



HOKKAIDO UNIVERSITY

Title	Seroepidemiological study for bat- and shrew-borne hantaviruses and analysis of renal pathology in hantavirus-infected mouse model simulating hemorrhagic fever with renal syndrome
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Degree Grantor	北海道大学
Degree Name	博士(感染症学)
Dissertation Number	甲第16660号
Issue Date	2025-09-25
DOI	https://doi.org/10.14943/doctoral.k16660
Doc URL	https://hdl.handle.net/2115/98270
Type	doctoral thesis
File Information	Emmanuel_Kodua.pdf, 全文



Seroepidemiological study for bat- and shrew-borne
hantaviruses and analysis of renal pathology in
hantavirus-infected mouse model simulating hemorrhagic
fever with renal syndrome

(コウモリおよびトガリネズミ由来ハンタウイルス
の血清疫学および腎症候性出血熱マウスモデルの腎
病理学的解析)

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Abbreviations

ALTV - Altai virus

ANDV – Andes virus

ASAV - Asama virus

B2m - Beta-2-microglobulin

BRNV - Brno virus

BSA - Bovine serum albumin

BSL-3 - Biosafety level 3

CBNV - Cao Bang virus

CDC - Centers for disease control and prevention

Ct - Cycle threshold (qPCR)

DKGV - Dakrong virus

dpi - Days post-infection

ELISA - Enzyme-linked immunosorbent assay

ERC - Ethical review committee

FFU - Focus forming units

GP - Glycoprotein

HFRS - Hemorrhagic fever with renal syndrome

HPS - Hantavirus pulmonary syndrome

HRP - Horseradish peroxidase

HTNV - Hantaan virus

IFAT - Indirect immunofluorescence antibody test

IgG - Immunoglobulin G

IPTG - Isopropyl β -D-1-thiogalactopyranoside

KHFV - Korean hemorrhagic fever virus

Mab - Monoclonal antibody

MCS - Multiple cloning site

N - Nucleocapsid protein

NVAV - Nova virus

ORF - Open reading frame

PAGE - Polyacrylamide gel electrophoresis

PBS – Phosphate-buffered saline

PCR - Polymerase chain reaction

PFU – Plaque-forming units

qPCR - Quantitative PCR

QZNV - Quezon virus

RBC - Red blood cell

RNA - Ribonucleic acid

ROBV - Robina virus

rN - Recombinant nucleocapsid protein

RT-PCR - Reverse transcription PCR

SDS - Sodium dodecyl sulfate

SNV - Sin Nombre virus

SWSV - Seewis virus

TPMV - Thottapalayam virus

WB - Western blot

XSV - Xuan Son virus

Notes

This thesis contains three chapters, and they have been published as follows:

1. Sarii RS, Kajihara M, Wei Z, Lokpathirage SMW, Muthusinghe DS, Mori-Kajihara A, et al. Development of a seroepidemiological tool for bat-borne and shrew-borne hantaviruses and its application using samples from Zambia. *PLoS Negl Trop Dis*, 18(11):e0012669, 2024 Nov., doi.org/10.1371/journal.pntd.0012669
2. Sarii RS, Senarathne P, Gamage C, Yoshimatsu K. Serological detection of antibodies against bat-borne and shrew-borne hantaviruses in Sri Lanka. *Jpn J Vet Res*, 73(2):51-58, 2025, Jul., doi.org/10.57494/jjvr.73.2_51
3. Sarii RS, Senarathne P, Yoshimatsu K. Urinalysis of a Hantaan virus-infected mouse model of hemorrhagic fever with renal syndrome. *J Vet Med Sci*, 87(7):727-734, 2025 Jul., doi:10.1292/jvms.25-0116

Preface

The emergence and re-emergence of zoonotic pathogens continue to challenge global public health systems, with hantaviruses serving as a particularly interesting case study in viral evolution and host adaptation. Hantaviruses are negative-sense RNA viruses of the family *Hantaviridae*. Their genomes consist of three main segments. Small (S), Medium (M), and Large (L). Hantaviruses have traditionally been linked to rodent reservoirs, causing two distinct clinical syndromes in humans: hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS). These emerging zoonotic pathogens pose significant public health risks worldwide.

HFRS is a zoonotic disease caused by Old World hantaviruses, including Hantaan virus (HTNV), Dobrava virus (DOBV), Seoul virus (SEOV), and Puumala virus (PUUV). They are primarily distributed across Europe and Asia. The disease onset is characterized by fever, chills, and aches, along with mid-phase renal signs such as proteinuria, oliguria, polyuria, and hypotension. Nausea, vomiting, neurological symptoms, and late-stage kidney failure may also occur. The most severe form of HFRS is caused by HTNV, which presents as thrombocytopenia, increased vascular permeability, and acute kidney damage. Although our understanding of HFRS pathophysiology is limited, immunological dysfunction and tissue damage caused by immune regulatory problems are thought to play a key role, with the endothelium regarded as the primary target of hantaviruses. Historically, HFRS has been a major public health issue in Korea.

The first case was documented in 1951, and the disease has remained a concern since then. The striped field mouse is one of the primary sources of HFRS in Korea, transmitting the virus to humans through contact with infected rodents. HFRS affects various countries worldwide, including China, Russia, South Korea, and parts of Europe.

China reports the highest number of cases, with tens of thousands each year. The disease tends to spread more readily in rural areas with poor sanitation and housing conditions. Russia also exhibits a high prevalence, particularly in European Russia and the Far East. After the initial outbreak, South Korea implemented public health measures to reduce rodent populations and educate the public about prevention. The South Korean government has established surveillance systems to monitor rodent populations and prevent the spread of hantaviruses.

In Europe, countries such as Sweden, Finland, and Germany have reported isolated cases of HFRS. These nations' public health authorities focus on early detection and treatment to reduce disease-related illness and death. Currently, HFRS remains a significant public health concern in many parts of the world. Ongoing research and surveillance efforts are essential for preventing future outbreaks and safeguarding public health.

HPS is as well severe and sometimes fatal respiratory illness caused by hantavirus infection. The viruses causing HPS (Sin Nombre [SNV], Monongahela [MNGV], Andes [ANDV], and Black Creek Canal [BCCV] viruses) are typically carried by rodents such as deer mice and can spread to people by contact with infected rodent urine, droppings, or saliva. HPS was initially detected in the United States in 1993, following an epidemic in the Four Corners region (southwestern corner of Colorado, southeastern corner of Utah, northeastern corner of Arizona, and northwestern corner of New Mexico). HPS symptoms often include fever, muscular pains, and exhaustion, which are followed by coughing and shortness of breath when the lungs swell with fluid. If not treated properly, the condition can advance quickly and cause respiratory failure.

The incidence of HPS varies by geography, with the majority of cases documented in the North and South American countries. According to the Centers for Disease Control

and Prevention (CDC), there have been around 728 verified cases of HPS in the United States since its discovery in 1993. The southwestern region of the United States has the highest prevalence of HPS, particularly in states such as New Mexico, Arizona, and Colorado. This is owing to the presence of deer mice, which are known carriers of the hantavirus. People who come into contact with rodent droppings or urine may get the virus and develop HPS.

Apart from rodent-borne hantaviruses, hantaviruses originating from bat and shrew origins have also been reported ⁴⁰, raising questions about their zoonotic potential and the need for expanded surveillance ^{22,91}. Despite their growing recognition, their antigenic properties and pathogenicity remain poorly understood ⁶. Until now, the infectivity of these emerging viruses to humans is unknown. Regarding infectivity in humans, the detection of shrew-borne hantaviruses, such as Altai virus (ALTV) and Thottapalayam virus (TPMV), has been reported in Sri Lanka and Thailand ⁵⁶. In addition, suggestive serological evidence of human Imjin viral infection has been reported in China ⁶¹. Their findings also revealed that shrew-borne viral infections may have affected humans in several different nations. However, apart from the work reported in this thesis, there is no other report on bat-borne hantavirus infection in humans. Figure 1 shows the phylogeny of rodent -, bat-, and shrew-borne hantaviruses based on their N sequences. Table 1 shows some selected viruses belonging to the subfamily *Mammantavirinae* of the family *Hantaviridae*.

This dissertation presents an integrated investigation of hantavirus biology through three complementary research strands, each employing innovative methodologies to address persistent knowledge gaps in the field. The work was conducted at the intersection of virology, immunology, and epidemiology, with findings that have important implications for both basic science and public health practice. It addresses

these gaps by developing novel serological tools for detecting bat- and shrew-borne hantaviruses and investigating their prevalence in Zambia and Sri Lanka. Additionally, it explores a mouse model of HFRS to elucidate renal pathology and evaluate the efficacy of neutralizing antibodies.

The first part of the study utilizes recombinant nucleocapsid (rN) proteins from 11 hantaviruses: five bat-borne viruses, Brno (BRNV), Dakrong (DKGV), Quezon (QZNV), Robina (ROBV), and Xuan Son (XNV) and six shrew-borne viruses, Asama (ASAV), ALTV, Cao Bang (CBNV), Nova (NVAV), Seewis (SWSV), and TPMV, to establish a multiplex indirect immunofluorescence antibody test (IFAT) for efficient screening ⁶⁴. Applying this tool to archived bat sera from Zambia (2006–2018) to establish a multiplex indirect IFAT, enabling efficient screening of diverse hantavirus antibodies. The findings reveal unexpected seropositivity patterns in African bats and Sri Lankan humans, suggesting cross-species transmission and the need for further research into these emerging viruses. The second part extends the serological investigation to human populations in Sri Lanka, where bat- and shrew-borne hantaviruses were previously undetected. Screening 336 human sera (168 febrile patients and 168 healthy controls), I identified antibodies against QZNV, ROBV, ASAV, ALTV, CBNV, and TPMV, with higher seropositivity in febrile cases. These findings suggest human exposure to diverse hantaviruses in Sri Lanka, warranting further studies to assess their clinical significance and ecological reservoirs. The last part of this work focuses on the Korean hemorrhagic fever virus (KHFV), a strain of HTNV, to model HFRS in immunocompetent mice. This section concentrates on characterizing renal dysfunction and testing the prophylactic and therapeutic potential of the neutralizing monoclonal antibody HCO2. This study provides insights into renal pathology and virus

tropism in the kidney and treatment strategies. The results underscore the utility of this model for antiviral and vaccine development.

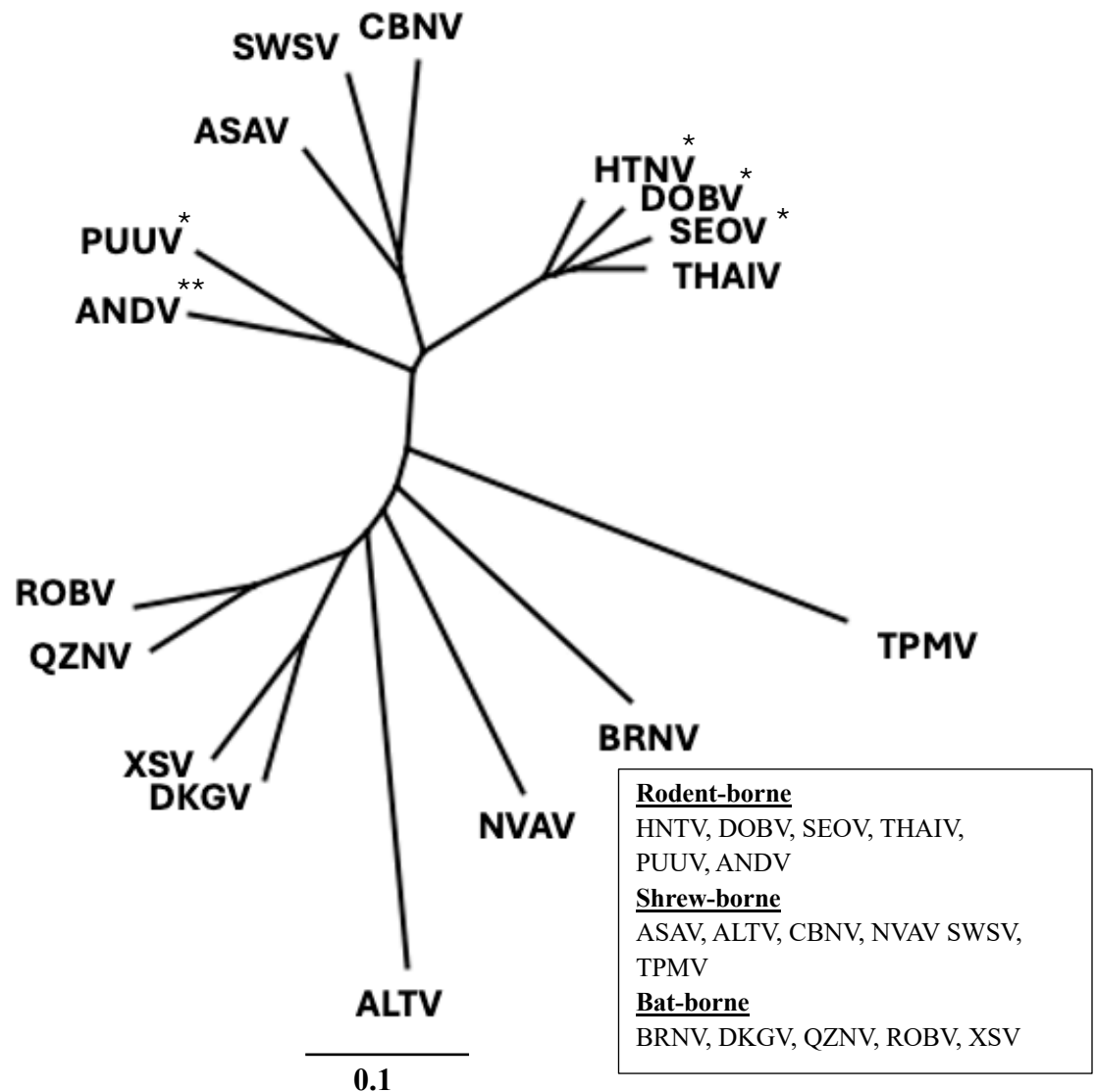


Figure 1. Phylogenetic tree of hantaviruses based on amino acid sequences of nucleocapsid protein (N)

Hantavirus N sequences were aligned using MAFFT, and the phylogenetic tree was constructed with the Neighbor-joining method in Geneious Tree Builder on Geneious Prime for macOS 15.3.1. The scale bar indicates a sequence divergence of 0.1. The sequences' accession numbers are listed below: HNTV (M14626.1), SEOV (AY273791.1), DOBV (CAC85163.1), THAIV (MZ343360.1), PUUV (MN832784.1), ANDV (AF291702.1), ASAV (ACI28511.1), SWSV (MT544324), TPMV (AY526097.1), ALT (QPK67294.1), CBNV (EF543524.1), NVAV (KX512329.1) (BRNV (KX845678.1), DKG (MG663534.1), QZNV (KU950713.1), ROBV (MK165655.1), XSV (WJV41803.1).

* Reported as the causative virus of HFRS, ** Reported as the causative virus of HPS

Table 1. Viruses belonging to the subfamily *Mamantavirinae* of the family *Hantaviridae*,

Genus	Virus	Host animal	Disease	Distribution of virus	Reference
<i>Orthohantavirus</i>	Hantaan (HTNV)	<i>Apodemus agrarius</i>	Severe HFRS KHF	Asia, Europe	Lee et al., 1978 ³⁸
<i>Orthohantavirus</i>	Seoul (SEOV)	<i>Rattus rattus</i> , <i>Rattus norvegicus</i>	Moderate HFRS	World-wide	Lee et al., 1982 ³⁷
<i>Orthohantavirus</i>	Dobrava (DOBV)	<i>Apodemus flavicollis</i>	Severe HFRS	Balkans, Russia, Estonia	Avsic-Zupanc et al., 1994 ¹¹
<i>Orthohantavirus</i>	Thailand (THAIV)	<i>Bandicota indica</i>	Unknown	Thailand	Pattamadilok et al., 2006 ⁵⁹
<i>Orthohantavirus</i>	Puumala (PUUV)	<i>Clethrionomys glareolus</i>	Mild HFRS/NE	Europe, Balkans, Russia	Brummer et al., 1980 ¹³
<i>Orthohantavirus</i>	Andes (ANDV)	<i>Oligoryzomys longicaudatus</i>	HPS	Argentina	Gorbunova et al., 2010 ¹⁹
<i>Orthohantavirus</i>	Asama (ASAV)	<i>Urotrichus talpoides</i>	Unknown	Japan	Arai S et al., 2008 ⁵
<i>Unclassified</i>	Altai (ALTV)	<i>Sorex caecutiens</i>	Unknown	Russia, Europe, Mongolia	Kang HJ et al., 2019 ²⁹
<i>Orthohantavirus</i>	Cao Bang (CBNV)	<i>Anourosorex squamipes</i>		Vietnam, China	Song JW et al., 2007 ⁶⁹
<i>Orthohantavirus</i>	Seewis (SWSV)	<i>Sorex araneus</i>	Unknown	Europe, Russia	Ling J et al., 2014 ⁴²
<i>Thottimovirus</i>	Thottapalayam (TPMV)	<i>Suncus murinus</i>	Unknown	India	Carey DE et al., 1971 ¹⁴
<i>Unclassified</i>	Altai (ALTV)	<i>Sorex caecutiens</i>	Unknown	Russia, Europe, Mongolia	Kang HJ et al., 2019 ²⁹
<i>Mobatvirus</i>	Nova (NVAV)	<i>Talpa europaea</i>	Unknown	Europe	Gu SH et al., 2015 ²¹
<i>Loanvirus</i>	Brno (BRNV)	<i>Nyctalus noctula</i>	Unknown	Czech Republic	Strakova P et al., 2017
	Dakrong (DKGV)	<i>Aselliscus stoliczkanus</i>	Unknown	Vietnam	Arai S et al., 2019 ³
<i>Mobatvirus</i>	Quezon (QZNV)	<i>Rousettus amplexicaudatus</i>	Unknown	Philippines	Arai S et al., 2016 ⁶
<i>Mobatvirus</i>	Robina (ROBV)	<i>Pteropus alecto</i>	Unknown	Australia	Unpublished
<i>Mobatvirus</i>	Xuan Son (XNV)	<i>Hipposideros pomona</i>	Unknown	Vietnam, China	Arai S et al., 2013 ⁴

**Chapter 1: Development of a seroepidemiological tool
for bat-borne and shrew-borne hantaviruses and its
application using samples from Zambia**

Introduction

Hantaviruses are enveloped single-stranded negative-sense RNA viruses. They are members of the *Elliovirales* order and the family *Hantaviridae*. Currently, rodent-borne orthohantaviruses are associated with 2 lethal illnesses in humans: HFRS, which is mostly distributed in Europe and Asia, and HPS, found in the Americas^{13,38,51}. While the epidemiology is still unclear, literature reports that hantaviruses can be transmitted to humans through the respiratory system via inhalation of aerosolized secretions or excretions^{76,85}. Person-to-person transmission is seldom observed, with a few cases of HPS related to the ANDV in Argentina and Chile serving as exceptions. The N plays an important role in viral replication and assembly and serves as a serodiagnostic antigen^{1,16,65,89}. Apart from in rodents, hantaviruses have also been detected in bats and shrews⁴⁰. However, the potential for these viruses to infect humans remains unclear.

With over 1400 species of bats (order *Chiroptera*) identified, bats constitute the second-largest order of mammals. Bats are present on every continent except Antarctica. Most bats are insectivorous or frugivorous. Bats are natural reservoirs of many microbiological organisms, and their social structure, lifespan, and flight facilitate the spread of zoonotic pathogens. Some bat-borne hantaviruses have been identified based on partial or complete S, M, and L genomic sequences⁷. The viruses used in this chapter were selected based on the availability of their full-length N sequences. These include BRNV (*Loanvirus brunaense*)^{17,71} detected from the noctule bat, *Nyctalus noctula* (Czech Republic), DKGV (*Mobatvirus dakrongense*) detected from Stoliczka's trident bat, *Aselliscus stoliczkanus* (Vietnam), QZNV (*Mobatvirus quezonense*)⁶ detected from Geoffroy's rousette, *Rousettus amplexicaudatus* (Philippines), ROBV (*Mobatvirus robinaense*)⁸¹ detected from the black fruit bat, *Pteropus alecto* (Australia), and XSV (*Mobatvirus xuansonense*)⁴ detected from the ashy roundleaf bat, *Hipposideros cineraceus* (Vietnam). Phylogenetic analysis showed a close relationship between QZNV and ROBV viruses, similar to that between the DKGV and XSV viruses. In

African countries, partial hantavirus sequences have been detected in bats captured in Sierra Leone ⁸², Côte d'Ivoire ³⁰, and Gabon ⁸³. However, full genome sequencing and isolation of these viruses have not yet been performed.

The prototype of shrew-borne hantaviruses is the TPMV (*Thottimvirus thottapalayamense*), isolated from the Asian house shrew, *Suncus murinus*, in India ¹⁴. TPMV is thought to occur in a wide range of Asian countries, including China ²², Vietnam ⁴⁴, Indonesia, and Thailand ⁵⁶. Shrews carry *Thottimovirus*, *Orthohantaviruses*, and other unclassified hantaviruses. SWSV is extensively dispersed in Europe and Siberia, and may naturally infect several species of Sorex shrews, such as *Sorex araneus*, *Sorex caecutiens*, *Sorex minutissimus* and *Sorex roboratus* ³². Some related viruses have been reported in Kenya ⁵⁷. Sorex shrews are also carriers of ALTV, which is extensively dispersed throughout Russia, Mongolia, and Europe ^{32,86}. ASAV (*Orthohantavirus asamaense*) ⁵ NVAV (*Mobatvirus novaense*) ²⁷, and CBNV (*Orthohantavirus caobangense*) ²⁰ have been detected in Japanese, European, and Chinese shrew moles (*Urotrichus talpoides*, *Talpa europaea*, and *Anourosorex squamipes*, respectively). In African countries, the shrew-borne hantaviruses Tanganya virus, Azagny virus, Kilimanjaro virus, and Ulugru virus (unclassified) have been reported in *Crocidura* and *Myosorex* shrews ³⁰. The shrew-borne viruses ALTV, CBNV, and ASAV are genetically distinct, and their classifications have not yet been approved.

Although many hantaviruses from bats and shrews have been identified, their antigenic differences have not been reported. Additionally, due to a lack of available tools, only a few small-scale studies have been reported on the potential for bat- and shrew-borne hantaviruses to infect humans. Cases of human seropositivity to TPMV, ALTV, and Imjin viruses have been reported ^{56,61}. In a previous study, a recombinant TPMV antigen diagnostic method was reported ⁵⁶. We have also reported diagnostic methods for SWSV, ALTV, and ASAV and have shown low cross-reactivity among the

N antigens of these viruses ¹⁰ . In this study, I expressed recombinant nucleocapsid protein (rN) of 5 bat-borne hantaviruses (BRNV, DKGV, QZNV, ROBV, and XSV) and 6 shrew-borne hantaviruses (ASAV, ALTV, CBNV, NVAV, SWSV, and TPMV) for use in serological assays. Antisera to each rN were produced, and their cross-reactivities were examined. Systematic serological screening methods were established and applied to serum samples from bats captured in Zambia between 2006 and 2018.

Materials and Methods

Ethics statement

All animal experiments were approved by the Animal Studies Ethics Committee of Hokkaido University (24–0026). Mice were treated according to the laboratory animal control guidelines of the Hokkaido University Institutional Animal Care and Use Committee.

Plasmids

The pCAGGS/MCS plasmid was used to express the rN of hantaviruses in mammalian cells⁵². For NUS-tag rN expression in *Escherichia coli*, the pET43.1 system (Takara Bio, Shiga, Japan; Novagen, Merck Millipore, Billerica, MA, USA) was used.

Sequence information of N of bat- and shrew-borne hantaviruses and construction of plasmids

Accession numbers of N genes used in the present study are shown in the right panel of Fig. 2A. The differences in amino acid among these viruses used in this study are shown on the left side of Fig. 2A. The open reading frames (ORFs) of the BRNV (KX845678), ROBV (MK165655), and CBNV (EF543524) genes were synthesized (Thermo Fisher Scientific). The ORFs of DKGV (MG663534), QZNV (KU950713), XSV (KC688335), and NVAV (KX512329) were amplified from RNA (extracted from lung tissue) using RT-PCR and PCR before inserting them into their respective backbone plasmids. ALTV, SWSV, ASAV, and TPMV N genes were previously prepared in both pCAGGS/MCS and pET43.1 plasmids^{63,79}. Similarly, plasmids encoding the Ns of BRNV, DKGV, QZNV, ROBV, XSV, CBNV, and NVAV were prepared to express rNs.

Expression of rNs in Vero E6 cells for the IFAT and in 293T cells for Western blot

An outline of the preparation and confirmation of the rN antigens is shown in Fig. 2B. Vero E6 cells expressing the respective rNPs were prepared and seeded onto 24-well glass slides for IFAT. Briefly, Vero E6 cells grown in a 6-well plate were transfected with 2 µg of the expression plasmid vector pCAGGS/MCS, prepared as described above, using the TransIT-LT1 transfection reagent (Mirus Bio LLC, USA) according to the manufacturer's instructions. Twenty-four hours after transfection, the cells were collected by trypsinization, seeded onto 24-well glass slides (Matsunami Glass IND., LTD., Tokyo, Japan), and incubated for 24 hours in a 5% CO₂ incubator at 37°C. The glass slides were washed with phosphate-buffered saline (PBS) and fixed with acetone for 10 min. After fixation, slides were briefly washed with distilled water and dried. The slides were stored at -80°C until use. Similarly, 293T cells were transfected, collected 2 days after transfection, and treated with 500 µL of 2× sample buffer (Fujifilm-Wako, Osaka, Japan) for Western blot.

Preparation of antisera to rNs

Rabbit anti-TPMV, anti-SWSV, and ALTV Ns previously prepared were used as described^{56,79}. Previously described, rNs fused with NUS and histidine-tagged proteins at the N-terminal end were expressed using the pET43.1 system in BL-21 *E. coli* cell lines and purified using His Trap HP columns (Cytiva, Tokyo, Japan) according to the manufacturer's instructions. The molecular weights of the purified antigens were estimated by Western blot using a mouse anti-NUS-tag monoclonal antibody (Takara Bio; Novagen).

The anti-NUS-tag antibody was used to confirm the preliminary expression of the various proteins in BL-21 cells before proceeding to produce the respective antisera by using horseradish peroxidase (HRP)-conjugated anti-mouse IgG (H+L) (Jackson

ImmunoResearch, USA) as the secondary antibody. Purified rN antigens of BRNV, DKGV, QZNV, ROBV, XSV, ASAV, CBNV, and NVAV were respectively used to immunize mice three times with a total dose of 120 mg (60, 30, and 30 mg) at 3-week intervals. Zeno particle adjuvant (Zenogen Pharma Co., Ltd., Tokyo, Japan) was used for the first 2 doses only. Alexa Fluor 488-labeled goat anti-mouse or anti-rabbit IgG antibodies (Thermo Fisher Scientific) were used as secondary antibodies for mouse and rabbit antisera. Subsequently, individual homologous antisera titration was performed, and cross-reactivity between rN was assessed.

Titration was performed using two-fold serial dilutions for each antigen, starting from 1:100 and continuing to the twelfth dilution. Each dilution was tested with IFAT, and the corresponding titer was recorded. Additionally, I examined the reactivity of a previously reported monoclonal antibody E5/G6 against the expressed bat- and shrew-borne rNs on IFAT.

Novel screening tool for bat and shrew-borne hantaviruses

In this study, a serological platform for screening antibodies against 5 bat and 6 shrew-borne hantaviruses using their rN antigens was developed. Vero E6 cells were co-transfected with the mixture of pCAGGS/MCS containing N genes of the selected bat-borne (BRNV, DKGV, QZNV, ROBV, and XSV) and shrew-borne (ASAV, ALTV, CBNV, NVAV, SWSV, and TPMV) viruses using the TransIT-LT1 transfection reagent (Fig. 2C). Vero E6 cells grown in 6-well plates were transfected with the plasmids (0.5 µg of each plasmid of bat-borne virus and 0.4 µg of each plasmid of shrew-borne virus). Transfected cells were seeded onto 24-well glass slides as described above and evaluated using IFAT.

Confirmation of the reactivity of secondary antibody anti-bat IgG

The reactivity of HRP-conjugated Protein A and G (Thermo Fisher Scientific) and HRP-conjugated Goat anti-Bat IgG (H+L) (Bethyl Laboratories, Inc., USA) was compared using ELISA as previously described³⁶. Serially diluted sera from three bat species (*Macronycteris vittatus*, *Eidolon helvum*, and *Rousettus aegyptiacus*) were coated onto an ELISA plate and blocked using 3% bovine serum albumin (BSA). Respective HRP-conjugated proteins were used as secondary antibodies, and o-phenylenediamine dihydrochloride (OPD) was used as a substrate for detection. Anti-bat IgG was more reactive against the Bat IgG for the three species tested. Hence, unlabeled Goat anti-Bat IgG (H+L), and 0.5 mg of the IgG fraction, was conjugated with Alexa 488 dye using an Alexa Fluor 488 protein labeling kit (Thermo Fisher Scientific). The conjugated anti-Bat IgG served as a secondary antibody for IFAT screening of bat serum samples at a dilution of 1:1000.

Bat serum samples

Archived serum samples from 1,764 bats in Zambia were used for serological testing. These samples were collected by Professor Ayato Takada, Associate Professor Masahiro Kajihara, and Mrs. Akina Mori-Kagihara from 2006 to 2018, with permission from the Department of National Parks and Wildlife, Ministry of Tourism and Arts, Zambia (DNPW8/27/1), and ethical approval from the University of Zambia Biomedical Research Ethics Committee (1382–2020). All serum samples underwent IFAT screening using a co-transfected system at a serum dilution of 1:300. Subsequently, IFAT with individually expressed antigens was employed to identify bound antigens. The selected samples were titrated based on the reaction pattern and cross-reactivity. Some positive samples were further confirmed for reactivity using Western blot assay, as described below. IFAT positivity was graded as +, ++, or +++ depending on the intensity and number of positive cells. Triplicate samples were applied

to 24-well glass slides for all IFAT analyses. This grading was done subjectively by two examiners.

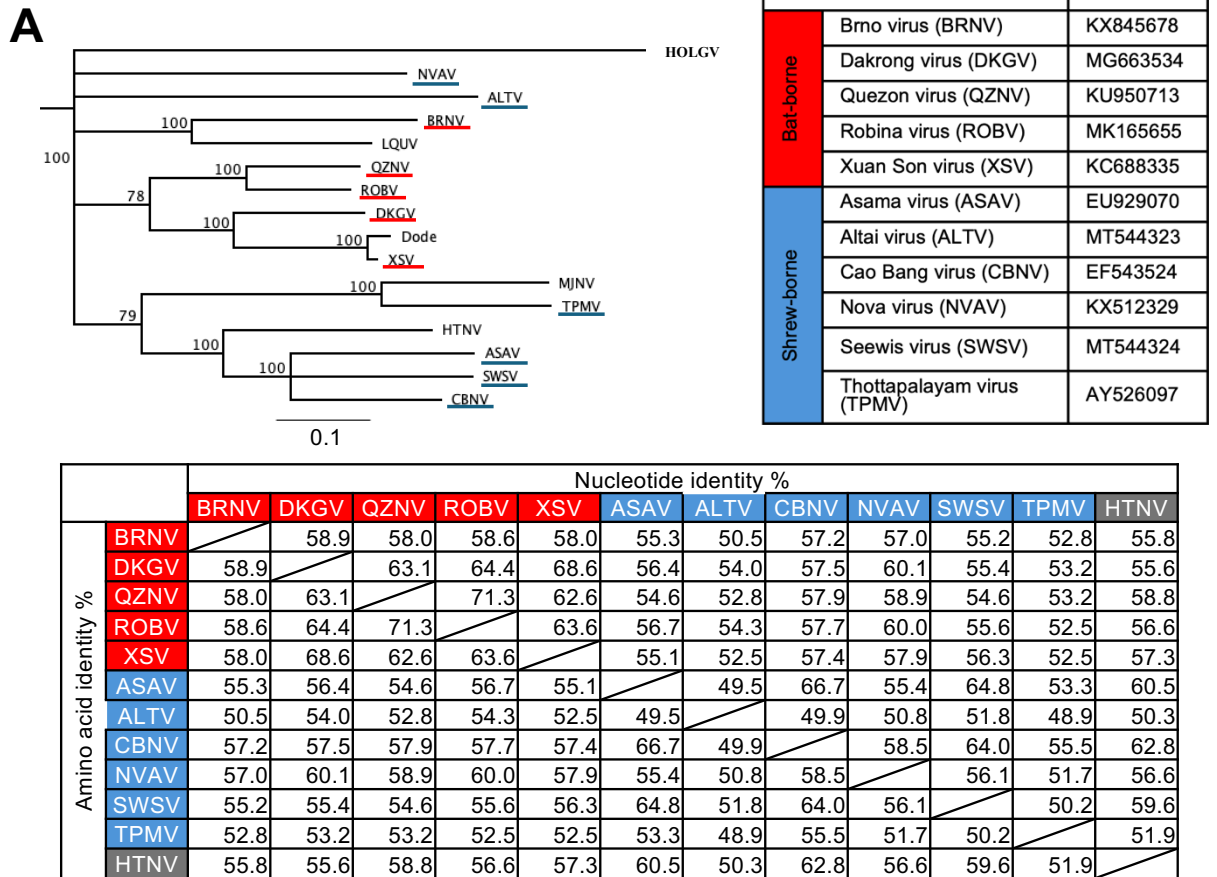


Figure 2. Outline of experiments.

(A) Phylogeny with nucleotide and amino acid sequence comparison

Bat- and shrew-borne hantaviruses were used in this study. The relationships between these viruses were determined through phylogenetic analysis based on their deduced amino acid sequences of the Ns. The phylogenetic tree was constructed using the neighbor-joining method with the MAFFT alignment tool in Geneious Prime 2024 (MDF; Tokyo, Japan). HTNV; Hantaan *Orthohantavirus* strain 76118 (M14626), MJNV; Imjin *Thottimvirus* (EF641804), LQUV; Longquan *Loanvirus* (JX465420), HOLGV; Hainan oriental lead-toed gecko *Reptillovirus* (MG599942), Dode; *Unclassified*. Other sequences are listed in the table. In the phylogenetic tree, bat-borne viruses are underlined in red, while shrew-borne viruses are underlined in blue. The lower table shows the pairwise nucleotide and amino acid identities generated in Geneious Prime 2024. ACC (accession)

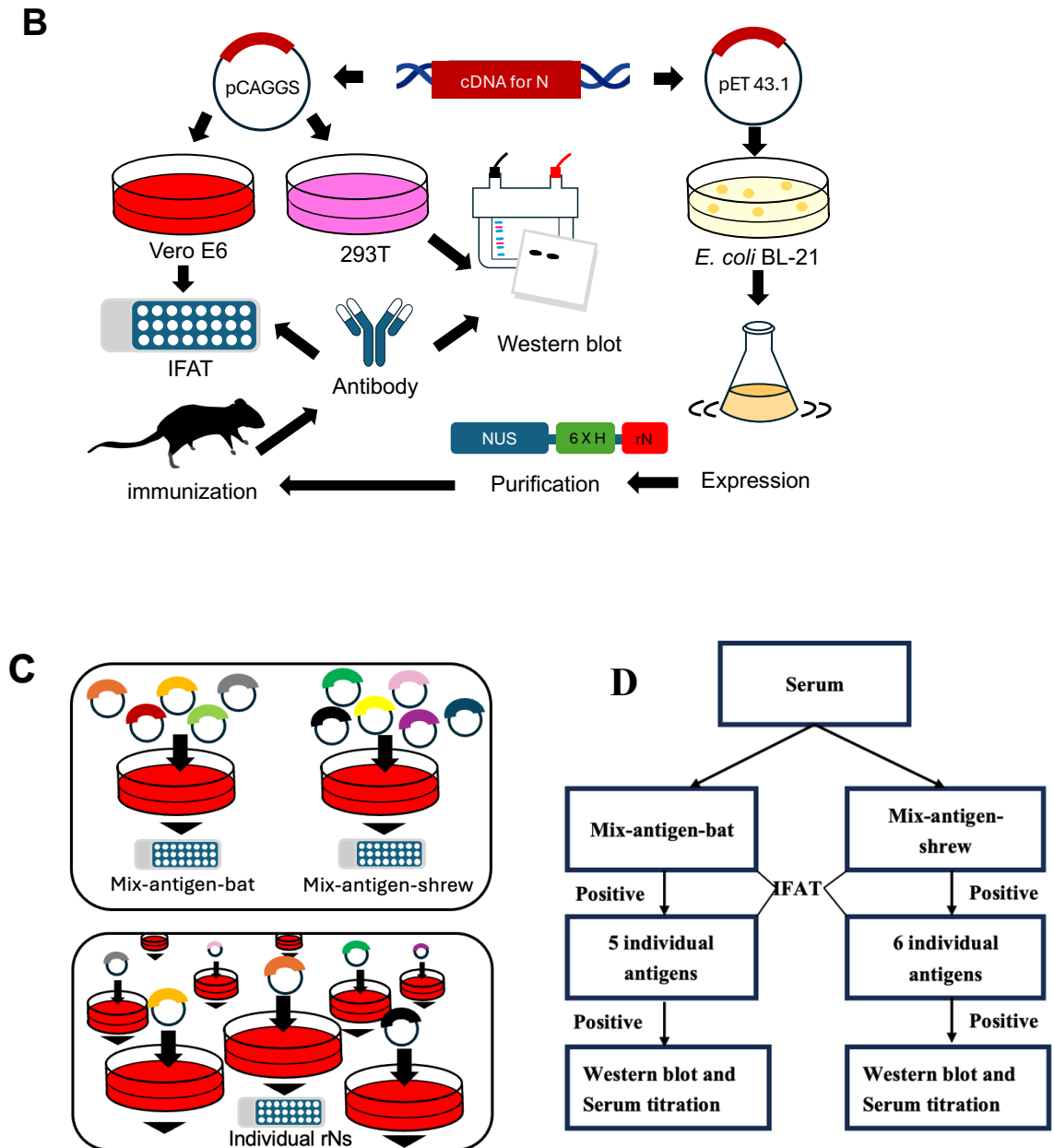


Figure 2. Outline of experiments (continuation from previous page).

(B) Expression of rNs as IFAT and Western blot antigens

Figure 2 shows an outline for constructing and generating antigens for IFAT and Western blot (pCAGGS), as well as antiserum from His Trap-purified proteins of the respective antigens.

(C) Preparation of antigens for serological screening by IFAT

A multiplex system consisting of five bat-borne antigens (Mix-antigen-bat) and six shrew-borne antigens (Mix-antigen-shrew) was prepared (upper panel). Additionally, individual antigens were also prepared (lower panel).

(D) A flow chart for the screening of bat serum samples

Serum samples are first screened using the mixed antigen assay. Positive sera are further screened on the individual antigen. Positive sera from individual screening are then analyzed by Western blot and titrated.

Western blot

10⁵ of 293T cells were transfected and collected 2 days after transfection and treated with 500 µl of 2x SDS-sample buffer (Fujifilm Wako) for sample preparation for sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE). 10 µL of cell lysate treated with 2x SDS-sample buffer was applied for electrophoresis. Proteins embedded into gels were then transferred to a PDVF membrane (Immobilon-P/IPVH, 0.45 µm, Sigma Aldrich). Rabbit and mouse immune serum (1:1000) and bat serum (1:500) were applied onto the membrane and incubated for 1 hour. Bound antibody was detected using secondary antibodies, HRP-conjugated anti-rabbit/HRP-conjugated anti-mouse IgG (H+L) (Jackson), and HRP-conjugated goat anti-bat (Bethyl) IgG (H+L), and Amersham ECL Prime Detection Reagent/detection machine: ImageQuant LAS 4000 mini (Cytiva). All secondary antibody dilutions were 1:5000.

Statistics

Fisher's exact test was performed using R software (R Foundation for Statistical Computing, Vienna, Austria) ⁷⁵. Relations between seropositivity were also examined by correlational analysis of the pairwise method on DATAtab ⁷⁴, and the correlation ratio was analyzed using the Microsoft Excel function "CORREL" (Microsoft Corporation, USA).

Results

Expression of rNs in Vero E6 cells and their antigenic similarity

To establish a serological screening assay based on IFAT, the rNs of hantaviruses were expressed in Vero E6 cells by transfection with mammalian cell expression vectors encoding N gene sequences. The expression of rNs was confirmed using homologous mouse or rabbit antiserum against the respective rN antigens expressed in the Vero E6 cell line. The expression of individual rN antigens in pET43.1 as well as 293T cells was confirmed by Western blot analysis (Fig. 3A). Strong signals from the rN-antisera reaction were detected at approximately 105 kDa for Nus-Tag fused antigens and 50 kDa, respectively, which are the expected molecular weights of the hantavirus Ns. The antisera showed specific fluorescence signals as shown in Fig. 3B. The absence of fluorescence in the mock-transfected cells indicates specific reactions of the antisera to rN-expressing cells.

Evaluation of titer and cross-reactivity

Confirmation of the individual rN was followed by homologous antibody titration testing for cross-reactivity between rNs (Table 2). No cross-reactivity was detected between these antigens and antibodies, except for QZNV and ROBV, where a tentative cut-off of 51200 was established. However, there was almost one-way cross-reactivity between the anti-ROBV antibody and QZNV antigen. Similarly, one-way cross-reactivity was observed between SWSV and CBNV.

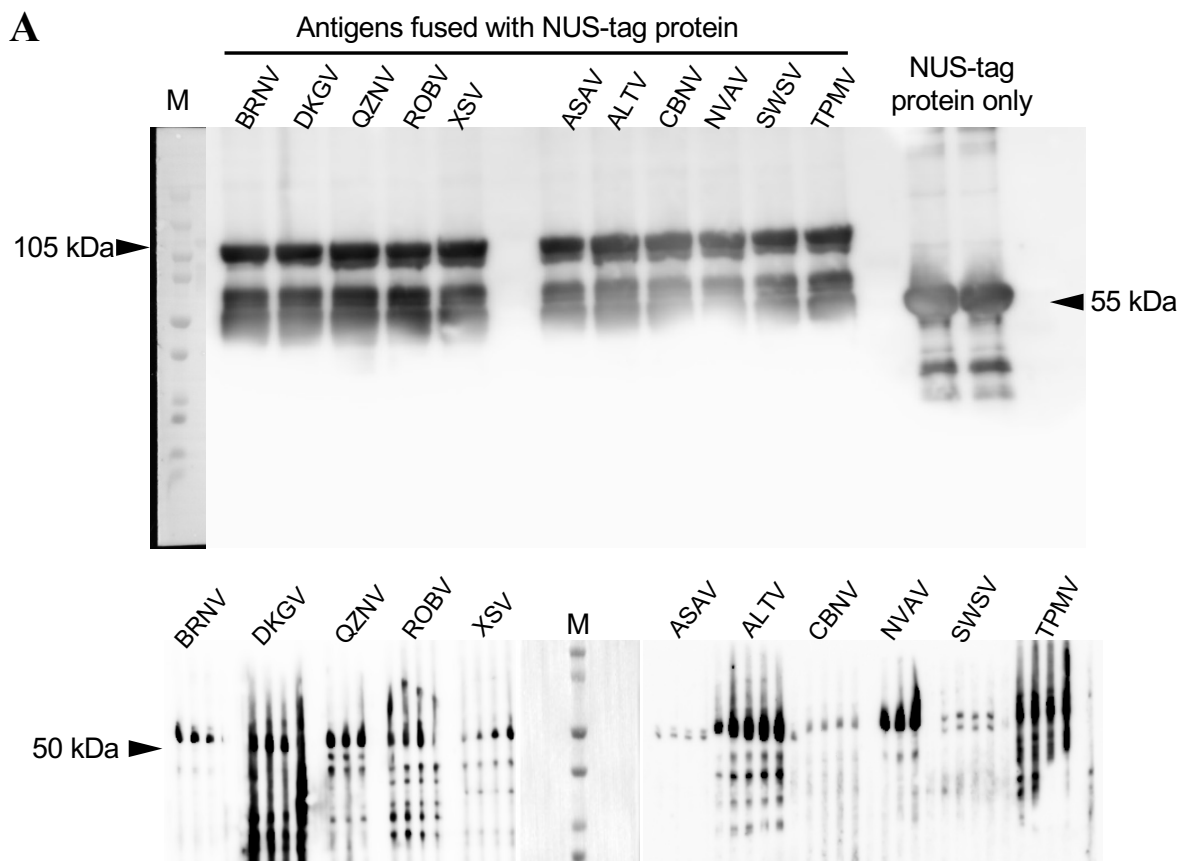


Figure 3. Expression of individual rNs of bat- and shrew-borne hantaviruses.

(A) Expression of rN fused with NUS-tag protein in *E. coli* by pET43.1 vector system (upper panel). The estimated size of the fusion protein was 105 kDa, detected with mouse anti-NUS-tag monoclonal antibody (Takara; Novagen). Expression of rN in 293T cells by pCAGGS/MCS plasmid vector (lower panels). The estimated molecular weights of rN were approximately 50 kDa, detected with corresponding specific antisera and applied to the 3-4 slits of Screener Blotter 28 (Sanplatec, Osaka, Japan). M: Molecular marker.

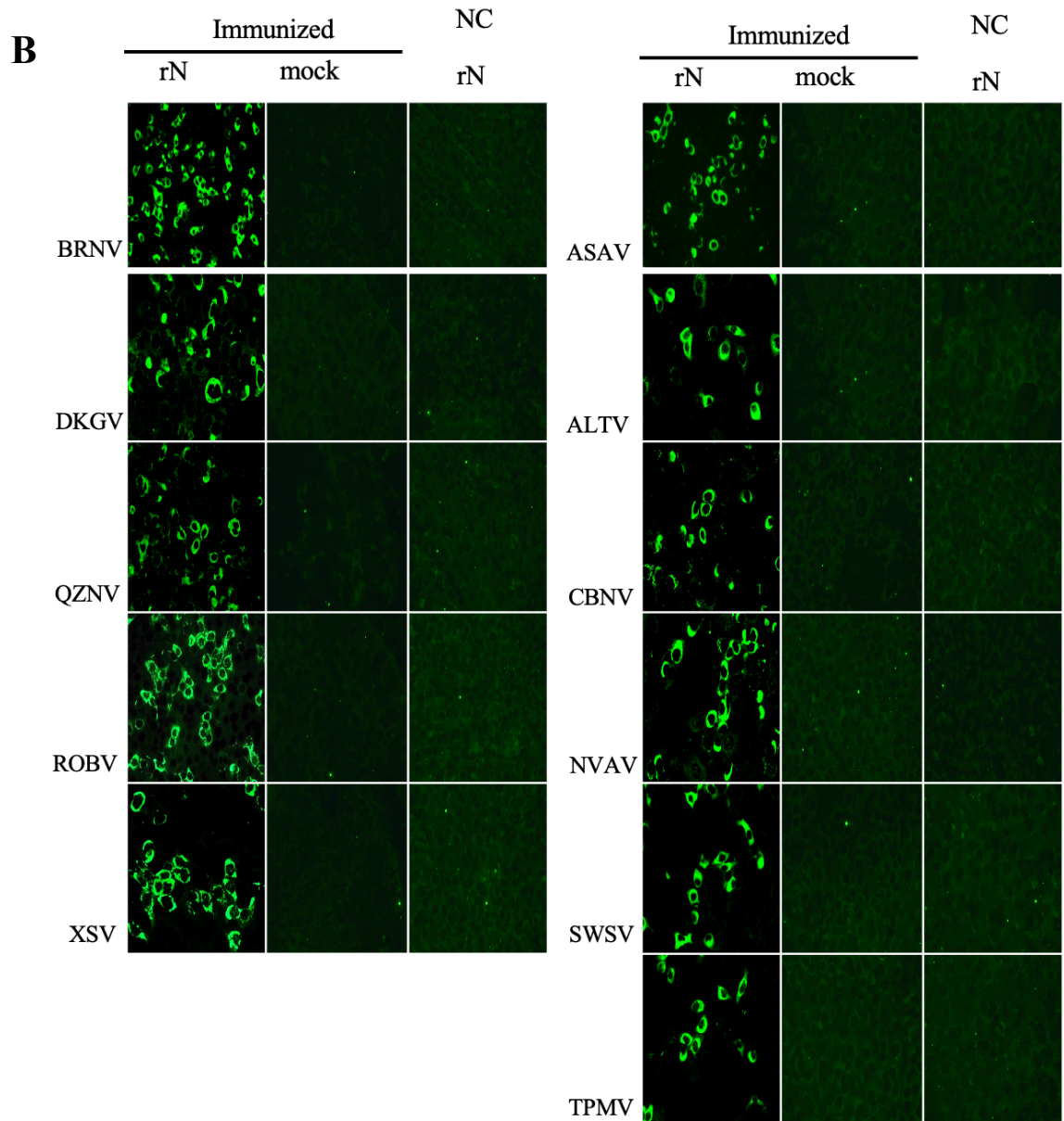


Figure 3. Expression of individual rNs of bat- and shrew-borne hantaviruses.

(B) IFAT profiles of individual rN antigens in individual antigens expressed in Vero E6 cells. Immunized and unimmunized mouse and rabbit sera (NC) were used at a dilution of 1:1000. Anti-mouse and anti-rabbit IgG Alexa Fluor 488 were used as secondary antibodies for the mouse and rabbit antisera, respectively. Mock-transfected Vero E6 cells were used as controls (second column).

Table 2. Cross-reactivities of antisera to rNs of bat- and shrew-borne hantaviruses.

rN antigen of		IFAT titers of polyclonal antisera to rN of										
		BRNV	DAGV	QZNV	ROBV	XSV	ASAV	ALTV	CBNV	NVAV	SWSV	TPMV
Bat-borne	BRNV	204800	-	-	-	-	-	-	-	-	3200	-
	DAGV	-	102400	-	-	-	-	-	-	-	-	-
	QZNV	-	-	204800	51200	3200	-	-	-	-	-	-
	ROBV	-	-	6400	204800	3200	-	-	-	-	3200	-
	XSV	-	3200	-	-	204800	-	-	-	-	-	-
Shrew-borne	ASAV	-	-	-	-	-	102400	-	-	3200	-	-
	ALTV	-	-	-	-	-	-	204800	-	-	-	-
	CBNV	-	-	-	-	-	-	-	204800	3200	-	-
	NVAV	-	-	-	-	-	3200	-	-	102400	-	-
	SWSV	-	-	-	-	-	-	-	25600	-	204800	-
	TPMV	-	-	-	-	-	-	-	-	-	-	102400

-; <100

Confirmation of simultaneous antigen expression of rNs of 5 bat-borne and 6 shrew-borne viruses

For serological screening using IFAT, simultaneous antigen expression of the rNs of 5 bat-borne viruses (Mix-antigen-bat) and 6 shrew-borne viruses (Mix-antigen-shrew) was performed. Antigen expression was confirmed by IFAT using homologous antisera (Fig. 4). Vero E6 cells without transfection did not react to any of the antisera.

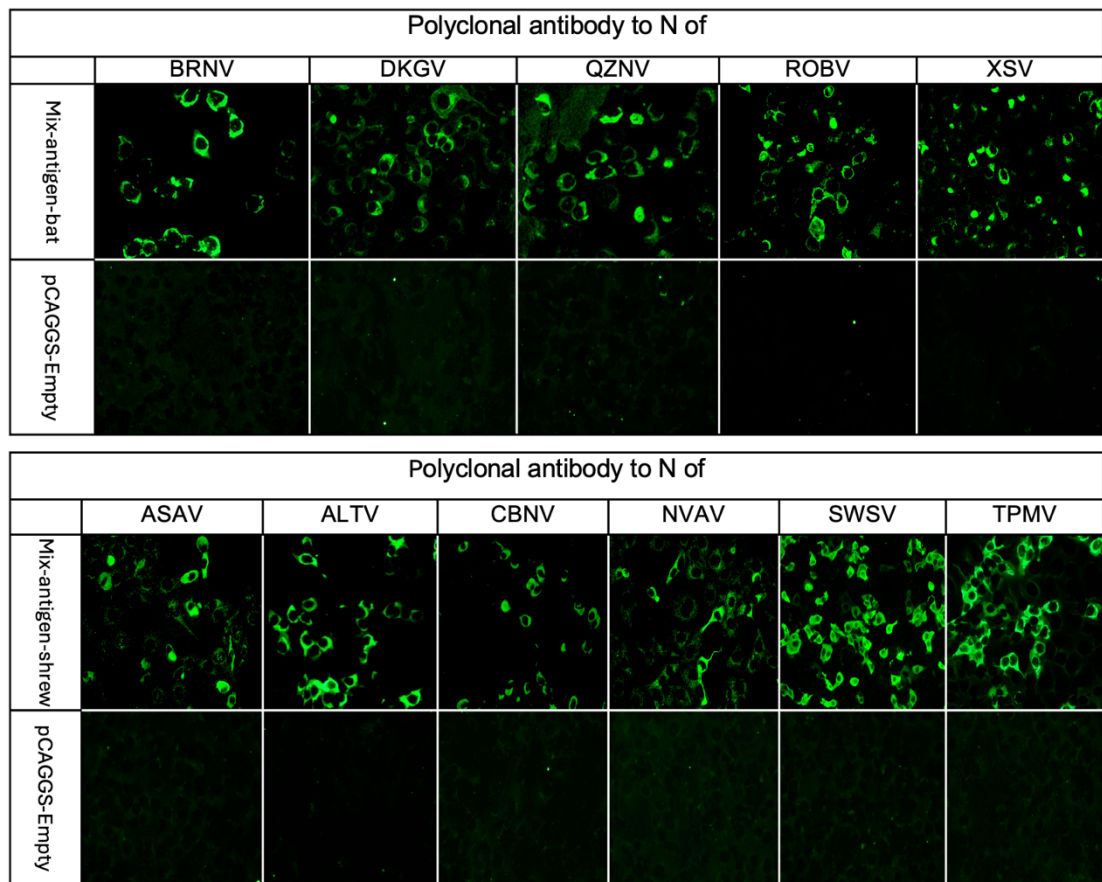


Figure 4. Expression of each antigen in Mix-antigen-bat and Mix-antigen-shrew.

Vero E6 cells expressing multiple rNs were incubated with respective antisera to confirm the expression of each rN. Figure 4 demonstrates that all rNs of BRNV, DKGV, QZNV, ROBV, and XSV for Mix-antigen-bat (upper part) and ASAV, CBNV, NVAV, ALTV, SWSV, and TPMV for Mix-antigen-shrew (lower part) produced strong fluorescent signals against their respective antisera when they were co-transfected in a single protocol, indicating the expression of each rN. In contrast, all antisera to rN did not bind to Vero E6 cells transfected with the empty pCAGGS/MCS plasmid DNA (second row from each part).

Seroprevalence of hantavirus infection in bats captured in Zambia

Of the 1,764 bat serum samples collected from the 11 bat species, 201 and 307 were positive for mix-antigen-bat and mix-antigen-shrew, respectively, exhibiting various fluorescence intensities at 1:300 dilution (Table 3). Of the 508 total positives, 107 were positive for both antigens, and 401 serum samples were positive for mix-antigen-bat or mix-antigen-shrew. These were further examined by IFAT using individual antigens. The degrees of reaction at 1:300 dilution were categorized as +, ++, and +++, respectively, for Mix-antigen-Bat and -Shrew. Therefore, 401 sera positive to either antigen were forwarded to further examination.

Table 3. Summary of Mix-antigen-bat and Mix-antigen-shrew screening by IFAT for bat sera from Zambia.

Species	Tested	Mix-antigen-bat				Mix-antigen-shrew				Specificities				
		Number of positives	IFAT intensity			Number of positives	IFAT intensity			Negative to		Positives to		
			+	++	+++		+	++	+++	both antigens	either antigen	both antigens	only bat antigen	only shrew antigen
<i>Eidolon helvum</i>	986	156	100	39	17	195	109	48	38	716	270	81	75	114
<i>Epomophorus crypturus</i>	84	7	7	0	0	14	11	3	0	66	18	3	4	11
<i>Hipposideros caffer</i>	2	0	0	0	0	0	0	0	0	2	0	0	0	0
<i>Macronycteris vittatus</i>	182	0	0	0	0	3	2	0	1	179	3	0	0	3
<i>Miniopterus</i> sp.	38	0	0	0	0	0	0	0	0	38	0	0	0	0
<i>Nycteris thebaica</i>	93	4	3	1	0	1	1	0	0	89	4	1	3	0
<i>Rhinolophus blasii</i>	1	0	0	0	0	0	0	0	0	1	0	0	0	0
<i>Rhinolophus swinnyi</i> .	4	0	0	0	0	0	0	0	0	4	0	0	0	0
<i>Rhinolophus</i> sp.	4	0	0	0	0	0	0	0	0	4	0	0	0	0
<i>Rousettus aegyptiacus</i>	370	34	22	9	3	94	46	24	24	264	106	22	12	72
Grand Total	1764	201 (11.4%)	132	49	20	307 (17.4%)	169	75	63	1363 (77.3%)	401 (22.7%)	107 (6.1%)	94 (5.0%)	200 (11.3%)

%= recorded value expressed as a percentage of 1764

Typical reaction patterns of bat serum in IFAT and Western blot

Generally, the reaction patterns of the bat serum sample on IFAT and WB showed varying degrees of reactivity to the respective rNs. For example, ZFB06-11 (ZFB = Zambian fruit bat, 06 = year, 11 = sample number) showed reactivity to DKGV and XSV antigens on individual IFAT but was reactive to XSV only on Western blot assay. Similarly, ZFB14-151 showed reactivity to both BRNV and ROBV on individual IFAT; however, only BRNV was reactive on Western blot. Other reaction patterns indicated mixed infection, as seen in ZFB11-40, where individual antigens showed reactivity to both BRNV and CBNV antigens and were detected on Western blot assays. Excerpts of the typical reaction patterns of bat serum using IFAT and Western blot are shown in Figs. 5 and 6. IFAT antibody titers of these sera are shown in Table 4.

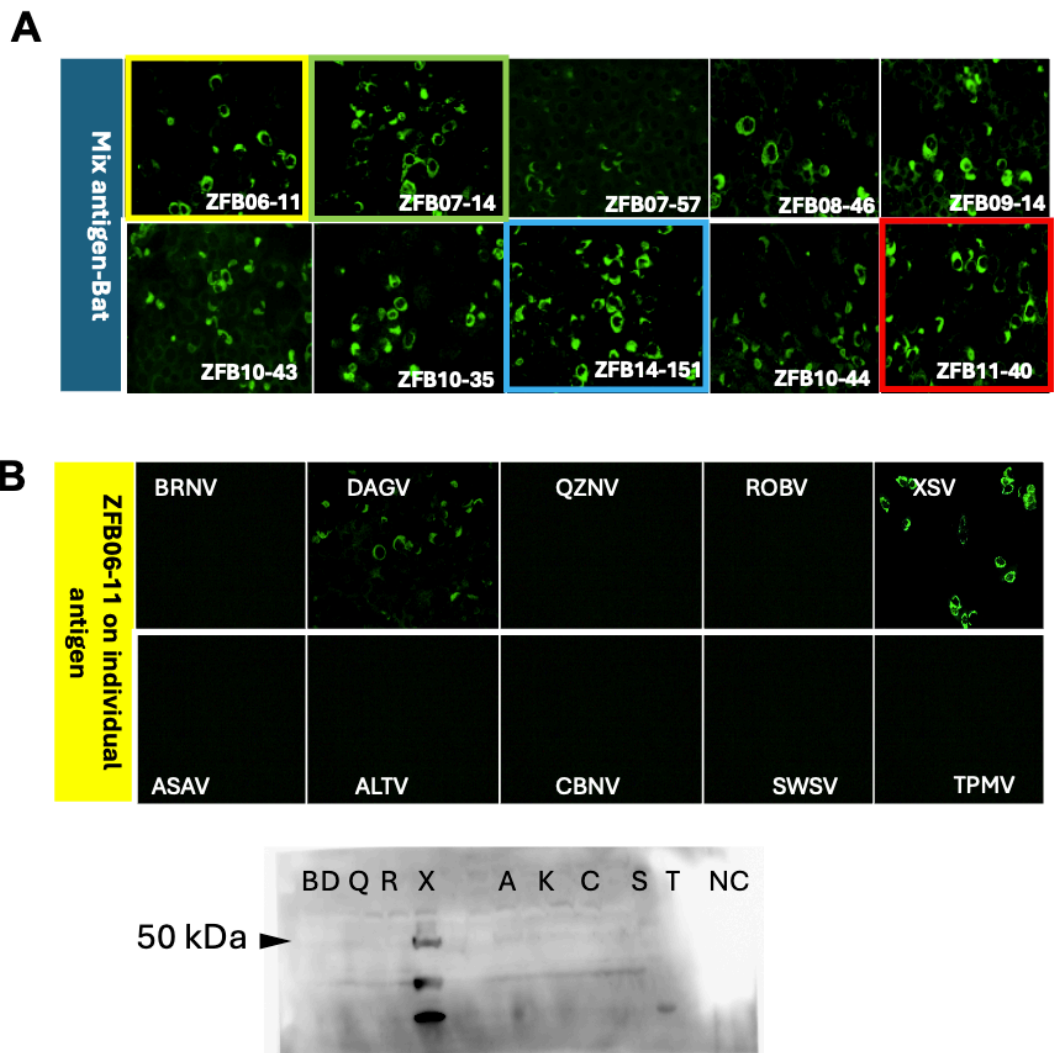


Figure 5. Typical reaction pattern of bat serum samples to mix-antigen-bat and individual antigen.

(A) Typical IFAT patterns of 10 selected bat serum samples for mix-antigen-bat.

(B) Individual Immune reaction profile of ZFB06-11 bat serum in IFAT and Western blot analyses.

Note: Letters on each panel denote the name of the respective viral antigen as previously described.

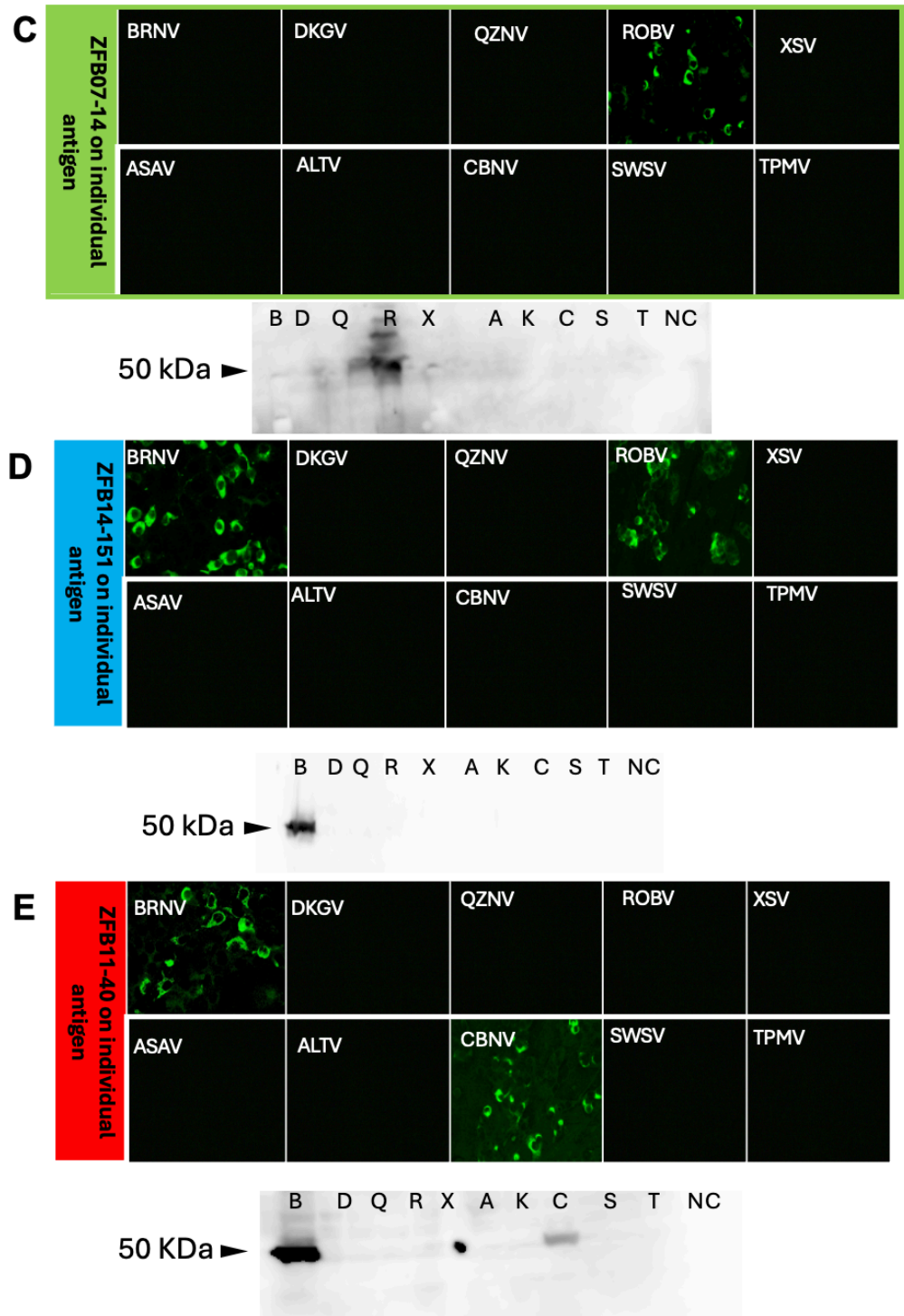


Figure 5. Typical reaction pattern of bat serum samples to mix-antigen-bat and individual antigen.

(C) IFAT and Western blot profiles of ZFB07-14 bat serum to individual antigens.

(D) IFAT and Western blot profiles of ZFB14-151 bat serum to individual antigens.

(E): IFAT and Western blot profile of ZFB11-40 bat serum to individual antigens.

Note: Letters on each panel denote the name of the respective viral antigen as previously described.

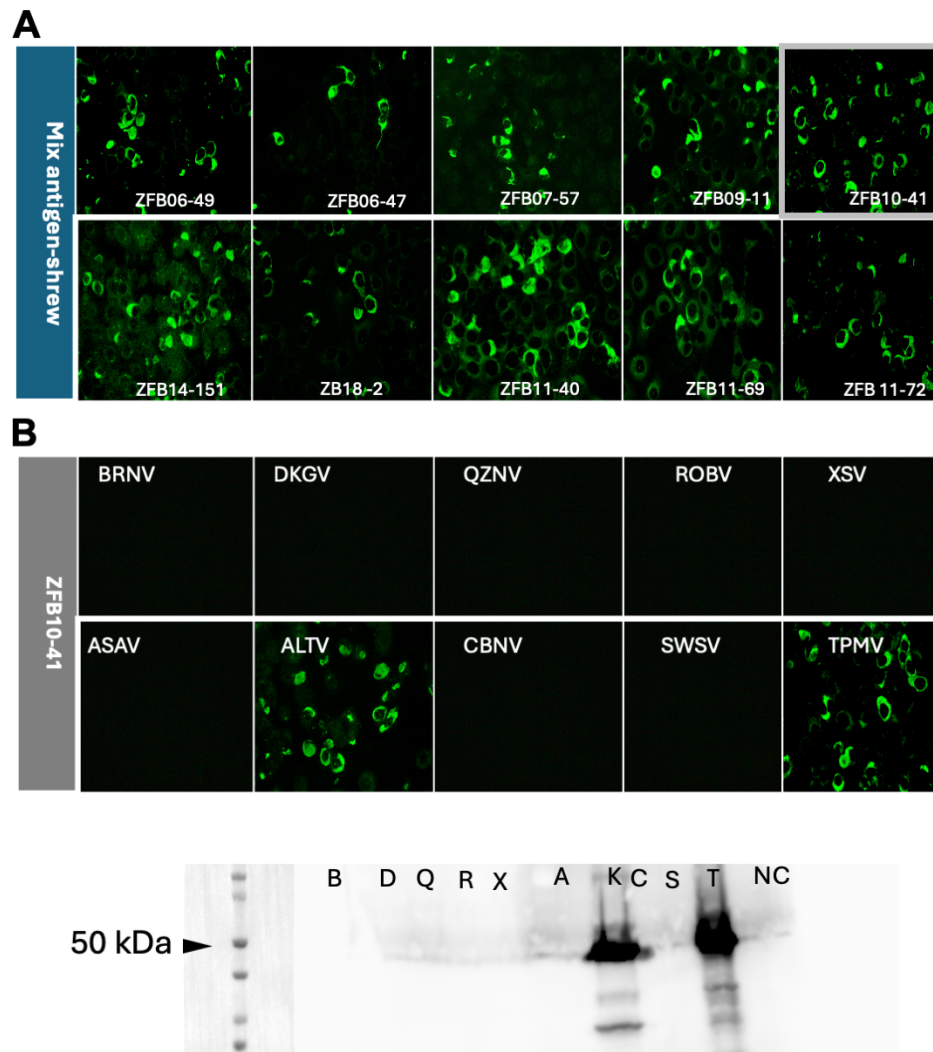


Figure 6. Typical reaction pattern of bat serum samples to mix-antigen-shrew and individual antigen.

(A) Typical IFAT patterns of 10 selected bat serum samples for mix-antigen-shrew.

(B) IFAT and Western blot profiles of ZFB10-41 bat serum to individual antigens. ZFB10-41 showed a positive reaction to both ALTV and TPMV and showed a strong positive reaction in the Western blot assay.

Note: Letters on each panel denote the name of the respective viral antigen as previously described.

Table 4. Field titer values of selected positive individual sera samples.

	Antigens	IFAT titers of bat sera					
		ZFB06-11	ZFB07-14	ZFB10-41	ZFB10-44	ZFB11-40	ZFB14-151
Bat-borne	BRNV	-	-	-	4800	19200	19200
	DKGV	600	-	-	-	-	-
	QNZV	-	-	-	-	-	-
	ROBV	-	19200	-	-	-	600
	XSV	19200	-	-	-	-	-
Shrew-borne	ASAV	-	-	-	-	-	-
	ALTV	-	-	9600	-	-	-
	CBNV	-	-	-	-	4800	-
	NVAV	-	-	-	-	-	-
	SWSV	-	-	-	-	-	-
	TPMV	-	-	19200	-	-	-

-; <300

Individual antigen screening

As shown above, bat serum samples positive for mix-antigen-bat and mix-antigen-shrew were examined for reactivity to individual rN antigens. Of the 201 samples positive for the mix-antigen-bat, 97, 15, 80, 38, and 41 samples reacted with the rNs of BRNV, DKGV, QZNV, ROBV, and XSV, respectively. Of the 307 samples positive for the mix-antigen shrew, 90, 90, 48, and 86 samples reacted to the rNs of ASAV, ALTV, CBNV, and TPMV, respectively. None of the bat serum samples reacted to the NVAV and SWSV antigens (Table 5).

Table 5. Seropositivity to Mix-antigen-bat and Mix-antigen-shrew, followed by IFAT with individual antigens.

Species	Tested	Mix-antigen-bat -positive	Individual antigens					Mix-antigen-shrew-positive	Individual antigens					
			BRNV	DKGV	QZNV	ROBV	XSV		ASAV	ALTV	CBNV	NVAV	SWSV	TPMV
<i>Eidolon helvum</i>	986	156 (15.8%)	78	13	65	27	35	195 (19.8%)	63	44	45	0	0	52
<i>Epomophorus crypturus</i>	84	7 (8.5%)	6	0	2	0	0	13 (15.9%)	4	3	2	0	0	5
<i>Hipposideros caffer</i>	2	0	-	-	-	-	-	0	-	-	-	-	-	-
<i>Macronycteris vittatus</i>	182	0	-	-	-	-	-	3 (1.6%)	0	1	0	0	0	1
<i>Miniopterus fraterculus</i>	38	0	-	-	-	-	-	0	-	-	-	-	-	-
<i>Nycteris thebaica</i>	93	4 (4.3%)	0	0	0	4	0	1 (1.1%)	0	1	0	0	0	0
<i>Rhinolophus blasii</i>	1	0	-	-	-	-	-	0	-	-	-	-	-	-
<i>Rhinolophus swinnyi</i>	4	0	-	-	-	-	-	0	-	-	-	-	-	-
<i>Rhinolophus</i> sp.	4	0	-	-	-	-	-	0	-	-	-	-	-	-
<i>Rousettus aegyptiacus</i>	370	34 (9.2%)	13	2	13	7	6	94 (25.4%)	23	41	1	0	0	28
Grand Total	1764	201 (11.4%)	97	15	80	38	41	307 (17.4%)	90	90	48	0	0	86

-, Not done, %= recorded value expressed as a percentage of 1764

Data analysis

A comparison of the seropositivity rates for Mix-antigen-bat and Mix-antigen-shrew antigens is shown in Fig. 7A. Serum samples from *Rousettus aegyptiacus* showed significantly low seropositivity to Mix-antigen-bat, according to Fisher's exact test. Three fruit bat species (*Eidolon helvum*, *Epomophorus crypturus*, and *Rousettus aegyptiacus*) showed higher seropositivity rates than those of the insectivorous bats *M. vittatus* and *Nycteris thebaica*. Samples from other bat species were not analyzed because of the low number of samples. Next, the seropositivity rates of individual antigens within each species were compared (Fig. 7B). The reaction profiles of fruit bats to the antigens of bat-borne hantaviruses were similar, as seen in *Eidolon helvum* with BRNV recording 7.9% (78) as the highest and 6.6% (65) for QZNV as the second highest. A similar pattern was observed in the *Rousettus aegyptiacus* species, as shown in Fig 7B. In contrast, reactivity to the antigens of the shrew-borne hantaviruses of *Rousettus aegyptiacus* showed a significantly lower seropositivity rate to the CBNV antigen, recording 1 positive out of 370 sera. In addition, a significant difference was detected between the seropositivity rates of the ASAV 6.2% (23) and ALTV 11% (41) antigens (Fig. 7C). Due to the inherent issues of non-equal sample sizes, comparisons between bat species were not performed. The secondary antibodies used in this study were not guaranteed to react equally with all bat species tested; therefore, no cross-species comparative analyses were performed.

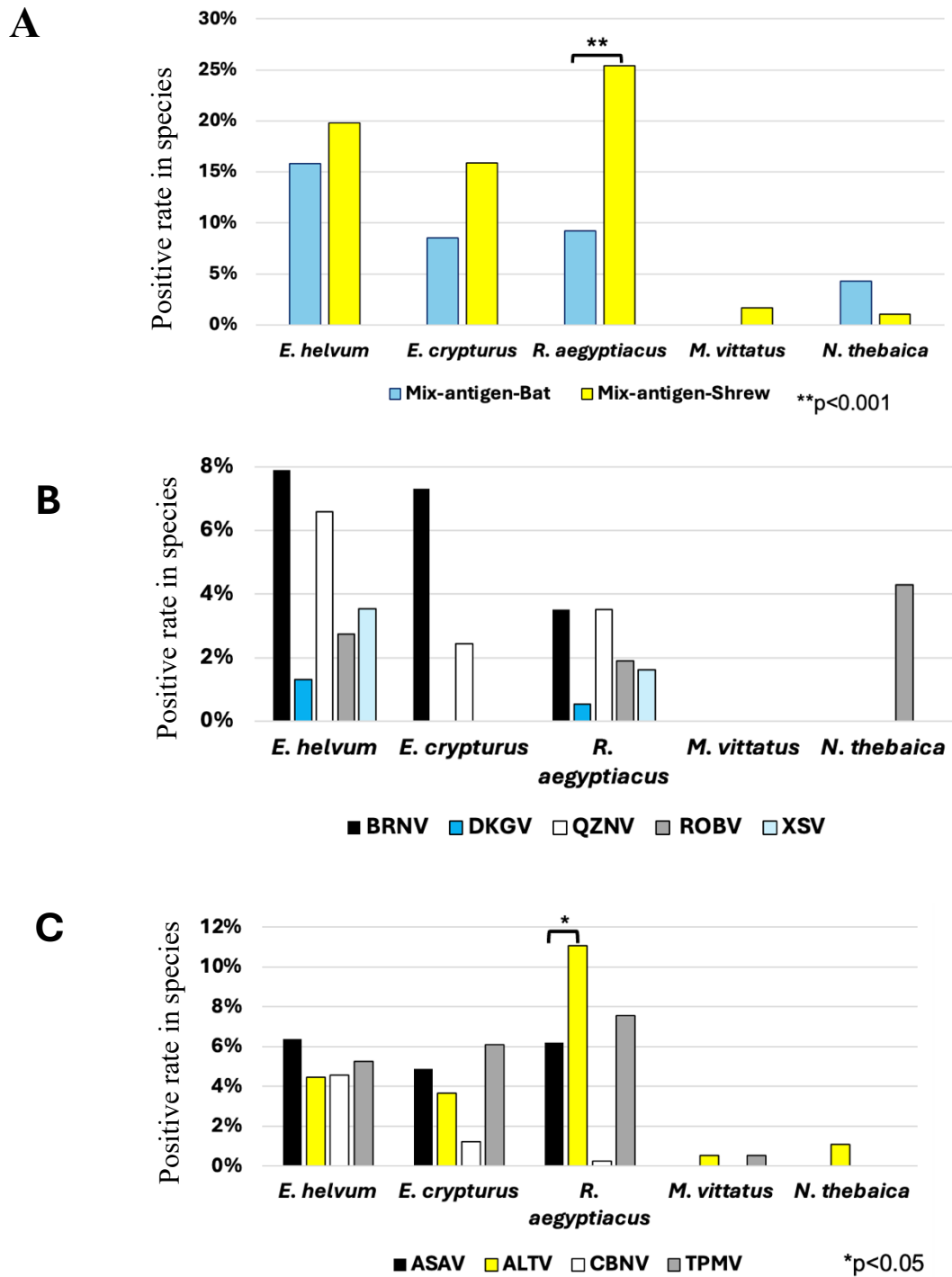


Figure 7. Antibody positivity rates for various antigens by bat species.

(A) To mix-antigens by bat species.

(B) To individual antigens of bat-borne viruses.

(C) To individual antigens of shrew-borne antigens.

Relationship between multiple reactivities

Next, cross-reactivities to the antigens of bat- and shrew-borne hantaviruses were assessed using bat serum reactivity. The relationships between the number of samples testing positive for each antigen were analyzed (Table 6). Then, bat serum samples showing multiple reactivities to different antigens were selected. A total of 138 bat serum samples were analyzed, and the relationship between the positive reactions is shown in Table 7. No significant association was found between seropositivity to any of the antigen combinations. These results suggest that there was no cross-reactivity between the antigens used in this study in the bat serum samples.

Reactivity of monoclonal antibody E5/G6

The reactivity of monoclonal antibody E5G6 to all eleven antigens based on the Haantan conserved region (76-118) was evaluated. Table 8 presents the screening results.

Table 6. Pair-wise comparison between individual antigens.

Combinations	t-test	p
BRNV - DKGV	1.04	0.298
BRNV - QZNV	0.17	0.862
BRNV - ROBV	0.35	0.729
BRNV - XSV	0.77	0.442
BRNV - ASAV	0.07	0.941
BRNV - ALTV	-0.46	0.647
BRNV - CBNV	0.66	0.511
BRNV - TPMV	-0.26	0.795
DKGV - QZNV	-0.87	0.386
DKGV - ROBV	-0.69	0.488
DKGV - XSV	-0.27	0.785
DKGV - ASAV	-0.97	0.334
DKGV - ALTV	-1.5	0.134
DKGV - CBNV	-0.38	0.701
DKGV - TPMV	-1.3	0.193
QZNV - ROBV	0.17	0.862
QZNV - XSV	0.59	0.552
QZNV - ASAV	-0.1	0.921
QZNV - ALTV	-0.63	0.528
QZNV - CBNV	0.48	0.629
QZNV - TPMV	-0.43	0.665
ROBV - XSV	0.42	0.674
ROBV - ASAV	-0.27	0.785
ROBV - ALTV	-0.81	0.421
ROBV - CBNV	0.31	0.757
ROBV - TPMV	-0.61	0.544
XSV - ASAV	-0.69	0.488
XSV - ALTV	-1.23	0.22
XSV - CBNV	-0.11	0.911
XSV - TPMV	-1.03	0.304
ASAV - ALTV	-0.53	0.594
ASAV - CBNV	0.58	0.56
ASAV - TPMV	-0.33	0.738
ALTV - CBNV	1.11	0.265
ALTV - TPMV	0.2	0.843
CBNV - TPMV	-0.92	0.359

Table 7. Correlation ratio between two antigens.

	ASAV	ALTV	CBNV	TPMV	BRNV	DKGV	QZNV	ROBV	XSV
ASAV	1	-0.45	0.11	-0.2	0.12	-0.12	0.02	-0.09	0.03
ALTV		1	-0.24	-0.08	-0.15	-0.02	-0.23	0.04	-0.09
CBNV			1	-0.13	-0.16	-0.14	-0.06	-0.13	-0.07
TPMV				1	-0.16	-0.04	-0.13	-0.02	-0.02
BRNV					1	-0.15	0.08	-0.08	-0.26
DKGV						1	0.04	0.05	-0.05
QZNV							1	-0.01	0.05
ROBV								1	-0.01
XSV									1

Table 8. Amino acid sequences of the epitope and corresponding sites of monoclonal antibody E5/G6.

Viruses	Site	Amino acid sequence of epitope										E5/G6 binding
HTNV 76-118	165-174	Y	E	D	V	N	G	I	R	K	P	+
BRNV	166-175	V	E	E	I	G	G	V	K	T	H	-
DKGV	166-175	F	F	E	K	G	G	I	K	V	P	-
QZNV	167-176	F	T	E	K	G	G	V	K	I	P	-
ROBV	166-175	F	T	E	K	N	G	V	K	T	P	-
XSV	166-175	F	V	E	K	G	G	I	K	V	P	-
ASAV	166-175	Y	I	E	V	N	G	I	R	K	P	+
ALTV	128-137	S	E	D	A	R	G	G	R	T	P	-
CBNV	166-175	F	I	E	E	N	G	I	R	K	P	+
VVAV	166-175	Y	T	E	K	G	G	V	K	V	P	-
SWSV	166-175	F	D	E	T	P	T	G	R	K	P	-
TPMV	175-185	M	E	D	R	N	G	I	K	Q	H	-

Discussion

Although the detection of bat- and shrew-borne hantaviruses has been increasing, serological screening assays for both bat- and shrew-borne hantavirus infections are not well established^{56,65}. Additionally, few studies have assessed the antigenicity of bat- and shrew-borne hantaviruses. In this study, I developed serological screening assays for these viruses and studied the antigenic similarities between representative bat- and shrew-borne hantaviruses. Serological screening of frugivorous and insectivorous bats in Zambia was conducted using the established assays. Although few reports on the antigenicity of the N of bat-borne and shrew-borne hantaviruses have been published, epitopes on the Ns of bat-borne hantaviruses have been analyzed only *in silico*, and common antigenic sites have been proposed¹⁸. In this study, I compared the antigenicity of Ns of novel members of the hantavirus recombinant protein expression system in a wet laboratory. No strong cross-reactivity was detected among the rN antigens of the 11 viruses; however, low cross-reactivity was found between highly immunized serum and the rN antigens. Common but not dominant epitopes might induce antibodies at a low frequency, leading to low-level cross-reactivity. However, by comparing their homologous titer values, it appeared that these rNs had different antigenicity. Therefore, I used all 11 rN antigens for serological screening. Two rNs of ROBV and QZNV appeared to have more similar antigenicity than those of other antigen combinations. Accordingly, these two viruses belong to the same phylogenetic group. However, none of the bat serum samples showed cross-reactivity to ROBV and QZNV, suggesting that cross-reactivity between ROBV and QZNV might be found only in hyperimmune serum.

Auxiliary to this study, the rNs of ASAV and CBNV reacted with the E5G6 monoclonal antibody, which recognizes an epitope located at amino acid positions 166–175 of the HTNV N. This epitope sequence is conserved among rodent-borne hantaviruses⁵⁵. The E5G6 epitope in rNs might not be immunodominant, and antibodies against this epitope are

unlikely to be induced during actual infection ⁸⁸. Hence, it is evident that some shrew borne hantaviruses may share antigenic epitopes with rodent-borne hantaviruses.

To efficiently screen the immune reactivity of bat serum samples for antigenically broad hantavirus antigens using IFAT, I attempted to express the rNs of 5 or 6 hantavirus species simultaneously. All the antigens were successfully expressed and detected using specific antisera. Samples positive for the mixed antigen were further tested by IFAT using rN antigens expressed individually in Vero E6 cells. In this step, some serum samples showed specific reactivity to 2 or 3 viral antigens. Therefore, serial dilutions were performed and titrated against the respective antigens. IFA antibody titers above 4800 were required for Western blot analysis of the positive bat serum samples.

The cut-off for cross-reactivity in laboratory-generated antisera, according to this manuscript, was estimated as 51200 titers. It was also clear that laboratory-generated sera titers were different from field antisera. While 4800 IFAT antibody titers of bat sera were also positive in Western blot, 51200 IFAT titers from the laboratory-generated antisera were only cross-reactive in IFAT and not in Western blot. Hence, a 51200 IFAT titer cut-off was used for the 11 antisera generated to estimate cross-reactivity. Generally, a 4-fold difference in antibody titer showed independent antigenicity. To evaluate true antigenic cross-reactivities, naturally infected animal sera are required. In this study, I did not detect cross-reactivity from bat sera captured in Zambia. Further careful study is needed to evaluate the cross-reactivity of bat-borne and shrew-borne hantavirus antigens. Regarding the reactivity of anti-bat IgG, it was designed to react with Bat IgG and as well as light chains common to other Bat immunoglobulins. The company showed confirmed reactivity to bat genus species *Pteropus vampirus*, *Desmodus rotundus*, *Eptesicus fuscus*, *Tadrida pumila*, *T. condylura*, *Hypsignathus monstrosus*, *Rosettus aegyptiacus*, *Epomorphus crypturus*, *Molossus species*, and *Phyllostomus species*.

All bat serum samples were analyzed sequentially, and 11.4% were positive for the Mix-antigen-bat. Among them, *Eidolon helvum* showed the highest positivity rate (17.4%). Three species of fruit bats, *Eidolon helvum*, *Epomophorus crypturus*, and *Rousettus aegyptiacus*, showed higher seropositivity than insectivorous bats. However, the affinities of the secondary antibodies used in this study varied with the immunoglobulins of the different bat species. Therefore, I could not compare seropositivity among bat species. However, differences in positive patterns within the same species can be compared. *Eidolon helvum* and *Rousettus aegyptiacus* showed similar positive patterns to the bat-borne hantavirus antigens. This may indicate that the opportunities for exposure to each virus were similar in the two fruit bat species. Thus, they may be terminal hosts infected by viral spillover, rather than reservoir hosts for these viruses. In contrast, the insectivorous bat *Nycteris thebaica* was positive only for ROBV at low frequencies. The insectivorous bats may have been exposed to the virus via a different transmission route from the route by which the fruit bats were infected.

BRNV or related viruses might be an important hantavirus source in Zambian fruit bats. BRNV was originally reported in Europe; however, DKGV, QZNV, ROBV, and XSV have been detected in Asian and Oceanian countries. The number of positives of the QZNV antigen was similar to that of the BRNV (80 and 97, respectively). This seroprevalence suggests the possibility of the circulation of QZNV-related viruses in Zambia. Recently, a novel hantavirus, Buritiense mobatvirus, which belongs to the same lineage as QZNV and ROBV, was discovered in bats in Brazil ¹². QZNV- and ROBV-related viruses may also be distributed in Africa, and not only in Asia, Oceania, and South America.

The number of bats positive for Mix-antigen-shrew was larger than that of bats positive for Mix-antigen-bat. The Japanese shrew mole (*Urotrichus talpoides*) is native to Japan. Therefore, ASAV carried by Japanese shrew moles has never been detected outside Japan. Surprisingly, I detected a high seroprevalence of Mix-antigen-shrew in bats, with a

particularly high positivity rate of the ASAV antigen in fruit bats. However, no bat serum samples were seropositive for the SWSV and NVAV antigens. According to phylogenetic analysis, ASAV is more closely related to SWSV than to ALTV and TPMV. Japanese shrew moles are not present in Zambia; therefore, alternative animal hosts must carry ASAV-related viruses.

Rousettus bats showed a different pattern against rN antigens of shrew-borne hantaviruses than that of the African fruit bat, *Eidolon helvum*. The Egyptian fruit bat, *Rousettus aegyptiacus*, showed a particularly high positivity rate for the ALTV antigen and a low positivity rate for the CBNV antigen. The classification of ALTV has not yet been confirmed. ALTV was originally detected in *Sorex* shrews in Eurasia ²⁹. However, its genetic characteristics are distinct from those of other *Sorex*-borne viruses, and it appeared to be an intermediate virus between shrew-borne and bat-borne hantaviruses (Fig. 2A). Therefore, the geographic distribution of ALTV and related viruses needs to be clarified. Further studies are needed to investigate the diversity of bat- and shrew-borne hantaviruses in Africa and to identify the reservoir hosts.

This retrospective study investigated the serological detection of bat- and shrew-borne hantaviruses circulating in Zambia. The presence of bats carrying viruses of zoonotic importance raises concerns about the possibility of human infection. Regarding human infectivity of shrew-borne hantaviruses, ALTV and TPMV seropositivity have been reported in humans in Sri Lanka and Thailand ^{56,79}. In addition, Imjin or Imjin-like viral infection has been detected in humans in China ⁶¹. These findings suggest that shrew-borne viral infections have the potential to infect humans in several countries.

The serological strategy established in this study presented some information on the possible distribution of bat- and shrew-borne hantaviruses in Zambia. It is not known how bats harbor antibodies to shrew-borne viruses. Tree-climbing shrews may have contributed

to their spread. Migratory fruit bats can cross highly contaminated areas during migration. It is also possible that shrew-borne viruses originated in bats. Further research is required to clarify the ecological dynamics of hantavirus transmission.

Previous reports have shown that bat-borne hantaviruses may have a worldwide distribution, except for Antarctica. Both partial and complete hantavirus sequences have been reported in Asia, Europe, and Africa. Further serological and molecular biological research is required to advance our understanding of the epidemiology of hantaviruses in bats, shrews, and humans in various regions.

This study therefore developed a serological system by expressing multiple antigens in a single transfection protocol to serve as a multiplex platform for testing five bat-borne and 6 shrew-borne antigens. Instead of testing all 1764 samples against individual antigens, which would have required 1,764 (5,292 in triplicate) wells expressing each antigen, the mixed antigen system reduces this to about 353 (1,059 in triplicate) and 294 (882 in triplicate) for the 5 bat- and 6 shrew-borne antigens, respectively. This significantly reduces the number of wells while yielding equal results. Hence, this system helps to effectively pool all positive samples with fewer wells, followed by specific individual antigen analysis based on the number of positives detected. Apart from animal samples, this system can be used to screen human serum samples while using the required secondary antibodies. We, therefore, intend to apply this system in screening human sera samples in the future.

Summary

Despite the two main diseases caused by rodent-borne hantaviruses, HFRS and HPS in humans, emerging hantaviruses have been identified in bats and shrews, raising concerns about their potential spillover into human populations. Despite their widespread distribution, the antigenicity, pathogenicity, and serological detection methods for bat- and shrew-borne hantaviruses remain poorly understood. This thesis addresses these gaps by developing a novel multiplex IFAT using rN from 5 bat-borne (BRNV, DKGV, QZNV, ROBV, XSV) and 6 shrew-borne (ASAV, ALTV, CBNV, NVAV, SWSV, TPMV) hantaviruses. Applying this tool to screen bat sera from Zambia, the following findings were revealed:

1. Low Cross-Reactivity: Antigenic analysis showed minimal cross-reactivity among hantaviruses, except between phylogenetically related QZNV and ROBV, supporting the assay's specificity.
2. High Seroprevalence in Zambian Bats: 11.4% (201/1,764) of bat sera reacted to bat-borne hantaviruses. 17.4% (307/1,764) reacted to shrew-borne hantaviruses. Fruit bats (*Eidolon helvum*, *Rousettus aegyptiacus*) showed the highest exposure rates.
3. Evidence of spillover: Bats in Zambia were exposed to both bat- and shrew-borne hantaviruses, suggesting cross-species transmission or the presence of unidentified reservoir hosts.

This study provides a critical tool for hantavirus surveillance and reveals unexpected exposure patterns in African bats, highlighting the need for expanded research on emerging zoonotic viruses.

Chapter 2: Serological detection of antibodies against bat-borne and shrew-borne hantaviruses in Sri Lanka

Introduction

Hantaviruses belong to the family *Hantaviridae* and are the causative agents of two rodent-borne zoonotic diseases: HFRS and HPS ^{38,51}. Many species of rodents carry their specific hantaviruses, suggesting that rodents and hantaviruses have coevolved ⁶⁰. However, hantaviruses have also been found in shrews, moles, and bats ⁸⁴. Thus, the acquisition of hantavirus diversity remains controversial ⁹¹.

From the aspect of public health, the infectivity and pathogenicity of these novel hantaviruses should be clarified. Serological screening of 375 human sera in Sri Lanka for 4 shrew-borne hantaviruses, ASAV ⁵, ALTV ²⁹, SWSV ⁴², and TPMV ¹⁴ by using recombinant antigens has been reported. Although three seropositive human sera, two to ALTV and one to TPMV, were found in the Girandurukotte area of Sri Lanka, the relationship between seropositivity and diseases was not found ⁷⁹.

Recently, numerous novel hantaviruses have been identified from various shrews, moles, and bats species. I developed a serological detection system to identify antibodies against bat-borne hantaviruses, including BRNV ^{17,71}, DKGV ³, QZNV ⁶, XNV ⁴, and ROBV ⁶⁸, shrew-borne hantaviruses, ASAV, ALTV, CBNV ²⁰, NVAV ²⁸, SWSV, and TPMV. This study aimed to determine the presence of antibodies against bat- and shrew-borne hantavirus in human sera from Sri Lanka. I attempted to elucidate the potential for human infections by these novel hantaviruses in Sri Lanka.

Materials and Methods

Following a previously reported protocol, serum samples were screened for bat-borne and shrew-borne hantavirus antigens using an IFAT ⁶⁴. Fixed Vero E6 cells expressing rN antigens for bat-borne hantaviruses, a Mix-antigen-bat (BRNV, DKGV, QZNV, ROBV, and XNV), and a Mix-antigen-shrew (comprising ASAV, ALTV, CBNV, NVAV, SWSV, and TPMV) were tested using an IFAT with Alexa488-conjugated goat anti-human IgG (H+L) (A11013; Invitrogen, Thermofisher Scientific) as the secondary antibody. According to the previous procedure, Mix-antigen-bat and shrew were used for the initial screening of the human sera in IFAT at a serum dilution of 1:200 with phosphate-buffered saline (PBS). Positive samples were then further screened by IFAT to determine the binding specificity with individual antigens. Positive reactions were further validated by a Western blot assay as previously reported ^{64,79}.

Serum samples from 168 hospitalized acute febrile patients, collected by the Department of Microbiology at the University of Peradeniya between 2011 to 2016, were used for the detection of hantavirus infection in this study. These patients experienced episodes of acute febrile illness of unknown origin. Serum samples collected from the community in the Girandurukotte region (n=168) were also included in this study. These control sera were the same as those from the previous study, but the number of sera was reduced in this current study due to availability.

Ethical approval for this study was obtained from the Institutional Ethical Review Committee (ERC) at the Faculty of Medicine, University of Peradeniya, Sri Lanka (Project No. 2015EC/SP/15 and Project No. 2016/EC/64 for the National Science Foundation – Sri Lanka [NSF-Sri Lanka] study). Written consents from all participants were preserved in the Department of Microbiology, Faculty of Medicine at the University of Peradeniya. The ERC at the Institute for Genetic Medicine, Hokkaido University, Japan (21-001) approved

the treatment of Sri Lankan human materials based on G0313-5, Project No. 2015EC/SP/15, Project No. 2016/EC/64, and 2021/EC/44.

Results and discussion

Mix-antigen assays

As shown in Table 9, 12 sera tested positive for Mix-antigen-bat (10 febrile patients and 2 healthy individuals), while 31 (2 double and 29 single positives for febrile patients) and sera tested positive for Mix-antigen-shrew. In this study, +, ++, and +++ represent the intensity of IFAT-positivity as previously reported ²¹. The number of seropositive sera for shrew-borne antigens was significantly higher than the number of positives for bat-borne antigens ($p < 0.01$ in Fisher's exact test, performed using R software (R Foundation for Statistical Computing, Vienna, Austria). Among these, two febrile specimens (173F and 305F) tested positive for Mix-antigen-bat and -shrew. The seropositivity to Mix-antigen-bat or Mix-antigen-shrew in febrile patients (39/168) was higher ($p < 0.01$ in Fisher's exact test) than in the control group (2/168). Control sera were collected from the Girandurukotte community, but the area of residence for the febrile cases was not identified. Therefore, the difference between the two groups, healthy controls and febrile patients, could not be directly compared.

Table 9. IFAT screening of sera from Sri Lanka by Mix-antigen-bat and -shrew.

		Mix-antigen-shrew			
		Community (n=168)		Febrile patients (n=168)	
		Positive	Negative	Positive	Negative
Mix- antigen-bat	Positive	0	2	2	8
	Negative	0	166	29	129

Individual antigen assays

The outcome of the individual antigen assays is shown in Tables 10 and 11. Table 10 shows the results for individual antigen-bat assays of control and febrile specimens, while Table 11 shows the individual antigen-shrew assays. Though 12 seropositives were recorded in the Mix-antigen-bat, 15 seropositives were seen in the individual bat-borne antigen assay due to multiple positives. Similarly, 31 seropositives for the Mix-antigen-shrew; however, 32 positives were recorded in the individual assay. No seropositivity was observed for BRNV and NVAV; this might be because these viruses were originally found in Europe. The geographical distribution of viruses might be related to seropositivity in Sri Lanka. The geographical distribution of viruses might be related to seropositivity in Sri Lanka. Sera with mix-antigen reactivity (+) produced no positive reaction in the individual antigen assay. This may be due to the presence of very low IgG for binding.

Table 10. IFAT results of individual antigens for sera positive in screening by Mix-antigen-bat.

Serum ID	IFAT intensity to Mix antigen-bat	IFAT intensity to individual antigen of					No. of positive antigens
		BRNV	DKGV	QZNV	ROBV	XNV	
40F	+++	-	-	++	-	+++	2
100F	++	-	-	-	++	-	1
164F	++	-	-	-	-	-	0
173F	+++	-	-	++	+++	++	3
227F	++	-	-	++	-	-	1
305F	++	-	-	-	++	-	1
416F	+++	-	-	++	+++	-	2
448F	++	-	-	-	-	++	1
569F	+	-	-	-	-	-	0
AIN 71F	+++	-	-	++	+++	-	2
G/Con/01	++	-	-	++	-	-	1
G/Con/09	++	-	-	++	-	-	1
Total	12	0	0	7	5	3	15

BRNV (Brno virus), DKGV (Dakrong virus), QZNV (Quezon virus), ROBV (Robina virus) XNV (Xuan Son virus).

Table 11. IFAT results of individual antigens for sera positive in screening by Mix-antigen-shrew.

Seru m ID	IFAT intensity to Mix- antigen- shrew	IFAT intensit to individual antigen of						No. of positive antigens
		ASA V	ALT V	CBN V	NVA V	SWS V	TPM V	
169F	++	++	-	-	-	++	-	2
173F	++	-	-	++	-	-	-	1
174F	++	-	++	-	-	-	-	1
175F	+++	-	+++	-	-	-	-	1
223F	+	-	-	-	-	-	-	0
233F	+++	-	++	-	-	-	-	1
236F	+	-	-	-	-	-	-	0
250F	+++	-	+++	-	-	++	-	2
253F	+	-	-	-	-	-	-	0
305F	+++	-	-	+++	-	+++	-	2
311F	+++	+++	-	-	-	-	-	1
313F	+	-	-	-	-	-	-	0
317F	+++	-	-	-	-	-	+++	1
328F	+++	-	-	+++	-	++	-	2
329F	+++	+++	-	-	-	-	-	1
333F	++	++	-	-	-	-	-	1
337F	+++	-	-	++	-	-	-	1
357F	+++	+++	-	+++	-	-	-	2
358F	+++	+++	-	-	-	-	-	1
413F	++	-	++	-	-	-	-	1
433F	+++	+++	-	++	-	-	+++	3
480F	+++	+++	-	-	-	-	-	1
501F	++	-	-	-	-	-	++	1
505F	+++	+++	-	-	-	-	-	1
507F	++	-	-	++	-	-	-	1
522F	+	-	-	-	-	-	-	0
545F	+	-	-	-	-	-	-	0
554F	+++	+++	-	-	-	-	+++	2
560F	++	-	-	++	-	-	-	1
578F	++	-	-	++	-	-	-	1
587F	+	-	-	-	-	-	-	0
Total	31	10	5	9	0	4	4	32

ASAV (Asama virus), ALTV (Altai virus), CBNV (Cao Bang virus), NVAV (Nova virus), SWSV (Seewis virus), TPMV (Thottapalayam virus)

Table 12. Host and virus distribution of individual antigens.

Individual antigen of	Original host	Virus distribution	No. of positive	
			/168 Control	/168 Febrile
Brno (BRNV)*	<i>Nyctalus noctula</i>	Czech Republic	0	0
Dakrong (DKGV)*	<i>Aselliscus stoliczkanus</i>	Vietnam	0	0
Quezon (QZNV)*	<i>Rousettus amplexicaudatus</i>	Philippines	2	7
Robina (ROBV)*	<i>Pteropus alecto</i>	Australia	0	5
Xuan Son (XSV)*	<i>Hipposideros pomona</i>	Vietnam/China	0	3
Asama (ASAV)**	<i>Urotrichus talpoides</i>	Japan	0	10
Altai (ALTV)**	<i>Sorex caecutiens</i>	Russia	0	5
Cao Bang (CBNV)**	<i>Anourosorex squamipes</i>	Vietnam/China	0	9
Nova (NVAV)**	<i>Talpa europaea</i>	Europe	0	0
Seewis (SWSV)**	<i>Sorex araneus</i>	Sweden, Russia, Japan, Americas	0	4
Thottapalayam (TPMV)**	<i>Suncus murinus</i>	India, China, Indonesia, Vietnam, Nepal	0	4
Bat-borne*, Shrew-borne**				

A previous study examined anti-ALTV, ASAV, SWSV, and TPMV antibodies in controls from Girandurukotte and found positives for TPMV antigens in 2 out of 271. Although not all control sera, including those previously positive, were available, the results were consistent with earlier studies that reported negative results for ASAV, ALTV, SWSV, and TPMV. Additionally, these sera were negative for other novel shrew-borne hantavirus antigens, CBNV and NVAV. However, this study identified two positives for the QZNV antigen, which is a bat-borne hantavirus.

The high seropositivity for ASAV among febrile patients raises a question regarding the host species. Because the original host animal for ASAV is the Japanese shrew mole (*Urotrichus talpoides*), which is endemic to Japan¹⁵. However, ASAV seropositive bats have also been found in Zambia⁶⁴. Since the Japanese shrew mole cannot be a reservoir of ASAV in Zambia and Sri Lanka, ASAV-related viruses must be carried by different species. It will be necessary to identify the host animal species in these areas.

TPMV was carried by an Asian house shrew (*Suncus murinus*) and distributed widely in Asian countries, India, China, Nepal, Vietnam, and Indonesia^{14,22,31,41,45,56}. Since the Asian house shrew is widespread in Sri Lanka, and antibody-positive humans with TPMV were detected in both this study and previous studies⁷¹. These observations suggest that a virus antigenically similar to TPMV is circulating in Sri Lanka. Without information on the genetic makeup of these suspected circulating viruses, I cannot definitively state that they are ASAV and TPMV viruses; doing so would be scientifically incorrect. These viruses may share a cross-reactive epitope.

Most of the seropositive reactions to bat-borne antigens were to QZNV and ROBV, which were originally found in fruit bats (Table 12). Sri Lanka is home to at least two species of fruit bats, *Pteropus medius* and *Rousettus leschenaulti*. Sri Lankan fruit bats may be reservoirs for QZNV and ROBV-related viruses. Further investigations are necessary to identify the reservoir species for hantaviruses.

As shown in Tables 10 and 11, individual antigen reactions were positive for QZNV, ROBV, ALTV, ASAV, CBNV, SWSV, and TPMV viruses. These positive reactions to QZNV and XNV (40F), ROBV and CBNV (173F), ASAV (317F), and TPMV (317F, 433F, and 554F) in IFAT (Figure 8) were also confirmed in the Western blot assay (Figure 9). On the other hand, four IFAT-positive sera to SWSV were not confirmed in the Western blot. It is unclear why responses to SWSV were not confirmed by Western blot, but not all antigens induce antibodies to linear epitopes detected in Western blot. Other methods are needed to confirm SWSV infection.

Sri Lanka is an island country at the intersection of the Indian Ocean and Asia; therefore, it has a rich and distinctive fauna. Two types of hantaviruses have been detected in rodents to date: the Lanka virus carried by *Mus booduga* and the Anjzorobe virus carried by *Rattus rattus*⁵⁰. There are many types and species of small mammals belonging to the order *Euonymus*, commonly called shrews and moles. Additionally, Sri Lanka is home to numerous types and populations of bats. However, no studies have been conducted to investigate the presence of hantavirus in these animals. Our findings suggest that bat- and shrew-derived hantaviruses could transmit to humans in Sri Lanka. Careful planning and implementation of studies to assess the pathogenicity of these viruses in Sri Lanka will be required.

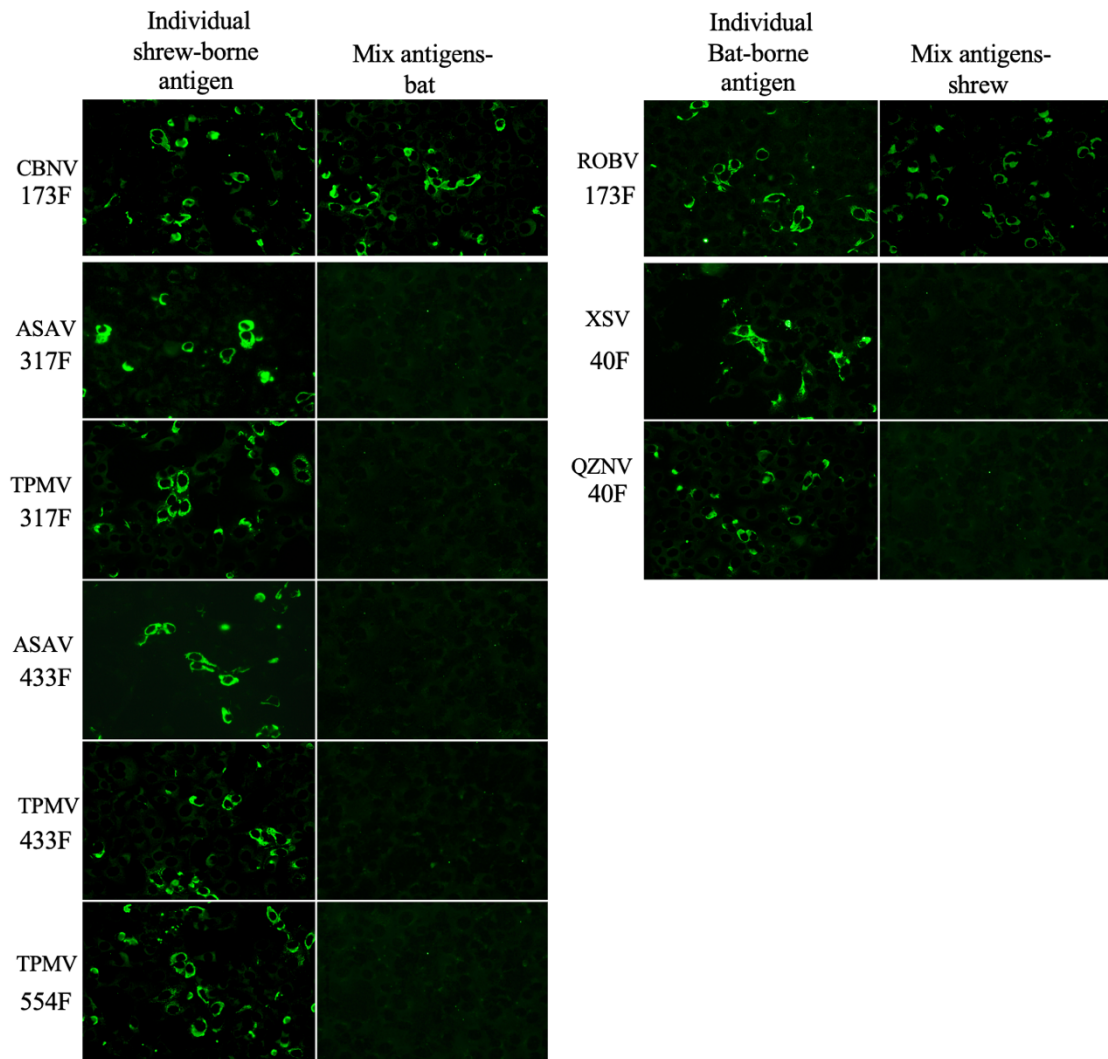


Figure 8. IFAT profiles of positive human sera to rN antigens of bat- and shrew-borne hantaviruses.

The left panel shows an individual shrew-borne antigen against its respective positive human serum, as well as its reaction on a Mix-antigen-bat. The right panel shows an IFAT profile of human sera against the respective bat-borne antigens and Mix-antigen-shrew. While 173F showed dual positive to Mix-antigen-bat and -shrew and showed positive to individual ROBV and CBNV antigens.

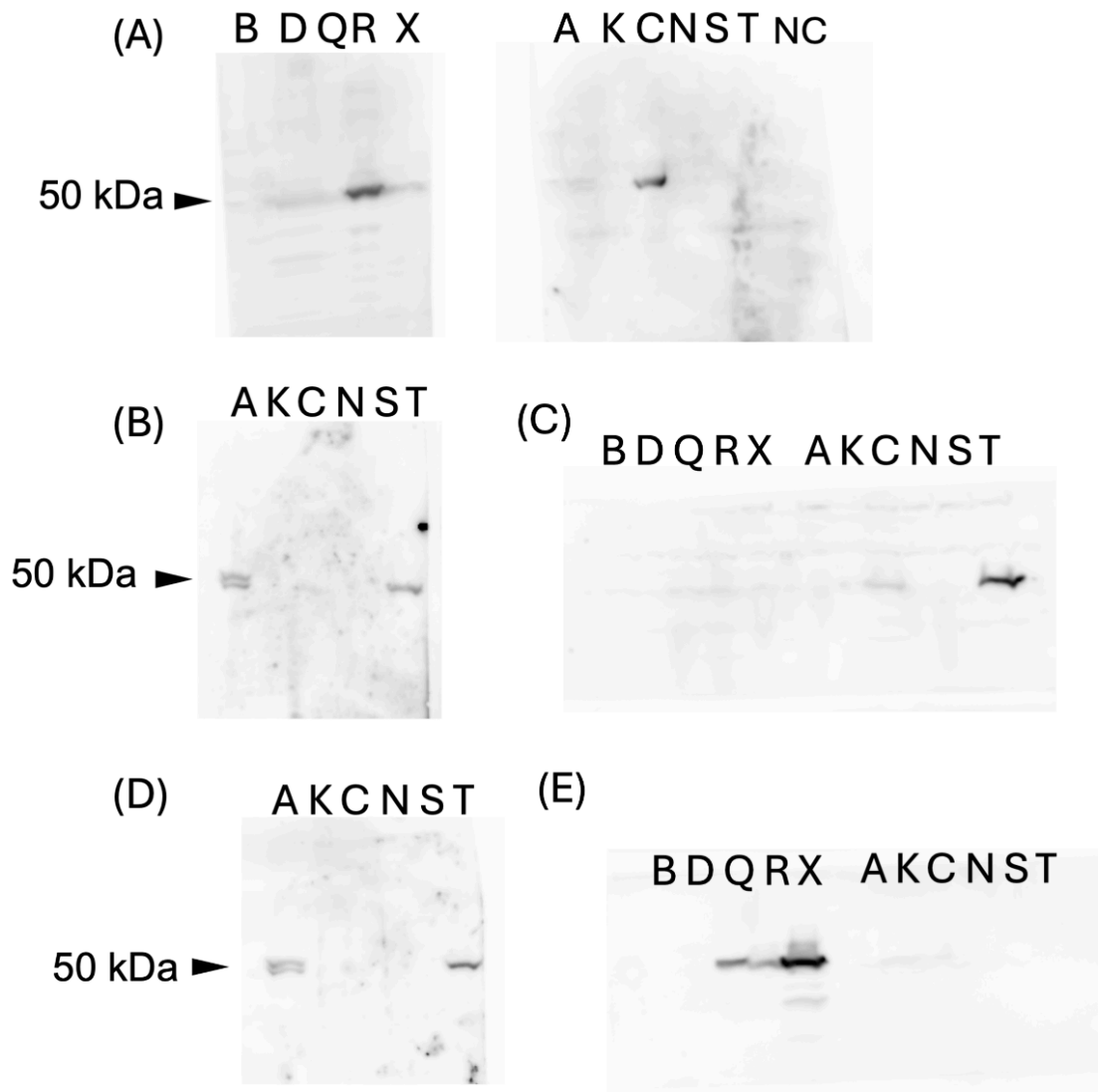


Figure 9. Western blot profiles of human sera to recombinant N antigens of bat- and shrew-borne hantaviruses.

Recombinant Ns expressed in 293T cell lysates were used as an antigen. B, BRNV; D, DKG V; Q, QZNV; R, ROBV; X, XSV; A, ASAV; K, ALTV; C, CBNV; N, NVAV; S, SWSV; T, TPMV; NC, 293T cell lysate without expression. Western blot profiles of febrile (F) patient serum IDs 173F (A), 433F (B), 554F (C), 317F(D), and 40F (E) were shown. A: 173F showed a positive reaction to ROBV (left) and CBNV (right). B and D: 433F and 317F showed double positives to ASAV and TPMV, C: 554F was positive to only TPMV. E: 40F showed a positive reaction to both QZNV and XSV antigens.

Summary

The study investigates the seroprevalence of bat- and shrew-borne hantaviruses in human sera from Sri Lanka using the IFAT and Western blot for detection. Using serological tools, researchers analyzed 336 human sera, including 168 healthy individuals and 168 febrile patients.

Key findings include:

1. Seropositivity rates: 12 sera were positive for bat-borne hantaviruses (Mix-antigen-bat). 31 sera were positive for shrew-borne hantaviruses (Mix-antigen-shrew). Seropositivity was significantly higher in febrile patients (39/168) compared to healthy controls (2/168).
2. Individual antigen reactions: Positive reactions were observed for QZNV, ROBV, ALTV, ASAV, CBNV, SWSV, and TPMV viruses. No seropositivity was detected for BRNV and NVAV viruses, likely due to their geographic origins (Europe).
3. Geographic and host considerations: The presence of antibodies to viruses such as ASAV (originally from Japan) and TPMV (widespread in Asia) suggests the potential for cross-reactive epitopes or related viruses circulating in Sri Lanka. Fruit bats (*Pteropus medius* and *Rousettus leschenaulti*) may be reservoirs for QZNV and Robina-like viruses.
4. Public health implications: The findings suggest potential human infections with bat- and shrew-borne hantaviruses in Sri Lanka, although the pathogenicity remains unclear. Further studies are needed to identify reservoir species and assess the health impact of these viruses.

The study highlights the presence of antibodies against diverse hantaviruses in Sri Lanka, suggesting zoonotic transmission. Future research should focus on virus

characterization and epidemiological studies to understand their clinical significance.

**Chapter 3: Analysis of renal pathology in a Hantaan
virus-infected mouse model of hemorrhagic fever with
renal syndrome**

Introduction

As indicated in chapter one, hantavirus is associated with two devastating rodent-borne zoonotic illnesses in humans: HFRS and HPS. HFRS is caused by Old World hantaviruses, including the HTNV³⁸, SEOV³⁷, DOBV¹¹, and PUUV¹³. Among the viruses mentioned above, millions of HTNV infections have been documented during the last 50 years, and it is regarded as one of the most significant viruses in East Asia. Still, specific antiviral medications have yet to be identified. Inactivated hantavirus vaccines have been produced in China and Korea, while clinical trials of DNA vaccines are ongoing in the United States^{24,26,43}. However, an approved vaccine is not available in most countries.

Animal models are useful for assessing viral pathogenicity and developing antiviral therapies, and evaluating vaccine efficacy. Laboratory mice are among the most useful experimental animals because they offer a wealth of background information and research tools. However, laboratory mice seldom develop symptoms mimicking HFRS after being inoculated with hantaviruses. Neonatal and severely immunodeficient mice frequently exhibit symptoms such as pulmonary edema, neutrophil depletion, encephalitis, pneumonia, and wasting syndrome, which are not characteristic of HFRS^{35,72,87}.

Previous studies demonstrated that the HTNV strain KHFV caused weight loss and renal medullary hemorrhage in immunocompetent mature mice, suggesting its potential as a model for HFRS^{67,80}. However, functional renal damage in this model has not yet been examined. In this work, I sought to clarify whether the KHFV pathognomonic lesion (renal hemorrhage) leads to renal damage and as well investigate the *in vivo* preventive and therapeutic trial of the neutralizing monoclonal antibody against disease progression.

Materials and Methods

Virus preparation and inoculation to mice

As a HTNV strain that induces HFRS-like symptoms in mice, KHFV was used as previously described^{9,67}. KHFV was propagated in Vero E6 cells, and virus infectivity titer was measured as Focus Forming Units (FFU) as previously described⁹. In the mock infection, the growth medium for Vero E6 cells was intravenously inoculated. Five-week-old female BALB/cCrSlc mice (SLC, Hamamatsu, Japan) were injected with 10⁵ FFU of KHFV via the tail vein. All animal experiments were approved by the Animal Studies Ethics Committee of Hokkaido University (24-0026). The mice were treated following the Laboratory Animal Control Guidelines of the Hokkaido University Institutional Animal Care and Use Committee. Viral infection experiments were carried out in a BSL-3 facility.

Renal damage in KHFV inoculated mouse (Exp. 1)

Mice in the positive control group were inoculated with KHFV as above and kept in a metabolic cage (CM-10S, CLEA-Japan, Tokyo, Japan) for serial collection of urine and fecal samples for nine days post-inoculation (dpi). Mock-infected mice were treated similarly. The daily weights of animals, urine, and fecal matter were recorded between 0 and 9 dpi. After nine days of observation, mice were moved to flat cages. At 14 dpi, mice were euthanized by cervical dislocation under 3% isoflurane anesthesia. Blood was collected to examine serum antibody induction, and organs were preserved in 4% phosphate-buffered paraformaldehyde (Fujifilm-Wako) for histological examination. Outline of the experiment is shown in Fig. 10A. All samples were stored at -80°C, except for formalin-preserved samples.

Monoclonal antibody and isotype control

HCO2 was previously produced and reported as neutralizing monoclonal antibody directed to HTNV, and its isotype was mouse IgG2b^{8,62}. Ascitic fluid induced by HCO2 hybridoma cells was supplied by Prof. Jiro Arikawa (Hokkaido University Graduate School of Medicine, Laboratory of Microbiology). According to the manufacturer's instructions, the Monoclonal antibody was purified from the ascitic fluid using a Protein A column (KANEKA, Tokyo, Japan). A Mouse IgG2b, kappa monoclonal antibody (MPC 11; Abcam, Cambridge, UK) was used as an isotype control.

Effect of neutralizing monoclonal antibody HCO2, prophylaxis group (Exp. 2)

In this part of the study, monoclonal antibody (Mab) HCO2 and isotype control were administered intraperitoneally at a dose of 0.035mg/0.15 mL/mouse. The next day, mice were challenged with 10⁵ FFU of KHFV. The outline of the experiment is shown in Fig. 11A. Similarly, sample collection and storage conditions were as previously described in Exp. 1.

Therapeutic effect of neutralizing monoclonal antibody HCO2 (Exp. 3)

In this experiment, three different groups were inoculated with KHFV but received 0.035 mg/mouse of HCO2 monoclonal antibody at different intervals: 1 dpi, 3 dpi, and 5 dpi (Fig. 12A). The selection of experimental intervals was based on the previously established onset of clinical symptoms on day 7 dpi, characterized by a peak in weight loss as observed in Exp. 1. Daily individual weights were measured for each group of animals over 9 days. The animals were euthanized after day 9, and serum and organ samples were collected for further analysis.

Humane endpoint

No death was recorded during the observation period, and death by cervical dislocation was set as the humane endpoint.

RNA extraction and genome quantification (Real-Time RT-qPCR)

Total RNA was extracted from harvested kidneys by using ISOGEN (Nippon gene, Tokyo, Japan) according to the manufacturer's instructions. Similarly, total RNA was extracted from the serially collected urine by using Isogen-LS (Nippon Gene). Total RNA fraction from fecal samples of 3 pellets from each day was also extracted. KHFV genome was detected by Real-time RT-PCR, which was performed using the 2x iTaq Universal Probe One-Step Kit (Bio-Rad Laboratories, Richmond, CA, USA) and the qPCR primers 5'-TGGACCAAAGGATTATTGTGC-3', 5'-CATCCCCTAAGTGAAGTTGTC-3', and TaqMan-probe 5'-FAM-CTCTTTATGGTAGCCTGGGGAAAG-MGB-3' (Thermo Fisher Scientific). As an internal control, the housekeeping gene mouse β 2-microglobulin was detected using primers 5'-ACAGTTCCACCCGCTCACATT-3', 5'-TAGAAAGACCAGTCCTTGCTGAAG-3', and TaqMan-probe 5'-VIC-CCTAAAGTAGTCAGATATGTCCTTC-MGB-3' (ThermoFisher Scientific). HTNV76–118 N coding construct served as a standard control to determine the copy number of the viral genome.

Detection of proteinuria

Fifty μ L of daily urine samples were resuspended in 50 μ L 2x SDS-PAGE sample buffer (Fujifilm-Wako) and incubated at 100 °C for 5 min. Fifteen μ L of each sample was loaded onto an SDS-PAGE gel (e-PAGEL 1020 L, Atto, Tokyo, Japan) and run. Gels were stained with Coomassie blue stain (Thermo Fisher Scientific), and protein bands

were compared. Similarly, gels were electroblotted onto a 0.45 μ m pore immunoblot PVDF membrane (Millipore). The membranes were incubated with rabbit anti-mouse albumin polyclonal antibody (Proteintech, Rosemont, IL, USA) and rabbit anti-hemoglobin alpha (14537-1-AP, Proteintech). HRP-conjugated goat anti-rabbit IgG antibody (Jackson Immuno Research Laboratories Inc., Baltimore, MD, USA, 111-035-003) was used to detect the bound antibodies, which were further reacted with Amersham ECL Prime (Cytiva, Tokyo, Japan) and detected with an ImageQuant LAS 4000 mini (Cytiva).

Histopathology

Tissues were fixed in a 4% paraformaldehyde phosphate buffer solution (Fujifilm-Wako) and stored at 4 °C. They were then embedded in paraffin within 14 days, sectioned, and stained with hematoxylin and eosin (HE) (Sapporo General Pathology Laboratory, Sapporo, Japan) to assess the extent of renal hemorrhage.

Serum antibody titer detection

Detection of anti-N antibody in mouse sera at 14 dpi was performed by an IFAT. Recombinant N of HTNV strain 76118 was expressed in Vero E6 cells by transfection of plasmid pCHTNS as previously described⁹⁰. Acetone-fixed smears of cells were used as an antigen. Serially diluted sera were applied to the antigen and then followed by Alexa488-conjugated goat anti-mouse IgG(H+L) (Invitrogen, Thermofisher Scientific). Antibody titers were determined as the reciprocal value of the highest dilution giving a positive reaction.

Results

Assessment of renal damage by urine albumin leakage: Exp. 1

As shown in Fig. 10B, KHFV-inoculated mice experienced weight loss peaking at 7 dpi, consistent with previous reports⁸⁰. Oliguria was observed between 5-9 dpi, along with renal hemorrhage that caused the urine to appear dark brown to chocolate-colored due to erythrocyte hemolysis, with gradual recovery seen by 9 dpi (Fig. 10C). Mock-inoculated mice that received only the medium did not exhibit oliguria or renal hemorrhage. To detect proteinuria, protein staining followed by SDS-PAGE was performed (Fig. 10D, left panel). Multiple protein bands appeared during 5-8 dpi, indicating protein leakage into the urine of KHFV-inoculated mice. Additionally, urine albumin was detected by Western blot, with albumin leakage peaking at 7 dpi (Fig. 10D, right panel). Hemoglobin alpha chain was detected by Western blot at 6 and 7 dpi (Fig. 10E). In contrast, mock-inoculated mice showed no increase in renal albumin or hemoglobin alpha chain.

Prophylaxis group: Exp. 2

Neutralizing Mab HCO2 and isotype control antibody were administered to mice one day before viral inoculation (Fig. 11A). Weight loss was not detected in HCO2-administered mice (Fig. 11B). The isotype control group mice showed the same pattern of renal output as KHFV-inoculated mice in Fig. 10. Mice administered with isotype control antibody showed oliguria, hematuria, and albuminuria, while the HCO2 Mab-administered mice had normal appearance and other comparable parameters (Fig. 11C and D).

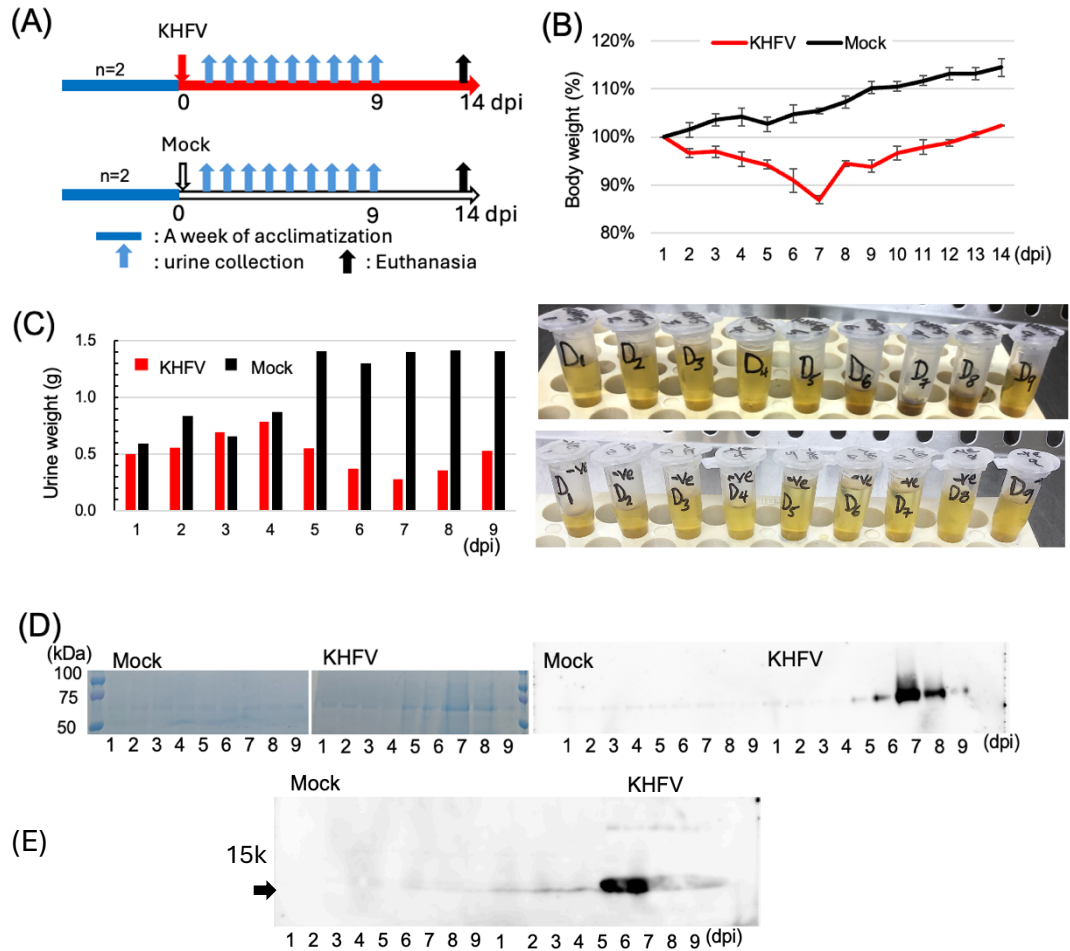


Figure 10. Characterization of renal dysfunction in Korean hemorrhagic fever virus (KHFV) inoculated mice.

- A. Outline of Exp.1
- B. Body weight fluctuation. The error bars represent the individual values.
- C. Fluctuation of daily urine weight (left panel) and gross appearance of urine (right panel).
- D. Proteinuria (left panel) was examined with Coomassie brilliant blue staining, and albuminuria (right panel) was stained with rabbit anti-mouse albumin polyclonal antibody in Western blot.
- E. Hemoglobin in urine was stained with rabbit anti-mouse albumin alpha chain polyclonal antibody in a Western blot.

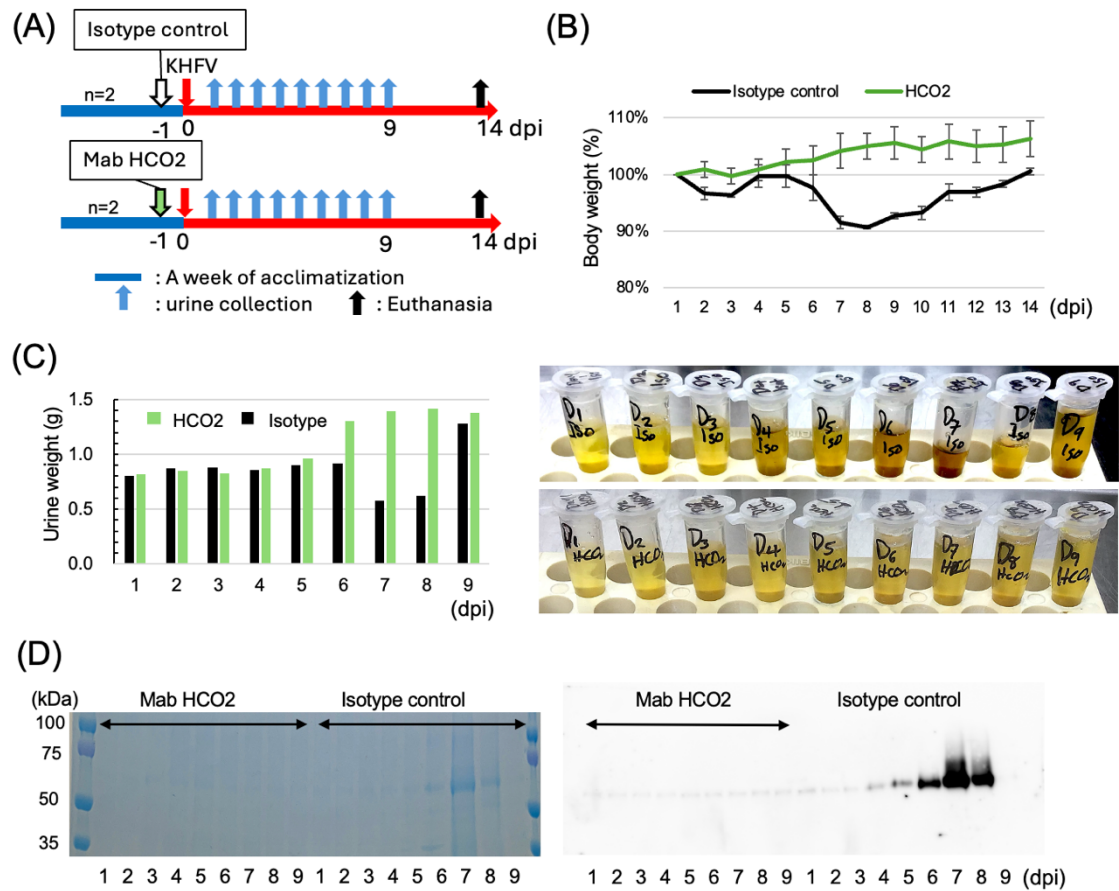


Figure 11. Prophylaxis effect of monoclonal antibody HCO2 on Korean hemorrhagic fever virus (KHFV) infection.

- Outline of Exp.2
- Body weight fluctuation. The error bars represent the individual values.
- Fluctuation of daily urine weight (left panel) and gross appearance of urine (right panel).
- Proteinuria (left panel) was examined with Coomassie brilliant blue staining, and albuminuria (right panel) by rabbit anti-mouse albumin polyclonal antibody in Western blot.

Treatment trial: Exp. 3

Monoclonal antibody HCO2 was administered to mice after KHFV inoculation at 1, 3, and 5 dpi (Fig. 12A). Mice administered with HCO2 at 1 dpi showed no weight loss. Similarly, 3 dpi-and 5 dpi-administrations showed early recovery of body weight (Fig. 12B). Mice were euthanized at 9 dpi, and no visible hemorrhage was observed in their kidneys.

Histopathological observation

Three organs (lung, liver, and kidney) were selected for histopathological analysis in the Exp. 2. Fig. 13A shows the histopathological findings in the kidney, liver, and lung samples. As expected, the isotype antibody did not affect disease progression. Extensive hemorrhages appeared in the kidney, particularly in the corticomedullary region (Fig. 13A panels a and b). However, renal hemorrhages were significantly affected by the presence of HCO2 Mab (Fig. 13A, panels c and d). The liver sections from the isotype group displayed perivascular aggregation of mononuclear lymphoid cells, especially around the hepatic triad (Fig. 13A, panels e and f), compared to the HCO2 Mab group (Fig. 13A, panels g and h). Likewise, thickened alveoli with pneumonic lesions were evident in the isotype control group (Fig. 13A, panels i and j) in contrast to the HCO2 Mab prophylaxis group (Fig. 13A, panels k and l). These findings suggest that the administration of HCO2 antibodies reduced pathological changes in the liver, kidneys, and lungs of infected mice. Furthermore, the treatment groups at 1 dpi, 3 dpi and 5 dpi indicated a positive prognosis for early detection and treatment with Mab HCO2 (Fig. 13B).

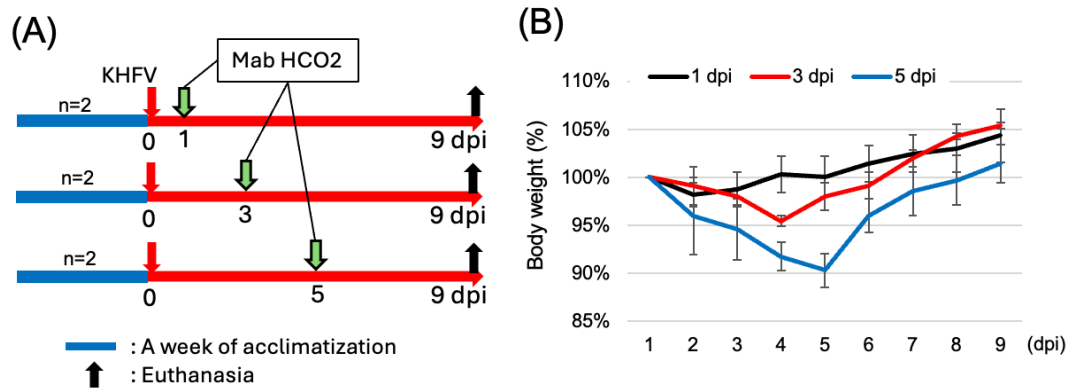


Figure 12. Therapeutic effect of monoclonal antibody HCO2 on Korean hemorrhagic fever virus (KHFV) infection.

A. Outline of Exp.3

B. Body weight fluctuation of 1 dpi, 3 dpi, and 5 dpi. The error bars represent the individual values.

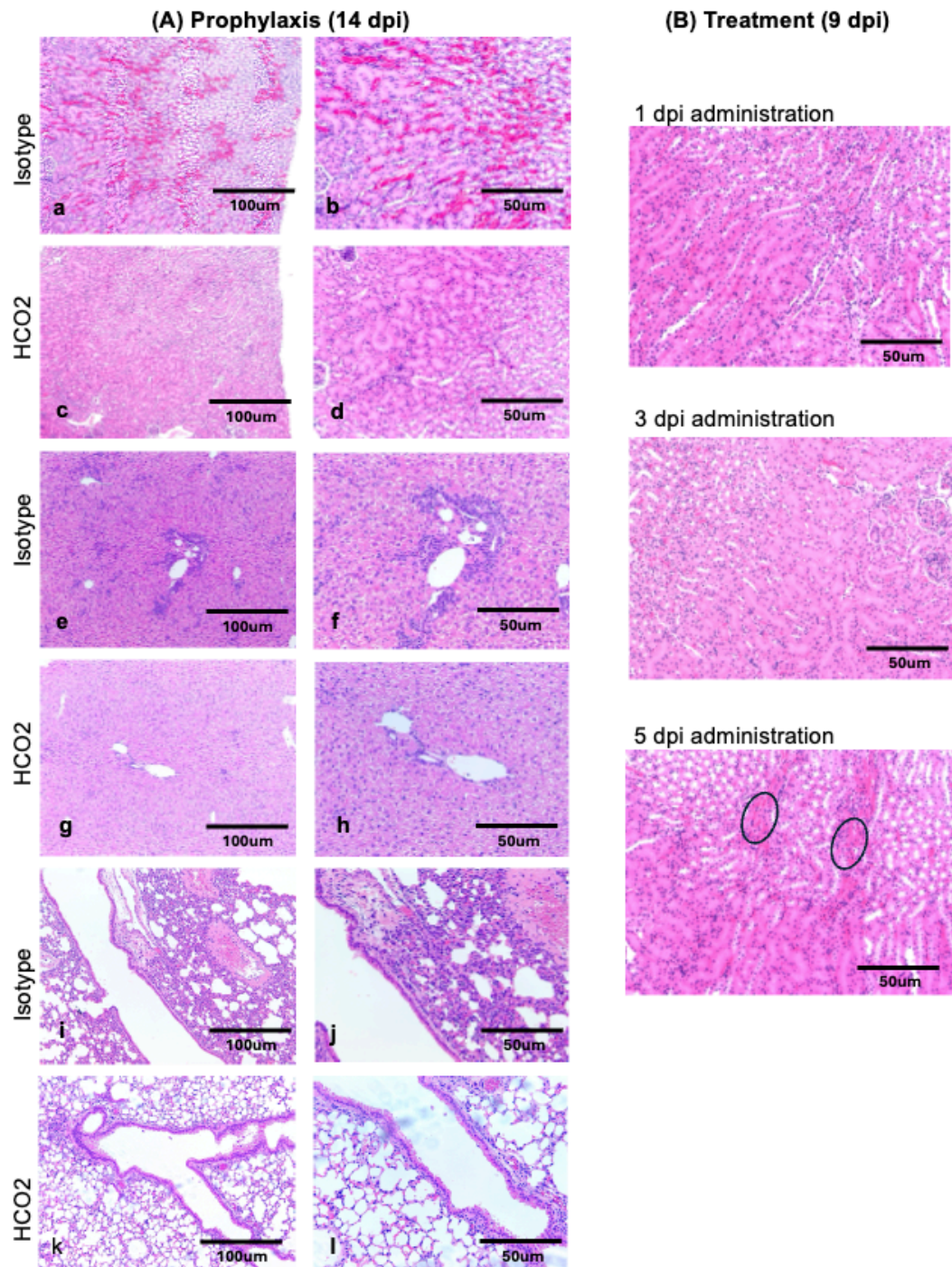


Figure 13. Histopathological analysis of prophylaxis (Exp. 2) and therapeutic (Exp. 3) trials with monoclonal antibody HCO2.

- A. Histological examination of the kidney (a-d), liver (e-h), and lung (i-l) in the prophylaxis group of Exp. 2 of monoclonal antibody HCO2 administrated mice and isotype antibody administrated mice.
- B. Histological examination of the kidneys from the three treatment groups of Exp. 3. Monoclonal antibody HCO2 significantly prevented hemorrhage appearance at 1, 3, and 5 dpi administration. Except that at 5 dpi, some degree of hemorrhages was still detected (ovals).

Viral load and replication in mice

As shown in Table 13, the viral genome was detected in urine and kidney by quantitative RT-PCR. In Exp. 1, the viral genome was detected in the urine of the KHFV-inoculated mouse but not in the urine from the mock-infected mouse. On the other hand, no viral genome was detected in feces (data not shown). Viral load in the mice of Exp. 2, HCO2 administrated mice had viral genome in kidney below the detectable range with ct values >40, and ct value for beta 2-microglobulin (B2m) internal control remained same as other kidney reactions, while mice of the isotype control had viral genome 5×10^7 copy/kidney, similar to KHFV-inoculated mice. These results indicate that HCO2 administration before viral inoculation reduced KHFV replication in the kidney.

Table 13. Viral load in urine and kidney.

Exp	Mouse ID	dpi	Sample	KHFV (FAM) Cq		Viral genome copy number	<i>B2m</i> (VIC) Cq	
Exp. 1	KHFV #1	1	Urine	-	-	-	ND	
		2		-	-	-		
		3		25.85	26.03	4.7 X 10 ⁴ /μl		
		4		-	-	-		
		5		-	-	-		
		6		-	-	-		
		7		25.29	25.61	4.4 X 10 ⁴ /μl		
		8		-	-	-		
		9		29.8	29.82	2.9 X 10 ² /μl		
Exp. 2	KHFV #1	14	Kidney	21.08	21.2	3.2 X 10 ⁷ /kidney	17.49	17.81
	KHFV #2	14		20.94	21.32	3.3 X 10 ⁷ /kidney	17.15	17.61
	Mock #1	14		-	-	-	17.77	18.42
	Mock #2	14		-	-	-	18.16	18.06
	Isotype #1	14		21.03	20.86	3.7 X 10 ⁷ /kidney	18.45	18.42
	Isotype #2	14		20.99	21	3.6 X 10 ⁷ /kidney	17.21	17.59
	HCO2 #1	14		-	-	-	18.40	17.46
	HCO2 #2	14		-	-	-	16.81	17.37

-; under detection, ND; not done

Serum antibody titers to recombinant N antigen

I attempted to examine seroconversion in mice after the challenge. Since mice received a monoclonal antibody to glycoprotein (GP), seroconversion was assessed using recombinant N antigen. IFAT titers in mice from Exp.1 and Exp.2 were analyzed (Table 14). The KHFV-administered group in Exp. 1 and the isotype control group in Exp. 2 had very high antibody titers of 200,000. In contrast, mock-inoculated mice showed antibody titers of less than 100. Mice given HCO2 antibody before challenge exhibited low antibody titers to the N antigen, which were induced by actual infection. This result suggests that although the infection was not completely prevented by HCO2, it hindered the disease from becoming severe. Therefore, virus inhibition may be directly proportional to the concentration of administered antibody.

Table 14. Serum IFAT antibody titers to rN antigen of HTNV at 14 dpi.

Experiment	Mouse ID	IFAT titer
Exp. 1	KHFV #1	204,800
	KHFV #2	204,800
	Mock #1	<100
	Mock #2	<100
Exp.2	Isotype control #1	204,800
	Isotype control #2	204,800
	HCO2 #1	12,800
	HCO2 #2	12,800

Discussion

Previous studies have shown that KHFV-inoculated mice showed hepatitis and peripheral neutrophilia, as observed in HFRS patients ^{48,53,54,73}. However, there was insufficient information on kidney dysfunction. In this study, kidney function was first analyzed by examining urine volume and urinary protein levels. Proteinuria has been reported to be seen in 100% of HFRS patients infected with HTNV, and 85% of HFRS patients showed hematuria. As a result, proteinuria and albuminuria were observed in this model. Oliguric phase is observed in 60% of patients 6–8-day onset, and the diuretic phase is observed in 95% of cases, with a 9–13-day onset ³⁹. This peak coincides with the time of bleeding in the renal medulla in this model, 6-9 dpi. Due to the 24-hour collection interval, urinary erythrocytes undergo hemolysis, giving the urine a darker appearance. These urinary findings in KHFV-infected mice were like those seen in HFRS. It has also been reported that hypoalbuminemia in the acute phase has been associated with the severity of HFRS in humans ³⁴. The amount of urine in KHFV-infected mice on 7 dpi was approximately four times lower than that of the control. However, it was more than 4 times as much albumin observed compared to that of the control. Therefore, urine albumin detected in KHFV-infected mice was not simply a concentration, but albumin leakage. The serum albumin level of infected mice was not examined in this assay, but albumin leakage into urine was observed, which might have led to hypoalbuminemia in mice.

When neutralizing antibodies were administered prophylactically before virus inoculation, no HFRS-like symptoms were observed. On the other hand, weight loss, decreased urine volume, and albuminuria were observed in the isotype control group. However, antibody seroconversion indicated that infection was not completely prevented even with the administration of neutralizing antibodies. Although the infection was not completely prevented due to the balance between the amount of

antibody administered and the amount of inoculated virus, prophylactic administration of antibodies was highly effective in reducing symptoms. It suppressed the viral load in the kidneys and mitigated pathological changes in the kidneys, lungs, and liver.

Since the Mab HCO2 was expected to reduce symptoms, I investigated its therapeutic effect after infection. Mice given at 1 dpi showed almost no symptoms. Administering the antibodies at 3 dpi and 5 dpi also helped decrease weight loss. When the mice were euthanized on 9 dpi and their kidneys examined, no visible bleeding in the renal medulla was seen in any of the mice. However, histological analysis revealed bleeding in the kidneys when the antibody was given at 5 dpi. My findings suggest that neutralizing antibodies can be effective in alleviating symptoms even if given late, but the effect is stronger when administered early.

The HCO2 used in this study is unique among neutralizing monoclonal antibodies. Hantavirus GP is the target molecule for neutralization and is composed of Gn and Gc molecules. Most monoclonal antibodies with neutralizing activity against hantaviruses are directed against the Gn molecule by affecting the low-pH-dependent membrane fusion process or receptor-binding process ⁷⁰. HCO2 was generated against HTNV in 1991, but it also exhibits neutralizing activity against SEOV ^{8,62}. Although it was reported as an anti-glycoprotein Gc antibody by immunoprecipitation assay, the amino acid related to neutralizing and HCO2-binding sites was found in both Gn and Gc through an escape mutant-generating assay ^{33,77}. Studies on PUUV and ANDV also suggest that antibodies targeting epitopes spanning Gn and Gc may have cross-serotype neutralizing activity by locking Gn/Gc ⁴⁹. Inducing antibodies against the epitope of Gn/Gc locking antibodies might be effective for eliciting protective immunity across different serotypes.

This study's limitation was the small number of experiments (n=2), which made it difficult to evaluate statistically. This was due to the limited availability of HCO2

antibody material. Additionally, handling hantavirus-infected mice in metabolic cages in a BSL3 facility requires extreme caution, making large-scale experiments challenging. Despite these limitations, the results of this experiment were clear and provided many insights for the development of the hantavirus infection model.

In conclusion, acute renal failure following KHFV infection in this HFRS model was shown in this study and may serve as a useful tool to evaluate antiviral drugs, antibodies, and vaccine efficacy.

Summary

The study used the KHFV, a strain of HTNV, to induce HFRS-like symptoms in immunocompetent mice. Symptoms included weight loss, renal medullary hemorrhage, oliguria, hematuria, and albuminuria, mimicking human HFRS. KHFV-infected mice exhibited oliguria (reduced urine output) and hematuria (blood in urine) at 5–8 dpi. Albuminuria (protein leakage into urine) was detected, peaking at 7 dpi, indicating kidney damage.

Prophylactic Use: Administering a neutralizing antibody HCO2 before infection prevented weight loss, renal hemorrhage, and urinary symptoms. Post-infection administration (1, 3, or 5 dpi) reduced disease severity, with earlier treatment showing better outcomes. HCO2 lowered viral loads in kidneys and mitigated histopathological damage in kidneys, liver, and lungs. Viral RNA was detected in the urine and kidneys of infected mice but not in feces. Seroconversion was observed in infected mice, but HCO2 administration reduced antibody titers, suggesting partial viral suppression. The KHFV mouse model replicates key HFRS symptoms, providing a tool for evaluating antiviral drugs, antibodies, and vaccines. HCO2 antibody shows promise for both prevention and treatment, supporting its potential clinical application. This study validates the KHFV mouse model for HFRS research and highlights the therapeutic potential of neutralizing antibodies. Further studies are needed to confirm therapeutic timing and dosage effects.

Conclusions

This thesis presents a comprehensive investigation into the seroepidemiology of bat- and shrew-borne hantaviruses and the renal pathology of hantavirus infections, focusing on their zoonotic potential and public health implications. The research is divided into three interconnected chapters, each addressing critical gaps in our understanding of hantavirus biology, diagnostics, and pathogenesis. The findings underscore the importance of expanded surveillance, improved diagnostic tools, and the development of therapeutic interventions for hantavirus infections. Key findings and contributions.

Development of a seroepidemiological tool for bat- and shrew-borne hantaviruses (Chapter 1)

The study successfully developed a multiplex IFAT using rNs from 11 hantaviruses (5 bat-borne and 6 shrew-borne). This tool demonstrated high specificity with minimal cross-reactivity, making it a valuable resource for large-scale serological screening. Application of this tool to bat sera from Zambia revealed unexpected seropositivity patterns, with 11.4% of bats reactive to bat-borne hantaviruses and 17.4% to shrew-borne hantaviruses. Notably, fruit bats exhibited higher exposure rates, suggesting potential spillover events or unidentified reservoir hosts. These findings highlight the need for further research into the ecological dynamics of hantavirus transmission in Africa and beyond.

Serological detection of bat- and shrew-borne hantaviruses in Sri Lanka (Chapter 2)

Screening of human sera from Sri Lanka revealed antibodies against bat- and shrew-borne hantaviruses, with higher seropositivity rates in febrile patients compared to healthy controls. Notably, antibodies against QZNV, ROBV, ASAV, and TPMV viruses were detected, suggesting human exposure to these emerging pathogens. The absence

of seropositivity for BRNV and NVAV viruses, which are geographically restricted to Europe, underscores the role of local reservoir hosts in virus transmission. These findings emphasize the need for further studies to identify reservoir species and assess the clinical significance of these infections in Sri Lanka.

Analysis of renal pathology in a Hantaan virus-infected mouse model (Chapter 3)

The study used a mouse model for HFRS using the KHFV. This model replicated key clinical features of HFRS, such as weight loss, renal hemorrhage, oliguria, and albuminuria. Prophylactic and therapeutic administration of the neutralizing monoclonal antibody HCO2 significantly reduced disease severity, lowering viral loads and histopathological damage in the kidneys, liver, and lungs. These results validate the KHFV mouse model as a valuable tool for assessing antiviral therapies and vaccines, while also highlighting the potential of neutralizing antibodies for clinical use.

✓ Broader implications

Research presented in this thesis has several important implications for public health and virology.

Diagnostic Advancements: The multiplex IFAT developed in this study provides a scalable and efficient method for screening hantavirus antibodies, facilitating early detection and surveillance in both animal and human populations.

Therapeutic Potential: The efficacy of the HCO2 monoclonal antibody in the KHFV mouse model highlights its promise as a prophylactic and therapeutic agent for HFRS, warranting further clinical evaluation.

Zoonotic Risk: The detection of antibodies against diverse hantaviruses in bats, shrews, and humans underscores the zoonotic potential of these viruses and the need for One Health approaches to mitigate spillover risks.

Global Relevance: The findings from Zambia and Sri Lanka reveal the widespread distribution of bat- and shrew-borne hantaviruses, challenging the traditional view of hantaviruses as primarily rodent-borne pathogens.

✓ **Future Directions**

Virus Isolation and Characterization: Isolating and sequencing hantaviruses from bats and shrews to better understand their genetic diversity and evolutionary relationships.

Pathogenicity Studies: Investigating the clinical impact of bat- and shrew-borne hantaviruses in humans to determine their role in febrile illnesses of unknown origin.

Ecological Studies: Identifying reservoir hosts and transmission pathways to inform targeted surveillance and control measures.

Vaccine Development: Utilizing the KHFV mouse model to test candidate vaccines and therapeutics for HFRS.

✓ **Final Remarks**

This thesis enhances our understanding of hantaviruses by providing a platform that can be further developed to improve hantavirus diagnostics, pathogenesis, and epidemiology. The tools and models created here establish a foundation for future research, while the findings emphasize the importance of vigilance against emerging zoonotic threats. By integrating serological, experimental, and ecological approaches, this work aids global efforts to mitigate the public health impact of hantaviruses and other emerging pathogens. Continued interdisciplinary collaboration will be vital to address the challenges posed by these viruses and protect human and animal health.

Acknowledgement

First and foremost, I would like to express my deepest gratitude to my supervisor, Kumiko Yoshimatsu, an associate professor at the Institute of Genetic Medicine, for her invaluable guidance, unwavering support, and insightful critiques throughout this research journey. Her expertise in virology and epidemiology has been instrumental in shaping this thesis, and her encouragement has consistently served as a source of motivation.

I am also sincerely thankful to the specially appointed Professor Hiroaki Kariwa, Prof. Ayato Takada, and Associate Professor Keita Matsuno for their constructive feedback and mentorship, which greatly enriched the quality of this work. Their dedication to scientific rigor and infectious enthusiasm for research has left a lasting impression on me.

This research would not have been possible without the collaboration and support of my colleagues, who are now alumni at the Graduate School of Infectious Diseases, Hokkaido University. I am particularly grateful to Wei Zhuoxing, who assisted me during my research student status and first year in my PhD. He never hesitated to share with me his acquired skills in molecular cloning and immunological assays. Devinda S. Muthusinghe assisted me in sequencing techniques and data processing using genetic tools, and Sithumini M.W. Lokupathirage helped me in cell culture maintenance and serology. I would also like to thank my new laboratory mate, Pavani Senarathne, for her contribution to my research papers. I thank all of them for their technical assistance, stimulating discussions, and camaraderie in the laboratory. Their contributions, whether in experiments, data analysis, or troubleshooting, were indispensable to the success of this work.

I would also like to acknowledge the invaluable contributions of our collaborators, the Graduate School of Infectious Diseases, associate professor Masahiro Kajihara, Mrs.

Akina Mori-Kajihara, and Professor Chandika D. Gamage, especially for providing essential samples and logistical support. Their efforts in field surveillance and sample collection were crucial to the success of this study. Another thanks goes to Professor Jiro Arikawa for supplying me with Mab HCO2 for the therapeutic study.

Special thanks to Mr. Hiroyuki Murota and Mr. Koki Otaki, staff members of the Laboratory for Animal Experimentation, for their technical support and for maintaining the infrastructure necessary for this research. I also appreciate the administrative staff at the Institute for Genetic Medicine and my graduate school for their help with documentation.

Scholarship for my PhD course and financial support for this research were generously provided by the Japan International Cooperation Agency (JICA), and I sincerely appreciate their investment in advancing scientific knowledge on emerging zoonotic viruses.

Finally, I owe an immeasurable debt of gratitude to my parents, Effah Kyere and Aisha Fosuaa Ibrahim, for their unconditional love, patience, and encouragement. Their unwavering belief in me, even during the most challenging phases of this journey, kept me motivated and resilient.

To all who have contributed, directly or indirectly, to this thesis, thank you. This work stands as a testament to the collective effort and support of many individuals, and I am deeply honored to have had such an incredible network behind me.

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日本語要旨

本論文は、コウモリおよびトガリネズミ由来ハンタウイルスの血清疫学とハンタウイルス感染症の腎臓病理について、人獣共通感染の可能性と公衆衛生への影響に焦点を当てた研究である。本研究は3つの相互に関連した章に分かれており、各章はハンタウイルスの生物学、診断、病因に関する理解における重要な課題を取り上げている。本研究の知見は、ハンタウイルス感染症に対するサーベイランスの拡大、診断ツールの改善、そして治療法の開発の重要性に貢献する。主な知見と貢献を以下に示す。

第1章：コウモリおよびトガリネズミ媒介性ハンタウイルスの血清疫学ツールの開発

本研究では、11種のハンタウイルス（コウモリ由来5種、トガリネズミ由来6種）の組換えNタンパク質抗原を用いたマルチプレックス抗原の開発に成功した。このツールは高い特異性と低い交差反応性を示し、大規模な血清学的スクリーニングのための貴重なツールとなると期待された。このツールを用いてザンビアで捕獲されたコウモリの血清に応用した。1764検体のコウモリの11.4%がコウモリ由来ハンタウイルス抗原に、17.4%がトガリネズミ由来ハンタウイルス抗原に反応した。特に、ストローオコウモリは数種の抗原に高い抗体陽性率を示しており、未確認の宿主からのスピルオーバー感染が示唆された。これらの知見は、アフリカおよびそれ以外の地域におけるハンタウイルス伝播の生態学的動態に関して更なる研究が必要であることを示す。

第2章：スリランカのヒトにおけるコウモリ由来ハンタウイルスおよびトガリネズミ由来ハンタウイルス抗原に対する抗体検出

スリランカのヒト血清のスクリーニングにより、コウモリ由来ハンタウイルスおよびトガリネズミ由来ハンタウイルスに対する抗体が検出され、発熱患者では健常対照群と比較して高い血清陽性率を示した。特に、Quezon、Robina、Asama、およびThottapalayamウイルスに対する抗体が検出され、これらの新興病原体へのヒトの曝露が示唆された。地理的にヨーロッパに限定されているBrnoおよびNovaウイルスに対する陽性例がなかったことは、現地に固有の宿主が存在しウイルスを伝搬していることを強く示唆する。これらの知見から、スリランカにおけるこれらの感染症の宿主を特定し、病原性を解明するために更なる研究が必要であると考えられる。

第3章：Hantaan ウイルス感染マウスモデルにおける腎病理の解析

本研究では、Hantaan ウイルス韓国出血熱ウイルス株(KHFV)を用いた腎症候性出血熱(HFRS)のマウスモデルを用いた。このモデルは、体重減少、腎出血、乏尿、アルブミン尿など、HFRSの主要な臨床的特徴を再現した。中和モノクローナル抗体HCO2の予防

的および治療的投与は、疾患の重症度を軽減し、ウイルス量と腎臓、肝臓、肺における組織病理学的変化を減少させた。これらの結果は、KHFV マウスモデルが抗ウイルス療法およびワクチンの評価に有用なツールであることを実証するとともに、中和抗体の臨床応用の可能性を示唆するものである。

✓この論文の影響

本論文で示された研究は、公衆衛生とウイルス学に重要な知見を与えると期待される。

診断の進歩：本研究で開発されたマルチプレックス蛍光抗体法(IFAT)は、ハンタウイルス抗体のスクリーニングのための拡張可能で効率的な方法を提供し、動物とヒトの両方の集団における感染の早期発見と監視を促す。

治療の可能性：KHFV マウスモデルにおける HCO2 中和モノクローナル抗体の効果は、HFRS の予防薬および治療薬としての有望であり今後さらなる評価が必要である。

人獣共通感染リスク：コウモリ、トガリネズミ、そしてヒトにおいて、多様なハンタウイルスに対する抗体が検出されたことは、これらのウイルスが人獣共通感染症の原因となる可能性と、スピルオーバーリスクを軽減するためのワンヘルスアプローチの必要性を示す。

世界的な意義：ザンビアとスリランカの知見は、コウモリとトガリネズミ由来ハンタウイルスの広範な分布を示すものであり、ハンタウイルスは主にげっ歯類媒介性病原体であるという従来の見解に疑問を投げかけている。

✓今後の課題

ウイルスの分離と特性解析：コウモリとトガリネズミからハンタウイルスを分離し、その遺伝的多様性と進化的関係をより深く理解する必要がある。

病原性研究：コウモリとトガリネズミが媒介するハンタウイルスのヒトにおける臨床的影響を調査し、原因不明の発熱性疾患の原因となっていないかどうか明らかにする。

生態学的研究：監視と制御対策に役立てるため、宿主と伝播経路を特定する。

ワクチン開発：KHFV マウスモデルを用いて HFRS の候補ワクチンと治療薬を試験する。

✓結論

本論文は、ハンタウイルスの診断、病因、疫学研究を改善するためのプラットフォームを提供するものである。ここで構築されたツールとモデルは、将来の研究の基盤を確立するものであり、同時に、新たな人獣共通感染症の脅威に対する警戒の重要性を強調するものである。本研究は、血清学的、実験的、生態学的アプローチを統合することで、ハンタウイルスやその他の新興病原体による公衆衛生への影響を軽減するための

世界的な取り組みに貢献する。継続的な学際的連携はウイルスがもたらす課題に対処し、人間と動物の健康を守るために不可欠である。