博士論文　(要約)

論文題目　Serological and epidemiological studies on zoonotic emerging infectious diseases in primates and bats in the Philippines

（フィリピンにおける翼手目及び霊長類の動物由来新興感染症に対する血清学的・疫学的研究）

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General introduction

Recently many viral emerging infectious diseases have been recognized around the world. “Emerging infectious disease” is defined as an infectious disease that is caused by an unidentified agent, which causes public health problems either locally or internationally. The typical examples of viral emerging infectious diseases are Ebola hemorrhagic fever (EHF), Lassa fever, Crimean-Congo hemorrhagic fever (CCHF), hemorrhagic fever with renal syndrome (HFRS), hantavirus pulmonary syndrome (HPS), acquired immunodeficiency syndrome (AIDS), and severe acute respiratory syndrome (SARS) and severe fever and thrombocytopenia syndrome (SFTS). The detailed information of viral emerging infectious diseases and their distribution are depicted in Figure 1.

Among the agents of emerging infectious diseases emerged in Asia, Reston virus (RESTV), CCHF virus (CCHFV), HFRS virus and SFTS virus (SFTSV) cause viral hemorrhagic fever (VHF) to humans or non-human primates (1-4). Under the Law concerning the Prevention of Infectious Diseases and Medical Care for Patients of Infectious diseases in Japan, EHF and CCHF are classified as Class 1 infectious diseases, and HFRS and SFTS are classified as Class 4 infectious diseases. Under the law, RESTV and CCHFV are categorized into Category 1 pathogens and are restricted to be manipulated in a Biosafety Level 4 (BSL-4) facility. HFRS virus and SFTSV are categorized into Category 3 pathogens and are restricted to be manipulated in a BSL-3 facility. Since there are no available BSL-4 facilities and a limited number of BSL-3 facilities in Japan, studies using these viruses are not allowed or are strictly restricted.

Ebolavirus belongs to a family *Filoviridae*, order Mononegavirales which is

characterized by a filamentous shape of the virion carrying an envelope originated from the cellular lipid membrane and a nonsegmented, negative-stranded (NNS) RNA genome (5). The family *Filoviridae* consists of two genera, *Ebolavirus* and *Marburgvirus* (MARV). Genus *Ebolavirus* has 5 species; *Zaire ebolavirus* (Ebola virus, EBOV), *Sudan ebolavirus* (Sudan virus, SUDV), *Taï Forest ebolavirus* (Taï Forest virus, TAFV), *Bundibungyo ebolavirus* (Bundibugyo virus, BDBV), and *Reston ebolavirus* (RESTV) (6). Recently, a new filovirus, named Lloviu virus, was identified in dead bats, *Miniopterus schreibersii*, in Cueva del Lloviu in southern Europe. Lloviu virus is proposed to be included in a species *Lloviu cuevavirus,* genus *Cuevavirus* in the family *Filoviridae* (7, 8).

Filoviruses are notorious because they cause highly lethal hemorrhagic illness in humans and nonhuman primates. Among filoviruses, MARV and four members of EBOV, SUDV, TAFV, and BDBV are known to cause severe disease in humans, while RESTV only causes severe disease in macaques (1). The first RESTV epizootic among cynomolgus macaques emerged in Reston, Virginia, U.S.A. in 1989 (9). The some monkeys imported from a primate breeding and export facility (facility A) located at Laguna in Luzon in the Philippines showed clinical signs of hemorrhagic fever during quarantine (1, 10). These macaques exhibited anorexia, decreased activity and hemorrhage. Splenomegaly and scattered petechial hemorrhages in a variety of organs were found at necropsy. Then the existence of ebolavirus was confirmed by electron microscopy (EM), serological test, and viral isolation. The virus was closely related but distinct from previously identified EBOV and SUDV. The virus was named RESTV

after the place of quarantine facility. Similar outbreak occurred among macaques in Philadelphia, Pennsylvania, too. These macaques were also received from the facility A. After these epizootics, there were several RESTV epizootics in Reston and Texas, U.S.A. in 1990 and in Italy in 1992 (11). In 1996, cynomolgus macaques, which were under quarantine in the Philippines before export to Texas, exhibited symptoms characterized by anorexia and lethargy, and died. RESTV infections in the macaques were confirmed by antigen-capture enzyme-linked immunosorbent assay (ELISA) and by EM. All the monkeys infected with RESTV in these epizootics were derived from the facility A. Epidemiological study in the facility A showed prevalence of RESTV infection among macaques (12). During the RESTV epizootics, any symptomatic RESTV infection among humans has not been reported while 4 (13) and 3 (14) antibody-positive individuals have been identified in U.S.A. and the Philippines, respectively. There have been no more cases of RESTV infection in macaques in the Philippines since all the monkeys were euthanized in the facility A, which was finally closed in 1997.

In 2008, RESTV infection was first detected in swine in several pig farms in the Philippines (15). RESTV infection was identified to be coinfected with porcine reproductive and respiratory syndrome virus (PRRSV) that causes a severe respiratory syndrome in swine. Swine sera and tissue samples were collected from five groups of swine at two commercial premises. Then several diagnostic investigations were conducted. In addition to PRRSV, RESTV was detected from a lymph node of a sick pig at Bulacan, near Manila in Luzon Island. The nucleotide sequence of the RESTV

isolate was more than 95% identical to the previously reported RESTV isolates from the macaques. Furthermore, RESTV infected pigs were discovered not only in Bulacan but also in Pangasinan, located outside Manila. During these swine RESTV epizootics, any symptomatic RESTV infection in humans has not been reported while 6 antibody-positive individuals associated with the epizootics have been identified.

Filovirus caused outbreaks were occurred mainly near the rain forest or mines and caves in Africa. Therefore many researchers, to elucidate this enzootic natural reservoir, investigated a variety of animals which live in forest, caves, and mines in Africa (16-21). However, in spite of many investigations since the first outbreaks of MARV and EBOV, the natural reservoirs of filoviruses have not been discovered. Recently, several reports finally demonstrated EBOV specific-IgG in fruit bats sera and EBOV genomes in the spleens and livers from four fruit bat species, *Rousettus aegyptiacus, Hypsignathus monstrosus*, *Epomops franqueti*, and *Myonycteris torquata*, in Africa (22-24). More recently, MARV was successfully detected and isolated from a fruit bat, *Rousettus aegyptiacus* (25, 26). Therefore, these fruit bats are thought to be natural reservoirs of EBOV and MARV.

SFTSV, another VHF agent detected in Asia, belongs to a genus *Phlebovirus*, family *Bunyaviridae* which is characterized by a spherical shape of the virion, with a diameter of 100 nm, carrying an envelop originated from the cellular lipid membrane and three segmented RNA genome (27). *Bunyaviridae* consists of five genera, *Phlebovirus*, *Hantavirus*, *Nairovirus*, *Orthobunyavirus* and *Tospovirus*. The genus *Phlebovirus* contains more than 80 viruses and most of them are associated with

phlebotomine sandflies, hence the genus name *phlebovirus*. There are several prominent exceptional virus members such as Rift Valley Fever virus (RVFV), which is associated with *Aedes* species mosquitoes, and Uukuniemi virus (UUKV), Bhanja virus (BHAV), SFTSV, and Heartland virus (HRTV), which are associated with ticks. The first SFTSV outbreak was recorded in Hubei Province in China in 2009 (4). Seventeen patients, five of whom died, showed fever, gastrointestinal symptoms, myalgia, and regional lymphadenopathy, and were suspected to contract human anaplasmosis (HA). However, gastrointestinal symptoms are inconsistent with HA and the antibodies against the HA pathogen, *Anaplasma phagocytophilum*, or its DNA were not detected in any of 17 patients. Since C-reactive protein (CRP) was not elevated in the patients, virus infection was suspected. Viral isolation using multiple cell lines was performed by researchers in Chinese Center for Disease Control and Prevention (China CDC), then a cell cytopathic effect (CPE) was observed in a dog macrophage cell line, DH82, and the virus was observed in their cells by EM. Genomic sequence analysis revealed that the agent was a novel bunyavirus. The clinical symptom of SFTS is characterized by high fever, gastrointestinal symptoms, myalgia, and regional lymphadenopathy. Besides, dizziness, joint pain, chills, and hemorrhage in mouth are also common in SFTS patients. Blood test is characterized by thrombocytopenia, leukocytopenia, and hepatic disorder. The novel bunyavirus causing SFTS was named SFTSV. After the identification of SFTSV, China CDC performed epidemiological surveillance from June 2009 through September 2010 (4). Hospitalized patients who met the case definition for SFTS in central and northeast China were examined, and SFTSV RNA or specific antibodies were detected

in 171 patients among the 241 patients. These patients included 43 in Henan, 52 in Hubei, 93 in Shandong, 31 in Anhui. The case fatality rate of SFTS ranged from 2% to 12%. In these regions, SFTSV RNA was also detected in ticks of the species *Haemaphysalis longicornis* at a rate of some 5.4%, and the ticks are shown to be associated with SFTSV transmission (4). In 2012 and 2013, SFTSV has been also discovered in Japan (28) and Korea (29), and SFTSV infection is considered to be a serious public health concern not only in China but also in all East Asia.

Many tick-borne phleboviruses (TBPVs) have been found all over the world (Figure 2). HRTV, genetically closed to SFTSV, was first detected in Missouri in US in 2009, and *Amblyomma americanum* is considered to be one of the vectors (30, 31). In India, Africa, and Europe, Bhanja virus serogroup (BHAV group) consisting of BHAV, Kismayo virus, Palma virus, and Forecariah virus have been detected (32-40). Phylogenetical and serological analyses have shown that these viruses constitute a novel clade of TBPVs (41, 42). Some of TBPVs including SFTSV are genetically close and cause severe febrile illness in humans. Several previous reports described a close antigenic relationship between SFTSV, HRTV, and viruses in BHAV group (42). UUKV, which has been used in a number of laboratory studies and causes disease in sea birds, is also included in TBPV (43).

Several epidemiological investigations targeting for these TBPVs including SFTSV were also conducted. SFTSV, viral RNAs and SFTSV-specific antibodies were detected in sheep, cattle, dogs, pigs, chickens, and hedgehogs in China (44, 45). On the other hand, there are no reports on the prevalence of SFTSV and the TBPVs in

Southeast Asia, even though Southeast Asia is located near China, Korea, and Japan.

Most emerging infectious diseases are also characterized as being zoonotic (46). Among the viral emerging infectious diseases shown in Figure 1, EHF, Marburg hemorrhagic fever, SARS, MERS, Nipha virus disease, and Hendra virus disease are recently recognized as bat associated infectious diseases (47-49). As bats are identified as natural reservoirs of several viruses, their unique characteristics and the role of bats as hosts for pathogens are getting revealed (50). They are the only flying mammal group of the order *Chiroptera*, which represents about 20% of all classified mammalian species worldwide and the number of species are the second biggest (47, 51). About 1,240 bat species are identified and divided into two suborders; *Megachiroptera* (fruit bat) and *Microchiroptera* (insectivore bat). They are distributed throughout most of the world except Antarctica and their geographical distribution is known to be broader than that of rodents. In addition to these characteristics, they have the excellence of their flying ability, the frequently great population densities, and long life span. As described above, bats have many favorable characteristics as host for pathogens, and they have many opportunities to be exposed to many pathogens as compared with other mammals. Actually, recent study indicates that many animal and human paramyxoviruses have originated from bats (52). Thus, many other viruses might have originated from bats in the past. In this regard, serological and epidemiological studies on emerging infectious diseases in wild bats in a particular region are useful approaches to elucidate the prevalence of agents of emerging infectious diseases in the region.

In the thesis study, I focused on RESTV and SFTSV which cause zoonotic

emerging infectious disease characterized by hemorrhagic fever in non-human primates and human, respectively. RESTV infection is only recorded in the Philippines and the exploration of their reservoir is not elucidated in detail. SFTSV is a novel bunyavirus discovered in China in 2011 and in Japan and Korea in 2013 and its geographical distribution and the involvement of wild animals in a lifecycle of SFTSV are also not elucidated. To elucidate these questions, for RESTV, I developed several serodiagnosis systems using recombinant proteins and performed serological studies on RESTV-infected non-human primates, which are considered to be terminal hosts, and wild bats, which are hypothesized to be natural hosts. Ebolaviruses including RESTV cannot be cultured in Japan, since any BSL-4 facilities are unavailable in the country. Therefore the studies were mainly performed by using recombinant RESTV proteins. For SFTSV, as described the efficiency of epidemiological studies on wild bats above, I investigated the prevalence of SFTSV infection in wild bats in the Philippines to assess the prevalence of SFTSV infection in wild animals in the Philippines. As shown in Figure 3, tick parasitism to bats is frequently observed. In this regard, serological studies on tick-borne Phleboviruses including SFTSV in wild bats are considered to be significant to elucidate the prevalence of SFTSV or its related viruses in South Asia.

Figure legends

Figure 1. Geographical distribution of viral emerging infectious diseases.

Diseases, marked "*" at the right upper, are categorized as viral hemorrhagic fever.

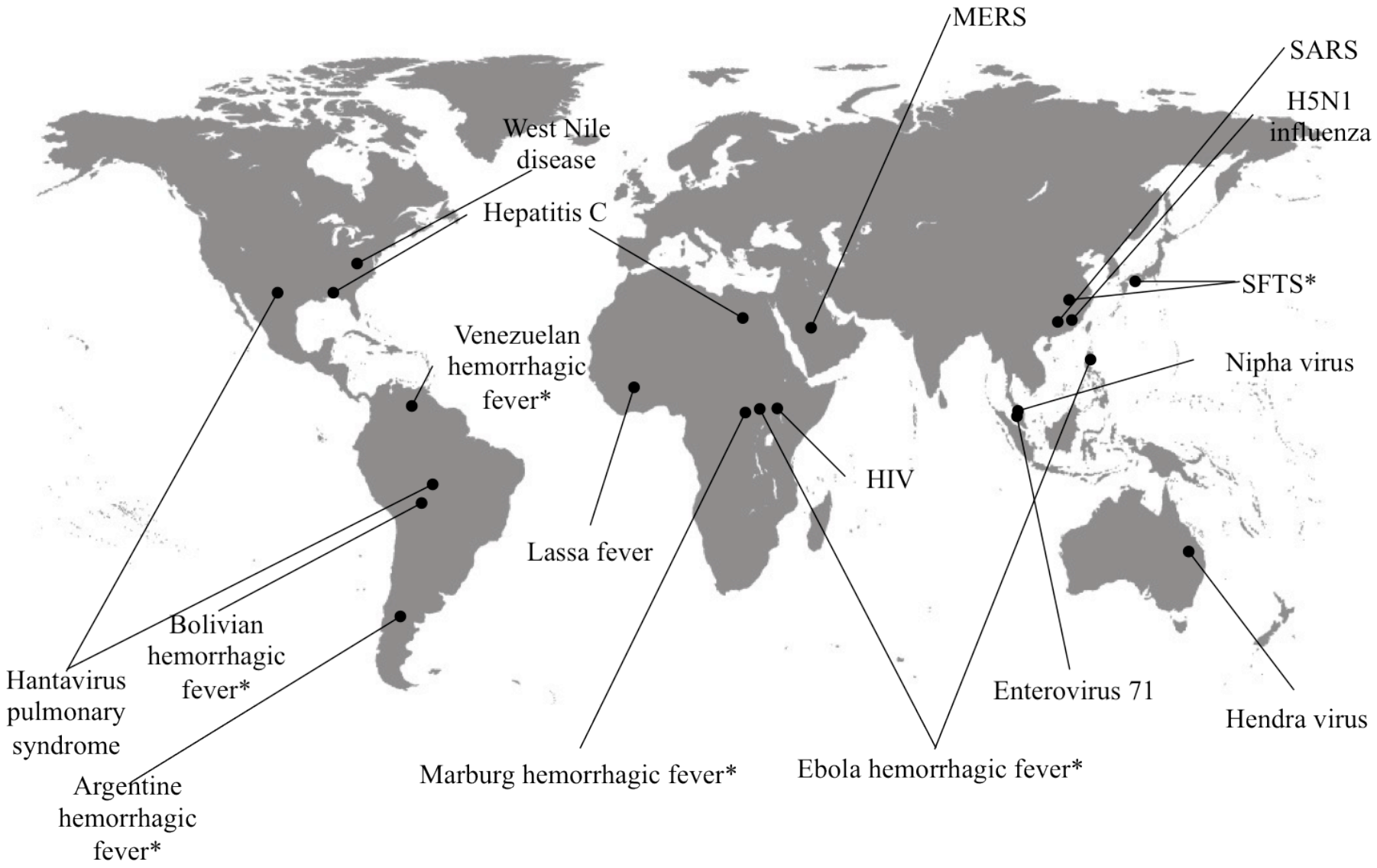
Figure 2. Geographical distribution of Tick-borne Phlebovirses.

The prevalence of severe fever with thrombocytopenia syndrome virus infection was recorded in the countries colored with red. The prevalence of Heartland virus infection was recorded in the country colored with blue. The prevalence of Bhanja virus group viruses (Bhanja virus, Kismayo virus, Palma virus, and Forecariah virus) infection was recorded in the countries colored with green.

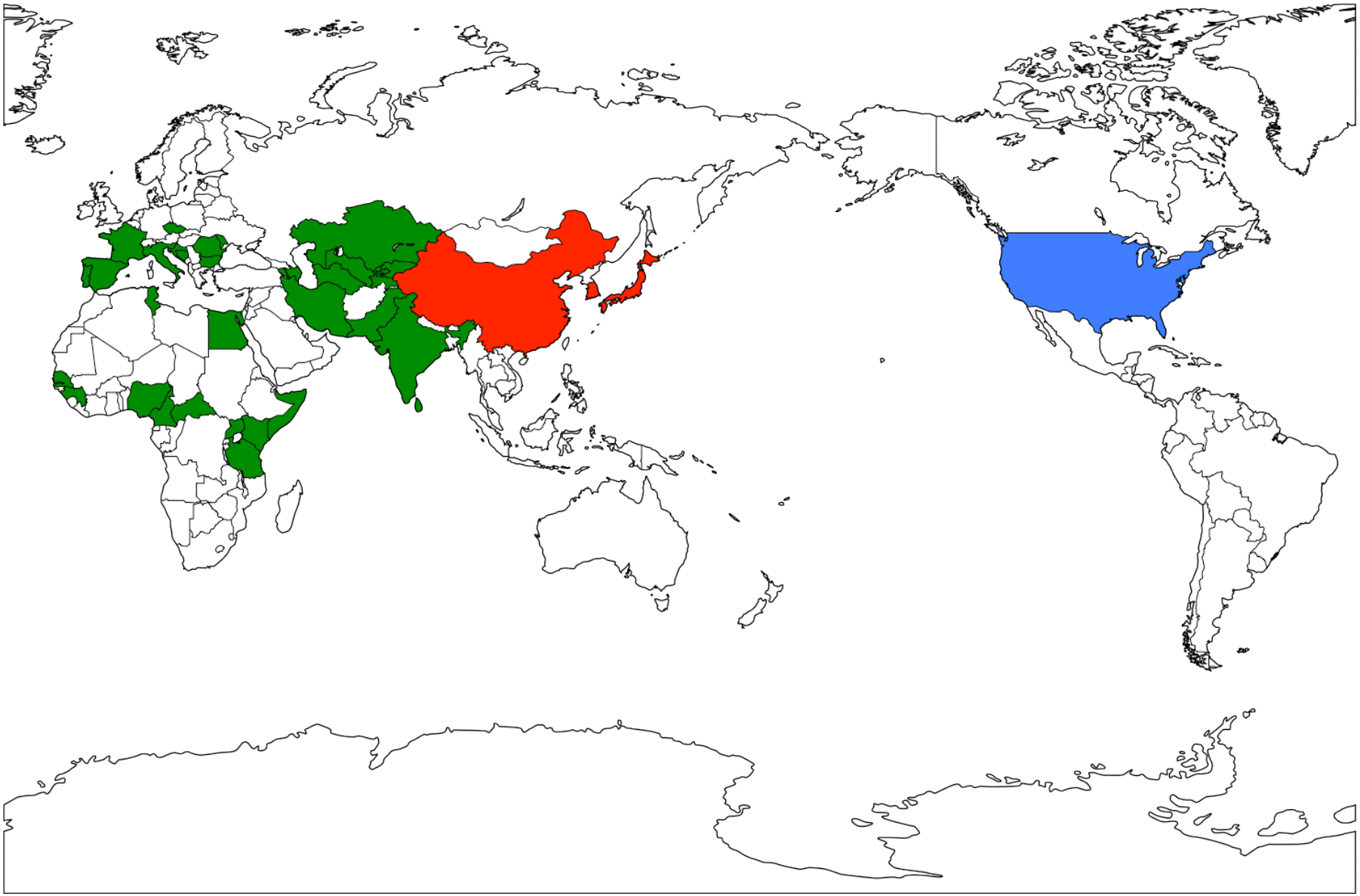
Figure 3. Tick parasitism to bats.

Arrows indicate ticks on the bat wing.

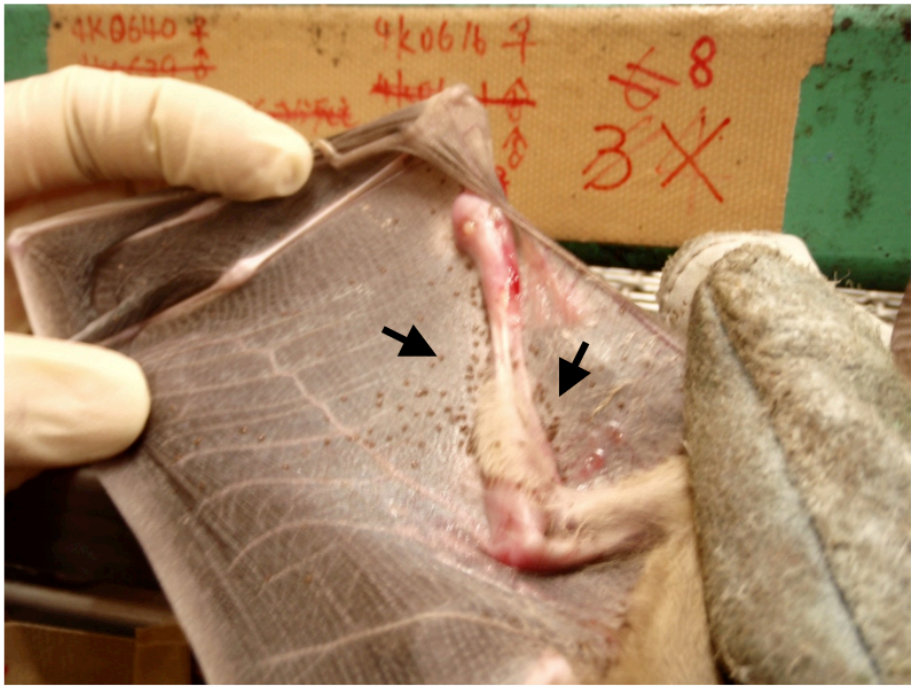
General introduction Figure 1.



General Introduction Figure 2.



General Introduction Figure 3.



Chapter 1

Analysis of the humoral immune responses among cynomolgus macaque naturally infected with Reston ebolavirus during the 1996 outbreak in the Philippines

Abstract

Ebolaviruses induce lethal viral hemorrhagic fevers (VHFs) in humans and non-human primates, with the exceptions of Reston virus (RESTV), which is not pathogenic for humans. In human VHF cases, extensive analyses of the humoral immune responses in survivors and non-survivors have shown that the IgG responses to nucleoprotein (NP) and other viral proteins are associated with asymptomatic and survival outcomes, and that the neutralizing antibody responses targeting ebolaviruses glycoprotein ($GP_{1,2}$) are the major indicator of protective immunity. On the other hand, the immune responses in non-human primates, especially naturally infected ones, have not yet been elucidated in detail, and the significance of the antibody responses against NP and $GP_{1,2}$ in RESTV-infected cynomolgus macaques is still unclear. In this study, I analyzed the humoral immune responses of cynomolgus macaque by using serum specimens obtained from the RESTV epizootic in 1996 in the Philippines to expand our knowledge on the immune responses in naturally RESTV-infected non-human primates. The antibody responses were analyzed using IgG-ELISA, an indirect immunofluorescent antibody assay (IFA), and a pseudotyped VSV-based neutralizing (NT) assay. Antigen-capture (Ag)-ELISA was also performed to detect viral antigens in the serum specimens. I found that the anti-$GP_{1,2}$ responses, but not the anti-NP responses, were closely correlated with the neutralization responses, as well as the clearance of viremia in the sera of the RESTV-infected cynomolgus macaques. Additionally, by analyzing the cytokine/chemokine concentrations of these serum specimens, I found high concentrations of proinflammatory cytokines/chemokines, such

as IFN-γ, IL-8, IL-12, and MIP-1α, in the convalescent phase sera. These results imply that both the antibody response to $GP_{1,2}$ and the proinflammatory innate responses play significant roles in the recovery from RESTV infection in cynomolgus macaques.

Introduction

As has been described in "General introduction", Filoviruses induce lethal VHFs in both humans and non-human primates, while RESTV infection in humans is probably subclinical, yet it also causes highly lethal VHF in cynomolgus macaques. In this study, I investigated the antibody responses of cynomolgus macaques that could be dead-end hosts for RESTV. Using serum specimens collected from cynomolgus macaques during a RESTV outbreak in the Philippines in 1996, I attempted to elucidate the significance of neutralizing antibodies to RESTV in viral clearance. An IgG-ELISA and an indirect immunofluorescent antibody assay (IFA) specific for RESTV nucleoprotein (NP) have been established previously (53-55). These assays are useful tools for investigating the signs of RESTV infection in cynomolgus macaques. In human cases, antibody responses against ebolaviruses have been analyzed extensively: IgG responses to NP and other structural proteins (e.g., VP40 and VP35) have been shown to correlate with asymptomatic and surviving cases, and neutralizing antibody responses targeting the ebolaviruses $GP_{1,2}$ appear to be the major indicator of protective immunity (56).

On the other hand, proinflammatory cytokines/chemokines are known to play a major role in the pathogenesis of ebolaviruses infections in various species. Previous studies have shown an uncontrolled secretion of proinflammatory cytokines/chemokines to contribute to a fatal outcome in EBOV-infected humans (57) and cynomolgus macaques (58). Strong proinflammatory cytokine/chemokine responses are also observed in convalescent or asymptomatic cases (59, 60). In RESTV-infected

cynomolgus macaques, high viremia has been shown to induce the secretion of proinflammatory cytokines/chemokines (61). However, there have so far only been a limited number of studies on the impact of proinflammatory cytokine/chemokine responses in the convalescent phase of RESTV infection.

In this study, I grouped the cynomolgus macaque samples based on the presence of RESTV NP-antigen in sera and analyzed the antibody reactions and cytokine/chemokine inductions to evaluate the presence of neutralizing antibody to RESTV. I found that the anti-$GP_{1,2}$ responses, but not the anti-NP responses, were closely correlated with the neutralization antibody responses, as well as the clearance of viremia, in the sera of RESTV-infected cynomolgus macaques. Additionally, a high concentration of proinflammatory cytokines/chemokines was detected in the convalescent phase specimens. These data suggest that both the anti-$GP_{1,2}$ responses and proinflammatory cytokines/chemokines play significant roles in the recovery from RESTV infection in cynomolgus macaques.

Materials and Methods

Sera

Twenty-seven cynomolgus macaque serum samples were obtained from the cynomolgus macaque facility in the Philippines where the 1996 RESTV epizootic occurred (14). The serum specimens in the affected facility were collected under quarantine of the Philippines. Nineteen of the 27 samples were previously subjected to an antigen capture ELISA. Nine of the samples were found to be RESTV antigen-positive, and the remaining 10 were considered to be antigen-negative (62). The serum specimens were treated at 56 °C for 30 min and virus in the cynomolgus macaque serum samples were inactivated. As negative controls for the IgG-ELISA and IFA, I used serum samples from 102 cynomolgus macaques collected at the Tsukuba Primate Research Center (TPRC) in Japan.

Two rabbits (six months old, specific pathogen free, female Japanese White, KITAYAMA LABES, Ina, Japan) were immunized four times with the histidine-tagged ectodomain of the RESTV $GP_{1,2}$ (RESTV $GP_{1,2}\Delta TM$) using IMJECT-ALUM (Pierce, Rockford, USA) as an adjuvant. The RESTV $GP_{1,2}\Delta TM$ of a 1996 RESTV (63) was prepared and purified as described below.

Hyperimmune anti-RESTV NP rabbit sera used for positive control sera in the IgG-ELISA for detection of RESTV NP antibodies were prepared previously (54). Briefly, rabbits were immunized with glutathione S-transferase (GST)-tagged partial recombinant NP of RESTV composed of carboxyl half (amino acids 360 to 739 out of 739 amino acids protein) for four times using IMJECT-ALUM as an adjuvant. Another

rabbit was immunized with His-RESTV NP which has 6 histidine-tags at its amino terminus. The sera were collected from the rabbits, inactivated at 56 °C for 30 min, and then stored at 4 °C until use.

The experiments with animals were performed in accordance with the Animal Experimentation Guidelines of the National Institute of Infectious Diseases. The protocol was approved by the Institutional Animal Care and Use Committee of the institute (Permit number: 990163 and 109075).

Recombinant baculovirus encoding $GP_{1,2}$ of RESTV

Recombinant baculoviruses encoding various forms of RESTV $GP_{1,2}$ were generated using cDNA of $GP_{1,2}$ gene derived from 1996 RESTV outbreak among cynomolgus macaques in the Philippines (GenBank accession no. AB050936).

The following plasmids were constructed, i.e., pAcYM1 vector (64) carrying 1) an entire RESTV $GP_{1,2}$ cDNA, 2) an entire RESTV $GP_{1,2}$ cDNA in which signal sequence was replaced with a honeybee melittin signal sequence, which can enable efficient translocation of proteins into the endoplasmic reticulum of insect cells (65), 3) an ectodomain region of RESTV $GP_{1,2}$ cDNA, and 4) an ectodomain region of RESTV $GP_{1,2}$ cDNA in which signal sequence was replaced with the melittin signal sequence. In these plasmid constructs, histidine-tag sequence was added at the 3'-terminus. Tn5 insect cells were co-transfected with a mixture of 200 ng of the plasmid, 20 ng of linearized baculovirus DNA (BacPAK^{TM}6 DNA, Clontech, CA, U.S.A.) and a transfection reagent, UniFECTOR^{TM} (B bridge inc., California, U.S.A.), then cultured at

26℃ in kanamycin free TC-100 medium (Gibco, CA, U.S.A.) supplemented with 10% FCS and 2% tryptose phosphate broth (Difco, BD, NJ, U.S.A.). The culture supernatants were harvested when cytopathic effects became evident. The resultant recombinant baculoviruses were designated as Ac RESTV $GP_{1,2}$ A8, Ac Mel RESTV $GP_{1,2}$ A8, Ac RESTV $GP_{1,2}$ ΔTM, and Ac Mel RESTV $GP_{1,2}$ ΔTM, respectively. Characteristics of these baculoviruses are described in Table 1. To confirm the expression of recombinant $GP_{1,2}$ of RESTV by these baculoviruses, the lysates of Tn5 cells infected with these viruses were analyzed in Western blotting method using antibody against histidine-tag.

Expression and purification of the recombinant RESTV $GP_{1,2}$ ectodomain

A recombinant baculovirus that expresses RESTV $GP_{1,2}$ ΔTM was used to prepare recombinant RESTV $GP_{1,2}$ for the IgG-ELISA. Tn5 cells were infected with Ac RESTV $GP_{1,2}$ ΔTM in cell culture dishes (Cat. No. 353003, Becton, Dickinson, Co., Franklin Lakes, NJ) at an m.o.i of 1 and cultured at 26 °C for 3 days. Then the cells were harvested, washed in PBS for three times and lysed in PBS containing 1% Nonidet P40 (NP-40) for 10 min on ice. Then the lysates were centrifuged at 8,000 rpm for 15 min and the supernatants (NP-40 lysate) were collected. The pellets were dissolved and sonicated in PBS containing 1% NP-40 and 8 M urea, and centrifuged at 8,000 rpm for 15 min. The supernatants (Urea lysate) were mixed with Ni^{2+}-agarose beads (QIAGEN, Hilden, Germany) and binding buffer (His Bind Kits, Novagen, Darmstadt, Germany). Then the histidine-tagged RESTV proteins were adsorbed to the Ni^{2+}-agarose beads at

4 °C for 2 hrs. Then the mixture was transferred to a column, and the beads were washed with two-fold bed volume of washing buffer (His Bind Kits, Novagen, Darmstadt, Germany) with 1% NP-40 and 4 M urea, five-fold bed volume of the washing buffer with 1% NP-40 and 1 M urea, and five bed volume of the washing buffer with 0.5% NP-40 and 0.5 M urea. Finally, the beads were washed with five-fold bed volume of the washing buffer and eluted 4 times with every 1 ml of elution buffer (His Bind Kits, Novagen, Darmstadt, Germany). Then I eluted 4 times with every 1 ml elution buffer containing 0.25% NP-40. These eluted fractions were collected every five min. I checked the expression and purification of recombinant RESTV $GP_{1,2}$ by 7.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Furthermore I used PD-10 desalting columns (GE healthcare,U.K.) to remove imidazole from the eluted purified $GP_{1,2}$ (RESTV $GP_{1,2}$ ΔTM). The purified recombinant RESTV $GP_{1,2}$ ΔTM was used for the IgG-ELISA specific for RESTV $GP_{1,2}$. Lysates of Tn5 cells infected with baculovirus with a deleted polyhedrin gene, Ac-ΔP, were similarly processed and then used as negative control antigen in the IgG-ELISA described below.

RESTV $GP_{1,2}$-specific IgG-ELISA

Ninety-six well plates were coated with the RESTV $GP_{1,2}$ ΔTM or with negative control antigen in 100 μl of PBS and incubated overnight at 4 °C. The amounts of the antigens were determined using hyperimmune rabbit sera to RESTV $GP_{1,2}$ as described below. The plates were washed three times with PBS containing 0.05% Tween 20 (PBS-T), and then 200 μl of PBS-T containing 5% skim milk (SKIM-PBS-T)

was added to each well and incubated at 37 °C for 2 hr. The cynomolgus macaque sera were diluted at 1:100, 1:400, 1:1,600, and 1:6,400 in SKIM-PBS-T, and the hyperimmune rabbit sera were four-fold serially diluted from 1:1,000 to 1:64,000 in SKIM-PBS-T. One hundred microliters of each serum dilution was added to the antigen-coated wells and incubated at 37 °C for 1 hr. After they were washed three times with PBS-T, the wells were further reacted with either HRP-conjugated goat anti-human IgG (H+L) (Lot:60504974, ZyMED) or HRP-conjugated goat anti-rabbit IgG (H+L) (Lot:398581A, ZyMED) at a dilution of 1:1,000 in SKIM-PBS-T. After being washed three times again with PBS-T, the ABTS substrate (Roche Diagnostics) was added to the wells. Then, the plates were incubated at 37 °C for 30 min, and the OD values of the wells at 405 nm were measured. Adjusted OD values were calculated by subtracting the OD value of the wells coated with the negative control antigen from that of the wells coated with RESTV $GP_{1,2}\Delta TM$.

Generation of recombinant baculovirus encoding RESTV NP with histidine-tag

RESTV whole NP cDNA clone, which is derived from 1996 RESTV outbreak among cynomolgus macaques in the Philippines (GenBank accession no. AB050936), was inserted in pKS336 plasmid (pKS336-RESTV-NP). The DNA of RESTV NP was subcloned into pAcYM1-C-His plasmid, a derivative of pAcYM1 plasmid carrying 8 histidine sequences just downstream of the cloning site of *Bam*HI. A resultant plasmid, designated as RESTV-NP-pACYM1C-His was used to generate a recombinant baculovirus expressing RESTV NP with histidine-tag at carboxyl terminus

in Tn5 insect cells.

Tn5 cells prepared in a 6 well plate was co-transfected with a mixture of RESTV-NP-pACYM1 C-His, linearized baculovirus DNA, BacPAK^{TM}6 DNA (Clontech, CA, U.S.A.), and UniFECTOR^{TM} (B bridge inc., California, U.S.A.) in kanamycin-free TC-100 medium supplimented with 10% FCS and 2% TPB. Five days later, Tn5 cells were passaged into a T75 flask (Cat, No. 353134, Becton, Dickinson, Co., Franklin Lakes, NJ). The culture supernatants were harvested when cytopathic effect caused by baculovirus became evident, then the supernatants were clarified by a centrifuge at 3,000 rpm for 10 min and used as the seed virus (Ac RESTV NP C-his).

Expression and purification of recombinant RESTV NP

Tn5 cells were infected with Ac RESTV NP C-his with an m.o.i. of 1 and cultured at 26 °C for 3 days. The cells were collected, washed with PBS for three times, and then lysed in PBS containing 1% NP-40 for 15 min on ice. Then I centrifuged the lysates at 8,000 rpm for 15 min to remove the undissolved fractions. The supernatants (designated as NP-40 lysate) were mixed with Ni^{2+}-agarose beads (QIAGEN, Hilden, Germany) and binding buffer (His Bind Kits, Novagen, Darmstadt, Germany). Then the mixture was gently rotated using an end-over-end shaker for 2 hr at 4 °C to absorb the dissolved proteins to beads. The beads were then transferred to a column, washed with six times bed volume of washing buffer (His Bind Kits, Novagen, Darmstadt, Germany) and the recombinant RESTV NP was eluted 6 times with every one ml of elution buffer containing imidazole (His Bind Kits, Novagen, Darmstadt, Germany). The expression

and purification of the recombinant NP was analyzed by SDS-PAGE. Furthermore, I used PD-10 desalting column to remove imidazole from the purified recombinat NP (RESTV NP). The lysates prepared from Tn5 cells infected with baculovirus lacking a polyhedrin gene, Ac-ΔP, were similarly processed and used as negative control antigen in the IgG-ELISA described below.

IgG-ELISA using purified C-His-tagged RESTV NP

The purified recombinant RESTV NP with a histidine-tag at the C-terminus, which has been previously reported (53) and described in above section was used for IgG-ELISA specific for RESTV NP.

OD index and determination of the cut-off value for the IgG-ELISA

The sum of the OD values of serum dilutions at 1:100, 1:400, 1:1,600, and 1:6,400 for each specimen was calculated and designated as an "OD index" in the IgG-ELISA (66). The mean plus three standard deviations (SD) of the ELISA indices for the IgG-ELISAs was calculated using serum samples from uninfected TPRC cynomolgus macaques and was used as the cut-off value for the IgG-ELISAs.

Indirect immunofluorescent antibody assay (IFA) specific for RESTV NP and $GP_{1,2}$

The IFA specific for RESTV NP was reported previously (55). In the present study, a RESTV $GP_{1,2}$-specific IFA was established using HeLa cells stably expressing RESTV $GP_{1,2}$. HeLa cell line was purchased from the American Type Culture

Collection and used. RESTV $GP_{1,2}$ was subcloned into a mammalian expression plasmid, pKS336, to generate pKS336-RESTV-$GP_{1,2}$. The HeLa cells expressing RESTV $GP_{1,2}$ were selected in a medium containing 2 μg/ml of blasticidin-S-hydrochloride (Sigma, St. Lois, MO) after transfection with pKS336-RESTV-$GP_{1,2}$ using the FuGENE HD Transfection Reagent (Roche Diagnostics, Germany). The cells were trypsinized, washed with PBS, and mixed with normal HeLa cells, and were then spotted on 14 well Teflon-coated glass slides (Cat. No. JL926101, AR Brown Co., Ltd., Tokyo, Japan), air dried, and fixed with acetone at room temperature for 5 min. The slides were stored at -80 °C until use.

The slides were thawed and dried just before use. The serum specimens were 2-fold serially diluted in PBS, and a 20 μl aliquot of each dilution was applied to the wells of the antigen slides and incubated at 37 °C for 1 hr in a humidified chamber. Then the antigen slides were washed with PBS and reacted with 20 μl per well of FITC-conjugated goat anti-human IgG (H+L) (ZyMax lot: 415460A, Invitrogen, CA, U.S.A.) for cynomolgus macaque sera and FITC-conjugated goat anti-rabbit IgG (H+L) (ZyMax lot: 402686A, Invitrogen, CA, U.S.A.) for rabbit hyperimmune sera at a dilution of 1:100. After incubation at 37 °C for 1 hr, the slides were washed with PBS and covered with cover glasses. The slides were examined for the staining pattern under a fluorescent microscope. The antibody titer in the IFA was determined as the reciprocal of the highest dilution showing positive staining.

RESTV neutralization (NT) assay using VSV-RESTV-$GP_{1,2}$/GFP

The VSV pseudotype bearing RESTV $GP_{1,2}$, VSV-RESTV-$GP_{1,2}$/GFP was generated essentially according to the method described for the VSV pseudotype bearing SARS-CoV S protein (67), except that pKS336-RESTV-$GP_{1,2}$ was used in the present study. Briefly, 293T cells were prepared in 24 well plates at 20-30% confluency. The cells were transfected with pKS336-RESTV-$GP_{1,2}$ using FuGENE HD. The cells were then cultured for 24 hr and inoculated with VSV ΔG*/GFP pseudotyped with the VSV-G protein at an m.o.i of 5, adsorbed at 37 °C for 1 hr, and then washed with DMEM-5% FCS and further cultured for 24 hr. The culture supernatants were collected and centrifuged at 1,000 rpm to remove cell debris. Thereafter, the supernatants were stored at -80 °C as VSV-RESTV-$GP_{1,2}$/GFP. The infectivity titer of VSV-RESTV-$GP_{1,2}$/GFP, harboring the VSV ΔG*/GFP genome, was determined by counting the number of GFP-positive cells under a fluorescent microscope upon infection into Vero E6 cells, as described previously. Briefly, VSV-RESTV-$GP_{1,2}$/GFP was 3.2 (0.5 $\log_{10}$)-fold serially diluted with DMEM-5% FCS and then inoculated to Vero E6 cells prepared in 96 well culture plates. The cells were incubated at 37 °C in a CO_2 incubator for 24 hr. Then, GFP-positive cells were counted under a fluorescent microscope (BZ-9000; KEYENCE, Osaka, Japan), and the infectious units (IU) of the pseudotyped VSV were calculated.

The serum samples were serially diluted in DMEM-5% FCS, and a 50 μl aliquot of each dilution was mixed with the same volume of DMEM-5% FCS containing 1,000 IU of VSV-RESTV-$GP_{1,2}$/GFP and incubated at 37 ºC for 1 hr. The

mixture was inoculated into Vero E6 cells and incubated for 24 hr. The number of GFP-positive infected cells was counted, and serum dilutions with 50% neutralization (NT_{50}) were identified.

Multiplex assay for cytokines and chemokines in the cynomolgus macaque sera

Eleven RESTV-infected cynomolgus macaque serum samples were inactivated at 56 °C for 30 min, diluted 1:10 in the assay diluent supplied with the Human Cytokine 25-Plex antibody bead kit (Invitrogen, CA), and were subjected to a multiplex cytokine analysis using a Luminex 100 instrument (Luminex Co., Austin, TX) according to the manufacturer's instructions. This Human Cytokine 25-Plex antibody bead kit was previously used to cynomolgus macaque sera and the cross-reactivity was confirmed (68). As negative controls, we used sera from 13 cynomolgus macaques bred at the TPRC and investigated the cytokine concentrations of these serum samples.

Results

Confirmation of expression of various forms of RESTV $GP_{1,2}$ by recombinant baculovirus

Recombinant baculoviruses that could express various forms of RESTV $GP_{1,2}$ were generated and the level of expression of RESTV $GP_{1,2}$ in Tn5 insect cells was compared upon infection with these recombinant baculoviruses. Among four recombinant baculoviruses, the highest level of expression of RESTV $GP_{1,2}$ was observed when Tn5 cells were infected with Ac RESTV $GP_{1,2}$ ΔTM and Ac Mel RESTV $GP_{1,2}$ ΔTM (Figure 1). Therefore I decided to use Ac RESTV $GP_{1,2}$ ΔTM throughout the present study.

Determination of the amounts of the antigens per well in RESTV $GP_{1,2}$-specific IgG-ELISA

To determine the optimal amount of the RESTV $GP_{1,2}$ ΔTM in IgG-ELISA, 1,000-fold diluted serum obtained from a cynomolgus macaque (#2180) which was previously shown to be infected with RESTV were reacted with 2-fold serial dilution of RESTV $GP_{1,2}$ ΔTM. As shown in Figure 2, antigen diluted 1:400 gave the highest signal. So that following experiments were performed using this antigen dilution.

RESTV NP- and $GP_{1,2}$-specific antibodies, neutralizing antibody responses, and the viral antigens in the cynomolgus macaque sera from the 1996 RESTV epizootic

Twenty-seven serum samples derived from cynomolgus macaques that were

either found already dead or had been euthanized at the facility were available. The presence of RESTV NP antigens was evaluated by antigen-capture ELISA (62) or immunohistochemistry (69), while that of anti-RESTV NP IgG was evaluated using IgG-ELISA and IFA methods (53-55). RESTV NP antigens were detected in the livers in # 2182, 2612, 2615, 2669, 2739, 2921, 2644 and 2728, while RESTV NP was detected by antigen capture ELISA in the sera of #2182, 2408, 2612, 2615, 2669, 2739, 2921, 2721 and 2972 (Table 2). I therefore assumed that these cynomolgus macaques had suffered from the acute viremic phase of the disease. Seventeen of the 27 samples (#2408, 2615, 2669, 2739, 2921, 2728, 2180, 2181, 2189, 2190, 2191, 2195, 2404, 2693, 2696, 2713 and 2194) were positive for anti-NP IgG in IgG-ELISA, while these samples all reacted in IFA (Table 2). On the other hand, two samples (#2644 and 2719) were only positive in IFA (Table 2). We considered the samples as anti-NP IgG-positive when either ELISA or IFA showed positive reaction. As a result, a total of 19 samples had anti-NP IgG. Cynomolgus macaques with anti-NP IgG consisted of NP antigen-positive (#2408, 2615, 2669, 2739, 2921, 2644 and 2728) and NP antigen-negative groups (#2180, 2181, 2189, 2190, 2191, 2195, 2404, 2693, 2696 and 2713).

In order to examine whether the sera contained anti-$GP_{1,2}$ antibodies, I employed a $GP_{1,2}$-specific IgG-ELISA and IFA. RESTV $GP_{1,2}$ ΔTM prepared by a baculovirus expression system and RESTV $GP_{1,2}$-expressing HeLa cells were used as antigens for $GP_{1,2}$-specific IgG-ELISA and IFA, respectively. Anti-RESTV $GP_{1,2}$ IgG were detected in 10 (#2180, 2181, 2189, 2190, 2191, 2195, 2404, 2693, 2696 and 2713)

out of the 27 serum samples by ELISA (37%), whereas the remaining 17 samples (63%) showed negative reactions. Nine serum samples positive for $GP_{1,2}$ antibodies in the IgG-ELISA also showed positive reactions in the IFA, while one serum sample (#2194) was only positive in the IFA. Serum samples showing positive reactions in either the $GP_{1,2}$-specific IgG-ELISA or IFA were considered to be anti-$GP_{1,2}$ positive (11/27, 41%, Table 2). Interestingly, the sera derived from cynomolgus macaques in the acute viremic phase did not contain any detectable anti-$GP_{1,2}$ IgG, although they often contained anti-NP IgG.

I next attempted to detect the neutralization (NT) antibody response in the sera of RESTV-infected cynomolgus macaques. The VSV pseudotype RESTV $GP_{1,2}$ (VSV-RESTV-$GP_{1,2}$/GFP) was used for the NT assay. Twelve serum samples (12/27, 44%) neutralized the VSV-RESTV-$GP_{1,2}$/GFP infection, with NT titers ranging from 80 and 640 (#2721, 2972, 2181, 2181, 2189, 2190, 2191, 2195, 2404, 2693, 2696 and 2713) (Table 2). The anti-$GP_{1,2}$ IgG were not detectable in #2721 and #2972 by IgG-ELISA, while those samples both had a neutralizing activity. These two specimens showed a positive response for viral antigen in the Ag-capture ELISA and were thus considered to be collected in an early seroconversion phase.

All cynomolgus macaques at the facility were euthanatized regardless of clinical status and there was a possibility that some of the cynomolgus macaques had combined infection with simian hemorrhagic fever virus (SHFV) in the animal facility (70). Therefore, immune responses against RESTV did not always reflect the clinical manifestation. For these reasons, I defined “convalescent” or “non-convalescent” based

only upon serological findings.

It is noteworthy that, among the serum samples that were positive for viral antigen and negative for the NT antibody (Ag +, NT -), all nine serum samples were negative for anti-$GP_{1,2}$ antibodies, whereas only two samples were negative for anti-NP antibodies. On the other hand, all of the ten serum samples that were negative for viral antigen and positive for the NT antibody (Ag -, NT +) were positive for both anti-$GP_{1,2}$ and anti-NP antibodies. This finding indicates that the anti-$GP_{1,2}$ antibody may therefore increase in cynomolgus macaques in the convalescent phase, while anti-$GP_{1,2}$ antibody is rarely detectable in the acute viremic phase of infection.

Multiplex assay for cytokines and chemokines in the cynomolgus macaque sera

Ebola virus infection triggers the expression of several proinflammatory cytokines/chemokines (58, 71, 72). To examine whether the convalescence from the RESTV infection correlates with the circulating proinflammatory cytokines/chemokines, eleven RESTV-infected cynomolgus macaque serum samples were subjected to a multiplex cytokine analysis. Since we do not know when the infection occurred for each cynomolgus macaque, it is still unclear whether the sera represented an early or late stage of infection. I used seven convalescent phase sera (Ag-, NT +: #2404, 2181, 2189, 2693, 2696, 2713, 2180, and four acute viremic phase sera (Ag +, NT -: #2182, 2612, 2739, 2921). Among the 27 serum samples, only these 11 serum samples were available for multiplex assay. Since the sera were heat-inactivated at 56 °C for 30 min prior to being subjected to the multiplex analysis, some cytokines, such as GM-CSF and IL-2,

which were previously shown to be elevated in some RESTV infected cynomolgus macaques (61), could not be measured in the assay.

I found that concentrations of several proinflammatory cytokines/chemokines (e.g., IFN-γ, IL-8, IL-12, IL-1ra, and MIP-1α) were significantly higher in convalescent than in acute phase sera (Figure 3). This observation is similar to the previous studies showing elevated concentrations of proinflammatory cytokines/chemokines in the convalescent or asymptomatic human cases (59, 60). In contrast, the concentrations of the five cytokines/chemokines (e.g., IFN-α, IP-10, MIP-1β, IL-6, and TNF-α) did not differ significantly between the two groups (Figure 4). Furthermore, the concentration of MCP-1, one of the proinflammatory chemokines, was lower in the convalescent than in the non-convalescent sera (Figure 4). These data suggested that IFN-γ, IL-8, IL-12, IL-1ra or MIP-1α might therefore be involved with the host immune responses in the convalescent phase of RESTV infection.

Discussion

RESTV NP-specific IgG-ELISA and IFA, that proved to be useful for the seroepidemiological studies of cynomolgus macaques during the RESTV epizootic in the Philippines in 1996, were previously developed (53-55). The assays based on recombinant NP are sensitive for the detection of RESTV-specific antibodies. On the other hand, anti-$GP_{1,2}$ antibodies are elicited in EBOV-infected human cases and are believed to have protective roles against lethal EBOV infection (73, 74). In the present study, in order to gain insight into the IgG responses during the recovery from infection with RESTV, anti-NP, anti-$GP_{1,2}$, and neutralizing antibodies and the level of viremia in the serum specimens were analyzed. The data presented herein showed that the anti-$GP_{1,2}$ response, rather than the anti-NP response, was correlated with both the lack in viremia and the neutralizing activities in the sera of RESTV-infected cynomolgus macaques. There may be at least two possibilities for the lack of anti-$GP_{1,2}$ IgG in the acute phase samples. It is known that soluble GP (sGP), which does not contain membrane anchor, is secreted during ebolaviruses infection, and it can adsorb the anti-$GP_{1,2}$ antibodies (75). The other possibility is that apoptosis of lymphocytes is induced during RESTV infection and the resulting host immune responses may thus be abrogated. Although the precise mechanism of action is still unknown, it is likely that no IgG responses to RESTV $GP_{1,2}$ are induced in the cynomolgus macaques during the acute phase of infection.

Since the cynomolgus macaques at the facility where the RESTV epizootic occurred were found dead or euthanized, sequential serum specimens from each

cynomolgus macaque were not available. It is also difficult to determine when each cynomolgus macaque became infected with RESTV. I found that three specimens that have anti-$GP_{1,2}$ antibodies were obtained from dead monkeys (#2180, 2181 and 2194), however, it is difficult to conclude whether RESTV infection caused their death because of the possibility of succumbing to infection by SHFV or some other agents. It is predicted that, among the serum samples examined here, nine were acute phase samples because they were positive in Ag-ELISA or immunohistochemistry (69) (Table 2). On the other hand, all but one (serum #2194) of the anti-$GP_{1,2}$-positive serum samples were Ag negative. Therefore, these cynomolgus macaques were considered to be in the convalescent phase of RESTV infection. In this regard, the presence of the anti-$GP_{1,2}$ antibody is thought to be a useful indicator for convalescence in cynomolgus macaques infected with RESTV.

Aberrant production of proinflammatory cytokines/chemokines are a significant factor implicated in the disease progression of EBOV- and Sudan virus (SUDV)-infected human cases and experimentally infected cynomolgus macaques (57, 58, 76). In addition, a balanced proinflammatory response is believed to be a critical factor for determining the disease outcome (77, 78). I focused on the circulating inflammatory cytokines/chemokines in RESTV-infected cynomolgus macaques and examined their relationship with convalescence. I thus found the concentrations of several proinflammatory cytokines/chemokines, such as IFN-γ, IL-8, IL-12, and MIP-1α, to be significantly higher in convalescent sera than in non-convalescent sera. Gupta et al. (2012) recently demonstrated that convalescent serum samples obtained

from BDBV-infected human cases include high concentrations of IL-1α, IL-1β, IL-6, TNF-α, and MCP-1 (60). Although the exact profiles of proinflammatory cytokines/chemokines shown in my study are different from those reported by Gupta et al., these differences are considered most likely to be due to differences among ebolaviruses (RESTV vs. BDBV), host species (cynomolgus macaques vs. humans), and differences related to the disease phase when the samples were obtained. It is possible that the upregulation of the proinflammatory innate immune responses contributed to the recovery from RESTV infection in cynomolgus macaques.

In rhesus monkeys experimentally infected with a lethal dose of EBOV, anti-inflammatory cytokines, such as IL-13 and IL-1ra, are highly elevated in the acute phase (58). In human Ebola VHF patients, increased concentrations of IL-10 and IL-1ra have been shown in fatal cases, thus suggesting that the mixed anti-inflammatory response syndrome (MARS) contributes to the pathogenesis of the hemorrhagic fever caused by ebolaviruses. Since all of the cynomolgus macaques involved in the epizootic were euthanized at the affected facility, the actual fate of the cynomolgus macaques was not clear, and some might have survived the infection. My data obtained using the sera from cynomolgus macaques in the RESTV epizootic showed higher IL-1ra responses in the convalescent phase than in the non-convalescent phase (Figure 3). There were no significant differences in the concentration of IL-10 between the two groups (Figure 5). This suggests that, unlike other ebolaviruses infections, RESTV does not induce MARS, which is characterized by an elevated induction of IL-1ra in the acute phase.

In conclusion, I have shown that the anti-$GP_{1,2}$ responses, rather than the

anti-NP responses, in cynomolgus macaques naturally infected with RESTV were specifically detected in the convalescent stage of RESTV infection. In addition, a high concentration of proinflammatory cytokines/chemokines was observed in the convalescent phase. Therefore, the anti-$GP_{1,2}$ response and the upregulation of the specific proinflammatory response might be useful indicators of convalescence from RESTV infection in cynomolgus macaques.

Conclusions

In this study, I analyzed the humoral responses in cynomolgus macaque serum samples collected during the 1996 RESTV outbreak in the Philippines and demonstrated that the anti-RESTV $GP_{1,2}$ response and the proinflammatory innate response play significant roles in the convalescence from RESTV infection in cynomolgus macaques.

Figure legends

Figure 1. Expression of recombinant Reston virus glycoproteins in Tn5 cells by baculovirus expression system.

(A) Analysis of the recombinant proteins in SDS-PAGE / CBB-staining. Lane 1 and 2: molecular weight markers, SDS-6H and SDS-7B (Sigma), respectively, lane 3 to 7: The cell lysates of Tn5 cells infected with Ac RESTV $GP_{1,2}$ A8, Ac Mel RESTV $GP_{1,2}$ A8, Ac RESTV $GP_{1,2}$ ΔTM, and Ac Mel RESTV $GP_{1,2}$ ΔTM, Ac NPV (wild type baculovirus) respectively. Lane 8: mock-infected Tn5 cell lysates. (B) Analysis of the recombinant proteins in Western blot analysis. Anti-penta-His mouse IgG was used as a primary antibody. The order of lanes is corresponding to that in (A).

Figure 2. Determination of optimal amounts of the antigens in Reston virus glycoprotein-specific IgG-ELISA.

Optimal quantity of the Reston virus glycoprotein-specific IgG-ELISA antigen per well was determined by using the heat-inactivated Reston virus infected cynomolgus macaque serum (#2180). The wells of ELISA plate were coated with antigens 2-fold serially diluted from 1 : 200 to 1 : 25,600 in PBS, and the wells of antigen coated plates were reacted with the cynomolgus macaque serum (#2180) at a dilution of 1 : 1,000 in IgG-ELISA. Then OD value of each well was recorded and compared. As a result, the optimal quantity of antigen was obtained by diluting the antigen at 1 : 400.

Figure 3. Profiles of the serum cytokine and chemokine concentrations in Reston virus-infected cynomolgus macaques.

The serum concentrations of IFN-γ, IL-8, IL-12, MIP-1α, and IL-1ra. The concentrations of these proinflammatory cytokines/chemokines (IFN-γ, IL-8, IL-12, MIP-1α) and the anti-inflammatory cytokine (IL-1ra) were significantly higher in convalescent (Ag - NT +) than in non-convalescent sera (Ag + NT -). Each dot represents one sample, and dashes (-) represent the median values. * indicates $p<0.05$, ** indicates $p<0.005$ (Mann Whitney test). Broken lines and the numbers written aside indicate the average concentrations of cytokine and chemokine in negative control cynomolgus macaques (n=13).

Figure. 4. Profiles of the serum cytokine and chemokine concentrations in Reston virus-infected cynomolgus macaques.

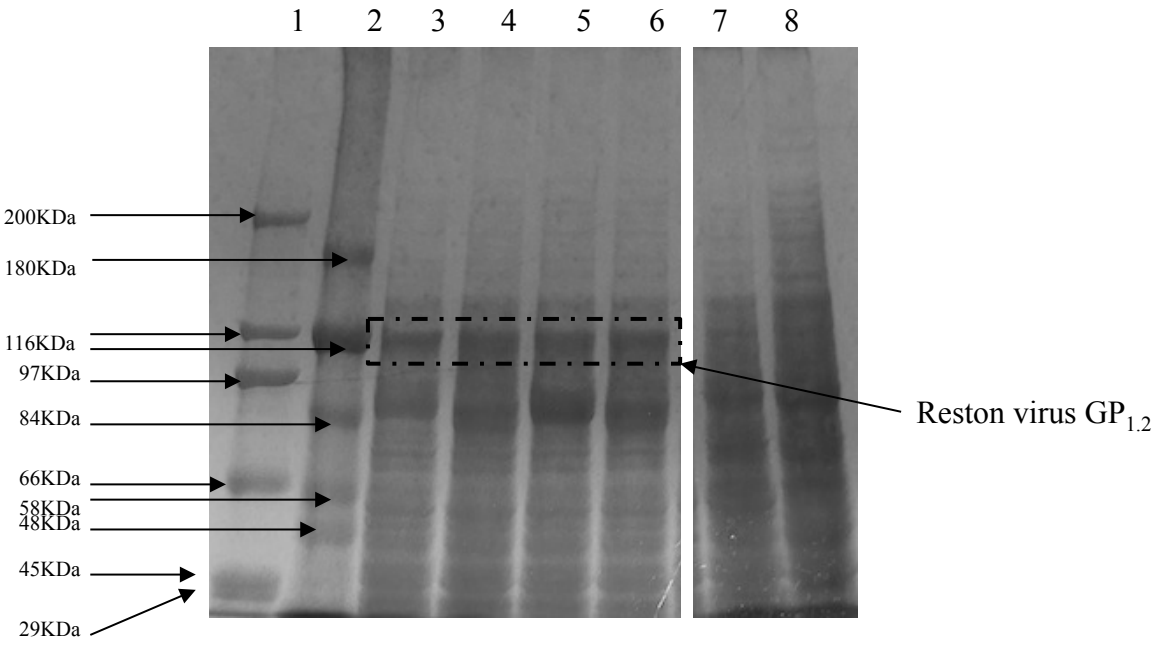
The IFN-α, IP-10, TNF-α, MIP-1β, IL-6 and MCP-1 concentrations. The serum concentrations of these cytokines/chemokines (IFN-α, IP-10, TNF-α, MIP1β) did not differ significantly between the convalescent cases (Ag - NT +) and the non-convalescent cases (Ag + NT -). The concentration of the anti-inflammatory cytokine, MCP-1, was lower in convalescent than in non-convalescent cynomolgus macaques (Mann Whitney test). Each dot represents one sample, and dashes (-) represent the median values. Broken lines and the numbers written aside indicate the average concentrations of cytokine and chemokine in negative control cynomolgus macaques (n=13).

Figure. 5. Profiles of the serum IL-10 concentration in Reston virus-infected cynomolgus macaques.

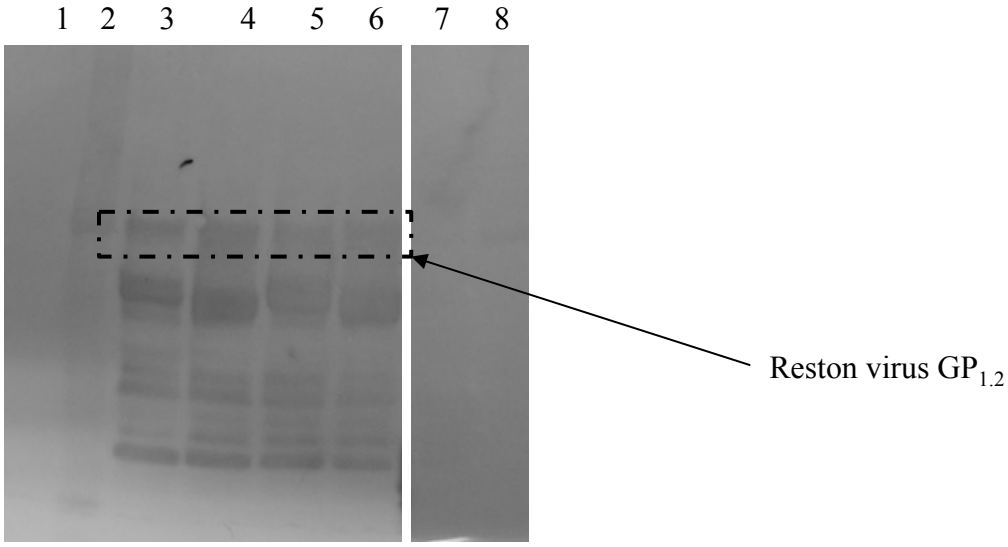
The serum concentration of IL-10 did not differ significantly between the convalescent cases (Ag - NT +) and the non-convalescent cases (Ag + NT -). Each dot represents one sample, and dashes (-) represent the median values. Broken lines and the numbers written aside indicate the average concentrations of IL-10 in negative control cynomolgus macaques (n=13).

Chapter 1 Figure 1.

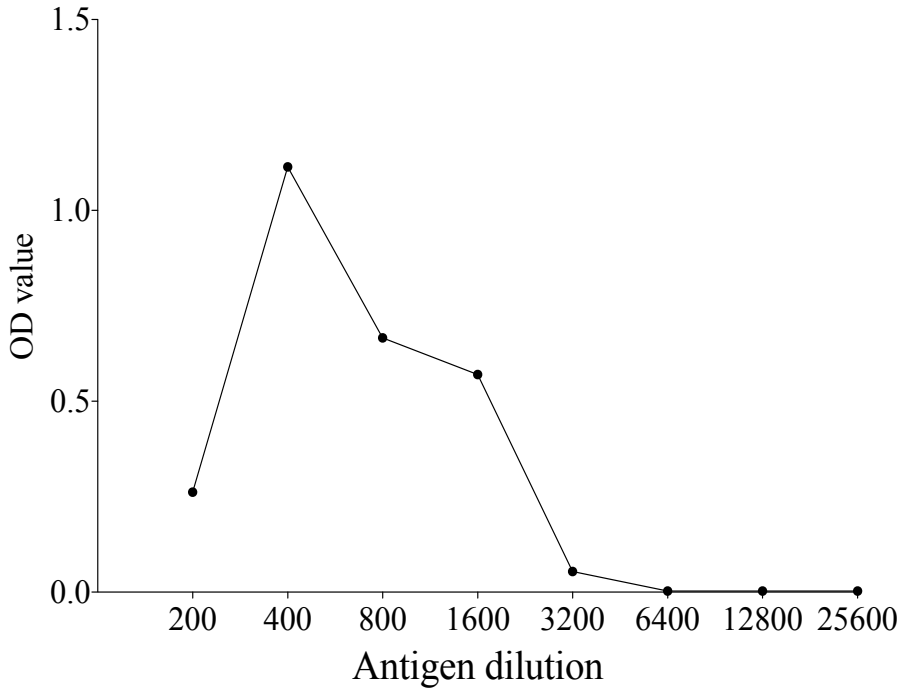
(A)



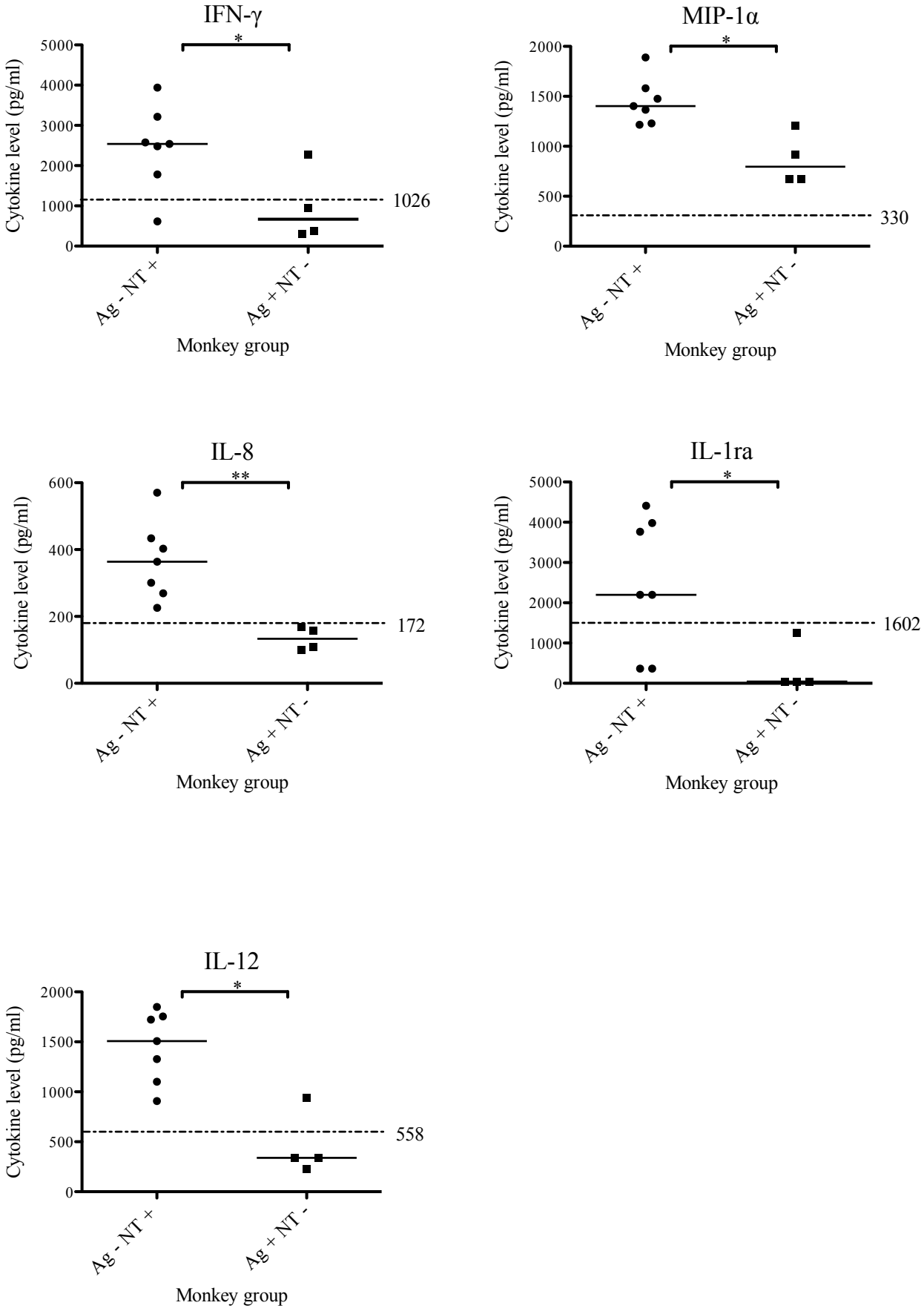
(B)



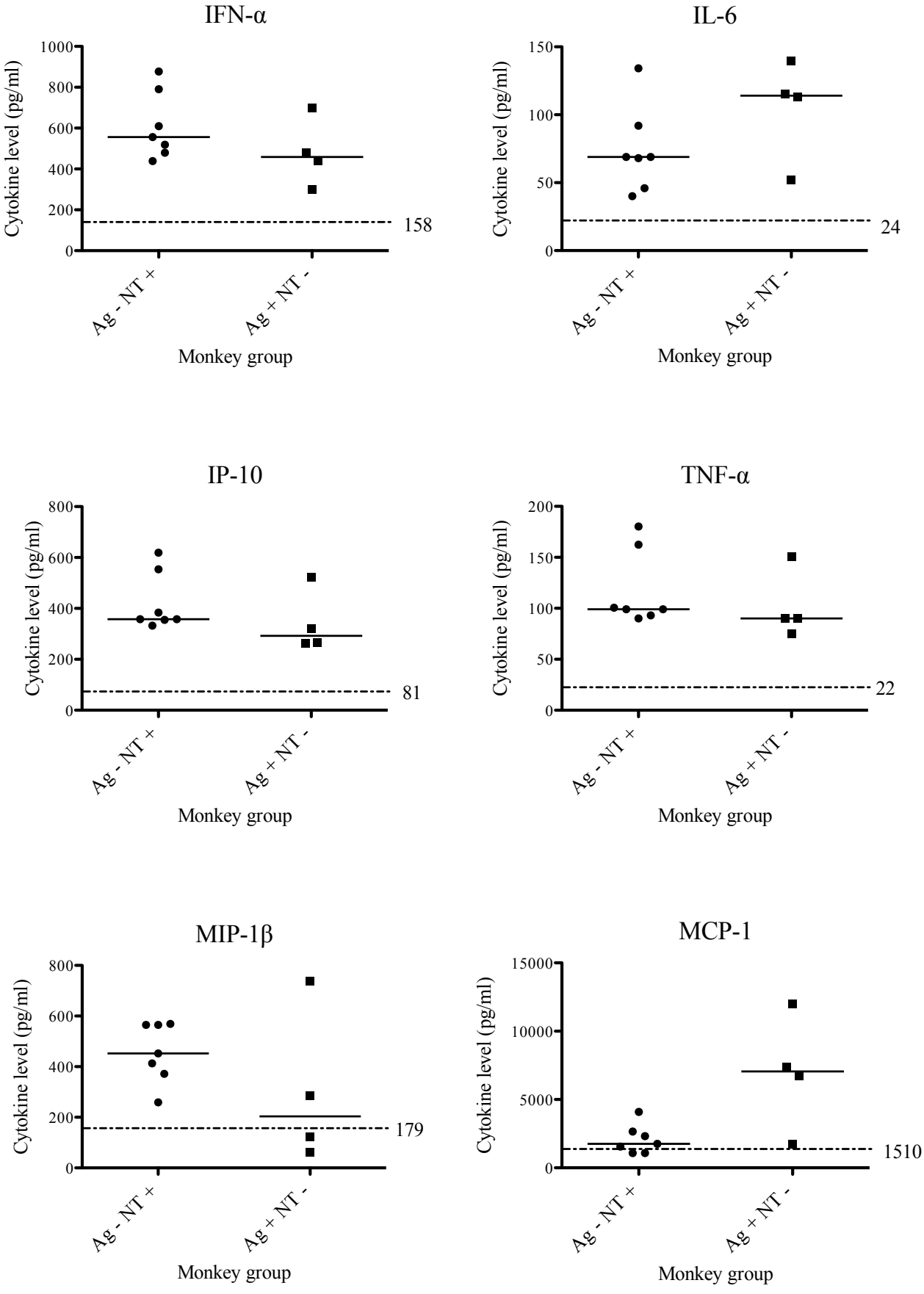
Chapter 1 Figure 2.



Chapter 1 Figure 3.



Chapter 1 Figure 4.



Chapter 1 Figure 5.

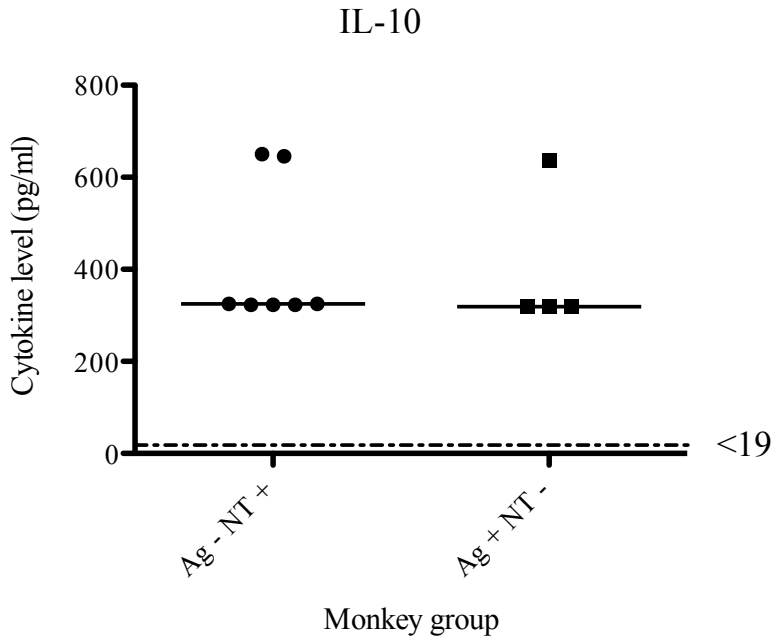


Table 1. The characteristic of recombinant baculoviruses expressing Reston virus glycoprotein

recombinant baculovirus	characteristic
Ac RESTVGP$_{1,2}$ A8	to express an entire Reston virus (RESTV) glycoprotein (GP$_{1,2}$)
Ac Mel RESTV GP$_{1,2}$ A8	to express an entire RESTV GP$_{1,2}$ with a mellitin signal peptide
Ac RESTV GP$_{1,2}$ ΔTM	to express an ectodomain of RESTV GP$_{1,2}$
Ac Mel RESTV GP$_{1,2}$ ΔTM	to express an ectodomain of RESTV GP$_{1,2}$ with a mellitin signal peptide

Table 2. Antibody responses and viremic status of Reston virus-infected cynomolgus macaques

Case ID	Anti-NP IgG		Anti-$GP_{1,2}$ IgG		NT	Ag-ELISA		Overall status	Dead or **euthanized
	ELISA	IFA	ELISA	IFA		liver	serum		
2182	-	<80	-	<80	-	+	+	Ag + NT -	euthanized
2612	-	<80	-	<80	-	+	+	Ag + NT -	euthanized
2408	+	10240	-	<80	-	*ND	+	Ag + NT -	#NR
2615	+	2560	-	<80	-	+	+	Ag + NT -	euthanized
2669	+	2560	-	<80	-	+	+	Ag + NT -	euthanized
2739	+	1280	-	<80	-	+	+	Ag + NT -	euthanized
2921	+	1280	-	<80	-	+	+	Ag + NT -	euthanized
2644	-	80	-	<80	-	+	ND	Ag + NT -	euthanized
2728	+	1280	-	<80	-	+	ND	Ag + NT -	euthanized
2721	-	<80	-	<80	80	+	+	Ag + NT +	euthanized
2972	-	<80	-	<80	160	+	+	Ag + NT +	dead
2180	+	1280	+	320	80	-	-	Ag - NT +	dead
2181	+	80	+	80	320	-	-	Ag - NT +	dead
2189	+	1280	+	320	320	-	-	Ag - NT +	NR
2190	+	160	+	640	640	-	-	Ag - NT +	NR
2191	+	640	+	320	320	-	-	Ag - NT +	NR
2195	+	2560	+	640	320	-	-	Ag - NT +	NR
2404	+	1280	+	160	160	-	-	Ag - NT +	dead
2693	+	160	+	<80	320	-	-	Ag - NT +	euthanized
2696	+	2560	+	160	320	-	-	Ag - NT +	euthanized
2713	+	5120	+	320	160	-	-	Ag - NT +	euthanized
2719	-	80	-	<80	-	ND		ND	NR
832	-	<80	-	<80	-	ND		ND	NR
888	-	<80	-	<80	-	ND		ND	NR
1134	-	<80	-	<80	-	ND		ND	NR
2636	-	<80	-	<80	-	ND		ND	euthanized
2194	+	5120	-	80	-	ND		ND	dead
No. of positive samples	17/27	19/27	10/27	10/27	12/27 (44%)	11/21 (52%)			
	19/27 (70%)		11/27 (41%)						

*ND: not determined, **euthanized: monkeys were euthanized regardless of clinical manifestation. #NR: not recorded, Ag +: antigen positive, Ag -: antigen negative, NT+: neutralization antibody positive, NT-: neutralization antibody negative. The specimens with case IDs written in italics were considered in acute phase. The specimens with case IDs written in bold were considered in convalescent phase.

Chapter 2

The detection of Reston virus antibodies in wild bats in the Philippines

Abstract

Natural reservoirs of Ebola virus (EBOV) and Marburg virus (MARV) have recently been thought to be African fruit bats. However, the natural reservoir of Reston virus (RESTV) in the Philippines is still unknown. In this study, I investigated the prevalence of RESTV antibody positive wild bats in the Philippines. Serum specimens from 298 wild bats were analyzed for the presence of RESTV specific IgG antibodies in IgG-ELISAs and IFAs. RESTV specific antibodies were detected only in a common species of fruit bat (*Rousettus amplexicaudatus*) in the Philippines. Several *R. amplexicaudatus* sera were positive in RESTV nucleoprotein (NP)-specific IgG-ELISA, RESTV NP-specific IFA, RESTV glycoprotein ($GP_{1,2}$)-specific IgG-ELISA, and RESTV $GP_{1,2}$-specific IFA. In Africa, *Rousettus aegyptiacus,* which is genetically close to *R. amplexicaudatus,* has been shown to be naturally infected with EBOV and MARV. Thus, *R. amplexicaudatus* is a possible natural reservoir of RESTV.

Introduction

As has been described in “General introduction”, Ebolavirus belongs to the family *Filoviridae*, order *Mononegavirales*. *Filoviridae* consists of two genera, *Ebolavirus*, and MARV. Recently a new filovirus, Lloviu virus, was detected in *Miniopterus schreibersii*, and proposed to be included in a new genus, *Cuevavirus* in the family *Filovirudae* (7, 8). Despite many epidemiological and ecological analyses to elucidate the enzootic natural reservoir, including a variety of animals that live in forests, caves, and mines in Africa, the natural reservoir remained obscure for some four decades since the first outbreaks of hemorrhagic fever (HF) caused by MARV and Ebolavirus (16-21). Recently, several reports finally detected the presence of EBOV-specific IgG in fruit bat sera and EBOV genomes in the spleens of fruit bats in Africa (22-24). More recently, MARV was successfully isolated from a fruit bat, *R. aegyptiacus* (25, 26). Therefore, these fruit bats are now thought to be the natural reservoirs of EBOV and MARV (79, 80). However, there are very few reports dealing with the epidemiologic investigations of RESTV (14).

In this study, I hypothesized for the first time that bats inhabiting the Philippines may be the natural reservoirs of RESTV. To investigate this hypothesis, I utilized several assays to detect RESTV specific antibodies in bats. First, to detect RESTV specific IgG in bat sera, I utilized IgG-ELISA using recombinant NP of RESTV (54, 81) and also IgG-ELISA using recombinant $GP_{1,2}$ of RESTV. The assays were slightly modified for bat specimens. The serum specimens showing a positive reaction in ELISA were further analyzed by IFAs specific to the RESTV NP and $GP_{1,2}$

using HeLa cells expressing the recombinant NP (55) and $GP_{1,2}$, respectively.

The natural reservoirs of EBOV and MARV are thought to be fruit bats. Thus, it should be possible to obtain a clue to understanding the etiology and the natural reservoir of RESTV by investigating whether Asian bats have the RESTV genome or antibodies.

Materials and Methods

Collection sites

Wild bats were captured at six locations in Luzon Island and one location in Panay Island in the Philippines (Figure 1). In Luzon Island, bats were captured at the forest of Los Baños, belonging to the University of the Philippines Los Baños campus (14°10′N; 121°14′E) (site A), at the forest and cave of Quezon National Park in the Province of Quezon (13°59′N; 121°48′E) (site B), at the forest of Atimonan in the Province of Quezon (13°59′N; 121°55′E) (site C), at the forest of Diliman, belonging to the University of the Philippines Diliman campus (14°39′N; 121°3′E) (site D), at the forest of Tanay in the Province of Calabarzon (14°31′N; 121°18′E) (site E) and at the forest of Santo Tomas (14°5′N; 121°10′E) (site F). In Panay Island, bats were captured at two caves located in the Province of Aklan (11°40′N; 122°20′E) (site G).

Collection periods

In site A, bats were collected in January 2007 (dry season), July 2008 (rainy season), and May 2011 (dry season). Bats were collected in site B and site C in March 2009 (dry season), in site D in July 2008 (rainy season), in site E and F in August 2010 (rainy season), and in site G in March 2008 (dry season)

Bat trapping and sampling

Before collecting bat specimens from the capture sites, a proposal and a request for Prior Informed Consent (PIC) to the Regional Office of the Protected Areas

and Wildlife Bureau (PAWB) under the Department of Environment and Natural Resources (DENR) was submitted, and the proposal was approved. After dissection of the captured bats, the carcasses were presented to the same Regional Office of the DENR for issuance of a transport permit.

Bats were trapped with mist nets. The nets were set up in the forest during the late afternoon just before sunset, and trapped animals were collected every night and the following morning before sunrise. Bats were captured by driving them into the nets in the caves. The captured bats were transported to a field laboratory, located near the collection sites, and they were euthanized by intraperitoneal inoculation of Zoletil (Virbac). Weight and body measurements, sexing, and species determination were performed at the field laboratory. Blood specimens were collected by cardiac puncture and stored at room temperature for at least 30 min. Then serum was obtained by centrifugation at 3,000 rpm for 20 min and stored at 4 °C.

Preparation of RESTV NP and $GP_{1,2}$ antibody positive sera

As has been described in "Chapter 1", recombinant RESTV $GP_{1,2}$ was expressed and purified from Tn5 cells infected with a recombinant baculovirus, Ac RESTV $GP_{1,2}\Delta TM$, which expresses the ectodomain of $GP_{1,2}$ with the histidine-tag at its C-terminus.

Bat hyperimmune anti-RESTV NP and $GP_{1,2}$ sera were prepared as follows. Four fruit bats, *Rousettus leschenaulti*, bred in Animal Resource Science Center, Graduate School of Agricultural and Life Sciences of the University of Tokyo, were

immunized with the RESTV NP using IMJECT-ALUM (Pierce, Rockford, USA) as an adjuvant. The bats were two- or three-year-old adult females. They were immunized with the RESTV NP four times, and two of these bats were further immunized with the RESTV $GP_{1,2}$ (referred to as anti-RESTV NP bat#1, anti-RESTV NP bat#2, anti-RESTV $NPGP_{1,2}$ bat#1, and anti-RESTV $NPGP_{1,2}$ bat#2, respectively) (Figure 2). Unimmunized bat serum was used as a negative control. These experiments were performed in accordance with the Animal Experimentation Guidelines of the University of Tokyo and were approved by the Institutional Animal Care and Use Committee of the Graduate School of Agricultural and Life Sciences, The University of Tokyo.

Serological studies using IgG-ELISA

Sera were inactivated by heating at 56 °C for 30 min and tested for the presence of anti-RESTV antibodies with the IgG-ELISAs. The antigens used for the IgG-ELISAs were RESTV NP and RESTV $GP_{1,2}$, respectively. These antigens were prepared in "Chapter 1". Bat sera were diluted at 1 in 100 and screened by the IgG-ELISAs specific for RESTV NP and $GP_{1,2}$. In the IgG-ELISAs for bat specimens, reactions were detected by anti-bat IgG rabbit IgG at a concentration of 10 μg/ml, followed by horseradish peroxidase conjugated anti-rabbit IgG at a dilution of 1 in 1,000 (Zymed, Invitrogen, CA). The anti-bat IgG rabbit IgG was prepared previously by immunizing rabbit with purified IgG of *R. aegyptiacus* and was shown to strongly cross-react to other bat species, including insectivorous bats, but not to other mammal IgG (82). Serum samples that showed relatively high OD values in the IgG-ELISA were

selected and further analyzed. These sera were four-fold serially diluted from 1 in 100 to 1 in 6,400 and were tested by IgG-ELISAs again.

OD index and determination of cut-off value

The adjusted OD values in the IgG-ELISAs were calculated by subtracting the OD value of a negative control antigen-coated well from that of a RESTV antigen-coated well. The sum of OD values of serum dilution at 1 in 100, 400, 1,600, and 6,400 for each specimen was calculated and designated as "OD index" in the IgG-ELISAs. The mean plus three standard deviations (SD) of OD indexes of the IgG-ELISAs were calculated using serum samples from RESTV antibody negative bats and were used as the cut-off values of the IgG-ELISAs.

Detection of RESTV NP antibodies in IFA

RESTV NP antigen slides were prepared as described in "Chapter 1". Two hyperimmune bat sera against RESTV NP (anti-RESTV NP bat#1,2) were used as antibody positive control sera. Serum specimens were two-fold serially diluted in PBS and were applied to the wells of the RESTV NP antigen slides and incubated at 37 °C for 1 hr in a humidified chamber. Then, the slides were washed in PBS and further reacted with anti-bat IgG rabbit IgG adjusted to a concentration of 10 μg/ml at 37 °C for 1 hr. The antigen slides were washed with PBS and further reacted with FITC-conjugated goat anti-rabbit IgG (H+L) at a dilution of 1 in 100 (ZYMED, Invitrogen, CA). After incubation at 37 °C for 1 hr, the slides were washed with PBS

and covered with micro cover glass (MATSUNAMI GLASS, Japan). The slides were examined for staining patterns under a fluorescent microscope. The antibody titer in the IFA was determined as the reciprocal of the highest serum dilution showing positive staining.

Detection of RESTV $GP_{1,2}$ antibodies in IFA

The sera that showed a high OD value in IgG-ELISA specific for RESTV $GP_{1,2}$ were analyzed in the RESTV $GP_{1,2}$-specific IFA. The ID numbers of these bat sera were 1539, 1632, 1642, 1643, 1651, 1657, and 1660. As positive control sera, I used the two hyperimmune bat sera against RESTV $GP_{1,2}$ (anti-RESTV $NPGP_{1,2}$ bat#1, 2). RESTV $GP_{1,2}$ antigen slides were prepared as described in "Chapter 1". The method of IFA specific for RESTV $GP_{1,2}$ is basically the same as that of RESTV NP.

Results

Reaction of hyperimmune bat sera to the recombinant NP and $GP_{1,2}$ of RESTV in IgG-ELISA

The four hyperimmune bat sera (anti-RESTV NP bat#1,2 and anti-RESTV $NPGP_{1,2}$ bat#1, 2) were analyzed in the RESTV NP-specific IgG-ELISA and found that they showed high OD values in the IgG-ELISA. In the RESTV $GP_{1,2}$-specific IgG-ELISA, specific antibodies were detected in the two hyperimmune bat sera (anti-RESTV $NPGP_{1,2}$ bat#1, 2) (Figure 3). On the other hand, the negative control bat serum showed low OD values in the both RESTV NP and $GP_{1,2}$-specific IgG-ELISAs. Thus, these IgG-ELISAs are considered to detect the antibodies to RESTV NP or $GP_{1,2}$ in bat sera.

Reaction of hyperimmune bat sera to the recombinant NP and $GP_{1,2}$ of RESTV in IFA

Anti-RESTV NP bat#1,2 obviously showed positive cytoplasmic granular staining pattern in RESTV NP-specific IFA. On the contrary, the negative control bat serum showed no reaction (Figure 4). The antibody titers in the IFA of both anti-RESTV NP bat#1 and anti-RESTV NP bat#2 were 2,560.

The antibody titers in the RESTV $GP_{1,2}$-specific IFA of anti-RESTV $NPGP_{1,2}$bat#1 and anti-RESTV $NPGP_{1,2}$bat#2 were 160 and 40, respectively. The cell surface was strongly stained in the IFA since the RESTV $GP_{1,2}$ expressed on the cell surface. On the contrary, the negative control serum showed no reaction (Figure 5).

Seroprevalence of RESTV in wild bats in the Philippines

A total of 298 wild bats belonging to 17 species, including one unknown species, were collected in the Philippines during 2007 and 2011. Among them, 75 bats were collected at site A (5 species), 29 at site B (6 species), 35 at site C (5 species), 29 at site D (6 species), 60 at site E (4 species), 50 at site F (3 species), and 20 at site G (7 species). The details of bat species and their collection sites are shown in Table 1 and Figure 1, respectively. Serum specimens were obtained from these bats.

Initially, all of the 298 sera were diluted at 1 in 100 and tested in the IgG-ELISAs specific for RESTV NP and $GP_{1,2}$. Since relatively high OD values were observed in sera from bats belonging mainly to *R. amplexicaudatus*, the 23 serum specimens from *R. amplexicaudatus* were further analyzed in the IgG-ELISAs specific for RESTV NP and $GP_{1,2}$. Cut-off values of OD indexes were first determined for each IgG-ELISA using serum specimens from RESTV antibody negative bats. The cut-off values of OD indexes, that is, the sum of OD values at serum dilutions of 1 in 100, 400, 1,600 and 6,400, for both IgG-ELISAs were determined to be 0.82. Among the 23 sera, five (21.7%) showed positive OD indexes in the IgG-ELISA using RESTV NP, and five (21.7%) showed positive OD indexes in the IgG-ELISA using RESTV $GP_{1,2}$. Among them, three bats (IDs 1643, 1651, and 1660) captured at site C showed positive reactions in the IgG-ELISAs using both NP and $GP_{1,2}$ (Table 2). Fifteen sera, which showed relatively high OD values in the screening test, collected from bats belonging to other bat species showed negative reactions in both IgG-ELISAs.

These bat sera were also tested in the IFAs specific to the RESTV NP and

$GP_{1,2}$ using HeLa cells expressing the NP and the $GP_{1,2}$, respectively. In the IFAs, two sera from *R. amplexicaudatus* showed high titers (1,280 and 640 respectively) of NP-specific antibodies, while one serum from *R. amplexicaudatus,* which showed the highest OD index in the IgG-ELISA using RESTV $GP_{1,2}$, showed a positive staining pattern up to a dilution of 1 in 20 (Table 2 and Figure 6). The other sera showed negative reactions in the IFAs. The RESTV NP-specific IFA used in the present study was previously established, and its efficacy was confirmed using cynomolgus macaque sera collected from the RESTV epizootic in the Philippines in 1996. Thus, the RESTV NP-specific IFA is also thought to be useful for seroepidemiological studies of RESTV infection among bats. Although IFA is thought to be less sensitive in detection of specific antibodies than IgG-ELISA, the IFA is useful for confirmatory assay for IgG-ELISA positive specimens. In the present study, several IgG-ELISA positive sera also showed positive reactions in the IFAs for both RESTV NP and $GP_{1,2}$. For instance, two bats, ID 1539 captured at site D and ID 1660 captured at site C, showed positive reactions in both NP-specific IgG-ELISA and IFA, while one bat, ID 1642 captured at site C, was $GP_{1,2}$ antibody positive in both assays. These bats were collected at sites relatively close to the monkey facility where the RESTV epizootic in macaques occurred and to the pig farms where the RESTV infection in swine was detected in Luzon Island. I performed highly sensitive hemi-nested RT-PCR specific for the RESTV NP gene for spleen specimens of all 23 *R. amplexicaudatus* bats, but I failed to detect any RESTV specific amplicon in the present study.

Discussion

In the Philippines, RESTV outbreaks in monkeys occurred several times in the 1990s, and RESTV infection was detected in swine in the Philippines in 2008. In these epizootics, there were no human patients showing manifestation of hemorrhagic fever; however, it remains unclear whether RESTV is avirulent in humans.

Since fruit bats are considered to be natural reservoirs for EBOV and MARV in Africa, it is crucial to conduct epidemiological surveillance of RESTV in wild bats in the Philippines. The epidemiological surveillance in wild bats using the several antibody detection systems established in this study showed that RESTV specific antibodies were prevalent in *R. amplexicaudatus*, indicating that this bat species may be one of the natural reservoirs, if any, of RESTV in the Philippines. However, only 23 specimens of *R. amplexicaudatus* were available in the present study, and it will be necessary to investigate more specimens of this species to detect the RESTV genome or antigens to conclude the bat is a natural reservoir of RESTV.

In the present study, bats were collected in Luzon Island and Panay Island. The RESTV antibody positive bats were captured at sites geographically close to the Bulacan and Facility A, where RESTV infection in pigs and cynomolgus monkeys, respectively, have previously been identified (Figure 1). One bat, ID 1539, which showed a relatively high titer of RESTV NP antibody was captured at site D, some 20 km southeast of Bulacan, while another six RESTV antibody positive bats, IDs 1632, 1642, 1643, 1651, 1657, and 1660, were captured at site C, some 40 km east of Facility A. Actually, bats, including *R. amplexicaudatus*, inhabit areas around Facility A and the

pig farms, so it is possible that the virus transmitted directly from the bats to these animals in the previous epizootics in the Philippines. In the present study, all the *R. amplexicaudatus* were captured in Luzon Island. However, the bats also inhabit Panay Island and other regions in the Philippines. Therefore, to understand the ecology of RESTV among bats in the Philippines, it is necessary to clarify if bat species, including *R. amplexicaudatus*, in other regions have anti-RESTV antibodies. RESTV antibody positive bats were captured in both dry and rainy seasons in this study. To understand seasonal pulses of RESTV circulation, it is necessary to investigate IgM antibody response in more bat serum specimens in both dry and rainy seasons.

Recently, EBOV specific antibodies have been detected in several bat species: *Hypsignathus monstrosus*, *Epomops franqueti*, and *Myonycteris torquata* (22). They are thought to represent the natural reservoirs of EBOV. The detection of EBOV and MARV specific antibodies in *R. aegyptiacus* was also reported (24). Furthermore, the MARV genome was detected and the virus was successfully isolated from *R. aegyptiacus* (25, 26, 80). Thus, *R. aegyptiacus* is a possible natural reservoir of both EBOV and MARV. *R. aegyptiacus* and *R. amplexicaudatus* are genetically very close. *R. aegyptiacus* populates mainly Africa, the Middle East, and west India. *R. amplexicaudatus* populates mainly the Philippines, Myanmar, New Guinea, and Indonesia, indicating that RESTV infection might be found not only in the Philippines, but also in Myanmar, New Guinea, and Indonesia. In this regard, further epidemiological investigation of RESTV in these countries is also necessary. On the other hand, another closely related bat species, *R. leschenaulti*, populates areas between

these two areas (Figure 7). Thus, it is of particular interest to elucidate whether the other *Rousettus* species, such as *R. leschenaulti,* have been naturally infected with filoviruses.

Conclusions

In this study, I describe *Rousettus amplexicaudatus,* a common species of fruit bat in the Philippines, has been naturally infected with RESTV. RESTV antibody positive bats were captured at the sites geographically close to the facilities, where RESTV infections in pigs and cynomolgus monkeys have previously been identified, thus the bat is a possible natural reservoir of RESTV. Further analysis to demonstrate the RESTV genome is necessary to conclude the bat is a reservoir of RESTV.

Figure legends

Figure 1. Bat collection sites and Reston ebolavirus epizootics in the Philippines.

(A) Bat collection sites are indicated by black rectangles. Bats were collected at five sites in Luzon Island and at two caves in the Province of Aklan in Panay Island.

(B) Detailed locations of bat collection sites in Luzon Island. Bats were collected at forests or a cave at the five sites, as indicated by black rectangles. Black crosses indicate the sites of swine ebolavirus epizootic (Pangasinan and Bulacan) and white cross indicate monkey breeding facility A, from where all of the Reston ebolavirus epizootics in macaques in 1989–1996 originated.

Figure 2. Schematic representation of immunization procedure of bats with the recombinant NP and $GP_{1,2}$ of Reston virus.

Two bats (anti-RESTV NP bat #1, 2) were immunized with the Reston virus (RESTV) NP for four times. The other two bats (anti-RESTV NP$GP_{1,2}$ bat #1,2) were immunized with both RESTV NP and $GP_{1,2}$ for four times, respectively. The blood specimens were collected by cardiac puncture.

Figure 3. Reaction of hyperimmune bat sera to the recombinant Reston virus glycoprotein $GP_{1,2}$ in the IgG-ELISA.

The reaction of anti-RESTV NP $GP_{1,2}$ bat#1, 2. Hyperimmune bat sera showed positive reaction in the IgG-ELISA using the Reston virus glycoprotein. Anti-RESTV NP $GP_{1,2}$ bat#1, 2 were two-fold serially diluted from 1 in 1,000 to 1 in 1024,000 and were tested

by IgG-ELISA.

Figure 4. Indirect immunofluorescent antibody (IFA) test using Reston virus NP expressing HeLa cells.

Reston virus NP-specific antibodies were detected in a hyperimmune bat serum (anti-RESTV NP bat #1) (A), but not in a negative control bat serum(B) in the REST NP-specific IFA.

Figure 5. Indirect immunofluorescent antibody (IFA) test using Reston virus $GP_{1,2}$ expressing HeLa cells.

Reston virus $GP_{1,2}$-specific antibodies were detected in a hyperimmune bat serum (anti-RESTV $NPGP_{1,2}$ bat #2) (A), but not in a negative control bat serum(B) in the RESTV $GP_{1,2}$-specific IFA.

Figure 6. Detection of RESTV NP and $GP_{1,2}$ antibodies by IFA.

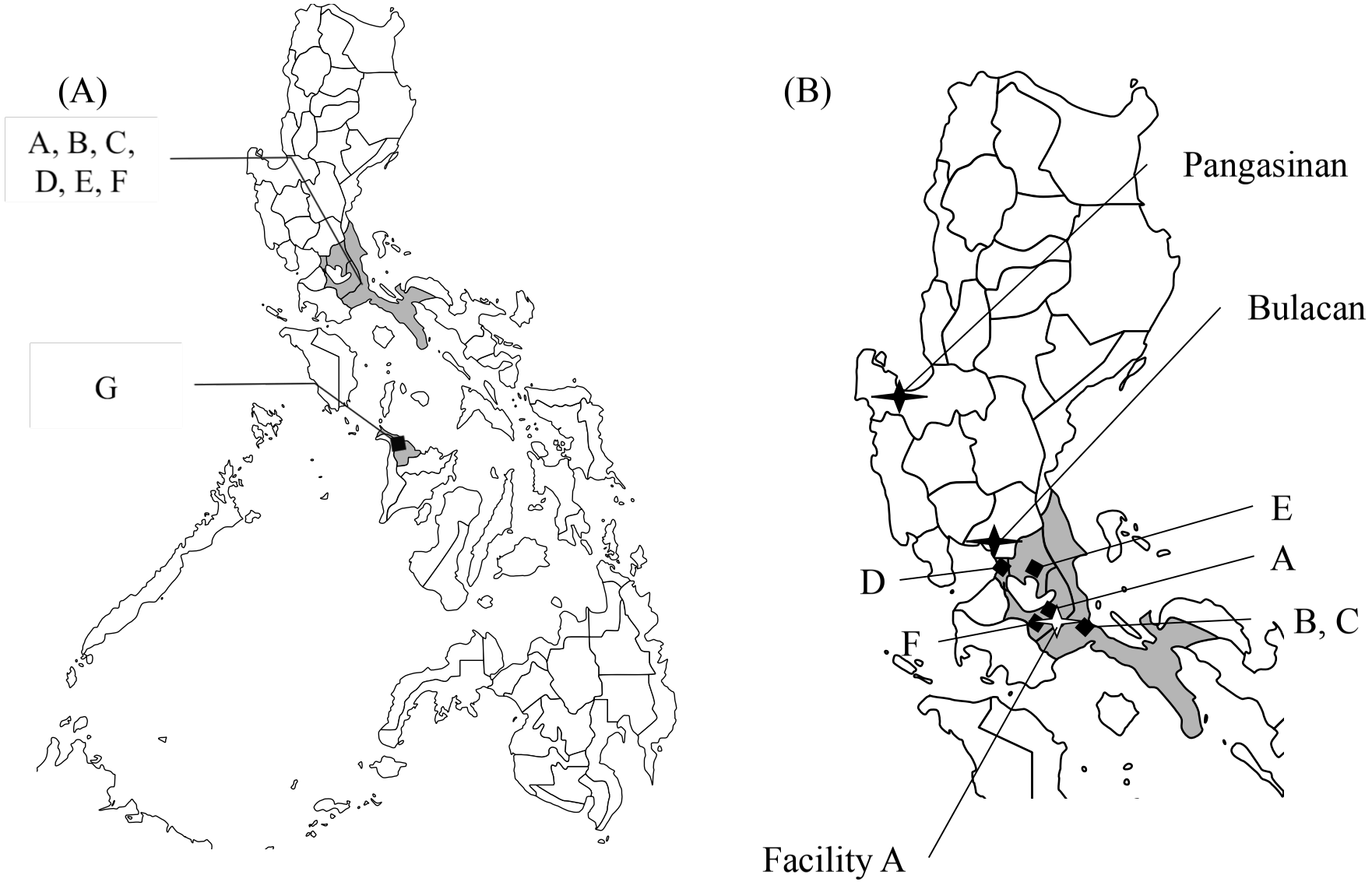
RESTV NP antibodies were detected in two bats, ID 1539 (A) and ID 1660 (B), in the RESTV NP-specific IFA test. RESTV $GP_{1,2}$ antibodies were detected in a bat, ID 1642 (C), captured in the Philippines in the $GP_{1,2}$-specific IFA.

Figure 7. The geographic distribution of Ebola haemorrhagic fever outbreaks and bats of *Rousettus spp.*

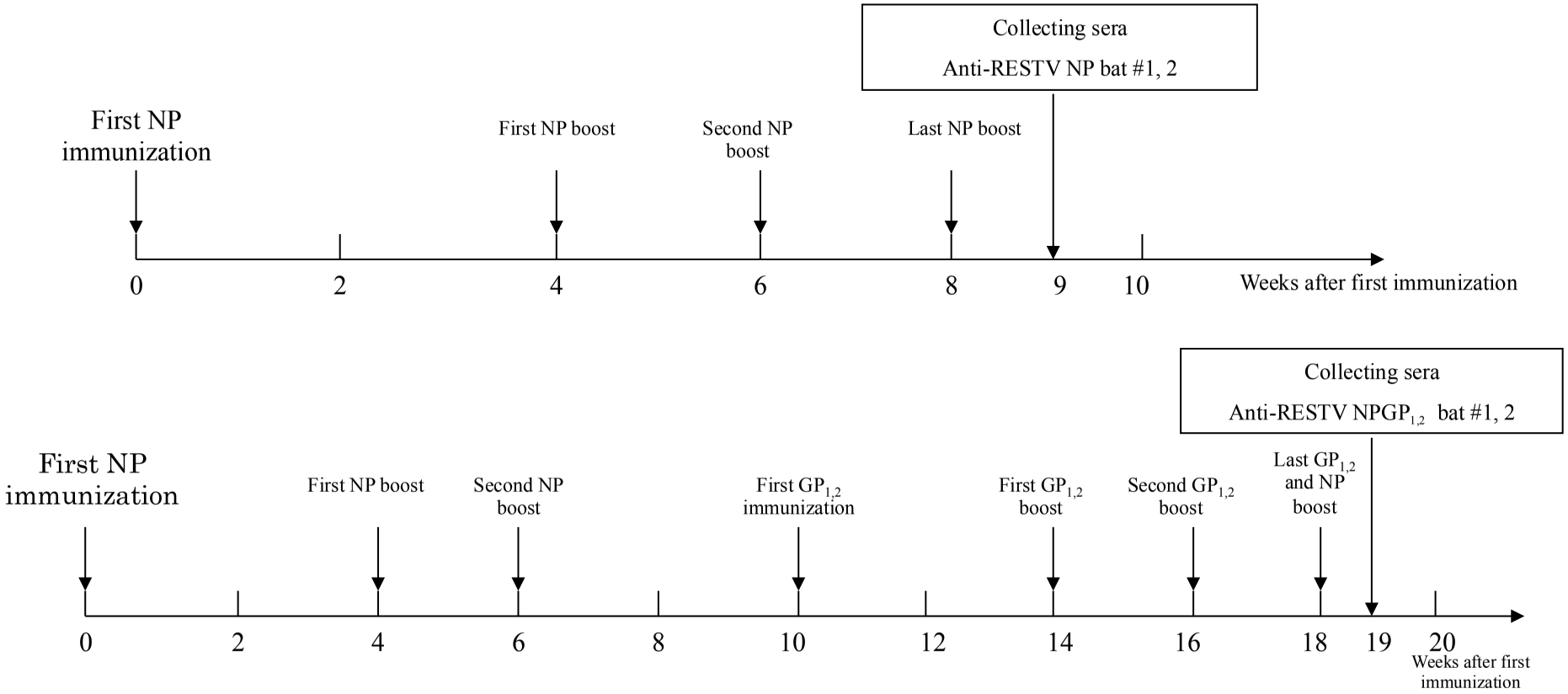
EBOV outbreaks have been reported in the areas shown in black. *Rousettus aegyptiacus*

inhabits mainly Africa, the Middle East, and west India, as shown in the circle at the left. *Rousettus amplexicaudatus* inhabits mainly the Philippines, Myanmar, New Guinea, and Indonesia, as shown in the circle at the right. Between these areas inhabited by *Rousettus aegyptiacus* and *Rousettus amplexicaudatus*, *Rousettus leschenaulti* inhabits mainly India and southeast Asia, as shown in the circle at the middle.

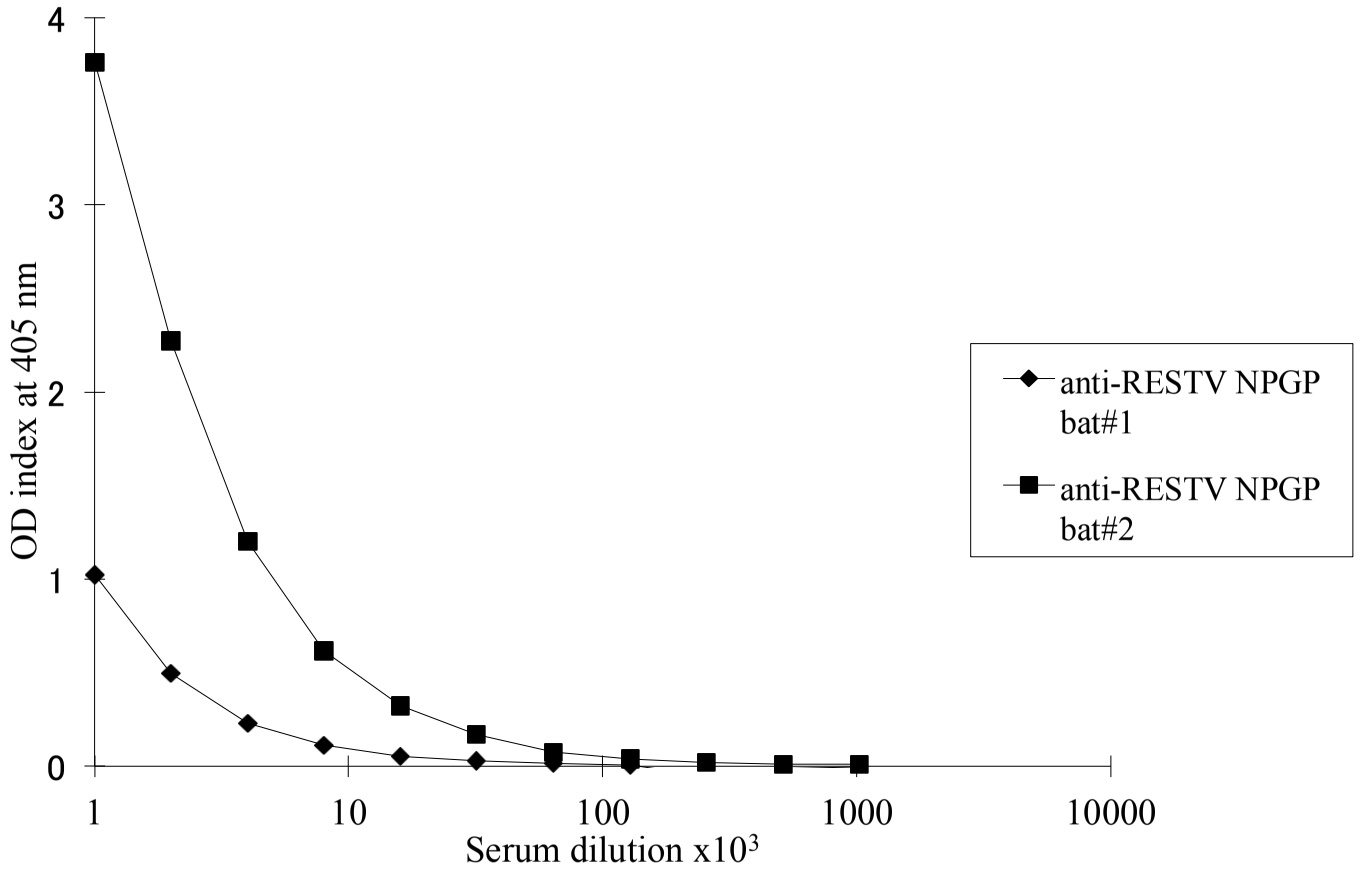
Chapter 2 Figure 1.



Chapter 2 Figure 2.

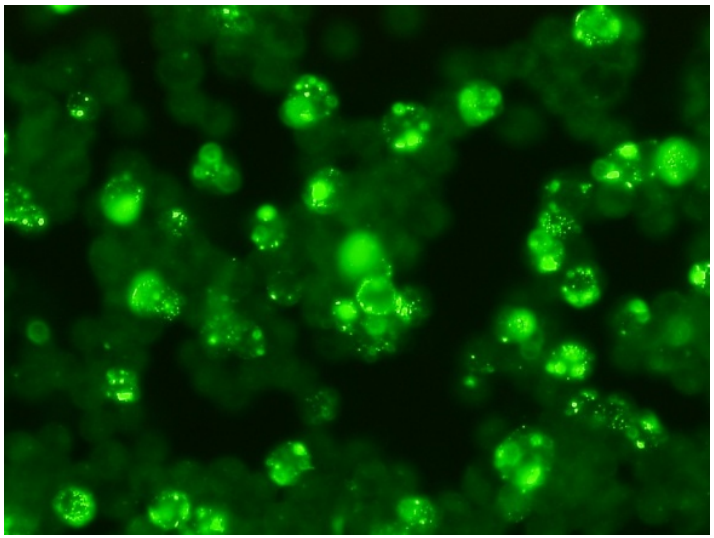


Chapter 2 Figure 3.



Chapter 2 Figure 4.

(A)



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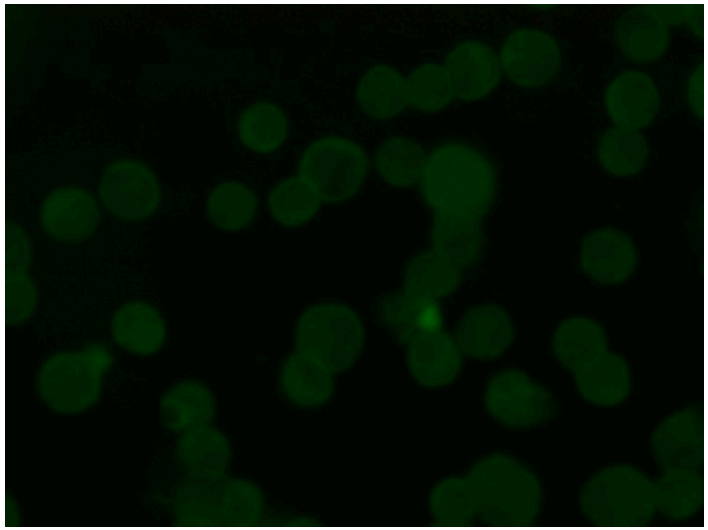
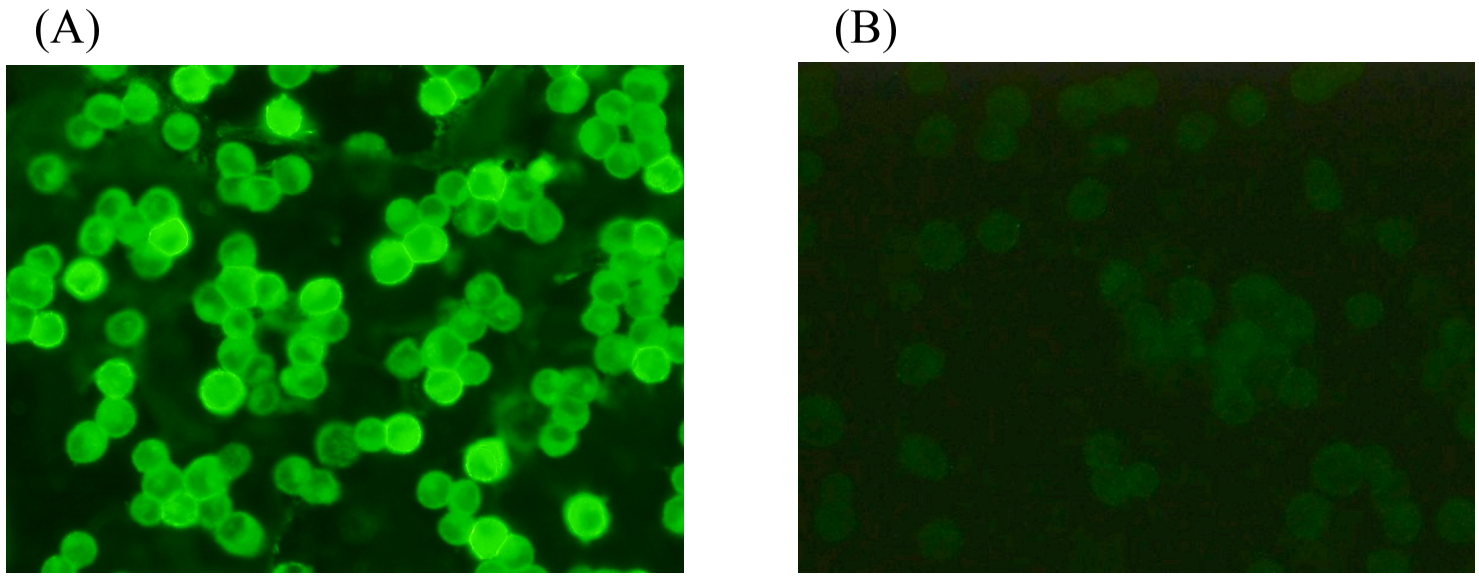
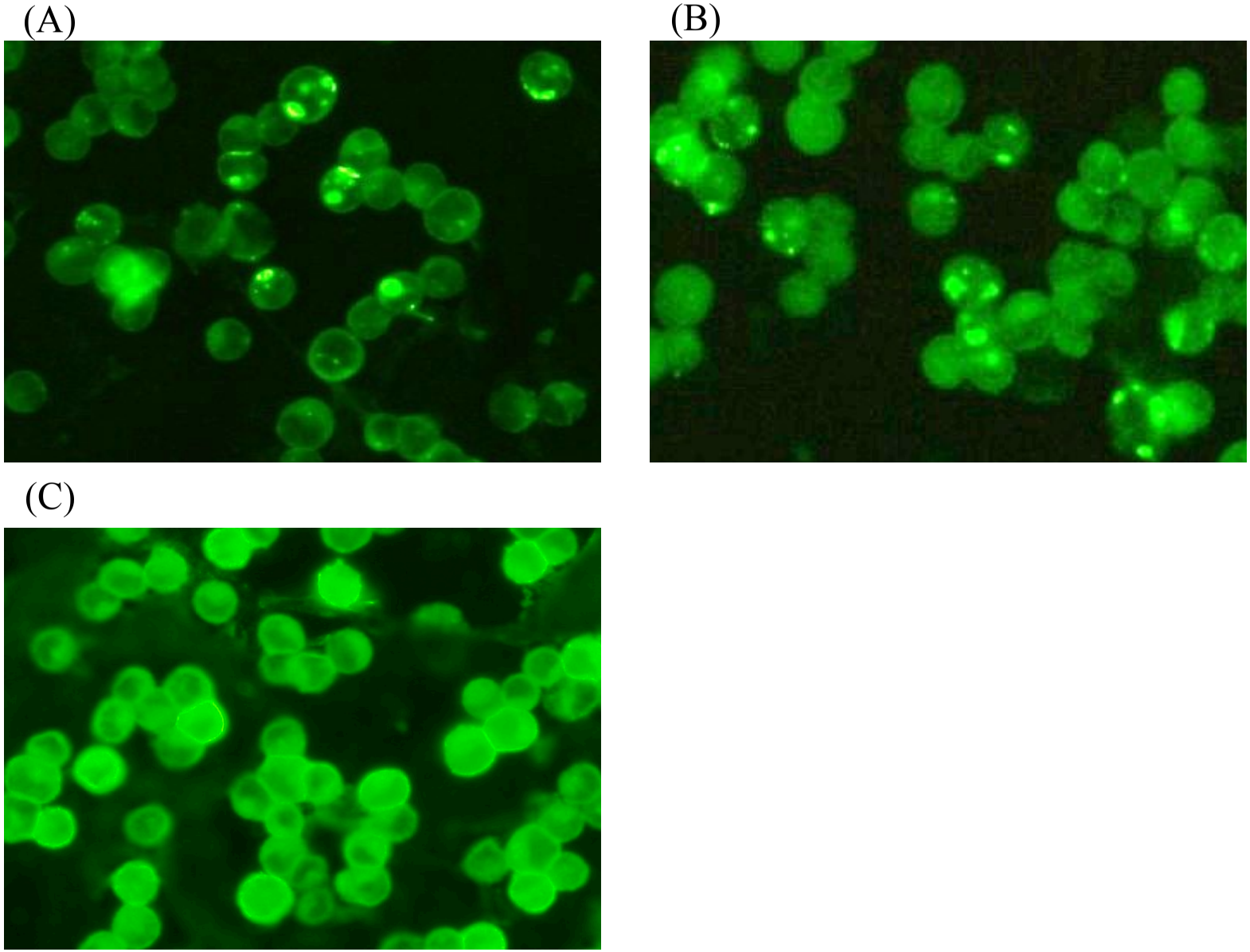


Chapter 2 Figure 5.



Chapter 2 Figure 6.



Chapter 2 Figure 7.

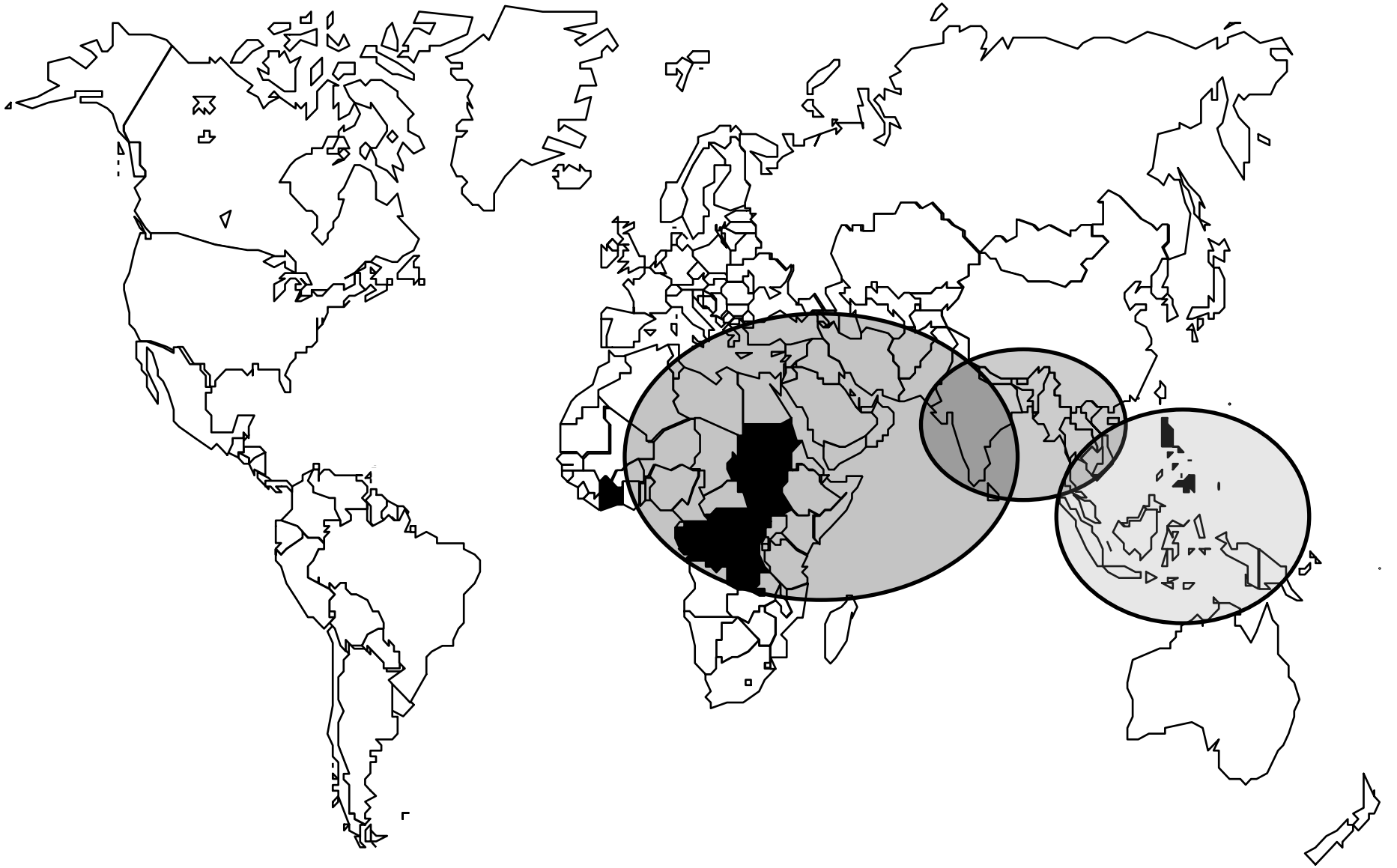


Table 1. List of bats captured in the Philippines

Species	Collection site*							
	A	B	C	D	E	F	G	total
Cynopterus brachyotis	22	2	10	18	44	28		124
Ptenochirus jagori	43	10	8	2	12	21	1	97
Rousettus amplexicaudatus	6		15	1	1			23
Eonycteris spelaea	3			2				5
Haplonycteris fischeri		6						6
Macroglossus minimus	1	2						3
Miniopterus schreibersi							8	8
Scotophilus kuhlii				5				5
Pipstrellus javanicus				1				1
Emballonura alecto		7						7
Rhinophus rufus		2						2
Rhinophus arcuatus			1					1
Hipposideros diadema			1		3	1	1	6
Myotis macrotarsus							1	1
Minipterus australis							7	7
Miniopterus tristis tristis							1	1
unknown							1	1
	75	29	35	29	60	50	20	298

*site A: forest of Los Banos, belonging to the University of Philippines Los Banos campus (14°10′N; 121°14′E)

site B: forest and cave of Quezon National Park in the Province of Quezon (13°59′N; 121°48′E)

site C: forest of Atimonan in the Province of Quezon (13°59′N; 121°55′E)

site D: forest of Diliman, belonging to the University of the Philippines Diliman campus (14°39′N; 121°3′E)

site E: forest of Tanay in the Province of Calabarzon (14°31′N; 121°18′E)

site F: forest of Santo Tomas (14°5′N; 121°10′E)

site G : two caves located in Province of Aklan in Panay Island (11°40′N; 122°20′E)

Table 2. Summary of IgG ELISA and IFA assay for detection of Reston ebolavirus antibodies in *Rousettus amplexicaudatus* in the Philippines

collecting site	bat ID	IgG-ELISA (OD index*)		IFA	
		RESTV NP	RESTV GP	RESTV NP	RESTV GP
A	n=6	average: 0.18	average: 0.00	<20	<20
C	1632	0.88	0.2	<20	<20
C	1642	0.36	5.22	<20	20
C	1643	1.26	0.92	<20	<20
C	1651	1.61	1.02	<20	<20
C	1657	0	1.69	<20	<20
C	1660	3.8	2.51	640	<20
C	others (n=9)	average: 0.17	average: 0.38	<20	<20
D	1539	2.13	0	1280	<20
E	2209	0.13	0	<20	<20
	control bat	0	0.07	<20	<20
total		5/ 23 (21.7%)	5/ 23 (21.7%)	2/ 23 (8.70%)	1/ 23 (4.35%)

*OD index: sum of OD values of serum dilution at 1 in 100, 400, 1,600, and 6,400

Chapter 3

The detection of Severe fever with thrombocytopenia syndrome virus antibodies in wild bats in the Philippines

Abstract

SFTS is an emerging viral infectious disease reported in China, Japan and Korea. A causative agent is SFTS virus (SFTSV), which is a novel bunyavirus discovered in 2011. Recently SFTSV and SFTSV-specific antibodies were detected in goats, sheep, cattle, dogs, pigs, and chickens in China. Furtheremore, a novel bat Phlebovirus, Malsoor virus, closely related to SFTSV and Heartland virus, was recently isolated in wild bats in West India. However geographical distribution of SFTSV and the involvement of wild animals in the SFTSV transmission cycle are unknown. In this study, I investigated the prevalence of SFTSV antibody positive wild bats in the Philippines. Serum specimens from 404 wild bats were analyzed for the presence of SFTSV specific IgG antibodies in IgG-ELISA and indirect immunofluorescent assay (IFA). SFTSV specific antibodies were detected in 3 species of fruit bat (*Cynopterus brachyotis, Ptenochirus jagori* and *Rousettus amplexicaudatus*) in the Philippines. Several sera were positive in both IgG-ELISA and IFA but were not positive for Rift Valley fever virus (RVFV) specific IFA. These results indicate that SFTSV or SFTSV-like virus exist in the Philippines. Recently Heartland virus in U.S.A., and Bhanja virus serogroup in Africa, Middle East and India were shown to be genetically and serologically related to SFTSV. Thus, further studies are required to clarify the prevalence of SFTSV or SFTSV-like virus in wild animals including bats in the Philippines.

本章の内容は、共著論文として学術雑誌に投稿される予定であり、また、インターネット公表に対する共著者全員の同意が得られていないため公表できない。本章の内容は今後共著論文として学術雑誌に投稿される予定である。

General Conclusion

In the thesis, I described the serological and epidemiological studies on zoonotic emerging infectious diseases in non-human primates and bats in the Philippines.

Recently many emerging viral infectious diseases derived from bats have been recognized around the world. Since EHF and Marburg hemorrhagic fever are one of the most lethal infectious diseases, among the bat associated infectious diseases, in humans and non-human primates, it is considered to be serious public health concern if these infectious diseases are introduced into Japan in association with import of non-human primates or with bats. Thus, import of bats became prohibited and import of non-human primates became highly restricted under the law concerning the prevention of infectious diseases and medical care for patients of infectious diseases. Filoviruses are endemic in Africa except for RESTV, which is only endemic in Asia, especially in the Philippines and causes lethal infection in macaque monkeys but not in humans.

In Chapter 1, I investigated the antibody and cytokine/chemokine responses of cynomolgus macaques that are thought to be dead-end hosts for RESTV. In human EHF cases caused by EBOV infection, humoral immune responses in survivors and non-survivors have been well analyzed, and the neutralizing antibody responses targeting ebolaviruses $GP_{1,2}$ are considered to be a major indicator of protective immunity (56). On the other hand, immune responses in non-human primates, especially naturally RESTV-infected cynomolgus macaques, had not yet been analyzed in detail, and the significance of antibody responses against NP and $GP_{1,2}$ in the RESTV-infected macaques was unclear. In this study, I found that anti-$GP_{1,2}$ antibody

response, but not anti-NP antibody response, was a critical key for the clearance of viremia in the sera of the RESTV-infected cynomolgus macaques. Additionally, I found high concentrations of proinflammatory cytokines/chemokines, such as IFN-γ, IL-8, IL-12, and MIP-1α, in the convalescent phase sera. These results imply that both the antibody response to $GP_{1,2}$ and the proinflammatory innate responses play significant roles in the recovery from diseases in RESTV infected cynomolgus macaques.

In Chapter 2, I hypothesized that bats inhabiting the Philippines may be the natural reservoirs of RESTV. To test this hypothesis, I utilized highly sensitive hemi-nested RT-PCR specific for detection of RESTV NP gene and serological assays, RESTV NP- and $GP_{1,2}$-specific IgG-ELISA, and IFAs for detection of RESTV antibodies. I could not detect RESTV genome in wild bats in the Philippines, but RESTV specific antibodies were detected only in a common species of fruit bat (*Rousettus amplexicaudatus*) in the Philippines. In Africa, *Rousettus aegyptiacus,* which is genetically close to *R. amplexicaudatus,* has been shown to be naturally infected with EBOV and MABV. Thus, *R. amplexicaudatus* is considered to be a possible natural reservoir of RESTV.

In chapter 3, I focused on serological surveillance of SFTSV, a novel bunyavirus, in wild bats in the Philippines and detected SFTSV specific antibodies in the 3 species (*Cynopterus brachyotis, Ptenochirus jagori* and *Rousettus amplexicaudatus*) of wild bats in the Philippines. SFTSV antibody positive bats were captured in all islands surveyed in the Philippines. These SFTSV antibody positive bat sera did not cross-react to RVFV. This is in consistent with the result that SFTS

convalescent patients sera with high titer of SFTSV antibodies do not cross-react RVFV (Dr. Shuetsu Fukushi, *personal communication*). However, some of the bat sera showed positive reaction to SFTSV antigen in the IgG-ELISA but not in the IFA, and *vice versa*, indicating the bats were infected with serologically cross-reactive but different from SFTSV. Thus it is possible that unknown SFTSV-like TBPVs exist in the Philippines. The isolation of Malsoor virus from bats in West India may support this hypothesis. In this regard, further molecular epidemiological investigation of TBPVs including SFTSV in the Philippines and in other Asian countries is also necessary.

As I described in general introduction, chapter 1, chapter 2, and chapter 3, I focused on two agents of emerging infectious disease, RESTV and SFTSV. These agents are categorized in BSL-4 or BSL-3 agents, respectively, in Japan. Since there is no BSL-4 laboratory in Japan, it is impossible to handle and culture RESTV. I solved this problem by using recombinant proteins of RESTV and revealed the importance of antibody response to $GP_{1,2}$ and the proinflammatory innate responses in recovery from diseases in naturally RESTV infected cynomolgus macaques. I also mentioned *R. amplexicaudatus* as a possible natural reservoir of RESTV. Recently RESTV specific antibody has also been detected in *R. leschenaultia*, closely related to *R. amplexicaudatus*, in China and in Bangladish (89, 90). Antibodies cross-reactive to RESTV, EBOV, SUDV, BDBV, TAFV and MARV have been detected in Indonesian orangutans (91). Besides, Lloviu virus, a newly discovered filovirus, has been detected in wild bat in Europe. These recent reports suggest that the existence of multiple species of filoviruses or unknown filovirus-related viruses around the world.

Since SFTSV is classified in BSL-3 pathogens, I could prepare the IgG-ELISA and IFA antigens using a live SFTSV infected cells in BSL-3 laboratory, and the antigens were inactivated and used in this study. However, SFTSV is highly virulent in human, it is desirable to prepare the antigens for serological diagnosis and serological surveillance using recombinant SFTSV proteins to avoid a risk of accidental infection of SFTSV. As mentioned above, the existence of SFTSV and/or SFTSV-like unknown TBPVs in the Philippines is suggested in this study. Antibody positive rate was highest in *Rousettus amplexicaudatus,* the only bat species carrying RESTV antibody, among the 3 species of the wild bats in the Philippines, thus the bat species may have been infected with viruses at risk to human and/or non-human primates. Further studies to elucidate the SFTSV or SFTSV-like TBPVs in the bats are necessary to understand the prevalence of these viruses and the risk to human in the Philippines. Such a study will also be important to elucidate the transmission cycles of TBPVs in the Philippines. In the near future, the extensive geographical distribution of filoviruses and TBPVs including SFTSV will be elucidated by extensive surveillance. The system established in the study will contribute to such surveillance.

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Abbreviation list

Ag: antigen

AIDS: acquired immunodeficiency syndrome

BDBV: Bundibugyo virus

BHAV: Bhanja virus

China CDC: Chinese Center for Disease Control and Prevention

CPE: cytopathic effect

DENR: Department of Environment and Natural Resources

EBOV: Ebola virus

EHF: Ebola hemorrhagic fever

ELISA: enzyme-linked immunosorbent assay

EM: electron microscopy

$GP_{1,2}$: glycoprotein

$GP_{1,2}$ ΔTM: ectodomain of the RESTV glycoprotein

HF: hemorrhagic fever

HRTV: Heartland virus

IFA: immunofluorescent antibody assay

MARS: mixed anti-inflammatory response syndrome

MARV: Marburg virus

NNS: nonsegmented negative-strand

N protein: nucleocapsid protein

NP: nucleoprotein

NP-40: nonidet P40

NT: neutralization

OD: optical density

PAWB: Protected Areas and Wildlife Bureau

PBS-T: PBS containing 0.05% Tween 20

PIC: Prior Informed Consent

PRRSV: porcine reproductive and respiratory syndrome virus

RESTV: Reston virus

RVFV: Rift Valley fever virus

SARS: Severe Acute Respiratory Syndrome

SD: standard deviation

SFTS: severe fever with thrombocytopenia syndrome

SFTSV: severe fever with thrombocytopenia syndrome virus

SHFV: simian hemorrhagic fever virus

SKIM-PBS-T: PBS-T containing 5% skim milk

S/N ratio: Signal/ Noise ratio

TAFV: Taï Forest virus

TBPV: tick-borne phlebovirus

TPRC: Tsukuba Primate Research Center

UUKV: Uukuniemi virus

VHF: viral hemorrhagic fever

VSV: vesicular stomatitis Indiana virus

VSV-RESTV-$GP_{1,2}$/GFP: VSV pseudotype bearing RESTV $GP_{1,2}$

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Summary in Japanese

論 文 の 内 容 の 要 旨

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論文題目　Serological and epidemiological studies on zoonotic emerging infectious diseases in primates and bats in the Philippines
(フィリピンにおける翼手目及び霊長類の動物由来新興感染症に対する血清学的・疫学的研究)

医学やウイルス学の発展とともに、エボラ出血熱や、クリミアコンゴ出血熱(CCHF)、ラッサ熱といった致死性の新興感染症が数多く同定されたが、2011 年に報告された重症熱性血小板減少症候群(SFTS)や、2012 年に報告された中東呼吸器症候群(MERS)を初め、今日でも多くの新興感染症が出現している。これらの新興感染症の多くはウイルス感染症であり、その大部分は野生動物が宿主として保有しているウイルスが、偶発的にヒトに感染したことで生じる動物由来感染症である。ウイルス性動物由来新興感染症の中でも出血熱を伴う感染症は、ヒト及び動物に対し高い致死率を示すことが多く、これらの感染症の制圧は公衆衛生上極めて重要である。アジアには、出血熱を伴う動物由来ウイルス感染症の原因ウイルスとして、レストンウイルス(RESTV)、CCHF ウイルス(CCHFV)、腎症候性出血熱ウイルス(HFRSV)、そして SFTS ウイルス(SFTSV)が存在する。これらのウイルスのうち、RESTV 及び CCHFV は感染症の予防及び感染症の患者に対する医療に関する法律により一種病原体等、HFRSV 及び SFTSV は三種病原体等に分類され、RESTV 及び CCHFV は BSL4 施設、HFRSV 及び SFTSV は指定された BSL3 施設でしか扱うことができない。日本には稼働している BSL4 施設はなく、BSL3 施設も限られるため、こ

れらのウイルスの研究は制限されている。
本研究では、RESTV 及び SFTSV に着目し、血清学的診断法の確立とそれを用いた血清学的な解析及び疫学的研究を行った。RESTV はフィロウイルス科に属し、過去に数回カニクイザルにおいて致死性のウイルス性出血熱の流行を引き起こしている。また、2008 年にはブタで RESTV 感染が確認されブタの輸出が禁止され畜産上でも大きな問題となった。RESTV が属するフィロウイルスの自然宿主は不明であるが、近年フィロウイルス科のマールブルグウイルス及びエボラウイルスでアフリカのオオコウモリより抗体及び遺伝子が検出され、コウモリが自然宿主であると考えられている。また、SFTSV は 2011 年に中国で初めて報告されたブニヤウイルス科に属するマダニ媒介性のウイルスであるが、疫学的な研究は開始されたばかりであり自然界での感染環、地理的な分布は不明である。
コウモリは哺乳類の中で唯一飛翔能力を持ち、げっ歯類に次いで種数が多く南極を除く全ての大陸に分布する。また、一部の種は数十万頭の巨大コロニーを形成する特性を持つ。近年、フィロウイルスだけではなく、ラブドウイルス、パラミクソウイルス、SARS コロナウイルス、MERS コロナウイルスがコウモリを自然宿主としていることが報告されている。私はコウモリの特性、そして多くのウイルスの自然宿主であるという点に着目し、RESTV 及び SFTSV の血清学的な調査を行った。また、1996 年の RESTV によるサル施設でのウイルス性出血熱流行時のカニクイザル血清を用いて、終宿主と考えられるカニクイザルにおける免疫応答について解析を行った。
各章の要約は以下の通りである。
(第 1 章)
1996 年の RESTV によるサル施設でのウイルス性出血熱流行時のカニクイザルにおける免疫応答について解析した。バキュロウイルスタンパク発現系により昆虫細胞で発現、精製した RESTV の核蛋白(NP)及び膜糖蛋白(GP)を抗原とした IgG-ELISA と、RESTV の NP 及び GP を発現した哺乳類細胞を抗原とした間接蛍光抗体法(IFA)を作製した。さらに RESTV GP を外套したシュードタイプ水胞性口炎ウイルスを作製し、これを代替ウイルスとして用いた中和試験法を確立した。生ウイルスを用いず BSL2 施設で実施可能なこれらの RESTV の抗体検出系と、既に報告されている RESTV NP の抗原検出 ELISA (Ag-ELISA)を用いて、RESTV 感染カニクイザル 27 検体の血清の抗体応答を解析した。また、これらの血清中の各種サイトカイン、ケモカインの値を測定した。その結果、血清 27 検体中 10 検体で NP、GP 両方に対する抗体の上昇がみられ、これらの血清は中和抗体陽性、ウイルス抗原陰性であった。一方、GP 抗体陰性の検体は、中和抗体が陰性、ウイルス抗原陽性であったことから、GP 抗体の有無と中和抗体、血清中ウイルス抗原の有無には強い相関が認められた。一方、血清中のウイルス抗原の有無と NP 抗体の有無には相関は認められなかった。ウイルス抗原陰性、中和抗体陽性の検体では IFN-γ、IL-8、IL-12、MIP-1α の有意な上昇が認められた。GP 抗体の上昇が中和抗体の上昇と血清中のウイルス抗原の陰性化と相関したことから、RESTV 自然感染カニクイザルにおいて、GP 抗体はウイルス排除に重要な免疫応答であることが明らかになった。ウイルス抗原陰性、中和抗体陽性検体において有意な上昇が認められたサイトカインは、RESTV 感染からの回復に重要な役割を果たすと考えられ、この結果はブンディブギョエボラウイルス感染症におけるヒトの回復期検体の報告とよく一致した。
(第 2 章)
アフリカでは、エボラウイルスやマールブルグウイルスの遺伝子や抗体がコウモリから検出さ

れており、フィロウイルスの宿主動物はコウモリである可能性が示唆されている。そこで私はRESTV の自然宿主も同様にコウモリであると仮定した。第 1 章で作製した RESTV 特異的IgG-ELISA、IFA を用い、2007 から 2011 年にフィリピンで捕獲された野生コウモリ 17 種、298 検体の RESTV 抗体保有状況を調査した。コウモリ血清中の抗体検出には、二次抗体として抗コウモリ IgG ウサギ免疫血清と、抗ウサギ IgG 標識抗体を用いた。また、東京大学農学部附属牧場で飼育されているデマレルーセットオオコウモリに、組換え RESTV GP 及び NP を免疫して陽性対照血清を作製した。フィリピンで捕獲されたコウモリ血清を対象に RESTV NP 及び GP 特異的 IgG ELISA を行った結果、ルーセットオオコウモリ属に属するジュフロアルーセットオオコウモリの血清 23 検体のうち、NP 特異的 IgG-ELISA で 5 検体、GP 特異的 IgG-ELISA で 5 検体が陽性を呈した。IgG-ELISA で NP 抗体、GP 抗体がともに陽性であった血清は 3 検体であった。これらのうち、NP 特異的 IgG-ELISA で高 OD 値を示した 2 検体と、GP 特異的 IgG-ELISA で高 OD 値を示した 1 検体は、IFA でも NP, GP に対して明瞭な抗体陽性反応を呈した。他のコウモリ種（16 種、275 検体）の血清は全て抗体陰性であった。マールブルグウイルスは、ルーセットオオコウモリ属（エジプトルーセットオオコウモリ）から検出されている。RESTV 抗体がフィリピンに分布するルーセットオオコウモリ属から検出されたことは、本コウモリが RESTV の自然宿主である可能性を示唆しており、今後遺伝子検出も含めた大規模な疫学的調査が必要であると考えられた。

(第 3 章)

SFTSV の流行地である中国では、SFTSV の感染環に動物（ヤギ、ヒツジ、ウシ、イヌ、ニワトリ等）とマダニが重要な役割を果たしている。SFTSV が、SFTF 患者の発生している中国、日本、韓国以外のアジア諸国に存在するのか、また、翼手目などがウイルスの感染環に含まれるか等は全く不明である。そこで私は、SFTSV のアジアにおける感染環の解明、翼手目における感染の有無を解明することを目的として、2007 年から 2013 年にフィリピンで捕獲した野生コウモリ 14 種、404 検体を対象に SFTSV に対する抗体保有状況を調査した。抗体検出法には、SFTSV 感染細胞ライセートを抗原とした IgG-ELISA と、ウイルス感染細胞を用いた IFA を作製した。また、SFTSV と同じフレボウイルス属に分類されるリフトバレー熱ウイルス(RVFV)感染細胞を抗原とした IFA も併せて行った。その結果、コイヌガオフルーツコウモリ、ジュフロアルーセットオオコウモリ、ビロードフルーツコウモリに IgG-ELISA 及び IFA で抗体陽性の個体が確認された。これらの抗体陽性血清は、RVFV 感染細胞を抗原とした IFA では全て陰性を示した。これらの結果から、SFTSV あるいは SFTSV に類似したウイルスがフィリピンにも分布する可能性が示唆された。今後、フィリピンを含む東南アジアにおいてコウモリを含む種々の野生動物における SFTSV あるいは SFTS 様ウイルスの感染実態を解明していく必要があると考えられた。

以上のように、血清学的診断系を用いたアプローチで、第 1 章では終宿主であるカニクイザルを対象に RESTV 自然感染時の免疫応答の解析、第 2 章では RESTV の自然宿主と考えられるコウモリを対象とした疫学的研究、第 3 章では SFTSV のアジアにおける感染環の解明、翼手目における感染の有無の解明を目的とした疫学調査を行った。エボラウイルスを実験感染させ免疫応答を解析した実験は多くなされているが、ヒト以外の霊長類における自然感染時の免疫応答を解析した報告はない。このため、第 1 章で行われた研究成果は今後への大きな知見になると考えられる。第 2 章ではコウモリを対象とした RESTV の疫学的研究から、ルーセットオオコウモリが RESTV の宿主動物である可能性を示唆する成果を得たが、他の研究者による調査により、中国のブタからも RESTV が検出され、中国及びバングラディッシュのルーセットオオコウモリ

から RESTV 抗体が検出されている。また、インドネシアのボルネオ島のオラウータンからもフィロウイルスに対する抗体が検出されている。RESTV の自然宿主はコウモリであると考えられたが、今後コウモリからの RESTV 遺伝子検出あるいは RESTV 分離がその証明には必要である。第３章では、フィリピンに SFTSV あるいはそれに近縁なウイルスが存在する可能性を示した。アメリカ大陸では SFTSV と近縁なハートランドウイルスが、インド、アフリカ及びヨーロッパではバンジャウイルスが分離されている。今後、東南アジアにおいて、SFTSV あるいはそれに近縁などのようなウイルスが存在するのかを解明するために、野生動物や家畜を対象としたウイルス遺伝子検出やウイルス分離が必要であると考えられる。その解明は、ヒトへのリスクを評価する上でも重要な知見となる。