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August 2016
Master's Degree Thesis

Vector-Borne Viral Infections in South-West Region of Korea

Graduate School of Chosun University

Department of Biomedical Sciences

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한국의 남서부 지역에서 매개체 관련 바이러스 질환

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Advisor: Prof. Dong-Min Kim, MD, PhD

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ABBREVIATIONS AND SYMBOLS

BLAST	basic local alignment search tool
C-RT-PCR	conventional reverse transcriptase polymerase chain reaction
C.I.	confidence interval
cDNA	complementary DNA
DENV	dengue virus
DNA	deoxyribonucleic acid
gDNA	genomic DNA
bp	base pairs
DOBV	dobra virus
e.g.	example given
EM	electron microscope
HTNV	hantaan virus
HFRS	hemorrhagic fever with renal syndrome
HCPS	hemorrhagic cardio pulmonary syndrome

JEV	Japanese encephalitis virus
Kb	kilobase (one thousand bases)
kDa	kilodalton
km^2	kilometre squared
L(K)	likelihood distribution; estimates number of clusters in STRUCTURE
L-segment	large segment
Mb	megabase pairs
M-segment	medium segment
NS5	nonstructural protein
OR	odds ratio
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PUUV	puumala virus
ROK	Republic of Korea
RNA	ribonucleic acid

spp	species
SEOV	seoul virus
SFTS	severe fever with thrombocytopenic syndrome
SFTSV	severe fever with thrombocytopenia syndrome virus
S-segment	small segment
Tm	melting temperature
VBVI	vector borne viral infections
VBVD	vector borne viral diseases
WNFV	west-nile fever virus

ABSTRACT

Vector-Borne Viral Infections in South-West Region of Korea

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Background

Vector-borne viral infections (VBVIs) are defined as viral infections transmitted by vectors such as mosquitoes, ticks, lice, biting flies, mites, and fleas. Vector-borne infections account for more than 17% of all infectious diseases, causing more than 1 million deaths annually. Severe fever with thrombocytopenia syndrome (SFTS) caused by severe fever with thrombocytopenia syndrome virus (SFTSV) and Hemorrhagic fever with renal syndrome (HFRS) caused by hantaviruses from the family *Bunyaviridae* are two of the emerging VBVIs worldwide. Similarly, Flavivirus of the family *Flaviviridae* transmitted by arthropod vectors especially by ticks and mosquitoes causing various infections such as West Nile fever, Yellow

fever, Dengue, etc are widely distributed . SFTSV, a novel *bunyavirus* reported to be endemic in central and northeastern China, was first detected in 2010 in China and later reported in other Asian and Mediterranean countries as well as in the USA. On the other hand Hantaviruses found global attention during the Korean War between 1951 to 1953, when more than 3000 United Nations and US soldiers experienced an acute febrile illness with acute renal failure and shock. In this study, we investigated the positive rate of SFTSV, Hantavirus and Flavivirus in humans and rodents in south-western region of ROK.

Methods

In 2015, all the suspected cases of SFTS and HFRS from two different University Hospitals located in Gwangju City, which is in south-west of ROK were investigated for these three clinical entities by different PCR techniques. We also collected rodents (striped field mouse; *Apodemas agrarius*) from the suburbs of Gwangju city to detect the presence of SFTSV and Hantavirus as wild rodents are largely considered as the natural reservoirs of these pathogenic viruses. A total of 267 patients were investigated for SFTSV by RT-PCR targeting 'M' segment and 295 patients for HFRS by RT-Nested PCR targeting 'L' segment. Similarly, we tried to investigate the presence of Flavivirus in the suspected cases by conventional RT-PCR targeting 'NS5 'gene. Also, 41 wild mice captured in August, October and November were investigated for the presence of both these viral pathogens by similar molecular methods as for human samples. The amplified products were sequenced and analyzed.

Results

In this study, 2.2 % (6/267) of patients' plasma sample were positive for SFTS and 3.1 % (9/295) were found positive for HFRS. On the other hand, none of the mouse samples were positive for SFTSV but a proportionally high positive rate of Hantavirus in mouse; 46.3 % (19/41) was found. This fact suggests that SFTSV

and hantavirus are prevalent in human population at a reasonably higher rate than thought previously.

Conclusions

SFTS and HFRS have been endemic to Korea, and these vector-borne infectious diseases have been circulating naturally within the country. Phylogenetic analysis of SFTS positive patient samples showed close proximity to human strains from China and Japan, similarly phylogenetic analysis of Hantavirus positive samples showed clustering in one group and showed close affinity to Hantaan virus.

Keywords: SFTS, HFRS, PCR, striped field mouse, Republic of Korea

한 글 요 약

한국의 남서부지역에서 매개체관련 바이러스 질환

Babita Jha

Supervisor: Prof. Dong-Min Kim, MD, PhD

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Background

바이러스성 매개체 감염이란 모기나 진드기, 이, 파리, 벼룩 등의 매개체에 의해 전파되는 바이러스 감염 질환이다. 매개체에 의한 감염은 모든 감염질환의 17% 이상을 차지하며, 연간 백만 명 이상이 사망한다. 중증 열성 혈소판 감소 증후군 바이러스(Severe fever with thrombocytopenia syndrome virus, SFTSV)에 의해 유발되는 중증 열성 혈소판 감소 증후군(Severe fever with thrombocytopenia syndrome, SFTS)과 Bunyaviridae 계열의 한타바이러스(Hanta virus, HTNV)에 의해 유발되는 신증후성 출혈열(Hemorrhagic fever with renal syndrome, HFRS)은 전 세계적으로 주목 받고 있는매개체 관련 바이러스성 질환이다. Flaviviridae 계열의 flavivirus 는 진드기나 모기와 같은 절지동물 매개체에 의해

전파되며, 웨스트나일열, 황열병, 뎅기열 등 전 세계적으로 퍼져있는 감염병을 유발한다. SFTSV 는 중국의 중부 및 북동부에서 발견된 새로운 bunyavirus 계열 풍토병으로, 2010 년 중국에서 처음 발견된 이래 다른 아시아 국가 및 지중해 국가, 그리고 미국에서도 그 존재가 확인되었다. 한타바이러스는 1951 년에서 53 년 사이 발발한 한국전 에서 전 세계적인 주목을 받은 바이러스로, 당시 3000 명 이상의 유엔 및 미군 병사들이 급성 신부전과 쇼크를 동반한 급성경련 질환을 겪었다. 이 논문은 한국의 서남부지역에서의 병원과 인근지역에서 야생설치류를 수집하여 인간과 야생쥐에서 SFTSV 와 Hantavirus, Flavivirus 의 양성율을 조사하였다.

Methods

2015 년 대한민국 서남부에 위치한 광주광역시 내 두 곳의 대학병원에서 SFTS 와 HFRS 로 의심되는 모든 환자들 PCR 기법을 사용하여 조사하였다. 또한 야생쥐들이 이 바이러스들의 자연적 숙주로 여겨지기에 광주광역시 교외에서 설치류 (등줄쥐; *Apodemas agrarius*) 포획하여 SFTSV 와 HTNV 의 존재유무를 조사하였다. 총 267 명의 환자는 'M' segment'를 감지하는 RT-PCR 기법을 사용하여 SFTSV 검사를 받았고, 295 명의 환자는 'L' segment 를 감지하는 RT-Nested PCR 기법을 사용하여 HFRS 검사를 시행하였다. 보편적인 RT-PCR 을 사용하여 flavivirus 의 NS5 유전자

존재유무를 확인하였다. 이와 동시에 8 월과 10 월과 11 월에 포획한 41 마리의 야생쥐들에서 SFTSV 와 Hantavirus 의 존재유무를 유사한 방법으로 확인하였다. PCR 에서 얻어진 산물들은 정제 후 염기서열을 분석하였다.

Results

이 연구에서 환자들 샘플의 2.3 % (6/267) 가 SFTS 양성으로, 3.05% (9/295) 가 HFRS 양성으로 판명되었다. 야생쥐 샘플의 경우 SFTS 양성은 없었으나 HFRS 의 경우 46.3 % (19/41) 가 양성반응을 나타내. 야생쥐에서의 Hantavirus 가 매우 높은양성율을 보였다.

Conclusions

SFTS 와 HFRS 는 모두 한국의 풍토병이며 이러한 매개체 감염질환들은비교적 높은 양성률을 보였다. SFTS 양성 환자의 계통발생 분석결과는 중국과 일본에서 얻어진 바이러스(human strain) 와 매우 유사하며, Hantavirus 의 계통발생 분석결과 역시 한 그룹에 집중되어 있고 동시에 Hantanvirus 와 매우 가까운 유사성을 나타내는 결과를 보였다.

Keywords: SFTS, HFRS, PCR, striped field mouse, Republic of Korea

"The stupidest virus is cleverer than the cleverest virologist.

"George Klein" (1925)

I. Introduction

Vector-borne viral infections

Generally, Vector-borne viral infections (VBVIs) are referred as viral infections transmitted by vectors such as mosquitoes, ticks, lice, biting flies, mites, and fleas. It accounts for more than 17% of all infectious diseases, causing more than 1 million deaths annually. Few of VBVIs, such as Dengue fever, have long been presented worldwide and some, such West Nile fever, are re-emerging as global public health threats. Other signigicant VBVIs include Japanese encephalitis, Chikungunya fever, Rift Valley fever, hemorrhagic fever with renal syndrome (HFRS), and severe fever with thrombocytopenia syndrome (SFTS). As a consequences of environmental changes and fast-paced globalization, many VBVIs pose increasing threats to human health and challenges to effective disease prevention and control.

Aim of this study

This study aimed to investigate the positive rate of emerging human pathogenic viruses, severe fever with thrombocytopenia syndrome virus (SFTSV) and Hantavirus and Flavivirus in patients admitted in two University Hospitals, namely Chosun University Hospital and Chonnam National University Hospital which are located in southwest region of Republic of Korea (ROK). We also investigated the positive rate of these viral pathogens in wild rodents (all of which were striped field mouse, *Apodemus agrarius*) collected from farmlands on the outskirts of the City. This study was a prospective study done from January 2015 to December 2015.

Severe Fever with Thrombocytopenia Syndrome (SFTS)

In the year 2010, SFTS was first discovered in China [1], followed by several reports from many other countries worldwide. SFTSV has been regarded as the causative agent of the disease and has been recognized as a public health threat. In the family *Bunyaviridae,* this novel Bunyavirus belongs to the *Phlebovirus* genus.

SFTS clinical features include fever, thrombocytopenia, leukocytopenia, and notable changes in several serum biomarkers. About 10 ~ 15% mortality rate, has been encountered in this disease, commonly because of multiorgan dysfunction. Many reports of SFTSV are received from the rural areas of Central and North-eastern China, with seasonal occurrence from May to September, mainly targeting those of ≥50 years of age. A wide variety of domesticated animals, including sheep, goats, cattle, pigs, dogs, and chickens have been proven seropositive for SFTSV. *Haemaphysalis longicornis*, ticks are suspected to be the potential vector, having a broad animal host range in ROK.

Genome

Like the normal genetic composition of Bunyaviridae family members, the genome of SFTSV also consists of three single-stranded negative sense RNA segments [large (L), medium (M), and small (S)] [1, 4 - 6]. The complete L segment consists of 6368 bp including a single ORF between positions 17–6271 that encodes 2084 aa residues forming the RNA-dependent RNA polymerase (RdRp), which functions as the viral transcriptase/replicase [1, 3, 4]. The total length of M segment is 3378 bp, forming an ORF between positions 19–3240 and encoding 1073 aa of membrane protein precursor, which matures to two envelopes,

a glycoprotein N (Gn) [19–1704 nucleotide (Nt)] and a glycoprotein C (Gc) (1705–3240 Nt) [1,10]. And thirdly, the S segment is 1744 Nts of ambisense RNA with two oppositely ORFs separated by a 54 bp Nt intergenic region, encoding two proteins, the Np (1702–965 Nt) in antisense orientation and the NS protein (29–910 Nt) in sense orientation [1,2,8]. Viral RNA encapsidation is facilitated by Np and is responsible for the formation of RNP complex [7]. A short noncoding sequences is possessed by the 5′ and 3′ termini of the L, M, and S segments. The 3′ noncoding regions are 100 Nt (L), 141 Nt (M), and 28 Nt (S) and the 5′ noncoding regions of SFTSV are 16 Nt (L), 18 Nt (M), and 42 Nt (S). (Figure 1)

Disease distribution

Previously, *Phlebovirus* was mainly circulating in some parts of Africa and Europe, Arabian Peninsula, the regions with the highest incidences of the *phlebotomine* sandfly transmitted viruses. Till SFTSV was identified in 2010, *Phlebovirus* has never been reported in China. After its identification in 2010 though SFTS have been found in some countries outside China, such as Korea, Japan and USA, the measure affected region still remains in China. The rural areas of eastern, central and north-eastern China are mainly affected by SFTS [1, 4, 9, 10].

Diagnostic methods

The diagnosis of SFTSV is based on viral nucleic acid tests and serological detection. Viral nucleic acids can be tested in the acute phase serum of patients. For

amplifying L, M, and S of SFTSV segments one-step TaqMan real-time assays were applied widely. The molecular techniques improved the detection of SFTSV. Yang et al. [11] and Xu et al. [12] established a modified, low cost, and rapid visualized one-step RT loop-mediated isothermal amplification method for the detection of RNA from the SFTSV. A two-tube multiplex real-time RT-PCR assay for the detection of SFTS, along with other closely related viruses revealed high sensitivity (10 copies/μL) with no cross reactivity between tested viruses indicating the high specificity of the assay [22]. Moreover, a very sensitive one-step real-time RT-PCR method using a minor groove binding probe was developed for detection and quantitation of SFTSV by Li et al. [13]. A unique assay developed by Cui et al. [14] involved a nearly instrument-free, simple molecular method that incorporates RT-cross-priming amplification coupled with a vertical flow visualization strip for rapid detection of SFTSV. This assay targets a conserved region of the M segment of the SFTSV genome and has a limit of detection of 100 copies per reaction, with no cross–reaction with other vector-borne *Bunyaviruses* and bacterial pathogens. The relative sensitivity and specificity of the assay were 94.1% and 100.0%, respectively. Furthermore, potential detection limit of 10 viral RNA copies/μl was achieved using quantitative real-time PCR technique utilizing primer–probe sets to detect L, M, and S genes of SFTSV, with 98.6% sensitivity and over 99% specificity [15].

Serological methods including the in-house Mac-EIA assay, indirect EIA assay, and double-antigen sandwich EIA assay have been also performed in testing virus specific IgM, IgG, and total antibodies in serum samples, respectively. Jiao et al. developed an improved EIA system utilizing N of SFTSV [16] with high sensitivity and specificity. Conventional methods like indirect immunofluorescence and the serum neutralization assays are still the key, however these methods are costly and need more time and well-trained personnel.

Reported SFTSV cases in ROK

Till 2013, a total of 36 SFTS case-patients were reported in South Korea. A retrospective study from 2012 identified the first SFTS case [17]. Thereafter, SFTS was diagnosed in 35 additional case-patients in South Korea. A different group diagnosed the first of the 35 cases in the country; one group of researchers diagnosed the other 34 cases, from which they isolated the 26 SFTSVs.

The SFTS case–fatality rate in ROK was 47.2 % (17/36), higher than that of the recent China cases (≈8.7%).Approximately 80% of patients were >50 years of age, defining it to be common in older age. Most patients were farmers approx. 70% including persons who cultivated vegetable gardens [18].

Hemorrhagic fever with renal syndrome (HFRS)

HFRS is a syndrome caused by viruses of the family *Bunyaviridae*, genus *Hantavirus*. Several different human pathogenic hantaviruses are known globally [19-21]. All hantavirus infections initial symptoms are similar, including an abrupt onset of high fever, malaise, myalgia and other flu-like symptoms. Increased vascular permeability leading to hypotension, thrombocytopenia and leukocytosis are common factors of HFRS. The Hantaan (HTN), Seoul (SEO) and Dobrova (DOB) are subtypes causing moderate to severe HFRS in Asia and Europe, whereas Puumala (PUU) causes a mild form of HFRS in central Europe and Scandinavia also referred to as nephropathia epidemica. This rodent-borne pathogens are normally transmitted to humans via aerosols generated from feces, urine, and saliva of infected rodents.

Hantavirus

With unknown pathogenic potentials, several novel hantavirus have been identified in a variety of insectivore hosts. New geographical distributions of hantaviruses have also been discovered and several new species were found in

Africa. Infections with hantavirus in humans can result in two clinical entities: HFRS and hantavirus cardiopulmonary syndrome (HCPS) caused by Old World and New World hantaviruses, respectively. The clinical features of HFRS varies from subclinical, mild, and moderate to severe, depending in part on the causative agent of the disease. Commonly, HFRS caused by Hantaan virus, Amur virus and Dobrava virus are more severe with mortality rates from 5 to 15%, whereas Seoul virus causes moderate and Puumala virus and Saaremaa virus cause mild forms of disease with mortality rates <1%.

Virology

Hantaviruses form a separate genus within the *Bunyaviridae* family and are enveloped RNA viruses, spherical in shapewith a diameter of 80 to 120 nm. The genome consists of three negative sense, single-stranded RNAs that share a 30 terminal sequence of the genome segments. The S (small), M (medium) and L (large) segments, encode the nucleoprotein (N), envelope glycoproteins (Gn and Gc), and the L protein or viral RNA-depended RNA polymerase, respectively [23]. Hantaviruses are readily inactivated by heat (30 min at 60°C), detergents, UV irradiation, organic solvents and hypochlorite solutions just like other enveloped viruses. By attaching the viral glycoprotein to the cell surface receptors, hantaviruses infects endothelial, epithelial, dendritic and lymphocyte cells.

Diagnosis

Acute hantavirus infections diagnosis is based on serology as virtually all patients have IgM and usually also IgG antibodies present in serum at the onset of symptoms. Serological tests which are commonly used include indirect IgM and IgG ELISA as well as IgM captures ELISAs, which have higher specificity than indirect ELISAs. IFA assays are also regularly used for diagnostics but have lower

specificity [24, 25]. Additionally, rapid 5-minute user-friendly immunochromatographic IgM-antibody tests have been developed and are available commercially [26, 27]. The confirmation of hantavirus infection can also be done by detection of hantavirus genome in blood or serum samples by RT-PCR. Traditional and quantitative, both RT-PCR are used to detect viremia [28-29]. Viral RNA can usually be detected if an acute sample is available even though the presence of viremia varies. Higher viremia is found in more severe hantavirus infections (DOBV, SNV, ANDV), compared with milder infections, caused by PUUV [31-34]. By detecting viral RNA, hantavirus infection has been confirmed even before the presence of specific antibodies [35].

Flaviviruses

Some of the most important arboviral pathogens of man are included in the genus *Flavivirus*, family *Flaviviridae*. This genus comprises over 70 viruses, many of which, such as the dengue (DEN) viruses, Japanese encephalitis (JE) virus,, and yellow fever (YF) virus are important human pathogens. The majority of exposed individuals will not develop disease, but a minority will develop a severe illness with a significant chance of permanent neurological damage or death. The components that determine this are many, involving complex interactions between virus and host and are still being actively uncovered. In numerous cases it appears that the immune response, while crucial to containing the virus and limiting spread to the brain, is also responsible for causing neurological damage. Innate responses can limit viral replication but may also be responsible for generating pathological levels of inflammation. Antibody responses which are neutralizing are protective but take time to develop. There is a role of T cells but it is less clear, and may be either protective or pathogenic.

Virology

The genome of Flavivirus is a single stranded, positive-sense, ~11 kb RNA genome with a single open reading frame that is directly translated into a polyprotein precursor [40]. Two surface viral proteins are present in the virus particles one is the E (envelope) glycoprotein, which is the major determining antigen and is involved in binding and fusion during viral entry, and the other is the M (membrane) protein, which is part of precursor prM, formed during the maturation of virus particle [40].

Epidemiology and clinical features

These viruses are known as arboviruses (arthropod-borne viruses) as they are transmitted by arthropods (insects or ticks), and except for dengue, they are all zoonotic (i.e. animal viruses that spill over into humans). JEV is transmitted principally among birds by *Culex* mosquitoes that breed in muddy water. Despite of being genetically closely related viruses, they are found in geographically different parts of the globe; however, they are tending to spread and can cause unexpected outbreaks. MVEV occurs in Australia and nearby islands, JEV occurs in the Asia-Pacific region and SLEV is confined to the Americas [41]; WNV was found in Africa and the Middle East, but in recent years has caused outbreaks in Southern Europe, and reached America, where it rapidly spread across the continent [42]. TBEV with a different ecology and epidemiology is transmitted between small mammals in the forests of central Europe and Russia by hard (ixodid) ticks. Various infections caused by these viruses are asymptomatic, or cause a non-specific febrile illness.

II. Materials and Methods

Study sites

The blood samples were collected from all the clinically suspected cases with SFTS, HFRS and Flavivirus from two different hospitals located in Jeollanamdo Province located in south-west region of ROK (34°10′N; 126°55′E) for one calendar year. The wild rodents were captured in a sylvatic habitat located within the west of the farmlands area in south-west of ROK, in the month of August, October and November using live-traps. The organ samples of the euthanized mice were stored at –20 °C until further experiment.

Clinical Case Definition

SFTS Case Definition

All clinically suspected cases of SFTS who had fever, vomiting, diarrhea with laboratory parameters showing leukopenia, and/or thrombocytopenia without another known acute infectious disease were included in this study. Acute-phase blood samples were obtained within 4-13 days after onset of illness.

HFRS Case Definition

All cases with features of fever, hemorrhage, and hypotension, renal failure with laboratory findings of leukocytosis, proteinuria and hematuria were clinically suspected cases and thus, samples were collected and analyzed.

Flavivirus Case Definition

The suspected flavivirus infected cases were screened for YFV virus, JEV and Dengue virus.

Extraction of Viral RNA

Total RNA was extracted from both whole blood and plasma of collected patient samples by using Viral Gene Spin™ Viral RNA Extraction Kit (iNTRON Biotechnology, Korea) according to manufacturer's instruction. Similarly the mice organ samples (lung) were homogenized by grinding with sterile Cell Strainer 70 µm and then total RNA was extracted as above.

Preparation of Viral cDNA

From the extracted viral RNA, cDNA was prepared using SuperScript VILO MasterMix (Invitrogen, Life technologies). cDNA was prepared in a total volume of 20 µl by mixing following volume: VILO™ MasterMix, 4 µl; RNA, 8 µl and distilled water 8 µl. The condition used was 25°C for 10 mins, followed by 42°C for 60 mins and 85°C for 5 mins.

PCR Amplifications

RT-PCR targeting 'M' segment for SFTSV detection

RT-PCR was performed using extracted RNA and specific primers – SFTS-F/SFTS-R for SFTSV targeting 'M' 'segment (Table 1). The PCR was performed in a total volume of 30 µL. Each PCR mixture consisted of 2x one step RT-PCR premix (Diastar, Solgent Co. Ltd) 15 µL, distilled water 8 µL, 1 µL of primers (10pmol/µL) and 5 µL of RNA template. The SFTS positive control was received from Centre For Disease Control (CDC,Korea) of 410-bp fragment . PCR cycling consisted of initial denaturation at 50◦C for 30 min, followed by annealing at 95°C for 15 m and then followed by 35 cycles of denaturation at 95 ◦C for 20 s, annealing at 58 ◦C for 40 s, and elongation at 72 ◦C for 30 s, and final extension at 72 ◦C for 5 min. The amplification was carried out in an AB thermal cycler (Applied Biosystem, Inc.). Table 2 shows the conditions of the PCRs. Amplified

products were separated by electrophoresis on 1.5% agarose gel and visualized by ethidium bromide.

RT-Nested PCR targeting 'L' segment for hantanvirus detection

RT-N-PCR was performed using prepared cDNA and specific primers HAN-L-F1/HAN-LR1 and HAN-L-F2/HAN-LR2 for Hantanvirus targeting 'L' segment (Table 1). The first and nested PCRs were performed in a total volume of 20 µl in AccuPower^{R} PCR PreMix (Bioneer Corp.). Each PCR mixture consisted of 16 µL of distilled water, 1 µL of each primer (10pmol/ µL) and 2 µL of cDNA template. The RT-N-PCR produced a 380-bp fragment. First PCR cycling consisted of initial denaturation at 95 ◦C for 7 min, followed by 35 cycles of denaturation at 95 ◦C for 30 s, annealing at 49 ◦C for 30 s, and elongation at 72 ◦C for 45 s. The second PCR cycle consisted of 95 °C for 5 min, followed by 35 cycles of denaturation at 95 ◦C for 20 s, annealing at 54 ◦C for 20 s, and elongation at 72 ◦C for 30 sec, and final extension at 72 ◦C for 7 min. The amplification was carried out in an AB thermal cycler (Applied Biosystem, Inc.). Table 2 shows the conditions of the PCRs. Amplified products were separated by electrophoresis on 1.5% agarose gel and visualized by ethidium bromide.

RT-PCR targeting 'NS5' gene for Flavivirus detection

RT-PCR was performed using prepared cDNA two specific primers (first set primers- Flavi all S and Flavi all AS 1, and second set- Flavi_PF1S and Flavi_PF2R) for Flavivirus targeting NS5 gene (Table 1). The PCR was performed in a total volume of 20 µL in AccuPower^{R} PCR PreMix (Bioneer Corp.). Each PCR mixture consisted of 16 µL distilled water, 1 µL of each primers and 2 µL of cDNA template. The first set primer produced 264 bp amplification and second set produced 272 bp fragment. PCR cycling consisted of initial denaturation at 95◦C for 10 min, followed by 35 cycles of denaturation at 95 ◦C for 20 s, annealing at 56 ◦C for 20 s for the first set and 50 °C for 20 s, and elongation at 72 ◦C for 20 s, and

final extension at 72 ◦C for 7 min. The amplification was carried out in an AB thermal cycler (Applied Biosystem, Inc.). Table 2 shows the conditions of the PCRs. Amplified products were separated by electrophoresis on 1.5% agarose gel and visualized by ethidium bromide.

Immunofluorescence Assay (IFA)

IFA for IgG titer estimation was done with wild mouse lung samples for detecting Hantavirus.

Nucleotide Sequencing

The nucleotide sequence of the amplified region of the 'M' segment for SFTS, 'L' segment for Hantan virus and 'NS5' gene for Flavivirus was determined by sequencing the positive isolates.The PCR products were purified with QIAquick PCR purification kits (Qiagen). Sequencing was performed at the Medical Research Institute (Daejong, Republic of Korea).

Phylogenetic analysis

Phylogenetic trees were constructed by the neighbor-joining (N-J) method using ClustalX software program Representative hantaviruses sequences from GeneBank, including Hantavirus isolates from China, Japan and previously identified HTNV sequences from ROK were included in the phylogenetic analysis. Similarly, representative SFTSV strains from China and Japan were included and the tree was constructed on the basis of nucleic acid sequences of the 'M' segment gene by using the neighbor-joining method. Genetic distances were computed using PAUP version 4.0b and topologies were evaluated by bootstrap analysis of 1000 iterations.

III. Results

In a calendar year January to December, from two University Hospital of Gwangju city namely Chosun University Hospital and Chonnam National University Hospital, we collected 267 blood samples from suspected cases of SFTS. Similarly for the year, we also collected 295 blood samples from patients admitted in these hospitals with the manifestation suspected of HFRS**.** We further screened for the presence of Flaviviruses including YFV, JEV and DENV in patients.

We also collected 41 wild mice (striped field mouse; *A. agrarius*) in the months of August, October and November, to detect the presence of SFTSV, Hantavirus and Flavivirus as wild mouse are largely considered as the natural reservoirs of these pathogenic viruses. All the three viral pathogens were investigated in the mouse organ samples (Lung) by molecular methods as for human samples**.**

RT-PCR targeting 'M' segment for SFTSV detection from patient whole blood and plasma samples

RT-PCR targeting the 'M' segment of the SFTSV detected six positive samples out of a total 267 patients.. All the positive results were detected from patient's plasma samples, while all the whole blood samples were negative. Chonnam National University Hospital samples which are positive is designated by JN. Primers and conditions used is mentioned in Table 1 and 2. These positive samples were sent for sequencing and all the 6 positive samples were identified as SFTSV (Table 3).

RT-Nested PCR targeting 'L' segment for hantavirus detection from patient whole blood and plasma

RT-N-PCR targeting the 'L' segment for Hantavirus detected eight positive samples out of a total 295 patients. One interesting finding was the detection of

hantavirus from one of the patient plasma as well as whole blood specimen ,rest of the 7 patients' samples which were detected positive were all from plasma specimens. All the positive samples were sequenced and all were found to be HTNV (Table 4).

RT-PCR targeting 'NS5' gene for Flavivirus detection from patients' whole blood and plasma

RT-PCR targeting the 'NS5' gene of the Flavivirus was carried out on to rule out viruses belonging to this family including Japanese Encephalitis, Dengue and Yellow fever viruses in a screening test. But none of the patients were found positive for this group of virus.

RT-PCR targeting 'M' segment for SFTSV detection from mouse organ

The wild mouse lung samples were investigated for SFTSV by targeting the 'M' segment using RT-PCR. All 41 mouse samples were found to be negative for SFTSV.

RT-N-PCR targeting 'L' segment for hantavirus detection from mouse organ

RT-N-PCR targeting 'L' segment detected 19 out of 41 wild mouse lung samples, positive for hantaviruses. All the positive samples were sequenced and all were found to be HTNV (Table 5).

RT-PCR targeting 'NS5' gene for Flavivirus detection from mouse organ

Using the same mouse lung samples RT-PCR targeting the 'NS5' gene was carried on to screen for the presence of DENV, JEV and YFV viruses belonging to

this group. All the mouse samples were found to be negative for this group of viruses.

IFA detection of mouse organ

When IFA was done with October and November mice lung sample 2 of the October and one of the November samples showed raised titer of HFRS Hantaan IgG (Table 6).

Phylogenetic tree analysis

Phylogenetic tree for partial M segment (480 bp) sequences of six isolates obtained from patients who were identified positive for SFTSV were compared with representative SFTS virus strains from China and Japan. The tree was constructed by using the neighbor-joining method. Phylogenetic analysis of SFTS positive patient samples showed close proximity to human strains from China and Japan (Figure 2).

Similarly, Phylogenetic tree for partial ‘ L’ segment (380 bp) sequences of eight positive isolates obtained from patients who were identified positive for hantavirus were compared with representative Hantaan virus, Seoul virus, Dobra virus, Muju virus, Imjin virus strains from different countries. All the detected strains in this study clustered around Hantaan virus and were in the same clad (Figure 3).

IV. Discussion

SFTS was first retrospectively isolated in China from in 2006; similarly the first case from Japan was reported in 2012. In ROK, the first clinically suspected patient was detected on Jeju Island on May 2013, and the first viral isolation was reported by Seoul National University Hospital from a retrospective study [67]. Till November 2013, a report from Korean Center for Disease Control indicated that cumulative confirmed cases of SFTS were 36 out of 404 (8.6%) suspicious cases, and fatal cases were 17 in South Korea [67]. Now considering our study, we detected 6 positive samples of SFTS out of 267 suspicious cases from two University Hospitals attributing to 2.2% positivity rate. Holding on to our finding ,it has become evident that SFTS is present in a noticeable number as SFTS endemic areas continue to expand out of China to nearby counties such as Korea and Japan.

As described in another report by Park et al, 2013, Japan isolates formed an independent cluster from the China isolates. In their study, SFTSV isolates formed 2 major clusters. Abundant of the Korea isolates formed a cluster with the Japan isolates, although some Korea and China isolates were included in the other group, perhaps not surprising given the geographic location of ROK between China and Japan. In this study also SFTSV positive isolates clustered around isolates from Japan and China as reported previously. (Figure 2)

One of the study by Xu et al, 2015 reported the presence of SFTSV by one-step real-time RT-PCR in rodent. They detected SFTSV genomic RNA in 2 of the 8 *A. agrarius*, but not in 40 *R. norvegicus* and 4 *R. losea.* Two of *A. agrarius lung* tissues were subjected to cell culture and SFTSV was detected at each passage of

cell cultures. But in this study we could not detect any SFTSV from wild rodent (*A. agrarius)* by RT-PCR. Difference in the molecular technique applied in our study and the variation of the geographical location and climatic factors may have attributed to our negative results .So more sensitive molecular technique and a larger number of samples must be investigated before reaching to any conclusion.

An etiologic agent of HFRS, HTNV, poses a serious health threat to military personnel training in field environments due to its mean duration of illness from the onset of symptoms to complete recovery, overall morbidity, and a mortality rate of 5–10% in the presence of good medical management [64]. Near the demilitarized zone (DMZ) in the ROK, HTNV transmission risks are especially high as rodent serological HTNV antibody positive (Ab+) rates were observed to be >60% during some survey periods [65]. Many hantavirus surveillance reports has previously been received from military training sites at elevations <50 m, which included host habitat characterization, seasonal small mammal population abundance and hantavirus Ab+ rates, and military activities that promoted increased infection risks [66].

A study by Choi et al, in 2013 described the epidemiology of HFRS in the past 10 year (2001-2010) in ROK. In this period, a total of 3,953 HFRS patients and an average prevalence rate of 0.81 per 100,000 populations were recorded, with a total of 40 fatal cases, corresponding to a case fatality rate of 1.01%. This study detected 8 positive samples out of 295 total suspicious cases of HFRS indicating the positivity rate of 3.1%. This finding suggests the presence of HFRS noteworthy in Korean population. The previous studies also reported the most prevalent Hantavirus causing HFRS as Hantaan virus which is similar to findings of our study too, which shows all positive samples to be Hantaan virus.

A. agrarius, is the natural host of HTNV in rural Korea. Ryou et al, 2011, reported the detection of partial S segment of Hantaan, Seoul, and Puumala viruses

in 766 lung samples of all captured animals and found HTNV RNA in 25 *A. agrarius* by using multiplex RT-PCR. Two isolates of HTNV were obtained from PCR-positive *A. agrarius* by cultivation in Vero E6 cells.

Out of 41 *A. agrarius* lung samples, 19 were positive for Hantaan virus by RT-N-PCR suggesting quiet a high rate of positivity in wild rodents in our study. This difference could be due to different geographical distribution, climatic variations or difference in molecular technique.

Although this study detected high rate of positivity of Hantaan virus in wild mice lung samples, the IFA for HFRS Hantaan IgG titer was seen to be raised only in 3 of the samples from October and November. Two of the IFA positive samples were also positive for RT-N-PCR for L segment, whereas other PCR positive samples showed less than 1:16 titre considering them to be negative by IFA (Table 6).

The genus *Flavivirus*, family *Flaviviridae*, contains some of the most important arboviral pathogens of man. The genus *Flavivirus* of the family *Flaviviridae* comprises over 70 viruses, many of which, such as the dengue DENV, JEV, and YFV are important human pathogens. In each case, the majority of exposed individuals will not develop disease, but a minority will develop a severe illness with a significant chance of permanent neurological damage or death.

Though Flaviviruses including DENV, JEV, and YFV are still circulating globally, its incidence is decreasing in ROK due to improved preventive measures including vaccination. In our study also we could not detect any positive patient or rodent samples suggesting the declining trend of Falvivirus infections.

V. Conclusion

In this study, 2.2 % (6/267) of patients' plasma samples were positive for SFTS and 3.1 % (9/295) were found positive for HFRS. We noted that plasma samples showed more sensitivity then the whole blood samples for detection of both SFTSV and HTNV. On the other hand none of the mouse lung samples were positive for SFTSV but a proportionally very high rate of Hantaviruse prevalence in mice; 46.3% (19/41) was found.

Phylogenetic analysis of SFTS positive patient samples showed close proximity to human strains from China and Japan as described in other reports, perhaps not surprising given the geographic location of South Korea between China and Japan.

Similarly, phylogenetic analysis of HFRS positive samples showed clustering in one group which showed close affinity to Hantaan virus, suggesting this virus as more prevalent hantaviruses from the family Bunyaviridae causing HFRS. Although we did not detect any positive result for SFTSVs and Flaviviruses in wild mouse lung samples, it may be, due, in part to small sample-size in this geographical region. This study suggests that SFTS and HFRS are prevalent in human population at a reasonable high rate than thought previously. Other amazing finding was high prevalence of HTNV in the wild mouse samples.

Thus, SFTS and HFRS have been endemic in Korea, and these vector-borne infectious diseases have been circulating naturally within the country in a considerable rate at the present environmental scenario.

Table 1. Oligonucleotide primers, and probes used in this study.

PCR assay	Primers and probe name (sequence)	Product size (bp)	Ref
SFTS RT-PCR	SFTS-F (5'-ACCTCTTTGACCCTGAGTTWGACA-3')	560	Von et al., (2009)
	SFTS-R (5'- CTGAAGGAGACAGGTGGAGATGA-3')		
HFRS RT- N-PCR (external primer)	HAN-L-F1 (5'-ATGTAYGTBAGTGCWGATGC-3')	450	Klempa et al., (2006)
	HAN-L-R1 (5'-AACCADTCWGTYCCRTCATC-3')		
HFRS RT- N-PCR (internal primer)	HAN-L-F2 (5'-TGCWGATGCHACIAARTGGTC-3')	380	Klempa et al., (2006)
	HAN-L-R2 (5'-GCRTCRTCWGARTGRTGDGCAA-3')		
Flavi C-RT-PCR	Flavi all S (5'-TACAACATGATGGGGAARAGAGARAA-3')	264	Patel et al., (2013)
	Flavi all AS 1 (5'-GTCCCANCCDGCKGTRTC-3')		

Table 2. PCR conditions used in this study.

PCR assay	Name of primers	PCR conditions				PCR Product Size (bp)
		Denaturation (°C/sec)	Annealing (°C/sec)	Extension (°C/sec)	Cycles	
SFTS RT-PCR	SFTS-F SFTS-R	95/20	58/40	72/30	35	510
HFRS RT- N-PCR (external primer)	HAN-L-F1 HAN-L-R1	95/30	49/30	72/45	35	450
HFRS RT- N-PCR (internal primer)	HAN-L-F2 HAN-L-R1	95/20	54/20	72/30	35	380
Flavi C-RT-PCR	Flavi all S Flavi all AS 1	95/30	56/30	72/30	35	264

Table 3. Sequencing result and identification of SFTSV from six patients' serum samples

Sample Name	Length	Sequence (SFTS-F/SFTS-R)	Blast result
2015-87	546	GTCCATGCTGATTCTAAACTTGTTTCGTGCAGGCAAGGGAGCG GAAATATGAAGGAATGTGTCACAACTGGGAGGGCGCTCCTTCC TGCGGTGAACCCAGGACAAGAGGCATGTCTGCACTTCACGGCA CCTGGGAGTCCGGACTCAAAATGTCTCAAAATCAAGGTTAAGA GGATCAACCTAAAATGTAAGAAGTCATCATCATATTTTGTTCCT GATGCTCGGTCCAGGTGTACATCTGTGAGGAGATGTCGCTGGG CAGGAGACTGCCAGTCTGGGTGCCCCTCTCATTTCACGTCCAAC TCCTTCTCTGATGATTGGGCAGGTAAAATGGATAGGGCTGGTTT AGGATTTAGTGGGTGCTCTGATGGGTGTGGAGGAGCAGCCTGC GGCTGCTTTAATGCGGCCCCTTCATGCATCTTTTGGAGGAAATG GGTAGAGAATCCACATGGGATCATCTGGAAAGTATCTCCATGT GCTGCATGGGTCCCTTCAGCAGTCATAGAGCTAACAATGCCCTC GGGGGAAGTGAGGACATTCCACCCC	Severe fever with thrombocytopenia syndrome virus strain Zhao segment M, complete sequence
2015-JN01	541	GTCCATGCTGATTCTAAGCTTGTCACATGCAGGCAAGGGAGCG GAAATATGAAGGAATGTGTCACAACTGGGAGGGCGCTTCTTCC TGCAGTGAACCCAGGTCAAGAGGCATGTCTTCACTTCACGGCA CCTGGGAGTCCGGACTCAAAATGTCTCAAAATTAAGGTTAAGA GGATCAACCTAAAATGTAAGAAGTCATCATCATATTTTGTTCCT GATGCCCGGTCCAGATGTACATCAGTGAGGAGATGTCGTTGGG CTGGAGACTGCCAGTCTGGGTGCCCCCCTCATTTCACATCCAAC TCCTTTTCTGATGATTGGGCAGGTAAGATGGACAGGGCTGGTCT AGGGTTCAGTGGCTGCTCTGATGGGTGTGGAGGAGCAGCCTGC GGCTGCTTTAATGCAGCCCCTTCATGCATC	Huaiyangshan virus isolate 2010-WWG segment M, complete sequence
2015-JN14	543	CATGCTGATTCTAAACTTGTTTCGTGCAAGCAAGGGAGCGGAA ATATGAAGGAATGTGTCACAACTGGGAGGGCGCTCCTTCCTGC GGTGAACCCAGGACAAGAGGCATGTCTGCACTTCACGGCACCA GGGAGTCCGGACTCAAAATGTCTCAAAATTAAAGTTAAGAGGA	Severe fever with thrombocytopenia syndrome virus viral cRNA, segment M, complete sequence, strain:

		TTAACCTGAAATGCAAGAAGTCATCATCATATTTCGTTCCTGAC GCTCGGTCCAGGTGTACATCTGTGAGGAGATGCCGCTGGGCAG GAGACTGTCAGTCTGGGTGCCCCTCTCATTTCACGTCCAACTCC TTTTCTGATGATTGGGCAGGTAAAATGGACAGGGCTGGTCTAG GATTCAGTGGGTGCTCTGATGGATGTGGAGGAGCAGCCTGCGG CTGCTTTAATGCGGCCCCTTCATGCATCTTCTGGAGGAAATGGG TAGAGAATCCACATGGGATCATCTGGAAAGTATCTCCATGTGC TGCATGGGTTCCATCAGCAGTCATAGAGCTAACAATGCCCTCA GGGGAAGTGAGGACATTCCCCCCC	SPL075A
2015-JN26	478	TAAACTTGTTTCGTGCAGGCAAGGGAGCGGAAATATGAAGGAG TGTGTCACAACTGGGAGGGCGCTCCTTCCTGCGGTGAACCCAG GACAAGAGGCATGTCTGCACTTCACTGCACCTGGGAGTCCGGA CTCAAAGTGTCTCAAAATCAAGGTTAAGAGGATCAACCTAAAA TGTAAGAAGTCATCATCATATTTTGTTCCTGATGCTCGGTCCAG GTGTACATCTGTGAGGAGATGTCGCTGGGCAGGAGACTGCCAG TCTGGGTGCCCCTCTCATTTCACGTCCAACTCCTTCTCTGATGAT TGGGCAGGTAAAATGGACAGGGCTGGTCTAGGATTCAGTGGGT GCTCTGATGGGTGTGGAGGAGCAGCCTGCGGCTGCTTTAATGC GGCCCCTTCATGCATCTTTTGGAGGAAATGGGTAGAGAATCCA CATGGGATCATCTGGAAAGTATCCCCATGTGCTGCATGGGTCCC T	Severe fever with thrombocytopenia syndrome virus strain ZJZHSH-LWL/China/08/2014 segment M, complete sequence
2015-JN30	522	TAAACTTGTTTCGTGCAGGCAAGGGAGCGGAAATATGAAGGAA TGTGTCACAACTGGGAGGGCGCTCCTTCCTGCGGTGAACCCAG GACAAGAGGCATGTCTGCACTTCACGGCACCTGGGAGTCCGGA CTCAAAATGTCTCAAAATCAAGGTTAAGAGGATCAACCTAAAA TGTAAGAAGTCATCATCATATTTTGTTCCTGATGCTCGGTCCAG GTGTACATCTGTGAGGAGATGTCGCTGGGCAGGAGACTGCCAG TCTGGGTGCCCCTCTCATTTCACGTCCAACTCCTTCTCTGATGAT TGGGCAGGTAAAATGGATAGGGCTGGTCTAGGATTTAGTGGGT GCTCTGATGGGTGTGGAGGAGCAGCCTGCGGCTGCTTTAATGC GGCCCCTTCATGCATCTTTTGGAGGAAATGGGTAGAGAATCCA CATGGGATCATCTGGAAAGTATCTCCATGTGCTGCATGGGTCCC TTCANCAGTCATAGAGCTAACAATGCCCTCGGGGGAAGTGAGG	Severe fever with thrombocytopenia syndrome virus strain Zhao segment M, complete sequence

2015-JN35	478	TAAACTTGTTTCGTGCAGGCAAGGGAGCGGAAATATGAAGGAG TGTGTCACAACTGGGAGGGCGCTCCTTCCTGCGGTGAACCCAG GACAAGAGGCATGTCTGCACTTCACTGCACCTGGGAGTCCGGA CTCAAAGTGTCTCAAAATCAAGGTTAAGAGGATCAACCTAAAA TGTAAGAAGTCATCATCATATTTTGTTCCTGATGCTCGGTCCAG GTGTACATCTGTGAGGAGATGTCGCTGGGCAGGAGACTGCCAG TCTGGGTGCCCCTCTCATTTCTCGTCCAACTCCTTCTCTGATGAT TGGGCAGGTAAAATGGACAGGGCTGGTCTAGGATTCAGTGGGT GCTCTGATGGGTGTGGAGGAGCAGCCTGCGGC	Severe fever with thrombocytopenia syndrome virus strain ZJZHSH-ZLD/China/06/2012 segment M, complete sequence

Table 4. Sequencing result and Identification of HFRS (HTNV) from eight patients serum samples and one whole blood sample (by HAN-L-F2/HAN-L-R1; HFRS RT-N-PCR)

Sample Name	Length	Sequence (HAN-L-F2/HAN-L-R1)	Blast result
Chosun 1 (plasma)	360	GAAATGGTCACCAGGAGATAACTCAGCAAAATTCAGGCGGTTCACATCTATGTTGCATAATGGCCTTCCCAATGATAAATTGAAAAATTGTGTTATTGATGCTTTGAAACAAGTATACAGAACTGATTTTTTTATGTCAAGGAAATTACGAAACTATATAGACAGTATGGAGAGTCATGATCCGCACATCAAGCAGTTCCTAGATTTCTTTCCTGACGGGCATCACGGGGAGGTTAAAGGAAACTGGTTACAAGGAAATCTTAATAAGTGTTCCTCACTCTTTGGAGTTGCAATGTCATTGCTGTTCAAGCAAGTATGGAACAATTTATTTCCAGAGCTAGATTGCTTTTTTGAGTTTGC	AB620033.1 Hantaan virus gene for RNA-dependent RNA polymerase, complete cds
Chosun 2 (plasma)	359	AAATGGTCACCAGGAGATAACTCAGCAAAATTCAGGCGGTTTACATCTATGTTGCATAACGGCCTTCCCAATGATAAATTAAAAAATTGTGTTATTGATGCCTTGAAACAAGTATACAGAACTGATTTTTTTATGTCAAGAAAATTACGAAACTATATAGACAGTATGGAGAGTCATGATCCGCACATCAAGCAGTTCCTAGATTTCTTTCCTGATGGGCACCACGGGGAGGTTAAAGGAAACTGGTTGCAAGGAAATCTTAATAAGTGTTCCTCACTATTTGGAGTTGCAATGTCATTGCTGTTCAAGCAAGTATGGAACAATTTATTTCCAGAATTAGATTGCTTTTTTGAGTTTGC	AB620033.1 Hantaan virus gene for RNA-dependent RNA polymerase, complete cds
Chosun 3 (plasma)	340	GAAATGGTCACCAGGAGATAACTCAGCAAAATTCAGGCGGTTTACATNTATGTTGCATAACGGCCTTCCCAATGATAAATTAAAAAATTGTGTTATTGATGCCTTGAAACAAGTATACAGAACTGATTTTTTTATGTCAAGAAAATTACGAAACTATATAGACAGTATGGAGA	AB620033.1 Hantaan virus gene for RNA-dependent RNA polymerase, complete cds

		GTCATGATCCGCACATCAAGCAGTTCCTAGATTTC TTTCCTGATGGGCACCACGGGGAGGTTAAAGGAA ACTGGTTGCAAGGAAATCTTAATAAGTGTTCCTCA CTATTTGGAGTTGCAATGTCATTGCTGTTCAAGCA AGTATGGAACAATTTATTTCCAGAATTA	
Chosun 3 (blood)	360	GAAATGGTCACCAGGAGATAACTCAGCAAAATTC AGGCGGTTTACATCTATGTTGCATAACGGCCTTCC CAATGATAAATTAAAAAAGTGTGTTATTGATGCCT TGAAACAAGTATACAGAACTGATTTTTTTATGTCA AGAAAATTACGAAACTATATAGACAGTATGGAGA GTCATGATCCGCACATCAAGCAGTTCCTAGATTTC TTTCCTGATGGGCACCACGGGGAGGTTAAAGGAA ACTGGTTGCAAGGAAATCTTAATAAGTGTTCCTCA CTATTTGGAGTTGCAATGTCATTGCTGTTCAAGCA AGTATGGAACAATTTATTTCCAGAATTAGATTGCT TTTTTGAGTTTGC	AB620033.1Hantaan virus gene for RNA-dependent RNA polymerase, complete cds
Chosun 4 (plasma)	360	GAAATGGTCACCAGGAGATAACTCAGCAAAATTC AGGCGGTTTACATCTATGTTGCATAACGGCCTTCC CAATGATAAATTAAAAAATTGTGTTATTGATGCCT TGAAACAAGTATACAGAACTGATTTTTTTATGTCA AGAAAATTACGAAACTATATAGACAGTATGGAGA GTCATGATCCGCACATCAAGCAGTTCCTAGATTTC TTTCCTGATGGGCACCACGGGGAGGTTAAAGGAA ACTGGTTGCAAGGAAATCTTAATAAGTGTTCCTCA CTATTTGGAGTTGCAATGTCATTGCTGTTCAAGCA AGTATGGAACAATTTATTTCCAGAATTAGATTGCT TTTTTGAGTTTGC	AB620033.1 Hantaan virus gene for RNA-dependent RNA polymerase, complete cds
Chosun 5 (plasma)	360	GAAATGGTCACCAGGAGATAACTCAGCAAAATTC AGGCGGTTTACATCTATGTTGCATAACGGCCTTCC CAATGATAAATTAAAAAATTGTGTTATTGATGCCT TGAAACAAGTATACAGAACTGATTTTTTTATGTCA AGAAAATTACGAAACTATATAGACAGTATGGAGA GTCATGATCCGCACATCAAGCAGTTCCTAGATTTC	AB620033.1 Hantaan virus gene for RNA-dependent RNA polymerase, complete cds

		TTTCCTGATGGGCACCACGGGGAGGTTAAAGGAA ACTGGTTGCAAGGAAATCTTAATAAGTGTTCCTCA CTATTTGGAGTTGCAATGTCATTGCTGTTCAAGCA AGTATGGAACAATTTATTTCCAGAATTAGATTGCT TTTTTGAGTTTGC	
Chosun 6 (plasma)	360	GAAATGGTCACCAGGAGATAACTCAGCAAAATTC AGGCGGTTTACATCTATGTTGCATAACGGCCTTCC CAATGATAAATTAAAAAATTGTGTTATTGATGCCT TGAAACAAGTATACAGAACTGATTTTTTTATGTCA AGAAAATTACGAAACTATATAGACAGTATGGAGA GTCATGATCCGCACATCAAGCAGTTCCTAGATTTC TTTCCTGATGGGCACCACGGGGAGGTTAAAGGAA ACTGGTTGCAAGGAAATCTTAATAAGTGTTCCTCA CTATTTGGAGTTGCAATGTCATTGCTGTTCAAGCA AGTATGGAACAATTTATTTCCAGAATTAGATTGCT TTTTTGAGTTTGC	AB620033.1 Hantaan virus gene for RNA-dependent RNA polymerase, complete cds
Chosun 7 (plasma)	360	GAAATGGTCACCAGGAGATAACTCAGCAAAATTC AGGCGGTTTACATCTATGTTGCATAACGGCCTTCC CAATGATAAATTAAAAAATTGTGTTATTGATGCCT TGAAACAAGTATACAGAACTGATTTTTTTATGTCA AGAAAATTACGAAACTATATAGACAGTATGGAGA GTCATGATCCGCACATCAAGCAGTTCCTAGATTTC TTTCCTGATGGGCACCACGGGGAGGTTAAAGGAA ACTGGTTGCAAGGAAATCTTAATAAGTGTTCCTCA CTATTTGGAGTTGCAATGTCATTGCTGTTCAAGCA AGTATGGAACAATTTATTTCCAGAATTAGATTGCT TTTTTGAGTTTGC	AB620033.1 Hantaan virus gene for RNA-dependent RNA polymerase, complete cds

Chosun 8 (plasma)	356	TGGTCACCAGGAGATAACTCAGCAAAATTCAGGCGGTTCACATCTATGTTGCATAATGGCCTTCCCAATGATAAATTAAAAAATTGTGTTATTGATGCTTTGAAGCAAGTATACAGAACTGATTTTTTTATGTCAAGAAAATTACGAAATTATATAGACAGTATGGAGAGTCATGATCCGCACATCAAGCAGTTCCTAGATTTCTTTCCTGATGGGCATCACGGGGAGGTTAAAGGAAACTGGTTGCAAGGAAATCTTAATAAGTGTTCCTCACTCTTTGGAGTTGCAATGTCATTGCTGTTCAAGCAAGTATGGAACAATTTATTTCCAGAGCTAGATTGCTTTTTGAGTTTGC	AB620033.1 Hantaan virus gene for RNA-dependent RNA polymerase, complete cds

Table 5. Sequencing result and Identification of HFRS (HTNV) mouse lung positive samples (by HAN-L-F2/HAN-L-R1; HFRS RT-N-PCR)

Sample Name	Length	Sequence	Blast result
10-1-M-L	356	TGGTCGCCAGGAGACAACTCAGCAAAATTCAGGCGGTTCACATCNATGTTG CATAATGGCCTTCCCAATGATAAATTAAAAAACTGTGTTATTGATGCTTTGAA ACAAGTATACAGAACTAATTTTTTTATGTCAAGGAAATTACGAAACTATATAG ACAGTATGGAGAGTCATGATCCGCACATCAAGCAGTTCTTAGATTTCTTTCCT GATGGGCACCACGGGGAGGTTAAAGGAAACTGGCTGCAAGGAAATCTTAA YAAGTGTTCCTCACTCTTTGGAGTTGCAATGTCATTGCTGTTCAAGCAAGTAT GGANCAATTTATTTCCNGAGCTAGATTGCTTTTTTGAGTTTGC	Hantaan virus XAAa10091712 nucleoprotein, glycoprotein, and putative RNA-dependent RNA polymerase genes, complete cds
10-9-M-L	311	ATGTTGCATAATGGCCTTCCCAATRATAAATTAAAAAACTGTGTTATTGATGCT TTGAAACAAGTATACAGAACTAATTTTTTTATGTCAAGGAAATTACGAAACTA TATNGACAGTATGGAGAGTCATGATCCGCACATCAAGCAGTTCTTAGATTTC TTTCCTGATGGGCACCACGGGGAGGTTAAAGGAAACTGGCTGCAAGGAAA TCTTAATAAGTGTTCCTCACTNTTTGGAGTTGCAATGTCATTGCTGTTCAANC AAGTATGGANCAATTTATTYCCNGAGCTNGATTGCTTTTTTGAGTTTGC	Hantaan virus XAAa10091712 nucleoprotein, glycoprotein, and putative RNA-dependent RNA polymerase genes, complete cds
11-1-M-L	358	AATGGTCGCCAGGAGACAACTCAGCAAAATTCAGGCGGTTCACATCTATGTT GCATAATGGCCTTCCCAATGATAAATTAAAAAACTGTGTTATTGATGCTTTGA AACAAGTATACAGAACTAATTTTTTTATGTCAAGGAAATTACGAAACTATATA GACAGTATGGAGAGTCATGATCCGCACATCAAGCAGTTCTTAGATTTCTTTC CTGATGGGCACCACGGGGAGGTTAAAGGAAACTGGCTGCAAGGAAATCTT AATAAGTGTTCCTCACTCTTTGGAGTTGCAATGTCATTGCTGTTCAAGCAAGT ATGGAGCAATTTATTTCCAGAGCTAGATTGCTTTTTTGAGTTTGC	Hantaan virus XAAa10091712 nucleoprotein, glycoprotein, and putative RNA-dependent RNA polymerase genes, complete cds
11-4-M-L	680	GGCCAGACAGCAGATTGGCTCAGTATTGTTATCTATCTAACATCATTTGTGGT TCCGATTCTTCTAAAGGCTCTATACATGCTAACAACACGAGGGAGGCAGACT ACAAAGGACAATAAAGGGACTAGGATCAGATTCAAGGATGATAGCTCTTTT GAGGATGTAAACGGAATTCGCAAGCCCAAACACCTTTATGTTTCGTTACCTA ATGCACAGTCTAGCATGAAAGCAGAGGAAATTACACCTGGCAGATACAGGA CAGCTGTTTGTGGACTTTATCCGGCCCAAATCAAGGCACGGCAAATGATTAG CCCTGTAATGAGTGTACTAGGATTTATGGCACTGGCTAAAGATTGGAGCGAA CGGATAGAGCAATGGTTAAGTGAACCCTGCAAACTTCTTCCTGACACATCGG CAGTCAGCCTCCTTGGTGGTCCAGCAACAAACAAGGATTACCTGCGGCAAA GACAGATTGCCTTAGGCAATATGGAAACCAAAGAGTCAAAAGCAATACGCC AACAAGCTGAGGCTGCAGGCTGCAGCATGATTGAGGACATTGATTCACCAT CATCAATTTGGGTCTTTGCTGGTGCACCAGATAGGTGCCCACCAACATGCCT TTTTATTGCAGGAATGGCAGAGCTTGGGGCATTCTTTTCTATTCTGCAGGAC ATGCGAAA	Hantaan virus strain Maaji-1 S segment nucleocapsid protein gene, complete cds

11-8-M-L	675	GGCCAGACAGCAGATTGGCTCAGTATTGTTATCTATCTAACATCATTTGTGGT TCCGATTCTTCTAAAGGCTCTATACATGCTAACAACACGAGGGAGGCAGACT ACAAAGGACAATAAAGGGACTAGGATCAGATTCAAGGATGATAGCTCTTTT GAGGATGTAAACGGAATTCGCAAGCCCAAACACCTTTATGTTTCGTTACCTA ATGCACAGTCTAGCATGAAAGCAGAGGAAATTACACCTGGCAGATACAGGA CAGCTGTTTGTGGACTTTATCCGGCCCAAATCAAGGCACGGCAAATGATTAG CCCTGTAATGAGTGTACTAGGATTTATGGCACTGGCTAAAGATTGGAGCGAA CGGATAGAGCAATGGTTAAGTGAACCCTGCAAACTTCTTCCTGACACATCGG CAGTCAGCCTCCTTGGTGGTCCAGCAACAAACAAGGATTACCTGCGGCAAA GACAGATTGCCTTAGGCAATATGGAAACCAAAGAGTCAAAAGCAATACGCC AACAAGCTGAGGCTGCAGGCTGCAGCATGATTGAGGACATTGATTCACCAT CATCAATTTGGGTCTTTGCTGGTGCACCAGATAGGTGCCCACCAACATGCCT TTTTATTGCAGGAATGGCAGAGCTTG	Hantaan virus strain Maaji-1 S segment nucleocapsid protein gene, complete cds
11-11-M-L	358	CTACGAAATGGTCGCCAGGAGACAACTCAGCAAAATTCNGGCGGTTCACAT CTATGTTGCATAATGGCCTTCCCAATGATAAATTAAAAAACTGTGTTATTGATG CTTTGAAACAAGTATACAGAACTAATTTTTTTATGTCAAGGAAATTACGAAAC TATATAGACAGTATGGAGAGTCNTGATCCGCACATCAAGCAGTTCTTAGATTT CTTTCCTGATGGGCACCACGGGGAGGTTAAAGGAAACTGGCTGCAAGGAA ATCTTAATAAGTGTTCCTCACTCTTTGGAGTTGCAATGTCATTGCTGTTCAAG CAAGTATGGAGCAATTTATTTCCAGAGCTAGATTGCTTTTTTGAGTTTGC	Hantaan virus XAAa10091712 nucleoprotein, glycoprotein, and putative RNA-dependent RNA polymerase genes, complete cds
11-13-M-L	359	AAATGGTCACCAGGAGATAATTCAGCAAAGTTCCGTAGGTTCACCT CCATGCTACATAACGGACTTCCCAATAATAAGCTAAAAAACTGTGTA ATTGATGCACTTAAACAAGTTTATAAGACAGATTTTTTTATGTCAAG GAAACTAAGGARTTATATTGACAGCATGGAAAGCCTTGACCCACAC ATCAAACAGTTTTTAGATTTTTTCCCTGATGGGCACCATGGGGAAGT GAAGGGAAACTGGCTGCAGGGTAACTTGAACAAGTGTTCTTCACT TTTCGGTGTTGCAATGTCATTACTATTTAAACAGGTATGGACTAACTT ATTCCCTGAGCTTGATTGTTTCTTTGAGTTTGC	Hantaan virus strain 76-118/POR segment L, complete sequence
11-15-M-L	325	GGCGGTTCACATCTATGTTGCATAATGGCCTTCCCAATGATAAATTAA AAAACTGTGTTATTGATGCTTTGAAACAAGTATACAGAACTAATTTT TTTATGTCAAGGAAATTACGAAACTATATAGACAGTATGGAGAGTCA TGATCCGCACATCAAGCAGTTCTTAGATTTCTTTCCTGATGGGCACC ACGGGGAGGTTAAAGGAAACTGGCTGCAAGGAAATCTTAATAAGT GTTCCTCACTCTTTGGAGTTGCAATGTCATTGCTGTTCAAGCAAGTA TGGANCAATTTATTTCCAGANCTNGATTGCTTTTTTGAGTTTGC	Hantaan virus XAAa10091712 nucleoprotein, glycoprotein, and putative RNA-dependent RNA polymerase genes, complete cds
8-3-M-L	362	ACGAAATGGTCGCCAGGAGACAACTCAGCAAAATTCAGGCGGTTC ACATCTATGTTGCATAATGGCCTTCCCAATGATAAATTAAAAAACTGT GTTATTGATGCTTTGAAACAAGTATACAGAACTAATTTTTTTATGTCA AGGAAATTACGAAACTATATAGACAGTATGGAGAGTCATGATCCGC ACATCAAGCAGTTCTTAGATTTCTTTCCTGATGGGCACCACGGGGA GGTTAAAGGAAACTGGCTGCAAGGAAATCTTAATAAGTGTTCCTCA CTCTTTGGAGTTGCAATGTCATTGCTGTTCAAGCAAGTATGGAGCA ATTTATTTCCAGAGCTAGATTGCTTTTTTGAGTTTGC	Hantaan virus XAAa10091712 nucleoprotein, glycoprotein, and putative RNA-dependent RNA polymerase genes, complete cds
8-8-M-L	362	ACGAAATGGTCGCCAGGAGACAANTCAGCAAAATTCAGGCGGTTC ACATCTATGTTGCATAATGGCCTTCCCAATGATAAATTAAAAAACTGT	Hantaan virus XAAa10091712

		GTTATTGATGCTTTGAAACAAGTATACAGAACTAATTTTTTTATGTCAAGGAAATTACGAAACTATATAGACAGTATGGAGAGTCATGATCCGCACATCAAGCAGTTCTTAGATTTCTTTCCTGATGGGCACCACGGGGAGGTTAAAGGAAACTGGCTGCAAGGAAATCTTAATAAGTGTTCCTCACTCTTTGGAGTTGCAATGTCATTGCTGTTCAAGCAAGTATGGAGCAATTTATTTCCAGAGCTAGATTGCTTTTTTGAGTTTGC	nucleoprotein, glycoprotein, and putative RNA-dependent RNA polymerase genes, complete cds
8-11-M-L	359	AAATGGTCGCCAGGAGACAACTCAGCAAAATTCAGGCGGTTCACATCTATGTTGCATAATGGCCTTCCCAATGATAAATTAAAAAACTGTGTTATTGATGCTTTGAAACAAGTATACAGAACTAATTTTTTTATGTCAAGGAAATTACGAAACTATATAGACAGTATGGAGAGTCATGATCCGCACATCAAGCAGTTCTTAGATTTCTTTCCTGATGGGCACCACGGGGAGGTTAAAGGAAACTGGCTGCAAGGAAATCTTAATAAGTGTTCCTCACTCTTTGGAGTTGCAATGTCATTGCTGTTCAAGCAAGTATGGAGCAATTTATTTCCAGAGCTAGATTGCTTTTTTGAGTTTGC	Hantaan virus XAAa10091712 nucleoprotein, glycoprotein, and putative RNA-dependent RNA polymerase genes, complete cds

Table 6. Comparison between results of IFA and RT-N-PCR of wild mice lung samples for the month of October and November

Wild mouse sample number	HRFS; Hantaan Virus IgG titre	RT-N-PCR targeting L segment
10-1	<1:16	Negative
10-2	<1:16	Negative
10-3	<1:16	Negative
10-4	<1:16	Negative
10-5	<1:16	Negative
10-6	**1:512 (Positive)**	Negative
10-7	<1:16	Negative
10-8	<1:16	Negative
10-9	<1:16	Negative
10-10	**1:512 (Positive)**	**Positive**
10-11	<1:16	**Positive**
10-12	<1:16	**Positive**
11-2	<1:16	**Positive**
11-6	<1:16	**Positive**
11-7	<1:16	Negative
11-8	<1:16	**Positive**
11-9	<1:16	**Positive**
11-10	<1:16	**Positive**
11-11	<1:16	**Positive**
11-12	<1:16	**Positive**
11-14	**1:2048 (Positive)**	**Positive**

Figure 1. Schematic diagram of the structure and genome of the SFTSV [1,10]. SFTSV genome included L, M, and S segments. The antisense RNA and the encoded ORFs are represented in orange and blue boxes respectively. In L, M, and S genomic RNAs, only the first 20 Nts of both the 5′ and 3′ termini of each segment are shown. The first 15 Nts of both the 5′ and 3′ termini of each segment are paired and conserved except for the mismatches A–C/G or C–U in red color and C–C or G–G in blue color. Note: The data were sourced from the report of Li et al. and processed in PHOTOSHOP 7.0 [1]

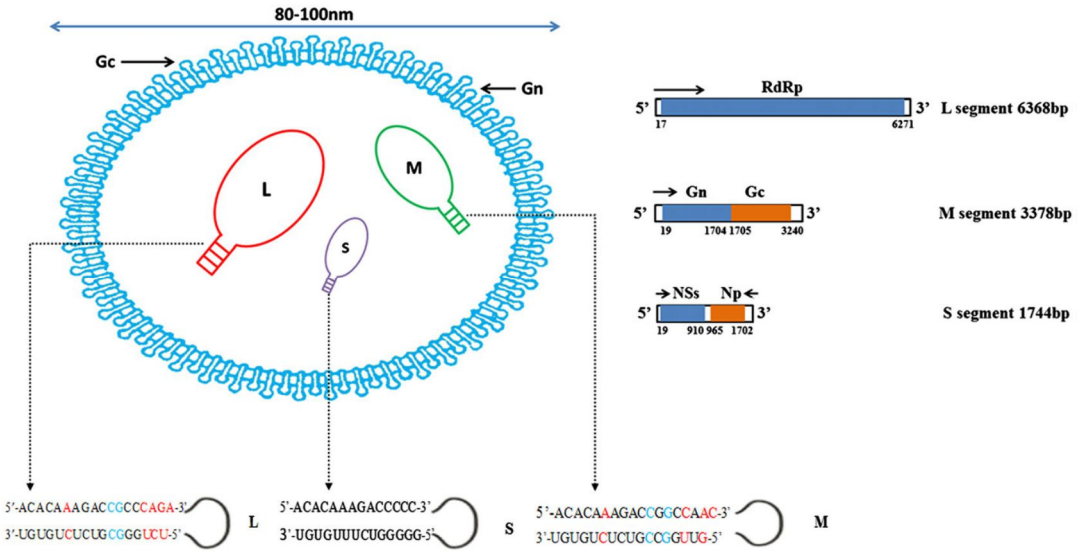


Figure 2. Phylogenetic tree based on M segment (480 bp) sequences identified positive for SFTSV (black arrow) compared with representative SFTS virus strains from China and Japan from Genebank. GeneBank accession number is indicated. Scale bar indicates 0.05% sequence distance. (Chonnam National University Samples are designated by JN).

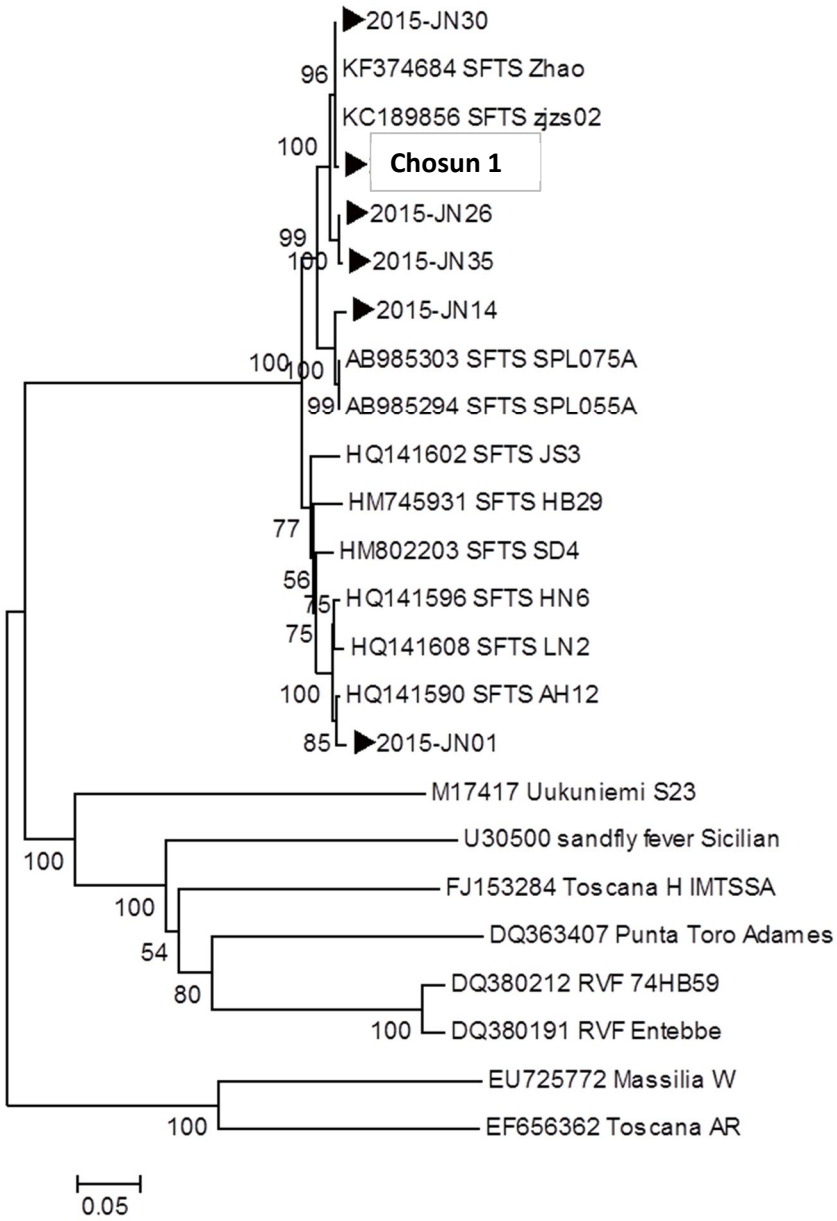


Figure 3. Phylogenetic tree based on L segment (380 bp) of eight isolates identified positive for HFRSV (black arrow) compared with hantaviruses sequences from Genebank. GeneBank accession number is indicated. Scale bar indicates 0.05% sequence distance.* All positive samples, Chosun 1 to Chosun 8 except for Chosun 3, are from PLASMA sample.

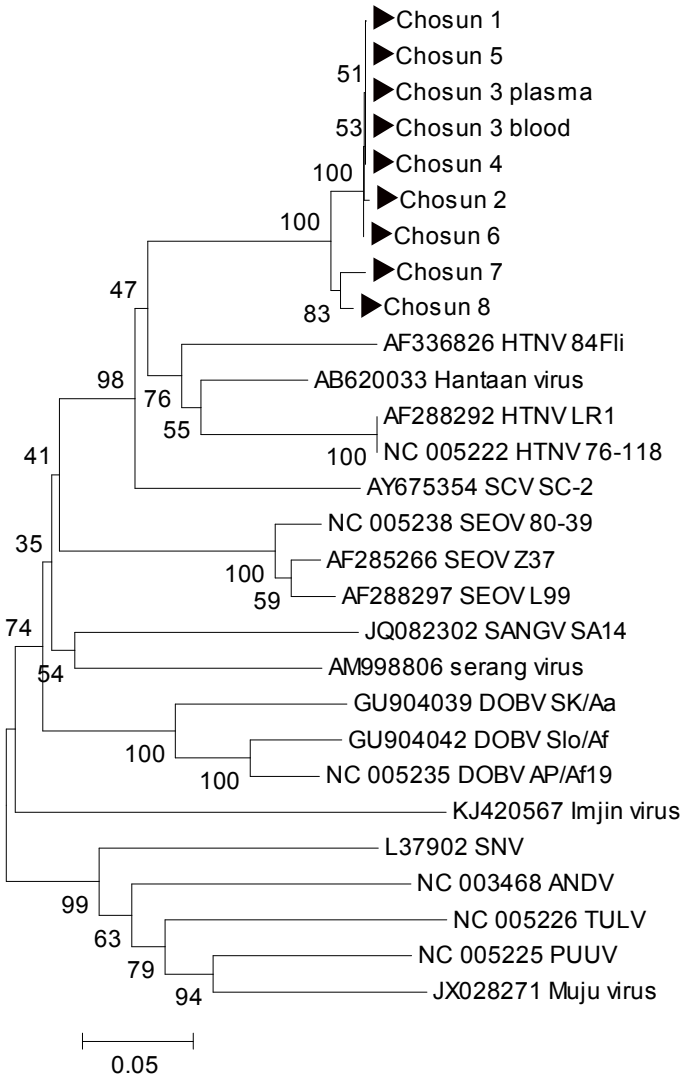


Figure 4. Agarose gel electrophoresis of the PCR product (560) after SFTS M segment targeting RT-PCR using patients specimens; Molecular marker (M), Negative control (Lane N), Positive control (Lane P), Chosun 1 (Lane 1), Chonnam 1 (Lane 2), Chonnam 2 (Lane 3), Chonnam 3 (Lane 4), Chonnam 4 (Lane 5), Chonnam 5 (Lane 6) (bands corresponds to the predicted product size).

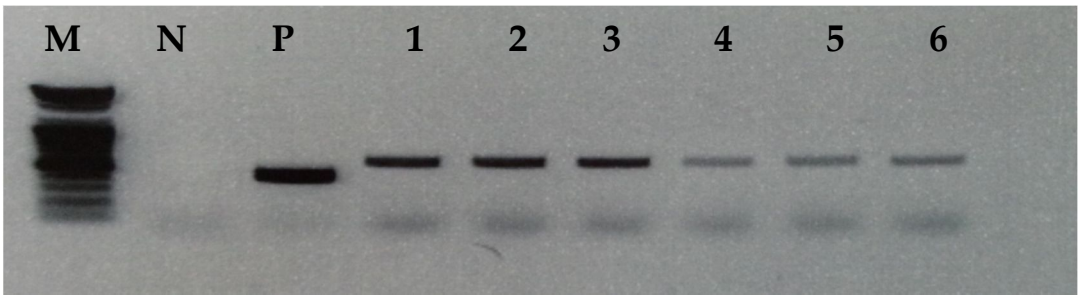


Figure 5. Agarose gel electrophoresis of the PCR product (380) after HFRSV L segment targeting RT-N-PCR using patients specimens; Molecular marker (M), Negative control (Lane N), Positive control (Lane P), Chosun 1 (Lane 1), Chosun 2 (Lane 2), Chosun 3 (Lane 3), Chosun 4 (Lane 4), Chosun 5 (Lane 5), Chosun 6 (Lane 6), Chosun 7 (Lane 7), Chosun 8 (Lane 8), Chosun 9 (Lane 9) (bands corresponds to the predicted product size).

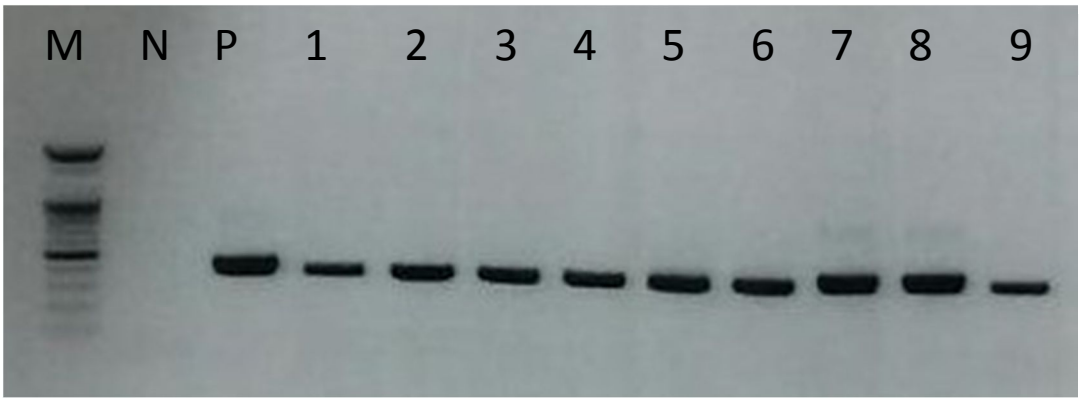


Figure 6. Agarose gel electrophoresis of the PCR product (380) after HFRSV L segment targeting RT-N-PCR using patients specimens; Molecular marker (M), Negative control (Lane N), Positive control (Lane P), 8-1 (Lane 1), 8 - 2 (Lane 2), 8 - 3 (Lane 3), 8 - 4 (Lane 4), 8 - 5 (Lane 5), 8 - 6 (Lane 6), 8 - 7 (Lane 7), 8 - 8 (Lane 8), 8 - 9 (Lane 9) 8 -10 (Lane 10), 8-11 (Lane 11), 8 -13 (Lane 12) (bands corresponds to the predicted product size).

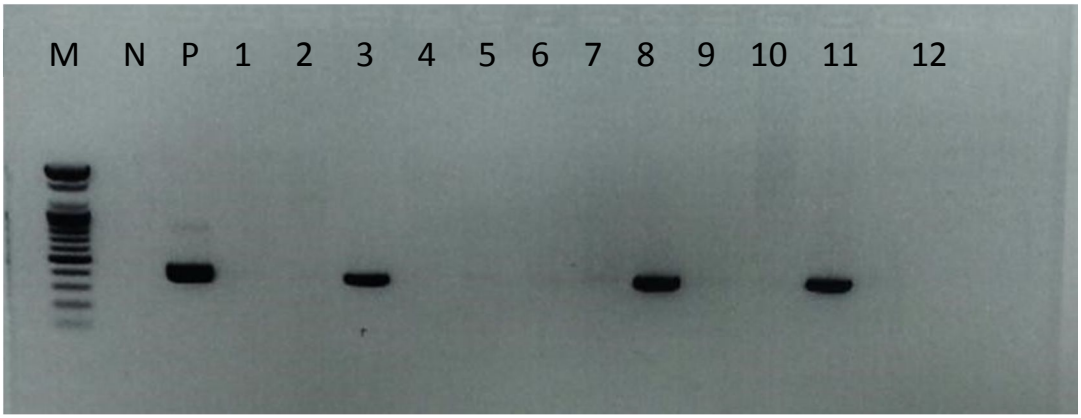


Figure 7. Agarose gel electrophoresis of the PCR product (380) after HFRSV L segment targeting RT-N-PCR using patients specimens; Molecular marker (M), Negative control (Lane N), Positive control (Lane P), 10-1 (Lane 1), 10 - 2 (Lane 2), 10 - 3 (Lane 3), 10 - 4 (Lane 4), 10 - 5 (Lane 5), 10 - 6 (Lane 6), 10 - 7 (Lane 7), 10 - 8 (Lane 8), 10 - 10 (Lane 9) 10 -11 (Lane 10), 10-12 (Lane 11), 11 -12 (Lane 12), 11 – 13 (Lane 13), 11 -14 (Lane 14) (bands corresponds to the predicted product size).

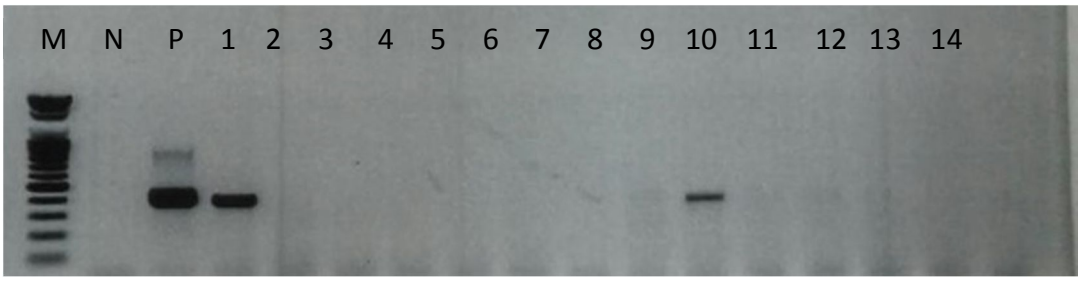
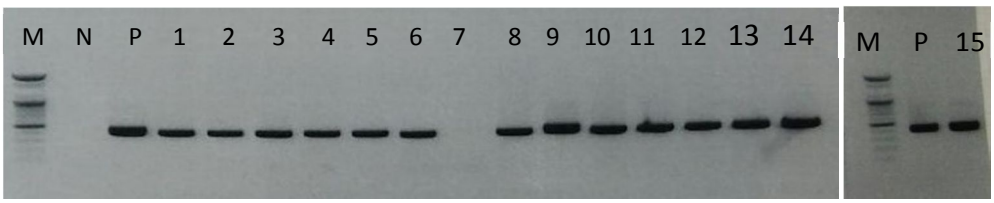


Figure 8. Agarose gel electrophoresis of the PCR product (380) after HFRSV L segment targeting RT-N-PCR using patients specimens; Molecular marker (M), Negative control (Lane N), Positive control (Lane P), 11-1 (Lane 1), 11 - 2 (Lane 2), 11 - 3 (Lane 3), 11 - 4 (Lane 4), 11 - 5 (Lane 5), 11 - 6 (Lane 6), 11 - 7 (Lane 7), 11 - 8 (Lane 8), 11 - 9 (Lane 9) 11 -10 (Lane 10), 11-11 (Lane 11), 11 -12 (Lane 12), 11 – 13 (Lane 13), 11 -14 (Lane 14), 11 -15 (Lane 15) (bands corresponds to the predicted product size).



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