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Sequence-Independent, Single-Primer Amplification Next-Generation Sequencing of Hantaan Virus Cell Culture–Based Isolates

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Abstract. Hantaan virus (HTNV), identified in the striped field mouse (*Apodemus agrarius*), belongs to the genus *Hantavirus* of the family *Bunyaviridae* and contains tripartite RNA genomes, small (S), medium (M), and large (L) segments. HTNV is a major causative for hemorrhagic fever with renal syndrome (HFRS) with fatality rates ranging from 1% to 15% in the Republic of Korea (ROK) and China. Defining of HTNV whole-genome sequences and isolation of the infectious particle play a critical role in the characterization and preventive and therapeutic strategies of hantavirus outbreaks. Next-generation sequencing (NGS) provides an advanced tool for massive genomic sequencing of viruses. However, the isolation of viral infectious particles is a huge obstacle to investigate and develop anti-virals for hantaviruses. Here, we report 12 HTNV isolates from lung tissues of the striped field mouse in the highly HFRS-endemic areas. Sequence-independent, single-primer amplification (SISPA) NGS was attempted to recover the genomic sequences of HTNV isolates. The nucleotide sequence of HTNV S, M, and L segments were covered up to 99.4–100%, 97.5–100%, and 95.6–99.8%, respectively, based on the full length of the prototype HTNV 76-118. The whole-genome sequencing of HTNV isolates was accomplished by additional reverse transcription polymerase chain reaction (RT-PCR) and rapid amplification cDNA ends (RACE) PCR. In conclusion, this study will lead to the attempt and usage of SISPA NGS technologies to delineate the whole-genome sequence of hantaviruses, providing a new era of viral genomics for the surveillance, trace, and disease risk management of HFRS incidents.

INTRODUCTION

Hantaviruses, the family *Bunyaviridae*, are an enveloped virus with single-stranded negative-sense RNA genomes consisting of small (S), medium (M), and large (L) segments.[1] The tripartite segments encode a nucleocapsid (N) protein, two envelope glycoproteins (Gn and Gc), and an RNA-dependent RNA polymerase (RdRp). Hantavirus infections pose a public health threat worldwide due to the lack of effective prophylactics and therapeutics.[2,3] The Old World hantaviruses, for example, Hantaan virus (HTNV), Seoul virus, Dobrava-Belgrade virus (DOBV), and Puumala virus (PUUV), cause hemorrhagic fever with renal syndrome (HFRS) in Asia and Europe. In contrast, the New World hantaviruses, for example, Sin Nombre virus (SNV), Andes virus (ANDV), and New York virus (NYV), develop hantavirus pulmonary syndrome (HPS) in the Americas. In particular, HTNV is geographically divergent and transmitted to humans from excreta of persistently infected striped field mice (*Apodemus agrarius*), which serve as a primary reservoir host.[4,5] HFRS is highly endemic in east Asia, with over 20,000 cases per year with mortality rates ranging from 1% to 15%.[6] The isolation and whole-genome sequencing of HTNV are required for the development of anti-viral strategies and the understanding of hantavirology.

The genomic sequencing of viruses plays important roles in discovery of a new virus, detection and tracking of the virus from an outbreak, and development of prophylactic and therapeutic vaccines.[7–10] Usage of molecular genomic amplification, for example, polymerase chain reaction (PCR), is a representative method to acquire viral genome sequences from specimens. However, it is time and effort consuming to obtain the complete genome sequences of ultra-low copy of viral RNA in tissues or clinical samples. Recently, next-generation sequencing (NGS) provides a robust tool for the acquisition of massive genomic information from a variety of specimens.[11,12] Using the NGS technology, the whole-genome sequencing of viruses, such as Schmallenberg virus, Dengue virus, enterovirus, and respiratory syncytial virus, has been completed.[13–15] This approach is cost effective and highly efficient for the recovery of viral genome sequences, taking advantage of the detection and characterization of viral genomes and viral metagenomics.[16,17] Sequence-independent, single-primer amplification (SISPA) is a methodology to synthesize and enrich viral genomes using random hexamer and SISPA sequences (5′-GCCGGAGCTCTGCAGATATC-3′).[18] SISPA NGS was used for defining RNA and DNA viruses including Rotavirus, Astrovirus, and Parvovirus.[19–21]

Here, we attempted to establish SISPA NGS for the rapid recovery of HTNV tripartite genomic sequences identified from highly HFRS-endemic areas. In addition, 12 HTNV strains were isolated using Vero E6 cell culture–based method, and whole-genome sequencing of the HTNV isolates was complete using SISPA NGS, reverse transcription (RT) PCR, and rapid amplification cDNA ends (RACE) PCR. In conclusion, this study will lead to the attempt and usage of SISPA NGS technologies to delineate the whole-genome sequence of hantaviruses, providing significant genomic information for the detection, characterization, and preventive and therapeutic strategies of hantavirus outbreaks.

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MATERIALS AND METHODS

Trapping. Striped field mice, *A. agrarius*, were captured in military training sites in Paju and natural trapping sites in Yeoncheon, Gyeonggi Province in the Republic of Korea (ROK), using Sherman traps (7.7 × 9 × 23 cm; H. B. Sherman Traps, Tallahassee, FL). Sera were collected by cardiac puncture under isoflurane anesthesia. Tissues including lung, liver, kidney, and spleen were collected from euthanized rodents. All samples were provided from the Department of Microbiology, College of Medicine, Korea University. All procedures of the sampling were approved by the Korea University Institutional Animal Care and Use Committee (KUIACUC, no. 2010–212) protocol.

Indirect immunofluorescence assay (IFA) test. Sera, diluted 1:32, were placed on acetone-fixed slide wells of HTNV-infected Vero E6 cells. After incubation at 37°C for 30 minutes, the slide was washed with phosphate-buffered saline (PBS). Fluorescein isothiocyanate (FITC)-labeled anti-mouse IgG (ICN Pharmaceuticals, Laval, Canada) was added to each well and incubated at 37°C for 30 minutes. Following washes, the slide was examined for virus-specific fluorescence using an Axioscope fluorescent microscope (Carl Zeiss AG, Oberkochen, Germany).

RNA extraction and RT-PCR. Total RNA was extracted from lung tissues of *A. agrarius* using a Hybrid R Kit (GeneAll Biotechnology, Seoul, ROK) according to the manufacturer's specifications. cDNA was generated using M-MLV (Promega, Fitchburg, WI) with random hexamer or OSM55 (5′-TAGTAGTAGACTCC-3′).[22] First and nested PCR were performed in 25-μL reaction mixtures containing 200 μM dNTP (Elpis Biotech, Daejeon, ROK), 0.25 U of Super-Therm Taq DNA polymerase (JMR Holdings, London, UK), 1.5 μL cDNA, and 5 pM of each primer. An initial denaturation was performed at 94°C for 4 minutes, followed by six cycles of denaturation at 94°C for 30 seconds, annealing at 37°C for 30 seconds, elongation at 72°C for 1 minute, followed by 32 cycles of denaturation at 94°C for 30 seconds, annealing at 42°C for 30 seconds, elongation at 72°C for 1 minute, and then elongation at 72°C for 5 minutes. DNA sequencing was performed in an automatic sequencer (Model ABI 3730XL DNA Analyzer; Thermo Fisher Scientific, Waltham, MA).

Virus isolation. Attempts to isolate HTNV from the lung tissues of *A. agrarius* were performed in Vero E6 cell culture, using a previously described method.[23] Vero E6 cells cultured in 25-cm^2 flasks were inoculated with 5% (v/w) lung tissue homogenates, prepared in Dulbecco's modified eagle's medium (DMEM) containing 5% fetal bovine serum (FBS). The cells were subcultured at 7- to 14-day intervals. An aliquot of the cells was identified for HTNV antigens by IFA test. Total RNA extracted from the cells or supernatants was examined using HTNV-specific primers by RT-PCR.

SISPA next-generation sequencing. cDNA was generated from total RNA of the cells identified for the isolation of HTNV strains using FR26RV-N (5′-GCCGGAGCTCTGCA GATATCNNNNNN-3′). The reaction was performed in a 20-μL volume containing 7 μL total RNA, 10 pM primer, 100 mM dithiothreitol, 25 mM $MgCl_2$, 10 mM of dNTP, 0.5 μL RNaseOUT, and 0.5 μL 10× Superscript III buffer (Life Technologies, Carlsbad, CA) in a Proplex thermocycler (Life technologies). The PCR program was at 25°C for 10 minutes, 50°C for 50 minutes, and 85°C for 10 minutes. Double-stranded (ds) cDNA was synthesized using 1 μL Klenow 3′ → 5′ exo DNA polymerase (Enzynomics, Daejeon, ROK) and 1 μL RNaseH (Invitrogen, San Diego, CA). The Klenow reaction was incubated at 37°C for 1 hour and 75°C for 15 minutes. The ds cDNA was cleaned using MinElute PCR purification kit (Qiagen, Hilden, Germany). Using the FR20RV (5′-GCCGGAGCTCTGCAGATATC-3′), ds cDNA was amplified in a 50-μL volume containing 10 μL ds cDNA template, 10 pM primer, and 2 × MyTaq Red (Bioline, Taunton, MA). Initial denaturation was at 98°C for 30 seconds, followed by 38 cycles of denaturation at 98°C for 10 seconds, annealing at 54°C for 20 seconds, and elongation at 72°C for 45 seconds.

Libraries were prepared using TruSeq Nano DNA LT sample preparation kit (Illumina, San Diego, CA) according to the manufacturer's instructions. ds cDNA was sheared using an M220 focused ultra-sonicator (Covaris, Woburn, MA). The ds cDNA was size-selected, followed by evaluating quality and concentration of the samples using an Agilent DNA 1000 chip kit or a high-sensitivity DNA chip kit on a bio-analyzer (Agilent Technologies, Santa Clara, CA). The libraries were poly-A tailed, ligated with a single index, and enriched by PCR. The libraries were quantified using the Library Quantification Kit for Illumina sequencing platforms (KAPA Biosystems, Wilmington, MA) and a Quantstudio 6 Flex Real-Time PCR System (Life Technologies). NGS sequencing was performed on a MiSeq benchtop sequencer (Illumina) with 2 × 150 base pairs (bp) using a MiSeq reagent V2 (Illumina).

NGS data analysis. Adaptor and index sequences of reads were trimmed and low-quality sequences were filtered using CLC Genomics Workbench version 7.5.2 (CLC Bio, Cambridge, MA). The tripartite genome sequence of HTNV 76-118 was used in a reference matching method. The read mapping to the reference genome sequence and the extraction of consensus sequences were performed.

Whole-genome sequencing of HTNV. To fill out the gap of HTNV genomic sequences obtained by NGS, conventional nested RT-PCR was performed. Total RNA was extracted from HTNV-isolated cells using a Hybrid R Kit (GeneAll Biotechnology). cDNA was generated using M-MLV (Promega) with random hexamer or OSM55. First and nested RT-PCR were performed using Solg™ 2× h-Taq PCR Smart mix (Solgent, Daejeon, ROK). An initial denaturation was performed at 95°C for 15 minutes, followed by 40 cycles of denaturation at 95°C for 20 seconds, annealing at 50°C for 40 seconds, and elongation at 72°C for 1 minute, and then a cycle of 72°C for 3 minutes. To obtain the genomic sequence of HTNV 3′- and 5′-ends, 3′ -and 5′-end RACE experiments were performed using 3′- and 5′-Full RACE Core Set (Takara, Shiga, Japan), respectively, according to the manufacturer's specifications. The primer sequences for both 3′- and 5′-end RACE experiments were shown in Supplemental Tables 1 and 2, respectively.

Phylogenetic analysis. Using the Bayesian method, the whole-genome sequence of HTNV isolates was aligned and implemented using MrBayes version 3.1 under the best-fit GTR+I+Γ model of evolution selected by hierarchical likelihood-ratio testing in MrModeltest version 2.3 and jModelTest version 0.1.[24–26] To generate phylogenetic trees, Bayesian Metropolis–Hastings Markov Chain Monte Carlo run was replicated twice. Each run consisted of four chains

TABLE 1
Summary of total reads and read mapping to the reference genomes

Hantaan virus strain	Total reads	S segment		M segment		L segment	
		Reads mapped to reference sequence (M14626)	Depth of coverage*	Reads mapped to reference sequence (M14627)	Depth of coverage	Reads mapped to reference sequence (X55901)	Depth of coverage
Aa 10-434	1,668,106	2,086	184.5	2,843	117.9	3,056	70.2
Aa 10-518	1,026,198	803	71.0	1,033	42.9	999	22.9
Aa 13-3	1,153,104	2,037	180.2	2,464	102.2	2,340	53.7
Aa 14-172	1,138,528	688	60.8	3,010	124.9	1,329	30.5
Aa 14-175	1,402,110	1,966	173.9	3,191	132.4	2,430	55.8
Aa 14-184	1,052,612	1,313	116.1	2,415	100.2	1,529	35.1
Aa 14-186	1,041,180	1,006	89.0	1,860	77.2	1,185	27.2
Aa 14-188	1,293,936	1,381	122.1	2,475	102.7	1,221	28.0
Aa 14-204	198,154	584	51.7	935	38.8	929	21.3
Aa 14-207	927,798	856	75.7	1,226	50.9	1,081	24.8
Aa 14-234	907,142	946	83.7	2,193	91.0	1,110	25.5
Aa 14-239	1,355,584	1,230	108.8	6,555	271.9	2,091	48.0

*Depth of coverage was calculated by the number of mapped reads (read length × number of reads matching to the reference/reference genome size).

of 10 million generations sampled every 100 generations with a burn-in of 5,000 (5%), resulting in 30,000 trees overall. The S, M, and L segments were individually treated in phylogenetic analyses. Topologies were evaluated by bootstrap analysis of 1,000 iterations, and posterior node probabilities were based on 2 million generations and evaluated sample sizes of over 100 (implemented in MrBayes).

Ethical approval. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

RESULTS AND DISCUSSION

The genomic sequencing and isolation of viruses enable not only the identification of emerging infectious diseases but also the development of prophylactic and therapeutic agents.[27] Recently, the usage of NGS has tremendously increased in defining viral genomic sequences, outbreaks, and effective therapeutics.[10] The viral genomic recovery of hantavirus-infected hosts or patients is critical for the characterization of an endemic outbreak and disease risk mitigation, given that hantavirus infections cause serious diseases, such as HFRS and HPS. This report presents the implementation of SISPA NGS for sequencing and the genomic characterization HTNV isolates from highly HFRS-endemic areas.

Of 884 *A. agrarius* captured in Gyeonggi province of the ROK in 2010 and 2013–2014, 154 (17.4%) rodents were seropositive for anti-HTNV IgG. Among 884 rodents including seropositive and seronegative samples, 137 (15.5%) harbored a partial M segment of HTNV. Vero E6 cell culture–based isolation of HTNV attempted using lung lysates from both IFA- and RT-PCR-positive rodents in the area, for example, Paju and Yeoncheon in Gyeonggi Province. A total of 12 HTNV strains succeeded to be isolated in

TABLE 2
Similarity (%) of nucleotide and amino acid sequences of HTNV 76-118 to 12 HTNV isolates and representative other rodent-borne hantaviruses

Virus	Strain	S segment		M segment		L segment	
		1,287 nt*	429 aa	3,405 nt*	1,135 aa	6,453 nt*	2,151 aa
HTNV	Aa 10-434	95.3	98.8	94.3	98.4	94.8	99.2
HTNV	Aa 10-518	95.5	99.1	94.4	98.4	94.9	99.3
HTNV	Aa 13-3	95.4	99.1	94.4	98.4	95.0	99.2
HTNV	Aa 14-172	95.3	98.8	94.1	98.3	95,2	99.3
HTNV	Aa 14-175	95.2	99.1	94.6	98.7	95.3	99.3
HTNV	Aa 14-184	95.2	99.1	94.4	98.5	95.4	99.3
HTNV	Aa 14-186	95.1	99.1	94.2	98.4	95.2	99.4
HTNV	Aa 14-188	95.2	99.1	94.2	98.4	95.1	99.3
HTNV	Aa 14-204	94.8	98.8	94.6	98.7	94.6	99.0
HTNV	Aa 14-207	94.9	98.6	94.6	98.7	94.7	98.8
HTNV	Aa 14-234	95.3	99.1	94.7	98.4	94.9	99.1
HTNV	Aa 14-239	95.2	96.5	94.9	98.6	94.8	99.3
SEOV	80-39	74.6	83.0	72.3	77.2	74.4	85.0
SOOV	SOO-1	83.1	96.5	80.7	91.2	81.4	95.6
DOBV	Greece	74.2	83.0	71.7	77.4	74.8	85.2
SNV	NMH10	62.6	62.9	57.6	54.5	65.8	69.3
ANDV	Chile9717869	63.1	65.0	58.0	54,7	66.0	68.7
PUUV	Sotkamo	61.3	61.1	57.7	53.2	65.9	69.1
TULV	M5302v	63.4	63.4	58.9	55.1	65.5	68.5
PHV	PH-1	62.9	62.5	57.5	54.0	64.9	68.1

aa = amino acids; ANDV = Andes virus; DOBV = Dobrava-Belgrade virus; HTNV = Hantaan virus; nt = nucleotides; PHV = Prospect Hill virus; PUUV = Puumala virus; SEOV = Seoul virus; SNV = Sin Nombre virus; SOOV = Soochong virus; TULV = Tula virus.
*The number of nucleotides indicates the length of open reading frames.

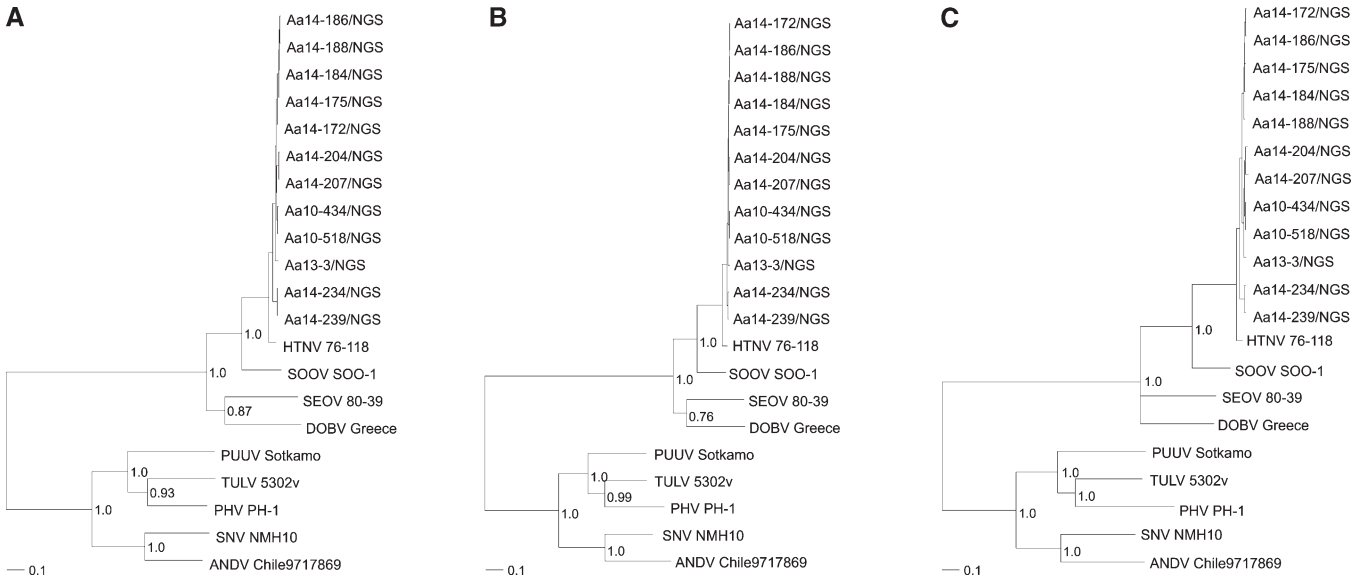

FIGURE 1. Phylogenetic analysis of Hantaan virus (HTNV) tripartite genomes acquired by sequence-independent, single-primer amplification next-generation sequencing (SISPA NGS). Phylogenetic trees generated by maximum likelihood and Bayesian methods using the GTR+I+Γ model of evolution and based on the alignment of the (**A**) S, (**B**) M, and (**C**) L segment sequences of HTNV strains. Since tree topologies were very similar using RAxML and MrBayes, the trees generated by MrBayes are displayed. The phylogenetic positions of HTNV isolates (Aa 10-434/NGS, S: KU207167, M: KU207160, L: KU207153; Aa 10-518/NGS, S: KU207168, M: KU207161, L: KU207154; Aa 13-3/NGS, S: KX687232, M: KX687227, L: KX687237; Aa 14-172/NGS, S: KU207169, M: KU207162, L: KU207155; Aa 14-175/NGS, S: KU207170, M: KU207163, L: KU207156; Aa 14-184/NGS, S: KU207171, M: KU207164, L: KU207157; Aa 14-186/NGS, S: KX687233, M: KX687228, L: KX687238; Aa 14-188/NGS, S: KU207172, M: KU207165, L: KU207158; Aa 14-204/NGS, S: KU207173, M: KU207166, L: KU207159; Aa 14-207/NGS, S: KX687234, M: KX687229, L: KX687239; Aa 14-234/NGS, S: KX687235, M: KX687230, L: KX687240; Aa 14-239/NGS, S: KX687236, M: KX687231, L: KX687241) are shown in relationship to the prototype HTNV (HTNV 76-118, S: M14626, M: M14627, L: X55901) and other rodent-borne hantaviruses, including Soochong virus (SOOV SOO-1, S: AY675349, M: AY675353, L: DQ056292), Seoul virus (SEOV 80-39, S: NC_005236, M: 005237, L: 005238), Dobrava (DOBV Greece, S: NC_05233, M: NC_005234, L: NC_005235), Andes virus (ANDV Chile9717869, S: NC_003466, M: NC_003467, L: 003468), Sin Nombre virus (SNV NMH10, S: NC_005216, M: NC_005215, L: 005217), Puumala virus (PUUV Sotkamo, S: NC_005224, M: NC_005223, L: NC_005225), Tula virus (TULV M5302v, S: NC_005227, M: NC_005228, L: NC_005226), and Prospect Hill virus (PHV PH-1, S: Z49098, M: X55129, L: EF646763). The numbers at each node are posterior-node probabilities based on 150,000 trees. The scale bar indicates nucleotide substitutions per site.

the cell culture–based method. The isolation of HTNV was confirmed by detecting viral antigen and RNA from 38 to 94 days after inoculation. Consistent with other rodent-borne hantaviruses, HTNV isolates did not show cytopathic effect in Vero E6 cells.

To perform genomic sequencing of HTNV using SISPA NGS, total RNA was extracted from the cell infected with HTNV isolates, followed by preparing paired-end libraries for Illumina MiSeq. Genomic sequencing of the HTNV tripartite RNA generated 198,154–1,668,106 reads with an average read length of 150 bp. The number of virus reads mapped to a reference sequence, the prototype HTNV strain 76-118, revealed 584–2,086 to the S segment, 935–6,555 to the M segment, and 929–3,056 to the L segment (Table 1). The appearance of the HTNV genomic sequencing demonstrated that 10 out of 12 HTNV S segments were completely recovered and the coverage rates were 99.4% up to 100%, based on the full length of S segment. Among 12 HTNV isolates, six of HTNV M segments were completely sequenced, and the whole length of M segment was nearly obtained from half of them, ranging from 97.5% to 100%. Any of HTNV isolates was not complete for the full length of L segment, but the L segment of HTNV was obtained with the coverage rates ranging from 95.6% to 99.8%. The longest genome of HTNV L segment showed lower coverage depth of viral reads than the S and M segments. The region of the L segment sequence, coordinated 1,500–2,100 nucleotides (nt), was not specifically covered due to the low quality and quantity of virus reads mapping to HTNV 76-118. The gap of genomic sequences of 12 HTNV isolates was filled out by performing Sanger sequencing. In addition, the tripartite genomic sequences of 3′- and 5′-ends were determined by 3′- and 5′-end RACE PCR experiments.

Among 12 HTNV isolates, the characterization of HTNV tripartite genomes, for example, Aa 14-172, demonstrated that the full-length S segment contained 1,696 nt, encoded as an N protein (429 amino acids [aa]). The complete M segment consisted of 3,616 nt, encoding two glycoproteins of 1,135 aa. The conserved WAASA amino acid motif of the M segment was found (coordinated to 644–648 aa). The entire length of Aa 14-172 L segment was defined as 6,530 nt, and the L segment encoded an RdRp of 2,151 aa. Based on 3′- and 5′-end RACE PCR, the termini of HTNV tripartite RNA were determined as 3′-AUCAUCAUCUGAGG-5′ and 5′-UAGUAGUAUGCUCC-3′, respectively. Compared with the 5′-end genomic sequence of HTNV 76-118, the termini of S, M, and L segments had incomplete complementarity, containing a mismatch at ninth and a noncanonical U-G pair at 10th nucleotides as previously described.[28] In the M segment, 12th nucleotide was defined as U instead of A from HTNV 76-118. The length at the 5'-end of Aa 14-172 L segment was 6,530 nt, which was three nucleotides shorter than that of HTNV 76-118. The discrepancy of L segments was also observed from Puumala virus, Sotkamo strain,

demonstrating that the "UAG" at the 5′-end was absent.[29] The termini of 3′- and 5′-ends of HTNV isolates were equivalent to those of HTNV strains directly identified in the lung tissues, suggesting no alteration of the end sequences occurred during cell culture–based passages.[30] The genomic configuration of each segment of other HTNV isolates was identical to Aa 14-172. Pairwise alignment and comparison of the full-length S, M, and L segments of HTNV isolates to HTNV 76-118 revealed high sequence similarities ranging from 94.0% to 95.5% and 98.2% to 99.4% at the nucleotide and amino acid levels, respectively (Table 2). Using maximum likelihood and Bayesian methods with the GTR+I +Γ model of evolution, phylogenetic trees of the tripartite genomes of 12 HTNV isolates attained by SISPA NGS and Sanger sequencing showed well-supported clades with the prototype HTNV 76-118 (Figure 1 A–C).

Here, SISPA NGS was applied to obtain the genome sequences of HTNV, and the genomic sequences were validated by forming phylogenetic clusters with HTNV 76-118, a representative rodent-borne hantavirus. This report provides a potential possibility that SISPA NGS will be a robust and effective approach to recover and characterize whole-genome sequences of hantavirus tripartite genomes from not only natural reservoirs but also HFRS or HPS clinical samples.

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REFERENCES

1. Elliott RM, 1990. Molecular biology of the Bunyaviridae. *J Gen Virol 71:* 501–522.
2. Bi Z, Formenty PB, Roth CE, 2008. Hantavirus infection: a review and global update. *J Infect Dev Ctries 2:* 3–23.
3. Schmaljohn C, Hjelle B, 1997. Hantaviruses: a global disease problem. *Emerg Infect Dis 3:* 95–104.
4. Lee HW, Lee PW, Johnson KM, 2004. Isolation of the etiologic agent of Korean hemorrhagic fever. 1978. *J Infect Dis 190:* 1711–1721.
5. Song JW, Baek LJ, Kim SH, Kho EY, Kim JH, Yanagihara R, Song KJ, 2000. Genetic diversity of *Apodemus agrarius*-borne hantaan virus in Korea. *Virus Genes 21:* 227–232.
6. Zhang WY, Yin WW, Hu WB, Magalhaes RJS, Ding F, Sun HL, Zhou H, Li SL, Haque U, Tong SL, Glass GE, Bi P, Clements ACA, Liu QY, Li CY, 2014. Spatiotemporal transmission dynamics of hemorrhagic fever with renal syndrome in China, 2005–2012. *PLoS Negl Trop Dis 8:* e3344.
7. Ladner JT, Wiley MR, Mate S, Dudas G, Prieto K, Lovett S, Nagle ER, Beitzel B, Gilbert ML, Fakoli L, Diclaro JW 2nd, Schoepp RJ, Fair J, Kuhn JH, Hensley LE, Park DJ, Sabeti PC, Rambaut A, Sanchez-Lockhart M, Bolay FK, Kugelman JR, Palacios G, 2015. Evolution and spread of Ebola virus in Liberia, 2014–2015. *Cell Host Microbe 18:* 659–669.
8. Neverov A, Chumakov K, 2010. Massively parallel sequencing for monitoring genetic consistency and quality control of live viral vaccines. *Proc Natl Acad Sci USA 107:* 20063–20068.
9. Prachi P, Donati C, Masciopinto F, Rappuoli R, Bagnoli F, 2013. Deep sequencing in pre- and clinical vaccine research. *Public Health Genomics 16:* 62–68.
10. Quinones-Mateu ME, Avila S, Reyes-Teran G, Martinez MA, 2014. Deep sequencing: becoming a critical tool in clinical virology. *J Clin Virol 61:* 9–19.
11. Barzon L, Lavezzo E, Costanzi G, Franchin E, Toppo S, Palù G, 2013. Next-generation sequencing technologies in diagnostic virology. *J Clin Virol 58:* 346–350.
12. Kim WK, Kim JA, Song DH, Lee D, Kim YC, Lee SY, Lee SH, No JS, Kim JH, Kho JH, Gu SH, Jeong ST, Wiley M, Kim HC, Klein TA, Palacios G, Song JW, 2016. Phylogeographic analysis of hemorrhagic fever with renal syndrome patients using multiplex PCR-based next generation sequencing. *Sci Rep 6:* 26017.
13. Rosseel T, Scheuch M, Höper D, De Regge N, Caij AB, Vandenbussche F, Van Borm S, 2012. DNase SISPA-next generation sequencing confirms Schmallenberg virus in Belgian field samples and identifies genetic variation in Europe. *PLoS One 7:* e41967.
14. Thorburn F, Scheuch M, Höper D, De Regge N, Caij AB, Vandenbussche F, Van Borm S, 2015. The use of next generation sequencing in the diagnosis and typing of respiratory infections. *J Clin Virol 69:* 96–100.
15. Prachayangprecha S, Schapendonk CM, Koopmans MP, Osterhaus AD, Schürch AC, Pas SD, van der Eijk AA, Poovorawan Y, Haagmans BL, Smits SL, 2014. Exploring the potential of next-generation sequencing in detection of respiratory viruses. *J Clin Microbiol 52:* 3722–3730.
16. Marston DA, McElhinney LM, Ellis RJ, Horton DL, Wise EL, Leech SL, David D, de Lamballerie X, Fooks AR, 2013. Next generation sequencing of viral RNA genomes. *BMC Genomics 14:* 444.
17. Barzon L, Lavezzo E, Militello V, Toppo S, Palu G, 2011. Applications of next-generation sequencing technologies to diagnostic virology. *Int J Mol Sci 12:* 7861–7884.
18. Djikeng A, Halpin R, Kuzmickas R, Depasse J, Feldblyum J, Sengamalay N, Afonso C, Zhang X, Anderson NG, Ghedin E, Spiro DJ, 2008. Viral genome sequencing by random priming methods. *BMC Genomics 9:* 5.
19. Lambden PR, Cooke SJ, Caul EO, Clarke IN, 1992. Cloning of noncultivatable human rotavirus by single primer amplification. *J Virol 66:* 1817–1822.
20. Matsui SM, Kim JP, Greenberg HB, Young LM, Smith LS, Lewis TL, Herrmann JE, Blacklow NR, Dupuis K, Reyes GR, 1993. Cloning and characterization of human astrovirus immunoreactive epitopes. *J Virol 67:* 1712–1715.
21. Jones MS, Kapoor A, Lukashov VV, Simmonds P, Hecht F, Delwart E, 2005. New DNA viruses identified in patients with acute viral infection syndrome. *J Virol 79:* 8230–8236.
22. Song JW, Gu SH, Bennett SN, Arai S, Puorger M, Hilbe M, Yanagihara R, 2007. Seewis virus, a genetically distinct hantavirus in the Eurasian common shrew (*Sorex araneus*). *Virol J 4:* 114.
23. Yanagihara R, Goldgaber D, Lee PW, Amyx HL, Gajdusek DC, Gibbs CJ Jr, Svedmyr A, 1984. Propagation of nephropathia epidemica virus in cell culture. *Lancet 1:* 1013.
24. Ronquist F, Huelsenbeck JP, 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics 19:* 1572–1574.
25. Posada D, Crandall KA, 1998. MODELTEST: testing the model of DNA substitution. *Bioinformatics 14:* 817–818.
26. Posada D, 2008. jModelTest: phylogenetic model averaging. *Mol Biol Evol 25:* 1253–1256.
27. Radford AD, Chapman D, Dixon L, Chantrey J, Darby AC, Hall N, 2012. Application of next-generation sequencing technologies in virology. *J Gen Virol 93:* 1853–1868.

28. Plyusnin A, Vapalahti O, Vaheri A, 1996. Hantaviruses: genome structure, expression and evolution. *J Gen Virol 77:* 2677–2687.
29. Piiparinen H, Vapalahti O, Plyusnin A, Vaheri A, Lankinen H, 1997. Sequence analysis of the Puumala hantavirus Sotkamo strain L segment. *Virus Res 51:* 1–7.
30. Kim JA, Kim WK, No JS, Lee SH, Lee SY, Kim JH, Kho JH, Lee D, Song DH, Gu SH, Jeong ST, Park MS, Kim HC, Klein TA, Song JW, 2016. Genetic diversity and reassortment of Hantaan virus tripartite RNA genomes in nature, the Republic of Korea. *PLoS Negl Trop Dis 10:* e0004650.