

博士論文

**Studies on characterization of nairovirus and  
establishment of laboratory diagnostic systems**

(ナイロウイルスの性状解析および実験室診断系の構築に関する研究)

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## **List of abbreviations**

**CCHF:** Crimean-Congo hemorrhagic fever

**NSD:** Nairobi sheep disease

**CCHFV:** Crimean-Congo hemorrhagic fever virus

**STBV:** Soft tick bunyavirus

**IKV:** Issyk-Kul virus

**HAZV:** Hazara virus

**NSDV:** Nairobi sheep disease virus

**VSV:** Vesicular stomatitis virus

**GP:** glycoprotein

**N:** nucleoprotein

**ELISA:** enzyme-linked immunosorbent assay

**IFA:** immunofluorescence assay

**BSL:** biosafety level

## **Preface: Aims and Scope of the thesis**

Nairoviruses are tick-borne viruses and include zoonosis pathogens such as CCHFV, NSDV, and IKV. One of the most notable pathogen in nairovirus is CCHFV. CCHFV causes severe hemorrhagic fever, CCHF, and the case-fatality rate is nearly 30% in humans. The incidence and geographic range of confirmed CCHF cases have increased. As CCHFV requires BSL-4 containment for being handled, the study of CCHFV and other nairoviruses has been limited. Laboratory diagnosis of CCHF has been also limited because viral isolation and neutralization assay could not be performed without BSL-4. For more exact diagnosis and further studies, the novel systems have been needed.

Nairoviruses comprise seven serogroups based on the antibody-cross reactivities. CCHFV belongs to CCHF group with HAZV. However, some other viruses including IKV, STBV, and other novel nairoviruses have not been classified. For further understanding of nairoviruses, the characterization and the serological study of these viruses are needed. Well understanding of these features will help the control of nairoviruses.

In the present thesis, for a further understanding of nairovirus, the author described the characterization of CCHFV and other nairoviruses including a novel nairovirus and the establishment of the serological assays.

In **General Introduction**, characteristics of the genus nairovirus and diseases caused by nairoviruses are summarized for the background of the present thesis.

In **Chapter 1**, the author generated CCHFV pseudotype virus and analyzed CCHFV entry mechanism by using it.

In **Chapter 2**, the author generated a novel serological diagnostic assay for CCHF using the pseudotype virus generated in Chapter 1. Human serum samples were tested by a neutralization assay using the pseudotype virus and the result was compared to that of a recombinant N-based immunoglobulin G (IgG) ELISA, a well-established assay for the serodiagnosis, or that of

neutralization assay using authentic virus.

In **Chapter 3**, the author compared the characteristics of three nairoviruses, HAZV, STBV, and IKV, for well-understanding of nairoviruses including a novel nairovirus. HAZV is genetically and serologically closely related to CCHFV and a relatively well-studied virus in nairovirus. STBV was recently isolated from tick in Japan. IKV is known as a pathogen of human. The author analyzed the virological and serological characteristics of these viruses.

In **General Conclusion**, the author discussed the data in the thesis collectively.

## **General Introduction**



## Nairovirus

Nairovirus is the genus of the family *Bunyaviridae*. *Bunyaviridae* includes the genus hantavirus, nairovirus, orthobunyavirus, phlebovirus, and tospovirus. Fig. I.1 shows phylogenetic tree of the members of nairovirus. Nairovirus includes pathogens to human such as CCHFV and IKV, and to livestock such as NSDV. Nairovirus comprises seven serogroups based on the antibody cross-reactivities, CCHF, NSD, Thiafora, Sakhalin, Dera Ghazi Khan, Hughes, and Qalyub group (Casals & Tignor, 1980; Lasecka & Baron, 2014).

Nairoviruses have an envelope and a tri-segmented, negative-sense, single-strand RNA genome. Nairovirus has the largest genome among *Bunyaviridae* and the L, M, and S segments encode the RNA dependent RNA polymerase (L), GP, and N, respectively (Fig. I.2). The premature GP is cleaved and modified to generate structural proteins, Gn and Gc, and one or more non-structural proteins (Walker *et al.*, 2015). Life cycle of nairoviruses is shown in Fig. I.3. Virus attaches to a cellular receptor (which has not yet been revealed) and enters a host cell by endocytosis-mediated manner. Upon reaching the low pH condition of the endosome, GP may change the conformation and viral ribonucleocapsids (RNPs) and polymerase are released to the cytoplasm. Viral replication occurs in the cytoplasm, and viral assembly and budding take place in the Golgi. Nairoviruses share many common features with other bunyaviruses such as replication in cytoplasm, budding in the Golgi, and RNA replication mechanisms. However, nairoviruses have unique features. For example, GP cleavage is involved in the cellular proteases, the subtilisin kexin isozyme-1/site-1 protease (SKI-1/S1P)-like proteases and furin (Sanchez *et al.*, 2002, 2006; Vincent *et al.*, 2003). Nairoviruses have protein domains which have not been seen in other bunyaviruses, such as the L protein ovarian-tumor (OTU) domain. Mucin-like domain of GP is considered to be related with the pathogenicity of the virus. While most other bunyaviruses are insect-borne, nairoviruses are tick-borne in general.

Nairoviruses have not been studied well while some notable pathogens are belonged to. Furthermore, many novel nairoviruses have been found in these years (e.g., Leopards Hill virus (Ishii *et al.*, 2014), Finch creek virus (Major *et al.*, 2009), and STBV (Oba *et al.*, 2015)). Now it is necessary to reveal the mechanisms of the viral infection and establish the system of diagnosis and treatment for nairovirus infections.

### **N of nairoviruses**

The N of nairoviruses is approximately 50 kDa. The N is the most abundant protein in the virion. The N is necessary for packaging of the viral genome into virions by encapsidating newly synthesized vRNA and cRNA (Bergeron *et al.*, 2010). The N of CCHFV and HAZV has endonuclease activity specific for dsDNA and ssDNA. However, that of Kupe virus has weak one and that of Erve virus has no activity (Wang *et al.*, 2015). The protein of CCHFV, HAZV, and Erve virus has caspase-3 cleavage site while that of Kupe virus does not have (Wang *et al.*, 2015). The biological significance of the cleavage could be interpreted as the host cell immunity against the viral infection or as CCHFV strategy preventing the cell apoptosis to act as a suicide decoy substrate.

### **GP of nairoviruses**

The full CCHFV GP has six hydrophobic regions, which could function as transmembrane region. These regions act as classic secretory signal peptides, membrane anchors, or a combination of both (Sanchez *et al.*, 2002). CCHFV GP cleavage to mucin-like domain, GP38, Gn, NSm, and Gc. Gn and Gc are structure proteins that form complexes on the envelope and interact with host receptor to initiate infection. The Gn and Gc proteins of nairoviruses are approximately 35 and 75 kDa, respectively. Mucin-like domain, GP38, and NSm are non-structural proteins. Protein cleavage

occurs in ER by cellular signalase and generates the preGn (containing the mucin-like domain, GP38, and Gn), the predominantly cytoplasmically oriented NSm, and the preGc. PreGn is cleaved to separate mucin-like domain/GP38 from Gn. The cleavage occurs through recognition of a conserved RRLL motif by SKI-1/S1P of host cell (Vincent *et al.*, 2003). It is predicted that preGc cleaved by a related subtilisin-like protease but not by SKI-1/S1P to generate the mature Gc at a similar motif (RKPL) exists in 41 amino acids downstream of the signalase cleavage site (Sanchez *et al.*, 2002; Vincent *et al.*, 2003). Gn interacts with Gc and translocated to the virus assembly site in Golgi (Sanchez *et al.*, 2006). In the trans-Golgi, mucin-like domain and GP38 are cleaved by a furin-like protein convertase at the RSKR site (Sanchez *et al.*, 2006). Although the function of NSm protein has not been clear, the fact that Gn maturation requires NSm suggests that NSm is necessary for CCHFV replication (Altamura *et al.*, 2007). However, it was recently reported that a double-membrane spanning domain between Gn and Gc that is corresponding region of CCHFV NSm was lacking from Leopards Hill virus, Erve virus, Thiafora virus, Yogue virus, Kasokero virus, Gossas virus, Keterah virus, and IKV (Walker *et al.*, 2015). These results show that there are some differences among nairoviruses. Glycosylation is an important post-translational modification of secreted and membrane proteins that can effect protein folding, transport, and function (Hauri *et al.*, 2000; Helenius & Aebi, 2001; Molinari & Helenius, 2000). It has been reported CCHFV GP is glycosylated (Sanchez *et al.*, 2002). The N-terminal part of CCHFV GP, which contains the mucin-like domain, is heavily O-glycosylated, in contrast GP38 has few O-glycosylated sites (Sanchez *et al.*, 2002, 2006). Both mucin-like domain and GP38 have N-glycosylated sites. Mature Gn and Gc have one and two N-glycosylated sites, respectively (Sanchez *et al.*, 2002). As Gn glycosylation is critical for Gn maturation and transport of itself and other glycoproteins, the correct glycosylation of Gn is essential for viral replication (Erickson *et al.*, 2007).

### **L protein of nairoviruses**

The L protein is approximately 450 kDa and contains the RNA-dependent RNA polymerase (RdRp). The catalytic region of the RdRp is the most conserved among nairoviruses (Honig *et al.*, 2004; Marriott & Nuttall, 1996). L protein contains three regions, N-terminal region, RdRp region and C-terminal region (Kinsella *et al.*, 2004). Function of N-terminal region and C-terminal region is unknown. N-terminal region contains OTU-like protease domain that is unique to this genus, while this protease activity is not necessary for viral genome replication (Bergeron *et al.*, 2007).

### **Diseases caused by nairoviruses**

#### **CCHF**

CCHF is a potentially fatal tick-borne infectious disease that has been reported in over 30 countries in parts of Africa, Eastern Europe, and Asia (Bente *et al.*, 2013; Mertens *et al.*, 2013; Messina *et al.*, 2015; Papa *et al.*, 2015; Whitehouse, 2004). Cattle, sheep, and other animals were infected with CCHFV through tick bite but the animals show no symptoms upon infection. Human infection can occur through the bite of virus-infected tick or through contact with the tissue or blood of viremic animals or CCHF patients. The case-fatality rate is nearly 30% (Ak,nc, *et al.*, 2013; Bente *et al.*, 2013; Mertens *et al.*, 2013; Papa *et al.*, 2015; Whitehouse, 2004). The virus has been isolated from at least 31 different species of *Haemaphysalis* and *Hyalomma* ticks (Mehravaran *et al.*, 2013). Both the incidence and geographic range of confirmed CCHF cases have increased. In Turkey, the first cases of CCHF were identified in 2002, after which the number of the patients increased and the number of the identified case was more than 9,000 to date (Papa *et al.*, 2015). In Iran, 1,017 cases have occurred between 2000 and 2014 (Papa *et al.*, 2015). In China, 286 cases were identified between 1965 and 1997 (Papa *et al.*, 2002). In Kazakhstan, during 2000 to 2013, 212 cases were reported (Nurmakhanov *et al.*, 2015). At present, there is no established countermeasure for

treating or preventing CCHF.

The clinical spectrum of CCHF varies from subclinical infection to severe disease and death. During the course of CCHF, there are incubation phase, pre-hemorrhagic phase, hemorrhagic phase, and convalescence phase. The incubation period of CCHF ranges from 1 to 13 days. The pre-hemorrhagic phase is characterized with influenza-like symptoms. In severe case, the hemorrhagic phase is developed 3 to 6 days after onset of the illness. The patient dead 5 to 14 days after onset of CCHF due to multiple organ failure and shock. Convalescent phase starts at 15 to 20 days after onset of the disease (Ak,nc, *et al.*, 2013; Ergönül, 2006; Oncü, 2013). The viral main target tissues are liver and spleen (Bente *et al.*, 2010). Amplification in macrophages and dendritic cells may facilitate spreading in the host (Connolly-Andersen *et al.*, 2009).

For experimental working, CCHFV requires BSL-4 containment in most countries. There are only approximately 50 BSL-4 containments in the world in this time. Therefore, the number of laboratories that can perform viral isolation, one of the standards for CCHF diagnosis, is limited. Moreover, the sensitivity of viral isolation is low and the result could not be certain. Other practical diagnostic methods are conventional and real-time quantitative reverse transcription PCR (RT-PCR and qRT-PCR) for genome detection (Burt *et al.*, 1998; Duh *et al.*, 2006; Kamboj *et al.*, 2014), antigen-capture ELISA for antigen detection (Logan *et al.*, 1993; Saijo *et al.*, 2005a; Saluzzo & Le Guenno, 1987), and IFA (Burt *et al.*, 1994; Ergunay *et al.*, 2014; Saijo *et al.*, 2002a; Vanhomwegen *et al.*, 2012) and ELISA (Burt *et al.*, 1993; Marriott *et al.*, 1994; Qing *et al.*, 2003; Saijo *et al.*, 2002b, 2005b) for detection of the specific antibody. Antibody to N becomes detectable from about 1 week of CCHF and specific IgG remains detectable for several years. The neutralizing antibody was developed in the second to third weeks after the disease onset. As viral isolation and neutralization assay require BSL-4 containment, these are not prepared in Japan in this time.

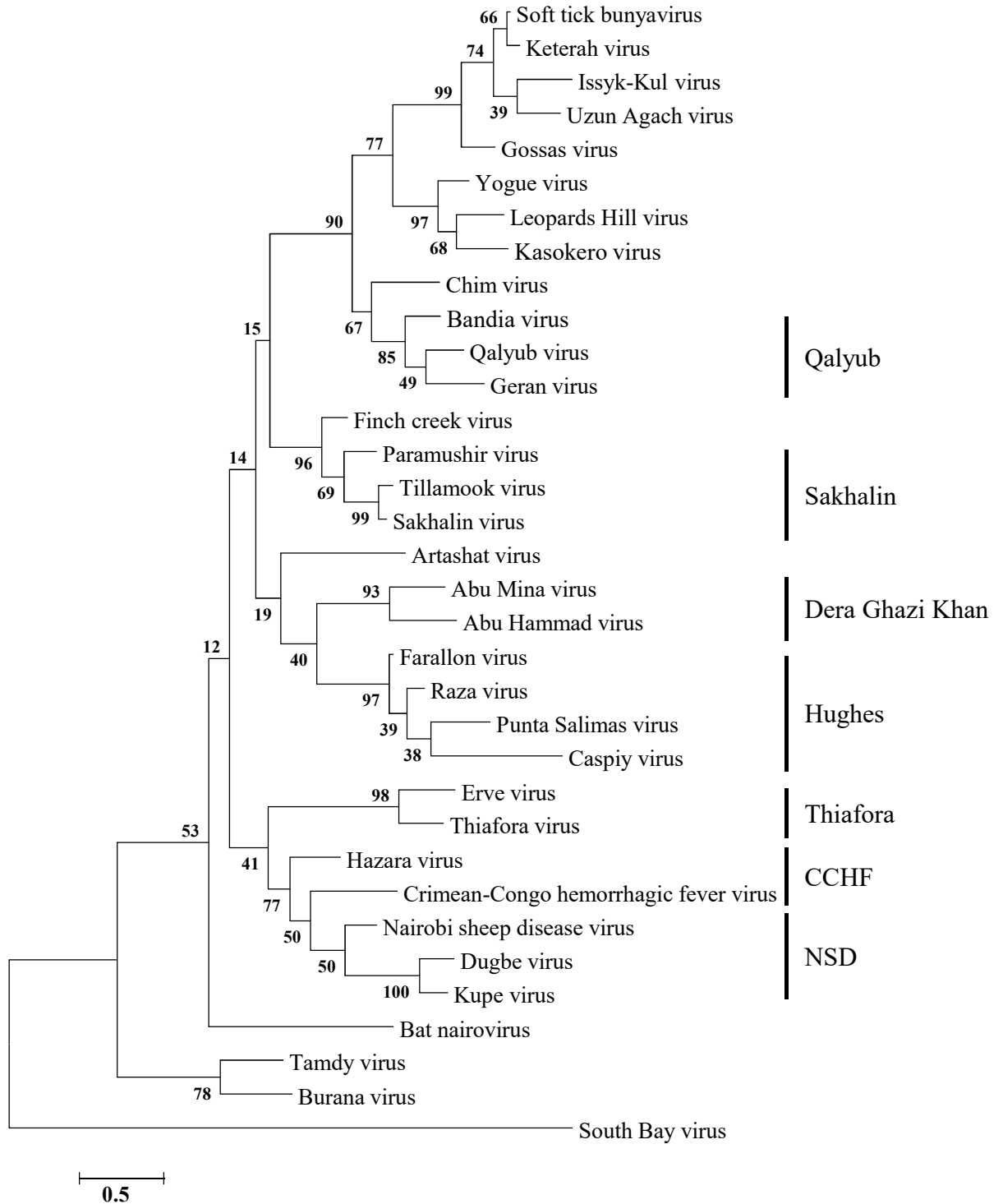
## Other diseases

NSDV causes NSD, a hemorrhagic gastroenteritis in sheep and goats. NSDV is also known as a zoonosis pathogen causing mild influenza-like symptoms in humans. NSDV is found in East and Central Africa and in India, where it is known as Ganjam virus (Marczinke & Nichol, 2002). NSDV transmitted through tick bites and *Rhipicephalus appendiculatus* is the most important vector.

Dugbe virus, which is belonging to NSD serogroup, was once described to relate a human case of thrombocytopenia (Burt *et al.*, 1996). Dugbe virus is isolated from ticks and cattle, and occasionally from humans in Africa.

Erve virus, which is related to Thiafora virus, may cause severe headache and neurological disorders in humans (Dilcher *et al.*, 2012; Treib *et al.*, 1998; Woessner *et al.*, 2000). Erve virus was firstly isolated from a white-toothed shrew collected in France and was detected in France, the Netherlands, Czech Republic, and Germany (Chastel, 1998).

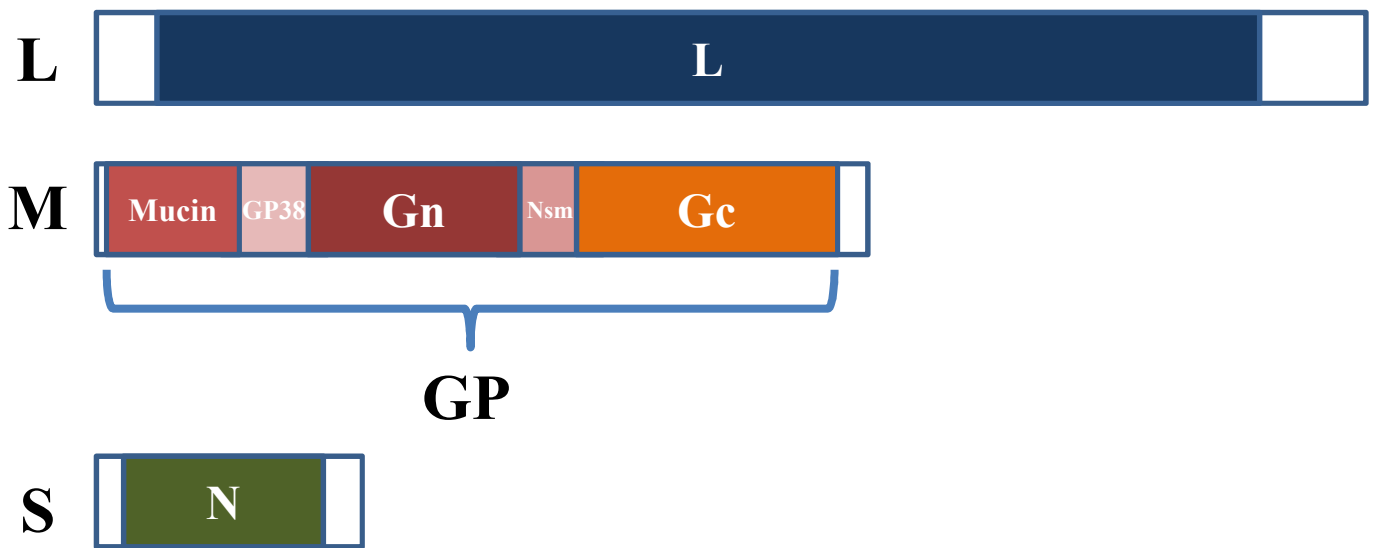
IKV causes fever, headache, dizziness, muscle pain, rash, and nausea in humans (Irina N, 2001; Løvv *et al.*, 1984). IKV was isolated from humans, ticks, bats, and birds in Kyrgyzstan, Tadzhikistan, and Kazakhstan.



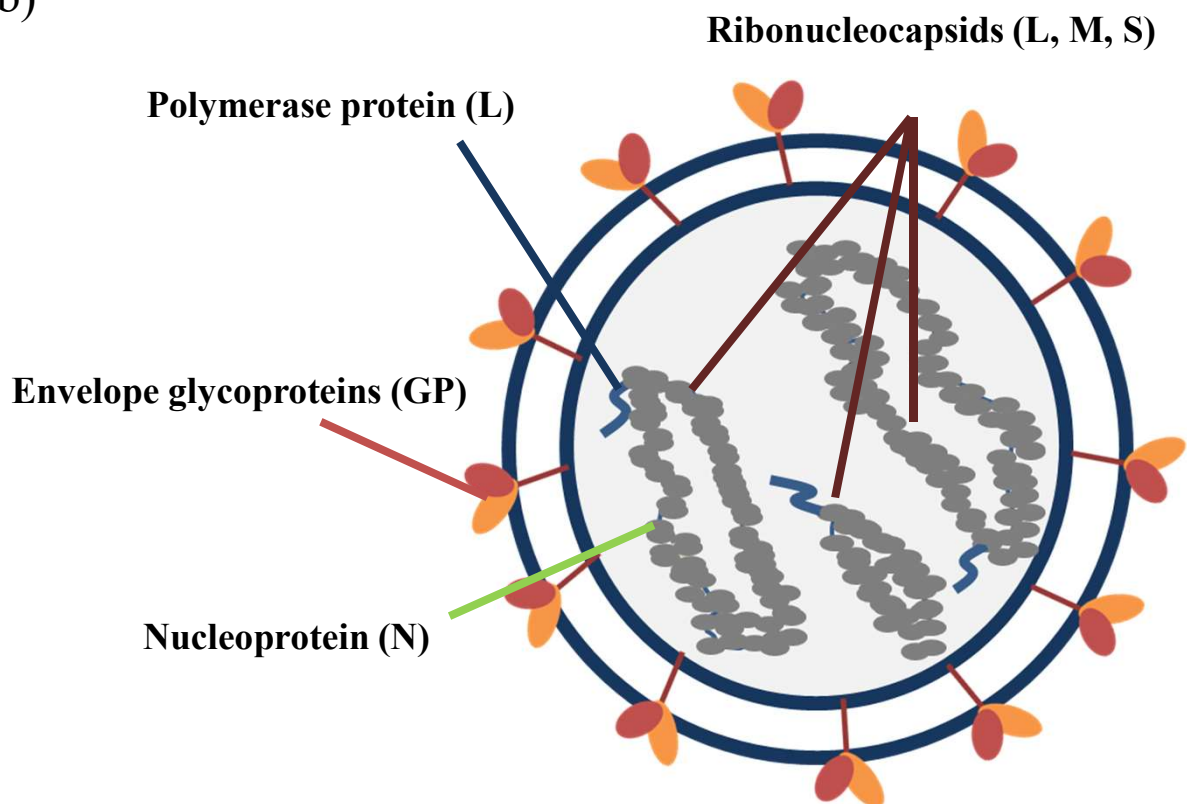
**Fig. I.1** Phylogenetic tree of nairovirus

Nucleotide sequence alignment was created from the available sequence data using ClustalW. The resulting sequence alignment of the conserved region of L segment included 34 taxa and was 358 nucleotides. The phylogenetic tree was constructed using MEGA6 by using Maximum Likelihood method based on the Tamura-Nei model. A discrete Gamma distribution was employed to model evolutionary rate differences among sites (5 categories). The phylogenetic robustness of each node was determined using 1,000 bootstrap replicates. Nairoviruses comprise seven serogroups, CCHF, NSD, Thiafora, Sakhalin, Dera Ghazi Khan, Hughes, and Qalyub group. GenBank accession numbers: Raza virus (AY359529), Farallon virus (AY359523), Erve virus (JF911697), Thiafora virus (KR537450), Artashat virus (KF801650), Tillamook virus (AY359530), Sakhalin virus (KF801659), Paramushir virus (KF801657), Finch creek virus (EU267169), Hazara virus (KP406723), NSDV (EU697951), CCHFV (NC\_005301), Dugbe virus (NC\_004159), Kupe virus (EU257628), Tamdy virus (KF801655), Burana virus (KF801651), Abu Mina virus (AY357716), Abu Hammad virus (AY357715), STBV (LC027465.1), Keterah virus (KR537447), IKV (KR709221), Uzun Agach virus (KJ744032), Gossas virus (KR534878), Chim virus (KF801656), Qalyub virus (AY3589528), Geran virus (KF801649), Bandia virus (AY357717), Leopards Hill virus (AB842088), Kasokero virus (KR537444), Yogue virus (KR537453), Bat nairovirus (KF170224), Punta Salimas virus (AY359527), Caspiy virus (KF801658), and South Bay virus (KM801658).

(a)



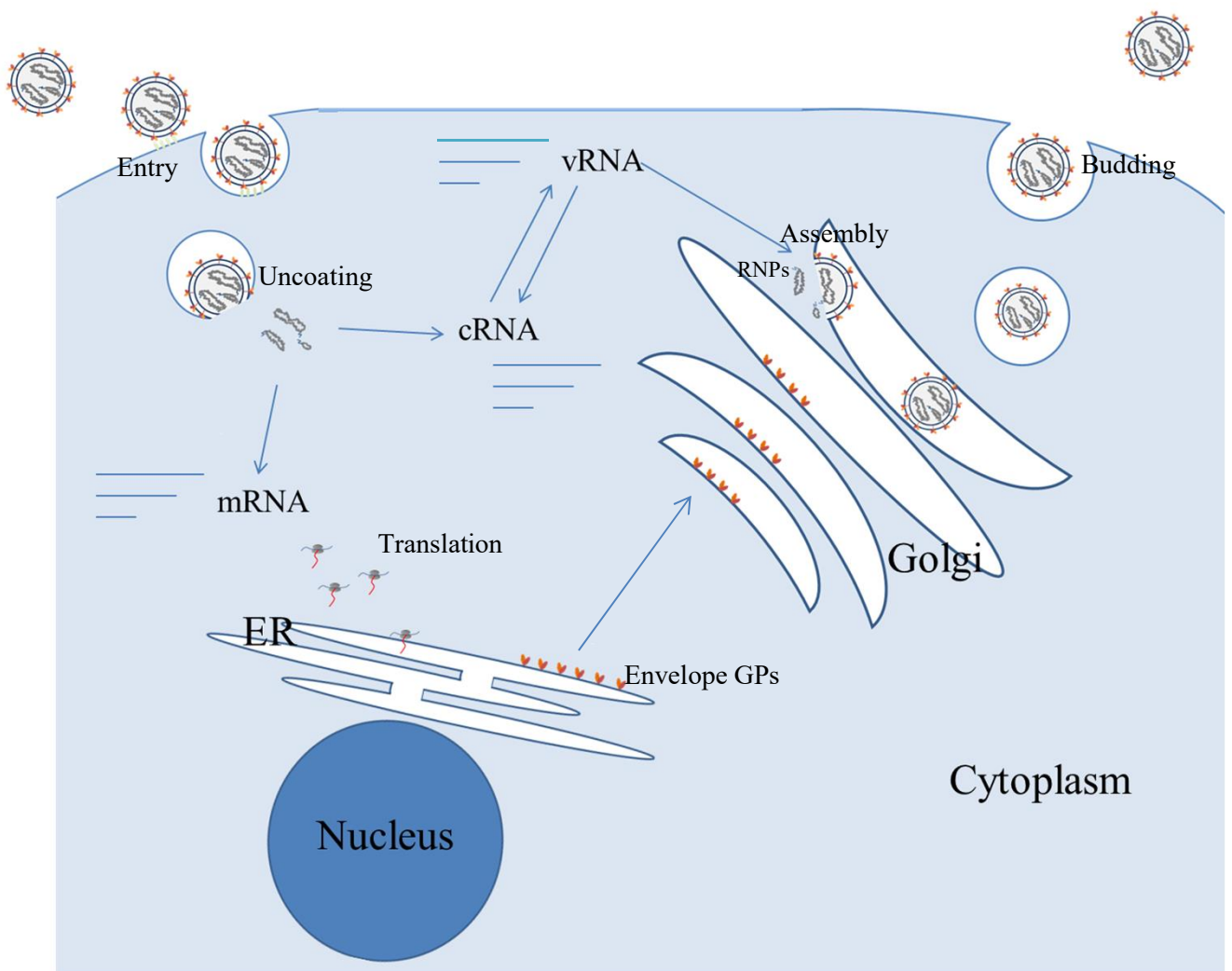
(b)



**Fig. I.2** Schematic structure of CCHFV proteins and CCHFV

(a) The L, M, and S segments encode the RNA dependent RNA polymerase, GP, and N, respectively. (b) The three genome segments make complex with Ns and are encapsidated. RNPs and the L protein are packaged with in the envelope. Gn and Gc, the proteins cleaved from GP, form complexes and are inserted into the envelope.





**Fig. I.3** Replication cycle of nairoviruses

Virus attaches to a cellular receptor(s). The virus is internalized into the cell via endocytosis. The endosome containing virus reaches the low pH environment and viral GP changes their conformation. The conformational change leads the release of the RNPs and the L proteins into cytoplasm. Viral RNA is transcribed into mRNA and viral proteins are synthesized. Viral RNA is transcribed into complementary RNA. Newly synthesized viral RNA is replicated using complementary RNA as the template. GP is synthesized as a single polypeptide, processed in the ER and Golgi into mature protein. Virion assembly occurs at the Golgi. The new virus buds into the Golgi and exits the host cell.

## **Chapter 1**

# **Analysis of Crimean-Congo hemorrhagic fever virus entry mechanism by use of vesicular stomatitis virus-pseudotyping system**

## **Abstract**

CCHF is a fatal tick-borne disease causing severe hemorrhagic symptoms. The experimental use of CCHFV, which causes CCHF, requires high BSL containment. In contrast, pseudotyping of various viral GPs onto VSV can be used in facilities with lower BSL containment and has facilitated studies on the viral entry mechanism and the measurement of neutralizing activities especially for the high pathogenic viruses. In this chapter, the author generated high titers of pseudotyped VSV bearing CCHFV envelope GP and analyzed the mechanisms of CCHFV infection. The partial deletion of the CCHFV GP cytoplasmic domain increased the titer of the pseudotyped VSV, which had similar entry mechanism to authentic virus. With the use of the pseudotype virus, DC-SIGN (a calcium-dependent (C-type) lectin cell surface molecule) was revealed to enhance the viral infection and act as an entry factor for CCHFV.

## Introduction

CCHF is a potentially fatal tick-borne infectious disease. CCHF is caused by infection with CCHFV and experimental use of CCHFV requires BSL-4 containment in most countries. Premature GP is cleaved and modified to generate structural proteins, Gn and Gc, and non-structural proteins, GP38 and NSm (Bergeron *et al.*, 2007; Sanchez *et al.*, 2002; Vincent *et al.*, 2003). As the structural proteins form complexes on the envelope and interact with the host receptors to initiate infection, they are the primary targets of the neutralizing antibodies (Bertolotti-Ciarlet *et al.*, 2005). It was previously reported that the early stage of CCHFV infection is pH- and cholesterol-dependent (Garrison *et al.*, 2013; Simon *et al.*, 2009). Furthermore, it was previously described that Gc cytoplasmic tail of some viruses in *Bunyaviridae* such as Rift Valley fever virus, Uukuniemi virus and Bunyamwera virus was crucial for the trafficking and localization of not only Gc but also Gn (Carnec *et al.*, 2014; Overby *et al.*, 2007; Shi *et al.*, 2007).

Vesicular stomatitis Indiana virus (VSV), a member of the family *Rhabdoviridae*, is a non-segmented, negative-sense, single-stranded RNA virus, which can be handled in facilities with BSL-2 containment. Pseudotyping of viral glycoproteins onto VSV have facilitated studies on viral entry and measurement of virus neutralizing activities in BSL-2 containment (Filone *et al.*, 2006; Fukushi *et al.*, 2005; Ogino *et al.*, 2003; Sayama *et al.*, 2012; Takada *et al.*, 1997; Tamin *et al.*, 2009; Tani *et al.*, 2007, 2014; Taniguchi *et al.*, 2012). As the number of BSL-4 containment is only about 50 in the world, these pseudotype viruses are valuable tool especially for the study and diagnosis for the high pathogenic viruses.

Although nucleolin has been reported to be a candidate receptor for CCHFV (Xiao *et al.*, 2011), the CCHFV entry factors remain unclear. Calcium-dependent (C-type) lectins recognize glycans, which exist as glycoproteins and/or glycolipids, and some lectins such as DC-SIGN, LSECtin, MGL and CLEC5A are known to be entry factors for several viruses, including human immunodeficiency

virus-1 (Geijtenbeek *et al.*, 2000), measles virus (de Witte *et al.*, 2008), dengue virus (Tassaneetrithep *et al.*, 2003), severe acute respiratory syndrome coronavirus (SARS-CoV), filoviruses (Marzi *et al.*, 2004), and Lassa virus (Shimojima *et al.*, 2012). In the family *Bunyaviridae*, it was revealed that some phleboviruses including Rift Valley fever virus, Uukuniemi virus (Lozach *et al.*, 2011), and severe fever with thrombocytopenia syndrome virus (SFTSV) (Hofmann *et al.*, 2013) used DC-SIGN as a receptor, and Uukuniemi virus directly bound DC-SIGN (Lozach *et al.*, 2011). Recognition of glycans on virions by C-type lectins results in the enhancement of viral endocytosis, in which the cellular and viral membranes fuse. CCHFV Gn and Gc have some N-glycosylated sites (Erickson *et al.*, 2007; Sanchez *et al.*, 2002) and likely bind to C-type lectin.

In the present study, in facilities with BSL-2 containment, we generated high titers of pseudotyped VSV bearing CCHFV envelope GP on its surface (CCHFVpv) and analyzed the mechanisms of CCHFV cell entry using the pseudotype virus.

## Materials and Methods

### Cells

293T cells, Vero cells, and VeroE6 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco). Jurkat cells were cultured in RPMI-1640 (Sigma) medium supplemented with 10% (v/v) FBS. Jurkat cells stably expressing with fCD2 CT, DC-SIGN, and LSECtin were prepared as described previously (Shimajima *et al.*, 2012). Briefly, pS-fCD2 CT, pS-DC-SIGN, or pS-LSECtin was mixed with p8.9QV and pCAG-VSV-G, and the mixtures were transfected into 293T cells. After 2 days, the cell culture supernatants containing generated lentiviral vectors were collected. The lentiviral vectors were infected with Jurkat cells and the lectin expression cells were generated.

### Plasmids

cDNAs encoding the open reading frame of CCHFV IbAr 10200 strain GP (NP\_950235) were cloned into pCAGGS using a Rapid DNA ligation Kit (Roche) to generate pC-CCHFV GP. For constructing plasmids coding mutant GPs in which the carboxyl terminal region was partially truncated, stop codons were introduced in pC-CCHFV GP by PCR-based mutagenesis to express the mutant GPs described in Table 1.1. All plasmid constructions were confirmed by sequencing with a DNA sequencer ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

### Production of pseudotyped VSV bearing CCHFV envelope GP

Pseudotyped VSV bearing CCHFV envelope GP was generated as described previously (Takada *et al.*, 1997; Tani *et al.*, 2007). Briefly, HEK293T cells transfected with CCHFV GP plasmids using TransIT-LT1 (Mirus) were infected with VSV G/GFP-\*G or VSV G/Luc-\*G, in which the G gene was replaced with the GFP or the luciferase gene, respectively, at a multiplicity of

infection of 0.1 - 1. The virus was adsorbed for 2 h at 37 °C and then extensively washed out four times with serum-free DMEM. After 24 h of incubation at 37 °C with the culture medium, the culture supernatants were centrifuged to remove cell debris and stored until use at -80°C. To produce a pseudotype virus (with the GFP reporter gene or the luciferase reporter gene) bearing no viral envelope proteins, empty plasmid pCAGGS, or GFP expression plasmid pC-GFP was used. VSVpv/Luc (pseudotyped VSV with the luciferase gene bearing VSV G), MLVpv/Luc (pseudotyped VSV with the luciferase gene bearing murine leukemia virus envelope proteins), Lassapv/GFP (pseudotyped VSV with the GFP gene bearing Lassa virus envelope protein), and EBOVpv/GFP (pseudotyped VSV with the GFP gene bearing Ebola virus envelope protein) were generated as described previously (Tani *et al.*, 2014).

#### Reporter assay

To quantitatively measure the infectivity of CCHFVpv, the reporter activity in inoculated cells was assayed at 1 day post-infection as follows. For the pseudotype virus with the luciferase gene, luciferase activity was measured using a Bright-Glo luciferase assay system (Promega) in accordance with the manufacturer's protocol with GloMax (Promega). For the pseudotype virus with the GFP gene, the supernatant was replaced with phosphate-buffered saline (PBS) and GFP-positive cells were counted under a fluorescence microscope (BZ-X710, KEYENCE). The infectious units (IU) were determined as the number of the GFP-positive cells.

#### Effects of ammonium chloride (NH<sub>4</sub>Cl) and methyl- $\beta$ -cyclodextrin (M $\beta$ CD) on the infectivity

Cells were treated with the indicated concentrations of NH<sub>4</sub>Cl for 60 min or M $\beta$ CD for 30 min at 37°C and then inoculated with the pseudotype viruses in DMEM supplemented with 2% (v/v) FBS. Infectivity was determined according to the above described method. For refilling, cells were

treated with 200 $\mu$ M exogenous cholesterol for 30 min after cholesterol removal.

#### Serum samples

The CCHF patient serum sample was obtained from the Center for Disease Control and Prevention (Atlanta, Georgia, USA). Two serum samples, which were collected from healthy Japanese adults, were used as controls. The usage of the sera was approved by the research and ethics committee of the National Institute of Infectious Diseases, Tokyo, Japan (Reference no. 439).

#### Neutralization assay

CCHFVpv was incubated with diluted human serum samples for 30 min at 37 °C and inoculated onto VeroE6 cells. At 1 day after inoculation, CCHFVpv infectivity was measured as described above.

#### Flow cytometry

Antibody staining and analyses were performed as described previously (Shimajima *et al.*, 2006, 2012). Briefly, Jurkat cells expressing lectins were incubated in ice-cold PBS containing 2% FBS, 0.02% sodium azide, and the antibodies (FACS buffer). After incubation for 30 min at 4°C, the cells were washed twice with FACS buffer and recovered in FACS buffer containing fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG antibody (Invitrogen). Cells were incubated for 30 min at 4°C and washed twice with FACS buffer. The cells were analyzed with a FACSCalibur (Becton Dickinson) and FlowJo 7.6.5 software (FlowJo, LCC). The antibodies used to detect the lectins were as follows; normal mouse IgG antibody (Mouse IgG Isotype Control) (R&D systems), anti-fCD2 antibody (Shimajima *et al.*, 2002), anti-DC-SIGN antibody (Clone DC28, R&D systems), and anti-LSEctin antibody (SOTO-1, Santa Cruz Biotechnology, Inc.).



### Statistical analysis

The differences in the infectivity were compared by Student's  $t$ -test.

## Results

### GP carboxyl terminal regions affected titers of the pseudotype virus

To generate pseudotyped VSV bearing CCHFV envelope GP, we firstly used the full-length GP as an envelope protein and VSV G/GFP-\*G, the reporter gene of which is GFP, as a seed virus. VeroE6 cells were inoculated with the resultant pseudotype virus and the number of cells with GFP was counted. The titer of the pseudotype virus with full-length GP was 200IU/ml (Table 1.1). Next, we examined the effects of deletion within the carboxyl terminal of GP on titers of the pseudotype virus because deletion of Gc cytoplasmic tail might change the trafficking of the envelope protein and it could affect the pseudotype virus production. Furthermore, it was previously reported that a pseudotyped VSV bearing the envelope protein of SARS-CoV with a truncation in the cytoplasmic domain was more efficiently incorporated in the viral particle than the full-length protein (Fukushi *et al.*, 2005). We used four mutant GPs (Table 1.1) as envelope proteins to generate the pseudotype virus. All of the cytoplasmic region-deleted GPs that we examined produced higher titers of the pseudotype virus than the full-length GP (Table 1.1). The highest titer, which was approximately 30 times that of the full-length GP, was obtained with the GP del10 mutant. The pseudotype virus with GP del10 was used for the subsequent experiments and named CCHFVpv. Consistent results were obtained when luciferase reporter was used instead of GFP reporter (Fig. 1.1). The luciferase activity obtained with CCHFVpv/Luc was approximately 30 times higher than in the cells incubated with the non-envelope pseudotype virus.

### Characteristics of CCHFVpv infection

We examined whether the CCHFVpv infection had the characteristics that were recognized in authentic CCHFV. Authentic CCHFV infection has been reported to be pH- and cholesterol-dependent (Garrison *et al.*, 2013; Simon *et al.*, 2009). First, we examined the effects of

NH<sub>4</sub>Cl, which increases pH of intracellular compartments, on CCHFVpv infection. As shown in Fig. 1.2, treatment with NH<sub>4</sub>Cl at each concentration, 12.5 mM and 25 mM, decreased CCHFVpv/Luc infection in a dose-dependent manner. NH<sub>4</sub>Cl treatment had a similar effect on the infection with VSVpv/Luc which requires a low pH step for the entry (Carneiro *et al.*, 2001; Garrison *et al.*, 2013). In contrast, the treatment did not affect the infectivity of MLVpv/Luc, which is independent of pH for the entry (Tani *et al.*, 2014). This indicated that CCHFVpv infection was pH-dependent. Next, the effects of M CD, which removes cholesterol from the cell membranes, were investigated. As shown in Fig. 1.3a, treatment with M CD at each concentration, 2.5 mM and 5 mM, decreased CCHFVpv/Luc infection in a dose-dependent manner. As shown in Fig. 1.3b, the M CD treatment decreased CCHFVpv/Luc infection, and the inhibitory effects were reduced when cells were refilled with exogenous cholesterol after the removal of cholesterol. This indicated CCHFVpv infection was cholesterol-dependent. Furthermore, to confirm dependency of CCHFVpv infection on CCHFV envelope GP, we used sera obtained from healthy donors and a CCHF patient in the convalescent phase. As shown in Fig. 1.4, the CCHF patient serum inhibited CCHFVpv/Luc, but not VSVpv/Luc infection. In contrast, the control sera from two healthy subjects did not affect the infection with either of the two pseudotype viruses. These results indicated that CCHFVpv infection was dependent on CCHFV envelope GP. These results show that the CCHFVpv has the characteristics of the entry mechanisms which are recognized in authentic CCHFV. It is therefore likely to be useful for investigating the entry mechanism of CCHFV.

#### Cell surface molecules involving CCHFV infection

Because the CCHFVpv titer measured in Jurkat cells was found to be quite low (5 IU/mL) in comparison to that in VeroE6 cells (6,250 IU/mL) (Table 1.1), Jurkat cells were used to investigate involvement of C-type lectins, cell surface molecules which have been well-investigated in studies

of various viral entry mechanisms and enhance the entry of Ebola virus and Lassa virus entry into cells (Shimojima *et al.*, 2006, 2012), in CCHFVpv. Expression of control molecule (fCD2ΔCT) and C-type lectins were confirmed by flow cytometry (Fig. 1.5a). As shown in Fig. 1.5b, CCHFVpv/GFP and Lassapv/GFP infections in Jurkat cells were enhanced by the expression of DC-SIGN. Whereas LSEctin expression enhanced Lassapv/GFP and EBOVpv/GFP infections (Fig. 1.5b), the lectin did not affect the CCHFVpv/GFP infection. The results showed that DC-SIGN, but not LSEctin, was related to CCHFVpv infection. It was previously reported that the binding of Lassapv to DC-SIGN, which mainly recognizes high-mannose-type glycans, was blocked by mannan, a polymer of mannose (Shimojima *et al.*, 2012). CCHFVpv/GFP infection in DC-SIGN-expressing Jurkat cells was inhibited by pretreatment with mannan, but not by GlcNAc 1-2Man, one of the high affinity ligands of LSEctin (Powlesland *et al.*, 2008; Shimojima *et al.*, 2012) (Fig. 1.5c). Furthermore, CCHFVpv/GFP infection in the cells was inhibited also by pretreatment with the antibody to DC-SIGN (Fig. 1.5d). These results show that CCHFV may preferentially infect cells that express DC-SIGN as a result of binding between carbohydrates on envelope GP and DC-SIGN.

## Discussion

A quantitative measurement of infection of a pseudotyped VSV, in which the viral envelope protein has been replaced with that of a non-VSV, is highly sensitive, rapid, and easy. We herein report the creation of a VSV pseudotype bearing the envelope protein of CCHFV, which requires high BSL containment for experimental use. Although pseudotyped VSV bearing full-length CCHFV envelope GP has recently been reported (Shtanko *et al.*, 2014), the intensity of luminescence (the expression of the reporter used in the experiment) in infected cells was only 8 times higher than background. However, the pseudotype virus with much higher titer would be needed to be used for a more certain study or diagnosis. We revealed that the use of CCHFV GP with a truncated carboxyl terminal resulted in a higher titer of pseudotyped VSV (30 times higher than background) (Fig. 1.1). Although the precise reason(s) why the truncation of the carboxyl terminal of CCHFV GP produced high viral titers is unclear, the altered localization of Gn and Gc and/or proper assembly with the VSV proteins may have been involved. Nonetheless, because of its high titer and characteristics that were recognized in authentic CCHFV in terms of the pH- and cholesterol-dependency of infection (Table 1.1, Figs. 1.2 and 1.3), the pseudotyped VSV with truncated CCHFV GP (e.g. del10 mutant in Table 1.1), is useful in studies on the entry mechanisms of CCHFV and sero-surveillance experiments, such as the measurement of neutralizing activities. As CCHFV requires high biosafety containment, this pseudotype virus will be powerful tool to study CCHFV.

Owing to the fact that the CCHFV envelope protein was a glycoprotein (Erickson *et al.*, 2007; Sanchez *et al.*, 2002) and some phleboviruses such as Rift Valley fever virus, Uukuniemi virus (Lozach *et al.*, 2011), and SFTSV (Hofmann *et al.*, 2013) used a C-type lectin, DC-SIGN, as a receptor, we expected C-type lectins to be receptors for CCHFV. In this study, we revealed that CCHFVpv efficiently infected DC-SIGN-expressing cells while we examined only two C-type

lectins (Fig. 1.5), indicating that DC-SIGN is a possible novel entry factor in CCHFV. The result suggests that the role of DC-SIGN in cell entry is conserved among the *Bunyaviridae* family. CCHFV initially replicates in dendritic cells and tissue resident macrophages, then the virus migrates to a broad range of tissues and organs including regional lymph nodes, spleen, and liver (Ak,nc, *et al.*, 2013; Burt *et al.*, 1997; Connolly-Andersen *et al.*, 2009). As DC-SIGN presents on the surface of dendritic cells (Geijtenbeek *et al.*, 2000, 2002) and macrophages (Zhang *et al.*, 2014), the result in this study agreed with the viral tropism and suggests that DC-SIGN has important role in the infection with dendritic cells and macrophages. However, as the inhibitory effect by mannan or anti-DC-SIGN antibody was only approximately 50% (Figs. 1.5c, d), the mechanism of the enhanced infection of CCHFVpv by DC-SIGN could not be specific. It was considered to be because a part of the mechanism of the enhancement was a binding via other than the carbohydrate recognition domain of DC-SIGN.

The effects of two C-type lectins on CCHFVpv infection differed to the effects on EBOVpv and Lassapv infection (Fig. 1.5b). CCHFVpv infection was enhanced by DC-SIGN but not by LSEctin. In contrast, Lassapv infection was enhanced by both lectins and EBOVpv infection was enhanced to a much greater extent by LSEctin than by DC-SIGN. Such different features suggest that the glycosylation status of CCHFV envelope GP is different to that in the Ebola and Lassa virus GPs. However, there are several possibilities for the reason why LSEctin expression does not enhance CCHFVpv infection. For example, the molecule does not bind CCHFVpv particle, resulting in no involvement (no enhancement) of the viral infection. Second possibility is that the molecule binds CCHFVpv particle but the binding does not result in enhancement of infection due to inadequate interaction with a putative (co-)factor(s) necessary for infection. Third possibility is that the binding of CCHFVpv particle with LSEctin enhances transportation to a degradation pathway(s).

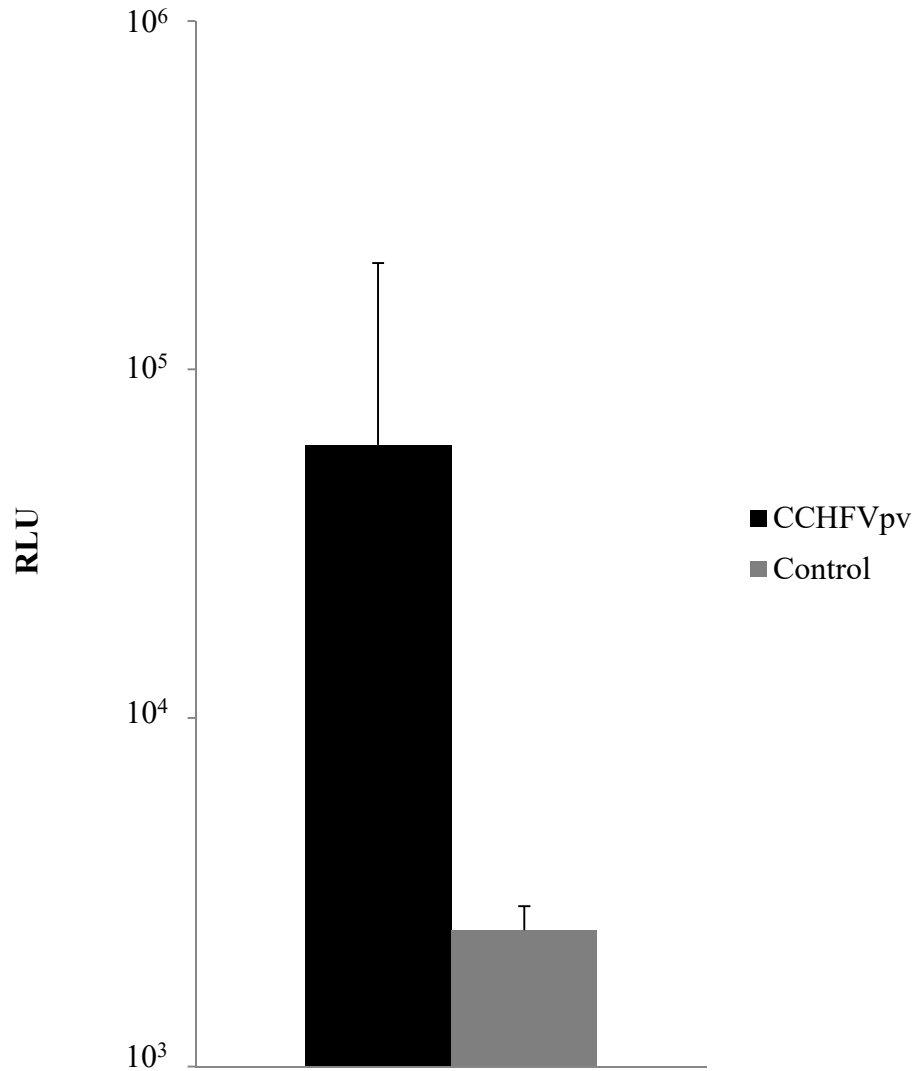
There is a possibility that other molecules which have not yet been examined may enhance CCHFV infection more than DC-SIGN. To reveal entry mechanism of CCHFV more precisely, further experiments including examination of other C-type lectins (such as mannose receptor, L-SIGN, or Langerin, etc.), identification of yet unknown receptor(s), and binding and internalization assays will be needed. In addition, candidate molecules/pathways which will be found to involve CCHFVpv entry into cells should be addressed with wild CCHFV because it is unclear whether entry mechanisms of CCHFVpv and CCHFV are completely identical. Nevertheless, this pseudotype virus showed novel insight of CCHFV infection mechanism and could develop the CCHFV study as a surrogate model.

**Table**

**Table 1.1.** CCHFV GP variants and pseudotyped VSV titers

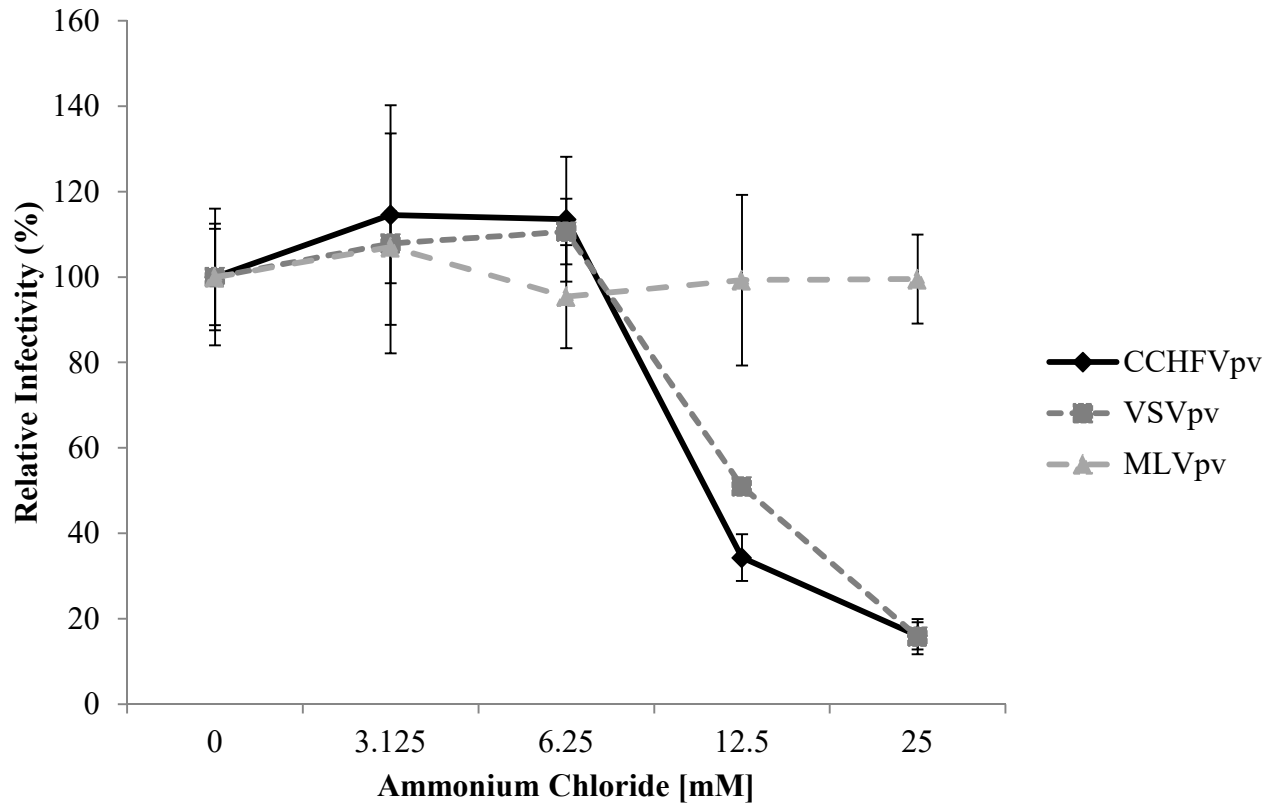
<b>Variants</b>	<b>The amino acid sequences of each variant cytoplasmic region</b>	<b>Titer (IU/mL)</b>
Full-length	<b>CFKCCRRTRGFLFKYRHLKDDEETGYRRIIEKLNNKKGKNK LLDGERLADRRRIAELFSTKTHIG</b>	200
del4	<b>CFKCCRRTRGFLFKYRHLKDDEETGYRRIIEKLNNKKGKNK LLDG</b>	1,150
del9	<b>CFKCCRRTRGFLFKYRHLKDDEETG</b>	1,700
del10	<b>CFKCCRRTRG</b>	6,250
del14	<b>C</b>	1,225





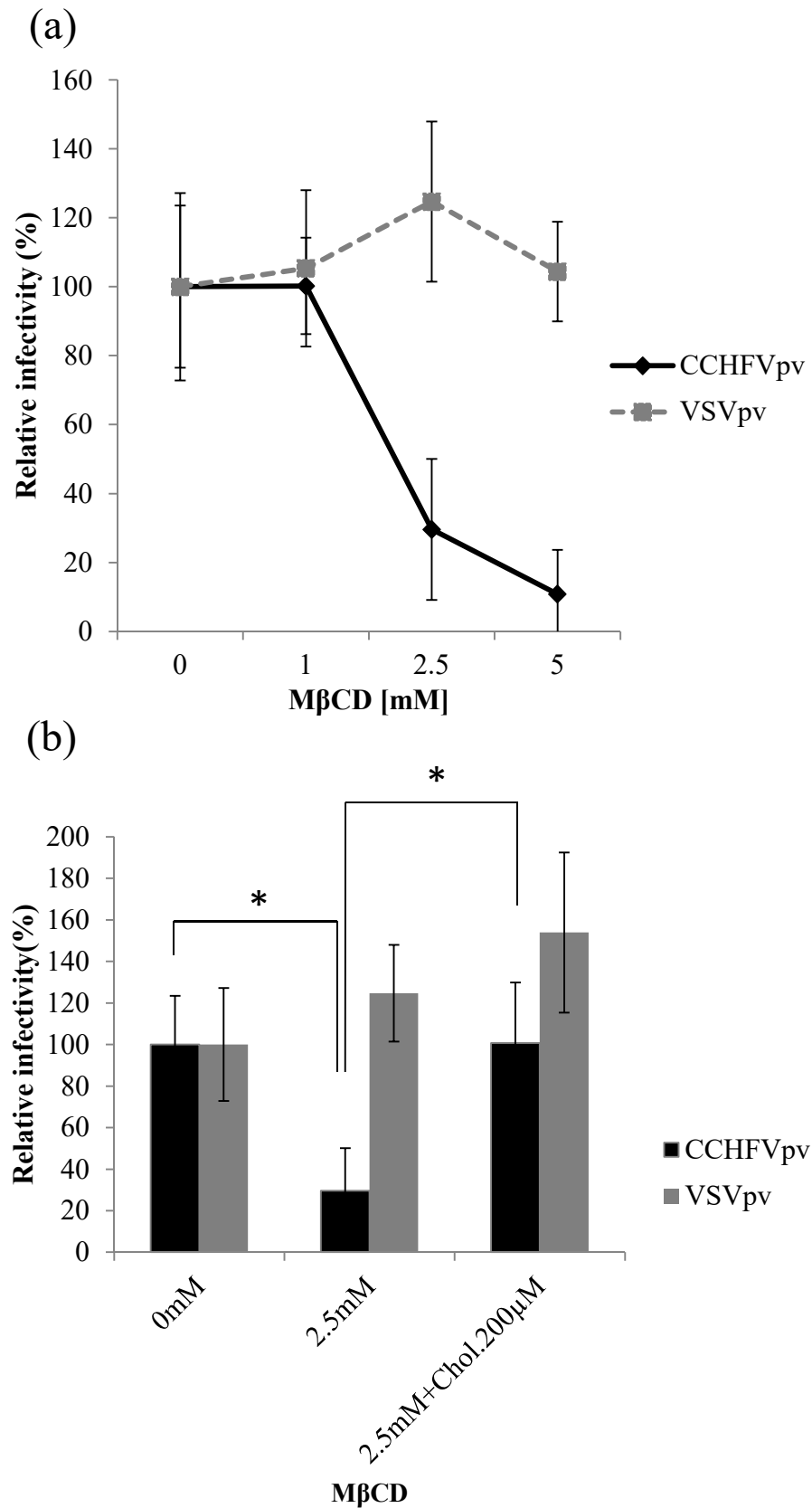
**Fig. 1.1** The CCHFVpv/Luc titer

To generate a pseudotype virus, HEK293T cells were transfected with pC-CCHFV GP del10 or pC-GFP as a control. After overnight incubation, the cells were infected with VSV $\Delta$ G/Luc-\*G. The collected supernatants were incubated with Vero cells to determine the titer of the pseudotype virus. Luciferase activity was measured as described in Materials and Methods at post-infection 1 day. The tests were performed in triplicate and means  $\pm$  standard deviations are shown. RLU, relative luciferase unit. Control indicates the titer of the pseudotype virus which was generated using pC-GFP.



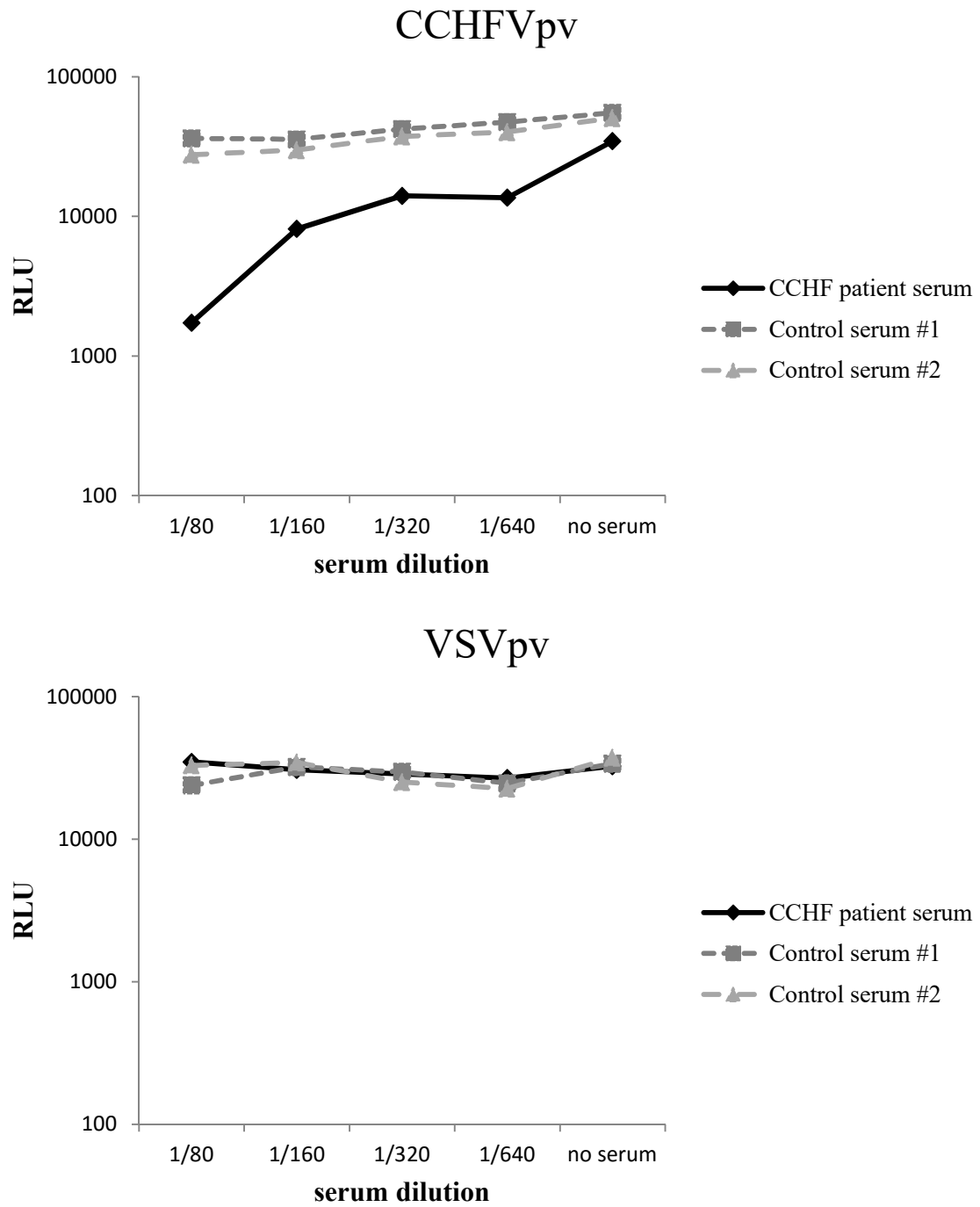
**Fig. 1.2** pH-dependency of CCHFVpv entry

VeroE6 cells were pretreated with  $\text{NH}_4\text{Cl}$  at the variously designated concentrations for 60 min before infection with CCHFVpv/Luc, VSVpv/Luc, or MLVpv/Luc. After 1 day, luciferase activity was measured as described in the Materials and Methods. The tests were performed in triplicate and means  $\pm$  standard deviations are shown.



**Fig. 1.3** Cholesterol-dependency of CCHFVpv entry

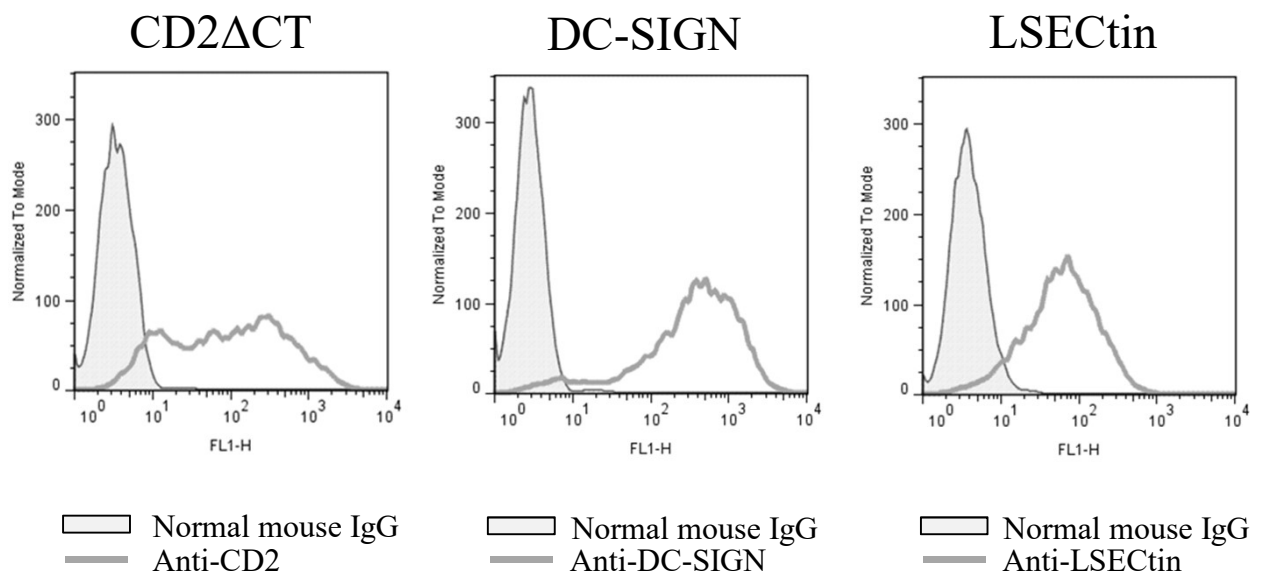
(a) VeroE6 cells were pretreated with variously designated concentrations of MβCD for 30 min before infection with VSVpv/Luc or CCHFVpv/Luc. After 1 day, luciferase activity was measured as described in Materials and Methods. (b) VeroE6 cells were depleted of cholesterol and infected with pseudotype viruses or depleted and refilled with cholesterol before infection in the presence of MβCD or cholesterol. The tests were performed in triplicate and means  $\pm$  standard deviations are shown. \*, indicates statistical significance ( $P < 0.05$ ).



**Fig. 1.4** Neutralization of CCHFVpv and VSVpv with CCHF patient's serum

A neutralization assay was performed by incubating VSVpv/Luc or CCHFVpv/Luc with the CCHF patient's serum or the sera collected from two healthy Japanese CCHFV antibody negative adults at the designated dilutions for 30 min. The mixtures were added to VeroE6 cells and luciferase activity was measured at 1 day post-infection. The tests were performed in duplicate and means are shown. RLU, relative luciferase unit.

(a)



**Fig. 1.5** Role of C-type lectin as an entry factor for CCHFVpv

(a) The expression of fCD2 $\Delta$ CT and C-type lectins was confirmed by flow cytometry.

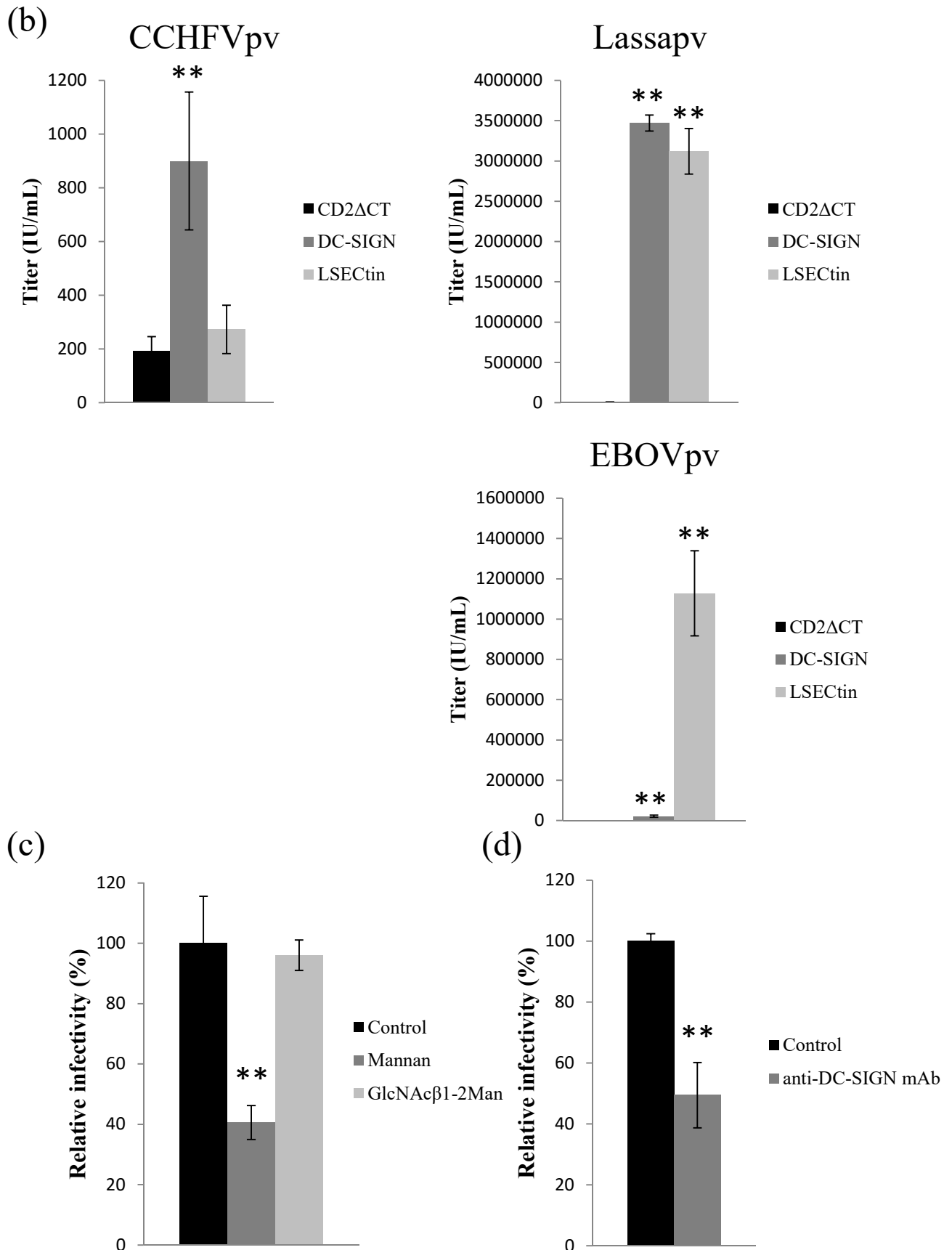


Fig. 1.5 Continued.

(b) Jurkat cells expressing fCD2ΔCT, DC-SIGN, or LSECtin were infected with CCHFVpv/GFP, Lassapv/GFP or EBOVpv/GFP. After overnight incubation, the number of GFP fluorescent cells was counted. (c) Jurkat cells expressing DC-SIGN were treated with mannose or GlcNAcβ1-2Man and then infected with CCHFVpv/GFP. After overnight incubation, the number of GFP fluorescent cells was counted. (d) Jurkat cells expressing DC-SIGN were pretreated with anti-DC-SIGN antibody (MAB161, R&D systems) or normal mouse IgG as a control and then infected with CCHFVpv/GFP. After overnight incubation, the number of GFP-positive cells was counted. The tests were performed in triplicate and means  $\pm$  standard deviations are shown. \*\*, indicates statistical significance ( $P < 0.01$ ) when compared with data from fCD2ΔCT (b) or Control (c), (d).

## **Chapter 2**

# **Development of a novel diagnostic assay using pseudotyped vesicular stomatitis virus for detection of neutralizing antibody to Crimean-Congo hemorrhagic fever virus**

## **Abstract**

CCHF is caused by CCHFV, which needs high BSL containments for experimental working. Serodiagnosis of CCHF is usually performed by ELISA and IFA. However, the cross-reactivity with the related viruses had been observed. Pseudotyping of various viral GPs onto VSV can be used in lower BSL containments and have facilitated studies on measurement of neutralizing antibodies to the viruses. In this chapter, the author established a novel neutralization assay using pseudotype virus bearing CCHFV envelope GP (CCHFVpv) developed in Chapter 1 for diagnosis of CCHFV infection. The serum samples containing CCHFV antibody were detected with high specificity in the neutralization assay using CCHFVpv. Moreover, the immunoreactivity in neutralization assays using CCHFVpv and CCHFV were similar. Thus, CCHFVpv can be used as a safe alternative tool in CCHF diagnosis.



## Introduction

CCHF is the most spreading notable tick-borne disease reported in over 30 countries throughout parts of Africa, Eastern Europe, and Asia. In Xinjiang Uygur Autonomous Region in China, sporadic outbreaks of CCHF have been reported (Saijo, 2007).

For detection of immunoglobulin G (IgG) antibodies to CCHFV, an ELISA using recombinant N (rN) is commonly used (Burt *et al.*, 1993; Dowall *et al.*, 2012a; Garcia *et al.*, 2006; Qing *et al.*, 2003; Saijo *et al.*, 2002a, 2005a, b; Samudzi *et al.*, 2012). In addition, IFA using rN (Saijo *et al.*, 2002a; Vanhomwegen *et al.*, 2012) and a commercial IFA Kit (Euroimmun, Luebeck, Germany) are also available to detect anti-CCHFV antibodies. However, NSD serogroup including NSDV and Dugbe virus, both of which cause disease in human and exist in Africa, where CCHF occurs (Burt *et al.*, 1996) shows cross-reactivities with CCHFV in ELISA, IFA, and indirect hemagglutination-inhibition (Casals & Tignor, 1980; Ward *et al.*, 1992). Moreover, the pathogenicity of HAZV, another CCHF serogroup member in Pakistan, is unclear. Therefore, it is needed to distinguish between CCHFV and HAZV. For precise diagnosis of CCHF and epidemiological studies of CCHFV infection, a neutralization assay should be done because the assay has high specificity (Casals & Tignor, 1980). Furthermore, the neutralization assay for CCHFV requires live virus, which needs BSL-4 containment in most countries for experimental working. Thus, in countries without BSL-4 laboratories, the neutralization assay for precise diagnosis/epidemiological study is impossible to be performed.

The author generated pseudotype virus bearing CCHFV envelope GP (CCHFVpv) in Chapter 1. The pseudotyped VSV is comparatively safe because of its inability to produce infectious progeny virus due to the lack of the envelope protein gene. Moreover, the infection of the pseudotyped VSV can be detected within 24 hrs because expression of reporter protein occurs within several hours after inoculation. Therefore, by using the pseudotyped VSV, it is possible to perform safe, rapid

neutralization assay in BSL-2 containments. In this study, the author reported a novel neutralization assay system using the pseudotyped VSV for diagnosis and/or epidemiological studies of CCHF.

## Materials and Methods

### Cells

Huh7 cells and Vero cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco).

### Production of CCHFV<sub>pv</sub>

CCHFV<sub>pv</sub> was generated as described in Chapter 1.

### Reporter Assay

Reporter assay for CCHFV<sub>pv</sub> was performed as described in Chapter 1.

### Serum samples

65 serum samples from the patients and the residents in the Xinjiang Uygur Autonomous Region in China were collected during an outbreak of the disease in 2001 (Saijo *et al.*, 2005b). The sera had been tested by the rN-based IgG ELISA and 22 samples were determined as antibody-positive. 6 serum samples from patients in Tajikistan (Tajik1, Tajik5, Tajik8, Tasjik10, Tajik11, and Tajik12) (Atkinson *et al.*, 2013), a serum from the convalescent patient of CCHF imported into United Kingdom (Conv22/7/14) (Lumley *et al.*, 2014), and a negative serum (SIGMA) as a negative control were used. The usage of the sera was approved by the research and ethics committee of the National Institute of Infectious Diseases, Tokyo, Japan (Reference no. 439).

### Statistical method

Receiver operating characteristics (ROC) curves and Spearman's rank Correlation Coefficient were analyzed using the statistical software R version 3.0.1.

#### Neutralization assay using CCHFVpv

CCHFVpv was incubated with diluted human serum samples for 30 min at 37°C and inoculated onto Huh7 cells. After 1 day of inoculation, infectivity of CCHFVpv was measured as described above. Relative infectivity (%) was calculated as follows: (luciferase activity in the presence of serum)/(luciferase activity in the absence of serum) x 100 for CCHFVpv/Luc or (the number of GFP-positive cells in the presence of serum)/(the number of GFP-positive cells in the absence of serum) x 100 for CCHFVpv/GFP. Reciprocal of the highest serum dilution which showed positive neutralization activities were used as neutralization antibody titers.

#### Neutralization assay using CCHFV

Neutralization assay using CCHFV was kindly performed by Drs. Stuart Dowall and Roger Hewson (Microbiology Services Division, Public Health England (PHE), United Kingdom) within the Containment Level 4 laboratory at PHE Porton. The diluted sera and virus (IbAr 10200 strain) mixture was incubated for 1 h at 37°C. Following this, the mixture was inoculated onto Vero cells. After 1 day of inoculation, for staining foci of infected cells, the cells were fixed in cold 1:1 acetone:methanol solution. Following this, the cells were treated with a sheep polyclonal antibody raised against Hazara N (kindly provided by University of Leeds) which cross-reacts with CCHFV N. Unbound antibody was removed and an anti-sheep IgG-FITC antibody (Vector Laboratories) was added. Unbound conjugated antibody was removed by further washing with PBS before cells were visualized using a fluorescence microscope. Foci of infected cells were quantified and focus-forming unit (FFU) ratios were calculated as follows: (the number of focus in the presence of serum)/ (the number of focus in the absence of serum) x 100.

## Results

### Serosurveillance by neutralization assay with CCHFVpv

The human sera collected at a CCHF-endemic area in China (the final dilution was 1:20) were tested in the neutralization assay using CCHFVpv/Luc (Fig. 2.1a). Compared with the results of rN-based ELISA (Saijo *et al.*, 2005b), the cut-off value was determined as 39.260 using ROC analysis (Fig. 2.1b). When relative infectivity was less than 39.260, the cut-off value, diluted serum was regarded as positive for neutralization activity. As a result, 13 samples (sample IDs: #2, #3, #4, #7, #10, #17, #24, #33, #39, #40, #43, #57, and #65) were determined as antibody-positive. The results were compared with that of rN-based ELISA (Saijo *et al.*, 2005b) (Table 2.1). The sensitivity of the neutralization assay was 59.1% and the specificity was 100% compared with the ELISA.

### Titration of the positive serum

Next, the author used those positive samples and titrated the neutralizing antibodies. The sera were serially diluted as indicated and used for the neutralization assay. The sera inhibited the viral infection as dose-dependent manner (Fig. 2.2). The antibody titers were determined using the cut-off value as described above. The determined antibody titers were shown in Table 2.2.

### Correlation between the neutralization titer and the O.D. value of the ELISA

The correlation between the neutralization titers and the O.D. values of the ELISA (serum dilution: 1:400) (Saijo *et al.*, 2005b) of 22 positive sera for the ELISA was shown in Fig. 2.3. The samples with higher O.D. values of the ELISA did not have always higher titers of neutralization assay. The Spearman's rank Correlation Coefficient was 0.38, which means weak correlation between the two values.

#### Neutralization assay using samples collected from the same patient

Samples collected from the same patient on days 1, 5, and 9 were tested in the neutralization assay using CCHFVpv/Luc (Fig. 2.4). The titers of each serum were <40, <40, and 80, respectively. The titer seemed to increase along with the days of onset.

#### Correlation between the neutralization titers using CCHFVpv and CCHFV

It was suggested that CCHFVpv can be a surrogate model for the neutralization assay. However, the sensitivity of the neutralization assay using the pseudotype virus was lower than that of the rN-based IgG ELISA (Table 2.1). This difference of the sensitivity could be due to the assay systems. Therefore, it remained to be revealed whether the titers using CCHFVpv were correlated to those using authentic virus. The sera collected in Tajikistan were performed in the neutralization assays using CCHFVpv and CCHFV (Fig. 2.5). The titers of the neutralization assay using CCHFVpv and CCHFV were shown in Table 2.3. The titers were similar between using CCHFVpv and CCHFV. This result suggests the titers of CCHFVpv were correlated to those of authentic virus. The O.D. values of ELISA seemed to be little correlated with the neutralization antibody titers (Table 2.3). These results suggested that the neutralization assay using CCHFVpv detected the neutralizing antibody properly.

## Discussion

The immunoreactivities of CCHFVpv and CCHFV were similar in the neutralization assay (Fig. 2.5, Table 2.3). This suggested that CCHFVpv can be utilized for the neutralization assay. The serum samples with CCHFV antibody were detected with high specificity in the neutralization assay using CCHFVpv because the specificity was 100% (Table 2.1). However, the sensitivity of the neutralization assay using the pseudotype virus was lower than that of the rN-based IgG ELISA (Table 2.1). Moreover, there was only weak correlation between the titers of the neutralizing antibody and the O.D. values of the ELISA (Fig. 2.3). This difference of the sensitivity between the neutralization assay and ELISA could be due to the antigens. It was previously reported that the reactivity of immunity in patients was different according to the antigens and neutralizing antibodies were produced later than anti-N antibodies (Shepherd *et al.*, 1989b). It was previously reported that the neutralizing antibody was developed in the second to third weeks after the disease onset though the antibody to N was developed in the first week after onset of the illness (Shepherd *et al.*, 1989b). In this study, the antibody titers of the sera from the same patient showed that the neutralizing antibody was not detected by day 5 (Fig. 2.4). However, rN-based IgG ELISA detected the antibody in the serum collected at days 5 and 9 (Saijo *et al.*, 2005b). This result was consistent with the previous report (Shepherd *et al.*, 1989b). Therefore, one possible reason of the difference of the sensitivity could be affected by the collected time of the serum from the infection. One more possible reason was that the antibody responses to the antigens were various among the patients. In bunyaviruses, although the antibodies against N protein were highly detected, the immune response against GP did not seem to be homogenous among the patients (Besselaar *et al.*, 1991; Di Bonito *et al.*, 2002). Most CCHF patients developed relatively low levels of neutralizing antibodies, but some developed high titer and maintained for several months (Shepherd *et al.*, 1989b). Moreover, in one fatal CCHF case, it was reported that anti-N antibody was lacking though anti-GP antibody was

detected in IFA (Ergunay *et al.*, 2014). Therefore, it is important to detect the antibodies to several antigens for precise diagnosis of CCHF although the N antigen seems to be useful for early and rapid diagnosis because of the high sensitivity. This neutralization assay could be valuable because this assay targets the envelope protein, the different antigen from that of rN-based IgG ELISA. Furthermore, neutralizing antibody reaction is one of the decisive factors to distinguish viruses. Although further study will be needed to see whether CCHFVpv is not neutralized by the antibody to other viruses, these results suggest that rN-based ELISA is useful for first screening and the neutralization assay reported in this study could be the advantage of distinction between CCHF and infection of viruses of NSD serogroup or HAZV. Furthermore, this assay has the advantage of safety and earliness compared with the neutralization assay using wild CCHFV. The neutralization assay using the authentic virus takes several days for viral replication. In contrast, the assay in this study took only one day after infection because of the early detection of the reporter protein expression.

It is known that CCHFV is a zoonotic pathogen and infects various wild and domestic animals such as cattle, sheep, goats, buffalo, hares, squirrels, mouse, rats, and hedgehogs (Mohamed *et al.*, 2008; Mourya *et al.*, 2012; Shepherd *et al.*, 1987, 1989a). While the further study will be needed to use the assay for animal sera, the neutralization assay reported in this study will be a strong seroepidemiological tool for CCHFV infection in animals and will be used for predicting the endemic.

This study revealed that CCHFVpv is a surrogate virus for the neutralization assay. It was demonstrated that this neutralization assay is worthwhile for the diagnosis of and seroepidemiological study on CCHF. More precise diagnosis of CCHF should be confirmed using rN-based ELISA in combination with neutralization assay using CCHFVpv.



## Tables

**Table 2.1** Relationship of the results between the rN-based ELISA and the neutralization assay using CCHFV<sub>pv</sub>

		rN-based IgG ELISA	
		(N=65)	
		Positive	Negative
Neutralization assay	Positive	13	0
	Negative	9	43

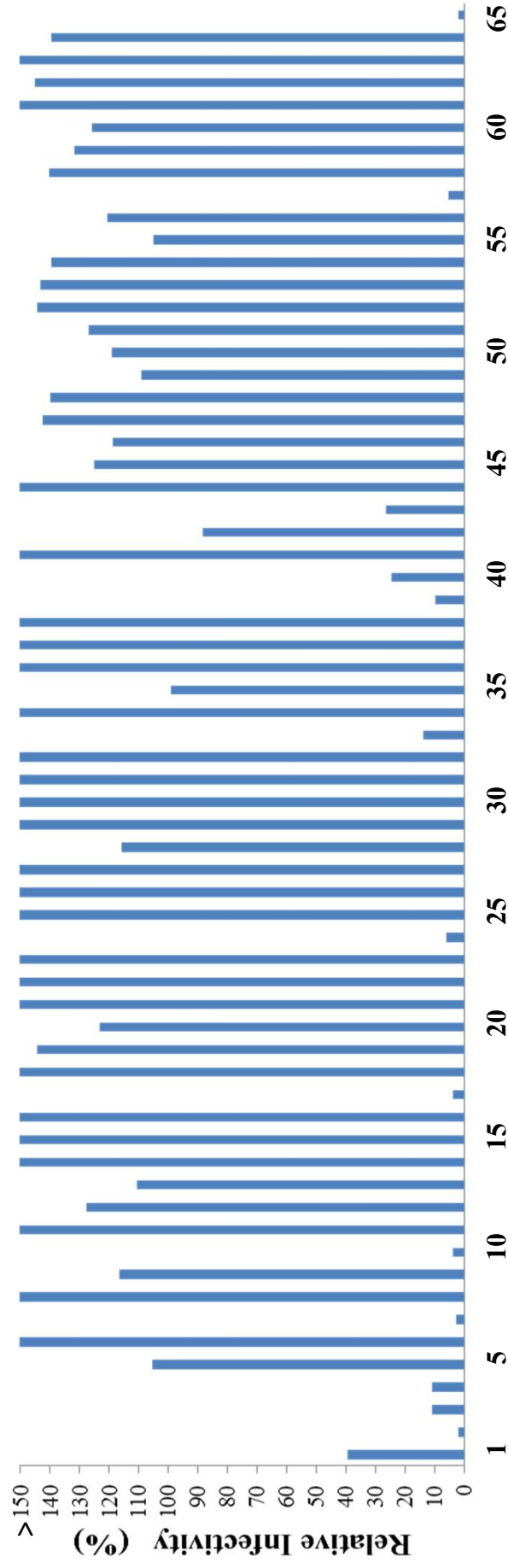
**Table 2.2** Titer of the neutralizing antibodies in the human sera

Sample ID	Titer
#2	40
#3	80
#4	20
#7	2560
#10	1280
#17	80
#24	80
#33	40
#39	320
#40	40
#43	40
#57	2560
#65	160

**Table 2.3** Titer of the neutralizing antibodies in the human sera and O.D. value of ELISA

Human Sera	Neutralizing antibody titer		O.D. value of ELISA
	CCHFVpv	CCHFV	
Tajik1	<20	<20	0.072
Tajik5	<20	<20	0.113
Tajik8	>1280	>2560	0.711
Tajik10	20	20	0.363
Tajik11	80	40	2.353
Tajik12	80	<20	1.172
Convalescent 22/7/14	>1280	>2560	2.741
Neg Serum SIGMA	<20	<20	0.131

(a)

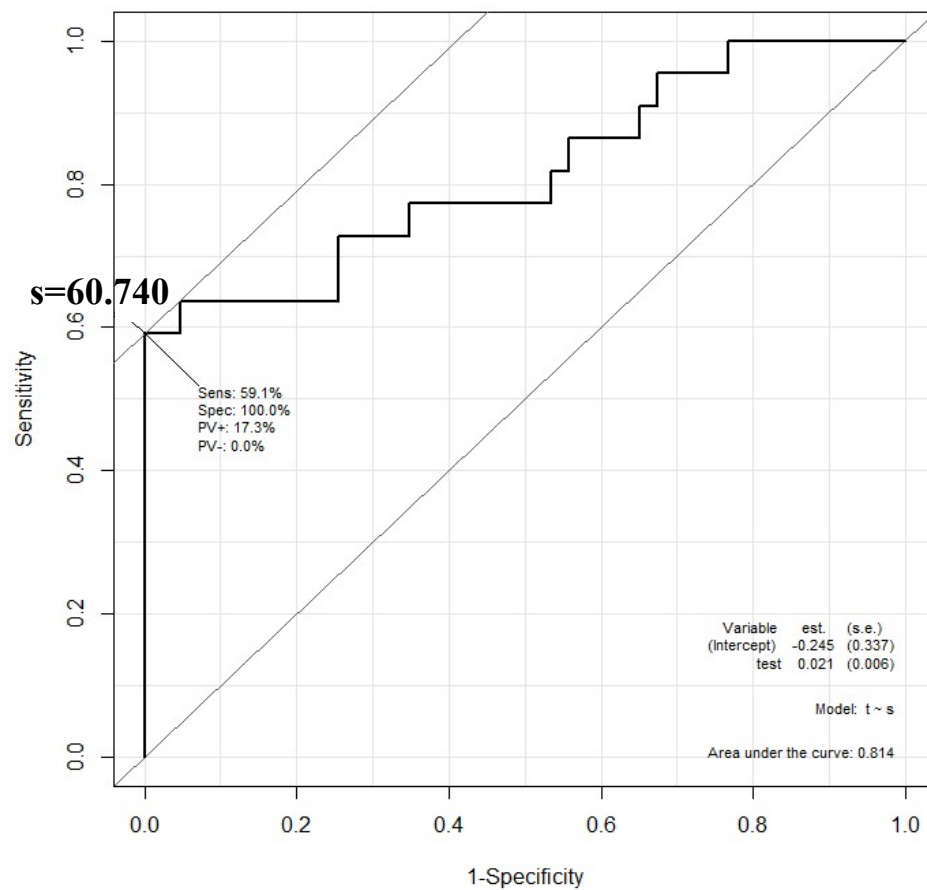


### The sample IDs

**Fig.2.1** Neutralization assay using CCHFVpv for human sera

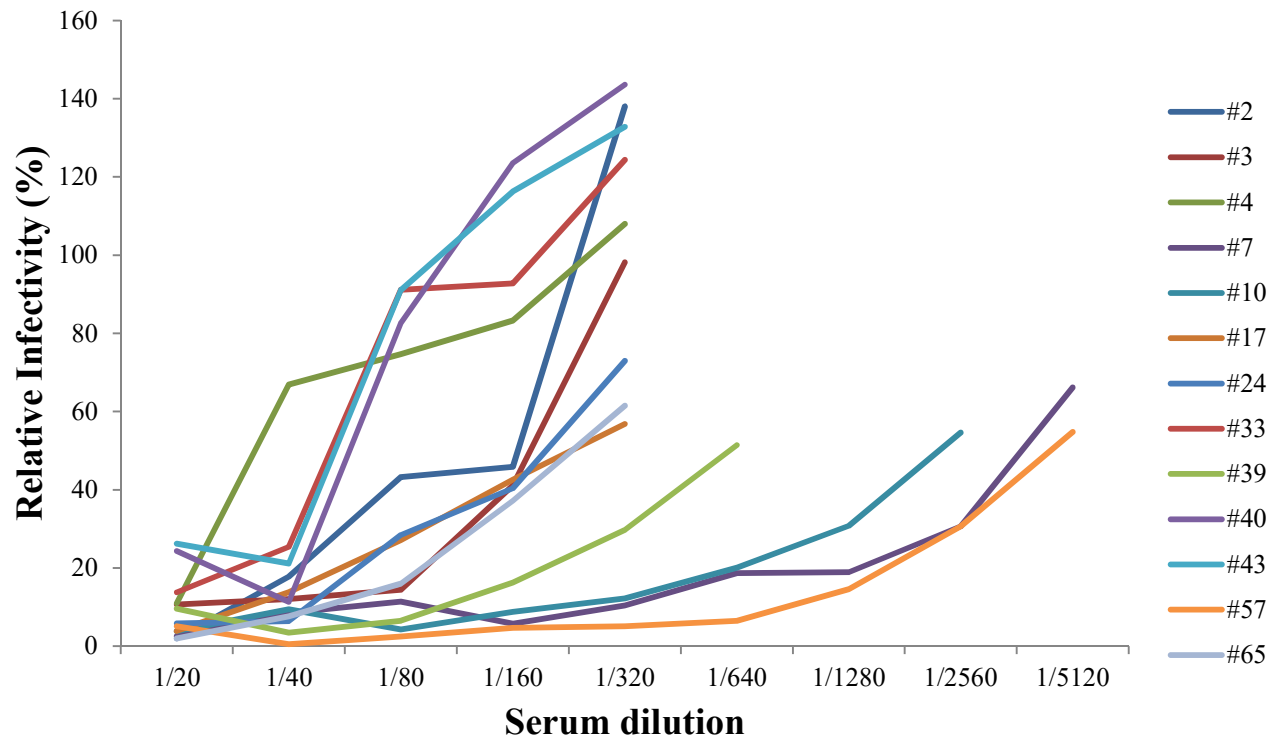
(a) A neutralization assay was performed by incubating CCHFVpv/Luc with the sera. CCHFVpv was reacted with the diluted human sera (the final dilution was 1:20) for 30 min. The mixtures were added to Huh7 cells and luciferase activity was measured at 1 day post infection (dpi). The values were showed as relative infectivity to the results of using no serum. The sample IDs of the serum were listed on the x-axis scale. The tests were performed in triplicate.

(b)



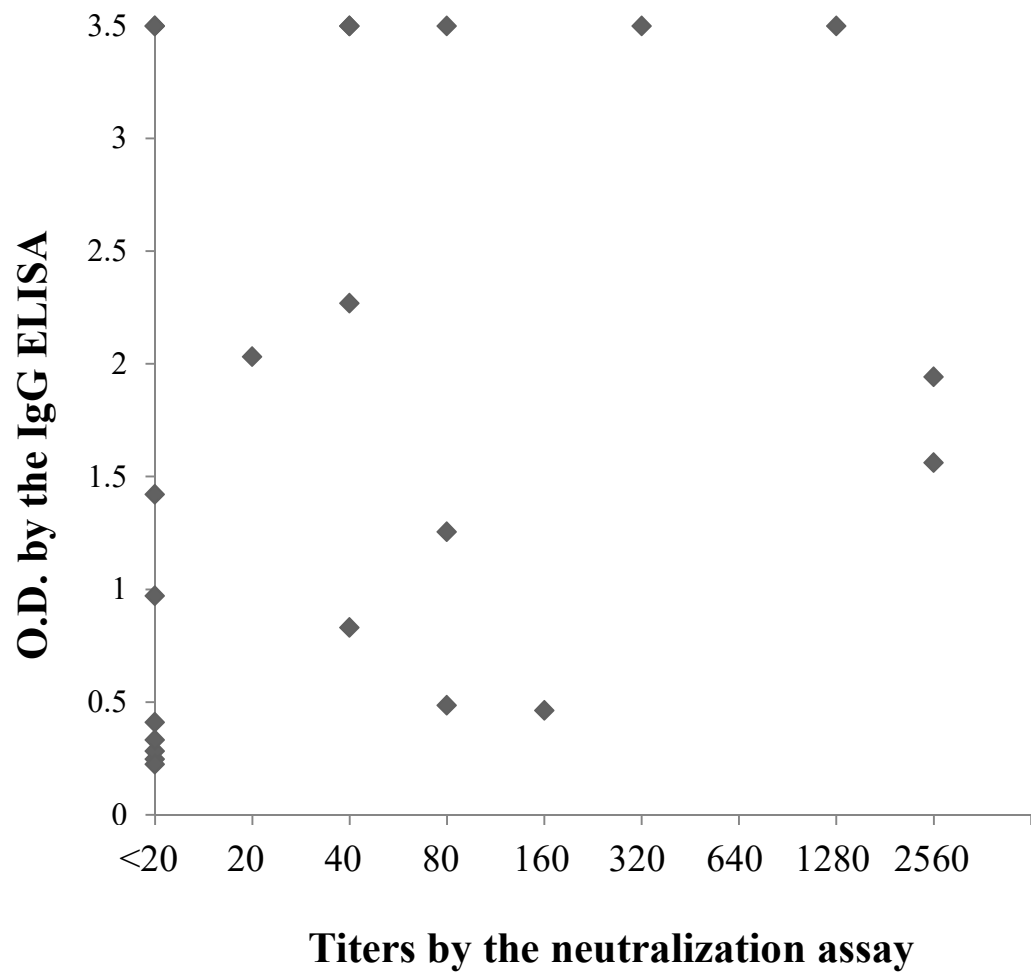
*Fig.2.1 Continued.*

(b) ROC analysis of the neutralization assay using CCHFVpv was carried out using the statistical software R version 3.0.1. The most suitable cut-off value was calculated as 39.260.



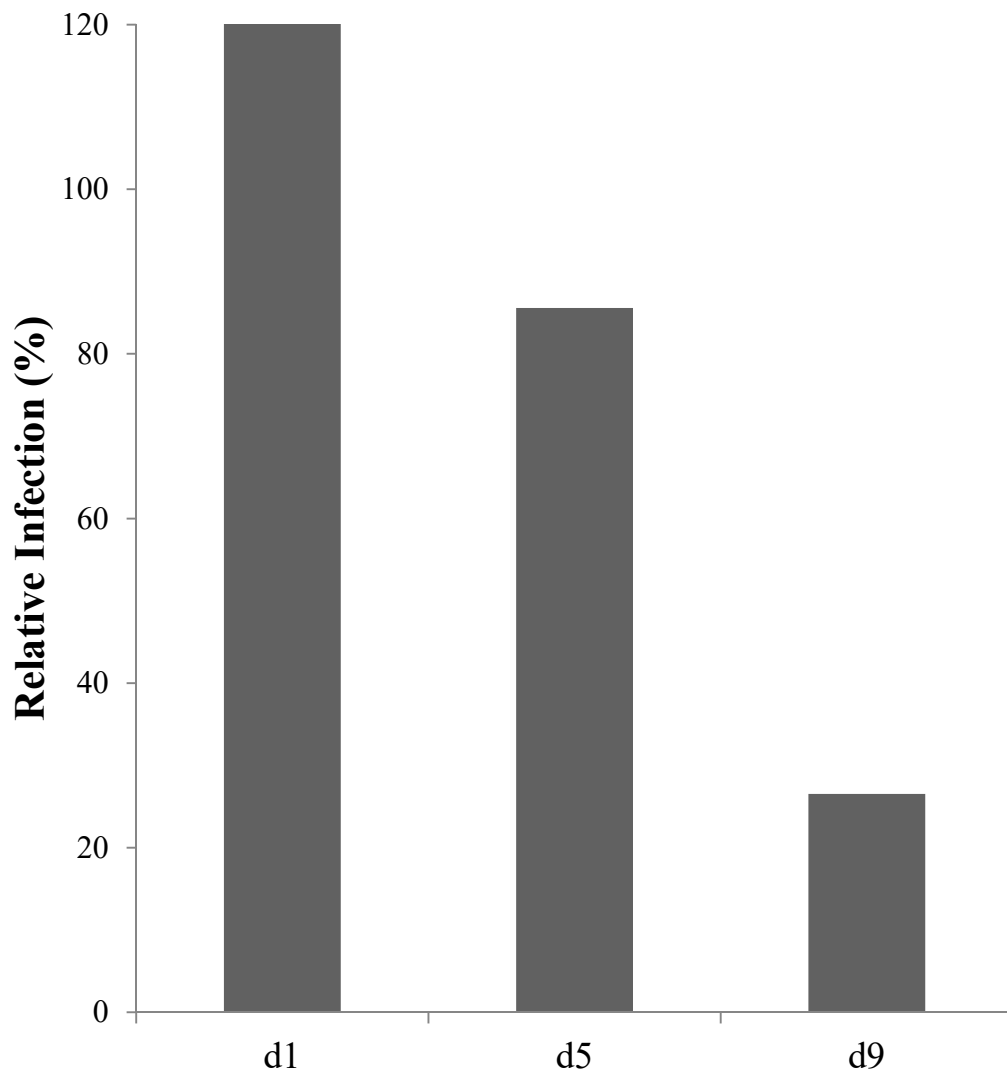
**Fig. 2.2** The neutralization assay using serially diluted sera

The sera which were positive at the screening were serially diluted as indicated and reacted with CCHFVpv for 30 min. The mixtures were added to Huh7 cells and luciferase activity was measured at 1dpi. The values were showed as relative infectivity to the results of using no serum. The tests were performed in triplicate.



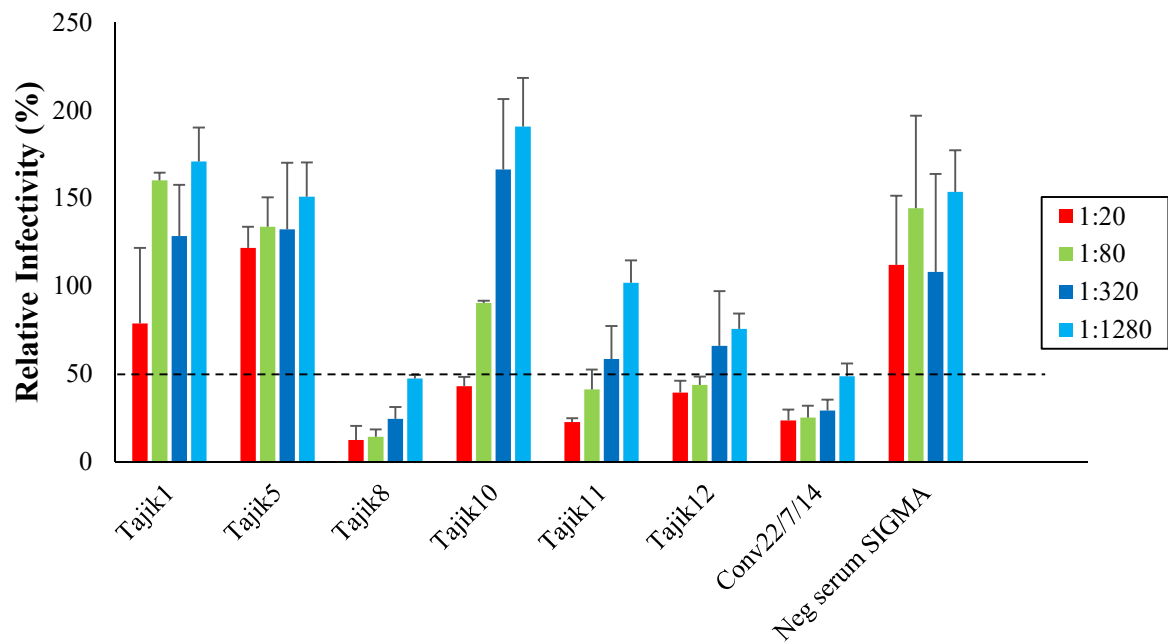
**Fig. 2.3** Correlation between the neutralization titers and the O.D. values of the ELISA

The titers with the neutralization assay were plotted on the x-axis scale and the O.D. values with the ELISA were plotted on the y-axis scale. The correlation coefficient was 0.38.

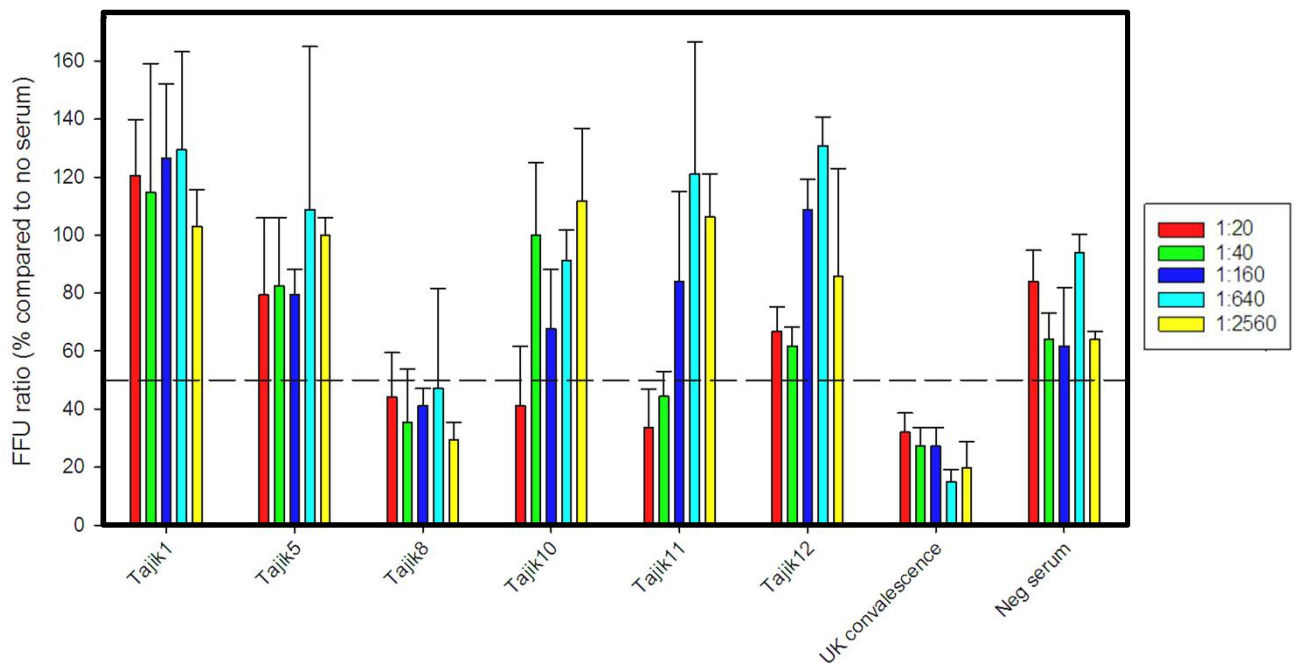


**Fig. 2.4** The neutralization assay for the samples collected from the same patient CCHFVpv was reacted with the diluted human sera (the final dilution was 1:40) for 30 min. The mixtures were added to Huh7 cells and luciferase activity was measured at 1 dpi. The tests were performed in triplicate.

(a)



(b)



**Fig. 2.5** The neutralization assay using CCHFVpv and CCHFV

(a) The sera were serially diluted as indicated and reacted with CCHFVpv for 30 min. The mixtures were added to Huh7 cells and GFP-positive cells were counted at 1 dpi under fluorescence microscopy. The values were showed as relative infectivity to the results of using no serum. The tests were performed in triplicate. (b) Neutralization assay using CCHFV. The diluted sera and virus (IbAr 10200 strain) mixture was incubated for 1 hour at 37° C. The mixture was inoculated onto Vero cells. At 1dpi, for staining foci of the infected cells, the cells were fixed and probed. The cells were visualized using a fluorescent microscope and the foci were quantified. The values were showed as relative infectivity to the results of using no serum. The tests were performed in triplicate.



## **Chapter 3**

**Comparative study on three nairoviruses:**

**Hazara virus, Soft tick bunyavirus,**

**and Issyk-Kul virus**

## **Abstract**

Nairovirus includes the important zoonotic pathogens such as CCHFV and NSDV, and many other poorly studied viruses. Recently, many novel nairoviruses have been found. STBV was novel nairovirus isolated from tick in Japan. In this chapter, to investigate the characteristics of the nairoviruses including the novel nairovirus, the author virologically and serologically compared three nairoviruses, HAZV, STBV, and IKV. HAZV is a virus closely related with CCHFV and requires BSL-2 containment for experimental working. HAZV was one of the relatively well-studied viruses in nairovirus besides CCHFV. IKV was a human pathogen but the study has not been done well. In this study, the author revealed that STBV and IKV were closely related virologically and serologically. In contrast, HAZV showed some different characteristics from these two viruses. To investigate the serological features of these viruses, the author generated recombinant N-based ELISA. These results and the generated serological assays will improve the study of the replication mechanisms, the diagnosis, and the serosurveillance of nairoviruses.

## Introduction

Recently, many novel nairoviruses have been found including Leopards Hill virus (Ishii *et al.*, 2014), Finch creek virus (Major *et al.*, 2009), and STBV (Oba *et al.*, 2015). Nairovirus comprises seven species based on the antigen cross-reactions. But at present, some viruses including Leopards Hill virus, Finch creek virus, IKV, and STBV have not been classified. It is necessary to investigate nairoviruses including novel nairoviruses for further understanding of nairovirus. STBV was isolated from *Argas vespertilionis* in Japan and was revealed to belong to *Bunyaviridae* nairovirus (Oba *et al.*, 2015). The cases of human bite of *Argas vespertilionis* were previously reported (Estrada-Peña & Jongejan, 1999; Hoogstraal, 1985). Therefore, although the pathogenicity of STBV is unknown in this time, STBV might have a potential of causing zoonosis. IKV was isolated from humans, bats, birds, ticks (*Argas vespertilionis* and *Ixoides vespertilionis*), mosquitoes, and tabanid in Kyrgyzstan, Tadjikistan, and Kazakhstan. IKV is likely to transmit to humans with tick bite, exposure to bat urine/feces, or a possible involvement of mites. IKV does not cause fatalities but produces fever, headache, dizziness, muscle pain, rash, and nausea in human (Irina N, 2001; Løvv *et al.*, 1984). Sporadic cases of this disease, Issyk-Kul fever, have been reported in south Tadjikistan in 1975 and 1978. Outbreaks of the disease occurred in the same region in 1982 and 1985 (Irina N, 2001; Løvv *et al.*, 1984). Although IKV causes these diseases, the characteristic of the virus has not been well-understood. HAZV, a virus belonging to the same serogroup with CCHFV, was isolated in Pakistan from *Ixodes redikorzevi* (Begum & Wissesman, 1970). HAZV, the pathogenicity of which against humans was not recognized, requires BSL-2 containment for experimental working. HAZV was one of the relatively well-studied viruses in nairovirus besides CCHFV (Buckley, 1974; Dowall *et al.*, 2012b; Flusin *et al.*, 2011; Foulke *et al.*, 1981; Smirnova, 1979; Smirnova *et al.*, 1977; Wang *et al.*, 2015). The well-understanding of the characteristics and the serological study will help the control of nairoviruses including pathogens. In this chapter, the author investigated the characteristic

of nairovirus and serological analysis using HAZV, STBV, and IKV.

## Materials and methods

### Cells

SW13 cells and Vero cells were cultured in DMEM supplemented with 10% (v/v) FBS. Jurkat cells and Daudi cells were cultured in RPMI medium supplemented with 10% (v/v) FBS. Jurkat cells expressing lectins were described in Chapter 1. The lectin expressing Daudi cells were generated by the infection of the lentiviral vectors for expression of the lectins as well as the lectin expressing Jurkat cells. Sf9 (*Spodoptera frugiperda*) cells were cultured in Sf900-II SFM (Gibco) supplemented with 10% (v/v) FBS. Tn5 cells were cultured in EX-CELL 405 Serum-Free Medium for Insect Cells (Sigma) supplemented with antibiotics (Gibco).

### Viruses

HAZV strain JC280 was kindly provided from Dr. Roger Hewson (Microbiology Services Division, Public Health England, United Kingdom). STBV was kindly provided from Dr. Shuji Ando (Department of Virology I, National Institute of Infectious Diseases (NIID), Japan). IKV strain LEIV 315K was kindly provided from Dr. Barbara W Johnson (Center for Disease Control and Prevention (CDC), USA). The virus titers were determined by plaque assay using SW13 cells as follow; SW13 cells were inoculated with the viruses for 1 h. and washed twice with DMEM supplemented with 2% (v/v) FBS. DMEM supplemented with 2% (v/v) FBS containing 1% SeaPlaque agarose (Lonza) was laid onto the inoculated cells. At 3 or 4 days post infection (dpi), the cells were stained with DMEM supplemented with 2% (v/v) FBS containing 0.01% neutral red. After 1 day, the number of the plaques was counted.

### Sequencing of STBV genome

Total RNA of STBV was extracted from the cell culture supernatant of the infected Vero cells

using High Pure Viral RNA Kit (Roche). The sequencing of STBV was kindly performed on MiSeq bench-top sequencer (Illumina) by Dr. Tsutomu Omatsu (Research and Education Center for Prevention of Global Infectious Diseases of Animals, Tokyo University of Agriculture and Technology, Japan). The open reading frame (ORF) of S segment was determined but those of M and L segments were uncertain because the 3' ends were not revealed. To investigate the ORFs of M and L segments, rapid amplification of cDNA ends (RACE) were performed. 3' linker ligation by miRNA Cloning Kit, High Efficient, DynaExpress (BioDynamics Laboratory) was performed with the purified viral RNA. The RNA was purified by NucleoSpin RNA clean-up XP (Takara Bio) and performed reverse transcription by SuperScript III Reverse Transcriptase (Invitrogen). The generated cDNAs were amplified by polymerase chain reaction (PCR) using the viral gene specific primers and the specific primer against the 3' linker. The PCR fragments were cloned into the vectors using Zero Blunt TOPO PCR Cloning Kit (Invitrogen). The plasmids were sequenced using the viral gene specific primers and the 3' ends of M and L segments of STBV genome were determined.

## Serum

BALB/c mice were infected with HAZV and STBV at  $2 \times 10^4$  PFU/head via intraperitoneal administration (i.p.), subcutaneous administration (s.c.) or intravenous administration (i.v.) (3 heads/route) (Table 3.1). After 4 weeks, the mice were sacrificed and the sera were collected. The collected sera were stored at  $-80^{\circ}\text{C}$ . The 1,000 human sera were obtained from the serum bank of NIID and 9 Japanese laboratory persons' sera were used as controls. The usage of the sera was approved by the research and ethics committee of the NIID (Reference no. 439).

## Phylogenetic analyses

Nucleotide and amino acid sequence alignments were created from the available sequence data

using ClustalW. The resulting sequence alignments of S, M, or L segment included 14 taxa and were 1,453, 3,508, or 11,356 nucleotides, respectively. The resulting sequence alignments of N, GP, or L protein included 14 taxa and were 464, 1,180, or 3,793 amino acids, respectively. The phylogenetic trees were constructed using MEGA6 by using Maximum Likelihood method based on the Tamura-Nei model of nucleotide substitution or based on the JTT matrix-based model of amino acid substitution. A discrete Gamma distribution was employed to model evolutionary rate differences among sites (5 categories). The phylogenetic robustness of each node was determined using 1,000 bootstrap replicates.

#### Growth kinetics

To examine the kinetics of viral replication, Vero cells, SW13 cells, Jurkat cells, and Daudi cells were infected with virus at a multiplicity of infection (m.o.i.) of 0.01. The virus was collected from the cell supernatant at various time points and the titers were determined by plaque assay using SW13 cells.

#### Flow cytometry

Antibody staining and analyses were performed as described in Chapter 1.

#### Predictions of glycosylation sites

The predictions of N-glycosylation sites were carried out using NetNGlyc 1.0 Server. The predictions of O-glycosylation sites were carried out using NetOGlyc 3.1 Server.

#### IFA

IF antigen slides were prepared as follows. SW13 cells were infected with HAZV, STBV, and

IKV at m.o.i. of 2 and collected at 1 dpi. HAZV, STBV, and IKV-infected SW13 cells were mixed with parent SW13 cells at ratio 1:2, washed with PBS, and spotted onto 24-well slide glasses. After air drying, slides were fixed with acetone at -30°C and stored. When used, cells were permeabilized with 0.2% Triton-X 100 in PBS for 30min. Then, test samples which were diluted with 5% skim milk in T-PBS were placed onto the slides. After 1 h incubation, the slides were washed with PBS and then treated with Alexa 488-conjugated goat anti-mouse immunoglobulin G (IgG) (Invitrogen) at a dilution of 1:400 for 1h. After another wash with PBS, the slides were examined under a fluorescence microscope (BZ-X710, KEYENCE). Reciprocal of the highest serum dilution which showed positive reactivity were used as antibody titers.

#### Neutralization Assay

HAZV, STBV, or IKV was reacted with the diluted mouse serum for 1 h at 37°C and inoculated onto SW13 cells. After 1 h inoculation, the cells were washed twice with 2% FCS DMEM and then overlaid with 1% SeaPlaque agarose in 2% FCS DMEM. After 3 or 4 days, the cells were stained with neutral red overnight and then counted the number of the plaque. Neutralizing titers were determined as the dilution of serum to reduce the number of the plaques by 50% compared with the number of the plaques of the control (PRNT<sub>50</sub>).

#### Immunoblotting

The samples were analyzed by SDS-PAGE, transferred to PVDF membranes, probed with antibodies, and detected by enhanced chemiluminescence (SuperSignal West Dura Extended Duration Substrate; Thermo Fisher Scientific). Antibodies used were horseradish peroxidase (HRP)-conjugated monoclonal antibody against mouse IgG (Invitrogen), mouse monoclonal antibody against penta-His (QIAGEN), and sera collected from the infected mouse. Images were



taken with an LAS-3000 image analyzer system (Fujifilm). SeeBlue Plus 2 Pre-stained Standard (Invitrogen) was used as a protein marker. The infected cell lysates were prepared as follow; SW13 cells were infected with the viruses at m.o.i. of 2 and at 3 dpi cells were collected. The cells were lysed using 1% Nonidet P-40 (NP-40) in PBS and centrifuged for removing debris. The supernatants were used as samples.

#### Expression of recombinant N (rN) of HAZV, STBV, and IKV

The rNs of the viruses were expressed in insect cells with recombinant baculoviruses. The recombinant baculoviruses, which expressed each N tagged with 8x histidine on C-terminus, were generated. A full-length cDNA of the N gene was originally cloned in the BamHI sites of plasmid vector pAcYM1. Transfer vector-recombinant DNA and linearized BaculoGold baculovirus DNA (PharMingen) were cotransfected into Sf9 cells according to the manufacture's protocol. After 7 days, the supernatant was collected, passaged, plaque purified, and used as a stock virus (Ac-HAZV/STBV/IKV N). A baculovirus which lacks polyhedrin expression (Ac- P) was used as a control virus. For expression of the recombinant proteins, Tn5 cells were infected with the recombinant baculovirus and incubated at 26°C for 3 days. Then, the cells were washed with PBS and lysed in 1% NP-40 in PBS containing Complete EDTA free (Roche). The cell lysate was centrifuged at 10,000rpm at 4°C for 10 min. The supernatant was collected and the pellet was lysed in 2M Urea in PBS and centrifuged. The supernatant was collected and the pellet lysed in 4M Urea in PBS and centrifuged. And then the supernatant was collected and the pellet was lysed in 8M Urea in PBS, centrifuged, and the supernatant was collected. The supernatant fractions were used as protein solutions. For checking the expressions and purifications, samples were analyzed by SDS-PAGE and stained with Bio-Safe Coomassie (Bio-Rad) as manufacture's protocol, and by immunoblotting. SeeBlue Plus 2 Pre-stained Standard was used as a protein marker.

## ELISA

MaxiSorp plates (Nunc) were coated with the predetermined optimal quantity of the rN (approximately 2,000 ng/well for HAZV rN and 500 ng/well for STBV and IKV rN) at 4°C overnight. Then, each well of the plates was inoculated with 5% skim milk in T-PBS (0.05% Tween20 in PBS) and incubated for 1 h at 37°C for blocking. The plates were washed three times with T-PBS and then inoculated with the test samples diluted in 5% skim milk in T-PBS at 37°C. After 1 h, the plate was washed three times with T-PBS and inoculated with goat anti-mouse or human IgG antibody labeled with HRP (Invitrogen). After 1 h, plates were washed and ABTS solution (Roche) was added to each well. The plates were incubated for 30 min at room temperature and the optical density (O.D.) was measured at 405 nm with reference at 490 nm.

## Results

### Phylogenic analyses

Firstly, the author analyzed the STBV genome sequence and the genetical relationships of HAZV, STBV, and IKV were investigated. The sequencing of STBV genome was performed as described in Materials and Methods. Phylogenic trees of nairovirus S, M, and L segments were shown in Fig. 3.1a. Phylogenic trees of N, GP, and L protein were shown in Fig. 3.1b. Genetically, in any segments, HAZV is closely related with CCHFV and NSDV. STBV is closely related with Keterah virus and IKV, but distantly related with HAZV.

### Growth kinetics

Next, the author examined whether the kinetics of the virus replications were different among the viruses or not. STBV was well replicated in Vero cells and SW13 cells, although not replicated in Daudi cells (Fig. 3.2). The highest titer was obtained at 4 dpi, the last day the author examined. The result of the growth kinetics of IKV replication was similar to that of STBV. HAZV was also well replicated in Vero cells and SW13 cells and the highest titer was obtained at 2 dpi and 3 dpi, respectively, and the titer was higher than that of STBV.

### Cell surface molecules involving the infections

The author examined the growth kinetics of HAZV, STBV, or IKV in the C-type lectin expression cells to reveal the effect of the lectins on the infections. Because the virus replication in Jurkat cells and Daudi cells was found to be low (Fig. 3.2), Jurkat cells and Daudi cells were used to investigate the involvement of C-type lectins. Expression of control molecule (fCD2 CT) and C-type lectins were confirmed by flow cytometry (Fig. 3.3a, Fig. 1.5a). In neither Jurkat cells nor Daudi cells, the replication of HAZV was enhanced by the expressions of the lectin (Fig. 3.3b, c).

Replication of STBV and IKV were superior in both DC-SIGN and LSECtin expressing Jurkat cells to control cells (Fig. 3.3b). However, the viruses did not replicate in Daudi cells regardless of expression of the lectins (Fig. 3.3c). Surprisingly, HAZV replication was not enhanced (Fig. 3.3b) though STBV and IKV replications were enhanced and DC-SIGN was suggested as an entry factor of CCHFV (Fig. 1.5b). Then the glycosylation sites of GP of these viruses were predicted, indicating that the number of the predicted either N- and O-glycosylation sites of HAZV GP was much fewer than those of CCHFV GP, STBV GP, and IKV GP (Fig. 3.3d, e).

#### Virus infection of mice

BALB/c mice were infected with HAZV and STBV as described in Materials and Methods. The body weights of the infected mice did not significantly change compared with mock-infected mice (Fig. 3.4). Antibodies in the sera of the infected mice were examined by IFA, showing that the specific antibodies were detected in all collected serum at 1:160 dilution. The highest titer to a HAZV was found in the serum collected from the HAZV-infected mouse via i.v. (#7) (antibody titer: 5,120) and to STBV was found in that from the STBV-infected mouse via i.p. (#10) (antibody titer: 5,120) (Fig. 3.5 and Table 3.2). The author used these sera for the subsequent experiments.

#### Cross-reactivity in IFA

To examine whether there is the cross-reactivity between HAZV, STBV, and IKV, the author firstly performed IFA. The antibody titer of the serum from the HAZV-infected mouse to HAZV was 5,120, while that to STBV was 80 and to IKV was 160. In contrast, the antibody titer of the serum from the STBV-infected mouse to STBV was 5,120 and to IKV was 2,560 though that to HAZV was 40 (Fig. 3.5 and Table 3.2). These results showed that there is strong cross-reactivity between STBV and IKV. The cross-reactivity between the two viruses and HAZV was relatively weak.

#### Neutralization assay for the nairoviruses

Next, the author performed neutralization assay to detect the neutralizing antibodies and investigate the cross-reactivity. Antibody titer of the serum from the HAZV-infected mouse to HAZV was 80 while those to STBV and IKV were not detected. In contrast, neutralizing antibody titers of the serum from the STBV-infected mice to STBV, IKV, and HAZV were not detected (Table 3.3).

#### Immunoblotting of the infected cell lysate

To detect which proteins were reacted with the antibodies, the author performed immunoblotting. The serum from the HAZV-infected mouse detected HAZV protein of approximately 45 kDa, which was considered as the N protein and weakly reacted with the protein of approximately 35 kDa, which was considered as the Gn protein (Fig. 3.6). The serum from STBV-infected mouse detected STBV and IKV proteins of approximately 45 kDa, which were considered as the N proteins (Fig. 3.6). The intensity of the reactivity seemed to be different between HAZV and STBV/IKV-infected cells but this could be because of the difference of the multiplication in the cells (Fig. 3.2).

#### Expression and purification of His-HAZV/STBV/IKV rN

The author generated rN-based IgG ELISA as another serological assay system. To establish the rN-based ELISA for HAZV, STBV, or IKV infection, rN of each virus was expressed using the baculovirus expression system. The expression and purification of HAZV/STBV/IKV rN in Ac-HAZV/STBV/IKV N-infected Tn5 cells were demonstrated by SDS-PAGE and immunoblotting (Fig. 3.7a, b). Immunoblotting with anti-penta-His antibody detected the protein band shown in Fig.

3.7b which was the expressed rN. The rN protein of HAZV was purified from 2M Urea in PBS and those of STBV and IKV were purified from 8M Urea in PBS.

## ELISA

The antigen HAZV/STBV/IKV rN was detected with anti-penta-His antibody by the ELISA to determine the optical concentration of the protein coating (Fig. 3.7c). All recombinant proteins were reacted with anti-penta-His antibody and the author decided that the amount of the antigen coating on ELISA plate was 2,000 ng/well for rN HAZV and 500 ng/well for rN STBV/IKV. Next, the serum from the mouse infected with HAZV or STBV was used in the ELISA system. HAZV rN was detected with the serum from the HAZV-infected mouse but not with the sera from the STBV-infected mouse and mock-infected mouse (Fig. 3.7d). In contrast, STBV rN was detected with the serum from the STBV-infected mouse but not with the serum from the HAZV-infected mouse (Fig. 3.7d). IKV rN was also detected with the STBV-infected mouse serum although the O.D. was lower than that in STBV rN-based ELISA (Fig. 3.7d). These results suggested that there is a little cross-reactivity between STBV and IKV in rN-based ELISA. In contrast, there was no cross-reactivity between HAZV and the two viruses.

## Serological surveillance

Here, the author generated rN-based ELISA for STBV infection. Finally, serological surveillance using the 1,000 human sera obtained from the serum bank of NIID and 9 Japanese healthy human sera as controls was performed. The author performed only rN-based ELISA for STBV infection because the sera were collected in Japan and HAZV and IKV have not been recognized in Japan. The sera were diluted (1:100) and used in the ELISA. The result was shown in Fig. 3.8a. The cut-off value in the ELISA was determined as 1.598, the value of mean+3SD of the

value of the 9 healthy human sera, and 13 samples (#30, #165, #228, #335, #352, #364, #390, #408, #422, #426, #427, #441, and #442) were positive for anti-STBV antibody in the ELISA. Using ELISA positive sera and the control samples, the author performed IFA and the neutralization assay. The sera were diluted (1:100) and performed in IFA. The examples of the result of IFA were shown in Fig. 3.8b. The neutralization assay was performed using the diluted sera (1:20). As the results, the immunoreactions of all sera were not detected in both IFA and the neutralization assay. Therefore, the author concluded that the all sera did not contain the specific antibodies to STBV.

## Discussion

STBV is a recently-discovered nairovirus isolated from tick in Japan (Oba *et al.*, 2015). IKV is a pathogen against human but its study was not performed well (Atkinson *et al.*, 2015; Irina N, 2001; Løvov *et al.*, 1984). HAZV is one of the relatively well-studied nairoviruses besides CCHFV (Buckley, 1974; Dowall *et al.*, 2012b; Flusin *et al.*, 2011; Foulke *et al.*, 1981; Smirnova, 1979; Smirnova *et al.*, 1977; Wang *et al.*, 2015) and requires BSL-2 containment for experimental working. It was previously reported that there was some variation of the features in N and GP among nairoviruses (Walker *et al.*, 2015). Therefore, further study and comparison will be needed to well-understanding of nairovirus. Nairovirus comprises seven serogroups and the serological study of nairoviruses is needed for the diagnosis of nairovirus infections in this time though some viruses have not been classified. Now, the author carried out the characterization and the serological analysis of HAZV, STBV, and IKV.

The phylogenetic analyses showed that STBV and IKV were genetically closely related (Fig. 3.1). Moreover, the growth kinetics of STBV was similar to that of IKV rather than that of HAZV (Fig. 3.2). The growth of HAZV (Fig. 3.2) was similar to those of Kupe virus and Dugbe virus (Crabtree *et al.*, 2009). These results showed that the virological features were different between HAZV and the two viruses.

Next, the author investigated the cross-reactivity between these viruses. The result of IFA suggested that the cross-reactivity between HAZV and STBV was much weaker than that between STBV and IKV (Table 3.2). The result of neutralization assay suggested that there is no cross-reactivity between HAZV and the two viruses (Table 3.3). The result of immunoblotting for the cell lysates also suggested that the cross-reactivity was shown between STBV and IKV but not between STBV and HAZV (Fig. 3.6). The author generated rN-based IgG ELISA and revealed the serum from STBV-infected mouse showed the reaction to IKV rN though the cross-reactivity was



weak (Fig. 3.7d). In contrast, no cross-reactivity was detected between HAZV and the two viruses by the ELISA. These results of serological studies suggested that STBV had similar characteristics to IKV but not to HAZV and was belonging to the same serogroup with IKV.

The productivity of the neutralizing antibody to the nairoviruses seemed to be low in mouse because the production of the antibody was weak or not detectable (Table 3.3). The antibody to Gn, the component of the envelope protein, was detected in the HAZV-infected mouse serum in immunoblotting but the production or the reactivity of the antibody seemed much weaker than that of anti-N antibody (Fig. 3.6). This suggested that main target of the antibody in the sera was N. It was previously reported that the reactivity of immunity in CCHF patients was different according to the antigens, and the production of the neutralizing antibodies was weaker than that of anti-N antibodies (Shepherd *et al.*, 1989b). The result in this study was consistent with the report and suggested that this feature was common in nairoviruses. The difference of the purification of rN from baculovirus expression system might indicate that there are different features and antigenicity between HAZV N and STBV/IKV N (Fig. 3.7a).

Some viruses in *Bunyaviridae* used DC-SIGN as an entry factor (Hofmann *et al.*, 2013; Lozach *et al.*, 2011). In nairovirus, the author showed that CCHFV might use DC-SIGN as an entry factor in Chapter 1. Here, the author revealed that the expression of DC-SIGN and LSECtin enhanced STBV and IKV replication (Fig. 3.3b). In contrast, the expression of the lectins did not affect HAZV replication (Fig. 3.3b). Further study will be needed to certain whether these molecules are actually involved in the viral infection *in vivo*, but these results suggested that the involvement of lectin in viral replication is various in nairoviruses. The prediction of the glycosylation sites revealed that HAZV had a fewer sites than CCHFV, STBV, and IKV (Fig. 3.3d, e). These results suggested that HAZV GP glycosylation was little and therefore the effects of the lectins were not shown. These results suggested that HAZV has not had completely similar entry mechanisms to CCHFV though

the viruses were genetically related well (Fig. 3.1). The prediction sites of STBV and IKV was similar to each other but that of CCHFV and the two viruses was not so similar (Fig. 3.3d, e). This might affect the result that LSECtin enhanced STBV and IKV replication but not CCHFV entry (Fig. 3.3b, Fig. 1.5). The result that STBV and IKV could not replicate in DC-SIGN or LSECtin expressing Daudi cells (Fig. 3.3c) suggested that Daudi cells do not have the replication factor(s) for the viruses or/and other essential entry factor(s).

The pathogenicity of STBV was not clear. Infection of mice with STBV in this study showed no significantly body weight changes (Fig. 3.4). This suggested that STBV has no pathogenicity or infectivity in mouse (BALB/c) as well as HAZV. However, as CCHFV have no pathogenicity in mouse (Bray, 2007), it is possible that STBV have pathogenicity in human or other animals. The serological diagnoses of IKV have been done by complement fixation tests and diffuse precipitation in agar reactions (Irina N, 2001). The serological assay the author generated in this study will be useful for a novel diagnostic tool for HAZV, STBV, and IKV infections. The cross-reactivity between STBV and IKV detected in the ELISA was weak compared with that in IFA and the immunoblotting. The reason was uncertain but use of the ELISA could distinguish these two virus infections in comparing the reactivity. The author did serological surveillance for STBV using Japanese human sera (Fig. 3.8). A total of 13 sera were positive by the ELISA but these were negative by IFA and the neutralization assay. The author concluded that the samples were all negative for STBV infection. This suggests that the human infection with STBV does not occur or does rarely in Japan. Recently a novel pathogen in *Bunyaviridae*, severe fever with thrombocytopenia syndrome virus (SFTSV), was found in clinical cases in Japan (Takahashi *et al.*, 2014). SFTSV has been considered to exist in Japan from long ago (Yoshikawa *et al.*, 2015) though the virus was found in 2013 (Takahashi *et al.*, 2014). If STBV has pathogenicity, like the SFTSV cases, STBV might be a pathogen of a neglected infectious disease. The further study will be needed

to clarify whether the virus cause disease. The author considered that this ELISA system could be a useful tool for surveillance of STBV infection and help the understanding of the infectious risk and the viral distribution.

In conclusion, the author revealed that STBV was closely related with IKV but not with HAZV virologically and serologically. The author generated the serological assays for STBV, IKV, and HAZV infections. These results and the generated serological assays will improve the understanding of the replication mechanisms, the diagnosis, and the serosurveillance of nairoviruses.

## Tables

**Table 3.1** List of the infected mouse IDs, the viruses and the infectious routes in the experimental infection

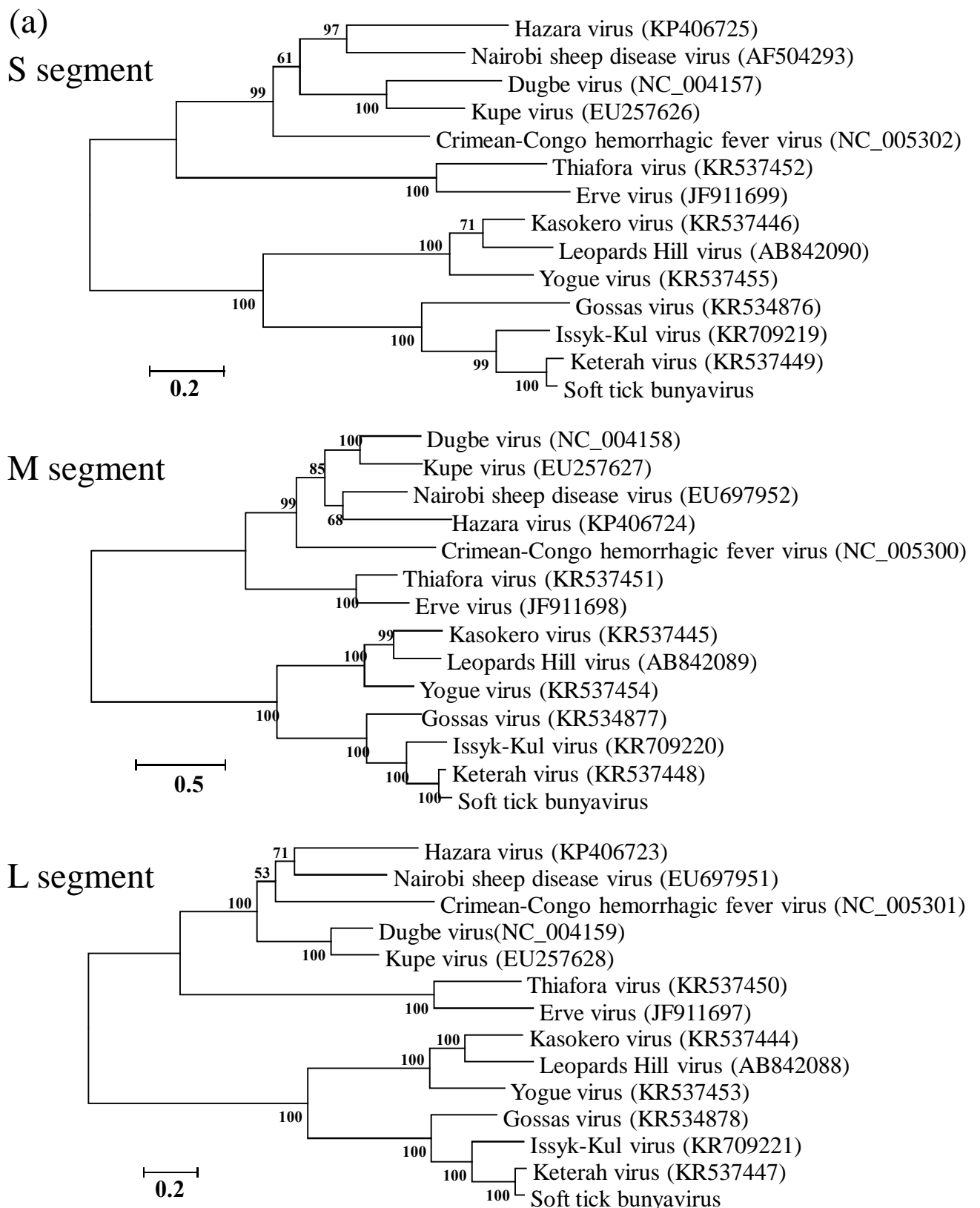
Mouse IDs	Virus	Route
#1 - 3	HAZV	i.p.
#4 - 6	HAZV	s.c.
#7 - 9	HAZV	i.v.
#10 - 12	STBV	i.p.
#13 - 15	STBV	s.c.
#16 - 18	STBV	i.v.
#19	mock	i.p.
#20	mock	s.c.
#21, 22	mock	i.v.

**Table 3.2** Antibody titers of the infected mouse sera in IFA

<b>Antiserum</b>	<b>Antigen (virus infected cells)</b>		
	<b>HAZV</b>	<b>STBV</b>	<b>IKV</b>
<b>HAZV-infected mouse serum</b>	5120	80	160
<b>STBV-infected mouse serum</b>	40	5120	2560

**Table 3.3** Antibody titers of the infected mouse sera in neutralization assay (PRNT<sub>50</sub>)

<b>Antiserum</b>	<b>Virus</b>		
	<b>HAZV</b>	<b>STBV</b>	<b>IKV</b>
<b>HAZV-infected mouse serum</b>	40	<20	<20
<b>STBV-infected mouse serum</b>	<20	<20	<20



**Fig. 3.1** Phylogenetic trees of nairovirus

Nucleotide and amino acid sequence alignments were created from the available sequence data using ClustalW. The resulting sequence alignments of S, M, or L segment included 14 taxa and were 1,453, 3,508, or 11,356 nucleotides, respectively. The resulting sequence alignments of N, GP or L protein included 14 taxa and were 464, 1,180, or 3,793 amino acids, respectively. The phylogenetic trees of the nucleotide sequences (a) and amino acid sequences (b) were constructed using MEGA6 by using Maximum Likelihood method based on the Tamura-Nei model of nucleotide substitution or based on the JTT matrix-based model of amino acid substitution. A discrete Gamma distribution was employed to model evolutionary rate differences among sites (5 categories). The phylogenetic robustness of each node was determined using 1,000 bootstrap replicates. GenBank accession numbers are shown in parenthesis.

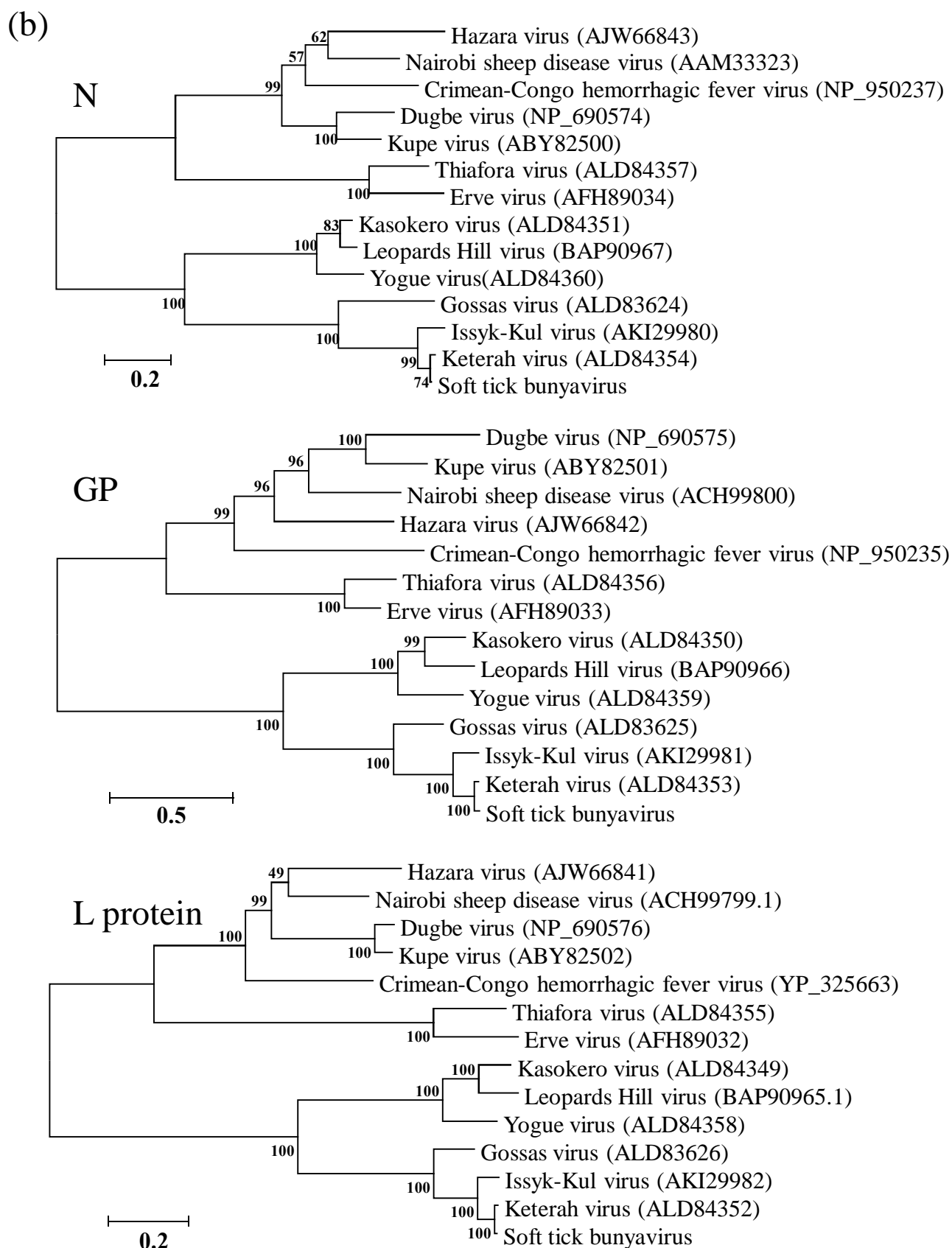
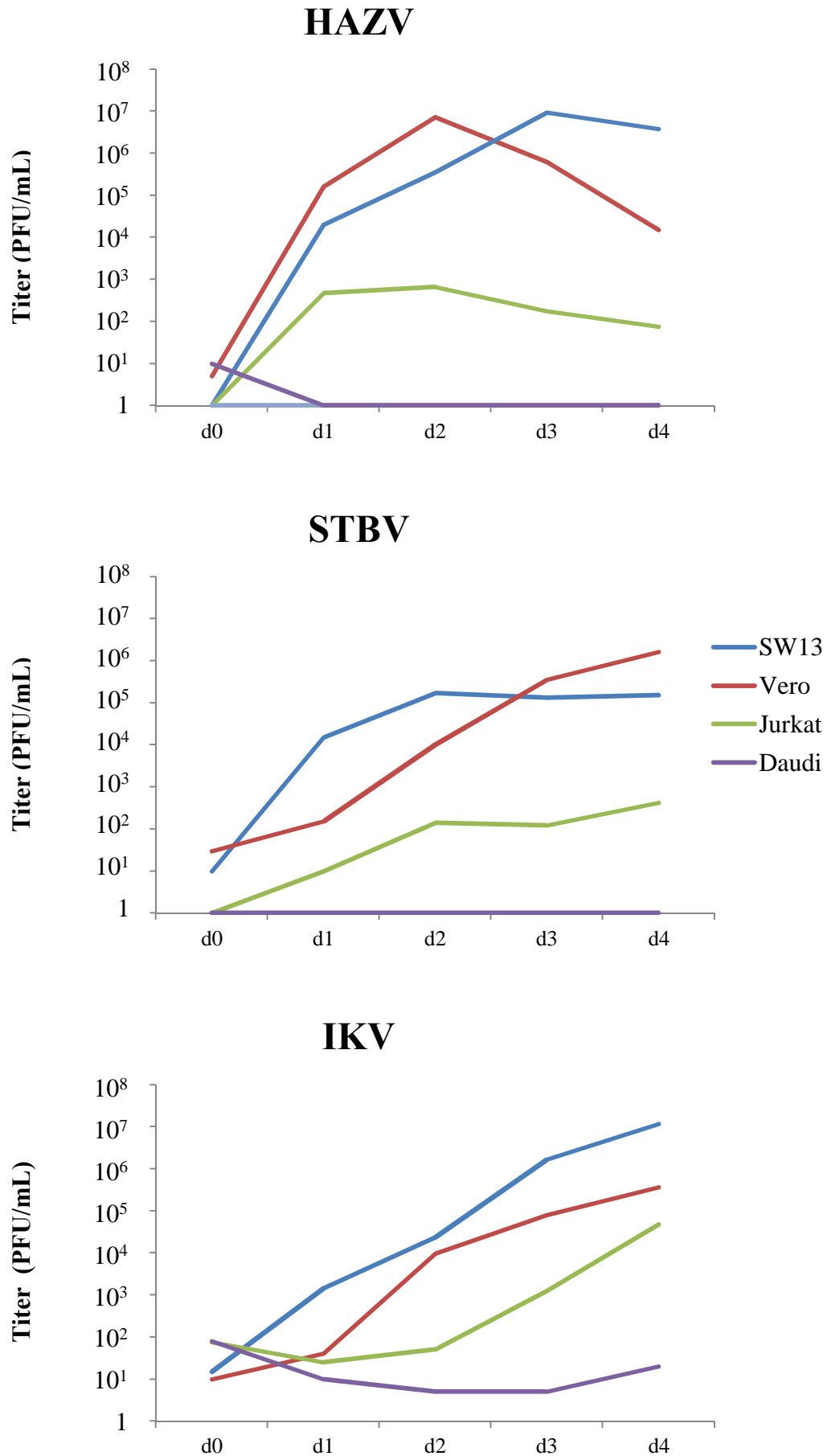


Fig. 3.1 Continued.

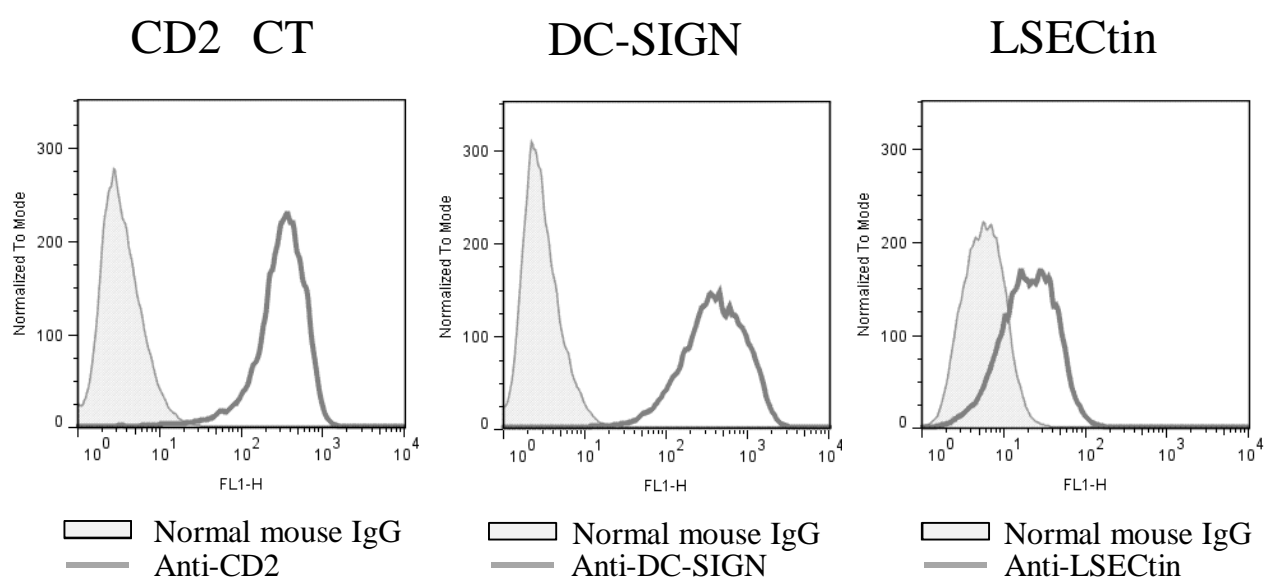


**Fig. 3.2** Growth kinetics of the nairoviruses

Vero cells, SW13 cells, Jurkat cells, and Daudi cells were infected with HAZV, STBV, and IKV at m.o.i. of 0.01. The viruses were collected from the cell supernatant at various time points and the titers were determined by plaque assay using SW13 cells.



(a)



**Fig. 3.3** Effects of C-type lectin on the nairoviruses replication

(a) The expression of fCD2 CT and C-type lectins on Daudi cells was confirmed by flow cytometry.

(b)

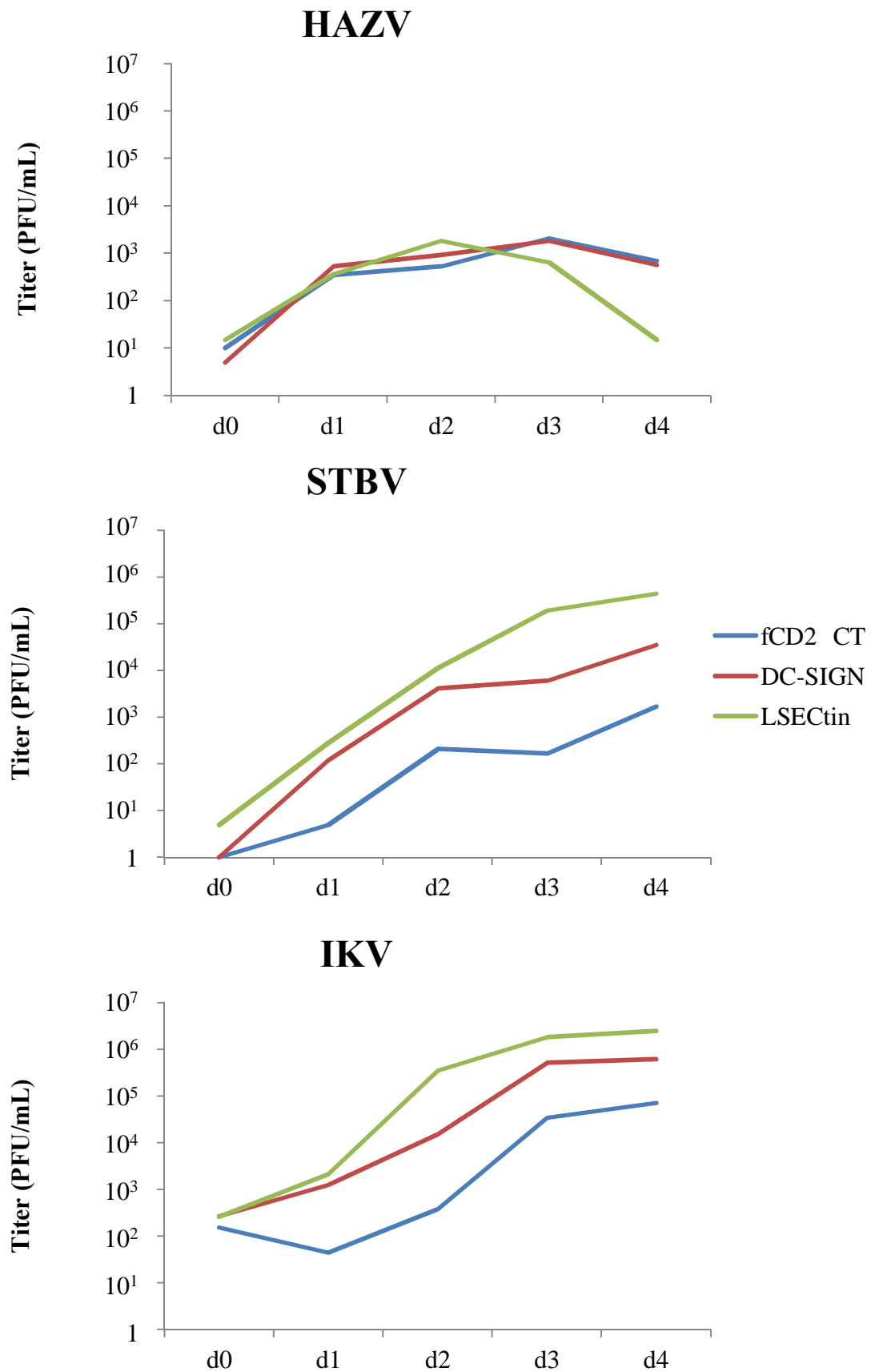


Fig. 3.3 Continued.

fCD2 CT-, DC-SIGN-, or LSECtin- expressing Jurkat cells (b) and Daudi cells (c) were infected with HAZV, STBV, and IKV at m.o.i. of 0.01. The cell supernatants at various time points were titrated by plaque assay using SW13 cells.

(c)

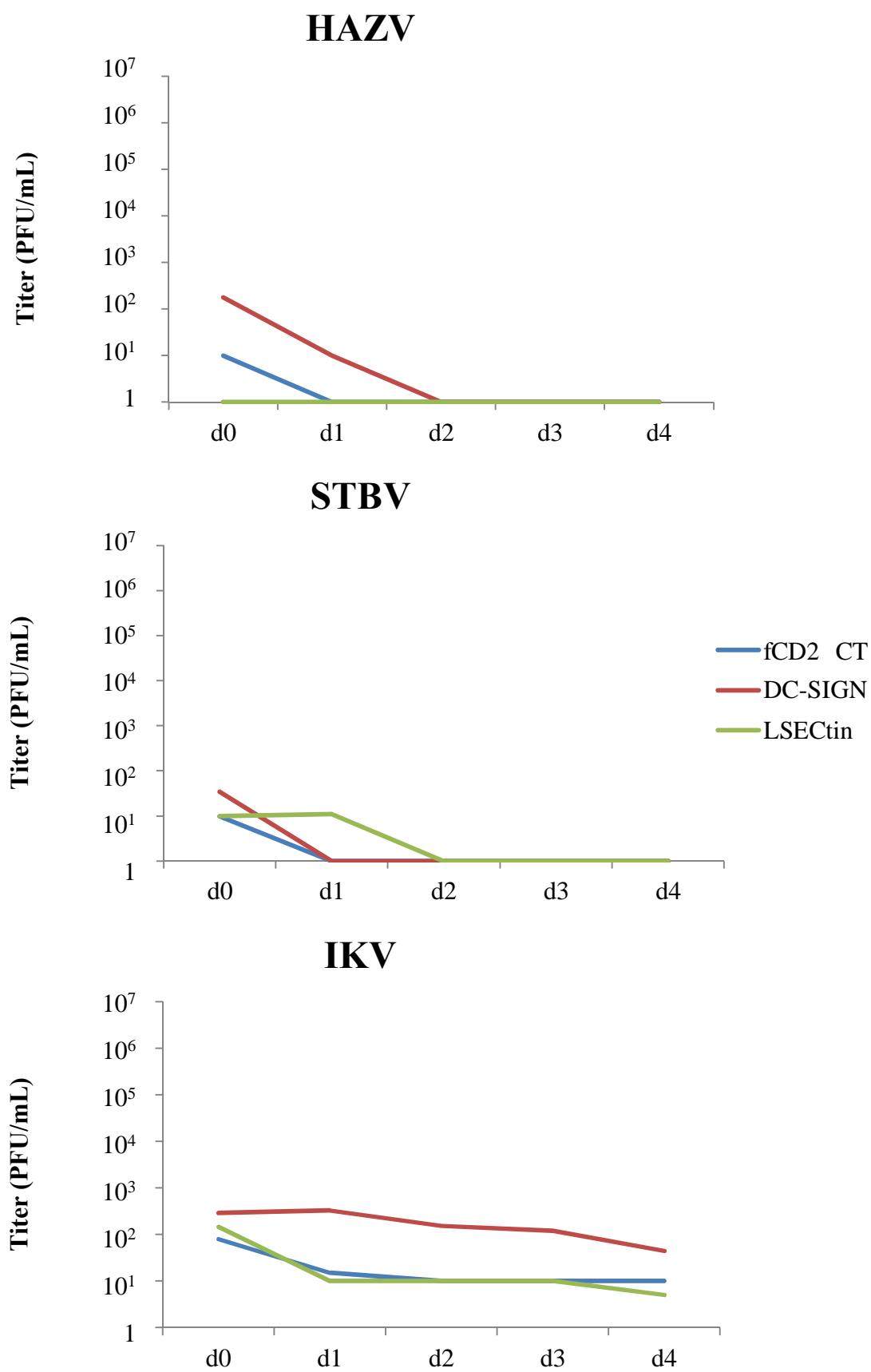
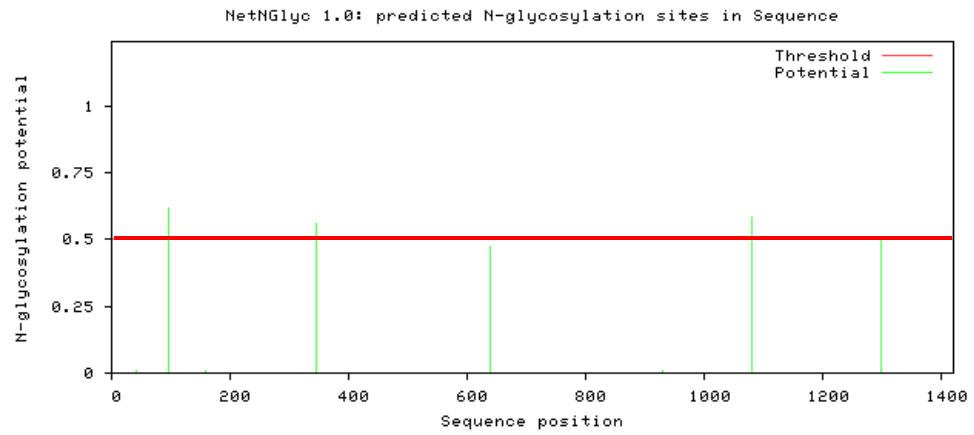


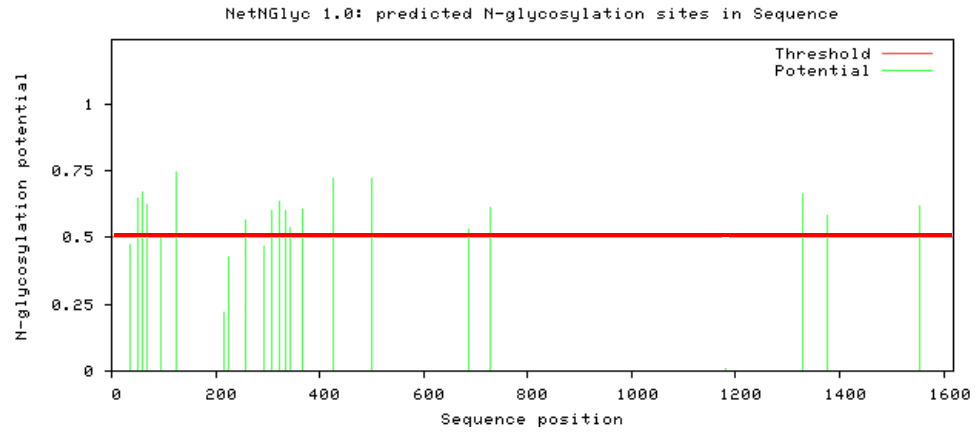
Fig. 3.3 Continued.

(d)

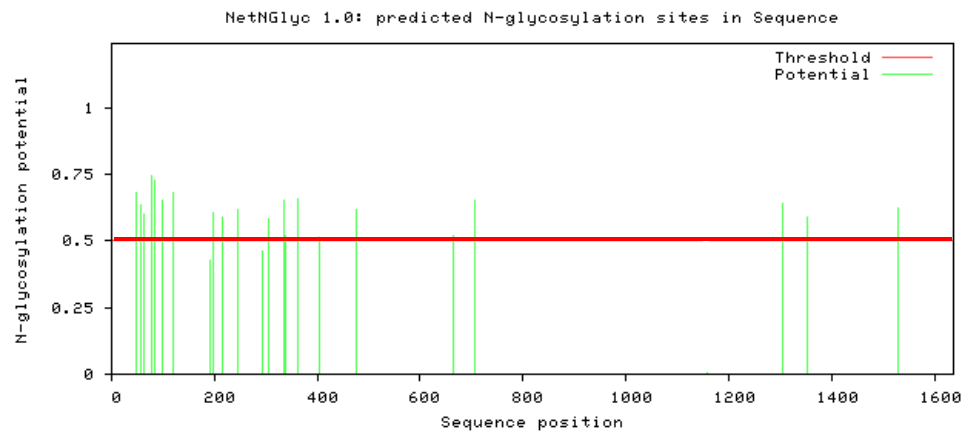
HAZV



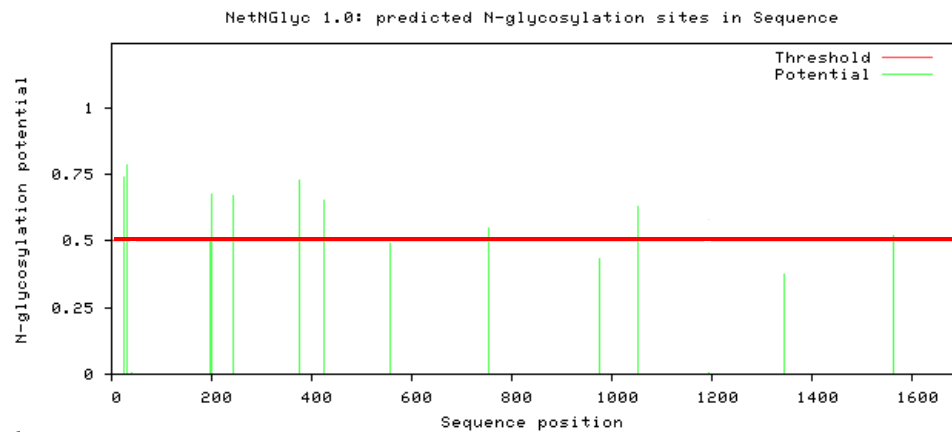
STBV



IKV



CCHFV

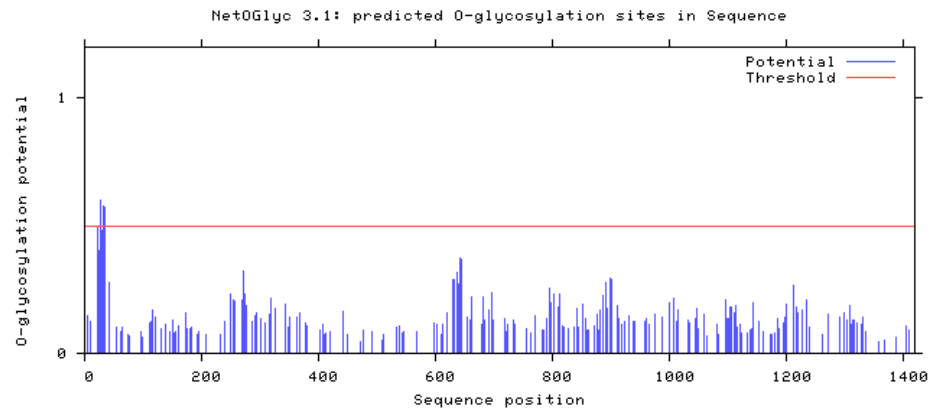


*Fig. 3.3 Continued.*

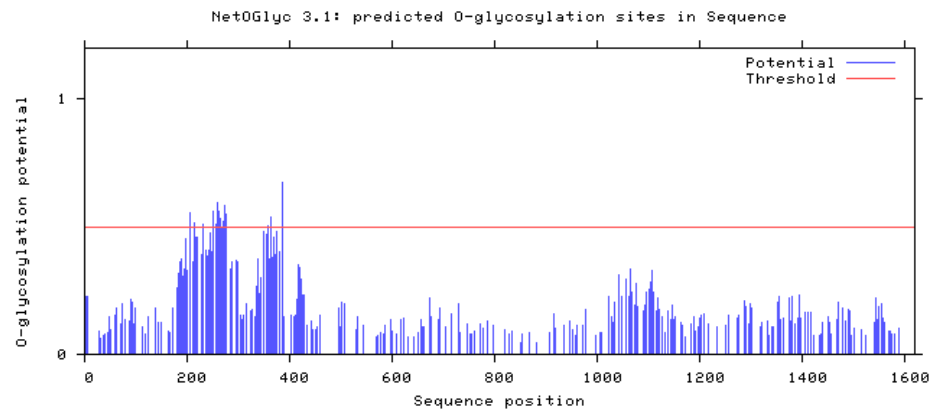
Prediction results of N-glycosylation sites (d) and O-glycosylation sites (e) in the nairovirus GPs were generated with the prediction server NetNGlyc 1.0 and NetOGlyc 3.1, respectively. Where the potential is greater than the threshold, the glycosylation is predicted for that site. The higher potential means the higher confidence of the prediction. The predicted data was partially modified to facilitate visualization.

(e)

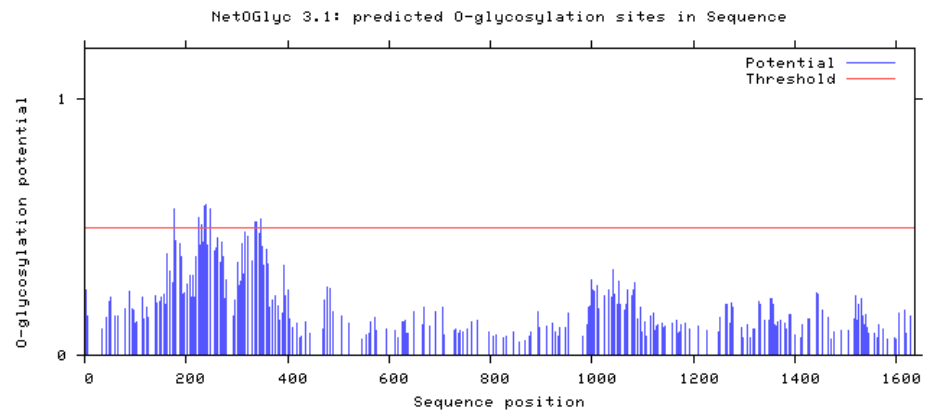
HAZV



STBV



IKV



CCHFV

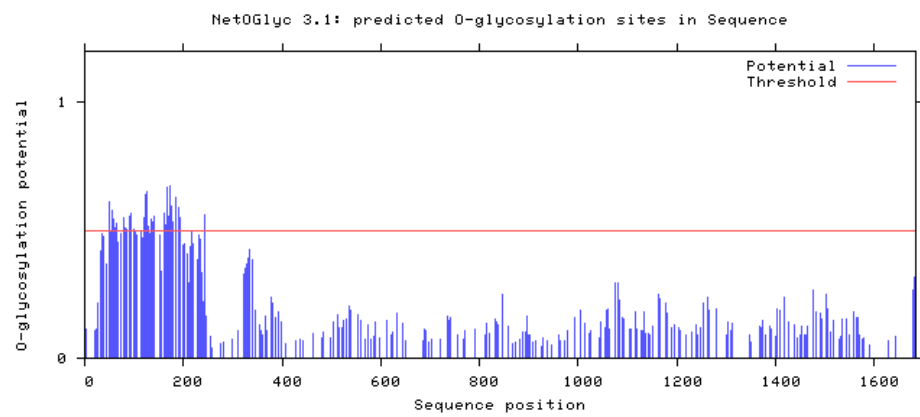
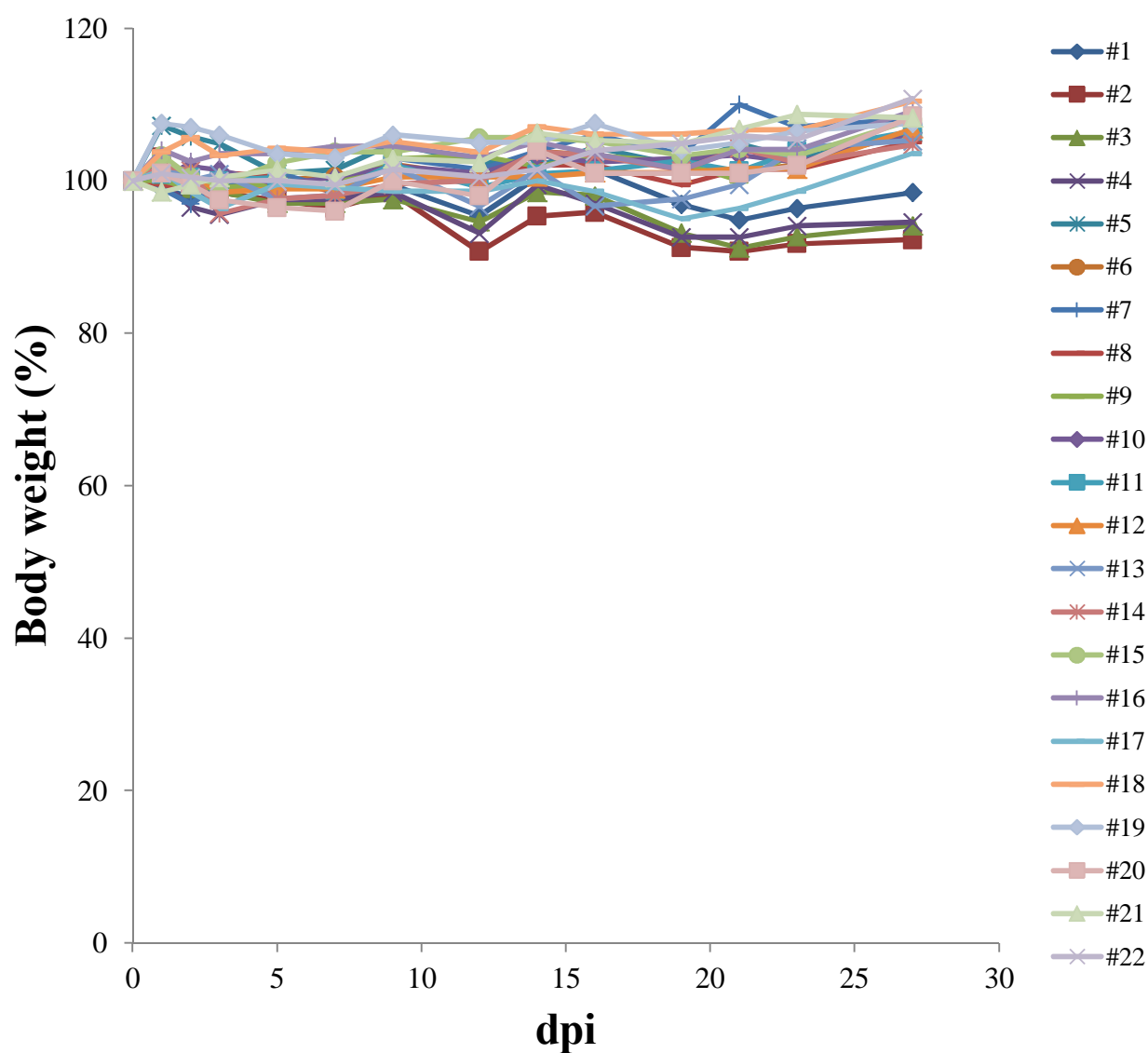
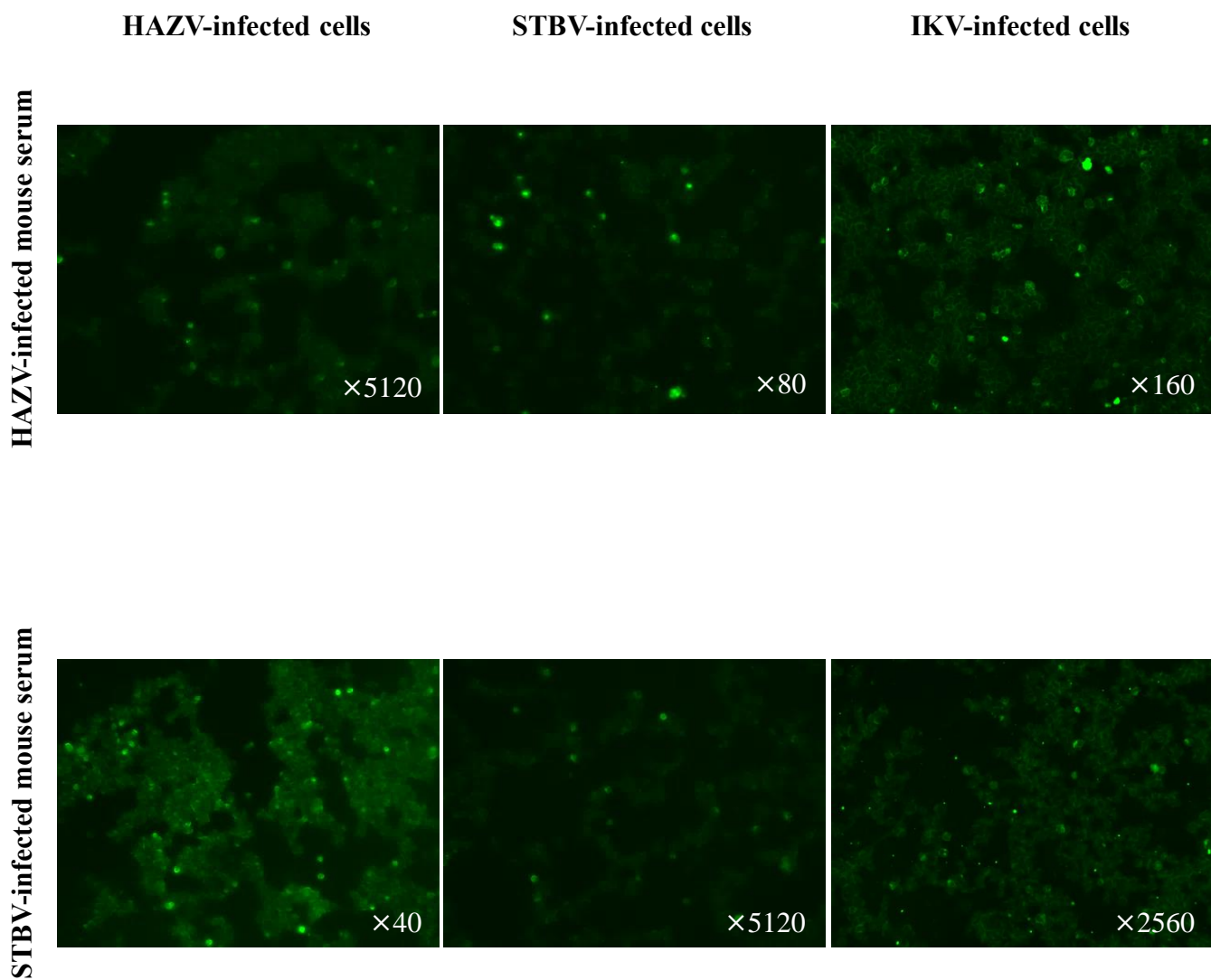


Fig. 3.3 Continued.



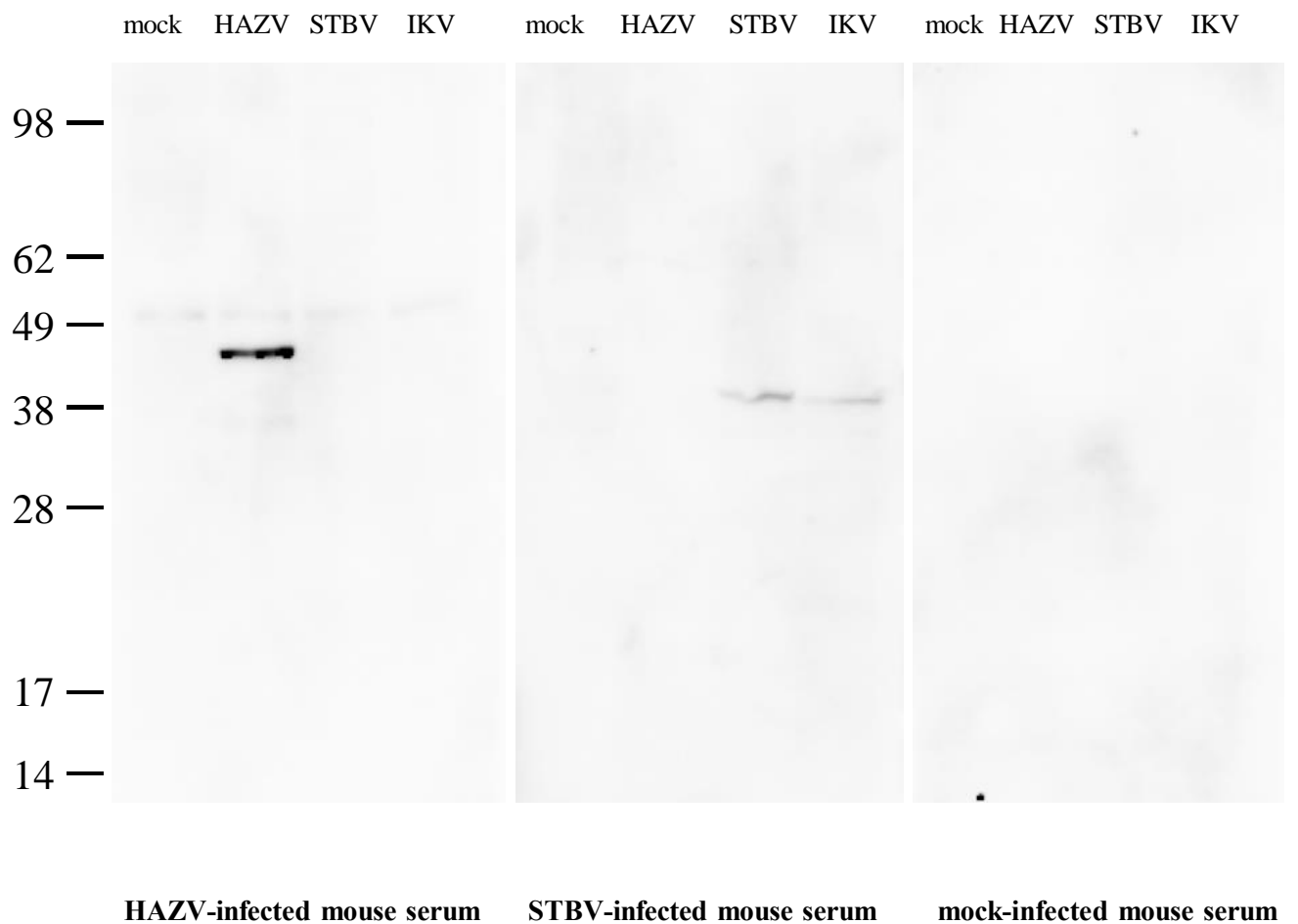
**Fig. 3.4** Body weight changes of the virus infected mice

The body weights of the mice infected with HAZV, STBV or mock were measured at 0, 1, 2, 3, 5, 7, 9, 12, 14, 16, 19, 21, 23, and 27 dpi. The values of (the body weight of the day/the body weight of day 0) x 100 were plotted on y-axis. The list of the infected mouse IDs, the viruses and the infectious routes was shown in Table 3.1.



**Fig. 3.5** IFA for the nairoviruses

The images of the results of IFA using the maximum diluted sera are shown. SW13 cells were infected with HAZV, STBV, and IKV at m.o.i of 2. After 1 day, the infected cells spotted onto 24-well slide glasses were fixed, permeabilized, probed, and examined under a fluorescent microscope.

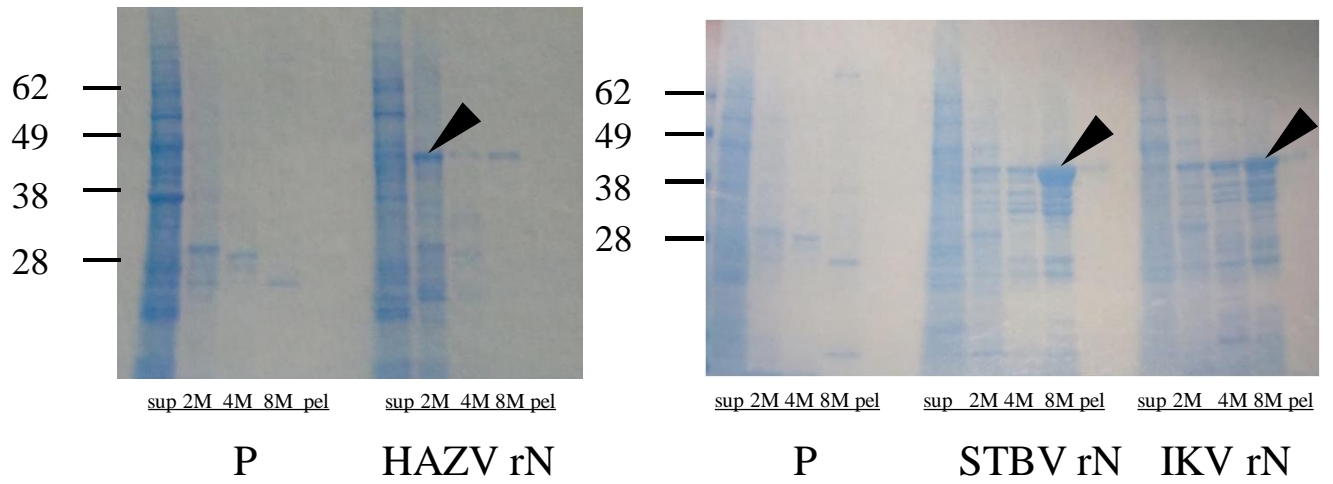


**Fig. 3.6** Immunoblotting for the nairovirus-infected cells

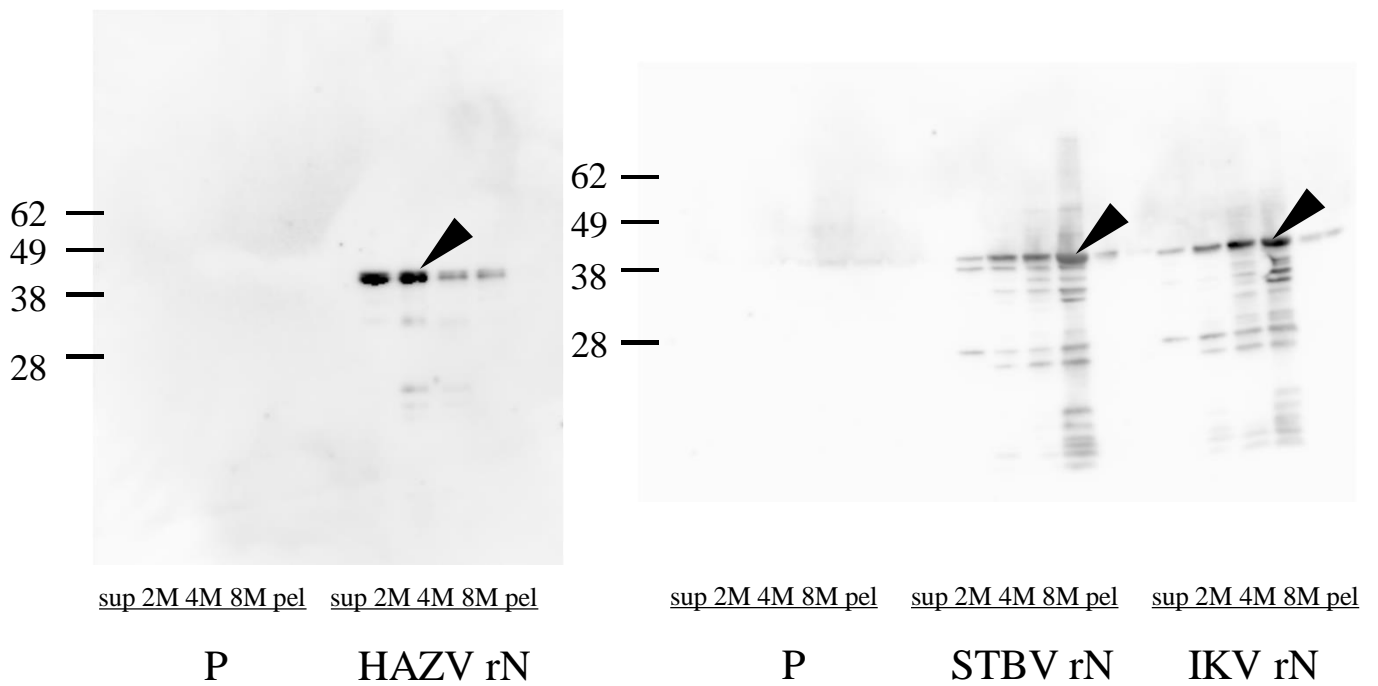
SW13 cells were infected with HAZV, STBV, and IKV at m.o.i. of 2. The cell lysates were prepared at 3 dpi, separated by SDS-PAGE, and electro-blotted to a PVDF membrane, followed by immunodetection with mouse serum infected with HAZV or STBV.



(a)



(b)



**Fig. 3.7** rN-based IgG ELISA for the nairoviruses

Tn5 cells were infected with the recombinant baculovirus and incubated at 26°C for 3 days. Then, the cells were lysed sequentially in 1% NP-40 in PBS, 2M Urea in PBS, 4M Urea in PBS, and 8M Urea in PBS, and the supernatant fractions were used as protein solutions. Samples were analyzed by SDS-PAGE and stained with Bio-Safe Coomassie (a) and by immunoblotting using anti-penta-His antibody (b). Arrow head indicated the rN protein.

(c)

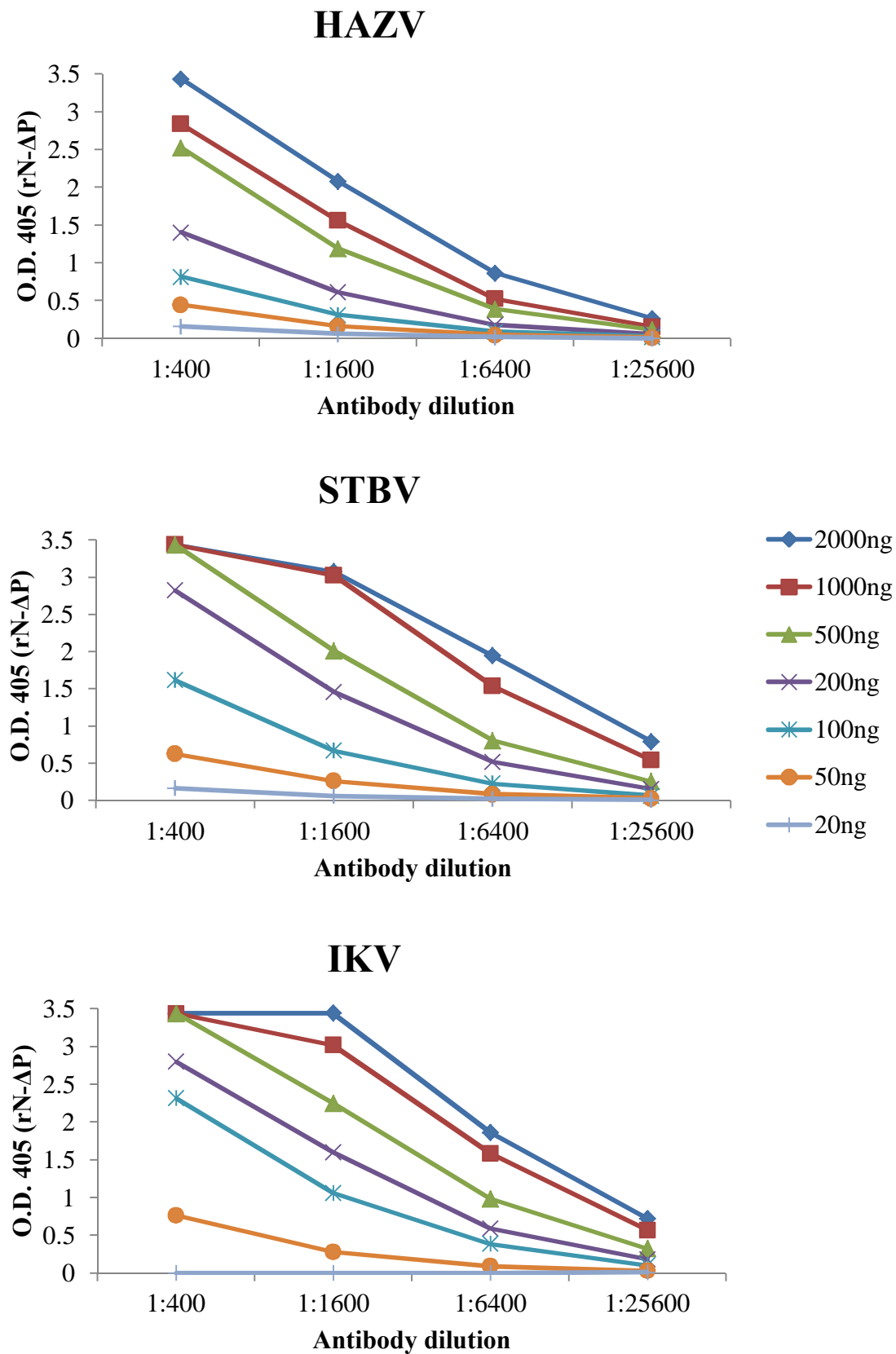


Fig. 3.7 Continued.

(c) The optical concentrations of protein coating were determined by the ELISA using anti-penta-His antibody. rN proteins were coated on the plate with various concentration as indicated. rN proteins were detected by anti-penta-His antibody diluted as indicated.

(d)

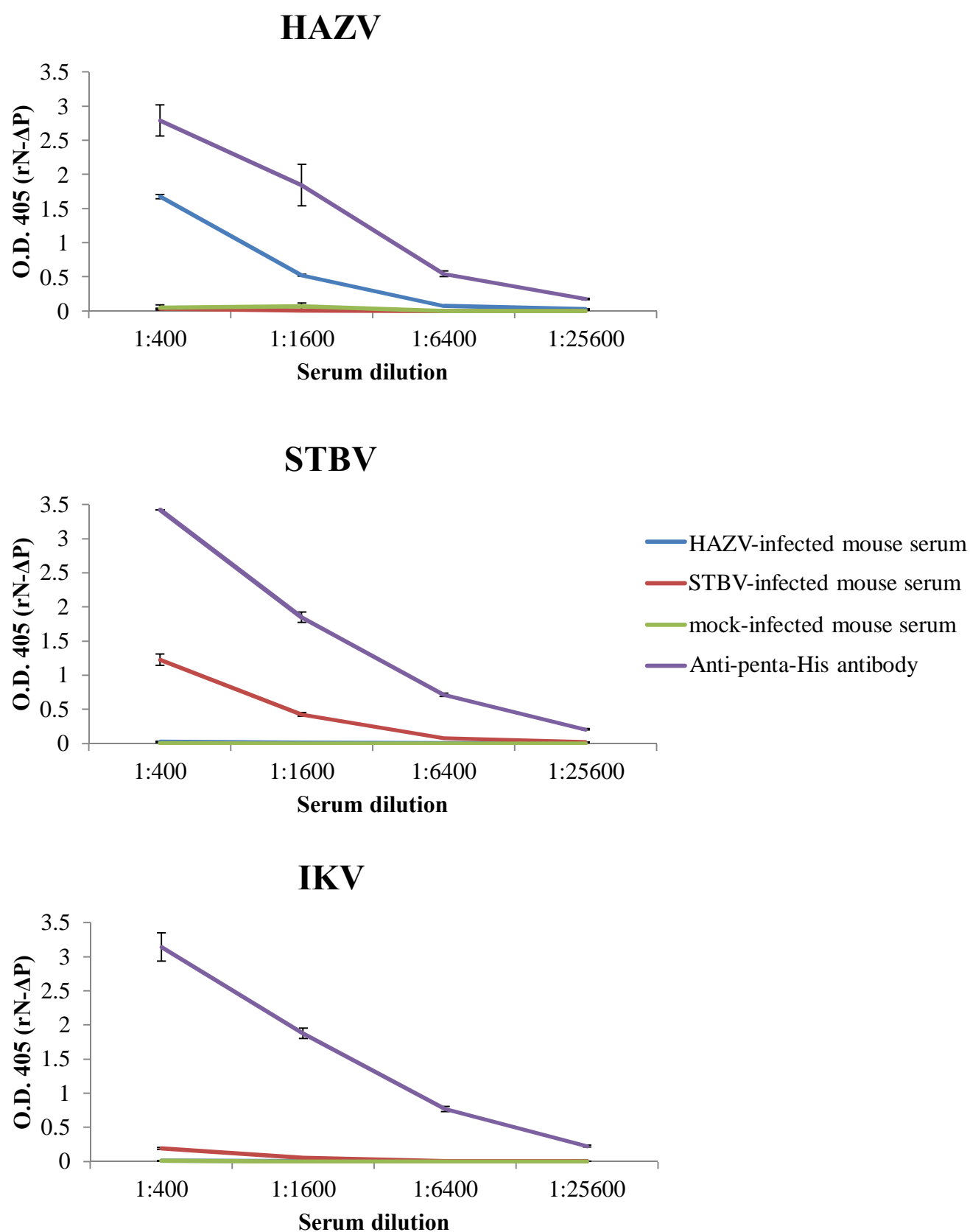
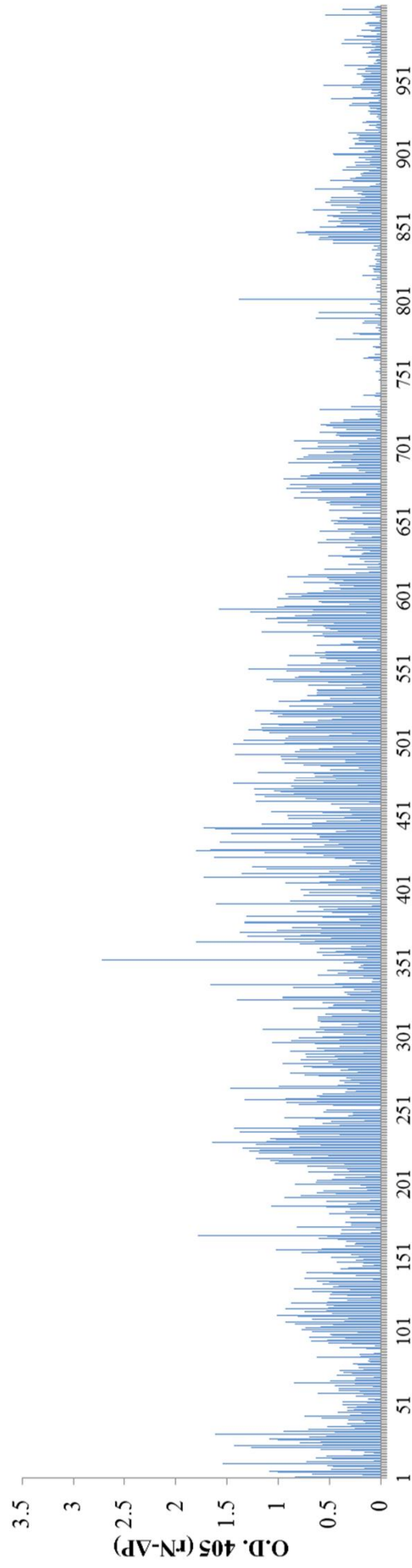


Fig. 3.7 Continued.

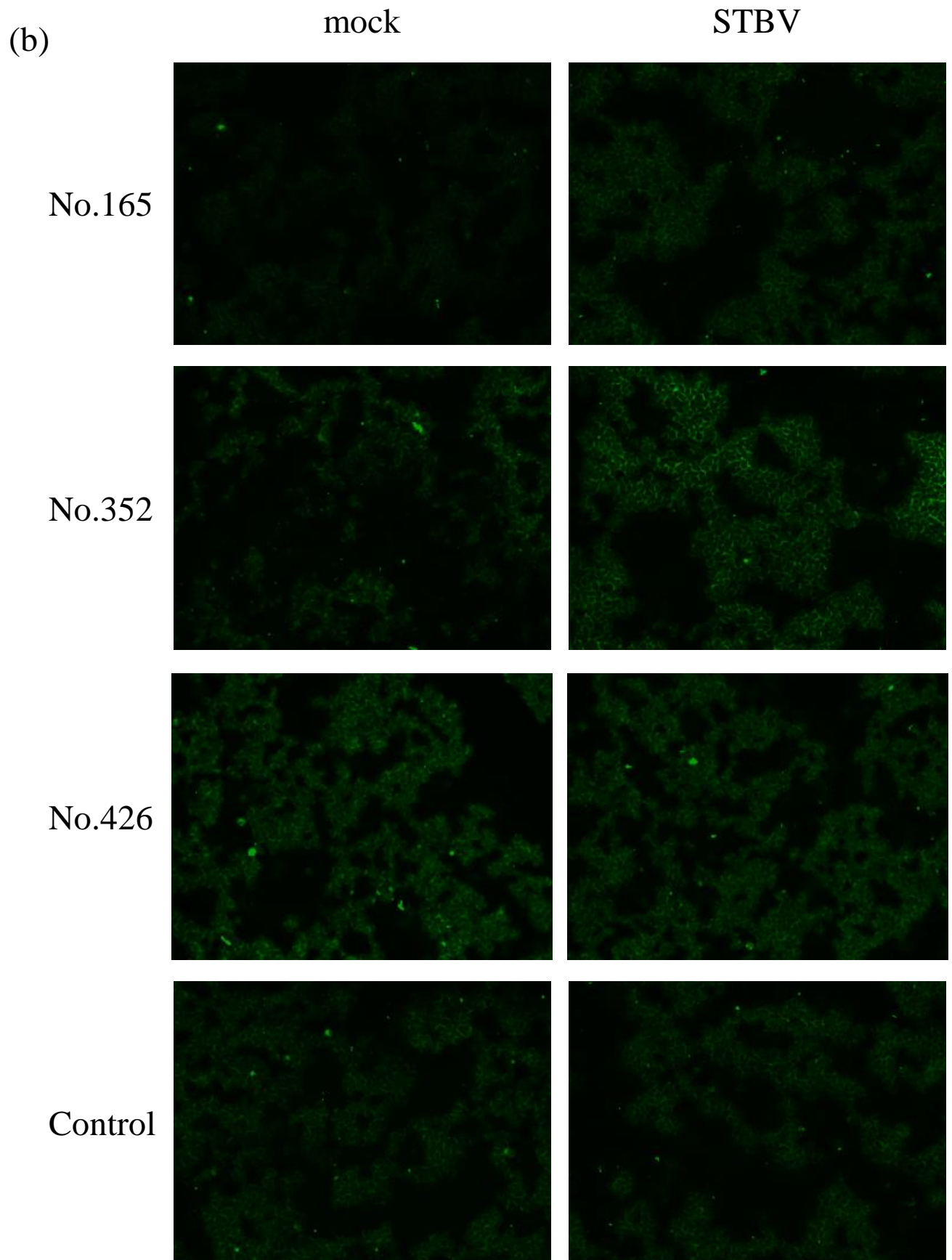
(d) rN proteins were coated on the plate with the predetermined optimal quantity of the rN (approximately 2,000 ng/well for HAZV rN and 500 ng/well for STBV and IKV rN). rN proteins were detected by the HAZV- or STBV- infected mouse serum diluted as indicated.

(a)



**Fig. 3.8** Surveillance for STBV infection

(a) STBV rN-based IgG ELISA was performed using the diluted human sera obtained from the serum bank of NIID (final dilution: 1:100).



*Fig. 3.8 Continued.*

(b) SW13 cells were infected with STBV at m.o.i. of 2 and collected at 1 dpi. The collected cells were spotted on the slide, fixed, permeabilized, probed with the ELISA positive sera, and examined under fluorescent microscope. The images of the result of three samples (No. 165, 352, and 426) and one negative control were shown as examples.

## **General Conclusion**

Nairovirus is the genus of *Bunyaviridae* including zoonotic pathogens such as CCHFV, NSDV, and IKV. CCHFV is a representative of the nairovirus but requires BSL-4 containment. The characteristics of CCHFV and other nairoviruses have not been well-understood. The laboratory diagnosis of CCHFV infection was also limited. Without BSL-4, viral isolation and neutralization assay could not be performed. As it is preferable that the diagnosis for highly pathogenic diseases should be determined by multiple diagnostic assays especially in non-endemic region including Japan, novel assay systems and tools for CCHFV diagnosis are required.

Nairovirus comprises seven serogroups based on the antigen cross-reactions. However, at present, many nairoviruses including IKV and STBV have not been classified. For the well-understanding, virological and serological studies for nairoviruses including the novel viruses are needed.

In this thesis, the author focused on the characterization of CCHFV and other nairoviruses and established novel serological assays for these virus infections.

For the first, in Chapter 1, the author generated the pseudotype virus bearing CCHFV envelope GP (CCHFVpv) and analyzed the entry mechanism of CCHFV using it. It was revealed that the partial deletion of the CCHFV GP cytoplasmic domain increased the titer of the pseudotype virus, which had an entry mechanism similar to that of authentic virus. Therefore, CCHFVpv was considered to be beneficial to the analysis of CCHFV entry mechanisms. With the use of the pseudotype virus, the author investigated the effect of the C-type lectins, DC-SIGN and LSECtin, on CCHFVpv entry. As a result, CCHFVpv infection was enhanced by expression of DC-SIGN and it was suggested that CCHFV uses DC-SIGN as an entry factor. This is the first report of the generation of the high titer CCHFVpv and the effect of DC-SIGN on CCHFVpv entry. This

pseudotype virus will improve the molecular study of the CCHFV infection mechanism.

In Chapter 2, the author applied CCHFVpv generated in Chapter 1 to neutralization assay. The assay detected the antibodies to CCHFV with high specificity and high sensitivity. The results of the neutralization assay using this pseudotype virus and authentic virus suggested that CCHFVpv detected the neutralizing antibody properly. The sensitivity of this assay was lower than that of the recombinant N (rN)-based immunoglobulin G (IgG) ELISA, which detects the antibody against CCHFV N. Correlation between the results in the neutralization assay and those in the ELISA suggested that the antibody-productivity varied in CCHF patients. This suggested that it was needed to use multiple assays with various antigens for more precise diagnosis. As the pseudotype-neutralization assay has advantages of safety and earliness in comparison with the existing methods, CCHFVpv can be a valuable surrogate model for the diagnosis measuring the neutralizing antibody in serum.

Recently, many novel nairoviruses such as STBV were found. The comparison of nairoviruses including the novel viruses will reveal the features of nairovirus in detail and the revealed features will be valuable for controlling nairovirus infections. In Chapter 3, the author compared the virological and serological characteristics among HAZV, STBV, and IKV. The sequencing of the STBV genome revealed that STBV and IKV, but not HAZV were genetically closely related to each other. The results of the growth kinetics suggested STBV and IKV have similar replication features *in vitro*. The cross-reactivity of serum prepared against STBV to IKV was observed in IFA, immunoblotting, and rN-based ELISA. This suggested that STBV and IKV were closely related to each other virologically and serologically. In contrast, the cross-reactivity of serum against STBV to HAZV was very weak. Therefore, it was considered that STBV and IKV belong to a serogroup



different from CCHF serogroup including HAZV. The expression of DC-SIGN and LSECtin enhanced the replication of STBV and IKV, in contrast, the replication of HAZV was not enhanced. CCHFVpv entry was enhanced by expression of DC-SIGN but not LSECtin (Chapter 1). These results together suggested that there is a variety of the rule of the lectins on their replications and that there is a variety in nairovirus characters of the replication.

Furthermore, the author generated serological assays for HAZV, STBV, and IKV infections. While it is uncertain that STBV has pathogenicity on human infection, the serosurveillance of STBV infection using the generated assays revealed that the risk of infection was no or rare case. The generated assays will be useful tools to investigate the infectious risk in detail.

In conclusion, the author performed characterizations of CCHFV and some other nairoviruses, and established the serological assays for these viruses. The pseudotype virus generated in this thesis is a powerful tool for analysis of CCHFV entry mechanisms and the serological diagnosis. The comparative study of the nairoviruses was valuable for understanding of nairoviruses and the generated serological assays will be utilized for the diagnosis and the serosurveillance of the infections. Using the generated diagnostic assays in combination with other diagnostic methods such as ELISA and IFA will make more precise results. The effects of the lectins on nairovirus infections were firstly reported in this study. It was suggested that the rule of the lectins and the entry mechanisms were various in nairoviruses.

Nairovirus is a complicated virus group because of the variety of the immunoresponse in the patients and the infected animals and of the divergence of the viral characteristics. The results of the viral characterization and the generation of the diagnostic assays in this thesis will make a substantial contribution to the control of nairovirus infections.

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## **Summary in Japanese**

論文の内容の要旨

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論文題目

**Studies on characterization of nairovirus and  
establishment of laboratory diagnostic systems**

(ナイロウイルスの性状解析および実験室診断系の構築に関する研究)

ナイロウイルスはブニヤウイルス科に属するウイルス属である。本ウイルス属には、人獣共通感染症の原因であるクリミア・コンゴ出血熱ウイルス（CCHFV）やナイロビ羊病ウイルス、Issyk-Kul ウイルス（IKV）といった病原体が属する。CCHFV はナイロウイルス属の代表的なウイルスであり、CCHF を引き起こす。CCHF はヒトでの致死率が 30% 近くにも上る重篤な出血熱であり、その症例報告は地域、数ともに増加しており問題となっている。一方、CCHFV はバイオセーフティレベル（BSL）4 施設での取り扱いが求められるウイルスであり、そのため CCHFV を含むナイロウイルスの研究は未解明な部分が多い。CCHF の診断も、ウイルス分離や中和試験は感染性ウイルスを扱うため、BSL-4 施設がなければ行えない。CCHF のような重要な疾病に対しては複数の実験系による診断が、非流行国である日本などでは望まれており、それに用いられる新規の実験系の構築や、研究に用いることができる新規のツールの構築が求められている。

ナイロウイルスは 7 つの血清型に分類される。例えば、CCHFV は Hazara ウイルス (HAZV) と CCHF グループを構成している。しかし、新規ウイルスの発見とともに IKV や Soft tick bunyavirus (STBV) など未分類のウイルスも多くなっている。ナイロウイルスの詳細な理解のためには、これらのウイルスの性状解析や血清学的研究が必要となり、その解析はナイロウイルス感染症の制御に貢献する。

本研究では、代表的なナイロウイルスである CCHFV および新規ナイロウイルスを含む他のナイロウイルスの性状解析と血清学的診断法の構築を行った。

第一章では、新規に作製した CCHFV のシュードタイプウイルスを用いて感染機構の解析を行った。シュードタイプウイルスとは、目的ウイルスのエンベロープ蛋白質を被ったウイルスであり、水疱性口炎ウイルス (VSV) やレトロウイルス、レンチウイルスなどがそのベースに用いられる。これらシュードタイプウイルスは、BSL-2 施設での目的ウイルスの侵入機構の解析、目的ウイルスそのものを用いない代替法としての中和試験による血清診断を可能とするものとして、これまで様々なウイルスで利用されてきた。しかし、これまでに高力価を示す CCHFV のシュードタイプウイルスの作製はなされていなかった。そこで、ウイルス粒子への取り込みを制御しうる CCHFV のエンベロープ蛋白質 (GP) の C 末端領域を一部欠損させたものを作製に用いることで、VSV ベースの高力価の CCHFV シュードタイプウイルスの作製を試みた。さらに、それを用いて代表的な C 型レクチンである DC-SIGN と LSECtin について、その CCHFV の細胞への感染における影響を解析した。その結果、CCHFV の GP の C 末端を 53 アミノ酸欠損させたものを作製に用いることで、高力価のシュードタイプウイルスの作製が可能となることが明らかとなった。作製したシュードタイプウイルスを用いた解析の結果、野生型の CCHFV と似た感染性状を示し、さらにその感染は DC-SIGN によって増強されたため、CCHFV の感染が DC-SIGN を発現する樹状細胞やマクロファージなどに起こりやすいことが示唆された。

第二章では、第一章で作製した CCHFV シュードタイプウイルスを用いて、血清診断法である中和試験系を構築した。中和試験は特異性が高く、ウイルスの同定にも利用される診断法である。CCHF 流行地で提供されたヒト血清を用いて中和試験を行い、その結果を現在確立されている診断法である組換え核タンパク質を抗原とした ELISA の結果と比較した。その結果、構築した中和試験系は、ELISA と比較すると感度は劣るものの、高い特異性で CCHFV 抗体を検出できることが示された。さらに CCHFV そのものを用いた中和試験と比較して、同一血清で得られる抗体力価に大きな差はなく、シュードタイプウイルスを用いた中和試験系は BSL-2 施設で可能な代替中和試験法として使用できることが明らかとなった。

近年、新規のナイロウイルスの分離報告が複数なされている。ナイロウイルス全体のさらなる理解には、新規ウイルスを含めた性状解析が必要となる。そこで第三章では、HAZV、STBV、IKV の 3 種類のナイロウイルスについて、そのウイルス学的、血清学的な性状を比較した。STBV は、2014 年に日本でコウモリマルヒメダニから分離された新規ナイロウイルスである。IKV は、ヒトに発熱やめまい、吐き気を引き起こす Issyk-Kul 熱の病原体であり、中央アジアでその存在が認められているが研究報告は極めて少ない。HAZV は、遺伝学的、血清学的に CCHFV に近縁だが非病原性で、BSL-2 施設での取り扱いが可能なウイルスである。血清学的な比較にあたり、新規に ELISA 系の構築も行った。STBV のゲノム解析から、STBV と IKV が遺伝学的に近縁であることが明らかとなった。培養細胞でのウイルス増殖性の比較の結果、STBV と IKV は類似の性状を示したが、HAZV ではこれらとは異なるウイルス増殖性が認められた。また、血清学的な解析においても、STBV と IKV の間には抗原交叉性が認められたが、HAZV との抗原交叉性は非常に低かった。これらの結果は、ナイロウイルス間におけるウイルス学的、血清学的多様性の存在を示してい

と考えられた。さらに、構築した血清診断系を用いてヒトの STBV 感染の血清疫学解析を行った。その結果、ヒトの STBV 感染リスクはない、もしくは極めて低いことが示された。構築した血清診断系は、今後のさらなる疫学解析や診断に有用なツールとなることが期待される。

本論文において、筆者はナイロウイルスの性状解析ならびに血清診断系の構築を試みた。構築したシュードタイプウイルスは高力価を示し、CCHFV の侵入機構の解析や、代替中和試験への利用などに強力なツールとなると期待される。また、新規ナイロウイルスを含む比較解析によりナイロウイルスの多様性が明らかとなったが、比較にあたって構築した ELISA 系などの血清診断系は、動物感染症学的かつ公衆衛生学的な見地において、ナイロウイルス感染症の診断や疫学解析に力を発揮することが期待される。以上のように、本論文で得られた知見は、ナイロウイルス感染症の理解と制御に貢献するものである。