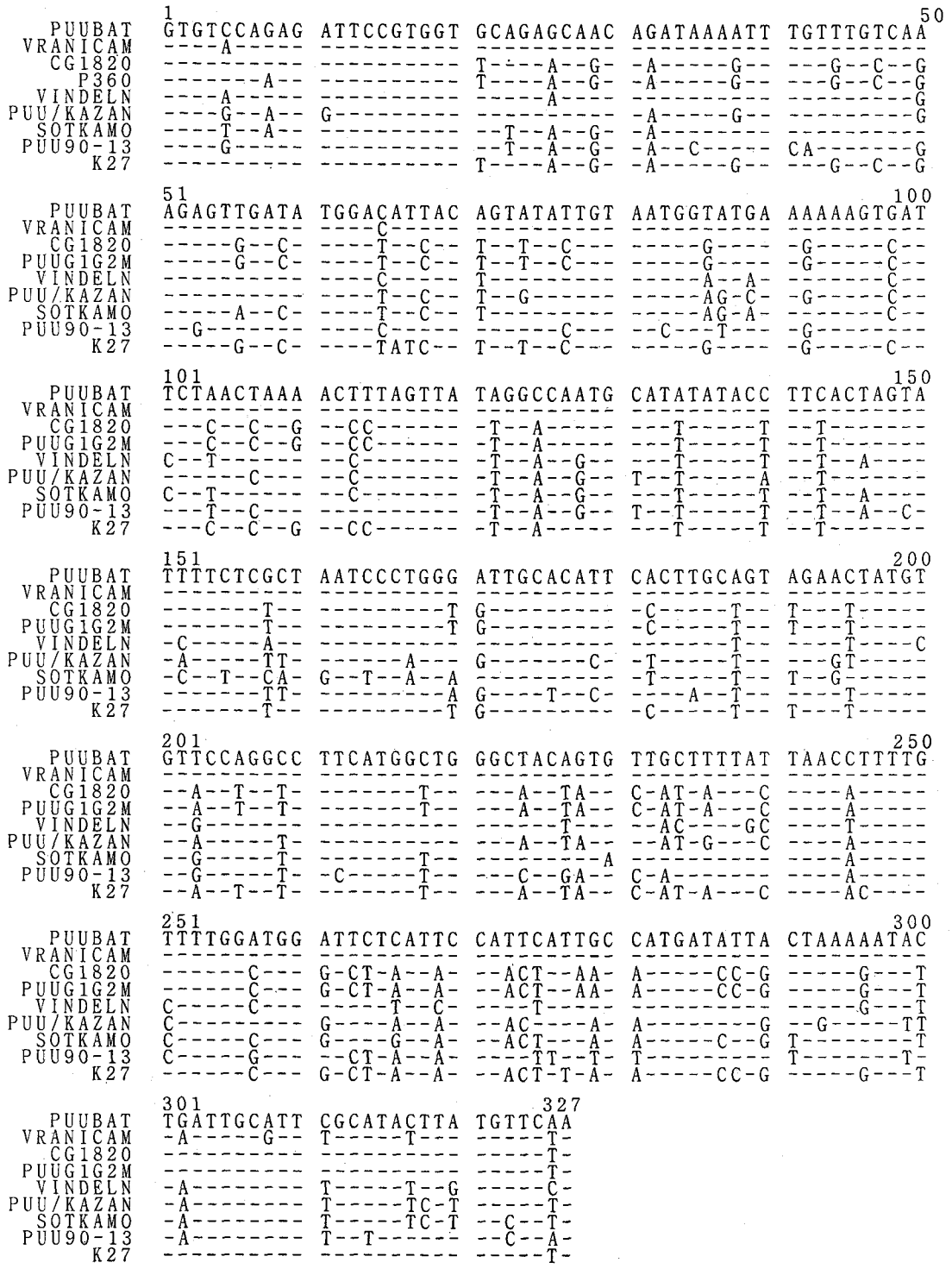

Figure 2. Nucleotide sequence comparison of PUUBAT with 8 puumala virus isolates. Nucleotide sequences were compared with GCG. The nucleotide sequence similarities are ranged from 71 to 97% among homologues. Vranicam shows the highest similarity (97%) and P360 shows the lowest nucleotide sequence similarity (71%). '-' indicates the same nucleotide with that of PUUBAT.

Table 1. Nucleotide sequence homologies between our sample (PBAT), VRANICAM, CG1820 (Hallnas), P360, Vindelin, Kazan, Sotkamo, PUU90-13, and K27 strains

	% nucleotide homology								
	PUUBAT	VRANICAM	CG1820	P360	VINDELN	KAZAN	SOTKAMO	PUU90-13	K27
PUUBAT	100%	97%	79%	79%	87%	80%	80%	81%	71%
VRANICAM	321	327	78%	78%	88%	82%	81%	81%	70%
CG1820	261	258	327	99%	81%	85%	82%	80%	89%
P360	260	257	327	327	81%	85%	82%	79%	89%
VINDELN	287	291	268	267	327	84%	85%	82%	72%
KAZAN	265	269	279	280	277	327	85%	81%	75%
SOTKAMO	264	268	269	270	282	281	327	79%	73%
PUU90-13	266	266	263	262	270	266	262	327	71%
K27	227	225	289	289	234	246	235	229	327

No of nucleotide homology

RT-PCR with the Puumala virus specific primer (PUUV) from a bat, *Rhinolophus ferrum-equinum* in Won-Joo city [14]. The infection rate of Puumala virus was 80%. This indicates that *Rhinolophus ferrum-equinum* is a natural reservoir of Puumala virus as well as Hantaan virus in Korea [5, 6]. A wild bird, *Paradoxomis webbiana* has been reported as a natural host for Puumala virus in Korea [2].

The 327 bp nested RT-PCR product was subcloned into pGem T easy vector (Promega) and the subclones were digested with EcoRI (Figure 1). The subclone #4 was sequenced and the sequence is shown in Figure 2. The DNA sequence has homology with published Puumala virus sequences. This indicates that the 327 bp nested RT-PCR product with Puumala virus specific primers is from Puumala virus in *Rhinolophus ferrum-equinum. Rhinolophus ferrum-equinum* is a natural host of Puumala virus.

The sequence of the nested RT-PCR product shows the highest DNA sequence identity with Puumala virus Vranicam strain

Database search found 8 Puumala virus strains homologous to the 327 bp sequence of nested RT-PCR product. Those Puumala virus strains are Vranicam, Vindelin, Kazan, Sotkamo, PUU90-13, CG1820 (Hallnas), K27, and P360. The nucleotide sequence homologies are ranged from 71 to 97% (Figure 2, Table 1). Vranicam showed the highest homology, 97%, and only 7 nucletides were different in the 327 bp nucleotide sequence. K27 showed the lowest homology, 71%. Vranicam was isolated from bank vole in Bosnia-Hercegovina and is known to related to HFRS in humans. K27 is a Puumala virus strain isolate from human for the first time with P360 in Russia. Vindelin shows 87% nucleotide sequence homology, and was isolated from a bank vole near Vindelin in Sweden. Sotkomo strain is the prototype of Puumala virus and shows 80% homology. Puu 90-13 was isolated from a human in northwestern France and a distinct western European virus. It shows 81% DNA sequence homology with PUUBAT. PUUCG1820 (Hallnas) is nephropathia epidemica virus (NEV) strain and the sequence identity is 79%. The sequence homology data shows that the PUUBAT is more closely related to the Puumala virus strain in bank vole from Russia than that from Europe or in human.

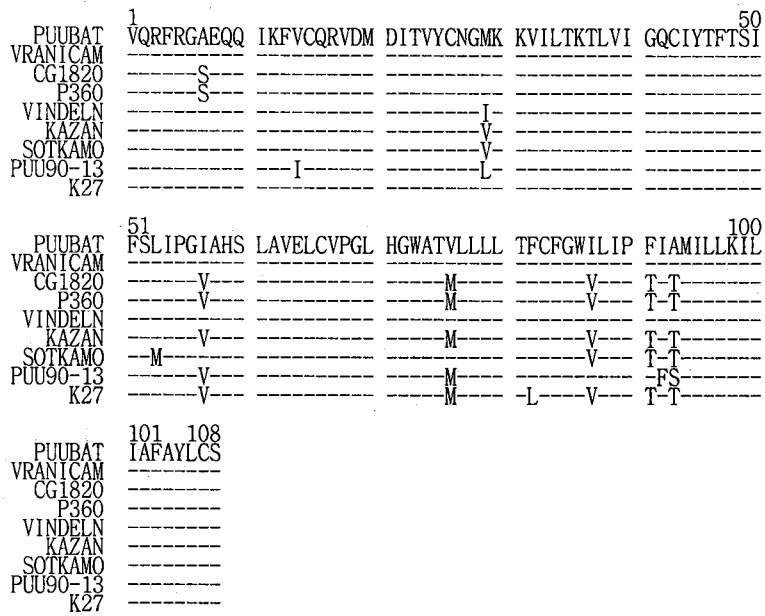

Figure 3. Amino acid sequence comparison between PUUBAT and 8 Puumala virus strains. The amino acid identities were ranged from 94 to 100%. '-' indicates the same amino acid with that of PUUBAT.

Table 2. Peptide sequence identities between our sample (PUUBAT), VRANICAM, CG1820 (Hallnas), P360, Vindeln, Kazan, Sotkamo, PUU90-13, and K27 strains

% amino acid identity

	PUUBAT	VRANICAM	CG1820	P360	VINDELN	KAZAN	SOTKAMO	PUU90-13	K27
PUUBAT	100%	100%	94%	94%	94%	95%	99%	94%	94%
VRANICAM	108	108	94%	94%	94%	95%	99%	94%	94%
CG1820	102	102	108	100%	98%	95%	93%	93%	98%
P360	102	102	108	108	98%	95%	93%	93%	98%
VINDELN	102	102	106	106	108	97%	94%	94%	98%
KAZAN	103	103	103	103	105	108	95%	91%	95%
SOTKAMO	107	107	101	101	102	103	108	94%	93%
PUU90-13	102	102	101	101	102	99	102	108	93%
K27	102	102	106	106	106	103	101	101	108

No of amino acid identity

Sequence comparison of deduced amino acid sequence

The deduced amino acid sequences of PUUBAT were compared with those of 8 Puumala virus strains (Figure 3, Table 2). As it mentioned before, G1 region of M segment was amplified and the 108 amino acid of amplified region was compared. The deduced amino acid sequence identities were ranged from 94% to 100% which were higher than nucleotide sequence homologies. The 108 amino acid sequence of Vranicam was 100% identical to PUUBAT and shows the highest amino

acid identity. This amino acid identity also confirms that PUUBAT is a Puumala virus detected from bat.

DISCUSSION

Rodents were found as the principle natural reservoir of hantavirus as a pathogene of two serious and often fatal human disease: hemorrhagic fever with renal syndrome (HFRS) and human pulmonary syndrome (HPS). HFRS by Hantaan virus are common in Asia, whereas Nephropathia epidemica are common in northern part of Europe. Hantaan, Seoul, and Puumala viruses are known to cause HFRS whose host are striped field mouse (*Apodemus agrarius*), rats (*Rattus norvigicus*) and bank vole (*Clethrionomys glareolus*) respectively. Clinical manifestations of HFRS are characterized by fever, renal failure and in severe cases hemorrhagic manifestations. The clinical manifestations of HFRS are generally more severe for infections caused by Hantaan virus, less severe for Seoul virus, and milder for Puumala virus. Sin Nombre virus, carried by the deer mouse (*Peromyscus maniculatus*) cause HPS in the Americas [21]. HPS are characterized by acute respiratory dysfunction, with mortality 50%.

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. No HPS cases have been reported yet in Korea. Recently, patients with light HFRS carried antibody against Puumala virus or show high antibody titre in Korea, according to unpublished data. This indicates the presence of Puumala virus in Korea. In central and northern Europe, Puumala virus is predominant causing influenza-like symptoms and renal dysfunction. However, the natural host and the infection routes of Puumala virus in Korea have 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 ferrum-equinum* and *Vespertilo abramus*) [17] and wild birds (*Paradoxmis webbiana* and *Emberiza elegans*) [9, 13] in Korea. Hantaan-like viruses were isolated and sequenced from bats, *Rhinolopus ferrum-equinum* and *E. serotinus* [5, 6]. Hantaan virus might be transmitted from *Apodemus* mice to bats by uncertain routes. The natural reservoir of Puumala virus in Europe is the rodents *Clethrionomys glareolus* [16]. Recently, antibodies against Puumala virus were detected in the sera of wild birds, *Paradoxmis webbiana* in Korea [2]. Previously, we reported that bats also carry Puumala virus in Korea for the first time [14].

The sequence analysis of the nested RT-PCR product was performed. As we expected, the 327 bp nested RT-PCR product showed 71～97% nucleotide sequence homology, and 94～100% amino acid sequence identities to Puumala virus strains. These proves that the nested RT-PCR product was amplified from Puumala virus in bats. Therefore, it confirms bat is a novel natural reservoir of Puumala virus. The nucleotide sequence of the 327 bp nested RT-PCR product (PUUBAT) was most close to Puumala virus Vranicam strain isolated from a bank vole in Bosnia-Hercegovina. PUUBAT was most distant from Puumala virus K27 strain isolated from human in Russia. It is hard to say the Puumala virus from bat is most close to Vranicam, because only part of the viral M segment from bat was sequenced. The infection route of Puumala virus to bats or wild birds has not been known yet. It is necessary to investigate the etiological relations among the natural Puumala virus reservoirs and life cycle of Puumala virus in nature.

SUMMARY

The nested RT-PCR product with Puumala

virus specific primer from bats were sequenced. The nucleotide sequence showed sequence homology, 71~97% with 8 Puumala virus strains, indicating that the nested RT-PCR product were amplified from Puumala virus in bats. This confirms our previous nested RT-PCR data with bats, that is bats are a natural reservoir of Puumala virus. The DNA and amino acid sequence comparisons indicate the possibility that Puumala virus from bat is closely related to a Puumala virus strain, Vranicam isolated from a bank vole in Bosnia-Hercegovina. For further study Puumala virus from bat, the virus (PUUBAT) needs to be isolated.

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