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ANNOTATED SEQUENCE RECORD

Characterization of the L genome segment of an orthohantavirus isolated from *Niviventer confucianus*

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Abstract

We sequenced and analyzed the L segment of the RNA genome of Hantaan virus (HTNV) strain NC167. This segment is 6,533 nucleotides in length and contains a single open reading frame (ORF) of 6,456 nucleotides in the antigenome sense that encodes the viral RNA-dependent RNA polymerase, which is 2,153 amino acids long with a predicted molecular mass of 246 kDa. The 5′ terminus of the viral RNA was found to contain the expected sequences that are conserved in orthohantaviruses. According to the phylogenetics and levels of sequence similarity, the L segment of HTNV NC167 is similar to but clearly distinct from the L segments of other orthohantaviruses.

Members of the genus *Orthohantavirus* cause two severe clinical syndromes: hemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome (HCPS) [1]. In contrast to viruses in other genera of the order *Bunyavirales*, orthohantaviruses are not transmitted by arthropods [2]. Orthohantaviruses are members of the family *Hantaviridae*, order *Bunyavirales* [3]. Four members of the genus Orthohantavirus, Hantaan virus (HTNV), Seoul virus (SEOV), Dobrava-Belgrade virus (DOBV), and Puumala virus (PUUV), are known to cause HFRS [2]. Sin Nombre virus (SNV) and Andes virus (ANDV) are the most common causes of HCPS in North and South America [4]. Orthohantaviruses establish a chronic infection that causes no apparent harm to their natural hosts, which are rodents of the family Muridae for HTNV, SEOV and DOBV, the family Arvicolinae for PUUV, and the subfamily Sigmodontinae for SNV and ANDV [5–7].

Orthohantaviruses possess a negative-sense RNA genome consisting of three segments: the large (L), medium (M), and small (S) segments. The L segment encode an RNA-dependent RNA polymerase (RdRp), which functions in replication and transcription of the genomic RNA segments. The M segment encode a glycoprotein precursor, which is cleaved to form the viral envelope glycoproteins (Gn and Gc), and the S segment codes for a nucleocapsid protein (NP) [8]. The 5′ and 3′ termini of orthohantavirus genome segments are highly conserved and complementary to each other [9]. The termini of RNA genomes of viruses of the order *Bunyaviridae* are exactly complementary for 8 or 9 nucleotides (nt). The L segments are of similar size among orthohantaviruses, varying from 6,530-6,562 nt. The RdRp is the RNA transcriptase and replicase, which transcribes mRNAs and replicates the genomic RNA using antigenomic RNA as an intermediate [10].

HTNV was first reported in Korea, and it was carried by *Apodemus agrarius* [11]. In a preliminary study in China, Chinese epidemiologists and virologists [12] reported the capture of antibody-positive Chinese white-bellied rats (*Niviventer confucianus*) in Shaanxi and Gansu provinces, and HTNV strain NC167 was isolated from *N. confucianus* caught in the mountainous region of Anhui Province.

In the present study, we report the detailed genomic and phylogenetic characterization of the full-length L segment sequence of HTNV-strain NC167 and compare the sequence of this strain to some other members of genus *Orthohantavirus*.

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Table 1 Primers used in the study

Name	Sequence (5′→3′)	Location
PL1	5'-TAGTAGTAGACTCCCTAAATAACAA-3'	1-25
PL2	5'-ATAAACTCCACTTGCACCAAC-3'	2120-2140
PL3	5'-ATAATATTAAGAGCCTGTTAGTTGC-3'	2022-2046
PL4	5'-CCTGAAAGTAACCCATTCTCCATCC-3'	4294-4318
PL5	5'-ATTCCGAAGGGCAGAGACAATCTT C-3'	4151-4175
L5470R	5'-AGGACACAGTTCTTATAAACA-3'	5470-5490
L5291F	5'-ATATTAGAAACATCGATTACACAAA-3'	5291-5315
PL6	5'-TAGTAGTAGTATGCTCCGGAAAATG-3'	6509-6533

We isolated HTNV-strain NC167 from lung tissues of a hantavirus-positive rat of the species *N. confucianus* (as determined by RT-PCR). Total RNA was extracted from the tissue using a QIAamp Viral Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's recommended procedures. cDNA was prepared using a SuperScript III First-Strand Synthesis System (Invitrogen) and random hexamer primers and/or an oligonucleotide primer (P14: 5'-TAG TAGTAGACTCC-3') [13] that was designed to include the conserved sequence at the 5' end of the S, M and L segments of hantaviruses. Primers for PCR were initially designed based on known L segment sequences of other hantaviruses from the GenBank database (Table 1). PCR amplifications were performed using L-specific primers in four overlapping parts, nt 1-2140, nt 2022-4318, nt 4151-5490, and nt 5291-6533, and a Platinum® Taq DNA Polymerase High Fidelity Kit (Invitrogen). To obtain the terminal sequence of the L segment, an RNA ligation reaction was performed. Briefly, purified DNA fragments were cloned into pGEM-T Easy Vector (Promega) using a TA Cloning Kit (Invitrogen). The terminal sequences of the L segment were cloned in two parts, which were sequenced from at least three clones for each part. Other virus nucleotide sequences were determined by direct sequencing of the PCR products to avoid potential sequence changes associated with isolation procedures. DNA sequencing was performed using an ABI Prism Dye Terminator Sequencing Kit and an ABI 373A Genetic Analyzer. All amplicons were sequenced twice on each strand.

Sequences were assembled using the DNAStar software package. Phylogenetic analysis was done in MEGA X [14], using the neighbor-joining (NJ) method with 1,000 replicates. Nucleotide phylogenetic analysis was also done using the maximum composite likelihood method [15], and amino acid sequence analysis was done using the Poisson correction method [16], both of which are included in MEGA X, using 1000 bootstrap replicates [17]. The neighbor-joining phylogenetic trees were obtained based on alignments of the nucleotide sequences and amino acid sequences of the corresponding sequences from different members of genus *Orthohantavirus* (the viruses and their GenBank accession numbers are listed in Fig. 1). Puumala virus CG1802 was included as an outgroup.

The large (L) genome segment of the hantavirus isolate NC167 was sequenced, and the sequence has been deposited in the GenBank database and assigned the accession number DQ989237. The L segment of NC167 is 6,533 nucleotides in length. This is comparable with other members of the genus *Orthohantavirus.* The nucleotide sequence identity values for the L segments of NC167 and HTNV, SEOV, and other selected strains are 78.2%-77.7%, 74.4%-74.1%, and 66.1%-65.8%, respectively. The sequence identity values for the encoded amino acid sequences of NC167, HTN viruses, and others were 92.2%-90.5%, and 85.8%~-68.8%, respectively. In terms of nucleotide and amino acid sequence similarity, the complete L segment of NC167 was most similar to that of HTNV. Although they were highly similar, distinct differences were found among the strains. The results confirmed that the NC167 L segment is most closely related to those of the HTNV isolates.

In a phylogenetic analysis of the L segment nucleotide and amino acid sequences of orthohantaviruses, NC167 virus showed the same relative clustering to the HTNV isolates for all sequences. The results showed that NC167 represents a distinct phylogenetic lineage that is closely related to other HTNV isolates (Fig. 1).

Based on the results of multiple sequence alignments, pairwise comparison and phylogenetic analysis, the L segment of NC167 is similar to but clearly distinct from those of other HTNV isolates. A previous report in which the entire S and M genome segment sequences were compared and used for phylogenetic analysis showed that NC167 is closely related to some HTNV isolates and distinct from the rest of the HTNV isolates [18]. Our studies confirm those earlier findings based on a phylogenetic analysis of the complete sequence of the L segment.

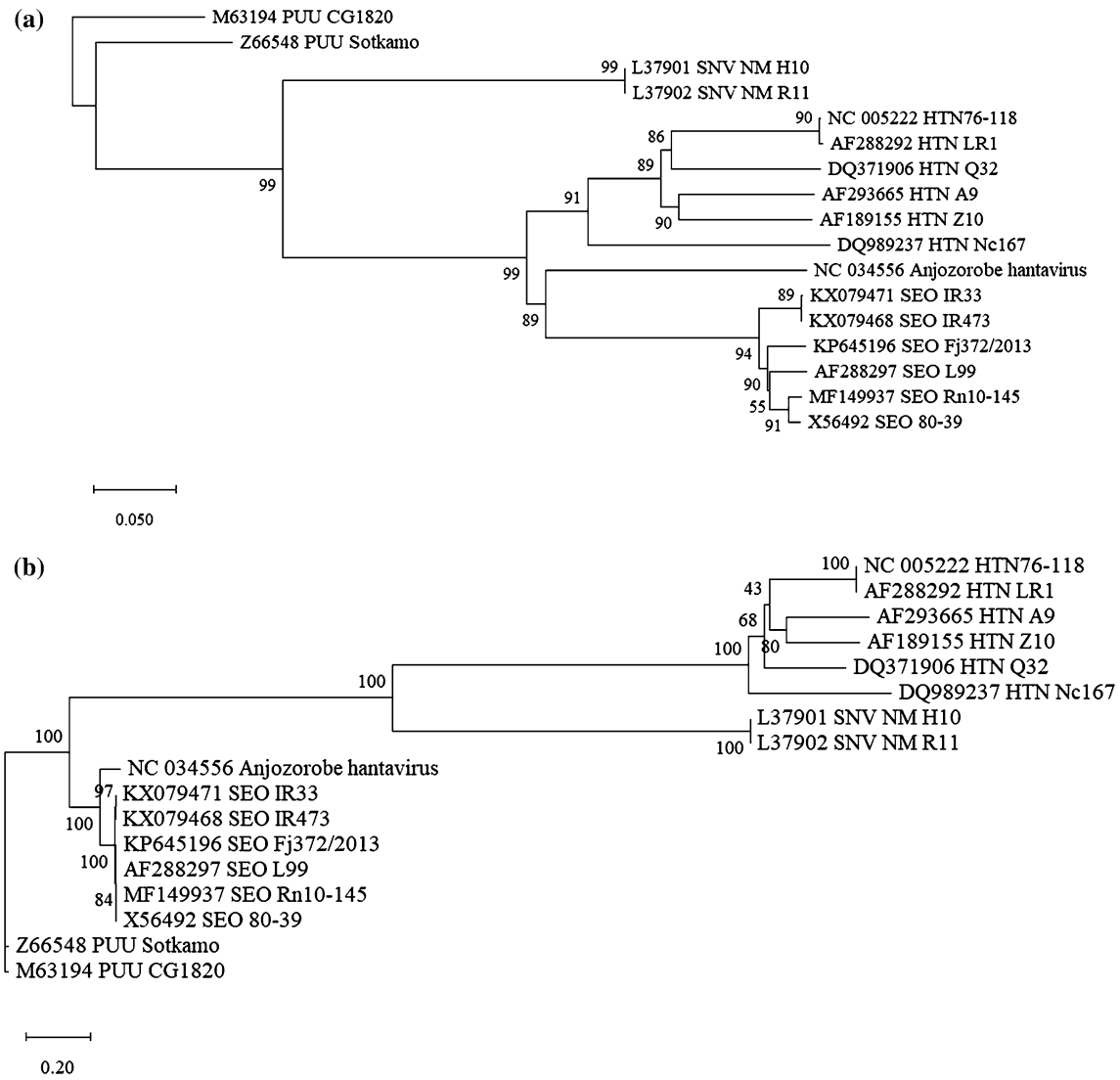

Fig. 1 Phylogenetic analysis based on the complete L segment sequence of NC67 and other published L segment sequences. Sequences of the following viruses were included in the analysis: Hantaan virus 76-118 (NC_005222 HTN76-118), Anjozorobe hantavirus (NC_034556 Anjozorobe hantavirus strain Anjozorobe/Em/MDG/2009/ATD49), hantavirus A9 (AF293665 HTNA9), hantavirus LR1 (AF288292 HTNLR1), hantavirus Z10 (AF189155 HTNZ10), Hantaan virus isolate Nc167 (DQ989237 HTN Nc167), Hantaan virus Q32 (DQ371906 HTNQ32), Seoul hantavirus isolate IR33 (KX079471SEO IR33), Seoul hantavirus isolate IR473 (KX079468 SEO IR473), hantavirus L99 (AF288297 SEOL99), Seoul orthohantavirus strain Rn10-145 (MF149937 SEO Rn10-145), Seoul virus 80-39 (X56492 SEO 80-39), Seoul virus isolate Fj372/2013(KP645196 SEOFj372/2013), Puumala virus CG1802(M63194 PUUCG1802), Sin Nombre virus NMH10 (L37901SNVNMH10), Puumala orthohantavirus Sotkamo (Z66548 PUUSotkamo), and Sin Nombre virus NMR11n (L37902 SNVNMR11). Neighbor-joining trees were constructed based on the L segment nucleotide sequences (A) and amino acid sequences (B). The scale bar represents changes per site

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Compliance with ethical standards

Conflict of interest We declare that we have no competing interests that might have influenced the performance or presentation of the work described in this report.

Ethical approval This study was performed in accordance with the Declaration of Helsinki and was approved by the Research Ethics Committee of Xi'an Jiaotong University, China.

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