Study on serological diagnostic assays for Crimean-Congo hemorrhagic fever

（クリミア・コンゴ出血熱の血清診断法に関する研究）

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Abbreviations

Arbovirus	arthropod-borne virus
BCA	Bicinchoninic Acid Assay
BSL4	Biosafety level 4
CBB	Coomassie brilliant blue
CCHF	Crimean-Congo hemorrhagic fever
CCHFV	Crimean-Congo hemorrhagic fever virus
cDNA	complementary deoxyribonucleic acid
Ch	chimeric
CI	confidence intervals
CO_2	carbon dioxide
Conc	concentrations
CsCl	cesium chloride
DAPI	4',6-diamidino-2-phenylindole, dihydrochloride
DMEM	Dulbecco's Eagle Medium
DMSO	dimethyl sulfoxide
DUGV	Dugbe virus/Dugbe fever virus
EBOV	Ebola virus
EDTA	ethylenediaminetetraacetic acid
E. coli	Escherichia coli
ELISA	Enzyme-Linked Immunosorbent Assay
EM	electron microscopy
FBS	fetal bovine serum
HCl	hydrochloric acid
HEK293T	human embryonic kidney 293T
HeLa cells	Henrietta Lacks' 'Immortal' Cells
HRPO	horseradish peroxidase-conjugated
IgG	immunoglobulin G
IgG(γ)	immunoglobulin G subclass gamma
IBC	Institutional Biosafety Committee
Mk	CCHF-infected monkey serum
mAb	monoclonal antibody
MIAF	mouse immune ascites fluids
Min	minute
NIAID	National Institute of Allergy and Infectious Diseases
NIH	National Institutes of Health
NP	nucleoprotein
NP-40	Nonidet P-40

NP-RNA	ribonucleic acid-nucleoprotein complex
NSDV	Nairobi sheep disease virus/Ganjam virus (Asia)
OD	optical density
PBS	phosphate-buffered saline
PBST	PBS containing 0.5% Tween-20
PCR	polymerase chain reaction
RNA	ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SOP	Standard operating procedure
SW-13	Scott and White n°13 cells
TEM	transmission electron microscopy
TMB	3,3',5,5' Tetramethylbenzidine
Tris- HCl	Tris (hydroxymethyl) aminomethane hydrochloride
UA	uranyl acetate
WHO	World Health Organization
WRCEVA	World Reference Center for Emerging Viruses and Arboviruses

Notes

The contents of chapter I have been published in the journal Nature Scientific Reports.
Lombe BP, Miyamoto H, Saito T, Yoshida R, Manzoor R, Kajihara M, Shimojima M, Fukushi S, Morikawa S, Yoshikawa T, Kurosu T, Saijo M, Tang Q, Masumu J, Hawman D, Feldmann H, Takada A. Purification of Crimean-Congo hemorrhagic fever virus nucleoprotein and its utility for serological diagnosis. Scientific Reports 11, 2324, 2021.

Preface

Crimean-Congo hemorrhagic fever (CCHF) is an important tick-borne zoonotic disease with a wide geographic distribution that affects people coming in contact with infected ticks and animals. CCHF is endemic in Africa, Asia, the Middle East, and Southern Europe [20, 41, 43]. It is caused by the CCHF virus (CCHFV) belonging to the genus *Orthonairovirus*, family *Nairoviridae*, order *Bunyavirales*. This genus also includes Nairobi sheep disease virus (NSDV) and Dugbe fever virus (DUGV), both of which are associated with human diseases [8, 26, 34, 63]. NSDV is an orthonairovirus of veterinary importance causing a severe hemorrhagic and abortive disease in sheep and goats [67].

CCHFV is transmitted by tick bites or direct contact with viremic animals and humans or contaminated materials. Human-to-human transmission occurs when proper infection control practices do not take place [44]. Depending on the route of infection, the incubation period is 2-7 days on average and the subsequent onset of symptoms is abrupt (less than one week). Although CCHFV infection is predominantly asymptomatic in animals and birds regardless of persistent viremia, infected subjects are potential sources of human infection. Furthermore, CCHFV has the potential for bioterrorism [5, 8, 22, 40, 63]. CCHFV infection of humans is often fatal (3-80%) with acute and severe hemorrhagic manifestations, but the initial symptoms are generally nonspecific (fever, fatigue, myalgia, headache, diarrhea, etc.) followed by progressive hemorrhage, shock and multiorgan failure in severe cases [18, 38, 45, 61, 63]. Fatal outcome is correlated to the increased viral load and dissemination, intravascular coagulopathy, and multi-organ failure [71]. CCHFV-specific vaccines and approved therapies are still unavailable, and supportive care remains the main stay of treatment [41, 71].

CCHFV is maintained in horizontal and vertical enzootic cycles involving *ixodes* spp ticks, ground frequenting birds and small mammalians [18]. It is continually spreading widely as evidenced by its association with the increasing distribution of its principal vector, *Hyalomma* ticks. CCHFV isolation and antibody detection are also continuously reported widely. CCHF was first described in the Crimean region in 1944-1945 and the causative virus was isolated for the first time in 1956 from a teenage boy in Kisangani in the Belgian Congo (the actual Democratic Republic of the Congo) [20, 64]. Since then, infectious CCHFV strains have been isolated from ticks and CCHF patients and CCHFV-specific antibodies have also been detected in birds (ostriches), domestic and wild animals such as sheep, goats, cattle, horses, donkeys, camels, pigs, hares, hedgehogs, and ground squirrels in different geographical regions, demonstrating

scientific evidence on vector, reservoir species, and virus dynamics in the nature [13, 18, 20, 38, 64]. Due to its potential for human-to-human transmission and the lack of prevention measures, the World Health Organization (WHO) ranks CCHF us a Blueprint priority disease necessitating research and development attention [38, 39].

CCHFV surveillance and early detection are essential at the human-animal-environment interface. As animals are CCHFV-amplifying hosts, serological surveillance of animals can shed light on the distribution of CCHFV and also serve as an indicator of CCHF risks for humans [13, 20, 59]. Unfortunately, in many endemic regions, CCHFV infection is not monitored due to the limited availability of diagnostic tools. Existing in-house molecular or genetic tools such as the reverse transcription polymerase chain reaction (RT-PCR) are often insensitive or virus lineage-specific (i.e., restricted to circulating regional strains) due to the genetic diversity of CCHFVs and limitedly used for the time point of acute infection [38]. In-house or commercialized serological tools are used only for humans and are not readily available in affected countries or not cost-effective for serological studies [38, 40]. Consequently, the accessibility of CCHFV serological assays for epidemiological studies remains limited in most of the endemic areas because of the absence or shortage of constant production and provision of antigens or specific monoclonal antibodies.

The aim of this study was to contribute to surveillance and epidemiological studies on CCHFV infection of animals and humans. Thus, its main objective was the development of a cost-effective, sensitive, and CCHFV-specific antibody detection assay. The present thesis comprises two chapters. The first chapter is devoted to purification of the CCHFV nucleoprotein and its utility for serologic diagnosis and the second focuses on the mapping of antibody epitopes on the CCHFV nucleoprotein.

Chapiter I: Purification of Crimean-Congo hemorrhagic fever virus nucleoprotein and its utility for serological diagnosis

Introduction

Crimean-Congo hemorrhagic fever virus (CCHFV) has tri-segmented negative-sense RNA genome that consists of, according to their sizes, Large (L), Medium (M), and small (S) segments encoding RNA-dependent RNA polymerase, glycoproteins Gn/Gc, and nucleoprotein (NP), respectively [11, 71].

CCHFV requires the highest level of biocontainment (Biosafety level 4), hampering its handling for experimental studies. Thus, there are only a few laboratories that can use infectious CCHFV for virus isolation and production of its whole native viral antigens for serological diagnostic assays that are expected to be less affected by genomic diversity of CCHFVs than genetic detection [21, 22, 38, 40].

Previous studies have shown that some of the CCHFV structural proteins including NP are the predominant components that are antigenically conserved among CCHFV strains [35, 42, 53] and induce a high immune response [15, 38]. Recombinant CCHFV NPs expressed in insect, bacterial, plant, and mammalian cells have been used as antigens for serological assays [9, 17, 53, 54]. However, the expression and purification processes often affect its conformation, structure, and antigenicity [3, 35, 52, 68]. Herein, I report a simple procedure for expression and purification of the recombinant CCHFV NP in mammalian cells and its utility as an antigen for host species-independent serological assays for detection of CCHFV-specific immunoglobulin G (IgG) in serum/plasma samples.

Methods

Plasmids and expression of CCHFV NP

CCHFV (strain IbAr10200) was propagated in Vero E6 cells in the BSL4 Laboratory of the Rocky Mountain Laboratories (RML), National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH). RNA extraction was performed according to standard operating protocols approved by the RML Institutional Biosafety Committee (IBC). Extracted RNA was used to synthesize complementary deoxyribonucleic acid (cDNA) using a SuperScript III reverse transcriptase kit (Invitrogen). The full-length NP gene was amplified using specific primers and cloned into a mammalian expression vector, pCAGGS/MCS. The NP sequence was confirmed by Sanger sequencing with a 3130xl/Genetic Analyzer (Hitachi/Applied Biosystems). The Nairobi sheep disease virus (NSDV) NP gene (GenBank Accession number: NC_034386.1) was synthesized in pUCFa vector (Fasmac CO., LTD) and similarly cloned into the pCAGGS/MCS vector. Human embryonic kidney 293T (HEK293T) cells seeded at 3-3.5 × 10^5 cells/ml were grown on a 12-well plate in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 0.1 mg/ml streptomycin at 37°C in a 5% CO_2 incubator for 24 h. The cells were transfected with the plasmid (1 µg) using a TransIT-LT1 Transfection Reagent (Mirus Bio LLC) and then incubated at 37°C for 48 h. The cells were washed 3 times with phosphate-buffered saline (PBS), treated with 250 µl of a lysis buffer [150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl (pH 8.0), 1.0% NP-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS) with cOmplete, Mini, EDTA-free protease inhibitor (Roche Diagnostics)] and incubated for 5 min on a swing rotator. The cell lysate was centrifuged at 13,000 *g* (TOMY MX-201 Micro centrifuge) for 10 min at 4°C. The supernatant was collected and used for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. The generation of recombinant CCHFV NP and NSDV NP was approved by the Ministry of Education, Culture, Sports, Science, and Technology, Japan. The generation of recombinant proteins was approved by the Ministry of Education, Culture, Sports, Science, and Technology, Japan (元受文科振 235 号).

Serum samples

A rabbit antiserum to CCHFV NP was used as a positive control serum [53]. A rabbit antiserum to Ebola virus (EBOV) NP [12] was also used as a negative control rabbit serum. Mouse antisera were obtained by immunizing animals intraperitoneally with

purified CCHFV and NSDV NPs (40-50 μg) twice at 4-week intervals followed by serum collection 2 weeks after the second immunization. CCHFV (Hoti)-infected and EBOV (Kikwit)-infected monkey sera were treated with gamma-irradiation according to the RML IBC-approved Standard operating procedure (SOP#4-28-27, -28, and -29) before use [19]. These sera were collected from nonhuman primates using animal study protocols approved by the RML Animal Use and Care Committee. Studies were performed in compliance with the Animal Welfare Act and other relevant statutes and regulations relating to animals and experiments involving animals and adhered to the principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 2011. A panel of serum samples previously collected from CCHF-suspected patients during an outbreak in the Xinjiang Uygur Autonomous Region of China was used for validation of assays [55]. The sera used in the present study were collected under informed consent. In the case of unconscious patients and children less than 20 years of age, informed consent was obtained from their family members and parents, respectively. The use of these human sera was approved by the medical research ethics committee of the National Institute of Infectious Diseases for the use of human subjects, Tokyo, Japan (No. 10). All animal experiments were conducted in strict accordance with the Guidelines for Proper Conduct of Animal Experiments of the Science Council of Japan. The protocol was approved by the Animal Care and Use Committee of Hokkaido University on March 30, 2018 (#18-0029). All methods were carried out in accordance with relevant guidelines and regulations.

Immunofluorescent assay

HEK293T cells were cultured for 24 h in a chambered cell culture slide glass coverslip (Thermo Fisher Scientific) pre-coated with Cultrex Poly-L-Lysine (Bio-techne) and then transfected with the NP-expression plasmid or negative control (empty pCAGGS) using TransIT-LT1 transfection reagent (Mirus Bio LLC). At 24 hours post-transfection, cells were washed with cold PBS and fixed with 4% paraformaldehyde for 20 min. After washing with PBS, the cells were incubated for 30 min with PBS supplemented with 1% bovine serum albumin. As primary and secondary antibodies, rabbit antiserum to CCHFV NP and Alexa Fluor 448-labeled donkey anti-rabbit IgG (H+L) (Aurion Immuno Gold Reagents & Accessories) were used, respectively. The cells were also stained with 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI). They were analyzed by confocal microscopy (Zeiss LSM 780) for image acquisition and processing using Zen software (Carl Zeiss Microscopy GmbH).

SDS-PAGE and Western blotting

Samples (i.e., cell lysates, fractions of cesium chloride [CsCl] gradient centrifugation, and purified NP) were mixed with Laemmli sample buffer (Bio-Rad) with 5% β-mercaptoethanol, boiled for 5 min, and then loaded for 12% SDS-PAGE, followed by Coomassie blue staining with Quick-CBB Plus (Fujifilm Wako Chemical Corporation) and Western blotting. For Western blotting, separated proteins were transferred onto a polyvinylidene membrane (Immobilon-P, Merck), followed by blocking with 3% skim milk in PBS. The membrane was then washed with PBS containing 0.05% Tween-20 (PBST) 3 times and soaked for 1 hours in 1% skim milk in PBST containing the anti-CCHFV NP rabbit serum. After washing with PBST 3 times, the membrane was incubated for 1 hours with horseradish peroxidase (HRPO)-conjugated goat anti-rabbit IgG antibodies (KPL), followed by washing with PBST 3 times. Bound proteins were visualized with a 3,3',5,5'-tetramethyl-benzidine (TMB) liquid substrate system for membrane (Sigma).

Purification of recombinant CCHFV NP

HEK293T cells were seeded at 3-3.5 × 10^5 cells/ml on 10 cm dishes. Twenty-four hours later, the cells were transfected with 10 µg of the plasmid encoding the CCHFV NP gene using TransIT-LT1. Transfected cells were harvested at 48-72 hours post-transfection and washed 3 times with cold PBS by centrifugation 2 times at 4°C. The cells were resuspended with PBS and transferred into a 1.5 ml tube and pelleted. Then PBS was removed, and the cells were stored at -80°C until use. Frozen cells were thawed on ice for 10 min and treated with 600 µl of lysis buffer (10 mM Tris-HCl, (pH 7.8), 0.15 M NaCl, 1.0 mM EDTA, and 0.25% NP-40) in the presence of Halt Protease Inhibitor Single Use Cocktail (Thermo Scientific). Cells were mixed by pipetting and then incubated at 4°C for 30 min with a SCINICS Revolution Mixing rotator (RVM-101). The lysate was centrifuged at 13,000 *g* (TOMY MX-201 Micro centrifuge) at 4°C for 10 min and the supernatant was collected. The supernatant was then loaded on the top of 20-50% (w/v) discontinuous CsCl gradients in Tris-buffered saline (TBS) layered from the top to bottom in 1 ml volumes of 20, 30, 40, and 50% CsCl in a 5 ml centrifuge tube (Beckman), followed by high-speed centrifugation using a SW55Ti rotor in a BECKMAN COULTER Optima L-100 XP ultracentrifuge at 280,000 × *g* at 4°C for 4 hours. Fractions (500 µl) were collected from the top to bottom and then analyzed by SDS-PAGE and Western blotting to check for the presence of CCHFV NP and its purity in collected fractions. CCHFV NP fractions were pooled and diluted with TBS and pelleted by centrifugation

280,000 × *g* for 30-60 min as previously described [6]. The supernatant was discarded and CCHFV NP was resuspended with 100 µl PBS with Halt Protease Inhibitor (Thermo Fisher Scientific). The concentration of purified NP was measured using a Nanodrop ND 1000 spectrophotometer (Thermo Fisher Scientific). Purified NP was re-analyzed by SDS-PAGE and Western blotting. The purified CCHFV NP was then stored at -80°C until use. For large-scale CCHFV NP purification, HEK293T cells were grown in a 150 × 25 mm dish. For purification, CsCl layers (2.2 ml each of 20, 30, 40, and 50%) in Ultra-Clear tubes (Beckman) and an SW41 rotor was used (210,000 × *g* at 4°C for 15 hours). Fractions (1 ml) were collected, and NP fractions were pooled and pelleted at 210,000 × *g* for 2 hours and processed as described above. NSDV NP was also purified in the same methods described above.

Transmission electron microscopy (TEM)

Freshly purified NP was dialyzed against TBS overnight using an EasySep dialysis membrane MD-014-50 (Tomy Seiko) according to the manufacturer's instructions. The protein was then concentrated using Amicon Ultra-0.5mL Centrifugal Filters, Ultracel-3 K (Merck Millipore). Concentrated CCHFV NP samples (5 µl) were applied on a collodion-coated copper grid (Nisshin EM) for 5 min then the excess sample was absorbed using filter paper. A 20 µl drop of 2% uranyl acetate (UA) solution was applied for 10 min. The grid was then treated with new drops of UA solution 2 times for each 1 min. TEM images were observed using a Hitachi H7650 TEM system (Hitachi High Technology Corporation). For immunogold staining, the grid was coated with freshly purified and dialyzed NP (5 µl) prior to blocking with bovine serum albumin. The rabbit antiserum to CCHFV NP and 5 nm gold-labeled goat anti-rabbit IgG (Biorbyt LLC) were used, followed by 2% UA staining as prior.

Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed as previously described [12]. Briefly, 96-well ELISA plates (Nunc Maxisorp) were coated with the purified recombinant NP antigen, inactivated whole viral antigens (gamma-irradiated supernatant from CCHFV-infected SW-13 cells), or mock supernatant (supernatant from mock-infected Vero E6 cells) overnight at 4°C, followed by blocking with 3% skim milk in PBS, and incubated with primary antibodies (i.e., rabbit, monkey, or human sera) in PBST containing 2% FBS for 1 hours at room temperature. Bound antibodies were visualized with HRPO-conjugated goat anti-rabbit IgG (H+L) (Rockland), goat anti-monkey IgG (γ) (Rockland), and goat anti-mouse IgG (H+L) (Jackson ImmunoResearch) antibodies or Purified Recomb

Protein A/G (Thermo Fisher Scientific)[49], and the TMB liquid substrate system for ELISA (Sigma). The reaction was stopped by adding 1 N phosphoric acid, and the optical density was measured. A commercially available ELISA-based serum diagnosis kit, Blackbox CCHFV IgG ELISA Kit (Blackbox-ELISA)[17] (Diagnostic Development Laboratory, Bernhard Nocht Institute for Tropical Medicine) was used according to the manufacturer's protocol. ELISA index values were calculated based on the values of optical density (OD) at 450 and 620 nm for both the CCHFV NP-based ELISA and Blackbox-ELISA according to the formula provided by the manufacturer.

Results

Expression and purification of CCHFV NPs

HEK293T cells were transfected with the pCAGGS plasmid (pCAGGS-CCHFV/NP) for expression of the recombinant CCHFV NP and the cellular expression of the recombinant NP was observed using immunofluorescence assays (Fig. 1). It was confirmed that CCHFV NP was successfully expressed by the transfection and formed densely stained inclusion body-like structures in the cytoplasm. The cytoplasmic and granular (i.e., inclusion body-like) localization of CCHFV NP expressed in the cells was similar to that expressed in transfected Hela cells or CCHFV-infected Vero E6 cells [2, 54]. The NP molecule was then purified from the transfected cells by equilibrium density gradient centrifugation in CsCl solution as described in "Methods". The presence of the NP molecules in each fraction obtained from the CsCl density gradient centrifugation was analyzed with SDS-PAGE followed by Coomassie blue staining and Western blotting. According to the electrophoretic mobility consistent with the expected molecular mass of CCHFV NP (about 52 kDa), the NP band was detected in almost all the fractions, suggesting the presence of multiple forms of the NP oligomer including non-homogenous aggregated polymers (Fig. 2). To observe the morphology of NP molecules in the fractions, TEM using the NP-rich fractions (e.g., fraction #8 in Fig. 2) was performed. TEM micrographs of the protein revealed that these fractions contained NPs in its oligomeric form showing ring- and helical-shaped architectures and their aggregates [68], suggesting NP-NP intermolecular interaction forming complex structures in the fractions (Fig. 3). Then the pooled NP-rich fractions (fractions #8, #9, and #10 in Fig. 2) were collected them as purified CCHFV NP as described in Methods. The average OD_{260}/OD_{280} ratio of purified NP fractions from multiple rounds of purification was 2.2, suggesting the presence of nucleic acids bound to the NP molecules.

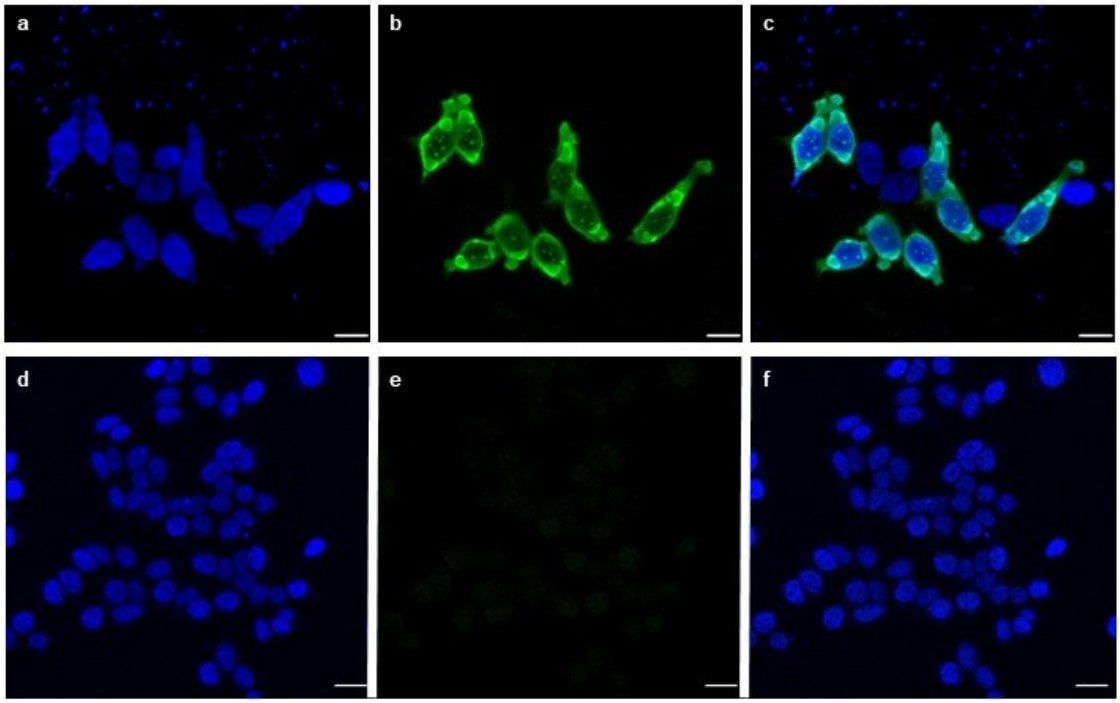


Figure 1: Cytoplasmic localization of expressed CCHFV NP

HEK293T cells transfected with the CCHFV NP-expressing plasmid (upper panels) or empty vector (lower panels) were stained with DAPI (shown in blue) (a,d) and the rabbit antiserum to CCHFV NP followed by the donkey anti-rabbit IgG (H+L) antibody conjugate (b,e). Merged images of the stained cells are also shown (c,f). Scale bars represent 20 µm.

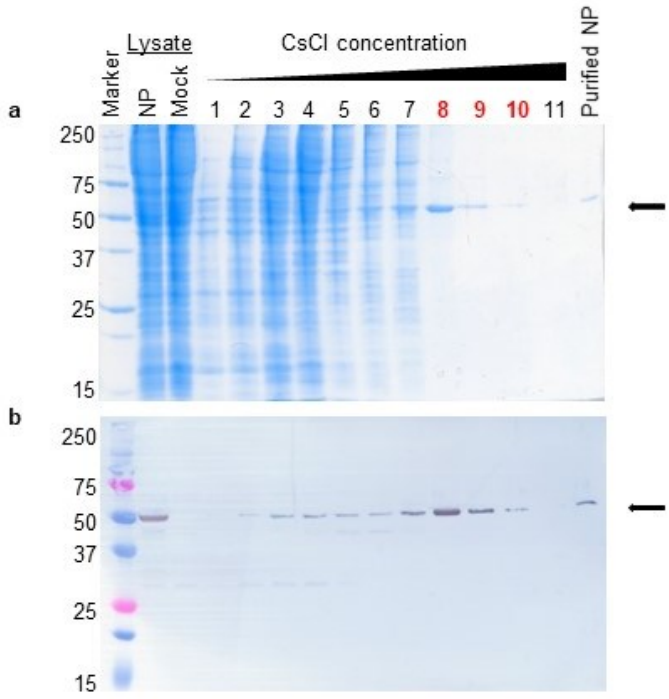


Figure 2: Purification of CCHFV NP by CsCl density gradient centrifugation
pCAGGS-CCHFV/NP-transfected HEK293T cells were lysed and fractionated through CsCl density gradient centrifugation as described in Methods. Each fraction was analyzed by SDS-PAGE. Arrow indicate CCHFV NP corresponding bands.

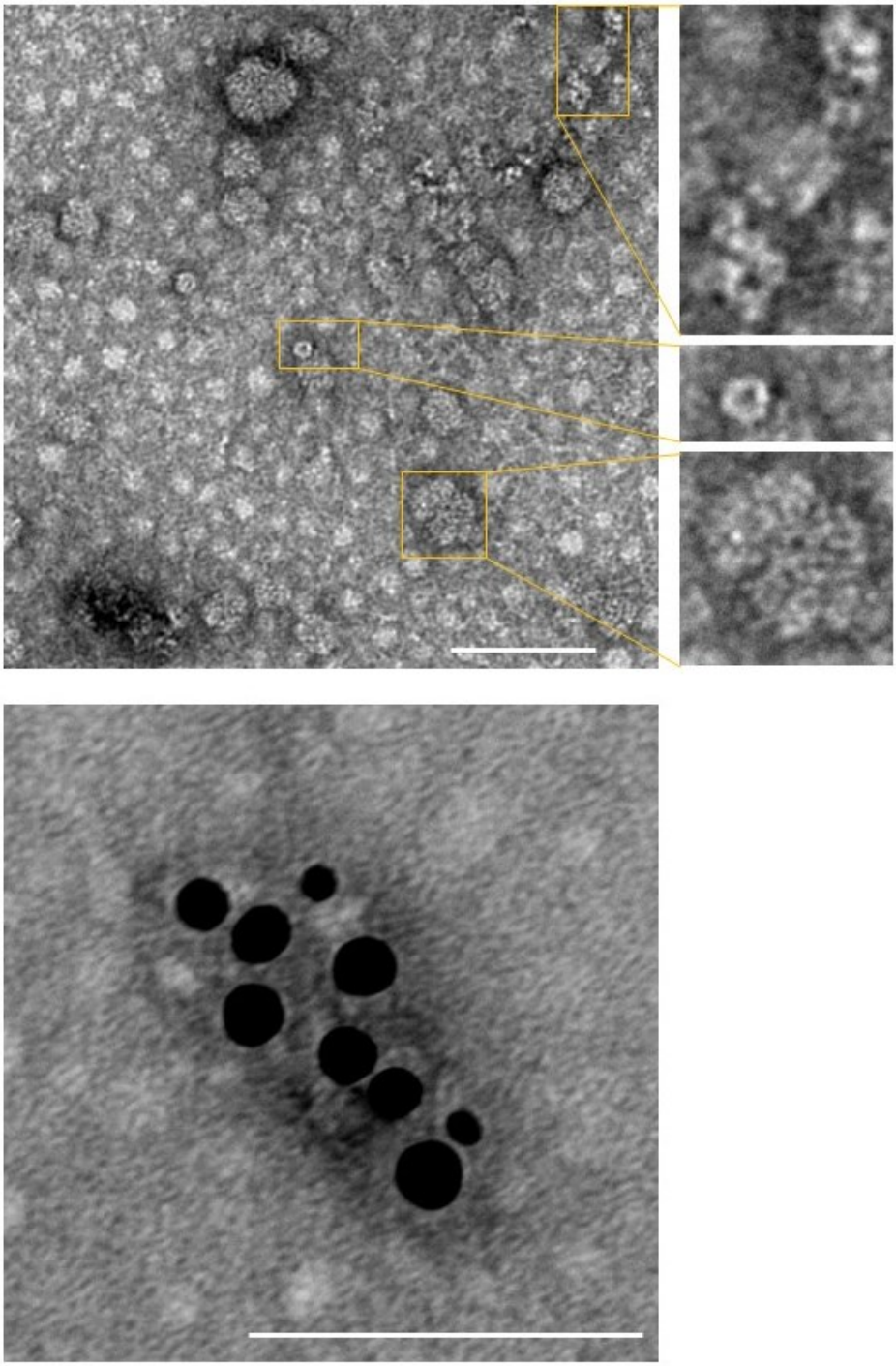


Figure 3: Electron micrographs of purified CCHFV NP

Purified CCHFV NP fractions were dialyzed overnight against TBS and then concentrated. Helical (upper right top panel), ring (upper right middle panel), and aggregated forms (upper right bottom panel) of the NP structures are shown. Polyclonal

goat anti-rabbit IgG antibodies labeled with 5 nm gold particles were used for immunoelectron microscopy (a helical form NP oligomer is shown) (bottom panel). TEM was operated at 80 kV, 1.0 μm, ×24.0 k magnification. Scale bars represent 100 nm.

Establishment of the purified recombinant CCHFV NP-based ELISA

Then, the purified CCHFV NP was used as an antigen for ELISA (5, 10, and 20 μg/ml) and investigated its utility to detect NP-specific IgG antibodies using the rabbit antiserum to CCHFV NP and CCHFV-infected monkey serum (Fig. 4a,d). The supernatants from CCHFV-infected Vero E6 cells and mock-infected supernatant were also used as positive and negative control antigens, respectively (Fig. 4b,c,e,f). In this experiment, host animal species-specific secondary antibodies (i.e., HRPO-conjugated anti-rabbit IgG and anti-monkey IgG antibodies) were used. IgG antibodies to the CCHFV NP antigen, as well as to whole CCHFV antigen in the infected cell culture supernatant, were clearly detected in the rabbit antiserum and CCHFV-infected monkey serum but not in the EBOV-infected monkey serum and negative control sera. The endpoint antibody titers of the rabbit antiserum and the CCHFV-infected monkey serum to the purified NP antigen were 25,600-102,400. There was no remarkable difference in the obtained OD values between 10 and 20 μg/ml concentrations of the NP antigen, although the 5 μg/ml concentration of the antigen gave slightly lower OD values.

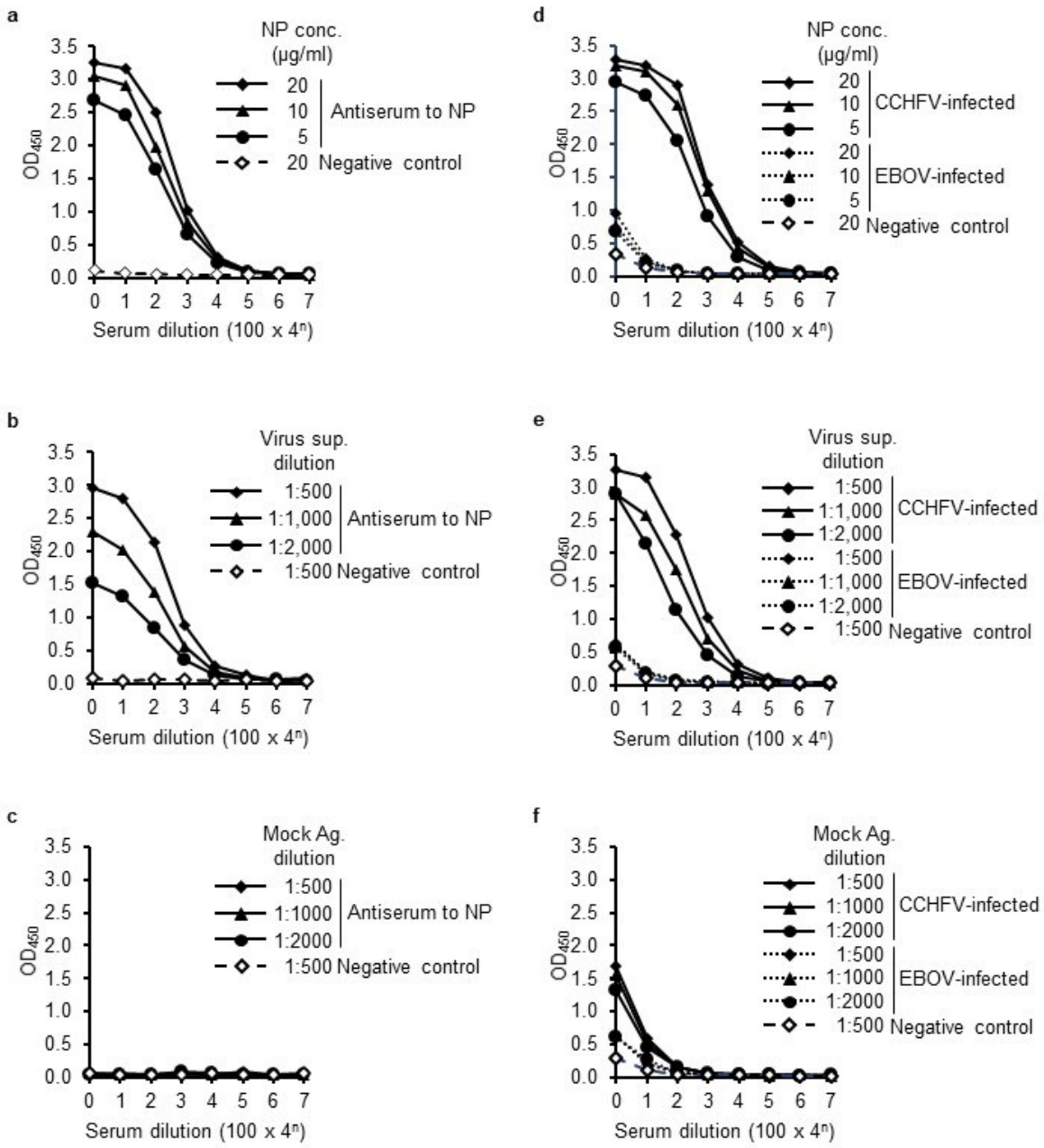


Figure 4: Detection of CCHFV NP-specific IgG in ELISA with purified CCHFV NP and whole CCHFV viral antigens

ELISA plates were coated with purified NP (5, 10, and 20 μg/ml) (a, d), serially diluted (1:500, 1:1,000, and 1:2,000) CCHFV-infected cell culture supernatant (Virus sup.) (b, e), or mock-infected supernatant (Mock Ag.) (c, f). Serial dilutions of the rabbit antiserum (a, b, c) and CCHFV-infected monkey serum (d, e, f) were used as primary antibodies, followed by detection with HRPO-conjugated anti-rabbit IgG and anti-monkey IgG

antibodies, respectively. Negative control rabbit and monkey sera and EBOV-infected monkey serum were also tested.

Modification of the CCHFV NP-based ELISA using HRPO-conjugated protein A/G

To modify the CCHFV NP-based ELISA to detect NP-specific antibodies from a wide variety of animals, the utility of HRPO-conjugated protein A/G in different conditions of ELISA were examined using the rabbit antiserum (Fig. 5). I tested 3 different concentrations of the purified NP antigen (2.5, 5, and 10 μg/ml) with serial dilutions of HRPO-conjugated anti-rabbit IgG antibody (host-specific) or HRPO-conjugated protein A/G (species-independent) reagent to detect bound IgG antibodies in the rabbit antiserum. It was found that 10 μg/ml gave the highest OD values for both HRPO-conjugated reagents and confirmed the dilution-dependent curves of the OD values, indicating that the HRPO-conjugated protein A/G reagent worked properly and could also be employed for the CCHFV NP-based ELISA. According to the curve trend, I used the antigen concentration of 10 μg/ml and 1:10,000 dilutions of HRPO-conjugated protein A/G, for the following experiment.

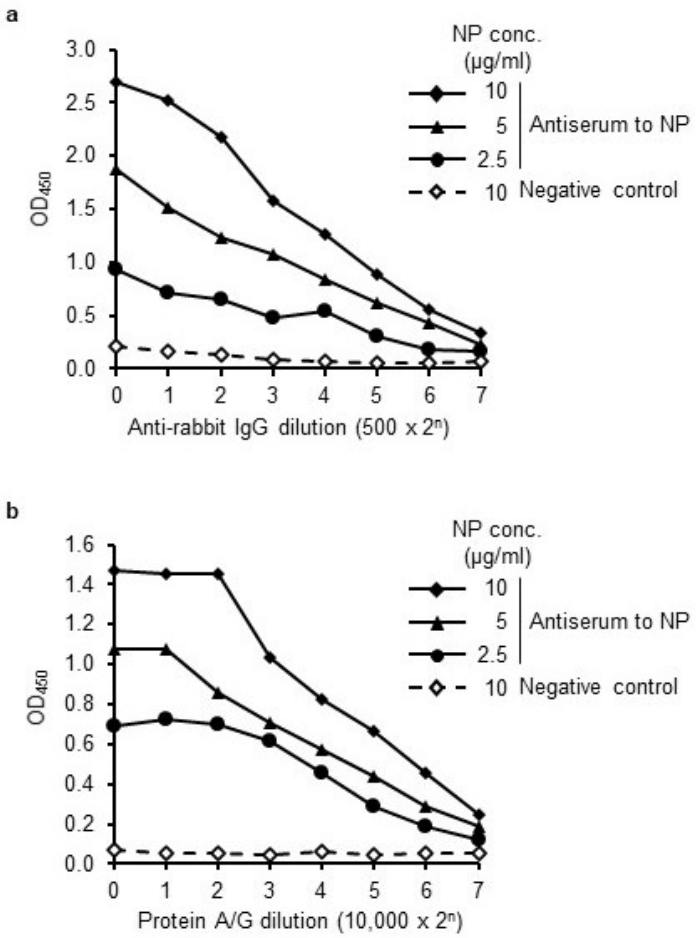


Figure 5: Detection of CCHFV NP-specific IgG using rabbit antiserum

ELISA plates were coated with the indicated concentrations (conc.) of purified NP and 1:1000 dilutions of the rabbit antiserum and negative control serum were used as primary antibodies. Serial dilutions of the HRPO-conjugated anti-rabbit IgG antibody (a) or the protein A/G reagent (b) were used for the detection of bound NP-specific antibodies.

The performance of the CCHFV NP-based ELISA compared to Blackbox-ELISA

To further confirm the utility of the CCHFV NP-based IgG ELISA with the HRPO-conjugated protein A/G reagent, its performance was compared to a commercially available kit, Blackbox-ELISA, which can be used for human serum samples. Forty-five human serum samples collected during an outbreak in a known CCHFV endemic area were simultaneously tested using the CCHFV NP-based ELISA established in this study and Blackbox-ELISA. Among the 45 patient serum samples, 16 sera were previously defined as CCHFV IgG positives [55]. The newly established CCHFV NP-based ELISA detected all 16 of those positive samples, whereas Blackbox-ELISA detected only 15 of the positive samples (Table 1). The OD index values obtained for the CCHFV NP-based ELISA and Blackbox ELISA showed a high positive correlation (correlation coefficient R^2 = 0.98), indicating that these ELISA procedures had similar capacities to detect CCHFV NP-specific antibodies (Fig. 6). The sensitivity and specificity of the established CCHFV NP-based ELISA were estimated by comparing the results to the Blackbox-ELISA and confidence intervals (CI) based on the Poisson-distribution approximation were calculated (Table 1). All 15 Blackbox-ELISA-positive samples were positive with the CCHFV NP-based IgG ELISA and most of the Blackbox-ELISA-negative samples (29/30) were also negative with CCHFV NP-based ELISA, which represented a sensitivity of 100% (15/15) (95% CI; 78.20-100) and a specificity of 96.6% (29/30) (95% CI; 82.78-99.92). Negative and positive predictive values were 100% (29/29) (95% CI; 88.06-100) and 93.8% (15/16) (95% CI; 69.76-99.84), respectively. The agreement rate with the Blackbox-ELISA was 97.8% (95% CI: 88.23-99.94).

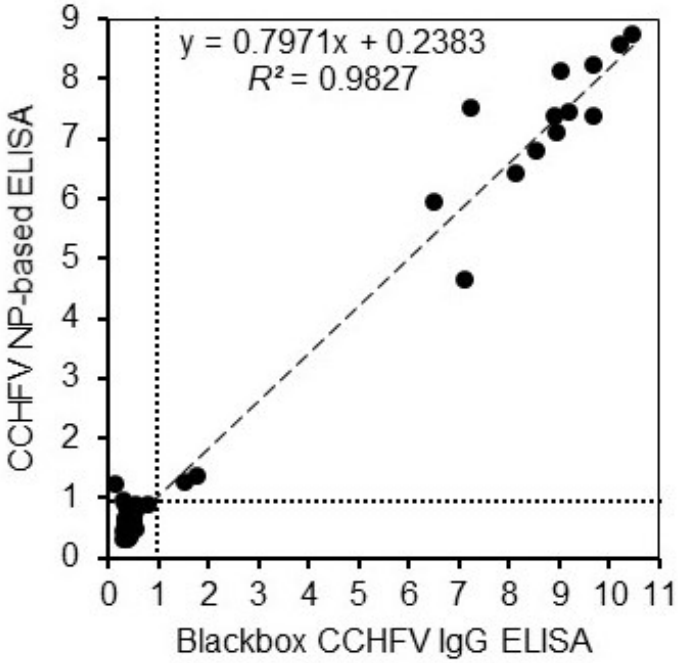


Figure 6: Correlation of IgG reactivity in human sera between the CCHFV NP-based ELISA and Blackbox-ELISA

ELISA plates were coated with purified NP (10 µg/ml). Serum samples were used at 1:100 dilution. The HRPO-conjugated protein A/G reagent (1:10,000 dilution) was used to detect bound antibodies. Scatter plots of the index values obtained by the CCHFV NP-based ELISA and Blackbox-ELISA are shown with the linear regression line (dashes). The coefficient of determination of R^2 and the cutoff lines (dotted lines) of both the CCHFV NP-based ELISA and Blackbox-ELISA are also shown.

Table 1: Performance of the CCHFV NP-based ELISA compared to Blackbox-ELISA

		Blackbox-ELISA		
		+	-	Total
CCHFV NP-based ELISA	+	15	1	16
	-	0	29	29
	Total	15	30	45

Sensitivity: 100% (15/15) (95% CI; 78.20-100)
Specificity: 96.6% (29/30) (95% CI; 82.78-99.92)
Negative predictive value: 100% (29/29) (95% CI; 88.06-100)
Positive predictive value: 93.8% (15/16) (95% CI; 69.76-99.84)
Agreement rate: 97.8% (44/45) (95% CI; 88.23-99.94).

Limited cross-reactivity among antisera to CCHFV and NSDV NPs

CCHFV patients and/or CCHFV-infected animals (e.g., sheep and goats) may have antibodies cross-reactive to NSDV, which is phylogenetically related to the CCHFV serogroup. Conversely, NSDV-infected animals may have cross-reactive antibodies to CCHFV. Thus, I produced mouse antisera to CCHFV and NSDV NPs and compared their reactivity to both antigens (Fig. 7) and found only a limited cross-reactivity among the antisera, suggesting that the CCHFV NP-based ELISA principally detected CCHFV-specific IgG antibodies.

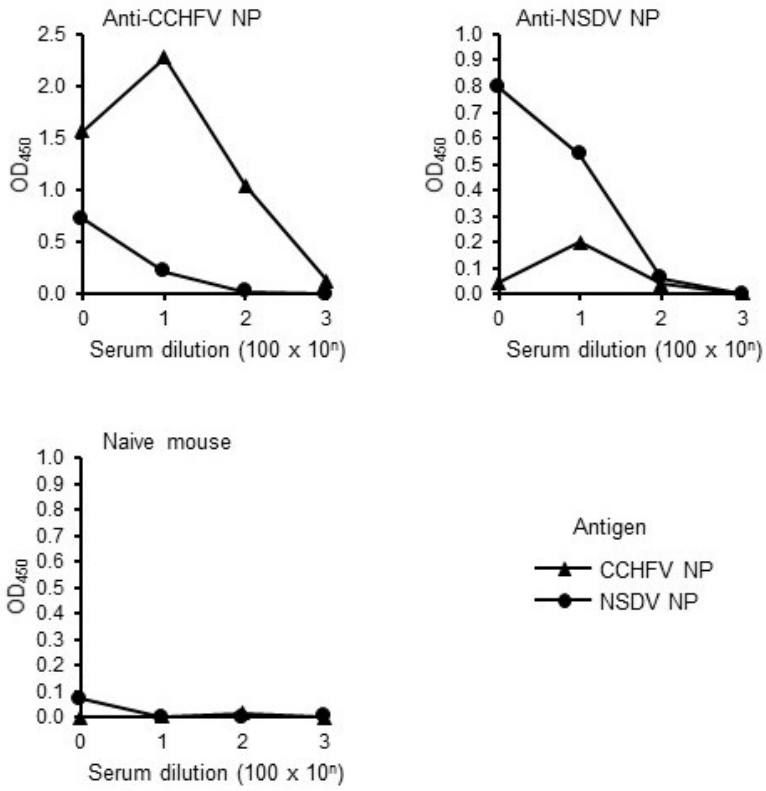


Figure 7: Cross-reactivity of antisera to CCHFV and NSDV NPs

ELISA plates were coated with 10 µg/ml of purified CCHFV NP, NSDV NP, or the mock antigen. Serial dilutions (1:100, 1:1,000, 1:10,000, and 1:100,000) of mouse antiserum to CCHFV, to NSDV NP, and naïve mouse serum were used as primary antibodies and incubated for 2 hours at 4°C. Peroxidase conjugate goat anti-mouse IgG (H+L) was used for detection of bound antibodies. To offset the nonspecific antibody reaction, the OD value of the mock antigen was subtracted for each dilution.

Discussion

Although CCHFV remains one of the priority pathogens needing urgent research and development of diagnostics, experimental studies involving live infectious CCHFV are restricted to the highest-level biosafety containment laboratories around the world [17, 21, 38]. Alternatively, recombinant CCHFV proteins have been used to study their functions or to develop serological diagnostic tools. For example, CCHFV NP has been expressed in insect, bacterial, mammalian, and plant cells and used as a protein antigen to detect virus-specific antibodies [4, 17, 22, 53, 54, 56]. However, accessibility to these serological diagnostic tools remains limited, probably because of the unavailability of the antigens or specific monoclonal antibodies for constant production and provision.

In this study, using the HEK293T cell line and pCAGGS plasmid, both of which are widely used for exogeneous protein expression in a large number of laboratories, full-length CCHFV NP was successfully expressed and purified. Importantly, I used the untagged NP construct and purified it without affinity chromatography procedures, which generally require multiple steps that should be optimized depending on each laboratory condition to obtain a pure protein [3]. Thus far, NP constructs with a histidine-tag at the N- and/or C-terminal have been shown to be purified through affinity tag-based chromatography and used for diagnostic purposes [4, 17, 55, 56] or biological studies [28]. However, these methods are often unavailable for some laboratories due to cost and/or laboratory equipment issues. In addition, it is conceivable that untagged NP is antigenically more native than tagged forms and some conditions during the affinity purification process (e.g., low pH) might negatively affect the structure and function of NP [3]. Indeed, a CCHFV NP fusion protein containing a 6 × histidine-tag purified under denaturing conditions has been shown to be relatively unstable, although it can detect CCHFV IgG [52]. Thus, I believe that, compared to affinity tag-based purification, the method described here is a simpler and more effective purification procedure that enables us to obtain recombinant CCHFV NP that is conformationally close to authentic NP produced in CCHFV-infected cells.

The essential nature of NP is to bind nucleic acids and to form a ribonucleoprotein (RNP) complex. Both termini of the CCHFV S segment are involved conjointly in this essential function, RNA binding [28]. Like other viruses in the genus *Orthonairovirus*, CCHFV NP binds to nucleic acids and then undergoes a conformational change [29, 68]. It was corroborated that his-tagged NPs purified by affinity chromatography with the OD_{260}/OD_{280} ratios 1.3 and 1.49 had high-ordered oligomeric RNP structures that exhibited head and stalk domains [68, 69]. When the RNP complex is formed, the stalk domain is thought to display a highly conserved epitope region that was suggested to be

used for a universal CCHF diagnostic approach [35, 53]. In the present study, the average ratio of OD_{260}/OD_{280} (2.2) of the purified CCHFV NP was greater than those described previously, suggesting that it contained more RNA associated with NP molecules. This difference may be explained by the functionality of both termini (i.e., with or without tag sequences) to form the RNP complex. These observations suggest that untagged CCHFV NP purified through CsCl density gradient centrifugation is an NP-RNA complex that may expose the stalk domain more efficiently than tagged NP, which might result in increased ELISA cross-reactivity through the universal CCHFV NP stalk domain's epitopes.

Commercially available CCHFV serological tools are specifically intended for the detection of human IgM or IgG (i.e., Blackbox CCHFV, VectoCrimean-CHF)[17, 66]. On the other hand, serological surveillance of CCHFV infection in animals is also important and serves as an indicator of CCHF risk to humans [13, 59]. Protein A/G is known to bind to multiple IgG classes of most mammalian species, including humans, domestic animals (cattle, goats, sheep, horses, rabbits, pigs, dogs, cats, alpacas, etc.), and wildlife. It has been widely used for purification of antibodies with affinity chromatography and, when conjugated with enzymes (e.g., HRPO), for routine immunoassays as an antibody detection tool [1, 7, 14, 27, 46, 49, 58, 65]. In this study, I demonstrated the utility of the purified CCHFV NP antigen together with the HRPO-conjugated protein A/G reagent. This procedure has the advantages of simplicity and versatility. For species whose IgG affinity to protein A/G is not proven, host-specific secondary antibodies may remain useful.

It has been suggested that CCHFV NP is the most conserved viral protein among members of the *Nairoviridae* family [35, 42, 53]. It is noteworthy that NP of the IbAr10200 strain was recognized by antibodies in a monkey infected with another CCHFV strain, Hoti, belonging to a different clade from IbAr10200 [57]. The detection of CCHFV NP-specific IgG in Chinese patients' sera using one of the African strains, IbAr10200, also demonstrated serological cross-reactivity among genetically distinct strains/lineages, suggesting potential worldwide application of the established CCHFV NP-based ELISA. On the other hand, it is important to pay attention to possible cross-reactivity of CCHFV NP antibodies to NSDV, which is a nairovirus in another serogroup, since some CCHFV-susceptible animals (e.g., sheep and goats) may have antibodies to this virus. However, it is assumed that there might be limited cross-reactivity since CCHFV NP was not recognized by antibodies directed against Dugbe virus belonging to the Nairobi sheep disease virus serogroup [25, 37]. Indeed, the present data also suggest that specificity of serum antibodies to CCHFV and NSDV could be distinguishable in

NP-based ELISA. In future studies, the purified CCHFV NP antigen still has to be evaluated for IgM detection, which is important for clinical use during acute CCHFV infection.

Summary

Crimean-Congo hemorrhagic fever virus (CCHFV) causes a zoonotic disease, Crimean-Congo hemorrhagic fever (CCHF) endemic in Africa, Asia, the Middle East, and Southeastern Europe. However, the prevalence of CCHF is not monitored in most of the endemic countries due to limited availability of diagnostic assays and biosafety regulations required for handling infectious CCHFV. In this study, I established a protocol to purify the recombinant CCHFV nucleoprotein (NP), which is antigenically highly conserved among multiple lineages/clades of CCHFVs and investigated its utility in an enzyme-linked immunosorbent assay (ELISA) to detect CCHFV-specific antibodies. The NP gene was cloned into the pCAGGS mammalian expression plasmid and human embryonic kidney 293T cells were transfected with the plasmid. The expressed NP molecule was purified from the cell lysate using cesium-chloride gradient centrifugation. Purified NP was used as the antigen for the ELISA to detect anti-CCHFV IgG. Using the CCHFV NP-based ELISA, CCHFV-specific IgG in anti-NP rabbit antiserum and CCHFV-infected monkey serum was efficiently detected. When compared to the commercially available Blackbox CCHFV IgG ELISA kit, the NP-based ELISA established in this study showed equivalent performance in detecting CCHFV-specific IgG in human sera. These results demonstrate the usefulness of the CCHFV NP-based ELISA for seroepidemiological studies.

Chapter II: Mapping of antibody epitopes on the Crimean-Congo hemorrhagic fever virus nucleoprotein

Introduction

CCHF is classified by the WHO as a prioritized diseases requiring research and development attention [38, 39]. The diagnostic methods for CCHFV include molecular techniques for viral RNA detection, serological assays for immunoglobulin detection, and virus isolation which is restricted to the highest containment laboratories (e.g., biosafety level 4). However, all these tests are carried out only in specialized laboratories in a limited number of countries [62, 64].

Like many hemorrhagic fevers, CCHF tends to be reported in remote areas lacking diagnostics capacity, which causes a delay in its clinical recognition, while retrospective serosurveys provide information on past exposure to the virus but not active infection [64]. Hence, there is a need to develop effective rapid diagnostic tools such as a lateral flow antigen detection kit that can be used for monitoring CCHFV in the field setting to limit its spread and epidemic potential [32, 35].

CCHFV NP, the most abundant and primary antigen detectable during CCHFV infection and which is antigenically conserved among the CCHFV clades, serves as the principal antigen for the development of serological assays [4, 17, 22, 35, 36, 53, 54, 56]. Previous studies showed the usefulness of purified recombinant CCHFV NP antigens in ELISA and its ability for detecting CCHFV NP-specific IgG directed against strains originating from different geographic regions [24, 31, 36, 53]. However, it is known that some anti-NP antibodies are cross-reactive to multiple orthonairoviruses in ELISA and Western blotting [24, 31, 36]. Considering the overlapping distribution of CCHFV-related orthonairoviruses such as Dugbe virus (DUGV) and Nairobi sheep disease virus (NSDV), also known as Ganjam virus in Asia, which is one of the most important human pathogens among nairoviruses circulating in Africa and Asia, the cross-reactivity of anti-NP antibodies is an important issue for serological diagnosis and surveillance of CCHF.

In general, mapping of antigenic sites on viral proteins is important for therapeutic, diagnostic, and vaccine development [12, 23, 47, 51]. The identification and characterization of CCHFV NP-specific epitopes will be useful for generating a modified CCHFV NP antigen that reduces the cross-reactivity or producing CCHFV NP-specific monoclonal antibodies that can be used for antigen detection assays. Several previous studies mapped a conserved and immunodominant region on the CCHFV NP molecule, which might be an attractive target for pan-CCHFV diagnosis [9, 35, 53, 70]. To date, however, it is unknown whether some epitopes on this region are common among other

nairoviruses, including NSDV and DUGV, and potentially induce cross-reactive antibodies [24, 36]. I, therefore, sought to identify the epitopes on the CCHFV NP molecule, using anti-CCHFV NP antisera, CCHFV-infected monkey serum, CCHFV patient sera, immune ascites fluids to the NSDV serogroup, and anti-CCHFV NP monoclonal antibodies (mAbs).

Methods

Construction of plasmids expressing CCHFV and NSDV NPs

A mammalian expression plasmid, pCAGGS, containing full-length NP genes of CCHFV strain IbAr10200 or NSDV isolate Jilin (GenBank Accession numbers KY484036.1 and NC_034386.1, respectively) was constructed as described previously [36]. A series of chimeric (Ch) NP genes between CCHFV and NSDV (Ch-NP1-5) were also constructed in an interwoven fashion (Fig. 8a). Designed fragments were PCR-amplified with a KOD One™ PCR master Mix (Toyobo) using specific primers. Amplified fragment genes were gel-purified (Wizard SV Gel and PCR Clean-Up System, Promega) and cloned into the pCAGGS plasmid using an In-Fusion® HD Multi-Insert Cloning Kit (Takara Bio). The sequences of the chimeric NP genes were confirmed using 3500xL Genetic Analyzer (Hitachi/Applied Biosystems). The generation of recombinant proteins was approved by the Ministry of Education, Culture, Sports, Science, and Technology, Japan (元受文科振 235 号).

Expression and purification of NPs

The recombinant NP antigens were purified as previously described [36]. Briefly, HEK293T cells were transfected with the pCAGGS plasmids encoding NPs, using TransIT-LT1 Transfection Reagent (Mirus Bio LLC). The cells were treated with lysis buffer [10 mM Tris-HCl (pH 7.8), 0.15 M NaCl, 1.0 mM EDTA, and 0.25% NP-40 in the presence of Halt™ Protease Inhibitor Single Use Cocktail (Thermo Scientific)] and recombinant NPs were purified from the cell lysate through ultracentrifugation with 20-50% (w/v) discontinuous CsCl gradients. The NP concentration was determined using the Bicinchoninic Acid Assay (Pierce Micro BCA Assay Kit-Perce) according to the company instructions and used for immunization.

Serum samples

Mouse immune ascites fluids (MIAFs) to NSDV/Ganjam virus and Dugbe fever virus were obtained from the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA), University of Texas Medical Branch, Galveston, USA. Rabbit and mouse polyclonal antisera raised against recombinant CCHFV NP (8402 strain and IbAr10200, respectively), CCHFV (strain Hoti)-infected monkey and 10 laboratory-confirmed CCHFV-infected patient serum samples that were used in this study have been described previously [36, 53]. Serum samples collected from healthy humans and monkeys without a history of CCHFV infections were also used as negative controls.

ELISA for mAb screening

ELISA was performed as described previously [12, 36]. Briefly, cell lysates (1:1,000 dilution) of HEK293T cells transfected with NP-expressing plasmids or purified NPs (10 µg/ml) diluted in PBS were used as antigens. Flat-bottom 96-well plates (Nunc®, Maxisorp) were coated with the antigens followed by blocking with 3% skim milk. After washing with PBST, 50µl supernatants of hybridomas were added. After washing with PBST, the plates were incubated with a secondary antibody; horseradish peroxidase (HRPO)-conjugated goat anti-mouse IgG (H+L) peroxidase-conjugated (Jackson ImmunoResearch). The bound antibodies were visualized with 50 µl of TMB substrate. The reaction was stopped by adding 1 N phosphoric acid and the OD at 450 nm was measured using a SpectraMAX 190 device (Molecular Devices).

ELISA with synthetic peptides as antigens

A library of 48 synthetic peptides spanning the entire CCHFV NP sequence of strain IbAr10200 was designed as described previously and produced by PEPscreen® Custom Peptide Libraries (Sigma) [16]. Each peptide had 20 amino acids including 10 overlapping residues with adjacent peptides except peptide 48 (12 amino acids) (Fig. 8b). The synthetic peptides were reconstituted in dimethyl sulfoxide (DMSO) (WAKO Pure Chemical Corporation) at a final concentration of 100 µg/ml. The reactivities of antibodies to synthetic peptides were assessed in ELISA. Flat-bottom 96-well plates (Nunc®) were coated overnight at 4°C with 50 µl of carbonate buffer (pH 8.0) containing 100 or 200 µg/ml of each peptide. The peptide solution was then replaced with 180 µl of 3% skim milk in PBS for blocking. After washing with PBST, the plate was incubated with appropriately diluted antibodies (i.e., mAbs, CCHFV-infected patients, and monkey' serum samples or MIAFs to NSDV and DUGV) for 1 hours at RT. The plate was washed 3 times with PBST and then incubated with corresponding secondary antibodies as described above or HRPO-conjugated protein A/G (Thermo Fisher Scientific). The bound antibodies were visualized with the TMB substrate and OD values were measured as described above.

Western blotting

The lysates prepared from HEK293T cells expressing NPs were subjected to SDS-PAGE. Rabbit antiserum to CCHFV NP, mouse antiserum to CCHFV NP, MIAFs to NSDV and DUGV, CCHFV-infected monkey serum, CCHFV patient sera, and purified mAbs were used as primary antibodies. HRPO-conjugated goat anti-rabbit IgG (H+L)

(KPL), goat anti-mouse IgG (H+L) (Jackson ImmunoResearch), goat anti-monkey IgG(γ) (Rockland), and goat anti-human IgG (H+L) (Jackson) were used as secondary antibodies and reactions were visualized with Immobilon® Western chemiluminescent HRPO substrate (Millipore Corporation).

Production and characterization of mouse mAbs to CCHFV NP

Anti-NP mAbs were prepared as described previously [12, 60]. Briefly, 5-week-old inbreed female BALB/c mice were immunized by intraperitoneal injection with 40-50 μg of purified CCHFV NP at 28-day intervals, followed by booster immunization with 100 μg of CCHFV NP 6 months after the second immunization. Three days after the booster, spleen cells were collected and splenocytes of the immunized mice were fused with P3-U1 mouse myeloma cells using polyethylene glycol. Hybridomas were selected in medium supplemented with hypoxanthine aminopterin thymidine and hypoxanthine thymidine. The supernatants of hybridomas were screened for the presence of anti-CCHFV NP mAbs in ELISA using cell lysates of transfected HEK293T cells expressing CCHFV, NSDV, and Ch-NPs or purified protein antigens. The cells producing CCHFV NP-specific mAbs were then cloned by a limiting dilution method [12, 33, 60]. Anti-NP mAbs were purified from culture supernatants using an Affi-Gel Protein A MAPS® kit (BIO-RAD) and their isotypes were determined using a mouse monoclonal antibody isotyping test kit (BIO-RAD). All animal experiments were conducted in strict accordance with the Guidelines for Proper Conduct of Animal Experiments of the Science Council of Japan. The protocol was approved by the Animal Care and Use Committee of Hokkaido University on March 30, 2018 (#18-0029).

Amino acid sequences analysis of cross-reactive peptides

To compare the amino acid sequences of the identified cross-reactive peptide regions, the NP amino acid residues of CCHFV IbAr10200 (KY484036.1), NSDV strain 6233 (HM991329.1), and DUGV strain Ib Ar 1792 (AMT75394.1) were retrieved from the National Center for Biotechnology Information GENBANK. Multiple sequence alignment was performed using the MUSCLE program through MEGA 10.2.2 software.

Statistical analysis

To determine the statistical significance in ELISA with synthetic peptides, the OD values obtained from PEPscreen reactions were assumed to follow the normal distribution. For CCHFV-infected patients and monkeys, the threshold defining positive reaction was obtained based on the average + 3 × standard deviation of all the OD values

(P1-P48) of negative control samples. Since MIAF-negative control samples were not available, the Smirnov-Grubbs rejection test, which is widely used to detect outliers that do not belong to the population consisting of all other values in the data set, was employed for the MIAFs to the NSDV serogroup. Briefly, if the highest OD value was considered to be an outlier, the T-value for the second highest OD value was similarly tested without the highest one. These steps were repeated until the T-value fell to below the level of statistical significance ($P < 0.01$).

Results

Reactivity of antisera/MIAF and CCHFV-infected monkey/human sera to CCHFV and NSDV chimeric NPs in Western blotting.

CCHFV and NSDV NP fragments were joined in an interwoven fashion in the pCAGGS plasmid. The chimeric proteins, Ch-NP1 to 5, gradually had 80 amino acid sequences of CCHFV NP from the N- to C-terminal while deleting those of NSDV NP and vice versa (Fig. 8a). All the chimeric proteins were expressed in 293T cells and antisera/MIAF and CCHFV-infected monkey sera were tested for their reactivities to wildtype CCHFV NP, NSDV NP, and CCHFV-NSDV chimeric NPs in Western blotting (Fig. 9a). Anti-CCHFV NP rabbit antiserum, CCHFV-infected monkey serum, and anti-CCHFV NP mouse serum all reacted to CCHFV NP, Ch-NP4, and Ch-NP5 but not to NSDV NP, Ch-NP1, Ch-NP2, or Ch-NP3. In contrast, anti-NSDV serum reacted to NSDV NP, Ch-NP1, and Ch-NP2, and CCHFV NP and the other chimeric NPs were almost undetectable with this serum. As well as anti-NSDV serum, anti-DUGV serum predominantly reacted to NSDV NP, Ch-NP1, and Ch-NP2. Interestingly, however, this serum showed little cross-reactivity to CCHFV NP. Despite high non-specific backgrounds, CCHFV-infected patient serum samples showed reaction patterns similar to those of anti-CCHFV NP rabbit antiserum, CCHFV-infected monkey serum, and anti-CCHFV NP mouse serum (i.e., they were generally reactive to CCHFV NP, Ch-NP4, and Ch-NP5 but not to NSDV NP and the other chimeric NPs) (Fig. 9b). Taken together, these results suggested that major the antigenic regions were different between CCHFV and NSDV NPs. Amino acids at positions 240-482 and 1-240 included dominant epitopes recognized by anti-CCHFV and anti-NSDV IgG antibodies, respectively.

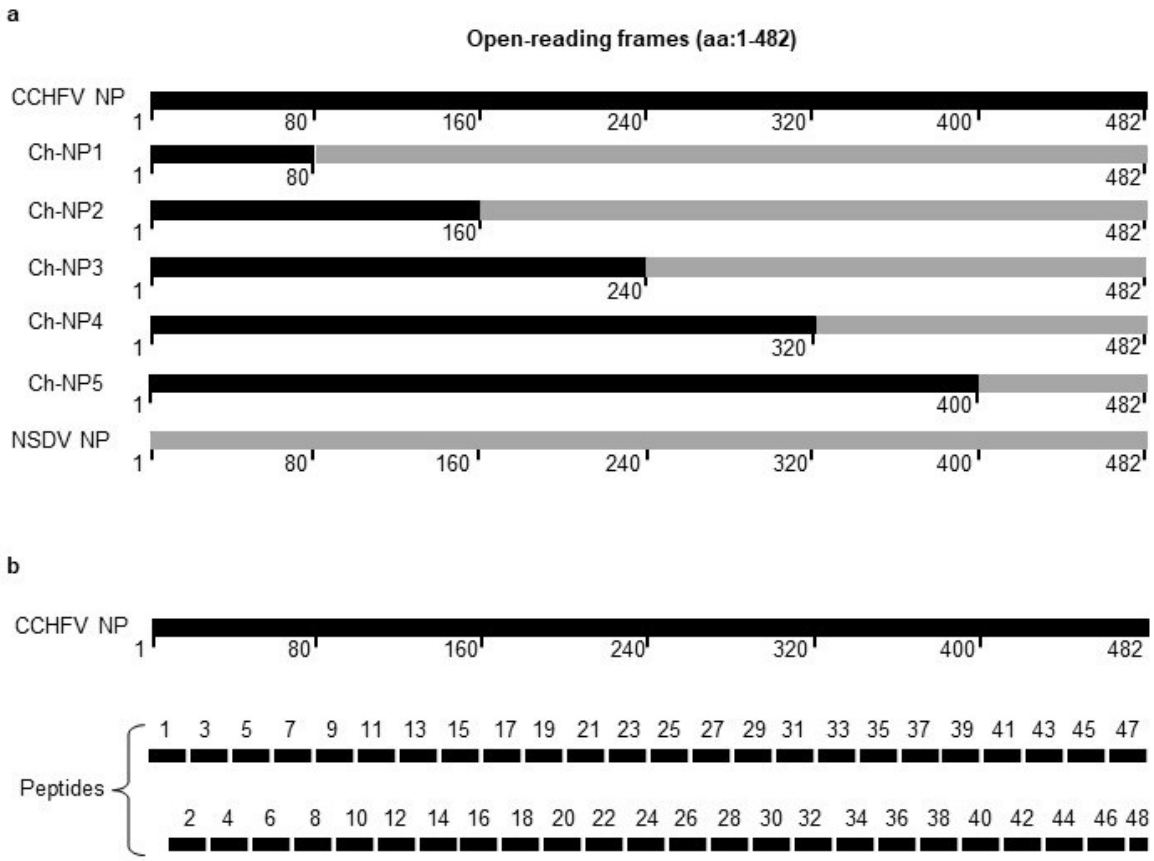


Figure 8: Schematic diagrams of CCHFV and NSDV NPs, their chimeric proteins, and CCHFV NP-based synthetic peptides

(a) An illustration representing the entire CCHFV (black) and NSDV (gray) NPs and constructed chimeric NPs (Ch-NP1-5) is shown. Amino acid positions are indicated below each bar. (b) Design and numbers (P1-P48) of 20 amino acid peptides overlapping by 10 amino acids with adjacent peptides, except P48 (12 amino acids) spanning the entire CCHFV NP.

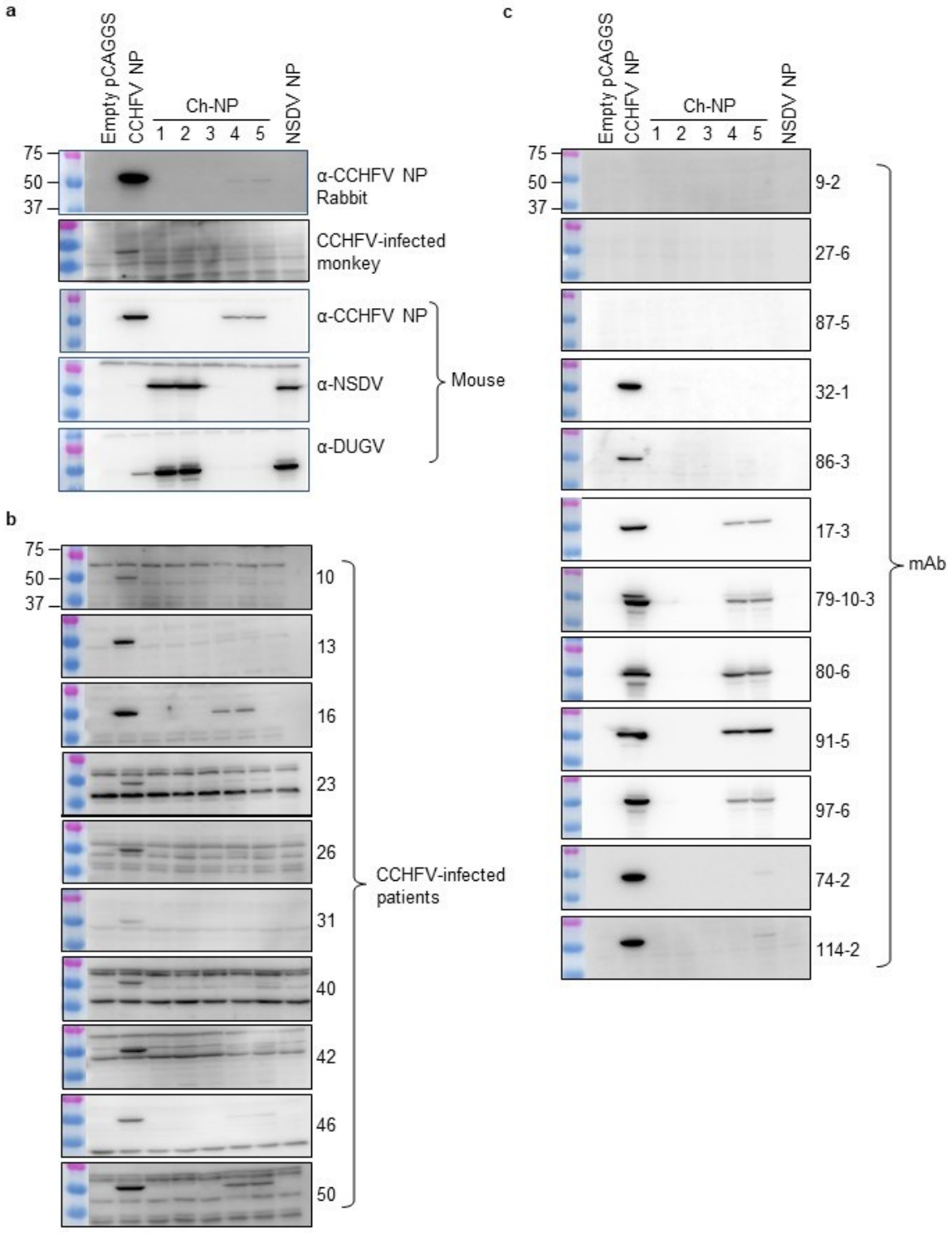


Figure 9: Reactivities of polyclonal and monoclonal antibodies to CCHFV NP, NSDV NP, and chimeric NPs in Western blotting

Western blotting was performed using lysates of HEK293T cells transfected with NP-expressing plasmids. Rabbit polyclonal antiserum to CCHFV NP (1:2,500), CCHFV-infected monkey (1:10,000), mouse antiserum to CCHFV NP (1:1,000), and MIAF to NSDV/DUGV (1:2,500) (a), CCHFV laboratory confirmed patient serum samples (b), and mAbs to CCHFV NP 2 μg (c) were used as primary antibodies.

Reactivities of anti-NP mAbs in Western blotting.

Then, 12 mAbs reactive to CCHFV NP were generated. The majority of the generated mAbs were IgGa or IgGb with a light chain kappa (k). These mAbs reacted to purified CCHFV NP but not to NSDV in ELISA (data not shown). Accordingly, none of the generated mAbs recognized NSDV NP in Western blotting, whereas CCHFV NP was recognized by 9 of the 12 mAbs either in nonreducing or reducing conditions (Fig. 9c). Of the mAbs reacting to CCHFV NP in Western blotting, mAbs 32-1 and 86-3 recognized none of the chimeric NPs. On the other hand, 5 mAbs (17-3, 79-10-3, 80-6, 91-5, and 97-6) recognized Ch-NP4 and Ch-NP5, although the band intensities relative to CCHFV NP varied. Although the band intensities were weak, mAbs 74-2 and 114-2 recognized Ch-NP5 but not Ch-NP4, which was different from the 5 mAbs that were reactive to both Ch-NP4 and Ch-NP5. Based on the reactivity pattern to a series of the NP antigens, the 12 mAbs were separated into 4 groups (Table 2). Reactivities of mAbs 17-3, 79-10-3, 80-6, 91-5, and 97-6 to the chimeric NPs suggested that these mAbs recognized epitopes on amino acid positions 240-320. Similarly, reactivities of mAbs 74-2 and 114-2 suggested that these MAbs recognized epitopes on amino acid positions 320-400

Table 2: Characterization and classification of mouse anti-CCHFV NP mAbs based on their reactivities in Western blotting.

Group	mAbs	CCHFV NP		Ch-NP					NSDV NP		Isotypes
		2ME (-)	2ME (+)	1 2ME (+)	2 2ME (+)	3 2ME (+)	4 2ME (+)	5 2ME (+)	2ME (-)	2ME (+)	
1	09-2	-	-	-	-	-	-	-	-	-	IgG2b kappa
	27-6	-	-	-	-	-	-	-	-	-	IgG2b kappa
	87-5	-	-	-	-	-	-	-	-	-	IgG2b kappa
2	32-1	±	+	-	-	-	-	-	-	-	IgG2b kappa
	86-3	±	+	-	-	-	-	-	-	-	IgG2b kappa
3	17-3	+	+	-	-	-	+	+	-	-	IgG2b kappa
	79-10-3	+	+	-	-	-	+	+	-	-	IgG2a kappa
	80-6	+	+	-	-	-	+	+	-	-	IgG2a kappa
	91-5	+	+	-	-	-	+	+	-	-	IgG2a kappa
	97-6	+	+	-	-	-	+	+	-	-	IgG2a kappa
4	74-2	±	+	-	-	-	-	±	-	-	IgG2b kappa
	114-2	+	+	-	-	-	-	±	-	-	IgG2b kappa

2ME: β-mercaptoethanol

(+) marked positive interaction; clear band detected

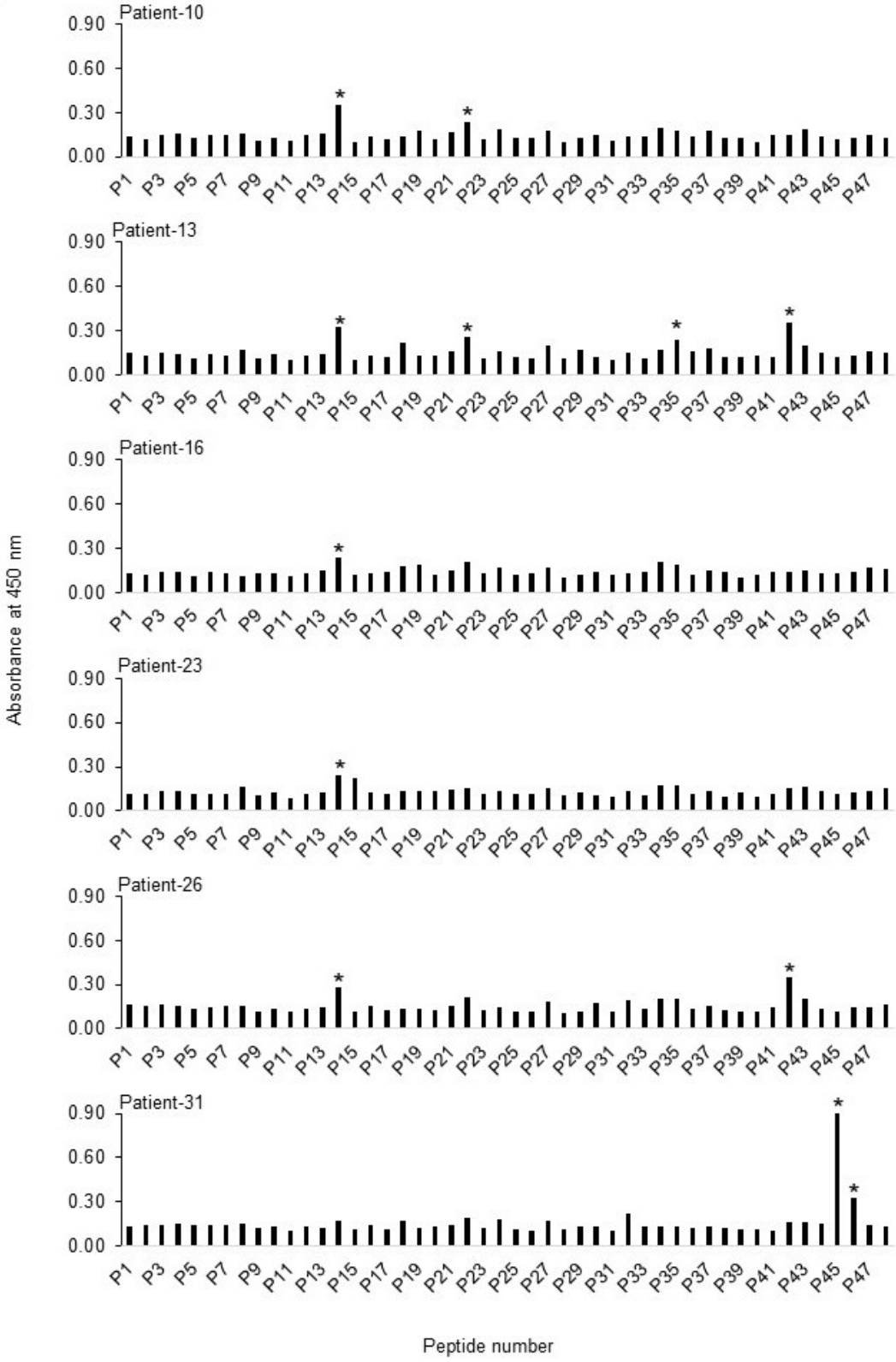
(-) negative: no detectable band

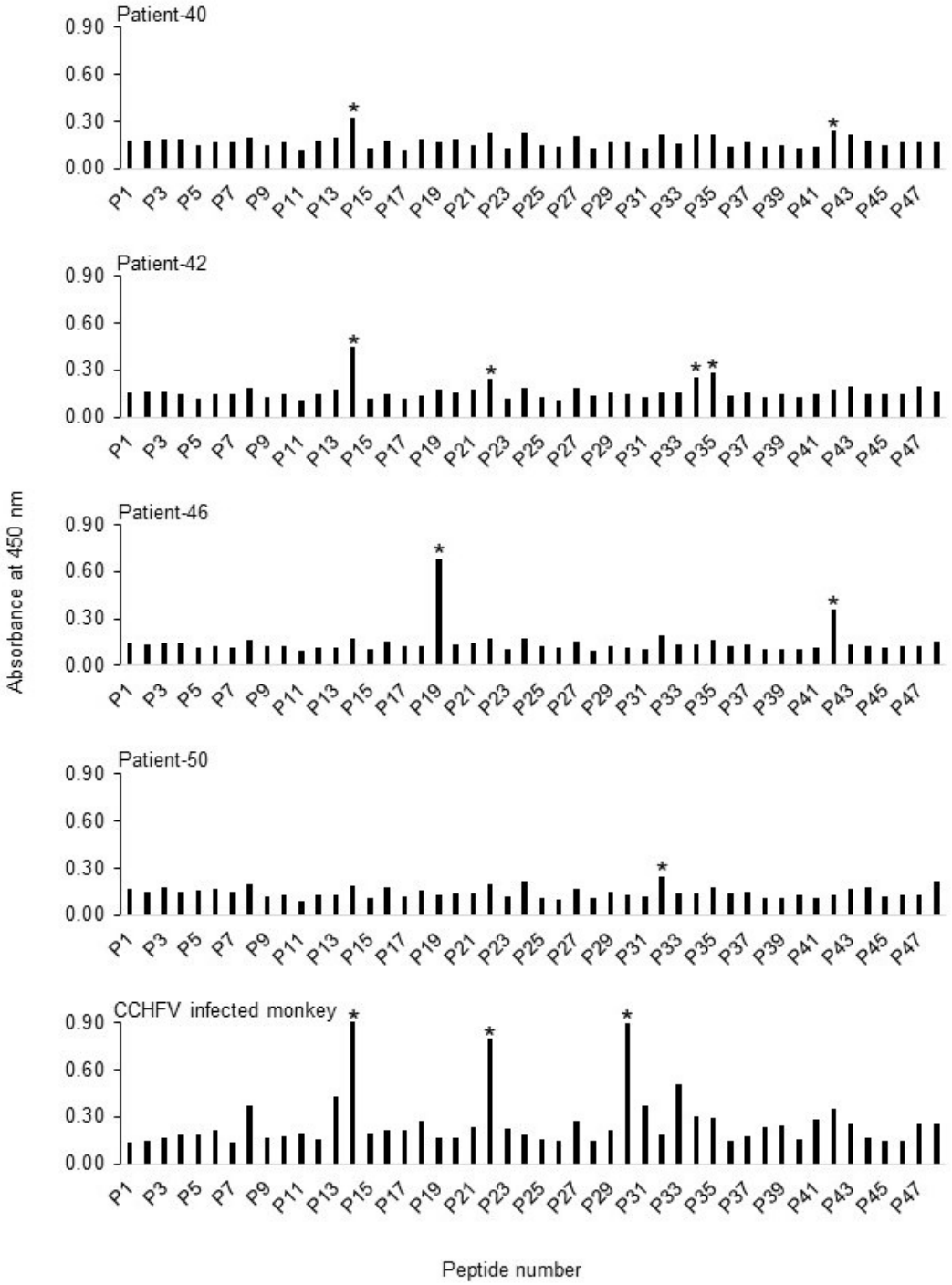
(±) plus/minus: band weakly seen

Identification of epitope peptides on the CCHFV NP molecule.

Next, I tried to determine CCHFV NP-specific epitopes using a peptide library based on the NP sequence of CCHFV strain IbAr10200 (Fig. 8b). To focus on epitopes for naturally induced antibodies, CCHFV-infected patient and monkey sera were tested for IgG antibodies reactive to each peptide. It was found that the reactive peptides were scattered from the N- to C-terminals over the length of the CCHFV NP sequence and the reaction patterns varied among individuals (Fig. 10a, Fig. 11, and Table 3). Peptides P14 and P22, corresponding to the amino acid residues at positions 131-150 and 211-230, respectively, were recognized by IgG antibodies in multiple patient and infected monkey sera. P42 also gave a positive reaction for 4 of the 10 patient sera. Some other peptides (P19, P30, P33, P34, P35, P45, and P46) were recognized by IgG antibodies in either patient or monkey sera. Of these, the positions of P30, P33, P34, P35, P42, P45, and P46 were consistent with the fragment recognized in Western blotting (i.e., Ch-NP4, Ch-NP5, and wildtype CCHFV NP). In contrast, all of the CCHFV NP mAbs generated in this study failed to recognize these synthetic peptides (data not shown). To further assess the existence of conserved epitopes among CCHFV, NSDV, and DUGV NPs, MIAF to NSDV and DUGV were also tested in ELISA using the PEPscreen library (Fig. 10b, Fig. 11, and Table 3). The two MIAFs recognized each two peptides within the region between P9 and P22 (i.e., P14/P22 for NSDV and P9/P22 for DUGV) spanning amino acids positions 81 to 230 of this antigen. This region was consistent with the fragment detected by these sera in Western blotting (i.e., Ch-NP1 and Ch-NP2).

a


Patient-10
Patient-13
Patient-16
Patient-23
Patient-26
Patient-31
0.90
0.60
0.30
0.00
Absorbance at 450 nm
P1 P3 P5 P7 P9 P11 P13 P15 P17 P19 P21 P23 P25 P27 P29 P31 P33 P35 P37 P39 P41 P43 P45 P47
Peptide number


Patient-40
Patient-42
Patient-46
Patient-50
CCHFV infected monkey
0.90
0.60
0.30
0.00
Absorbance at 450 nm
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Peptide number

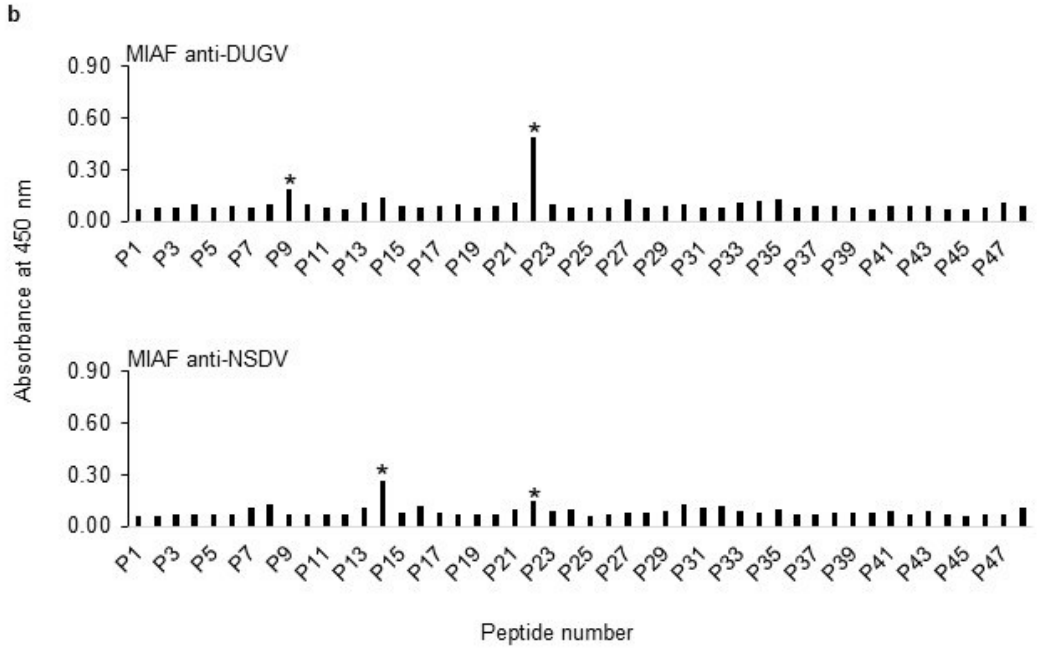

Figure 10: Reactivities of polyclonal antibodies to the PEPscreen library of CCHFV NP.

The peptides are numbered according to their positions (see also Fig. 8 and Table 3). (a) The serum samples of CCHF patients and CCHFV-infected monkeys or MIAF to NSDV and DUGV (b) were diluted at 1:500 and used as primary antibodies. Experiments were duplicated, and the averages are shown.

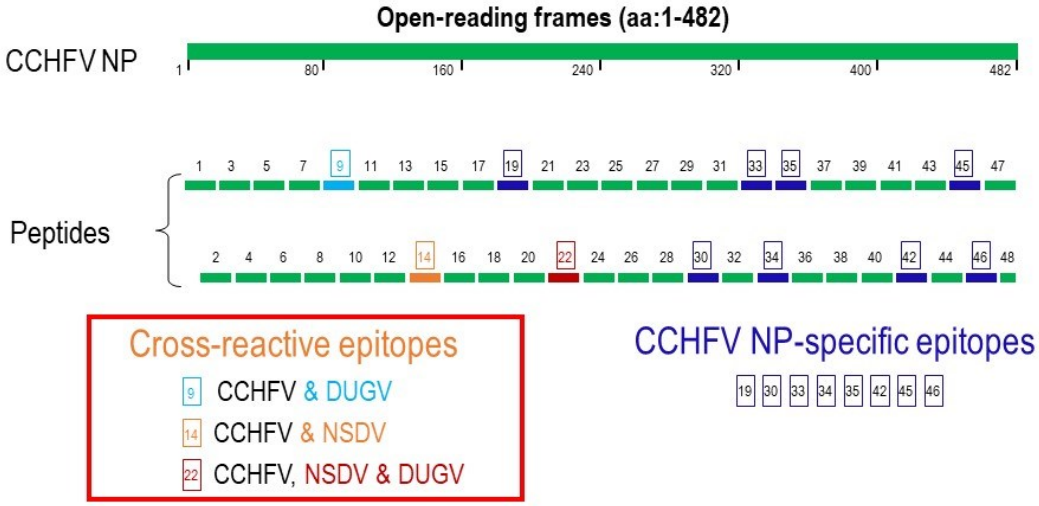

Figure 11: Summary of reactivity of antibodies to synthetic peptides in ELISA. Potential epitope peptides scattered from the N- to C-terminal within the CCHFV NP molecule.

Table 3: Synthetic peptides to which anti-CCHFV NP or anti-NSDV serogroup antibodies bound

Peptide name and aa position	CCHFV-infected patient										Mk	MIAF	
	10	13	16	23	26	31	40	42	46	50		NSDV	DUGV
P9 (81-100)	-	-	-	-	-	-	-	-	-	-	-	-	+
P14 (131-150)	+	+	+	+	+	-	+	+	-	-	+	+	-
P19 (181-200)	-	-	-	-	-	-	-	-	+	-	-	-	-
P22 (211-230)	+	+	-	-	-	-	-	+	-	-	+	+	+
P30 (291-310)	-	-	-	-	-	-	-	-	-	-	+	-	-
P33 (321-340)	-	-	-	-	-	-	-	-	-	+	-	-	-
P34 (331-350)	-	-	-	-	-	-	-	+	-	-	-	-	-
P35 (341-360)	-	+	-	-	-	-	-	+	-	-	-	-	-
P42 (411-430)	-	+	-	-	+	-	+	-	+	-	-	-	-
P45 (441-460)	-	-	-	-	-	+	-	-	-	-	-	-	-
P46 (451-470)	-	-	-	-	-	+	-	-	-	-	-	-	-

Mk: CCHFV-infected monkey serum

Amino acid sequence comparison for the common epitope peptides

The amino acid sequences of the peptides that were commonly recognized by IgG antibodies to CCHFV and NSDV serogroups (i.e., P9, P14, and P22) were compared among CCHFV, NSDV, and DUGV NPs (Fig. 12). As expected, these peptides had relatively conserved amino acid sequences among the viruses. The amino acid sequence similarity of P9 between CCHFV and DUGV was higher (60%) than those between CCHFV and NSDV (50%). The similarity of P14 between CCHFV and NSDV was 60% whereas that between CCHFV and DUGV was 45%. These differences were correlated with the cross-reactivity of the MIAFs to the respective peptides. The P22 peptide, to which anti-CCHFV, -NSDV, and -DUGV IgG antibodies all bound in ELISA, had 15 conserved amino acid residues, which was also consistent with cross-reactivity of the antibodies. These data suggested that the amino acid similarities of these peptides were correlated with the serological cross-reactivity.

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                 **   *** * * * ** **
P9     CCHFV     AWVSSTGIVKKGLEWFEKNA
       NSDV      AWTSCTGMIQRGLDWFDNNG
       DUGV      AWTCSTGVVQKSLSWFDKNK

                 ******     * *****
P14    CCHFV     LKWRKDIGFRVNANTAALSN
       NSDV      LKWRKDTKYGINKNTAALAA
       DUGV      QKWRKDVGYEINQFTRSLTH

P22    CCHFV     MAFNPPWGDINKSGRSGIAL
       NSDV      VVFNPPWGDINKAGKSGIAL
       DUGV      VVFNPPWGDINKCGKSGIPL
```

Figure 12: Amino acid sequence comparison among CCHFV, NSDV, and DUGV
Amino acid sequences of CCFHV, NSDV, and DUGV NPs corresponding to the P9, P14, and P22 peptides are shown. Amino acid residues conserved among all three viruses are shown in red. Amino acid residues shared between CCHFV and DUGV NPs and between CCHFV and NSDV NPs are indicated with asterisks for P9 and P14, respectively.

Discussion

Antibodies to CCHFV NP are known to potentially cross-react to other orthonairoviruses in the NSDV serogroup [24, 36]. Although CCHFV NP epitopes have been mapped in previous studies [9, 35, 42, 70], detailed information on the epitopes on orthonairoviruses is still needed. To discern and identify antigenic regions on CCHFV and NSDV NPs, CCHFV-NSDV chimeric NPs were produced and used for Western blotting analyses in this study. IgG antibodies produced against CCHFV mostly bound to Ch-NP4 and Ch-NP5, whereas those against NSDV and DUGV virus bound to Ch-NP1 and Ch-NP2. These results suggested that there might be a limited number of conserved epitopes among these viruses. The Western blotting analysis further suggested that CCHFV- and NSDV-specific epitopes were located in the amino acid regions at positions 240-482 and 1-240, respectively.

Accordingly, none of the 12 mAbs generated in this study reacted to NSDV NP in Western blotting analyses. Consistent with the data of polyclonal antibodies to CCHFV, 5 mAbs (17-3, 79-10-3, 80-6, 91-5, and 97-6) reacted to Ch-NP4 and Ch-NP5, suggesting that these mAbs recognized the amino acid region at positions 240-320. Although the reactivity was weak, mAbs 74-2 and 114-2 only bound to Ch-NP5, suggesting different epitope recognition (amino acids at positions 320-400) from the above 5 mentioned mAbs. Two mAbs (32-1 and 86-3) reacted to wildtype CCHFV NP and did not react to any chimeric NPs, suggesting that these mAbs recognized epitopes different from those of the other 7 mAbs that reacted to Ch-NP4 and/or Ch-NP5. Overall, these results demonstrated the presence of at least 4 distinct antigenic regions on the CCHFV NP molecule.

Previous papers by others have demonstrated that the amino acid region at positions 183-305 of CCHFV NP contains major antibody epitopes [9, 35, 37, 53, 70]. However, the N- and C-termini of NP have also been shown to include some epitopes [9, 42]. Consistent with the results of these studies, the polyclonal and monoclonal antibodies used in the present study reacted to the amino acid regions including positions 240-482 of CCHFV NP. PEPscreen library-based ELISA to further identify the epitope sequences revealed at least 11 epitope peptides scattered along the length of the CCHFV NP molecule. Distinct antibody repertoires among CCHFV-infected individuals were also suggested, consistent with previous studies [9, 37]. The epitope peptides for CCHFV-specific IgG were located in a wide region of CCHFV NP spanning amino acid positions from 131 to 470, which overlapped with the previously reported region consisting of amino acid residues at positions 123 to 396 [9]. However, the approaches in the present

study (i.e., Western blotting and short peptide antigens) has a limitation in that conformational epitopes cannot be identified.

None of the generated anti-NP mAbs bound to the synthetic peptides tested in this study. This probably indicates that they recognized conformational epitopes. Although some of the mAbs reacted to CCHFV NP in Western blotting, it is conceivable that the synthetic peptides used (20 amino acid-length) might not be able to form proper structures. It has also been shown that a predicted biosynthetic CCHFV NP linear antibody epitope expressed in *E. coli* (DH5α) is not recognized by mAbs or polyclonal anti-CCHFV [50, 70]. Since these antibodies showed no cross-reactivity to NSDV NP, it is likely that they are CCHFV NP-specific, and their epitope recognition is different from the majority of anti-NSDV NP antibodies as shown by the reactivity pattern to the chimeric NPs. Structural analyses using an antibody-NP complex will be required for further detailed epitope mapping of CCHFV NP.

Although the genetic diversity among nairovirus nucleoproteins is significant [67], the viruses within NSDV and CCHFV serogroups are closely related [26, 48]. Previously, a linear epitope was predicted within the P22 sequence region of CCHFV NP (strain SPU 415/85) and the antigenic similarity between CCHFV and DUGV has been reported [9, 35, 48]. The present study also pointed out the sequence similarity in some of the peptide sequences among CCHFV, NSDV, and DUGV, and suggested the potential cross-reactivity of antibodies to NSDV and CCHFV serogroups. Importantly, however, the present study suggests that although there are some common epitopes between NSDV/DUGV and CCHFV, such cross-reactive epitopes are not dominant as indicated by little cross-reactivity of the respective antibodies in Western blotting.

In this study, I focused on antibody epitopes on CCHFV NP and the presence of shared epitopes with NSDV/DUGV NPs. However, it is also important to analyze cross-reactivities of antibodies to Hazara virus, which belongs to the CCHFV serogroup but, is reported to be nonpathogenic to humans [10, 30]. Although several serological diagnostic assays for CCHF have been previously established, the cross-reactivity issue is still a potential weakness of the assays since the whole NP molecule is used as an antigen. Fine epitope mapping of CCHFV NP is expected to improve the serological methods that can be used to establish a rapid point-of-care detection assay. In addition, the mAbs produced in this study are useful for the development of immunochromatography-based rapid diagnostic tests.

Summary

The CCHFV NP is the most used antigen for serological screening of CCHFV infection of animals and humans. To gain insight into antibody epitopes on the NP molecule, I produced recombinant chimeric NPs between CCHFV and NSDV, which is another nairovirus, and tested rabbit and mouse antisera/immune ascites, anti-NP monoclonal antibodies, and CCHFV-infected animal/human sera for their reactivities to the NP antigens. It was found that the amino acids at positions 240-482 might include some epitopes recognized by anti-CCHFV IgG antibodies. In contrast, IgG antibodies to NSDV reacted to the region consisting of amino acid positions 1-240. Their binding capacities were further tested using a series of synthetic peptide whose sequences were derived from CCHFV NP. IgG antibodies in CCHFV-infected monkeys and patients were reactive to some of the synthetic peptide antigens (e.g., amino acid residues at positions 131-150 and 211-230). Only a few cross-reactivities to CCHFV NP were seen in the anti-NSDV serum. These results provide useful information to improve NP-based antibody detection assays as well as antigen detection tests relying on anti-NP monoclonal antibodies.

Conclusion

CCHFV is maintained in ticks and amplified in animals. Its infection of humans is potentially associated with fatal hemorrhagic fever. Serological surveillance of CCHFV infection of animals serves as an indicator of public health risks, and early detection of human infection is important for prevention of viral spread. However, CCHFV infection is not serologically monitored due to the limited availability of diagnostic tools. Existing serological diagnostics are human-specific and not readily available in CCHFV-affected areas. Handling live infectious CCHFVs requires a high biocontainment facility, limiting the capacity of serological diagnosis such as neutralizing tests. Hence, there is a need to improve serological assays for CCHFV surveillance and epidemiology studies to prevent potential outbreaks.

In the first chapter, I describe a simple procedure for expression and purification of the recombinant CCHFV NP and its utility as an antigen for ELISA for the detection of CCHFV-specific IgG in serum/plasma of humans and animals. I cloned cDNA of the open reading frame of the CCHFV NP, the predominant component expressed during infection, which is antigenically conserved among CCHFV clades. The recombinant NP was expressed in a human cell line and purified for the establishment of an ELISA protocol that can be used for screening of human and animal serum samples. In addition to its animal species-independent utility, the established CCHFV NP-based ELISA showed abilities comparative to ELISA with whole CCHFV particle antigens and a commercialized ELISA kit for the detection of CCHFV NP-specific IgG. These results demonstrate the usefulness of the CCHFV NP-based ELISA for seroepidemiological studies.

In the second chapter, I seek to identify antibody epitopes on the CCHFV NP molecule. It was found that major antigenic regions are different between CCHFV and NSDV NPs: amino acids at positions 240-482 and 1-240 include dominant epitopes recognized by anti-CCHFV and anti-NSDV IgG antibodies, respectively. Accordingly, all of the anti-CCHFV NP mAbs generated in this study recognized the antigenic region consisting of the amino acids at positions 240-482. ELISA with a series of synthetic peptides based on the CCHFV NP sequence revealed that IgG antibodies in CCHFV-infected monkeys and patients were reactive to some of the peptide antigens. Interestingly, some of these peptides had relatively conserved amino acid sequences among the orthonairoviruses tested. Fine epitope mapping of CCHFV NP and production of CCHFV-specific mAbs are expected to improve the virus-specific serological methods that can be used to establish a rapid point-of-care detection assay.

In this study, I focused on improving serological diagnostic assays for CCHFV infection. These findings not only provide useful information to improve the sensitivity and specificity of serological tests for CCHF but also help to enhance surveillance systems of CCHFV infection of both animals and humans through the development of rapid detection tests such as an immunochromatography-based diagnostic assay. These may contribute to CCHF prevention and control by reducing animal-to-human and human-to-human transmission of the virus.

Acknowledgements

Thanks, and glory to the heavenly Father for his endless blessing on me.

My deepest gratitude goes to Prof. Ayato TAKADA, for being a wonderful supervisor, for his helpful guidance, advice, and endless patience.

All my gratitude to Prof. Yasuhiko SUZUKI, Prof. Hirofumi SAWA, and Prof. Yoshihiro SAKODA for valuable supports and advice more than a necessity during the experimental part as well as during thesis redaction to improve and expand my aptitude and skill as Ph.D.

My deep gratefulness to Prof. Manabu IGARASHI, Specially Appointed Assistants Prof.: Reiko YOSHIDA, Rashid MANZOOR, and Prof. Masahiro KAJIHARA friendly Kaji san, for their advice, technical and academic assistance.

All my gratitude to Dr. Masayuki SAIJO for providing me with rabbit serum anti-CCHFV NP and human serum samples.

All my deep gratitude to Prof. Jean-Jacques MUYEMBE TAMFUM for providing me this Ph.D. opportunity. May God bless you.

Thanks to Profs. Justin MASUMU and Sheila MAKIALA for their support and assistance. I also thank Ms. Julienne Sumbu and Mr. Constant Sibitali for their support.

All my gratitude to Ms. Hiroko MIYAMOTO, Ms. Kanako IBARAKI and Ms. Akina MORI-KAJIHARA for all their assistance and advice.

My special thanks to the members of the division of global epidemiology: Dr. Takeshi SAITO, Dr. Tatsunari KONDOH, Dr. Masahiro SATO, Dr. Yoshihiro TAKADATE, Dr. Kosuke OKUYA, Dr. Mao ISONO, Mr. Suguru TAGA, Ms. Yurie KIDA, Ms. Nodoka KASAJIMA, Mr. Takanari HATTORI, Mr. Yuji TAKAHASHI, Ms. Risa HAYASHI, and Mr. Yannick MUNYEKU, and all members of IIZC and university colleagues for their kindness, and friendship.

I am indebted to my wife Therese KANDJI, my children: Nicolas YANGA, Pauline ADIYO, Charles EHESE, Moïse PONGOMBO, Yves SHUNGU, and Mylda DIHANDJO for their understanding, so many deprivations, endless support, and patience during the long period of my absence. Thank you for every kind word and encouragement and for believing in me.

Thanks to Prof. Celestin PONGOMBO, Prof. Victor OKOMBE, Mr Charles EHESE, Me Mike PUNGU, Mr. Emile WEMBO, Mr. Armand WEMBANDAJTE, Mg Thomas OTSHUDI, Mr. Coco OKUNDJI, Prof. David ONGOMBE and Uncle François UKUNDJI for being brothers and friends and for supporting and encouraging me.

May my parents, sisters, family, friends, children, and all those who in one way or another have contributed to my doctoral training be reassured of my gratitude.

My special thanks to Science and Technology Research Partnership for Sustainable Development (SATREPS), the Japan Agency for Medical Research and Development (AMED) and Japan International Cooperation Agency (JICA) for financial support.

My deepest thanks to the following institutions:

Leading Program Office for their help during my doctoral course works and the International Institute for Zoonosis Control for providing me the opportunity to study virology and Zoonosis Diseases Control.

Institut National de Recherche Biomédicale for the collaboration, le Laboratoire Vétérinaire Central de Kinshasa and la Faculté de Médecine Vétérinaire de l'Université Pédagogique National for assistance and motivation and for allowing me to study abroad and improve myself.

Finally, this work is dedicated to the honor and the pain of my late mother, Germaine AMBOKAWA, and my brother Georges TSHUND'OLELA who despite the encouragement to endure and to study could not live these moments. May their souls rest in peace.

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