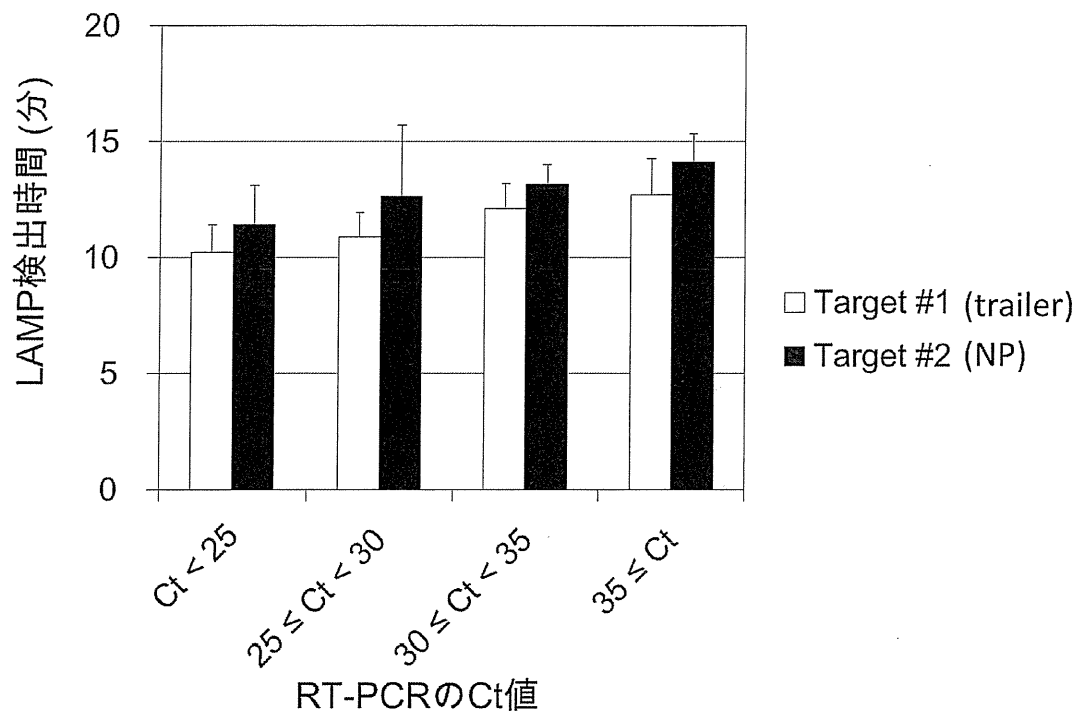

図２、リアルタイム RT-PCR と RT-LAMP の検出時間の相関
横軸：RT-PCR で陽性と判定されたサンプルのサイクル数（Ct)、縦軸：RT-LAMP で陽性と判定された時間

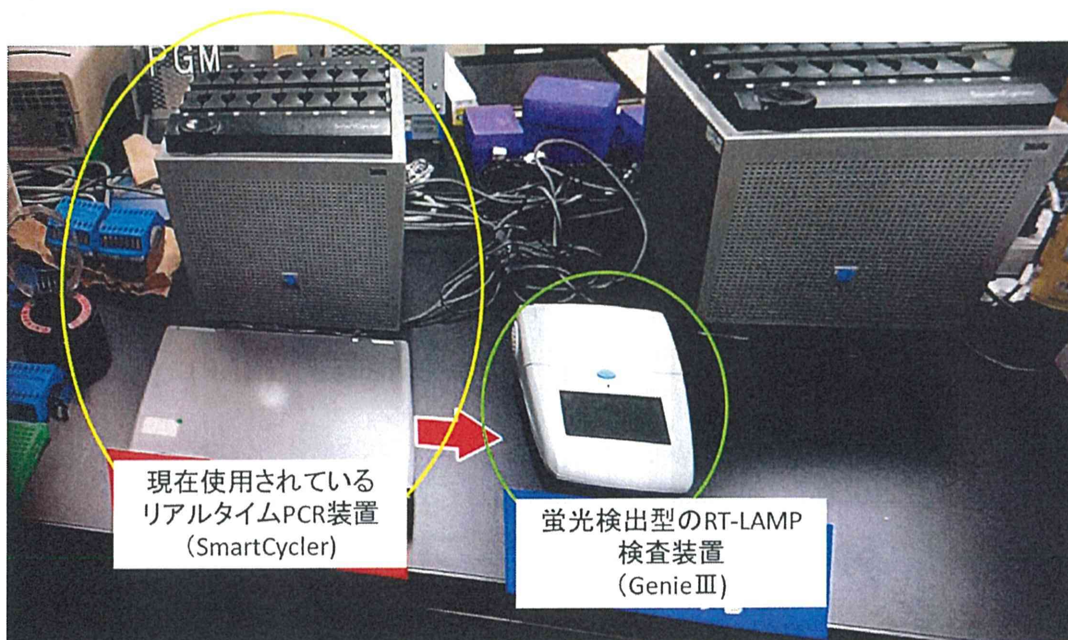

写真１、リアルタイム PCR 装置と RT-LAMP 装置の比較

III. 学会等発表実績

学 会 等 発 表 実 績

委託業務題目「エボラ出血熱に対する治療薬、診断薬の開発に関する研究」

1．学会等における口頭・ポスター発表

発表した成果（発表題目、口頭・ポスター発表の別）	発表者氏名	発表した場所（学会等名）	発表した時期	国内・外の別
Establishment of a rapid diagnosis of Ebola virus with a portable device （ポスター）	Yohei Kurosaki, Olamide Oloninyi, Saori Sakabe, Ayato Takada Jiro Yasuda	7th International Symposium on Filovirus	2015.3.25-29	国外
R&D Effort with Antibodies for Ebola Virus Disease（口頭）	Takeyoshi Yamashita Ayato Takada	2015 U. S. - JAPAN Medical Biodefense Research Symposium, Rockville, Maryland, USA	February 12-13, 2015	国外
Rapid and Simple Detection of Ebolaviruses（口頭）	Jiro Yasuda	2015 U. S. - JAPAN Medical Biodefense Research Symposium, Rockville, Maryland, USA	February 12-13, 2015	
R&D Efforts on Diagnostic Tools for Ebola（口頭）	Masayoshi Takahashi Hiroaki Goto	2015 U. S. - JAPAN Medical Biodefense Research Symposium, Rockville, Maryland, USA	February 12-13, 2015	
Development of T-705 (Favipiravir) in Japan and Current Agenda for the Treatment of Ebola Virus Diseases（口頭）	Koichi Yamada	2015 U. S. - JAPAN Medical Biodefense Research Symposium, Rockville, Maryland, USA	February 12-13, 2015	
Overview of Current International Collaborative Research Framework of Japan on Ebola Virus Diseases（口頭）	Yuki Maehira	2015 U. S. - JAPAN Medical Biodefense Research Symposium, Rockville, Maryland, USA	February 12-13, 2015	
Challenges in Medical Countermeasures and Preparedness for An Ebola Outbreak in Japan（口頭）	Tomoya Saito	2015 U. S. - JAPAN Medical Biodefense Research Symposium, Rockville, Maryland, USA	February 12-13, 2015	

2．学会誌・雑誌等における論文掲載

掲載した論文（発表題目）	発表者氏名	発表した場所（学会誌・雑誌等名）	発表した時期	国内・外の別
Seroepidemiological Prevalence of Multiple Species of Filoviruses in Fruit Bats (Eidolon helvum) Migrating in Africa	Ogawa H, et al.	J Infect Dis	印刷中	国外
Host Cell Factors Involved in Filovirus Infection	Kajihara M and Takada A	Curr Trop Med Rep	Feb. 2015	国外

IV. 研究成果の刊行物・別刷

Journal of Infectious Diseases Advance Access published March 18, 2015

SUPPLEMENT ARTICLE

Seroepidemiological Prevalence of Multiple Species of Filoviruses in Fruit Bats (*Eidolon helvum*) Migrating in Africa

Hirohito Ogawa,[1,2] Hiroko Miyamoto,[5] Eri Nakayama,[5] Reiko Yoshida,[5] Ichiro Nakamura,[2,6] Hirofumi Sawa,[2,7,9] Akihiro Ishii,[1,2] Yuka Thomas,[1,2] Emiko Nakagawa,[1,2] Keita Matsuno,[5,9] Masahiro Kajihara,[5] Junki Maruyama,[5] Naganori Nao,[5] Mieko Muramatsu,[5] Makoto Kuroda,[5] Edgar Simulundu,[2] Katendi Changula,[3,10] Bernard Hang'ombe,[3,10] Boniface Namangala,[3] Andrew Nambota,[2] Jackson Katampi,[4] Manabu Igarashi,[5,9] Kimihito Ito,[8,9] Heinz Feldmann,[11] Chihiro Sugimoto,[2,6,9] Ladslav Moonga,[1,3] Aaron Mweene,[2,10] and Ayato Takada[2,5,9]

[1]Hokudai Center for Zoonosis Control in Zambia, and Departments of [2]Disease Control, and [3]Paraclinical Studies, School of Veterinary Medicine, University of Zambia, Lusaka; [4]Zambia Wildlife Authority, Chilanga; Divisions of [5]Global Epidemiology, [6]Collaboration and Education, [7]Molecular Pathobiology, [8]Bioinformatics, Research Center for Zoonosis Control, and [9]Global Station for Zoonosis Control, Global Institution for Collaborative Research and Education, Hokkaido University, Sapporo, Japan; [10]Southern African Centre for Infectious Disease Surveillance, Chuo Kikuu, Morogoro, Tanzania; and [11]Laboratory of Virology, Division of Intramural Research, National Institutes of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, Montana

Fruit bats are suspected to be a natural reservoir of filoviruses, including Ebola and Marburg viruses. Using an enzyme-linked immunosorbent assay based on the viral glycoprotein antigens, we detected filovirus-specific immunoglobulin G antibodies in 71 of 748 serum samples collected from migratory fruit bats (*Eidolon helvum*) in Zambia during 2006–2013. Although antibodies to African filoviruses (eg, *Zaire ebolavirus*) were most prevalent, some serum samples showed distinct specificity for *Reston ebolavirus*, which that has thus far been found only in Asia. Interestingly, the transition of filovirus species causing outbreaks in Central and West Africa during 2005–2014 seemed to be synchronized with the change of the serologically dominant virus species in these bats. These data suggest the introduction of multiple species of filoviruses in the migratory bat population and point to the need for continued surveillance of filovirus infection of wild animals in sub-Saharan Africa, including hitherto nonendemic countries.

Keywords. Ebola virus; Marburg virus; filovirus; specific antibody; fruit bat; Zambia.

Ebola and Marburg viruses belonging to the family Filoviridae cause severe hemorrhagic fever in humans and nonhuman primates. Whereas the genus *Marburgvirus* consists of a single species, *Marburg marburgvirus*, 5 distinct species are known in the genus *Ebolavirus*: *Zaire ebolavirus*, *Sudan ebolavirus*, *Taï Forest ebolavirus*, *Bundibugyo ebolavirus*, and *Reston ebolavirus* [1]. Findings of previous studies have suggested that these filoviruses infect several species of animals, such as fruit bats, dogs, duikers, and pigs [2–5]. In particular, some species of fruit bats are suspected to be the natural reservoir of Ebola and Marburg viruses [6–8].

Based on virus isolation and nucleotide sequence analyses, the cave-dwelling Egyptian fruit bat (*Rousettus aegyptiacus*) was identified as a source of a Marburg virus disease outbreak in Uganda in 2007 [6, 8]. By contrast, infectious Ebola viruses have never been isolated from any fruit bat species, though small amounts of viral RNA fragments (*Z. ebolavirus*) and virus-specific antibodies were detected in some fruit bat species (*Hypsignathus monstrosus*, *Epomops franqueti*, and *Myonycteris torquata*) captured around endemic areas during the 2001–2003 Ebola virus disease outbreak in Gabon and the Democratic Republic of the Congo (DRC) [4, 7].

The filovirus genomes encode at least 7 structural proteins. Of these, the viral surface glycoprotein (GP) is responsible for receptor binding and fusion of the viral envelope with host cell membranes [9, 10] and is

Correspondence: Ayato Takada, DVM, PhD, Division of Global Epidemiology, Research Center for Zoonosis Control, Hokkaido University, Kita-20, Nishi-10, Kita-ku, Sapporo 001-0020, Japan (atakada@czc.hokudai.ac.jp).

The Journal of Infectious Diseases®
© The Author 2015. Published by Oxford University Press on behalf of the Infectious Diseases Society of America. All rights reserved. For Permissions, please e-mail: journals.permissions@oup.com.
DOI: 10.1093/infdis/jiv063

Downloaded from http://jid.oxfordjournals.org/ by guest on March 19, 2015

therefore the main target of neutralizing antibodies. Most antibodies induced against filovirus GPs recognize epitopes in the variable regions of the protein [11]. Nakayama et al [11] have established elsewhere an enzyme-linked immunosorbent assay (ELISA) using GP antigens, which enable us to detect filovirus species-specific antibodies, and have shown that GPs of all known species of filoviruses are serologically distinguishable, mirroring the phylogenetic relationship among filovirus species.

Zambia has borders with the DRC, Zimbabwe, and Angola, all of which have suffered outbreaks of Ebola or Marburg virus disease, whereas there has been no report of filovirus infection so far in any animal species, including humans, in Zambia. However, considering its geographic position, Zambia seems to be a high-risk country that could suffer an incursion of filovirus infection. Moreover, Zambia and the surrounding countries, such as the DRC and Angola, probably share the large common ecosystem providing habitats for various wild animals, including nonhuman primates and fruit bats, both of which are known to be susceptible to filovirus infection [4, 6, 8].

In the present study, we focused on migratory fruit bats (*E. helvum*), which are commonly found in Africa [12] and could likely be infected with Ebola virus, as suggested by a previous study initially demonstrating Ebola virus–specific antibodies in this bat species [13]. We carried out a serological survey to detect filovirus-specific antibodies using GP antigens of all known virus species of the genera *Ebolavirus* and *Marburgvirus*.

MATERIALS AND METHODS

Animals and Serum Samples

A total of 748 serum samples were collected from wild healthy straw-colored fruit bats (*E. helvum*; 263 male, 485 female) [12] caught in Central Province and Copperbelt Province in Zambia from December 2006 to December 2013 (Supplementary Table 1). Captured bats were euthanized with diethyl ether, and blood and tissue samples were collected for antibody detection and reverse-transcription polymerase chain reaction (RT-PCR) assays, respectively. Dissection and tissue processing were carried out in a biosafety level 3 containment facility at the Hokudai Center for Zoonosis Control in Zambia, belonging to the University of Zambia. All these activities were performed under the research project "Molecular and Serological Surveillance of Viral Zoonoses in Zambia," approved by the Zambia Wildlife Authority of the Republic of Zambia (Act No. 12 of 1998).

ELISA Protocol

Filovirus GP-based ELISA was performed as described elsewhere [11]. Briefly, His-tagged soluble recombinant GPs of strains Mayinga (Zaire), Boniface (Sudan), Cote d'Ivoire (Taï Forest), Bundibugyo (Bundibugyo), Pennsylvania (Reston), and Angola (Angola), representing the filovirus species *Z. ebolavirus*, *S. ebolavirus*, *Taï Forest ebolavirus*, *B. ebolavirus*, *R. ebolavirus*, and *M. marburgvirus*, respectively, were purified from the supernatants of human embryonic kidney 293T cells transfected with pCAGGS expressing each GP using the Ni-NTA Purification System (Life Technologies). ELISA plates (Nunc MaxiSorp) were coated with the GP antigens (100 ng of GP; 50 µL per well) or control antigens (fetal calf serum–derived proteins nonspecifically bound to the nickel beads), followed by blocking with 3% skim milk (150 µL per well). Serum samples diluted at 1:100 or 4-fold serially diluted from 1:100 were added and incubated for 1 hour at room temperature. The bound antibodies were visualized with a goat anti-bat immunoglobulin (Ig) G heavy- and light-chain antibody conjugated with horseradish peroxidase (Bethyl Laboratories) and 3,3′,5,5′-tetramethylbenzidine (Sigma-Aldrich). The reaction was stopped by adding 1N sulfuric acid, and the optical density (OD) at 450 nm was measured. To offset the nonspecific antibody reaction, the OD value of the control antigen was subtracted from that of each sample. Assays were conducted in duplicate or triplicate, and means were used for further data analyses.

Western Blotting

Serum samples were analyzed by Western blotting, as described elsewhere [14]. To generate viruslike particles, 293T cells were transfected with plasmids encoding filovirus (Zaire, Sudan, Taï Forest, Bundibugyo, Reston, and Angola) GP, viral nucleoprotein, and matrix protein (VP40) genes. At 48 hours after transfection, these particles were recovered from the pellets after centrifugation at 28 000*g* and 4°C for 1.5 hours through a 25% sucrose cushion. Supernatants from 293T cells transfected with an empty vector, pCAGGS, were used as a negative control. Viruslike particles were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis under nonreducing conditions on 5–20% SuperSep gel (Wako) and blotted on a polyvinylidene difluoride membrane (Millipore). Bat serum samples diluted at 1:100 were used as primary antibodies, followed by detection with goat anti-bat IgG heavy- and light-chain antibody conjugated with horseradish peroxidase (Bethyl). Mouse monoclonal antibodies ZGP42/3.7 to Ebola virus GPs and AGP127-8 to Marburg virus GP were used as positive control antibodies, followed by detection with goat anti-mouse IgG heavy- and light-chain antibody conjugated with horseradish peroxidase (Jackson ImmunoResearch) [14]. The bound antibodies were visualized with Western Lightning Plus-Enhanced Chemiluminescence (PerkinElmer) and detected with an ImageQuant LAS 4000 imaging system (GE Healthcare).

RT-PCR Protocol

RT-PCR assay was performed as described elsewhere [15]. Briefly, total RNA was extracted from 140 µL of 10% (wt/vol) homogenates of spleens and/or livers from individual fruit bats (367 bats captured in 2010–2013) with a QIAamp Viral

Downloaded from http://jid.oxfordjournals.org/ by guest on March 19, 2015

RNA Mini Kit (Qiagen) according to the manufacturer's instructions. One-step RT-PCR targeting the filovirus nucleoprotein gene was carried out using a Qiagen OneStep RT-PCR kit (Qiagen), according to the manufacturer's instructions. The filovirus-specific universal primers FiloNP-Fm, FiloNP-Rm, FiloNP-Fe, and FiloNP-Re were used [15]. The 1-step RT-PCR program consisted of reverse transcription at 50°C for 30 minutes and initial PCR activation at 95°C for 15 minutes, followed by 50 cycles of denaturation at 94°C for 15 seconds, annealing at 53°C for 30 seconds, extension at 72°C for 30 seconds, and final extension at 72°C for 7 minutes.

Statistical Analysis

All OD values obtained by GP-based ELISA (748 bats for 6 GP antigens) were analyzed concurrently. Smirnov–Grubbs rejection tests were employed as described elsewhere [14]. Briefly, the highest OD value was first picked up, and the T value ($T_{\mathrm{ODhighest}} = |\mathrm{OD}_{\mathrm{highest}} - \mathrm{OD}_{\mathrm{mean1\text{-}4488}}|/\mathrm{OD}_{\mathrm{SD1\text{-}4488}}$, where SD indicates standard deviation) was calculated for its statistical significance based on the critical values given by the Smirnov–Grubbs test ($n = 4488$; $T = 4.23$, $P < .05$). If it was considered an outlier, the T value for the second highest OD value was then similarly tested without the highest one ($T_{\mathrm{OD2nd\ highest}} = |\mathrm{OD}_{\mathrm{2nd\ highest}} - \mathrm{OD}_{\mathrm{mean1\text{-}4487}}|/\mathrm{OD}_{\mathrm{SD1\text{-}4487}}$). These steps were repeated until the T value fell below the level of statistical significance ($P < .05$).

RESULTS

Screening of Filovirus-Specific IgG Antibodies by ELISA

Fruit bat serum samples were screened for IgG antibodies specific to the known species of filoviruses (Figure 1), and the OD values obtained by GP-based ELISA were analyzed statistically as described in "Materials and Methods" section. Because there were no control serum samples either positive or negative for filovirus antibodies in this fruit bat species, it was not possible to set the cutoff value for the OD based on such control populations. Instead, to determine statistical significance of each OD value, we employed the Smirnov–Grubbs rejection test, which is widely used to detect significantly higher or lower values (ie, outliers) that do not belong to the population consisting of all other values in the data set. Based on the distribution of the samples (Supplementary Figure 1), we detected statistical outliers and reasonably assumed that the big peak represented the negative sample population and that the outliers ($P < .05$) with significantly higher OD values did not belong to the negative group. Thus, these statistical outlier samples were considered positive.

Filovirus Species Specificity of Serum IgG Antibodies Detected in Fruit Bats

ELISA-positive samples were analyzed for species specificity among filoviruses by comparing the OD values for each GP antigen. Representative data are shown in Figure 2. We found that the majority of the positive samples showed exclusive specificity for one of the antigens. Antibodies to African filoviruses were predominant (ie, Zaire, Sudan, Taï Forest, Bundibugyo, or Angola), but some of the positive samples showed distinct reactivity to the antigen derived from Reston virus, which has thus far been found only in Asia (ie, the Philippines and China) [3, 16]. Specificities of representative samples positive for each antigen were confirmed by Western blotting (Figure 3). Although 6 of the positive samples showed cross-reactivity to multiple virus species (eg, ZFB10-19 and ZFB09-35), there was little cross-reactivity across the genus (ie, *Ebolavirus* vs *Marburgvirus*), consistent with previous findings [11, 14].

Filovirus-specific IgG antibodies were detected continuously in this fruit bat species in Zambia during the years 2006–2013 (Table 1). In total, 2.5% (19 of 748), 2.5% (19 of 748), 1.2% (9 of 748), 1.1% (8 of 748), and 1.2% (9 of 748) of the serum samples showed the highest reactivity to Zaire, Sudan, Taï Forest, Bundibugyo, and Reston virus antigens, respectively. Overall, 8.6% (Ebola) and 0.9% (Marburg) of the samples were found to be IgG positive for filovirus GP antigens (Table 2). End point antibody titers of positive samples ranged between 1:100 and 1:6400 (Supplementary Table 2). No significant difference was found in the overall positivity between sexes (data not shown). Filovirus RNA genomes were not detected in spleens and livers of the bats captured in 2010–2013 (data not shown).

Tracing the History of Outbreaks of Filovirus Diseases and Serologically Dominant Filovirus Species in Bats

Since 2000, outbreaks of Ebola virus diseases caused by several virus species have been reported (Supplementary Table 3) [17, 18]. We compared the filovirus species that caused outbreaks in Central and West Africa and virus species for which specific antibodies were predominantly detected in the corresponding years (Figure 4). Ebola virus (species *Z. ebolavirus*) frequently appeared in the 2000s, but there were no reported outbreaks due to this virus species between 2009 and 2013. Interestingly, antibodies specific for *Z. ebolavirus* were predominantly detected in the bats until 2010, but none of the samples collected in 2011 and 2012 were positive for this species. Antibodies specific for *Z. ebolavirus* were then detected again in bats collected in 2013.

In contrast, epidemics caused by Sudan virus (species *S. ebolavirus*), which were seen only twice in the 2000s, occurred through 3 independent introductions into humans in 2011–2012. Correspondingly, while the presence of the Sudan virus-specific antibody in bats was comparatively minor until 2008, the antibody positivity to Sudan virus increased and became dominant in 2010. Bundibugyo virus (species *B. ebolavirus*), which was first found in 2007, caused an outbreak again in 2012, and the antibody positivity to Bundibugyo virus, which

Downloaded from http://jid.oxfordjournals.org/ by guest on March 19, 2015

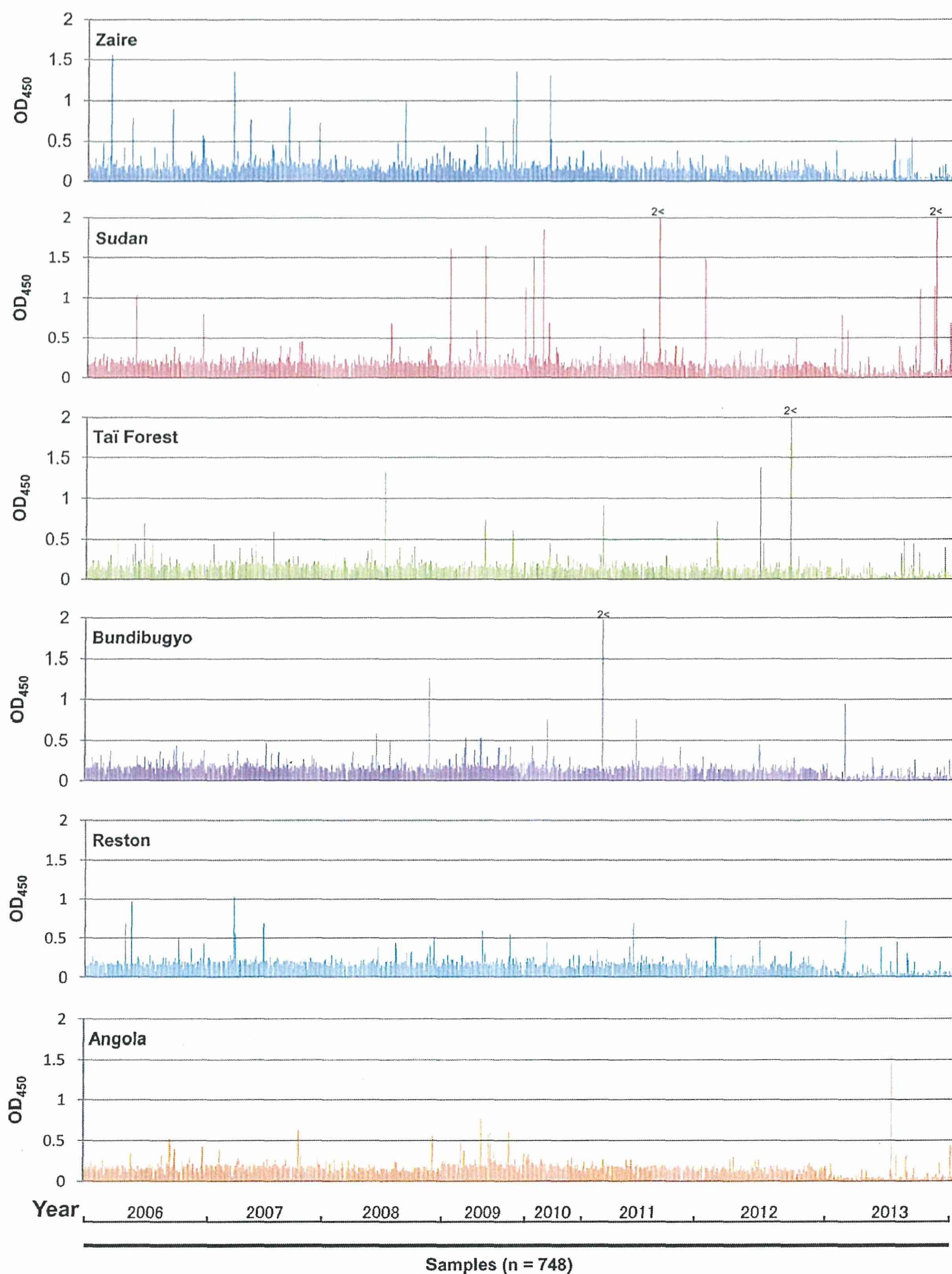

Figure 1. Immunoglobulin (Ig) G antibodies detected in the serum samples collected from *Eidolon helvum* in Zambia. Serum samples were tested (1:100 dilution) for IgG antibodies specific to Zaire, Sudan, Taï Forest, Bundibugyo, and Reston viruses and Angola Marburg virus by glycoprotein-based enzyme-linked immunosorbent assay. All optical density (OD) values were subjected to the Smirnov–Grubbs rejection test to discriminate the positive (ie, significantly higher OD values) from the negative population (Supplementary Figure 1).

Downloaded from http://jid.oxfordjournals.org/ by guest on March 19, 2015

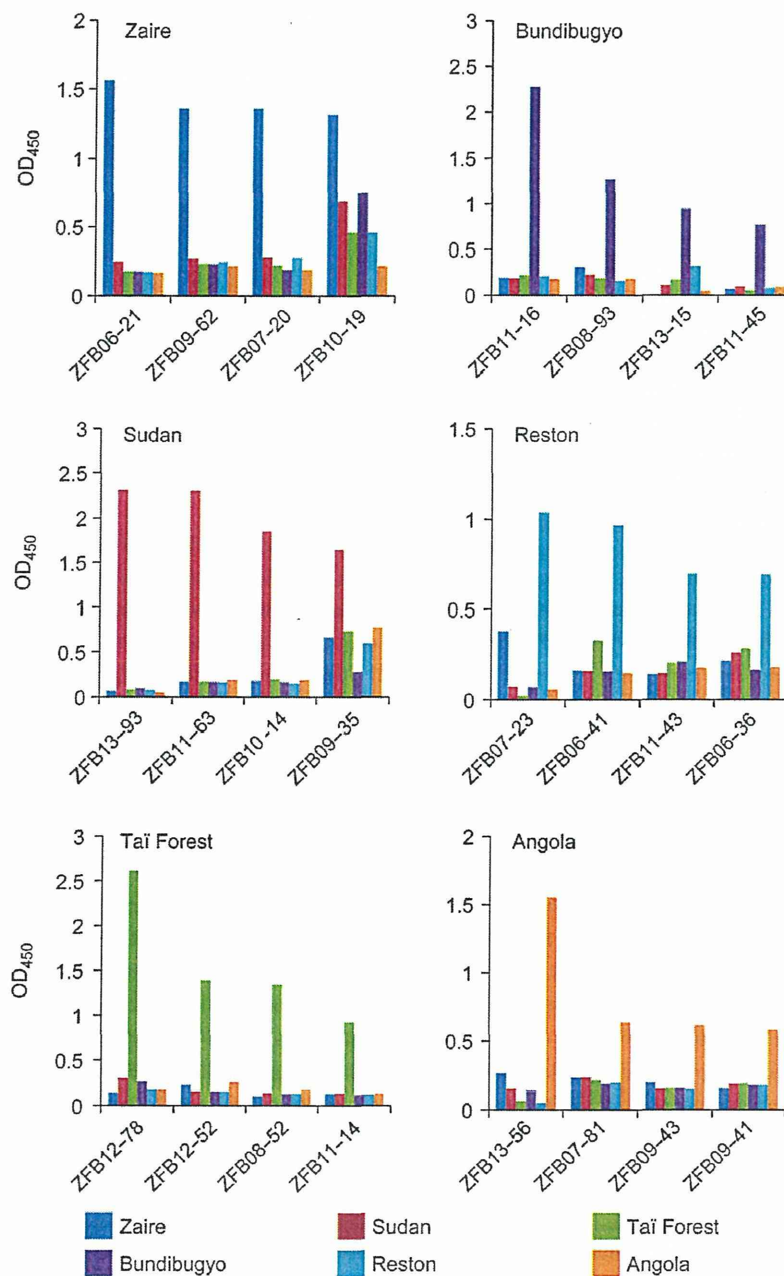

Figure 2. Filovirus species specificity of immunoglobulin (Ig) G antibodies in glycoprotein (GP)–based enzyme-linked immunosorbent assay (ELISA). Serum samples diluted at 1:100 were tested for IgG antibodies reacting with GP antigens in ELISA. Optical density (OD) values obtained for all filovirus antigen were compared. Four representative data are shown for each filovirus antigen. Sample identification numbers are shown on the horizontal axis.

was minor in 2006–2007, became prevalent in 2008 and 2011, a finding that seemed to be synchronized with 2 outbreaks caused by this virus in 2007–2008 and 2012. Taken together, the trend of the emerging filovirus species causing outbreaks in Central and West Africa seemed parallel to the proportion of seropositivity to each filovirus species in fruit bats tested in this study.

DISCUSSION

Although fruit bats have been suspected to play some roles in the ecology of filoviruses [7, 8, 19], it is still elusive whether fruit bats act as reservoirs continuously maintaining the virus in nature. Although multiple strains of Marburg viruses were

Downloaded from http://jid.oxfordjournals.org/ by guest on March 19, 2015