[UCI]I804:24011-200000267088

Feb 2019

Master's Degree Thesis

Viral Hemorrhagic Fevers in Korea

Supervised by Professor Dong-Min Kim

A thesis submitted in partial fulfillment of the requirements for the Master of Biomedical Science

Graduate School of Chosun University

Department of Biomedical Sciences

Sehrish Jalal

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국내의 매개체 관련 바이러스성 출혈열

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Sehrish Jalal의 석사학위논문을 인준함

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2018년 11월

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ABBREVIATIONS AND SYMBOLS

bp	base pairs
cDNA	complementary DNA
C-RT-PCR	conventional reverse transcriptase polymerase chain reaction
DOBV	dobrava virus
DW	distilled water
FITC	fluorescein isothiocyanate
HFRS	hemorrhagic fever with renal syndrome
HPS	hemorrhagic pulmonary syndrome
IFA	immunofluorescence assay
IgG	immunoglobulin G
IgM	immunoglobulin M
L-segment	large segment
M-segment	medium segment
NC	negative control
ORF	open reading frame
PBS	phosphate buffer saline
PC	positive control

PCR	polymerase chain reaction
RNA	ribonucleic acid
ROK	Republic of Korea
RT	reverse transcription
SEOV	Seoul virus
SFTS	severe fever with thrombocytopenic syndrome
SFTSV	severe fever with thrombocytopenia syndrome virus
S-segment	small segment
TBE	tick borne encephalitis
TBEV	tick borne encephalitis virus
Tm	melting temperature
VHF	viral hemorrhagic fever

ABSTRACT

Viral Hemorrhagic Fevers in Korea

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Background

Most of the viruses associated with viral hemorrhagic fevers (VHF) are zoonotic, dependent on their hosts for overall survival and further replication. Rodents and arthropods are the main reservoirs for viruses causing VHFs. Hantaviruses and severe fever with thrombocytopenia syndrome virus (SFTSV) are endemic in the Republic of Korea (ROK), yet the knowledge remains scanty in many fields on these infections. Till date, no study is conducted for SFTSV prevalence in rodents at ROK. Moreover, tick borne encephalitis virus (TBEV) has been identified in ticks and rodents, although no human case of TBE is confirmed in ROK. Presented study aims to investigate positive rate of emerging human pathogenic viruses in wild rodents collected in Korea, which serves as the reservoir and the carrier for the potential pathogenic viruses. In addition, this study compared the sensitivity of

different reverse transcription polymerase chain reactions (RT-PCR) conducted for detection of SFTSV presence.

Methods

Wild rodents were captured during 2017 and 2018 at sylvatic habitats in Boseong-gun, Jeollanam-do and Jeju Island, respectively. All rodents were euthanized under animal use protocol and guidelines. Organs like spleen, kidney and lungs were subjected to total RNA extraction. PCRs were performed for the detection of viruses including hantavirus, SFTSV and TBEV using a specific set of primers for each viral genome amplification. Serological analysis was performed using mice sera.

Results

In total 21 wild mice were captured in Boseong-gun in 2017 and 57 wild mice were captured at Jeju Island in 2018. All of wild mice captured at both locations were *Apodemus agrarius*. Hantavirus was detected in 24% (5 out of 21) of *A.agrarius* captured at Jeollanam-do (Boseong-gun) and 17.54% (10/57) for Jeju samples by nested RT-PCR (RT-N-PCR) targeting the L segment of hantavirus. SFTS RT-N-PCR targeting M-segment of the virus detected SFTSV in 5% (1/21) Boseong-gun samples and 5.3% (3/57) samples collected at Jeju Island. TBEV RT-N PCR performed for all the samples collected at Boseong-gun and Jeju were negative. Forty seven sera were available for serological analysis collected from Jeju, among which 5 (11%) of the *A.agrarius* sera were positive for Immunoglobulin G (IgG) antibodies against hantavirus and 2 (4.2%) for IgG antibodies against SFTSV.

Conclusions

Our findings includes the first detection of SFTSV in *A.agrarius* in ROK. This is the first report for occurrence of co-infection of SFTSV and hantavirus in *A.agrarius* and support the idea that such viruses can co-infect humans as well, provided the fact that they share the same routes of transmissions. Moreover, the study confirms the circulation of hantavirus in ROK through *A.agrarius* and thus, virus shedding from *A.agrarius* can increase the risk of human contracting HFRS. Although no TBEV was identified in this study, yet it poses a serious threat to human health in future. First study conducted for SFTS presence in rodents in ROK and positive result found in this study highlights the importance of continued surveillance to find these viral infections possible routes to humans and their prevention.

Keywords: HFRS, SFTS, TBEV, *Apodemus agrarius*, Coinfection, Republic of Korea

초록

국내의 매개체 관련 바이러스성 출혈열

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연구 배경

바이러스성 출혈열(VHF)과 관련된 대부분의 바이러스는 동물에 존재하고, 생존 및 복제를 위해 숙주에 의존하며, 특히 설치류와 절지동물은 VHF 바이러스의 주된 저장소다. 바이러스성 출혈열 의 원인이 되는 바이러스는 한타바이러스(Hantavirus), 중증열성혈소판감소증후군바이러스(SFTSV), 진드기매개뇌염 바이러스(TBEV) 등이 있으며, 한타바이러스와 중증열성혈소판감소증후군바이러스 는 한국에서 지역 풍토적으로 발견된다. 그러나 이 바이러스 감염과 관련된 연구는 아직까지 부족한 상태이며, 지금까지 국내 설치류에서 SFTSV 유병률을 확인하는 연구는 수행되지 않았다. 또한 TBEV 는 진드기와 설치류에서 확인되는데, 국내에서 진드기매개뇌염의 인체 감염 사례는 보고되지 않았다. 따라서 본 연구는

국내에서 잠재적인 병원성 바이러스에 대한 저장소 및 운반자 역할을 하는 야생 설치류에서 병원성 바이러스의 양성률을 조사하고자 수행되었으며, SFTSV 를 검출하기 위해 수행된 다양한 역전사 중합 효소 연쇄반응(Reverse Transcription Polymerase Chain Reactions, RT-PCR)의 민감도를 비교하였다.

연구 방법

2017 년과 2018 년 전라남도 보성군과 제주도에서 야생 설치류를 수집하였고, 모든 설치류는 동물실험 지침에 따라 안락사 시켰다. 비장, 신장 및 폐 조직에서 total RNA 를 추출하였고, 한타바이러스, SFTSV, TBEV 의 각 바이러스에 특이적인 유전자를 검출하기 위해 nested RT-PCR (RT-N-PCR)을 수행하였다. 또한 포획된 설치류의 혈청을 이용하여 한타바이러스와 SFTSV 에 대한 항체가 분석을 수행하였다.

연구 결과

2017 년 보성군에서 21 마리, 2018 년 제주도에서 57 마리의 야생 들쥐가 포획되었고, 두 지역에서 포획된 야생 들쥐는 모두 등줄쥐(*Apodemus agrarius*)로 확인되었다. 한타바이러스는 L segment 를 타깃으로 nested RT-PCR (RT-N-PCR)을 수행하였으며, 보성군 샘플에서 24% (5/21), 제주 샘플에서 17.54% (10/57)로 확인되었다. SFTSV 의 경우 M segment 를 타깃으로 RT-N-PCR 을 수행하였을 때 민감도가 가장 높았으며, 보성군 샘플에서 5% (1/21), 제주 샘플에서 5.3% (3/57)로 확인되었다. TBEV 의 RT-N-PCR 결과는 두 지역 샘플에서 모두 음성이었다. 제주에서 포획된 47 마리의 야생 들쥐 혈청을 이용한 항체가 조사 결과, 한타바이러스에 대한

IgG 양성이 11% (5/47)로 확인되었고, SFTSV 에 대한 IgG 양성은 4.2% (2/47)로 확인되었다.

연구 결론

본 연구를 통해 국내의 등줄쥐에서 SFTSV 의 감염을 처음 확인하였고, 또한 한 마리의 등줄쥐에서 SFTSV 와 한타바이러스가 동시 감염(co-infection) 되었음을 최초로 확인하였다. 이 결과는 바이러스가 숙주로 감염되는 경로를 공유하므로, 인체에도 마찬가지로 바이러스의 동시 감염이 일어날 수 있음을 시사한다. 또한 본 연구는 국내에서 등줄쥐의 SFTSV 와 한타바이러스 감염을 확인하였으며, 이는 바이러스의 인체 감염으로 질병 발생 위험이 증가할 수 있음을 시사한다. TBEV 는 본 연구결과 확인되지 않았지만, 추후에는 인체 건강에 심각한 위협이 될 수 있으므로 꾸준한 감시 및 모니터링이 필요하다. 본 연구는 국내의 설치류에서 SFTS 존재를 확인한 첫 결과이며, 이러한 설치류의 바이러스 감염이 인체 감염 전파 경로에 대한 이해와 예방법을 찾아내기 위해 설치류 바이러스의 지속적인 감시가 중요하다.

I. Introduction

Viral Hemorrhagic Fevers (VHFs)

Viral hemorrhagic fevers (VHFs) refer to a group of infectious diseases that causes a severe multisystem syndrome (affecting the multiple organ systems). Characteristically, the overall vascular system is damaged, and the body's ability to regulate itself is impaired. These symptoms are often accompanied by hemorrhage (bleeding) with a high case fatality rate. Some hemorrhagic fever viruses can cause relatively mild illnesses, while other causes severe life-threatening disease. Most of the viruses associated with VHFs are zoonotic, dependent on their hosts for overall survival and further replication. Rodents and arthropods are the main reservoirs for viruses causing VHFs.

Transmission in Humans

The route of transmission varies by specific virus. Typically transmission of viruses causing hemorrhagic fever occurs when the activities of infected reservoir hosts or vectors and humans overlap. The viruses associated with arthropod vectors are spread most often when the vector like mosquito, flea or tick bites a human. However, some of these vectors may spread virus to animals, livestock and then humans may become infected when they care for or slaughter the animals. Humans can get directly infected with the viruses carried by the rodent reservoirs by having contact with urine, fecal matter, saliva, or other body excretions from infected rodents. A few varieties of viruses can be even inhaled from infected rodent's excreta. Rodent-borne viruses can also be indirectly spread to humans as they serves as amplifying host of the viruses, when an ecto-parasite arthropod vectors like tick, mite or flea feed on rodents they become infected with viruses as well and then they

can bring those into direct contact with humans through bite. The multimammate rat, cotton rat, deer mouse, house mouse, and other field rodents are examples of reservoir hosts for VHF viruses (VHFV).

Hantaviruses

Hantaviruses belonging to the family Hantaviridae represent the unique genus *Hantavirus*. Hantaviruses are emerging viruses with capacity to cause substantial morbidity and mortality in humans. The virus genome consists of three segments of negative-stranded RNA; the large (L) segment which encodes the viral RNA polymerase, the medium (M) segment that is a glycoprotein precursor which is co-translationally cleaved into the envelope glycoproteins Gc and Gn, and the small (S) segment the nucleocapsid protein (N) (Krüger et al. 2011). During past decades, isolation of antigenically and genetically distinct Hantaviruses like Soochong virus (SOO) and Muju virus (MUJV) were reported in Korea from *Apodemus peninsulae* and *Myodes regulus*, respectively (Beak et al., 2006; Song et al. 2007). A prototype Shrew-borne Hantavirus, Thottapalayam virus and Imjin virus from the Ussuri white-toothed shrew (Crocidura lasiura) were also reported in Korea (Song et al., 2007; Song et al. 2009).

Hantavirus infections

Hantaviruses act as etiological agents for two human disease syndromes: hemorrhagic fever with renal syndrome (HFRS), alternatively known as Korean hemorrhagic fever with a mortality rates of up to 15%, and hantavirus cardiopulmonary syndrome (HCPS) with mortality rates of up to 40% (Holmes & Zhang 2015). Viral transmission to human occurs via aerosolized dried rodent urine, saliva, and feces inhaled by humans and rarely by rodent bites (Ryou et al. 2011). Hantaviruses causes a chronic infection in their natural hosts like small mammals and rodents with no apparent harm (Krüger et al. 2011).

Hantaviruses exhibit co-evolution and co-speciation with specific rodent species, and a particular hantavirus is transmitted only by one or a few closely related rodent/insectivore species (Goeijenbier et al. 2013; Krüger et al. 2001). Thereby, the natural host of each hantavirus indicates, although is not necessarily determined by, its geographical range (Krüger et al. 2001). This association is also reflected in their phylogeny; three major evolutionary clades are defined for rodent associated hantaviruses including their natural hosts from three Muridae subfamilies. Hantaan virus (HTNV), Seoul virus (SEOV) and Dobrava-Belgrade virus (DOBV) are most important examples of pathogenic Murinae-associated hantaviruses, Puumala virus(PUUV) belongs to the Arvicolinae-associated hantaviruses while Sin Nombre virus (SNV) and Andes virus (ANDV) causing HCPS in Americas are members of Sigmodontinae-associated hantaviruses (Krüger et al. 2011).

Hantaviruses in ROK

Hantaan virus is the primary etiologic agent of HRFS in ROK, which was first isolated from lung tissue of a striped field mouse (*Apodemus agrarius*) in 1976 in Songnae-ri, Gyeonggi Province, ROK. There are approximately 100-300 annually reported cases of HFRS in ROK with a mean mortality rate of 4.5%. Approximately 70% of the reported HFRS patients are infected with Hantaan virus, Seoul virus infects approximately 20%, and the remaining 10% of cases are due to unidentified agents (Baek et al. 2006). Significant increases in human HFRS incidence have been recorded in the past quarter century in ROK. Annually, 344 (2014), 384 (2015), 575 (2016) and 531(2017) cases were reported to the Korea Centers for Disease Control and Prevention (KCDC) from the whole country while in Jeju reported cases were as following: 2(2014), 0(2015), 3(2016) and 1(2017) (KCDC 2018).

Severe fever with thrombocytopenia syndrome (SFTS) Virus

In 2009, a novel virus-causing severe fever with thrombocytopenia syndrome (SFTS) belonging to the family Bunyaviridae was identified in China (Kim et al. 2013) and was subsequently reported in 2011 in Japan and in 2013 in ROK (Yun et al. 2016). Like other members of the Bunyaviridae, SFTSV is an enveloped, negative-stranded RNA virus. Its genome consists of three RNA segments: small (S), medium (M), and large (L). The L segment encodes the RNA-dependent RNA polymerase. The M segment has a length encodes for a Glycoprotein N and C (GnGc) precursor. The S segment coding for a neucleocapsid (N) and nonstructural (NS) protein. The broad geographic distribution of the virus across China, Korea and Japan, its high fatality rate, and the potential for human-to-human transmission led this virus to be seen as an increasingly important threat to regional and global health (Zhang et al. 2016).

SFTS infection

SFTSV is transmitted to humans through tick bites, and *Haemaphysalis longicornis* is known to be a main vector. Tick nymphs become infected while feeding on viremic hosts, and the virus can then be transmitted to humans through host-seeking nymphs, larvae and adult ticks. SFTSV is thought to circulate in an enzootic tick–vertebrate–tick cycle (Liu et al. 2014). Apart from infecting humans, SFTSV widely infects domesticated animals. Wild animals and some bird species have also been infected and proven to be seropositive for SFTSV. Studies reported SFTSV prevalence in goats, cattle, dogs, chicken, pig, deer and elk (Niu et al. 2013; Liu et al. 2014; Li et al. 2014Wu et al. 2013). SFTSV infection has also been investigated in rodents as possible hosts of this virus and also found to carry SFTSV in eastern China (Liu et al. 2013).

SFTS in ROK

Since first recognized in 2013 in Korea, the importance of SFTSV has been increasingly emphasized due to the significant rise in the number of SFTS cases reported in ROK annually, from 36 in 2013, to 55 in 2014, 79 in 2015, 165 in 2016 and to 272 in 2017 (KCDC, 2018) (Yun et al. 2016). The case–fatality rate for SFTS reported in South Korea was 47.2% (17/36) in 2013, which was much higher than that of reported in china (8.7%) in same year, declaring it a serious public health concern (Park et.al 2014). KCD report documented the mortality rate as 29%, 27% and 12% in 2014, 2015 and 2016 respectively, and then 20 % in 2017 (KCDC 2018). A group of researchers detected SFTSV presence in ixodid ticks collected from six provinces and 4 metropolitan areas in ROK which further provide evidence for circulation of SFTSV through ticks across the country (Yun et al. 2016). A recent study conducted for seroprevalence of SFTSV antibodies in rural areas of 3 provinces in ROK reported the positivity rate of 4.1% in general population (Han et al. 2018) indicating widespread of the SFTSV throughout the country. Being home to one of the largest populations of migratory birds on the Korean peninsula, Jeju Island is a considered as a high-prevalence region for SFTSV. Studies suggests a strong relationship between migratory birds and *H. longicornis* which is the most common vector for SFTSV transition in ROK (Yun et.al 2015).

Tick-borne encephalitis virus (TBEV)

Tick-borne encephalitis virus (TBEV), the etiological agent of tick-borne encephalitis (TBE) from family Flaviviridae is one of the most medically important viral diseases in Europe and Asia. TBEV is endemic to Eurasia and has been

categorized in three subtypes: European (Western), Far- Eastern and Siberian (Yun et.al 2016, Yoshii et.al 2017). Flaviviruses, including TBEV genome comprises a single-stranded, positive-sense RNA of approximately 11 kb in length, containing a 5´ cap and lacking a polyadenylate tail. The 5´ cap is important for mRNA stability and translation. The single open reading frame (ORF) encodes three structural proteins – capsid protein C, membrane protein M (formed by cleavage from its precursor prM) and the large envelope glycoprotein (E) – along with seven non-structural proteins – NS1 (glycoprotein), NS2A, NS2B (protease component), NS3 (protease, helicase and NTPase activity), NS4A, NS4B and NS5 (RNA-dependent polymerase). The 5´ and 3´ ends of the genome are non-coding regions (Mansfield et.al 2009).

TBEV infection

Tick-borne encephalitis Virus (TBEV) is the causative agent of tick-borne encephalitis (TBE), a potentially fatal neurological infection affecting the human central nervous system. TBEV infection usually presents as a moderate febrile illness that is followed by encephalitis in ~40% of patients. TBE human disease was first described in 1927 in Austria (Yoshii et.al 2017). Rodents/insectivores (mammalian subgroup) and birds (seabird subgroup) are the natural hosts of all tick-borne flaviviruses known thus far (Dobler 2010).

TBEV in ROK

Till date no human cases of TBE have been reported or confirmed in the ROK. Despite of that TBE poses a serious potential threat to human health, as it has been

detected in ticks infesting wild and domestic animals. Over the last decade, various studies have been carried out to determine whether TBEV is present in ROK. In 2008 TBEV strains was first isolated in ROK from *A.agrarius*, which serves as a potential maintenance host for TBEV (Yun et al. 2016). The virus has been identified in ixodid ticks collected from vegetation in ROK as well providing the fact that virus is circulating in ROK. Contrary to the expectations, phylogenetic analysis based on the complete E gene or full genome of TBEV revealed that the TBEV isolates from ROK belonged to the Western subtype of TBEV, while in the neighboring countries, including China, Japan and Russia, only the Far-Eastern subtype was isolated (Yun et al. 2012)

Aim of this study

Presented study aims to investigate positive rate of emerging human pathogenic viruses— HFRSV, SFTSV and TBEV in wild rodents collected at different locations in Korea, which serves as the reservoir and the carrier for the potential pathogenic viruses. In addition, this study compared the sensitivity of medium (M) segment targeting conventional and nested PCR and small (S) segment targeting nested PCR for detection of SFTSV. This study will help better understanding the rodent's reservoirs contributing to disease outspread and provide further insight regarding their circulation to aid in preventing these human disease in ROK.

II. Materials and Methods

Rodent Trapping

The rodents were captured at two sylvatic habitats, Boseong-gun (Jeollanam-do) and Jeju Island located in the south-west and south of the ROK. Capturing period was from October 2017 to November 2017 at Boseong-gun, and April 2018 to October 2018 at Jeju using live-traps. Wild rodents were trapped monthly using Sherman live traps baited with peanut butter–covered biscuits at five different sites including a rice paddy, a field, a hill, a reservoir and near dyke. The captured rodents were identified and numbered sequentially according to capturing location. All rodents were euthanized in accordance with an approved animal use protocol, and blood and organ samples like spleen and kidneys were stored at -80°C until further experiments.

Viral RNA Extraction

The mice organ samples (lung/ spleen/ kidney) were homogenized by grinding with a sterile 70-µm cell strainer. Total RNA was extracted from the samples homogenized using a Viral Gene Spin^{TM} Viral RNA Extraction Kit (iNTRON Biotechnology, Korea) according to the manufacturer's instructions.

Reverse Transcription (RT reaction)

Single-stranded RNA was reverse transcribed into complementary DNA (cDNA), using total cellular RNA extracted, with SuperScript VILO MasterMix (Invitrogen, Life Technologies). cDNA was prepared in a total volume of 20 µL by mixing the following volumes: 4 µL VILO^{TM} MasterMix, 8 µL RNA, and 8 µL distilled water. The conditions for reverse transcription were 25°C for 10 min, followed by 42°C for 60 min and 85°C for 5 min in a Veriti 96 Well Thermal Cycler (Applied Biosystems, Foster, CA, USA).

Hantavirus PCR

Reverse transcription-nested polymerase chain reaction (RT-N-PCR) was performed using the Hantavirus specific primers HAN-LF1/HAN-LR1 and HAN-L-F2/HAN-LR2 targeting the L segment of the virus. Additionally our designed RT-N-PCR targeting S segment of the hantavirus was also conducted with specific set of primers HFRS-S-2F/ HFRS-S-2R and HFRS-S2nd-1F/ HFRS-S2nd-1R.

Both first and the nested PCR were performed in a total volume of 20 µL in AccuPowerR PCR PreMix (Bioneer Corp.). Each PCR mixture consisted of 16 µL distilled water, 1 µL each primer F/R (10 pmol/µL) and 2 µL cDNA template (Table-1).

SFTS PCR

To detect the presence of SFTSV, One-step reverse transcription-polymerase chain reaction (RT-PCR) using extracted RNA and specific set of primers (SFTS-F/SFTS-R) targeting the M segment of the virus was conducted. PCR experiment was performed in a total volume of 30 µL. Each PCR mixture consisted of 15 µL

2x One-step RT-PCR Premix (Diastar, SolGent Co., Ltd)., 8 µL distilled water, 1 µL of each primer (10 pmol/µL) and 5 µL RNA template. The SFTS positive control 410-bp fragment was received from Korea Centre for Disease Control (KCDC).

To enhance the sensitivity of the SFTSV M segment targeting PCR, specific pair of primers (SFTS-1st F/SFTS-1st R) was designed for the primary round and its amplified product was used as a template subjected to the second round (conditions and primers from former RT-PCR described for M-segment amplification). Both first and the nested PCR were performed in a total volume of 20 µL in AccuPowerR PCR PreMix (Bioneer Corp.). Each PCR mixture consisted of 16 µL distilled water, 1 µL each primer F/R (10 pmol/µL) and 2 µL cDNA template (Table-1).

To detect S segment of SFTSV RT-N-PCR was performed with specific set of primers SFTS-S-NP-2F/SFTS-S-NP-2R and SFTS-S-N2F/ SFTS-S-N2R. Both first and the nested PCR were performed in a total volume of 20 µL in AccuPowerR PCR PreMix (Bioneer Corp.). Each PCR mixture consisted of 16 µL distilled water, 1 µL each primer F/R (10 pmol/µL) and 2 µL cDNA template (Table-1).

TBEV PCR

To detect TBEV, RT-N-PCR using specific primers TBE913F/ TBE1738R and TBE1192F/TBE1669R targeting TBEV envelope (E) gene. Both first and the nested PCR were performed with the total volume of 20 µL in AccuPowerR PCR PreMix (Bioneer Corp.). Each PCR mixture consisted of 16 µL distilled water, 1 µL each primer F/R (10 pmol/µL) and 2 µL cDNA template (Table-1).

During each run of PCR, a suitable positive control (Hantavirus, TBEV and SFTS) and molecular grade water as a negative control were included. All the PCR mixes

were prepared in a PCR hood at a room separated from that used to extract RNA, the post-PCR area was also physically separated from the amplification area in order to minimize the contamination. The conditions used for PCRs and the PCR-amplified product sizes are listed in Table 1. All amplifications were carried out in an AB thermal cycler (Applied Biosystem, Inc.). Amplified products were separated by electrophoresis on 1.5% agarose gel and visualized by ethidium bromide.

Nucleotide Sequencing

The nucleotide sequences of the amplified PCR products were determined by direct sequencing with the PCR primers. The PCR products were purified using QIAquick PCR purification kits (Qiagen). Sequencing was carried out at Macrogen Inc. (Seoul, Republic of Korea).

Phylogenetic Analysis

The phylogenetic trees were constructed by the neighbor joining (N-J) method using ClustalX software program. Representative Hantavirus sequences from GenBank, including Hantavirus isolates from other countries, and previously identified Hantavirus sequences from ROK were included in the phylogenetic analysis. Same was performed for SFTS isolates. Genetic distance was computed using PAUP version 4.0b, and topology was evaluated by bootstrap analysis of 1000 iterations.

Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics 23 for Windows to determine the relationship between virus positivity rate and the month of capturing the rodent. A chi square test was employed to the samples. Significance was set as $p \leq 0.05$.

Serological analysis

Slides used for indirect immunofluorescence assays (IFA) were kindly donated by KCDC to detect anti-HTNV immunoglobulin G (IgG) and anti-SFTSV immunoglobulin M (IgM) and IgG in mice sera.

IFA was performed according to KCDC protocol instructions. Briefly, sera from wild mice were serially diluted two fold from 1:32 to 1:1024 in sterilized phosphate-buffered saline (PBS). 20 µL of each diluted sera were added to the slide and incubated for 30 min in humid chamber at 37 °C. Next, slides were washed three times with PBS followed by three times washing with distilled water (DW) and air drying. Slides were treated with Alexa Fluor 488 goat anti-mouse IgG (H+L) and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgM. The slides were incubated for 30 min at 37 °C under humid chamber, washed with three changes of PBS followed by three times DW washes and then air-dried. The slides were read under a fluorescence microscope (U-LH100HG, Olympus corp. Tokyo, Japan) for specific fluorescence. A titer of 1:32 was taken as the cut-off for screening the positive candidate.

III. Results

Wild Mice Captured and Location

In total 21 wild mice were captured in Boseong-gun area in the year 2017. During the month of October, 10 mice were captured, while rest of the 11 wild mice were captured during the month of November.

In 2018 a total of 57 wild mice were captured in Jeju Island. 15 mice were captured during the April month, 7 were captured in the May, followed by 8 mice in the June, 5 mice in the July and 7, 9 and 6 mice in August, September and October, respectively.

All of the wild mice captured at both locations were *A.agrarius*. Geographical location for capturing site is shown in Figure-1.

Molecular Detection of Hantavirus

Hantavirus was detected in 24% (5 out of 21) of *A.agrarius* (organ samples) collected in Boseong-gun by RT-N-PCR targeting the L segment of Hantavirus. Monthly PCR positivity rates for mice captured in October 2017 and November 2017 at Boseong-gun were 30% (3/10) and 18% (2/11), respectively.

Same samples while subjected to RT-N-PCR targeting the S segment of Hantavirus yielded a decrease in sensitivity by giving only 20.0% (2/10) positivity for October mice and 9.1% (1/11) for November mice. With regards to monthly prevalence, these results were statistically not significant (Table-2).

Results by RT-N-PCR targeting the L segment of Hantavirus for wild mice captured in the year 2018 at Jeju showed overall 17.5% (10/57) positivity for Hantavirus. A large proportion of Hantavirus positive mice (10.5% of the total) were collected in the month of May with 85.7% (6/7) positivity rate within a month. Followed by 50% (3/6) positivity rate for mice collected in October and 14.3% (1/7) positive for August samples. All other samples from April, June, July and September were Hantavirus negative. Statistical analysis for monthly positivity rate of Hantavirus in this case provided results that were significant with a p-value <0.001 (Table-3).

Using 2018 mice from Jeju Island, RT-N-PCR targeting the S segment of hantavirus yielded a decrease in sensitivity as well, by giving only 5.3% (3/57) positivity rate. Positive samples amplified for S-segment were from May, August and October (1 for each month). Notably all the three samples identified by S-segment were expressed by targeting L-segment as well, suggesting higher sensitivity of L-segment PCR used in the study. Moreover, statistical results were significant as well (p value= 0.04) (Table-3)

Molecular Detection of SFTS virus

All of the collected mice samples were subjected to SFTS RT-PCR targeting M-segment of the SFTS virus. None of the sample was positive.

Similarly, all the samples were negative while SFTS RT-PCR targeting S segment of the SFTSV was conducted.

Samples collected from Boseong-gun subjected to SFTS-RT-N-PCR rendered 5% (1/21) SFTS positivity. The only positive sample was collected in the November. Although the results did not reach the statistical significance (Table-2).

A total of 9% (3/35) samples collected from Jeju Island in 2018 were SFTS virus positive by RT-N-PCR. All of the positive samples were from the month of April. Monthly positivity rate was 20% (3/15) for the April while no positive results were obtained from samples collected from May till October (Table-3).

Results indicated that nested PCR used for M-segment of the SFTSV was much more sensitive as compared to conventional as well as S-segment targeted N-PCR (Table-2 and 3). In case of Jeju wild mice collected during 2018, S-segment RT-N-PCR and M-segment conventional RT-PCR were not conducted for the samples collected from May till October for the reason of their low sensitivity shown by prior samples tested (2017 all samples and 2018 April samples) in this study. Only if samples were found positive by M-segment RT-N-PCR then they were subjected to S-segment RT-N-PCR and M-segment conventional RT-PCR.

Molecular Detection of TBE virus

TBEV RT-N PCR performed for all the samples collected at Boseong-gun showed negative results for the TBE virus presence.

Similarly all the samples collected at Jeju were also negative for TBEV.

Phylogenetic Analysis of Hantavirus based on L segment

Phylogenetic tree was constructed for partial L segment (360bp) sequences of five positive isolates collected from Boseong-gun in 2017 and ten positive isolates from Jeju Island in 2018 compared with representative sequences of

Hantaan virus, Sochong virus, Seoul virus, Dobra virus, Pumala virus and others, from Korea's neighboring countries such as Japan, China and different other countries. All the positive isolates from this study clustered near to a previously reported Hantaan virus isolate from Korea (Figure-2).

Phylogenetic Analysis of Hantavirus based on S segment

Phylogenetic tree was constructed for S segment (650bp) sequences of three positive isolates collected from Boseong-gun in 2017 and three positive isolates from Jeju Island in 2018 compared with representative Hantaan virus, Sochong virus, Seoul virus, Dobra virus, Pumala virus and others, from Korea's neighboring countries such as Japan, China and different other countries. All the positive isolates from this study clustered near to a previously reported Maaji virus isolate of Hantaan virus from Korea which does not group with other Haantan virus cluster including isolated from Korea and China (Figure-3). NCBI nucleotide blast results for positive isolates from Boseong-gun showed 96% similarity with Hantaan orthohantavirus isolate CUH15-126 from a HFRS patient's blood at Gwangju, ROK and 90% similarity to Hantaan virus strain Maaji-1 isolate from *A.agrarius*, ROK. Similarly, positive isolates from Jeju showed 96-98% similarity with Hantaan orthohantavirus isolate CUH15-126 and 88-90% similarity to Hantaan virus strain Maaji-1 isolate.

Phylogenetic Analysis of SFTSV based on M segment

Phylogenetic tree constructed for M segment (480 bp) sequences of one positive isolate collected from Boseong-gun in 2017 and three positive isolates from

Jeju Island in 2018 compared with representative SFTSV strains from different countries including China and Japan showed that all the positive isolates from this study clustered with previously reported SFTS virus isolate from China (Figure-4).

Serological results for hantavirus antibodies

In our study positive serological results were obtained for 5 (11%) of the *A.agrarius* sera collected from Jeju. Monthly prevalence rate for IgG antibodies against hantavirus was highest 2(33.3%) in May, 1 (14.3%) in August, 1 (11.1%) in September and 1 (17%) in October. Notably, September sample positive for hantavirus IgG antibodies was positive for IgG antibodies against SFTSV as well. Four out of five (80%) seropositive samples were positive by RT-N-PCR targeting L segment of the hantavirus as well. Similarly, three out of five seropositive samples were positive by RT-N-PCR targeting S segment of the hantavirus. Serology results of hantavirus IgG are presented in Table-4 and positive antibodies fluoresce detected is shown in Figure-5.

Serological results for SFTSV antibodies

In total, positive serological results were obtained for 2(4.2%) of the *A.agrarius* sera collected from Jeju in 2018. Both samples were positive for IgG antibodies against SFTSV with titer of 1:256 and 1:64 and were obtained from September samples only. All samples tested for SFTSV IgM antibodies were negative. None of the serologically positive sample was positive by PCRs conducted. Serology results of SFTSV IgM and IgG are presented in Table-4 and positive antibodies fluoresce detected is shown in Figure-6

IV. Discussion

Rodents can bring pathogens into contact with human either directly by serving as amplifying hosts of pathogens and then shedding viruses in environment or by means of an ectoparasitic arthropod vectors like tick, mite or fleas, which feeds on the infected rodent's blood and then transfer the pathogens to humans through bite. As rodents act as natural reservoir and amplifying host for SFTSV and TBEV, while HFRS is a rodent-borne disease, thus we investigated the occurrence of these infectious viruses in wild rodents to provide further insight regarding their circulation in our ecosystem and to aid in preventing human infections in ROK.

Hantaan virus the prototype Hantavirus circulating in ROK is distributed through the *A.agrarius* in China, Russia, and ROK (Meerburg et al. 2009). A study conducted in Korea including 766 rodents captured in five provinces collected over the period of year (January-December 2011) reported the detection of a partial S segment of Hantaan, Seoul, and Puumala viruses in rodents lung samples of found Hantaan virus RNA in 25 out of 766 (3.3%) *A.agrarius* samples by using multiplex RT-PCR and highest proportion was contributed from rodents caught in autumn season (Ryou et al. 2011). In our study, 24% (5 out of 21) in Boseong-gun and 18 % (10 out of 57) *A.agrarius* lung/spleen samples were positive for Hantaan virus using RT-N-PCR targeting L segment of Hantaviruses, suggesting a higher rate of positivity in *A.agrarius*. While subjected to S segment amplifying PCR, the positive number dropped to only 3 (14.3%) in case of samples collected in 2017 and 3 (5.3%) for 2018 samples. These differences among the results of our study targeting two different positions of the virus and from the previous study conducted, could be due to the fact that the mice were captured in the autumn season (peak of HFRS cases) in our study in Boseong-gun. Additionally, we choose a different targeting site of the virus (L segment) in our study than the previous study conducted (partial S segment) which seems to be

more sensitive as compared to our second target PCR (S segment) conducted in this study. Different rodent trapping site, geographical distributions, climatic variations or molecular techniques can also contribute to different results presented by different studies.

Another group of researchers provided epidemiological data on HFRS human cases reported between 2001 and 2010 in ROK, which analyzed HFRS cases by season, geography, and resident area of individuals. The HFRS cases were occurring predominantly during the last quarter of the calendar year (October, November, and December). Presumably, higher incidence of HFRS in the autumn versus other seasons were attributed to the high numbers of hantavirus-infected rodents in Korea during this period (Lee et al. 2013). Contrary to that, in our study a higher proportion of HFRS positive rodent's data from Jeju was comprised of May samples followed by October samples with the statistical significance (p value <0.001). The prevalence of HFRS depends on the viability of the pathogen in relation to the climate, human activity, landscape and seasonality in different areas (Joshi et al., 2017). Given the fact that Jeju climate somehow differs from rest of the ROK may have attributed to our different results.

In our study positive serological results were obtained for 5 (11%) of the *A.agrarius* sera collected from Jeju. Notably, September sample positive for hantavirus IgG antibodies was positive for IgG antibodies against SFTSV as well.

Interestingly, until 2013 two different studies documented only one Human case of HFRS from Jeju Island among total of 3,953 HFRS cases reported in almost a decade in ROK (Lee et al. 2013, Han et al., 2013). While another report published in 2017 referred the positive HFRS human cases from Jeju as five, from data collected during 2001-2009 (Joshi et al., 2017). High positive incidence among rodents captured in our study from Jeju Island suggests that the Hantavirus infection outbreak can occur in future, as virus-bearing rodents act as

the primary source of spreading infection to human. Further studies are needed to evaluate this aspect of our study.

The tick-borne SFTSV is known to be present in the ROK and significant increase in human cases are reported every year, although there are no reports for SFTS prevalence in rodents including *A.agrarius* from ROK. Moreover, no information is provided on *A.agrarius* role in spreading the SFTS infection. A previous study conducted in China reported the presence of SFTSV by one-step real-time RT-PCR in rodents (Ni et al. 2015). They detected SFTSV genomic RNA in 2 of the 8 *A.agrarius* strains, but not in 40 *R. norvegicus* and 4 *R. losea* strains. Furthermore, the two isolated strains of SFTSV from *A.agrarius* showed significant homologies to the strains isolated from local patients, indicating that *A.agrarius* is a natural host of SFTSV (Ni et al. 2015). Another group of researchers provided positive RT-PCR results and serologic evidence for shrews and rodents including house mice and striped field mice carrying SFTSV in eastern China (Liu et al. 2013).

To best of our knowledge, till date there are no reports from ROK confirming the SFTSV isolation directly from Rodents. In this study, we detect 5% (1/21) SFTSV positivity in Boseong-gun samples while 9% (3/35) SFTSV in wild rodent (*A.agrarius*) sample collected from Jeju via our designed RT-N-PCR. Positive results were seen for the rodents collected in April at Jeju. Although the conventional PCR (M segment) and N-PCR targeting S segment of SFTSV did not amplify any of the positive sample the results were confirmed through sequencing the positive isolates. Our study result suggests that with conventional SFTS-RT-PCR targeting M-segment we did not detect any positive sample, so we should use nested RT-PCR for M-segment detection of SFTSV in *A.agrarius*. All the amplified products sent for sequencing resulted in SFTS virus sequences highly similar to the previous SFTSV isolate sequences submitted to NCBI.

There are multiple reports describing co-infections of animal vectors and humans previously. In general, multiple infections in humans can present with more severe clinical picture though how they affect the host rodent or animals is not known. Co-infections symptoms of are often nonspecific, and therefore makes diagnosis difficult. Rodents infected with multiple pathogens at the same time may present a special threat to human health because the possibility of humans getting infected with many pathogen is higher. Co-infection of rodents with hantaviruses, *Leptospira*, and *Babesia* is reported. Multiple co-infections with all three pathogens were found in 3 of 28 (11%) *A. flavicollis*, suggesting that several pathogens can infect the same rodent host at the same time. Dual infections with hantaviruses and Leptospira were found in 7 of 44 (16%) rodents while dual infection with hantaviruses and Babesia in 2 rodents (5%) (Tadin et al. 2012).

It is noteworthy that one of the rodent collected from Boseong-gun presented co-infection with SFTSV and Hantavirus. This sample was confirmed by subjecting to all PCRs twice and through sequencing as well. Another sample collected in 2018 at Jeju was serologically positive for both hantavirus IgG and SFTSV IgG antibodies although negative by PCR for these viruses. Therefore, it is the first study from ROK confirming the SFTSV presence in rodents (*A.agrarius)* as well as reporting a new possible co-infection that can infect humans in a similar way. Although in ROK, *A.agrarius* role in spreading SFTSV to human is not defined yet, this report suggests it is a potential reservoir and carrier of SFTSV and can play role in spreading the virus to no-endemic areas as well. Further studies are needed to evaluate this aspect as well. Awareness of such co-infections presence among wild rodents (*A.agrarius*) will aid predicting co-infections in the human population as well. Testing for the presence of all described pathogens in patients should be considered in regions where co-infections in rodents have been detected.

Rodents play an essential role in the transmission of tick-borne encephalitis virus between ticks. Several rodents such as the bank vole (*Myodes glareolus*), the field mouse (*Apodemus agrarius* Pallas), and the red vole (*Myodes rutilus*) are known hosts of ticks that can cause TBE (Meerburg et al. 2009). Although human cases of TBE have not been reported in ROK, TBE is considered to be a potential threat to human health (Yun et al. 2016). Previous studies reported the Korean isolates show clustering with the European or Western TBEV subtype with 98-99% identity, that differs from the subtype (Far eastern subtype) isolated in the neighboring countries such as Japan, Russia and China (Kim et al. 2008; Yun et al. 2016). There is a chance that other subtypes of TBEV are also co-circulating in Korea, hence more extensive surveys should be conducted to find whether other types are present. Evidence of TBEV identified in ticks infesting wild and domestic animals, ixodid ticks collected from vegetation in ROK (Yun et al. 2016) and TBEV strains isolated from striped field mice, *A.agrarius*, in ROK (Kim et al. 2008) suggest that it is a potential emerging infectious disease that could threaten ROK in the future. In this study we did not detected any positive sample for TBEV. As changes in human behavior (particularly land use) and climate are predictors for the distribution of cases or outbreaks of rodent-borne pathogens outside their current areas of endemicity (Viel et al. 2011), a continuous surveillance of rodents is extremely important for preventing virulent pestilence in the future.

More extensive surveys should be conducted to determine the route and carriers of these infections to humans. Continuous surveillance of rodent populations will help to better predict future disease occurrence, the possibility of co-infections, novel rodent-borne diseases and their prevention. This study have few limitations, for instance small sample size collected in 2017 and capturing monotype rodents (*A.agrarius*). Additionally, we did not performed serological

tests for 2017 samples collected from Jeollanam-do and few of the 2018-Jeju samples due to lack of the blood samples collection in that case.

V. Conclusions

Till date there are no reports on SFTS isolation from rodents in ROK, our findings includes the first detection of SFTSV in *A.agrarius* in ROK and suggests that is a potential reservoir and carrier for SFTSV in ROK which can play significant role to spread the infection to non-endemic areas as well. Our findings based on molecular methods and serology, for the very first time reported the occurrence of co-infection of SFTSV and hantavirus in *A.agrarius* and support the idea that such viruses can co-infect the humans as well, provided the fact that they share the same routes of transmissions. Nested PCR targeting M-segment of SFTV (with our designed pair of primer for first round) was found to be more sensitive as compared to other PCRs for SFTSV detection from different studies. Moreover, the study confirms the circulation of hantavirus in ROK through *A.agrarius* and thus, virus shedding from *A.agrarius* can increase the risk of human contracting HFRS. Results for samples collected in 2017 at Boseong-gun showed comparable positivity rate in October and November. Contrasting to the previous findings of various studies conducted at ROK, Jeju samples from 2018 had a high proportion of positive samples from May. This demands the need for continuing further research in this field. Although no TBEV was identified in this study, yet it poses a serious threat to human health in future. First study conducted for SFTS presence in rodents in ROK and positive result found in this study highlights the importance of continued surveillance to find these viral infections possible routes to humans and their prevention.

VI. References

Amada, T., Yoshimatsu, K., Yasuda, S. P., Shimizu, K., Koma, T., Hayashimoto, N Gamage, C.D., Nishio, S., Takakura, A. & Arikawa, J. (2013). Rapid, whole blood diagnostic test for detecting anti-hantavirus antibody in rats. Journal of virological methods, 193(1), 42-49.

Baek, L. J., Kariwa, H., Lokugamage, K., Yoshimatsu, K., Arikawa, J., Takashima, I.,Kang, J.I., Moon, S.S., Chung, S.Y., Kim, E.J. & Kang, H. J. (2006). Soochong virus: an antigenically and genetically distinct hantavirus isolated from Apodemus peninsulae in Korea. *Journal of medical virology*, *78*(2), 290-297.

Dobler, G. (2010). Zoonotic tick-borne flaviviruses. *Veterinary microbiology*, *140*(3), 221-228.

Galeno, H., Mora, J., Villagra, E., Fernandez, J., Hernandez, J., Mertz, G.J. and Ramirez, E., (2002). First human isolate of Hantavirus (Andes virus) in the Americas. *Emerging infectious diseases*, *8*(7), p.657.

Goeijenbier, M., Wagenaar, J., Goris, M., Martina, B., Henttonen, H., Vaheri, A., Reusken, C., Hartskeerl, R., Osterhaus, A. & Van Gorp, E. (2013). Rodent-borne hemorrhagic fevers: under-recognized, widely spread and preventable–epidemiology, diagnostics and treatment. *Critical reviews in microbiology*, *39*(1), 26-42.

Hwang, J., Kang, J.G., Oh, S.S., Chae, J.B., Cho, Y.K., Cho, Y.S., Lee, H. and Chae, J.S., 2017. Molecular detection of severe fever with thrombocytopenia syndrome virus (SFTSV) in feral cats from Seoul, Korea. Ticks and tick-borne diseases, 8(1), 9-12.

Han, M.A., Kim, C.M., Kim, D.M., Yun, N.R., Park, S.W., Han, M.G. and Lee, W.J., 2018. Seroprevalence of Severe Fever with Thrombocytopenia Syndrome Virus Antibodies in Rural Areas, South Korea. *Emerging infectious diseases*, *24*(5), 872.

Han, S.S., Kim, S., Choi, Y., Kim, S. and Kim, Y.S., 2013. Air pollution and hemorrhagic fever with renal syndrome in South Korea: an ecological correlation study. BMC public health, 13(1), p.347.

Heo, S. T., Yoo, J. R., Lee, K. H., & Ko, K. S. (2015). The First Case of Non-retrospective Clinical Identification of Severe Fever with Thrombocytopenia Syndrome Patient in 2013 in South Korea. *Journal of Bacteriology and Virology*, *45*(2), 155-158.

Holmes, E. C., & Zhang, Y. Z. (2015). The evolution and emergence of hantaviruses. *Current opinion in virology*, *10*, 27-33.

Joshi, Y.P., Kim, E.H. and Cheong, H.K., 2017. The influence of climatic factors on the development of hemorrhagic fever with renal syndrome and leptospirosis during the peak season in Korea: an ecologic study. BMC infectious diseases, 17(1), p.406.

Korea Centers for Disease control and Prevention (KCDC) (2018). Infectious disease surveillance Yearbook. 2017. Retrieved from http://www.cdc.go.kr/npt/biz/npp/nppMain.do. Accessed November 2018.

Kim, K. H., Yi, J., Kim, G., Choi, S. J., Jun, K. I., Kim, N. H., Choe, P.G., Kim, N.J., Lee, J.K. & Oh, M. D. (2013). Severe fever with thrombocytopenia syndrome, South Korea, 2012. *Emerging infectious diseases*, *19*(11), 1892.

Kim, S. Y., Yun, S. M., Han, M. G., Lee, I. Y., Lee, N. Y., Jeong, Y. E., Lee, B.C., & Ju, Y. R. (2008). Isolation of tick-borne encephalitis viruses from wild rodents, South Korea. *Vector-borne and Zoonotic Diseases*, *8*(1), 7-14.

Klempa, B., Fichet-Calvet, E., Lecompte, E., Auste, B., Aniskin, V., Meisel, H., Denys, C., Koivogui, L., ter Meulen, J. & Krüger, D. H. (2006). Hantavirus in African wood mouse, Guinea. *Emerging infectious diseases*, *12*(5), 838.

Krüger, D. H., Ulrich, R., & Lundkvist, Å. (2001). Hantavirus infections and their prevention. *Microbes and infection*, *3*(13), 1129-1144.

Lee, S.H., Chung, B.H., Lee, W.C. and Choi, I.S., 2013. Epidemiology of hemorrhagic fever with renal syndrome in Korea, 2001-2010. Journal of Korean medical science, 28(10), 1552-1554.

Li, Z., Hu, J., Bao, C., Li, P., Qi, X., Qin, Y., Wang, S., Tan, Z., Zhu, Y., Tang, F. and Zhou, M., 2014. Seroprevalence of antibodies against SFTS virus infection in farmers and animals, Jiangsu, China. *Journal of Clinical Virology*, *60*(3), 185-189.

Liu, J.W., Wen, H.L., Fang, L.Z., Zhang, Z.T., He, S.T., Xue, Z.F., Ma, D.Q., Zhang, X.S., Wang, T., Yu, H. and Zhang, Y., 2014. Prevalence of SFTSV among Asian house shrews and rodents, China, January–August 2013. Emerging infectious diseases, 20(12), 2126.

Liu, K., Cui, N., Fang, L. Q., Wang, B. J., Lu, Q. B., Peng, W., Li, H., Wang, L.Y., Liang, S., Wang, H.Y. & Zhang, Y. Y. (2014). Epidemiologic features and environmental risk factors of severe fever with thrombocytopenia syndrome, Xinyang, China. *PLoS neglected tropical diseases*, *8*(5), e2820.

Liu, Q., He, B., Huang, S. Y., Wei, F., & Zhu, X. Q. (2014). Severe fever with thrombocytopenia syndrome, an emerging tick-borne zoonosis. *The Lancet Infectious Diseases*, *14*(8), 763-772.

Mansfield, K.L., Johnson, N., Phipps, L.P., Stephenson, J.R., Fooks, A.R. and Solomon, T., 2009. Tick-borne encephalitis virus–a review of an emerging zoonosis. *Journal of general Virology*, *90*(8), 1781-1794.

Meerburg, B. G., Singleton, G. R., & Kijlstra, A. (2009). Rodent-borne diseases and their risks for public health. *Critical reviews in microbiology*, *35*(3), 221-270.

Ni, H., Yang, F., Li, Y., Liu, W., Jiao, S., Li, Z., Yi, B., Chen, Y., Hou, X., Hu, F. & Ding, Y. (2015). *Apodemus agrarius* is a potential natural host of severe fever with thrombocytopenia syndrome (SFTS)—causing novel bunyavirus. *Journal of Clinical Virology*, *71*, 82-88.

Niu, G., Li, J., Liang, M., Jiang, X., Jiang, M., Yin, H., Wang, Z., Li, C., Zhang, Q., Jin, C. & Wang, X. (2013). Severe fever with thrombocytopenia syndrome virus among domesticated animals, China. *Emerging infectious diseases*, *19*(5), 756.

Park, S.W., Han, M.G., Yun, S.M., Park, C., Lee, W.J. and Ryou, J., 2014. Severe fever with thrombocytopenia syndrome virus, South Korea, 2013. *Emerging infectious diseases*, *20*(11), 1880Patel, P., Landt, O., Kaiser, M., Faye, O., Koppe, T., Lass, U., Sall, A.A. & Niedrig, M. (2013). Development of one-step quantitative reverse transcription PCR for the rapid detection of flaviviruses. *Virology journal*, *10*(1), 58.

Ryou, J., Lee, H. I., Yoo, Y. J., Noh, Y. T., Yun, S. M., Kim, S. Y., Shin, E.H., Han, M.G. & Ju, Y. R. (2011). Prevalence of hantavirus infection in wild rodents from five provinces in Korea, 2007. *Journal of wildlife diseases*, *47*(2), 427-432.

Song, J.W., Baek, L.J., Schmaljohn, C.S. and Yanagihara, R. (2007). Thottapalayam virus, a prototype shrewborne Hantavirus. *Emerging infectious diseases*, *13*(7), p.980.

Song, JW, Hae, JK, Se, HG, Sung, SM, Bennett, SN, Song, KJ, Luck, JB, Kim, HC, O'Guinn, ML, Chong, ST, Klein, TA & Yanagihara, R (2009). 'Characterization of Imjin virus, a newly isolated hantavirus from the Ussuri white-toothed shrew (Crocidura lasiura)' *Journal of Virology*, vol. 83, no. 12, 6184-6191.

Song, K.J., Baek, L.J., Moon, S., Ha, S.J., Kim, S.H., Park, K.S., Klein, T.A., Sames, W., Kim, H.C., Lee, J.S. and Yanagihara, R. (2007). Muju virus, a novel Hantavirus harboured by the arvicolid rodent Myodes regulus in Korea. *Journal of General Virology*, *88*(11), 3121-3129.

Tadin, A., Turk, N., Korva, M., Margaletić, J., Beck, R., Vucelja, M., Habuš, J., Svoboda, P., Županc, T.A., Henttonen, H. and Markotić, A., 2012. Multiple co-infections of rodents with hantaviruses, Leptospira, and Babesia in Croatia. Vector-Borne and Zoonotic Diseases, 12(5), 388-392.

Viel, J. F., Lefebvre, A., Marianneau, P., Joly, D., Giraudoux, P., Upegui, E., Tordo, N. & Hoen, B. (2011). Environmental risk factors for haemorrhagic fever with renal syndrome in a French new epidemic area. *Epidemiology & Infection*, *139*(6), 867-874.

Wu, T., Guo, X. L., Peng, H. Y., Chen, Y. I., Zhao, K. C. C., Cui, L. B., GE, Y., SHI, Z. Y., QI, X. & LIU, D. P. (2013). Isolation and analysis of genomic sequences of SFTS virus from goat and tick attached to its surface. *Jiangsu Journal of Preventive Medicine*. 24, 7–10.

Yoshii, K., Song, J.Y., Park, S.B., Yang, J. and Schmitt, H.J., 2017. Tick-borne encephalitis in Japan, Republic of Korea and China. *Emerging microbes & infections*, *6*(9) 82.

Yun, S. M., Lee, Y. J., Choi, W., Kim, H. C., Chong, S. T., Chang, K. S., Coburn, J.M., Klein, T.A. & Lee, W. J. (2016). Molecular detection of severe fever with thrombocytopenia syndrome and tick-borne encephalitis viruses in

ixodid ticks collected from vegetation, Republic of Korea, 2014. *Ticks and tick-borne diseases*, *7*(5), 970-978.

Yun, S.M., Song, B.G., Choi, W., Park, W.I., Kim, S.Y., Roh, J.Y., Ryou, J., Ju, Y.R., Park, C. and Shin, E.H., 2012. Prevalence of tick-borne encephalitis virus in ixodid ticks collected from the Republic of Korea during 2011–2012. *Osong public health and research perspectives*, *3*(4), 213-221.

Yun, Y., Heo, S.T., Kim, G., Hewson, R., Kim, H., Park, D., Cho, N.H., Oh, W.S., Ryu, S.Y., Kwon, K.T. and Medlock, J.M., 2015. Phylogenetic analysis of severe fever with thrombocytopenia syndrome virus in South Korea and migratory bird routes between China, South Korea, and Japan. *The American journal of tropical medicine and hygiene*, *93*(3) 468-474.

Zhang, Y.Z. and Xu, J., 2016. The emergence and cross species transmission of newly discovered tick-borne Bunyavirus in China. *Current opinion in virology*, *16*, 126-131.

Table 1. Oligonucleotide primers and conditions for polymerase chain reactions used in this study.

PCR Assay	Primers & Probe 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) (external primer	HAN-L-F1 (5'-ATGTAYGTBAGTGCWGATGC-3')	95/30	49/30	72/45	450	Klempa, et al., 2006
	HAN-L-R1 (5-AA CCADTCWGTYCCRTCATC-3')					
HFRS RT-N-PCR (internal primer)	HAN-L-F2 (5-TGCWGATGCHACIAARTGGTC-3')	95/20	54/20	72/30	380	Klempa, et al., 2006
	HAN-L-R2 (5'-GCRTCRTCWGARTGRTGDGCAA-3)					
HFRS RT-N-PCR (S segment) (external primer	HFRS-S-2F (5'-ARARRTCARBVCTHAGBTAYG-3')	95/30	44/30	72/60	1,009	In this study
	HFRS-S-2R (5-TGRTTVGAKATTTCCTTSAC-3')					
HFRS RT-N-PCR (internal primer)	HFRS-S2nd-1F (5-GAYATTGAWGAACCWASWGGVC-3')	95/30	50/30	72/60	725	In this study
	HFRS-S2nd -1R (5'GAHGCCATKATKGTRTTYCKC-3)					
SFTS RT-N-PCR (M segment) (external primer)	SFTS-M 1st-F (5'-TCATCCTGACYTATTYTGCAATWG-3')	95/20	58/40	72/30	510	In this study
	SFTS-M 1st-R (5'- TAAGTYACACTCACACCCTTGAA-3')					
SFTS RT-PCR (internal primer)	SFTS-F (5'- ACCTCTTTGACCCTGAGTTWGACA-3')	95/20	58/40	72/30	560	Heo, et al., 2015
	SFTS-R (5'- CTGAAGGAGA CAGGTGGAGATGA-3')					
SFTS RT-N-PCR (S segment)	SFTS-S-NP-2F (5'-CATCATTGTCTTTGCCCTGA-3')	94/20	53/20	72/30	461	Hwang, et al., 2017

(external primer)	SFTS-S-NP-2R (5'-AGAAGACAGAGTTCACAGCA-3')					
SFTS RT-PCR (internal primer)	SFTS-S-N2F (5'-AAYAAGATCGTCAAGGCATCA-3')	94/20	57/20	72/30	346	Hwang, et al., 2017
	SFTS-S-N2R (5'-TAGTCTTGGTGAAGGCATCTT-3')					
TBE RT- N-PCR (external primer)	TBE-913F (5’-TGCACACAYYTGGAAAACAGGGA-3’)	94/30	52/30	72/60	854	(Yun et.al., 2012)
	TBE-1738R (5’- TGGCCACTTTTCAGGTGGTACTTG-3’)					
TBE RT- N-PCR (internal primer)	TBE-1738R (5’- TGGCCACTTTTCAGGTGGTACTTG-3’)	94/20	62/10	68/20	506	(Yun et.al., 2012)
	TBE-1192F (5’-CAGAGTGATCGAGGCTGGGGYAA-3’)					

bp: base pair

°C/sec: degree Celsius per seconds (time)

Table 2. Monthly prevalence of hantavirus and severe fever with thrombocytopenia syndrome virus (SFTSV), detected by reverse transcription nested polymerase chain reaction (RT-N-PCR) targeting L and S segments of hantavirus and M segment of SFTSV captured in 2017 at Boseong-gun.

Month	Examined No. of mice	PCR positivity	Hantavirus-L (N-PCR)	Hantavirus-S (N-PCR)	SFTSV-M (N-PCR)	SFTSV-M (C-PCR)	SFTSV-S (N-PCR)	TBEV- E (N-PCR)
October	10	No. within month (%)	3 (30.0%)	2 (20.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
		% of Total	14.30%	9.50%	0.00%	0.00%	0.00%	0.00%
November	11	No. within month (%)	2 (18.2%)	1 (9.1%)	1 (9.10%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
		% of Total	9.50%	4.80%	4.80%	0.00%	0.00%	0.00%
Total	21	Positive No. (%)	5 (23.8%)	3 (14.3%)	1 (4.8%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
P value			0.52	0.47	0.32	-	-	-

Table 3. Monthly prevalence of Hantavirus and Severe fever with thrombocytopenia syndrome virus (SFTSV), detected by RT-N-PCR targeting L and S segments of Hantavirus and M segment of SFTSV captured in 2018 at Jeju Island.

Month	Examined No. of mice	PCR positivity	Hantavirus-L (N-PCR)	Hantavirus-S (N-PCR)	SFTSV-M (N-PCR)	SFTSV-M (C-PCR)	SFTSV-S (N-PCR)	TBEV- E (N-PCR)
April	15	No. within month (%)	0 (0%)	0 (0%)	3 (20%)	0 (0%)	0 (0%)	0 (0%)
		% of Total	0.0%	0.0%	5.3%	0.0%	0.0%	0.0%
May	7	No. within month (%)	6 (85.7%)	1 (14.3%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
		% of Total	10.5%	1.8%	0.0%	0.0%	0.0%	0.0%
June	8	No. within month (%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
		% of Total	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
July	5	No. within month (%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
		% of Total	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Aug	7	No. within month (%)	1 (14.3%)	1 (14.3%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
		% of Total	1.8%	1.8%	0.0%	0.0%	0.0%	0.0%
Sep	9	No. within month (%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
		% of Total	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%

Oct	6	No. within month (%)	3 (50%)	1 (16.7%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
		% of Total	5.3%	1.8%	0.0%	0.0%	0.0%	0.0%
Total	57	No. (%)	10 (17.5%)	3 (5.3%)	3 (5.3%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
P value			<0.001	0.04	0.18	-	-	-

Table 4. Monthly seroprevalence of hantavirus and Severe fever with thrombocytopenia syndrome virus (SFTSV), detecting hantavirus Immunoglobulin G (IgG) and SFTSV IgM and IgG antibodies in wild mice sera captured in 2018 at Jeju Island.

Month	Examined No. of mice	Sero-positivity	Hantavirus IgG positivity	SFTSV IgM positivity	SFTSV IgG positivity
April	6	No. within month (%)	0 (0%)	0 (0%)	0 (0%)
		PCR positive	0	0	0
May	6	No. within month (%)	2 (33.3%)	0 (0%)	0 (0%)
		PCR positive	2 (100%)	0	0
June	8	No. within month (%)	0 (0%)	0 (0%)	0 (0%)
		PCR positive	0	0	0
July	5	No. within month (%)	0 (0%)	0 (0%)	0 (0%)
		PCR positive	0	0	0
Aug	7	No. within month (%)	1 (14.3%)	0 (0%)	0 (0%)
		PCR positive	1 (100%)	0	0
Sep	9	No. within month (%)	1 (11.1%)	0 (0%)	2 (22.2%)
		PCR positive	0	0	0
Oct	6	No. within month (%)	1 (17%)	0 (0%)	0 (0%)
		PCR positive	1 (100%)	0	0
Total	47	No. (%)	5 (11%)	0 (0%)	2 (4.2%)

PCR positive: PCR positive within serological positive samples

Figure 1: Geographical location of wild mice capturing site. Province boundaries are shown with colored area as collection site of this study.

Figure 2: Phylogenetic tree based on the L segment (360 bp) of isolates identified positive for Hantavirus from Boseong-gun Jeollanam-do (JN) in 2017 (filled circle) and from Jeju (JJ) in 2018 (filled arrow) compared with Hantaviruses sequences from Genebank. The Genebank accession number is indicated. Scale bar indicates 0.05 (nucleotide substitution per site) sequence distance.

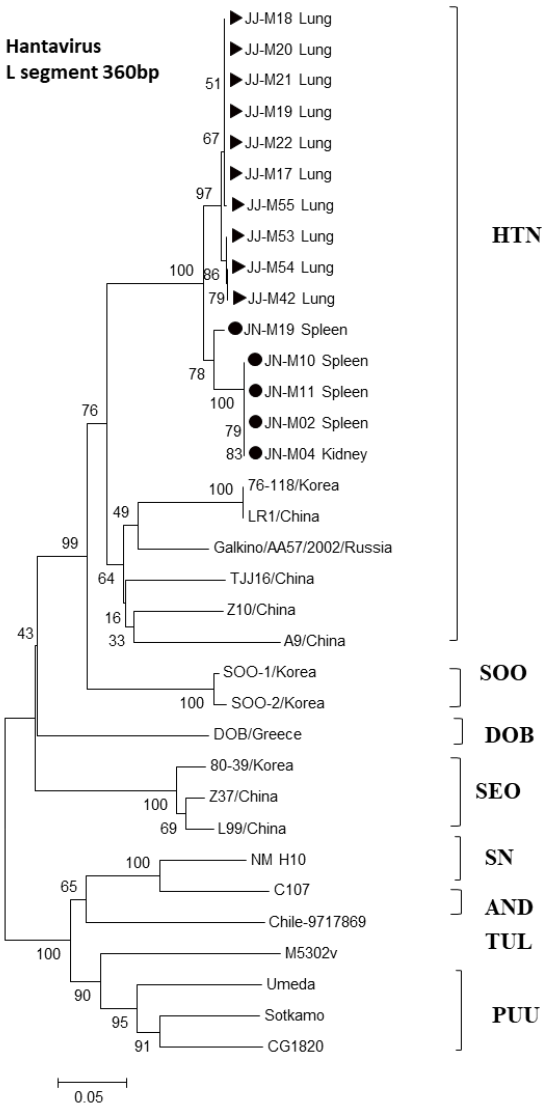


Figure 3: Phylogenetic tree based on the S segment (650 bp) of isolates identified positive for Hantavirus from Boseong-gun Jeollanam-do (JN) in 2017 (filled circle) and from Jeju (JJ) in 2018 (filled arrow) compared with Hantaviruses sequences from Genebank. The Genebank accession number is indicated. Scale bar indicates 0.05 (nucleotide substitution per site) sequence distance.

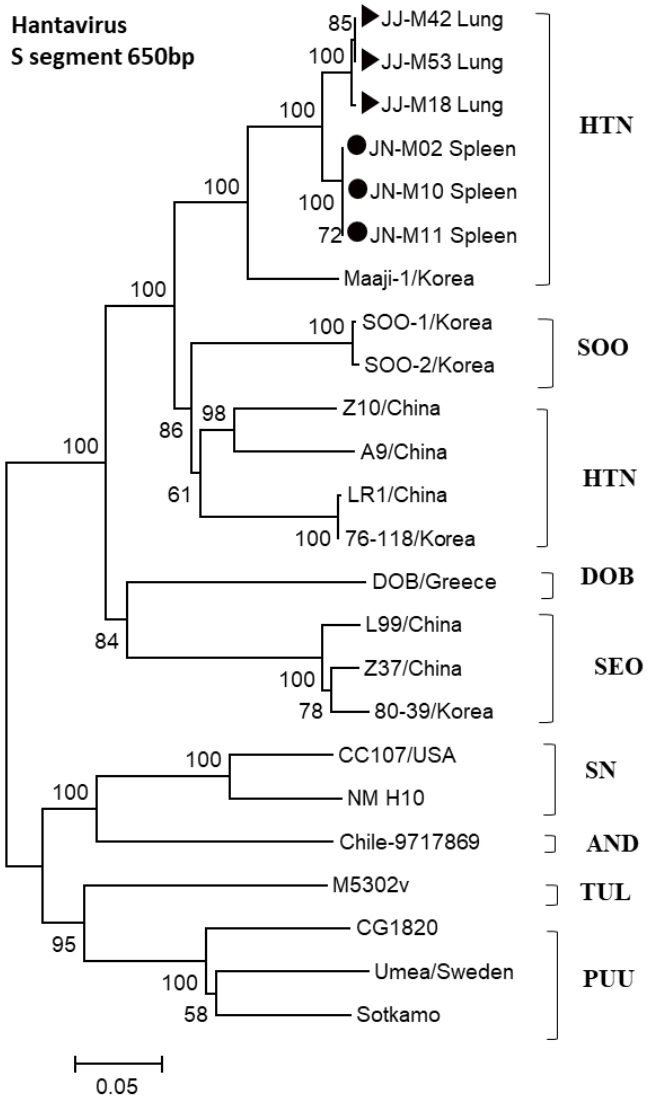


Figure 4: Phylogenetic tree based on the M segment (477 bp) of isolates identified positive for SFTSV from Boseong-gun Jeollanam-do (JN) in 2017 (filled circle) and from Jeju (JJ) in 2018 (filled arrow) compared with SFTSV sequences from Genebank. The Genebank accession number is indicated. Scale bar indicates 0.05 (nucleotide substitution per site) sequence distance.

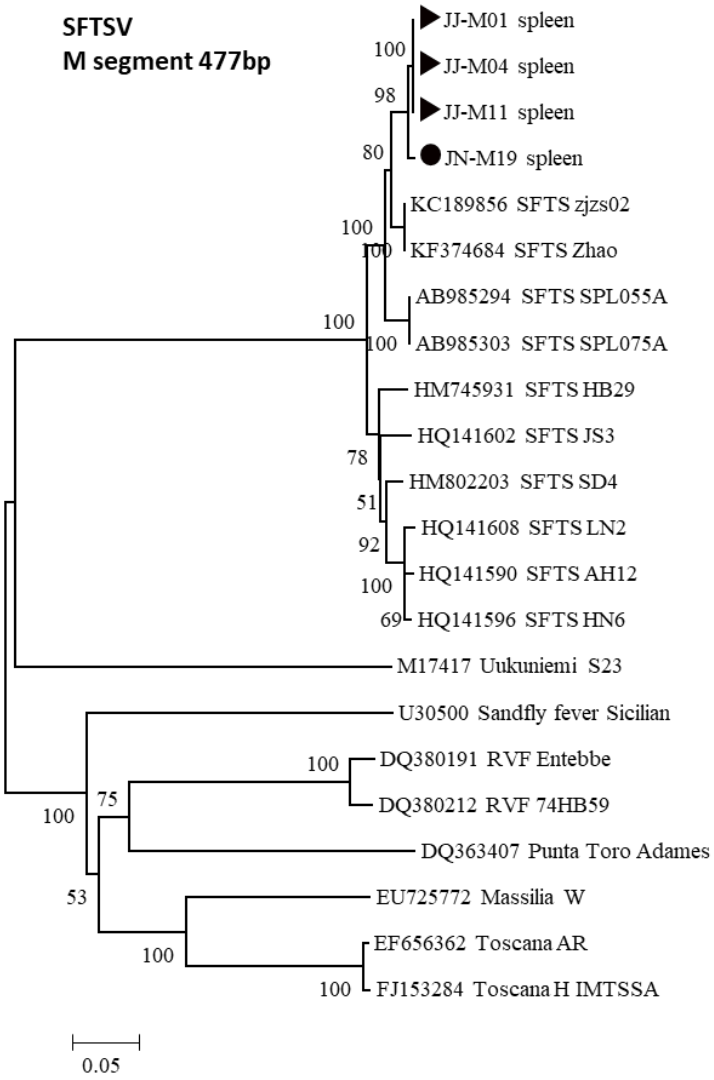
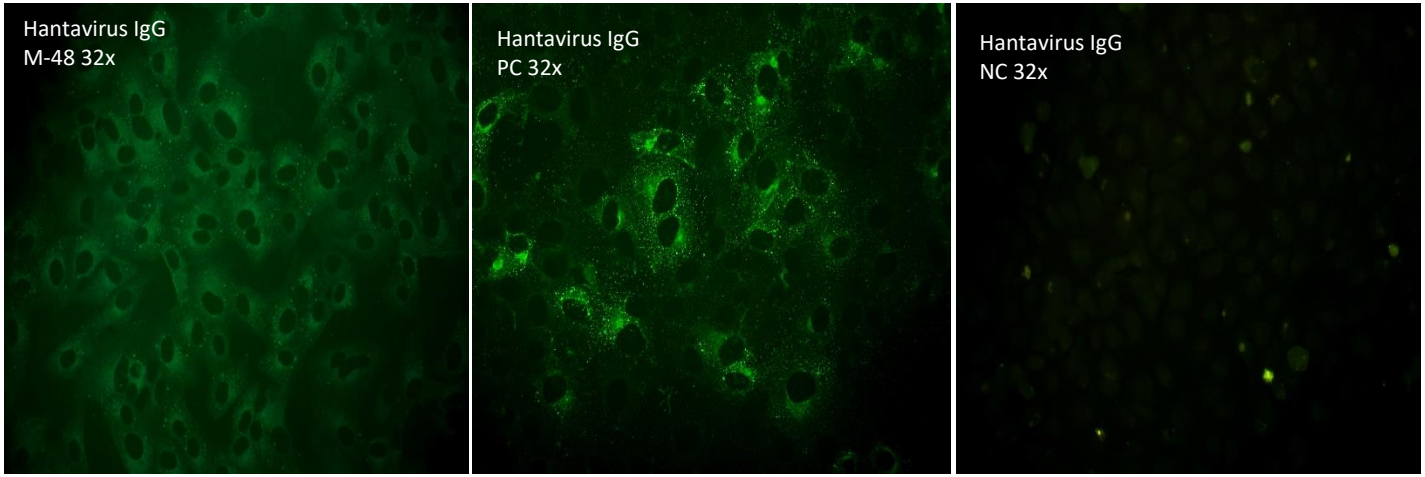


Figure 5: Serological results for Immunofluorescence assay (IFA) conducted for the presence of hantavirus specific antibodies Immunoglobulin G (IgG) detection using 2018-Jeju wild mice sera.

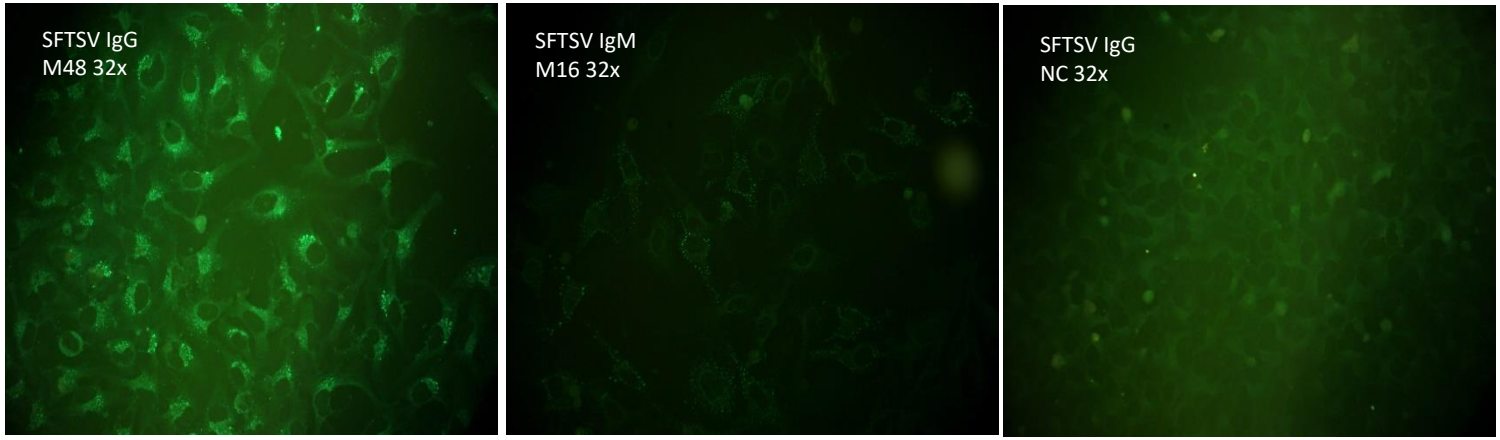


PC: positive control

NC: negative control

32X: 1/32 dilution factor (titer)

Figure 6: Serological results for Immunofluorescence assay (IFA) conducted for the presence of severe fever with thrombocytopenia syndrome virus (SFTSV) specific antibodies Immunoglobulin G (IgG) and Immunoglobulin M (IgM) detection using 2018-Jeju wild mice sera.



PC: positive control

NC: negative control

32X: 1/32 dilution factor (titer)

ACKNOWLEDGEMENT

I am grateful to God for the good health and wellbeing that were necessary to conduct this research and it is a pleasure to thank many people who made this thesis possible.

Firstly, I would like to express my sincere gratitude to my supervisor and advisor Prof. Dong Min Kim for providing me with an opportunity to join their team, for continuous support during my master's study and related research, for his patience, motivation, and immense knowledge. I could not have imagined having a better advisor and mentor for my graduate study. Besides my advisor, my sincere thanks also go to the rest of my thesis committee: Prof. Choon Mee Kim, for sharing her expertise, sincere and valuable guidance and encouragement throughout my research period, which steered me in the right direction. Prof. Geon Park for his insightful comments, critical review and the willingness to serve on my thesis committee.

I would like to thanks department of biomedical sciences, collage of medicine, Chosun University for giving access to the laboratory, research facilities and funding. I am also grateful to Gwangju Institute of Health and Environment and Gwangju Health University for the collection of specimens used in this study.

I am extremely thankful and indebted to Miss Yumi Lee and Miss Miseon Bang for providing me extensive personal and professional guidance. Without your precious support and friendship, my stay at Korea won't be this smooth. I am grateful to all of our lab members with whom I have had the pleasure to work during this and other related projects.

I must express my very profound gratitude to my parents, my family, my beloved daughter and my husband Inayat Ullah for providing me unconditional

love with unfailing support and continuous encouragement throughout my years of study. This accomplishment would not have been possible without them. Thank you.

And finally, last but by no means least, to all my teacher at Pakistan who enabled me to stand in place and my dear friends.

Thanks for all your encouragement!

Sehrish Jalal

February, 2019

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