# Geographical clustering of hantavirus isolates from Apodemus agrarius identified in the Republic of Korea indicate the emergence of a new hantavirus genotype

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**Research**

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## Abstract

Background Several studies on hantavirus evolution have shown that genetic reassortment plays an important role in the evolution and epidemiology of this disease. Hantaan virus, a prototype hantavirus carried by Apodemus agrarius, is found throughout China, Russia, and Korea. The aim of this study was to investigate the distribution of hantaviruses in rodents in the Republic of Korea (ROK) and perform phylogenetic comparisons using the geographical distribution of their natural reservoir rodent hosts as a point of reference.

Methodology To understand the genetic epidemiology of human pathogenic hantaviruses, we examined viral isolates from rodent reservoirs, captured at three different locations in the ROK, between 2017 and 2018. Each sample collected was subjected to reverse-transcription nested polymerase chain reaction (RT-N-PCR) targeting the L- and S-segments of the hantavirus genome. Positive isolates from Gwangju, Boseong-gun (Jeollanam-do Province), and Jeju Island were confirmed as Hantaan virus using DNA sequencing.

Results Phylogenetic analysis showed that all isolates grouped together as Hantaan virus. The isolates from Jeju, Boseong-gun, and Gwangju tended to cluster together, but with each region forming a distinct cluster. In addition, these three clusters were distinct from other Hantaan isolates reported in previous studies from Korea and its neighboring countries China and Russia. This suggests the emergence of a new hantavirus genotype in southwestern ROK.

Conclusion Hantaan viruses exhibit a considerable degree of geographical clustering, and there may be a novel Hantaan genotype in southwestern ROK. This study helps expand our knowledge regarding the emergence of new hantavirus strains and their degree of geographical variation.


## Background

Hantaviruses, members of the *Hantaviridae* family, are enveloped, tri-segmented, negative-stranded RNA viruses. The viral genome is made up of the large (L) segment, encoding a viral RNA-dependent RNA polymerase; the medium (M) segment, encoding a glycoprotein precursor that is cleaved into envelope glycoproteins Gc and Gn; and the small (S) segment, encoding a nucleocapsid protein (N). This virus establishes a persistent but asymptomatic infection in its primary rodent host [1]. In contrast, it is an etiological agent for two fatal human diseases: hemorrhagic fever with renal syndrome (HFRS), also known as Korean hemorrhagic fever, and hantavirus cardiopulmonary syndrome (HCPS) [2]. Viral transmission to humans occurs via the inhalation of aerosolized rodent urine, saliva, and feces and rarely via rodent bites [3]. Hantaan virus is the primary etiologic agent of HFRS in the Republic of Korea (ROK) and infects around 70% of HFRS patients [4]. It was first detected in striped field mouse (*Apodemus agrarius*) lung tissues in 1976 in Songnae-ri, Gyeonggi Province, ROK. A significant increase in the number of human HFRS infections has been observed in recent years with the number of reported cases steadily increasing from 344 in 2014 to 531 in 2017. Infections were not geographically isolated, and cases were reported from across the country to the Korean Centers for Disease Control and Prevention (KCDC) [5].

Antigenically and genetically distinct hantaviruses, including Soochong virus (SOO) from *A. peninsulae* and Muju virus (MUJV) from *Myodes regulus,* have been isolated in the ROK [4, 6]. Hantaviruses co-evolve and co-speciate within specific rodent species and a particular hantavirus is transmitted by only one or a few closely related rodent/insectivorous species [1, 7]. Studies have shown that reassortment plays an important role in the evolution, pathogenesis, and epidemiology of many segmented viruses, including hantaviruses. Genetic reassortment between closely related Sin Nombre virus (SNV) strains within local rodent populations has been described in North America [8]. Further, reassortment can occur between genetically distant hantaviruses when they infect the same rodent host or the same cell; this phenomenon has been described for reassortments between SNV and Andes viruses (ANDV) [9], Hantaan virus and Seoul orthohantavirus (SEOV) [10], and even in the more distant hantaviruses like Hantaan virus and Prospect Hill virus (PHV) [11].

The aim of this study was to investigate the distribution of hantaviruses in rodents in the ROK and describe the phylogenetic differences between these viruses. Here, we focused on regional differences in viruses isolated from wild rodents that act as the natural reservoir and carrier of this pathogenic virus. This study will help us understand the ecology of hantaviruses and their

interactions with their natural hosts, which is vital for preventing and controlling future viral outbreaks. In addition, the observation of RNA virus evolutionary patterns could help us to predict the emergence of pathogenic variant strains that may pose a risk to human health.

# Materials And Methods

### Sample collection and viral RNA extraction

Rodents were captured at three sylvatic habitats, Gwangju, Boseong-gun (Jeollanam-do Province), and Jeju Island, situated in the south and southwest of the ROK. Rodents were captured between September and November (2017) in Gwangju, October and November (2017) in Boseong-gun, and April to November (2018) in Jeju. Wild rodents were trapped using Sherman live traps baited with peanut butter-covered biscuits at five different sites including a rice paddy, field near a tomb, hill, reservoir, and area near a dyke (Table 1). The captured rodents were identified at the species level and numbered consecutively. All rodents were euthanized, and the blood, spleen, kidney, and lungs were harvested and stored at -80°C until further experiments were performed. Mice organ samples were homogenized with DPBS (150 µL) by grinding with a sterile 70-µm cell strainer. Viral RNA was extracted from the samples and homogenized using a Viral Gene Spin$^{TM}$ Viral RNA Extraction Kit (iNTRON Biotechnology, Korea) according to the manufacturer's instructions.

### PCR amplification

For the reverse-transcription nested polymerase chain reaction (RT-N-PCR) targeting L- and S-segments of the Hantavirus, we used SuperScript VILO MasterMix (Invitrogen, CA, USA) to synthesize complementary DNA (cDNA). AmpliTaq Gold 360 Master Mix (Applied Biosystems, Foster City, CA, USA) and the Veriti™ 96-well thermal cycler (Applied Biosystems, Foster City, CA, USA) were used to perform N-PCR using the Hantavirus-specific primers targeting L- [12] and S-segments of the virus. N-PCR was performed using the same reaction solution as the initial PCR, with the initial PCR product used as the template. With each run of PCR, a positive and a negative control (molecular grade water) were included. Hantaan virus 76-118 cDNA served as the positive control. To reduce PCR contamination risk, RNA extraction and the PCR mix were prepared in separate rooms, and the post-PCR area was physically separated from the amplification area. The primers, PCR conditions, and the PCR-amplified product sizes are listed in Table 2. PCR products were evaluated by gel electrophoreses on a 1.5% agarose gel visualized by ethidium bromide.

### Nucleotide sequencing and phylogenetic analysis

PCR products were purified using QIAquick PCR purification kits (QIAGEN, Hilden, Germany) and then sent for sequencing using the PCR primers at Macrogen Inc. (Seoul, ROK). Phylogenetic trees were constructed based on partial sequences of L- (360 bp) and S-segments (650 bp) of the hantavirus. Hantavirus sequences obtained from this study were submitted to National Center for Biotechnology Information (NCBI) and compared to sequences from GenBank using the neighbor joining (N-J) method on the LaserGene 6 Program (DNASTAR, Madison, WI, USA) of the ClustalX software program. Representative hantavirus sequences from GenBank, including hantavirus isolates from other countries and previously identified hantavirus sequences from other provinces of the ROK were included in the phylogenetic analysis. Genetic distance was computed using PAUP version 4.0b, and topology was evaluated by bootstrap analysis following the creation of 1000 trees. Nucleotide and amino acid pair distances and percent identity were computed using the Clustal W method on the MegAlign software, LaserGene 6 Program.

### Serological analysis

Indirect immunofluorescence assays (IFA) to detect anti-Hantaan virus immunoglobulin G (IgG) in *A. agrarius* sera was performed using the KCDC protocol. Briefly, sera from *A. agrarius* were serially diluted two-fold from 1:32 to 1:1024 in sterile phosphate-buffered saline (PBS). A total of 20 µL of each dilution was reacted with a Hantaan virus antigen slide, in a humidified chamber at 37 °C for 30 min. Next, the slides were washed three times with PBS and then another three times with distilled water (DW) and air dried. Slides were treated with Alexa Fluor 488 goat anti-mouse IgG (H+L) (ThermoFisher Scientific,

USA) and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgM (MP Biomedicals, Ohio, USA), respectively. The slides were incubated for 30 min at 37 °C in a humidified chamber, washed three times with PBS followed by three washes with DW, and then air-dried. The slides were then visualized on a fluorescence microscope (U-LH100HG, Olympus corp. Tokyo, Japan) under 400× magnification. A titer value of 32 was set as the cut-off for a positive result. Hantaan virus antigen slides used for IFA were kindly donated by the Korean Centre for Disease control (KCDC).

### Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics version 23 for Windows to determine the virus positivity/infection rate in relationship with the month in which rodents were captured during the study. We used the chi square test and statistical significance was set as $p \leq 0.05$.

## Results

### Rodents captured and molecular identification of Hantavirus

In total of 105 wild mice were caught during the study period, 30 at Gwangju, 21 at Boseong-gun, and 64 at Jeju Island. All captured mice were *A. agrarius.* The geographical locations of the capture sites are shown in Figure 1.

The hantavirus L- and S-segment RT-N-PCRs were conducted on the blood, spleen, kidney, and lung samples from wild rodents. RT-N-PCR targeting the hantavirus L segment detected the virus in 6.7% (2 out of 30) of all the *A. agrarius* captured at Gwangju, 23.8% (5 out of 21) of the Boseong-gun samples, and 15.6% (10 out of 64) of the Jeju samples. A statistically significant ($p < 0.01$) proportions of Hantavirus-positive samples from Jeju were collected in May, with an 85.7% (6/7) positivity rate for samples collected in this month. However, RT-N-PCR targeting the hantavirus S-segment indicated a decrease in sensitivity, as demonstrated by a drop in the positivity rate for samples from Boseong-gun to 14.3% (3/21) and for Jeju to only 4.6% (3/64). In the case of Gwangju samples, the proportion of positive samples was identical for both L- and S segments—6.7% (2/30). Notably, all samples positively identified by RT-N-PCR of the S-segment were also positive in the L-segment PCR. This suggests that the L-segment PCR has a higher sensitivity than the S-segment. DNA sequence analysis of all positive samples confirmed the presence of Hantaan virus in *A. agrarius* captured at Gwangju, Boseong-gun, and Jeju Island. We examined the prevalence rates for hantavirus infection by month, and for Jeju isolates, high prevalence rates were observed in May at 85.7% and in October at 50%, while 14.3% samples from August were shown as positive. Gwangju isolates had a positive rate of 7.7% in September and 10% in November. Boseong-gun showed a prevalence rate of 30% in October and 18.2% in November. These results are presented in Table 3. To the best of our knowledge, this is the first report of Hantaan virus detection in *A. agrarius* collected from Jeju Island.

### Sequence similarity and phylogenetic analysis of hantavirus isolates

All positive isolates were confirmed through DNA sequencing. Phylogenetic trees were constructed based on partial nucleotide sequences of L-segment (360 bp) of hantavirus isolates collected at Gwangju, Boseong-gun, and Jeju Island and compared with representative sequences of Hantaan virus collected from different provinces of the ROK, as well as strains isolated from neighboring countries like Russia and China. Analysis included comparisons of isolates with Soochong virus, Seoul virus, Dobra virus, Puumala virus, and others. All isolates from this study were grouped with previously reported Hantaan virus isolates from China; however, they created a distinct geographical cluster when compared with Korean Hantaan virus isolates (Figure 2). Phylogenetic trees based on partial S-segment (650 bp) sequences of Hantavirus-positive isolates were constructed in a similar manner. All positive isolates from this study clustered near a previously reported Maaji virus strain from the ROK that did not group with other Hantaan viruses, including isolates from the ROK and China (Figure 3).

To study geographical clustering, we compared sequences obtained from wild rodents and hantavirus isolates reported from different regions of the ROK. The percentage similarities of S- and L-segment nucleotide and amino acid sequences are shown in Table 4 and Table 5, while sequence similarity and amino acid identity among sequences isolated in this study are presented in Table 6. Based on partial S-segment sequences, Hantaan virus isolates had an 87.4–89.9% nucleotide similarity and 98.1–

98.6% amino acid identity with the Maaji virus strain reported from the ROK (Table 4). Sequence comparisons of the partial L-segment revealed that isolates from this study have a 70–83.9% nucleotide similarity and 94.1–97.5% amino acid identity with previously isolated hantaviruses from other provinces of the ROK (Table 5). We did not compare our L-Segment sequences to those of the Maaji virus as the latter sequences were not available on the NCBI site. We further characterized genetic distances among isolates obtained in this study. Sequence comparison of the L-segment sequence from Boseong-gun isolates demonstrated a minimum of 95.3% nucleotide similarity and 98.3% amino acid identity with Gwangju isolates but varied greatly from some Jeju isolates, showing a nucleotide similarity of only 82.8% and amino acid identity of only 85.6%. Similarly, Gwangju isolates also varied from Jeju isolates with only an 85.2% nucleotide similarity and 86.6% amino acid identity (Table 6).

### Serological results for Hantavirus

A total of 54 *A. agrarius* sera samples collected from Jeju were used for serological testing. Of these, only 6 (11%) of the sera were identified as positive using the IFA method. The monthly prevalence rate for hantavirus-specific IgG antibodies was 2 (33.3%) in May, 1 (14.3%) in August, 1 (11.1%) in September, 1 (17%) in October, and 1 (14.3%) in November. Four of the six (67%) seropositive samples were identified as positive by RT-N-PCR targeting the L-segment. Similarly, three out of five seropositive samples were identified as positive by RT-N-PCR targeting the S-segment of the Hantavirus. The serology results of hantavirus IgG are shown in Table 3, and positive antibody detection is shown in Figure 4.

Serum samples from Gwangju and Boseong-gun were not available for serological testing.

## Discussion

Hantaan virus, a prototype Hantavirus, is found in *A. agrarius* throughout China, Russia, and the ROK [13]. In this study, we conducted RT-N-PCR targeting the L- and S-segments of the hantavirus genome and demonstrated that on the same samples, L- and S-segments had different sensitivities. When we compared the results, there was an almost 10% increase in positive PCR results when using the L-segment. In our previous ROK study, rodents were captured in Gwangju (Buk-gu and Gwangsan-gu). These samples had a high rate of infection, with 46% of lung samples testing positive for Hantaan virus using RT-N-PCR targeting the L-segment [14]. Another group of researchers from the ROK reported a detection rate of 3.3% for hantaviruses in *A. agrarius* using a partial S-segment in multiplex RT-PCR [3]. Different rodent trapping sites, geographical distributions, climatic variations, and differences in molecular techniques used in these studies may have resulted in the variance observed in the results. To study whether geographical distribution can be used as a surrogate for genetic divergence, we collected rodents from different geographical locations and compared their sequences.

The prevalence of HFRS depends on pathogen viability; this includes adaptation to the climate, human activity, landscape, and seasonality in various regions [15]. A previous study conducted on hantaviruses prevalence provided epidemiological data on human HFRS cases (reported between 2001 and 2010) in the ROK. This study analyzed cases by season, geography, and the residential area of affected individuals. In this study, the majority of the HFRS cases were reported in the last quarter of the calendar year (October, November, and December). Presumably, the higher incidence of HFRS in these months can be attributed to the higher numbers of hantavirus-infected rodents in the ROK during this period [16]. Here, we found that a significantly ($P < 0.01$) higher proportion of HFRS-positive rodents was collected in Jeju during May (85.7%) and October (50%). Table 6 shows the nucleotide sequence similarity and amino acid identity between the Hantaan virus isolates collected at the three geographical locations shown in Figure 1. The distance between Gwangju and Boseong-gun is only 47.8 km, while the distance from Jeju to Boseong-gun and Gwangju is 150 km and 187 km, respectively. We were able to show that the nucleotide and amino acid identity among study locations mirrored the geographical distance between them. Although the study locations are not far apart, geographical clustering was shown by the isolates. Relatively high nucleotide similarity (95.3%) and amino acid identity of 98.3% was observed between isolates collected at the geographically closer locations of Boseong-gun and Gwangju. On the contrary, while comparing Jeju isolates with isolates from relatively distant locations—Boseong-gun and Gwangju—a lower nucleotide similarity of 82.2% (amino acid identity of 85.6%) and 85.2% (amino acid identity of 86.6%) was recorded, respectively. Given that the climate in Jeju differs from the rest of the Korean Peninsula, it may have contributed to the

increased nucleotide and amino acid divergence and the difference in seasonality. Previous studies have suggested that climate change can affect rodent distributions in a given area and consequently, the spread of zoonotic diseases [17]. Further studies are needed to evaluate this aspect of our study.

Additionally, serological results were obtained for 5 (11%) of the *A. agrarius* serum samples collected from Jeju. Interestingly, until 2013, only a single human HFRS case was documented from Jeju Island, amongst a total of 3,953 HFRS cases reported over almost a decade in the ROK [16, 18]. Another report, published in 2017, referenced five human HFRS cases from Jeju over a period from 2001 to 2009 [15]. The high hantavirus incidence among *A. agrarius* captured from Jeju Island along with the season variation suggests that this region may be at risk for a viral outbreak in the future. Further investigations are needed to better understand the hantavirus prevalence and seasonal variation in Jeju.

One of the major findings of our study was the geographical clustering of the isolates. These isolates grouped with the known Hantaan viruses but appeared to be distinct from the other Hantaan viruses isolated in different regions of the ROK. Genetic reassortment may explain this observation and amino acid divergence. Reassortment appears to occur frequently within similar hantavirus strains, especially those that share the same rodent host. On the contrary, reassortment between genetically distinct hantaviruses seems to occur infrequently even if they occasionally infect the same rodent host. It has been suggested that the reassortment between closely related strains of the same hantavirus group could lead to the emergence of unique strains with new virulence characteristics or host ranges [19]. A study conducted at Guizhou, China, presented a phylogenetic analysis of the S-, M-, and L-segments of hantavirus isolates and stated that although the S-segment of the two viruses belonged to the Hantaan virus group, both the M and L sequences were more similar to those of SEOV, indicating that a reassortment had occurred spontaneously at some point in their evolution [8]. Similarly, a Korean group reported spontaneous genetic exchanges in Hantaan virus genomes. This study reported that Hantaan virus isolates from Gangwon and Gyeonggi provinces of the ROK (areas where HFRS is highly endemic) show a high molecular diversity with geographically distinct clustering. Moreover, reassortment analysis demonstrated that these Hantaan virus isolates were heterogeneous for the L-segment but homogeneous for the M- and S-segments [20]. In our study, we compared the Gangwon and Gyeonggi Hantaan virus isolates with our isolates from south Jeolla and Jeju. Hantaan virus L-segment sequences obtained from the Jeju isolates demonstrated 70–83.9% nucleotide similarity and 96.2–97.5% amino acid identity. Gwangju isolates shared 78.1–81.9% nucleotide similarity and 95.8– 96.6% amino acid identity. Boseong-gun, Jellanamdo Province isolates shared a 77.2–81.9% nucleotide similarity and 94.9–96.6% amino acid identity with the isolates from Gangwon and Gyeonggi provinces (Table 5). When we compared S-segment sequences of Hantaan viruses from this study, we observed an amino acid identity of 95.3–96.3% when compared to the isolates from Gangwon and Gyeonggi provinces (Table 4). Moreover, phylogenetic analysis using these isolates as comparators based on both the partial S- and L-segments demonstrated distinct geographical clustering of isolates from different regions of the ROK. Figures 2 and 3 show that Hantaan virus isolates from Jeju formed a separate cluster from that of Boseong-gun, and similarly, the Gwangju isolates formed another distinct cluster. These three clusters were distinct from the Hantaan isolates reported in previous studies from Gyeonggi province (Hantaan virus/76-118–a Korean prototype Hantaan virus isolated in Uijeongbu, Yeoncheon, Pocheon, Paju) and Gangwon province (Hwacheon, Yanggu, Cheorwon). All of our Hantaan virus strains also clustered separately from isolates from neighboring countries including China and Russia. Although our results were based on partial segments of the viral genome, they suggest the emergence of a new hantavirus genotype in the southwest region of the ROK. This suggestion is supported by the high degree of reassortments shown to take place within Hantaan virus strains. Further studies based on full genome sequences are required to confirm the emergence of a new genotype of hantavirus.

This study has a few limitations, and the genetic distances were computed for only partial segments of the genome. Additionally, we did not perform serological tests on the Gwangju and Boseong-gun samples, as they were not collected in these regions. Further large-scale studies are required to understand the geographical clustering and seasonal variation of hantaviruses.

Environmental and genetic factors that mold the diverse evolutionary patterns observed in RNA viruses and illustrate the complexity of these systems can make it difficult to predict future viral disease emergence [21]. Therefore, timely surveillance of these pathogens in their hosts and carriers is essential.

## Conclusion

Our study suggests that Hantaan viruses show a considerable degree of geographical clustering, which may allude to the development of a new genotype variant in the southwestern region of the ROK. This was supported by the genetic diversity observed among the isolates in this study. This study will help to increase our understanding of geographical variance within hantaviruses and its role in the emergence of new viral strains.

## Abbreviations

ROK - Republic of Korea

RT-N-PCR - Reverse-transcription nested polymerase chain reaction

HFRS - Hemorrhagic fever with renal syndrome

HCPS - Hantavirus cardiopulmonary syndrome

KCDC- Korean Centers for Disease Control and Prevention

SOO - Soochong virus

MUJV - Muju virus

SNV - Sin Nombre virus

ANDV - Andes viruses

SEOV - Seoul orthohantavirus

PHV - Prospect Hill virus

IFA - Indirect immunofluorescence assays

NCBI - National Center for Biotechnology Information

PBS - Phosphate-buffered saline

## Declarations

### Ethics approval and consent to participate

This study was approved by the institutional review board (IRB) of Chosun University. All rodents were euthanized in accordance with an approved animal use protocol and evaluated by the Chosun University Institutional Animal Care and Use Committee (CIACUC), approval number CIACUC2017-S0014. This study adheres to the Korean Animal Protection Act (2007) and the Institutional Animal Care and Use (IACUC) committee guidelines and protocols.

### Consent for publication

Not applicable.

### Availability of data and materials

Data and materials are available upon request to the corresponding author.

### Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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**Authorship**

All authors has made substantial contributions in the conception and design of the study, acquisition of data, analysis and interpretation of data and drafting the article or revising it critically for important intellectual content. All authors has read and approved the final version to be submitted.

Sehrish Jalal, Choon-Mee Kim contributed equally to this work.

Dong-Min Kim contributed equally to this work.

**Authors' contributions**

SJ and CMK performed experiment, collected data, wrote the manuscript, and revised the draft during the course of submission. DMK designed and coordinated the study and contributed to drafting and reviewing the manuscript during the course of submission. CMK, HJS were responsible for the experiment and collected the data. JCL, MYS and HCL were directly responsible for the patient and performed the clinical examinations. All the authors read and approved the final version of the manuscript.

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## Tables

**Table 1.** Capture locations for *A. agrarius* and GPS coordinates of the sites used in the study

| *A. agrarius* capturing City | Site | Coordinates |
|---|---|---|
| Jeju (Seogwipo-si) | Rice paddy | 33°15'4.43"N / 126°32'34.53"E |
| | Field | 33°15'2.63"N / 126°32'38.54"E |
| | Reservoir | 33°15'13.09"N / 126°32'47.09"E |
| | Dyke | 33°15'3.25"N / 126°32'39.12"E |
| | Hill | 33°18'41.87"N / 126°34'26.02"E |
| Boseong-gun, Jeollanam-do (Seokho village, Hwabang-ri, Miryeok-myeon) | Rice paddy | 34°49'16.4"N / 127°07'06.8"E |
| | Field | 34°49'16.8"N / 127°07'08.6"E |
| | Reservoir | 34°49'18.9"N / 127°07'05.6"E |
| | Dyke | 34°49'17.7"N / 127°07'01.0"E |
| | Hill | 34°49'18.7"N / 127°07'08.9"E |
| Gwangju Metropolitan City (Buk-gu) | Rice paddy | 35° 13' 51.7"N, 126° 54' 23.8"E |
| | Dyke | 35° 13' 51.7"N, 126° 54' 23.8"E |
| | Hill/forest | 35° 13' 51.7"N, 126° 54' 23.8"E |
| | Around a tomb | 35° 13' 51.7"N, 126° 54' 23.8"E |
| Gwangju Metropolitan City (Gwangsan-gu) | Rice paddy | 35° 09' 19.2"N, 126° 45' 05.4"E |
| | Reservoir | 35° 09' 19.2"N, 126° 45' 05.4"E |
| | Dyke | 35° 09' 19.2"N, 126° 45' 05.4"E |
| | Hill/forest | 35° 09' 19.2"N, 126° 45' 05.4"E |
| | Around a tomb | 35° 09' 19.2"N, 126° 45' 05.4"E |

**Table 2.** Primers and PCR conditions used in this study.

| PCR Assay | Primers name (Sequence) | PCR conditions (35 cycles) | | | Product size (bp) | References |
|---|---|---|---|---|---|---|
| | | Denaturing (°C/sec) | Annealing (°C/sec) | Extension (°C/sec) | | |
| HFRS RT-N-PCR (L segment) | HAN-L-F1 (5′-ATGTAYGTBAGTGCWGATGC-3′) | 95/30 | 49/30 | 72/45 | 450 | [12] |
| (external primer | HAN-L-R1 (5′-AA CCADTCWGTYCCRTCATC-3') | | | | | |
| HFRS RT-N-PCR (L segment) | HAN-L-F2 (5′-TGCWGATGCHACIAARTGGTC-3') | 95/20 | 54/20 | 72/30 | 380 | [12] |
| (internal primer) | HAN-L-R2 (5'-GCRTCRTCWGARTGRTGDGCAA-3′) | | | | | |
| HFRS RT-N-PCR (S segment) | HFRS-S-2F (5′-ARARRTCARBVCTHAGBTAYG-3′) | 95/30 | 44/30 | 72/60 | 1,009 | This study |
| (external primer | HFRS-S-2R (5′-TGRTTVGAKATTTCCTTSAC-3') | | | | | |
| HFRS RT-N-PCR (S segment) | HFRS-S2nd-1F (5′-GAYATTGAWGAACCWASWGGVC-3') | 95/30 | 50/30 | 72/60 | 725 | This study |
| (internal primer) | HFRS-S2nd -1R (5'GAHGCCATKATKGTRTTYCKC-3′) | | | | | |

Cf > bp: base pair

**Table 3.** Hantaan virus detected by reverse transcription nested polymerase chain reaction (RT-N-PCR) targeting hantavirus L- and S-segments in *A. agrarius* captured at Jeju Island, Gwangju, and Boseong-gun. Seroprevalence of immunoglobulin G (IgG) antibodies against hantavirus in sera samples of *A. agrarius* captured at sites in Jeju.

| Capturing Location | Month | No. of mice captured | PCR positivity within month no. (%) | | IFA seropositive | |
|---|---|---|---|---|---|---|
| | | | RT-N-PCR targeting L segment | RT-N-PCR targeting S segment | No. of sera tested | No. of IFA seropositive (%) |
| Jeju | April | 15 | 0 (0%) | 0 (0%) | 6 | 0 (0%) |
| | May | 7 | 6 (85.7%) | 1 (14.3%) | 6 | 2 (33.3%) |
| | June | 8 | 0 (0%) | 0 (0%) | 8 | 0 (0%) |
| | July | 5 | 0 (0%) | 0 (0%) | 5 | 0 (0%) |
| | August | 7 | 1 (14.3%) | 1 (14.3%) | 7 | 1 (14.3%) |
| | September | 9 | 0 (0%) | 0 (0%) | 9 | 1 (11.1%) |
| | October | 6 | 3 (50%) | 1 (16.7%) | 6 | 1 (17%) |
| | November | 7 | 0 (0%) | 0 (0%) | 7 | 1 (14.3%) |
| | Total | 64 | 10 (15.6%) | 3 (4.6%) | 54 | 6 (11%) |
| | P-value | | <0.01 | 0.43 | | |
| Gwangju | September | 13 | 1 (7.7%) | 1 (7.7%) | — | — |
| | October | 7 | 0 (0.0%) | 0 (0.0%) | — | — |
| | November | 10 | 1 (10%) | 1 (10%) | — | — |
| | Total | 30 | 2 (6.7%) | 2 (6.7%) | — | — |
| | P-value | | 0.52 | 0.47 | | |
| Boseong-gun | October | 10 | 3 (30.0%) | 2 (20.0%) | — | — |
| | November | 11 | 2 (18.2%) | 1 (9.1%) | — | — |
| | Total | 21 | 5 (23.8%) | 3 (14.3%) | — | — |
| | P-value | | 0.70 | 0.70 | | |

Cf > IFA, Indirect immunofluorescent assay

**Table 4.** Percent similarity based on S-segment nucleotide and amino acid sequences between Hantaan virus isolates from *A. agrarius* captured in Gwangju (GJ), Boseong-gun (JN), and Jeju (JJ), and reference Hantaan viruses from GenBank

| Referenced/ Isolate (Accession #) | Hantaan virus/76-118 Uijeongbu* (M14626) | | Hantaan virus/Maaji-1 ROK (AF321094) | | Hantaan virus/Aa03-161 Yeoncheon* (KT935024) | | Hantaan virus/Aa09-948 Pocheon* (KT935034) | | Hantaan virus/Aa14-204 Paju* (KT935045) | | Hantaan virus/Aa14-266 Hwacheon† (KT935047) | | Hantaan virus/Aa14-362 Cheorwon† (KT935049) | | Hantaan virus/Aa14-408 Yanggu† (KT935054) | | Soochong virus/SOO-1 Inje-gun† (AY675349) | | Hantaan virus/Z10 Zhejiang,China (AF184987) | | Hantaan virus/AA57 Russia (AB620031) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | nt | aa | nt | aa | nt | aa | nt | aa | nt | aa | nt | aa | nt | aa | nt | aa | nt | aa | nt | aa | nt | aa |
| GJ-M06 sp (MN507691) | 80.6 | 95.3 | 87.9 | 98.1 | 81.2 | 95.3 | 81.7 | 95.8 | 81.4 | 95.3 | 80 | 95.8 | 81.4 | 95.3 | 81.4 | 95.8 | 79.4 | 93.5 | 80 | 93.9 | 82.2 | 95.3 |
| GJ-M30 sp (MN507693) | 80.2 | 95.8 | 87.9 | 98.6 | 80.8 | 95.8 | 81.2 | 96.2 | 80.9 | 95.8 | 79.5 | 96.2 | 80.9 | 95.8 | 80.9 | 96.2 | 79.4 | 93.9 | 79.5 | 94.4 | 81.7 | 95.8 |
| GJ-M30 Lg (MN507692) | 80 | 95.8 | 87.4 | 98.6 | 80.6 | 95.8 | 81.1 | 96.2 | 80.8 | 95.8 | 79.4 | 96.2 | 80.8 | 95.8 | 80.8 | 96.2 | 78.9 | 93.9 | 80 | 94.4 | 81.9 | 95.8 |
| JN-M02 sp (MN507697) | 80.6 | 95.8 | 89.8 | 98.6 | 81.4 | 95.8 | 81.9 | 96.3 | 81.2 | 95.8 | 80.5 | 96.3 | 81.6 | 95.8 | 81.2 | 96.3 | 79.1 | 93.9 | 80.5 | 94.4 | 82.6 | 95.8 |
| JN-M10 sp (MN507698) | 80.6 | 95.8 | 89.8 | 98.6 | 81.4 | 95.8 | 81.9 | 96.3 | 81.2 | 95.8 | 80.5 | 96.3 | 81.6 | 95.8 | 81.2 | 96.3 | 79.1 | 93.9 | 80.5 | 94.4 | 82.6 | 95.8 |
| JN-M11 sp (MN507699) | 80.5 | 95.8 | 89.6 | 98.6 | 81.2 | 95.8 | 81.7 | 96.3 | 81.1 | 95.8 | 80.3 | 96.3 | 81.4 | 95.8 | 81.1 | 96.3 | 78.9 | 93.9 | 80.3 | 94.4 | 82.5 | 95.8 |
| JJ-M18 Lg (MN507694) | 80.3 | 95.3 | 88.1 | 98.1 | 80.9 | 95.3 | 81.4 | 95.8 | 81.1 | 95.3 | 79.7 | 95.8 | 81.1 | 95.3 | 81.1 | 95.8 | 79.5 | 93.5 | 80 | 93.9 | 81.9 | 95.3 |
| JJ-M42 Lg (MN507695) | 80.5 | 95.8 | 87.9 | 98.6 | 81.1 | 95.8 | 81.6 | 96.3 | 81.2 | 95.8 | 79.8 | 96.3 | 81.2 | 95.8 | 81.2 | 96.3 | 79.7 | 93.9 | 79.8 | 94.4 | 81.7 | 95.8 |
| JJ-M53 Lg (MN507696) | 80.8 | 95.8 | 88.2 | 98.6 | 81.4 | 95.8 | 81.9 | 96.3 | 81.6 | 95.8 | 80.2 | 96.3 | 81.6 | 95.8 | 81.6 | 96.3 | 80 | 93.9 | 80.2 | 94.4 | 82 | 95.8 |

Cf > †Gangwon province isolates; * Gyeonggi province isolates
Lg, lung; sp, spleen; nt, nucleotide; aa, amino acid

**Table 5.** Percent similarity based on the L-segment nucleotide and amino acid sequences between Hantaan virus isolates obtained from *A. agrarius* captured in this study at Gwangju (GJ), Boseong-gun (JN), and Jeju (JJ), and reference Hantaan viruses from GenBank.

| Referenced/ Isolate (Accession #) | HNTV/76-118 Uijeongbu* (NC005222) | | HNTV/Aa03-161 Yeoncheon* (KT934956) | | HNTV/Aa09-948 Pocheon* (KT934966) | | HNTV/Aa14-204 Paju* (KT934977) | | HNTV/Aa14-266 Hwacheon† (KT934979) | | HNTV/Aa14-362/Cheorwon† (KT934981) | | HNTV/Aa14-408/Yanggu† (KT934986) | | SoochongVirus/ SOO-1Inje† (DQ056292) | | HNTV /Z10 Zhejiang,China (AF189155) | | HNTV /AA57 Russia (AB620033) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | nt | aa | nt | aa | nt | aa | nt | aa | nt | aa | nt | aa | nt | aa | nt | aa | nt | aa | nt | aa |
| GJ-M06 sp (MN507673) | 80 | 96.6 | 79.2 | 96.6 | 80.3 | 96.6 | 79.2 | 95.8 | 80.8 | 96.6 | 78.9 | 95.8 | 81.9 | 96.6 | 80 | 95 | 81.9 | 97.5 | 82.8 | 97.5 |
| GJ-M30 Lg (MN507674) | 79.4 | 95.8 | 78.6 | 96.6 | 79.7 | 96.6 | 78.6 | 95.8 | 80.3 | 96.6 | 78.3 | 95.8 | 81.4 | 96.6 | 79.4 | 94.9 | 81.7 | 96.6 | 82.5 | 97.5 |
| GJ-M30 sp (MN507675) | 79.2 | 95.8 | 78.3 | 96.6 | 79.4 | 96.6 | 78.3 | 95.8 | 80 | 96.6 | 78.1 | 95.8 | 81.1 | 96.6 | 79.2 | 94.9 | 81.4 | 96.6 | 82.2 | 97.5 |
| JN-M02 sp (MN507686) | 80.6 | 95 | 79.2 | 96.6 | 79.7 | 96.6 | 78.6 | 95.7 | 80.3 | 96.6 | 78.3 | 95.7 | 81.9 | 96.6 | 79.4 | 94.9 | 81.9 | 97.5 | 83.3 | 97.4 |
| JN-M04 kd (MN507687) | 80 | 95 | 78.6 | 96.6 | 79.2 | 96.6 | 78.1 | 95.7 | 79.7 | 96.6 | 77.8 | 95.7 | 81.4 | 96.6 | 78.9 | 94.9 | 81.4 | 96.6 | 82.8 | 97.4 |
| JN-M10 sp (MN507688) | 78.3 | 96.6 | 77.2 | 96.6 | 78.1 | 96.6 | 77.2 | 95.8 | 78.9 | 96.6 | 76.9 | 95.8 | 80 | 96.6 | 78.9 | 95 | 80.8 | 95.8 | 81.7 | 97.5 |
| JN-M11 sp (MN507689) | 79.2 | 95.8 | 77.8 | 96.6 | 78.3 | 96.6 | 77.2 | 95.8 | 78.9 | 96.6 | 76.9 | 95.8 | 80.6 | 96.6 | 78.1 | 94.9 | 80.3 | 95.8 | 81.7 | 97.5 |
| JN-M19 sp (MN507690) | 79.7 | 95 | 78.3 | 95.8 | 78.9 | 95.8 | 77.8 | 94.9 | 79.4 | 95.8 | 77.5 | 94.9 | 81.1 | 95.8 | 78.6 | 94.1 | 80.8 | 95.8 | 82.2 | 96.6 |
| JJ-M17 Lg (MN507676) | 81.7 | 97.5 | 80.8 | 97.5 | 81.9 | 97.5 | 80.8 | 96.6 | 82.5 | 97.5 | 80.6 | 96.6 | 83.6 | 97.5 | 80.6 | 95.8 | 83.3 | 98.3 | 84.4 | 98.3 |
| JJ-M18 Lg (MN507677) | 71.4 | 84.9 | 70.6 | 97.1 | 71.7 | 97.1 | 70.6 | 96.2 | 71.9 | 97.1 | 70.6 | 96.2 | 72.8 | 97.1 | 70.8 | 95.2 | 72.8 | 85.7 | 73.9 | 98.1 |
| JJ-M19 Lg (MN507678) | 81.7 | 97.5 | 80.8 | 97.5 | 81.9 | 97.5 | 80.8 | 96.6 | 82.5 | 97.5 | 80.6 | 96.6 | 83.6 | 97.5 | 80.6 | 95.8 | 83.3 | 98.3 | 84.4 | 98.3 |
| JJ-M20 Lg (MN507679) | 80.3 | 95.8 | 79.4 | 97.4 | 80.6 | 97.4 | 79.4 | 96.6 | 81.1 | 97.4 | 79.2 | 96.6 | 82.2 | 97.4 | 79.2 | 95.7 | 81.7 | 96.6 | 82.8 | 98.3 |
| JJ-M21 Lg (MN507680) | 81.7 | 97.5 | 80.8 | 97.5 | 81.9 | 97.5 | 80.8 | 96.6 | 82.5 | 97.5 | 80.6 | 96.6 | 83.6 | 97.5 | 80.6 | 95.8 | 83.3 | 98.3 | 84.4 | 98.3 |
| JJ-M22 Lg (MN507681) | 81.7 | 97.5 | 80.8 | 97.5 | 81.9 | 97.5 | 80.8 | 96.6 | 82.5 | 97.5 | 80.6 | 96.6 | 83.6 | 97.5 | 80.6 | 95.8 | 83.3 | 98.3 | 84.4 | 98.3 |
| JJ-M42 Lg (MN507682) | 70.8 | 84.9 | 70 | 97.1 | 71.1 | 97.1 | 70 | 96.2 | 71.4 | 97.1 | 70 | 96.2 | 72.2 | 97.1 | 70.6 | 95.2 | 72.5 | 85.7 | 73.3 | 98.1 |
| JJ-M53 Lg (MN507683) | 81.1 | 97.5 | 80.3 | 97.5 | 81.4 | 97.5 | 80.3 | 96.6 | 81.9 | 97.5 | 80 | 96.6 | 83.1 | 97.5 | 80.3 | 95.8 | 83.1 | 98.3 | 83.9 | 98.3 |
| JJ-M54 Lg (MN507684) | 80.6 | 97.5 | 79.7 | 97.5 | 80.8 | 97.5 | 79.7 | 96.6 | 81.4 | 97.5 | 79.4 | 96.6 | 82.5 | 97.5 | 79.7 | 95.8 | 82.5 | 98.3 | 83.3 | 98.3 |
| JJ-M55 Lg (MN507685) | 81.4 | 97.5 | 80.6 | 97.5 | 81.7 | 97.5 | 80.6 | 96.6 | 82.2 | 97.5 | 80.3 | 96.6 | 83.9 | 97.5 | 80.8 | 95.8 | 83.6 | 98.3 | 84.7 | 98.3 |

Cf > † Gangwon province isolates;* Gyeonggi province isolates

HNTV, Hantaan virus; Lg, lung; kd, kidney; sp, spleen; nt, nucleotide; aa, amino acid

**Table 6.** Percent similarity based on the L- and S-segment nucleotide and amino acid sequences among the Hantaan virus isolates obtained from *A. agrarius* captured at Gwangju (GJ), Boseong-gun, Jeollanam-do Province (JN), and Jeju (JJ). Lowest aa similarity values are in bold.

| | | GJ-M06 sp | | GJ-M10 sp | | GJ-M10 Lg | | JN-M02 sp | | JN-M04 kd | | JN-M10 sp | | JN-M11 sp | | JN-M19 sp | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | nt | aa | nt | aa | nt | aa | nt | aa | nt | aa | nt | aa | nt | aa | nt | aa |
| L-seg | JJ-M17 Lg | 97.2 | 99.2 | 97.5 | 99.2 | 97.5 | 99.2 | 95.5 | 99.2 | 95.5 | 99.2 | 95.6 | 99.2 | 95.6 | 99.2 | 95.3 | 98.3 |
| | JJ-M18 Lg | **85.2** | **86.6** | **85.9** | **87.3** | **86** | **87.3** | **84.5** | **88.1** | **84** | **88.1** | **82.8** | **86.6** | **83.6** | **87.4** | **83** | **85.6** |
| | JJ-M19 Lg | 97.2 | 99.2 | 97.5 | 99.2 | 97.5 | 99.2 | 95.5 | 99.2 | 95.5 | 99.2 | 95.6 | 99.2 | 95.6 | 99.2 | 95.3 | 98.3 |
| | JJ-M20 Lg | 95.8 | 97.5 | 96.6 | 98.3 | 96.6 | 98.3 | 95.2 | 98.3 | 94.7 | 98.3 | 93.6 | 97.5 | 93.6 | 97.5 | 93.6 | 96.6 |
| | JJ-M21 Lg | 97.2 | 99.2 | 97.5 | 99.2 | 97.5 | 99.2 | 95.5 | 99.2 | 95.5 | 99.2 | 95.6 | 99.2 | 95.6 | 99.2 | 95.3 | 98.3 |
| | JJ-M22 Lg | 97.2 | 99.2 | 97.5 | 99.2 | 97.5 | 99.2 | 95.2 | 98.3 | 95.2 | 98.3 | 95.6 | 99.2 | 94.7 | 98.3 | 95.3 | 98.3 |
| | JJ-M42 Lg | **85.2** | **86.6** | **85.9** | **87.3** | **86** | **87.3** | **84.2** | **87.3** | **83.7** | **87.3** | **82.8** | **86.6** | **82.8** | **86.6** | **83.3** | **85.6** |
| | JJ-M53 Lg | 97.2 | 99.2 | 97.5 | 99.2 | 97.5 | 99.2 | 95.2 | 98.3 | 95.2 | 98.3 | 95.6 | 99.2 | 94.7 | 98.3 | 95.5 | 98.3 |
| | JJ-M54 Lg | 96.9 | 99.2 | 97.2 | 99.2 | 96.9 | 99.2 | 94.6 | 98.3 | 94.7 | 98.3 | 95 | 99.2 | 94.2 | 98.3 | 95.5 | 98.3 |
| | JJ-M55 Lg | 96.9 | 99.2 | 97.2 | 99.2 | 97.2 | 99.2 | 94.9 | 98.3 | 94.9 | 98.3 | 95.3 | 99.2 | 94.4 | 98.3 | 95 | 98.3 |
| S-seg | JJ-M18 Lg | 98.2 | 98.1 | 99.1 | 99.5 | 99.2 | 99.5 | 97 | 99.5 | – | – | 96.9 | 99.5 | 97 | 99.5 | – | – |
| | JJ-M42 Lg | 98 | 98.6 | 99.1 | 100 | 99.2 | 100 | 96.9 | 100 | – | – | 96.7 | 100 | 97 | 100 | – | – |
| | JJ-M53 Lg | 98.3 | 98.6 | 99.1 | 100 | 99.2 | 100 | 97 | 100 | – | – | 96.9 | 100 | 97 | 100 | – | – |

Cf > Lg, lung; kd, kidney; sp, spleen; nt, nucleotide; aa, amino acid

## Figures

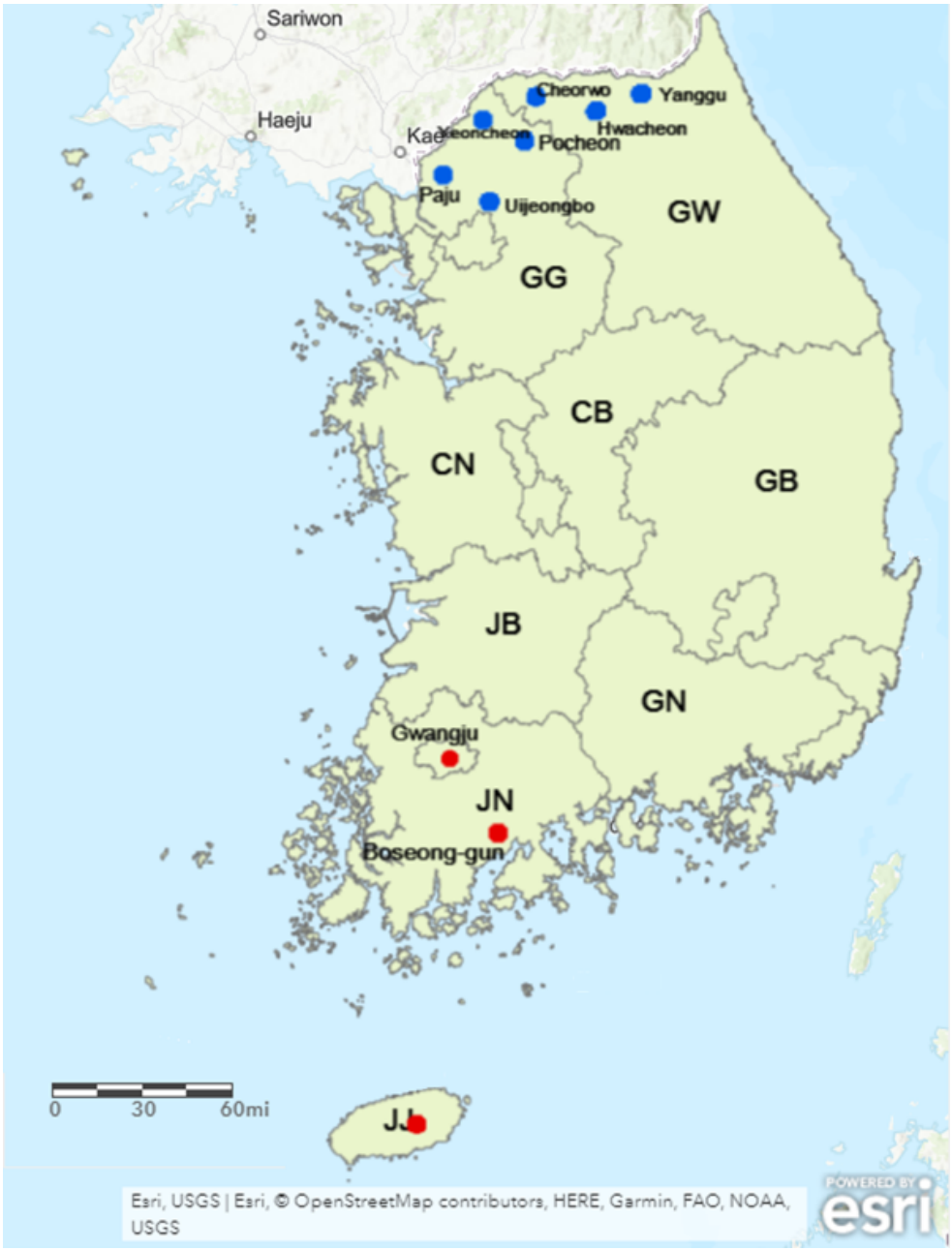
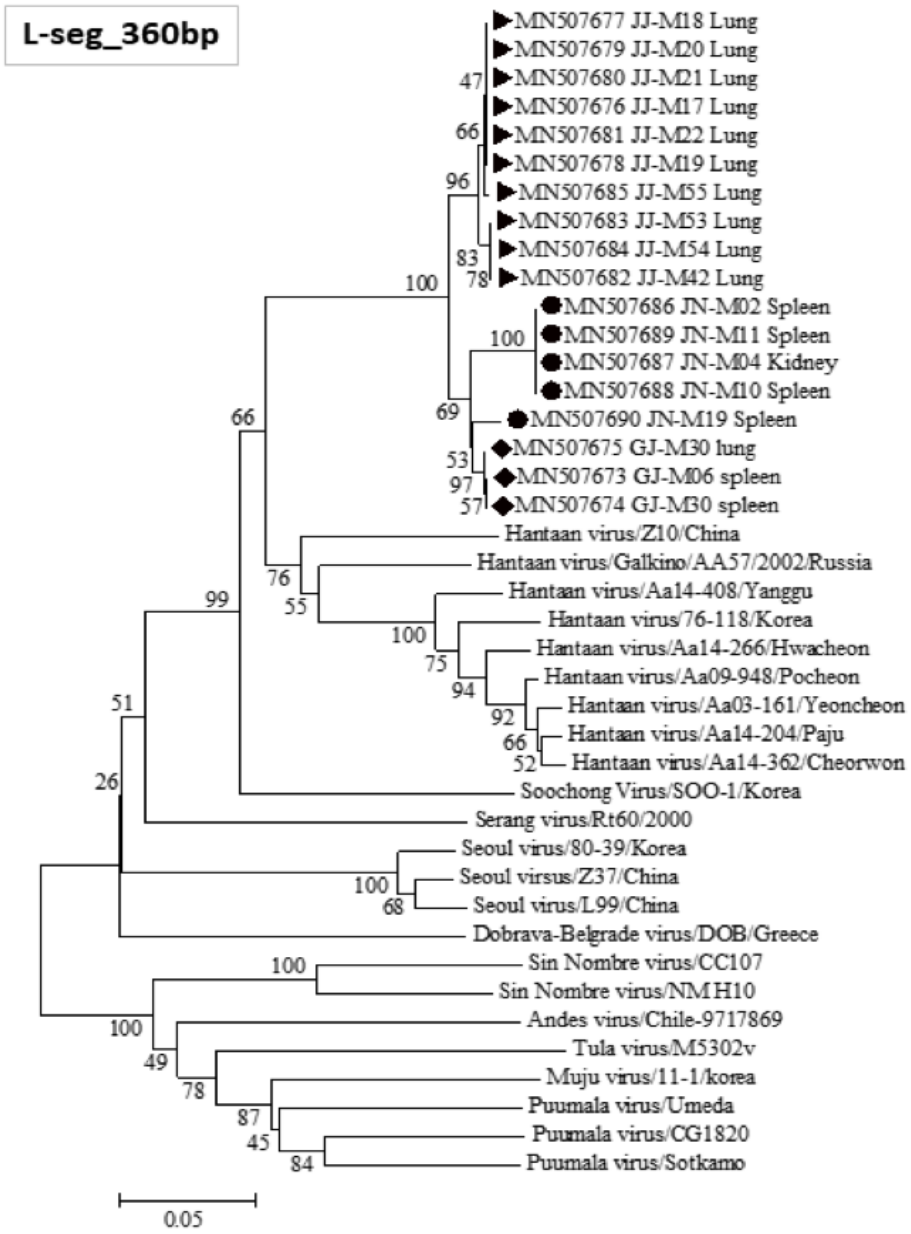


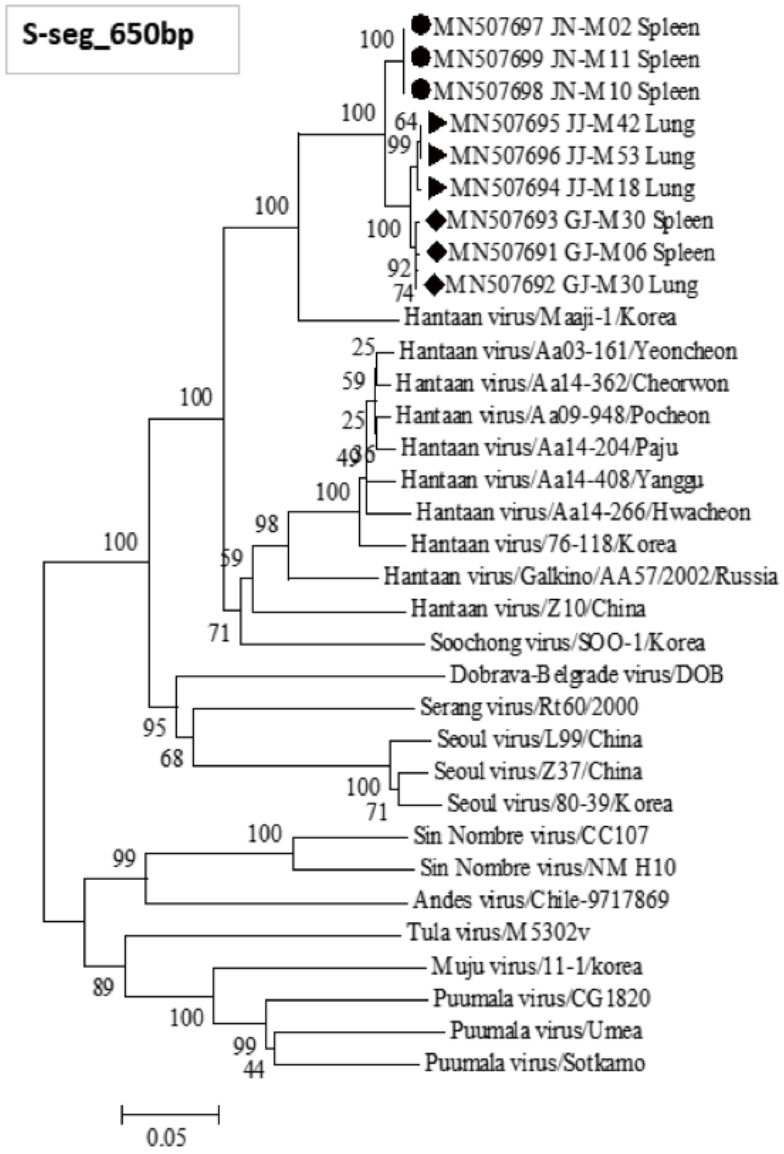

**Figure 1**

Geographical location of A. agrarius capture sites, Gwangju, Boseong-gun, and Jeju in the Republic of Korea (ROK). Red dots mark the collection sites in this study, and blue dots mark isolation sites of previously described hantavirus strains from the ROK.




## Figure 2

Phylogenetic tree based on the hantavirus L-segment (360 bp) of positive isolates identified from Gwangju-GJ (diamonds), Boseong-gun Jeollanam-do-JN (circles), and Jeju-JJ (arrow) compared with hantavirus sequences obtained from GenBank. The GenBank accession number is indicated. Scale bar indicates a 0.05 (nucleotide substitution per site) sequence distance.




## Figure 3

Phylogenetic tree based on the hantavirus S-segment (650 bp) of positive isolates identified from Gwangju-GJ (diamonds), Boseong-gun Jeollanam-do-JN (circles), and Jeju-JJ (arrow) compared with hantavirus sequences obtained from GenBank. The GenBank accession number is indicated. Scale bar indicates a 0.05 (nucleotide substitution per site) sequence distance.

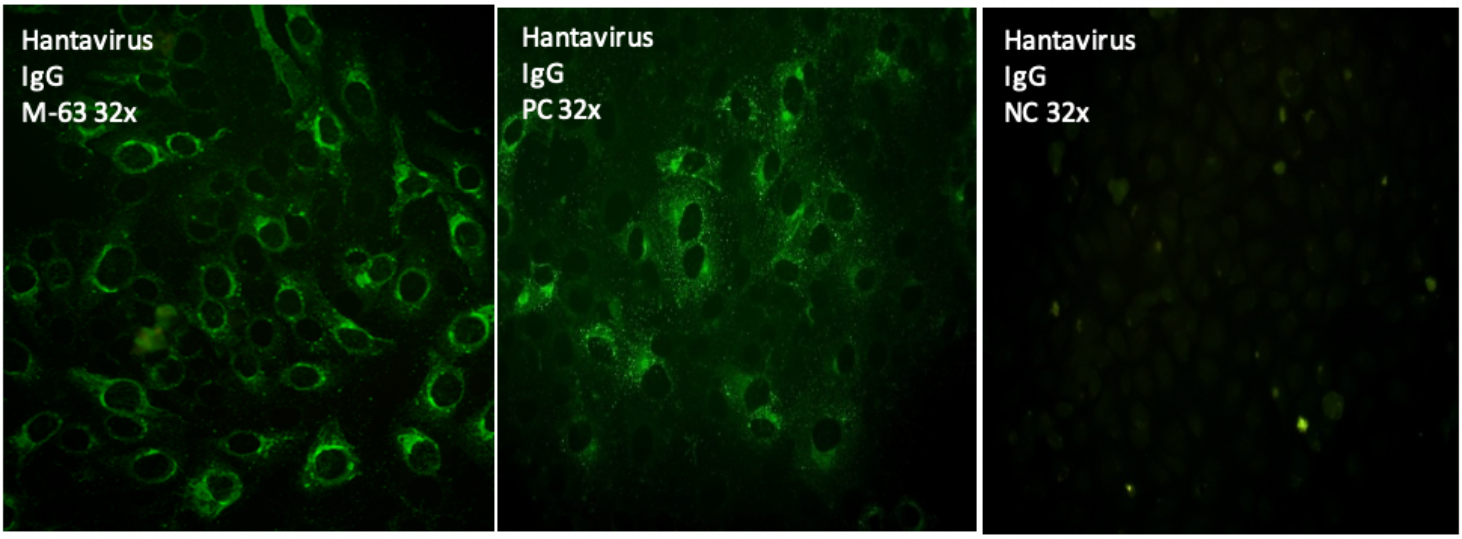



**Figure 4**

Immunofluorescence assay results showing the presence of hantavirus-specific antibody immunoglobulin G (IgG) using A. agrarius sera collected at Jeju. PC: positive control NC: negative control 32X: 1/32 dilution factor (titer)

## Supplementary Files

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