# Seroepidemiological prevalence of multiple species of filoviruses in fruit bats (*Eidolon helvum*) migrating in Africa

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

**Author affiliations:**

[1] Hokudai Center for Zoonosis Control in Zambia, School of Veterinary Medicine, University of Zambia, P.O. Box 32379, Lusaka, Zambia.

[2] Department of Disease Control, School of Veterinary Medicine, University of Zambia, P.O. Box 32379, Lusaka, Zambia.

[3] Division of Global Epidemiology, Research Center for Zoonosis Control, Hokkaido University, Sapporo 001-0020, Japan.

[4] Division of Collaboration and Education, Research Center for Zoonosis Control, Hokkaido University, Sapporo 001-0020, Japan.

[5] Division of Molecular Pathobiology, Research Center for Zoonosis Control, Hokkaido University, Sapporo 001-0020, Japan.

[6] Global Station for Zoonosis Control, Global Institution for Collaborative Research and Education, Hokkaido University, Sapporo 001-0020, Japan.

[7] Department of Paraclinical Studies, School of Veterinary Medicine, University of Zambia, P.O. Box 32379, Lusaka, Zambia.

[8] Southern African Centre for Infectious Disease Surveillance, P.O. Box 3297, Chuo Kikuu, Morogoro, Tanzania

[9] Zambia Wildlife Authority, Private Bag 1, Chilanga, Zambia

[10] Division of Bioinformatics, Research Center for Zoonosis Control, Hokkaido University, Sapporo 001-0020, Japan.

[11] Laboratory of Virology, Division of Intramural Research, National Institutes of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, Montana 59840-2932, USA

**Corresponding author:** Ayato Takada

Tel.: +81-11-706-9502

Fax: +81-11-706-7310

E-mail: atakada@czc.hokudai.ac.jp

**Running Title:** Anti-filovirus IgG in Zambian fruit bats

The character and space count of the title: 116

The character and space count of the running title: 40

The word count of the abstract: 148

The word count of the main text: 2805

**Abstract**

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. Though antibodies to African filoviruses (e.g., *Zaire ebolavirus*) were most prevalent, some of the sera 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 appeared 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

## Introduction

Ebola and Marburg viruses belonging to the Family *Filoviridae* cause severe hemorrhagic fever in humans and nonhuman primates. While the genus *Marburgvirus* consists of a single species, *Marburg marburgvirus*, five distinct species are known in the genus *Ebolavirus*: *Zaire ebolavirus*, *Sudan ebolavirus*, *Taï Forest ebolavirus*, *Bundibugyo ebolavirus*, and *Reston ebolavirus* [1]. Previous studies have suggested that these filoviruses infect several different species of animals such as fruit bats, dogs, duikers, and pigs [2-5]. Particularly, 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 (*Zaire 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 seven 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 therefore the main target of neutralizing antibodies. Most antibodies induced against filovirus GPs recognize epitopes in the variable regions of the protein [11]. We have previously established an

enzyme-linked immunosorbent assay (ELISA) using GP antigens, which enable us to detect filovirus species-specific antibodies, and shown that GPs of all known species of filoviruses are serologically distinguishable and it mirrors the phylogenetic relationship among filovirus species [11].

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 geographical position, Zambia seems to be a high risk country that potentially could suffer an incursion of filovirus infection. Moreover, Zambia and the surrounding countries such as the DRC and Angola likely 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 this study, we focused on migratory fruit bats (*Eidolon helvum*), which are commonly found in Africa [12] and could likely be infected with Ebola virus as suggested by the previous study initially demonstrating Ebola virus-specific antibodies in this bat species [13], and a serological survey was carried out to detect filovirus-specific antibodies using GP antigens of all known virus species of the genera *Ebolavirus* and *Marburgvirus*.

## Materials and Methods

### Animals and sera

Seven hundred forty-eight serum samples (from 263 males and 485 females) were collected from wild healthy straw-colored fruit bat (*Eidolon helvum*) [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

Filovirus GP-based ELISA was performed as described previously [11]. Briefly, His-tagged soluble recombinant GPs of strains Mayinga (Zaire), Boniface (Sudan), Cote d'Ivoire (Tai Forest), Bundibugyo (Bundibugyo), Pennsylvania (Reston) and Angola (Angola), representing the filovirus species *Zaire ebolavirus*, *Sudan ebolavirus*, *Taï Forest ebolavirus*, *Bundibugyo ebolavirus*, *Reston ebolavirus*, and *Marburg marburgvirus*, respectively, were purified from the supernatants of human embryonic

kidney 293T cells transfected with pCAGGS expressing each GP using a Ni-NTA Purification System (Life Technologies). ELISA plates (Nunc MaxiSorp) were coated with the GP antigens (100 ng of GP/50 μl/well) or control antigens (FCS-derived proteins non-specifically bound to the Ni-beads), followed by blocking with 3% skim milk (150 μl/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 G (IgG)-heavy and light chain antibody conjugated with horseradish peroxidase (Bethyl Laboratories, Inc.) and 3,3',5,5'-tetramethylbenzidine (Sigma-Aldrich). The reaction was stopped by adding 1 N 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 averages were used for further data analyses.

**Western blotting**

Serum samples were analyzed by western blotting as described previously [14]. 293T cells were transfected with plasmids encoding filovirus (Zaire, Sudan, Tai Forest, Bundibugyo, Reston, and Angola) GP, viral nucleoprotein (NP) and matrix protein (VP40) genes to generate virus-like particles (VLPs). At 48 hours post-transfection, VLPs were recovered from the pellets after centrifugation at 28,000 X *g* at 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. VLPs were subjected to

148 sodium dodecyl sulfate-polyacrylamide gel electrophoresis under non-reducing
149 conditions on 5-20% SuperSep (Wako) and blotted on a polyvinylidene difluoride
150 membrane (Millipore). Bat serum samples diluted at 1:100 were used as primary
151 antibodies, followed by detection with goat anti-bat IgG-heavy and light chain antibody
152 conjugated with horseradish peroxidase (Bethyl). Mouse monoclonal antibodies
153 ZGP42/3.7 to Ebola virus GPs and AGP127-8 to Marburg virus GP were used as
154 positive control antibodies, followed by detection with goat anti-mouse IgG-heavy and
155 light chain antibody conjugated with horseradish peroxidase (Jackson
156 ImmunoResearch) [14]. The bound antibodies were visualized with Western Lightning
157 Plus-ECL (PerkinElmer) and detected by an ImageQuant LAS4000 (GE Healthcare).
158
159 **RT-PCR**
160 RT-PCR assay was performed as described previously [15]. Briefly, total RNA was
161 extracted from 140 μl of 10% (w/v) homogenates of spleens and/or livers of individual
162 fruit bats (367 bats captured in 2010-2013) with QIAamp Viral RNA Mini Kit
163 (QIAGEN) according to the manufacturer's instructions. One-step RT-PCR targeting the
164 filovirus nucleoprotein gene was carried out using a QAIGEN OneStep RT-PCR kit
165 (QIAGEN) according to the manufacturer's instructions. The filovirus-specific
166 universal primers FiloNP-Fm, FiloNP-Rm, FiloNP-Fe, and FiloNP-Re were used [15].
167 The one-step RT-PCR program consisted of reverse transcription at 50°C for 30 min and
168 initial PCR activation at 95°C for 15 min, followed by 50 cycles of denaturation at 94°C
169 for 15 s, annealing at 53°C for 30 s, extension at 72°C for 30 s and final extension at

72°C for 7 min.

**Statistics**

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 previously [14]. Briefly, the highest OD value was first picked up, and the T value ($T_{OD\ highest} = |OD_{highest} - OD_{Average1\text{-}4488}|/OD_{Standard\ deviation1\text{-}4488}$) was calculated for its statistical significance based on the critical values given by the Smirnov-Grubbs test ($n = 4488$; $T = 4.23$, $P < 0.05$). If it was considered to be an outlier, the T value for the second highest OD value was then similarly tested without the highest one ($T_{OD\ 2nd\ highest} = |OD_{2nd\ highest} - OD_{Average1\text{-}4487}|/OD_{Standard\ deviation1\text{-}4487}$). These steps were repeated until the T value fell to below the level of statistical significance ($P < 0.05$).

## 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. Since 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 (i.e., 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 ($P < 0.05$) 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 (i.e., Zaire, Sudan, Tai 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 (i.e., 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 (e.g., ZFB10-19 and ZFB09-35), there was little cross-reactivity across the genus (i.e., *Ebolavirus* vs. *Marburgvirus*), consistent with previous studies [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% (19/748), 2.5% (19/748), 1.2% (9/748), 1.1% (8/748), and 1.2% (9/748) of the serum samples showed the highest reactivity to Zaire, Sudan, Tai Forest, Bundibugyo, and Reston, respectively. Overall, 8.6% (Ebola) and 0.9% (Marburg) of the samples were found to be IgG-positive for filovirus GP antigens, respectively (Table 2). Endpoint 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 genders (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 the bats.**

Since 2000, outbreaks of Ebola virus diseases caused by several different 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 *Zaire 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 *Zaire ebolavirus* were predominantly detected in the bats until 2010; however, none of the samples collected in 2011 and 2012 were positive for this species. Antibodies specific for *Zaire ebolavirus* were then detected again in bats collected in 2013. In contrast, epidemics caused by Sudan virus (species *Sudan ebolavirus*), which were seen only twice in the 2000s, occurred through three 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 *Bundibugyo ebolavirus*), which was first found in 2007, caused an outbreak again in 2012, and the antibody positivity to Bundibugyo virus, which was minor in 2006-2007, became prevalent in 2008 and 2011, which seemed to be synchronized with two outbreaks caused by this virus in 2007-8 and 2012. Taken together, the trend of the emerging filovirus species causing outbreaks in Central and West Africa appeared to be parallel to the proportion of seropositivity to each filovirus species in fruit bats tested in this study.

## Discussion

While 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 isolated from wild-caught and apparently healthy cave fruit bats (*Rousettus aegyptiacus*), which are common throughout Africa with distribution into the eastern Mediterranean and Middle East [8], infectious Ebola viruses have never been isolated from any bat species. Moreover, despite epidemiological efforts to discover the filovirus genome in fruit bats, currently used RT-PCR methods have failed to detect even small amounts of viral RNA [20] except for one report [7]. We also utilized universal primer sets for RT-PCR to detect all known species of filoviruses [15], but were not able to find any filovirus RNA genome in spleens and livers of the bats captured in 2010-2013 (data not shown). Thus, no infectious Ebola virus has yet been found in fruit bats and the presence of the viral RNA genome has not been fully proven.

Serological studies have been conducted for various fruit bats, including *Eidolon helvum*; however, most of them focused mainly on the *Zaire ebolavirus* [13, 20-22]. Our results showed that IgG antibodies specific to various filovirus species were detected in the sera of this fruit bat species by using GP-based ELISA. In particular, it is noteworthy that IgG antibodies specific to Reston virus, which has been believed to be a virus of Asian origin, were often detected during the years 2006-2013, suggesting the existence of Reston or Reston-like viruses in Africa. This hypothesis may be supported by the phylogenetic relationships among virus species (i.e., *Reston ebolavirus* and

*Sudan ebolavirus* cluster together with similar phylogenetic distances to the other known African filoviruses). Conversely, recent serological studies demonstrated that IgG antibodies specific to filoviruses other than Reston virus **(**e.g., *Zaire ebolavirus*) were detected in the sera of orangutans in Indonesia and fruit bats in Bangladesh [14, 21]. These reports suggest that filoviruses might be more widely distributed than assumed hitherto. The present study also suggests the existence of multiple species of filoviruses or unknown filovirus-related viruses in nonendemic areas in Africa.

*Eidolon helvum* is a migratory bat flying between the tropical forests of Central and West Africa (endemic areas of filovirus diseases) and north-central Zambia during October-December [12, 23]. Interestingly, filovirus species causing outbreaks in Central and West Africa during 2005-2012 appeared to shift from *Zaire ebolavirus* to *Sudan ebolaviruses* and *Bundibugyo ebolavirus*, synchronistically with the change of the serologically dominant virus species in these bats. Although none of the samples collected in 2011 and 2012 showed specificity for *Zaire ebolavirus*, antibodies to this filovirus species were detected again in those collected in 2013, which corresponded to the most recent West Africa outbreak caused by *Zaire ebolavirus* [24]. It is interesting to hypothesize that the seroprevalence in this bat species might be influenced by the overall activity and prevalence of filovirus species circulating in the natural reservoir(s) in the central African area and that this might also be stochastically linked to the probability of virus transmission into humans and nonhuman primates. If these bats act as the reservoir of filoviruses, the seroprevalence of each filovirus species might simply be a reflection of the shift of the proportion of multiple filoviruses maintained in the

290 reservoir bat population. It is also conceivable that these bats do not act as filovirus
291 reservoirs but are frequently exposed to spillover of the viruses from other animals (i.e.,
292 authentic reservoirs) that continually produce infectious filoviruses in central Africa. In
293 the latter case, these migratory bats may be infected only transiently with filoviruses in
294 the endemic area and do not carry the virus to Zambia in October-December.

295 However, filovirus activities in nature are largely unknown and remain speculative.
296 Continuous surveillance of filovirus infection not only in this single species of fruit bats
297 but also in many other wild and domestic animals will be needed to fully understand
298 how filoviruses are perpetuated and circulating in nature. Our serological data raised the
299 possibilities that antibodies could be detected due to the potential infections by
300 unknown filoviruses that have similar antigenicities to either of known species, and/or
301 some antibodies are undetected since the GP antigenicity of such viruses is likely to be
302 distinct from those of known species. Therefore, further studies for virus isolation
303 and/or viral RNA detection from bats or other wild animals are needed.

304 It is possible that filoviruses consist of diverse members with different
305 pathogenicities and different perpetuation mechanisms. Indeed, a new filovirus, named
306 Lloviu virus, was detected in long-fingered bats (*Miniopterus schreibersii*) in Spain [25].
307 The role of domestic animals, especially pigs, in the ecology of filoviruses has also been
308 suggested [2, 3]. Although filovirus infection has been reported neither in humans nor
309 animals in Zambia, our findings point to the need to enhance the diagnostic capacity and
310 to continue the surveillance of filovirus infection of humans and nonhuman primates, as
311 well as wild and domestic animals, in nonendemic areas in Africa.

**Acknowledgments**

We thank Ms. Madoka Kurata, Mr. Bwalya Chisha, Dr. Hideaki Higashi, Zambian Wildlife Authority, and Dr. Frank Willems, Kasanka Trust, and PhD students and postdoctoral fellows in Research Center for Zoonosis Control for technical assistance in capturing the animals and Kim Barrymore for editing the manuscript.

**Financial support**

This work was supported by the Japan Initiative for Global Research Network on Infectious Diseases (J-GRID) and by the Japan Science and Technology Agency (JST) and Japan International Cooperation Agency (JICA) within the framework of the Science and Technology Research Partnership for Sustainable Development (SATREPS). Funding was also provided by Global COE Program "Establishment of International Collaboration Centers for Zoonosis Control" (grant F-001) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan.

**Potential conflict of interest**

All authors: No reported conflicts.

**Corresponding author contact information:** Ayato Takada

Division of Global Epidemiology, Research Center for Zoonosis Control, Hokkaido University, Kita-20, Nishi-10, Kita-ku, Sapporo 001-0020, Japan.

Tel.: +81-11-706-9502

Fax: +81-11-706-7310

E-mail: atakada@czc.hokudai.ac.jp

## References


1. Kuhn JH, Bào Y, Bavari S, et al. Virus nomenclature below the species level: a standardized nomenclature for filovirus strains and variants rescued from cDNA. Arch Virol **2014**; 159:1229-37.

2. Allela L, Boury O, Pouillot R, et al. Ebola virus antibody prevalence in dogs and human risk. Emerg Infect Dis **2005**; 11:385-90.

3. Barrette RW, Metwally SA, Rowland JM, et al. Discovery of swine as a host for the *Reston ebolavirus*. Science **2009**; 325:204-6.

4. Leroy EM, Rouquet P, Formenty P, et al. Multiple Ebola virus transmission events and rapid decline of central African wildlife. Science **2004**; 303:387-90.

5. Swanepoel R, Leman PA, Burt FJ, et al. Experimental inoculation of plants and animals with Ebola virus. Emerg Infect Dis **1996**; 2:321-5.

6. Amman BR, Carroll SA, Reed ZD, et al. Seasonal pulses of Marburg virus circulation in juvenile *Rousettus aegyptiacus* bats coincide with periods of increased risk of human infection. PLOS Pathog **2012**; 8:e1002877.

7. Leroy EM, Kumulungui B, Pourrut X, et al. Fruit bats as reservoirs of Ebola virus. Nature **2005**; 438:575-6.

8. Towner JS, Amman BR, Sealy TK, et al. Isolation of genetically diverse Marburg viruses from Egyptian fruit bats. PLOS Pathog **2009**; 5:e1000536.

9. Manicassamy B, Wang J, Rumschlag E, et al. Characterization of Marburg virus glycoprotein in viral entry. Virology **2007**; 358:79-88.

10. Takada A, Robison C, Goto H, et al. A system for functional analysis of Ebola virus

359 glycoprotein. Proc Natl Acad Sci U S A **1997**; 94:14764-9.
360 11. Nakayama E, Yokoyama A, Miyamoto H, et al. Enzyme-linked immunosorbent
361 assay for detection of filovirus species-specific antibodies. Clin Vaccine Immunol
362 **2010**; 17:1723-8.
363 12. DeFrees SL, Wilson DE. *Eidolon helvum*. Mamm Species **1988**; 312:1-5.
364 13. Hayman DT, Emmerich P, Yu M, et al. Long-term survival of an urban fruit bat
365 seropositive for Ebola and Lagos bat viruses. PLOS ONE **2010**; 5:e11978.
366 14. Nidom CA, Nakayama E, Nidom RV, et al. Serological evidence of Ebola virus
367 infection in Indonesian orangutans. PLOS ONE **2012**; 7:e40740.
368 15. Ogawa H, Miyamoto H, Ebihara H, et al. Detection of all known filovirus species
369 by reverse transcription-polymerase chain reaction using a primer set specific for
370 the viral nucleoprotein gene. J Virol Methods **2011**; 171:310-3.
371 16. Pan Y, Zhang W, Cui L, Hua X, Wang M, Zeng Q. Reston virus in domestic pigs in
372 China. Arch Virol **2014**; 159:1129-32.
373 17. Centers for Disease Control and Prevention. Outbreaks. Available at:
374 http://www.cdc.gov/ncezid/dhcpp/vspb/outbreaks.html. Accessed 26 January 2015.
375 18. World Health Organization. Global Alert and Response. Available at:
376 http://www.who.int/csr/disease/en/. Accessed 26 January 2015.
377 19. de Jong C, Field H, Newman SH, Epstein JH. Emerging infectious diseases. In:
378 Newman SH, Field HE, de Jong CE, Epstein JH, eds. Investigating the role of bats
379 in emerging zoonoses: Balancing ecology, conservation and public health interests.
380 Rome: Food and Agriculture Organisation of the United Nations, **2011**:1-13.

20. Pourrut X, Souris M, Towner JS, et al. Large serological survey showing cocirculation of Ebola and Marburg viruses in Gabonese bat populations, and a high seroprevalence of both viruses in *Rousettus aegyptiacus*. BMC Infect Dis **2009**; 9:159.

21. Olival KJ, Islam A, Yu M, et al. Ebola virus antibodies in fruit bats, Bangladesh. Emerg Infect Dis **2013**; 19:270-3.

22. Pourrut X, Délicat A, Rollin PE, Ksiazek TG, Gonzalez JP, Leroy EM. Spatial and temporal patterns of *Zaire ebolavirus* antibody prevalence in the possible reservoir bat species. J Infect Dis **2007**; 196 Suppl 2:S176-83.

23. Richter HV, Cumming GS. First application of satellite telemetry to track African straw-coloured fruit bat migration. J Zool **2008**; 275:172-6.

24. WHO Ebola Response Team. Ebola virus disease in West Africa--the first 9 months of the epidemic and forward projections. N Engl J Med **2014**; 371:1481-95.

25. Negredo A, Palacios G, Vázquez-Morón S, et al. Discovery of an ebolavirus-like filovirus in europe. PLOS Pathog **2011**; 7:e1002304.

**Table 1. Filovirus species-specificity of the serum immunoglobulin G antibodies detected in *Eidolon helvum* in Zambia**

| | Positive rates (no. positive/no. total)[a] to the respective antigens | | | | | |
|---|---|---|---|---|---|---|
| Year | Zaire | Sudan | Tai Forest | Bundibugyo | Reston | Angola |
| 2006 | 4.7 (5/107) | 1.9 (2/107) | 1.9 (2/107) | 0 (0/107) | 2.8 (3/107) | 0.9 (1/107) |
| 2007 | 5.1 (5/99) | 0 (0/99) | 1.0 (1/99) | 0 (0/99) | 3.0 (3/99) | 1.0 (1/99) |
| 2008 | 1.9 (2/103) | 1.0 (1/103) | 1.0 (1/103) | 2.9 (3/103) | 0 (0/103) | 1.0 (1/103) |
| 2009 | 4.2 (3/72) | 5.6 (4/72) | 0 (0/72) | 2.8 (2/72) | 0 (0/72) | 4.2 (3/72) |
| 2010 | 3.9 (2/51) | 3.9 (2/51) | 0 (0/51) | 0 (0/51) | 0 (0/51) | 0 (0/51) |
| 2011 | 0 (0/95) | 2.1 (2/95) | 1.1 (1/95) | 2.1 (2/95) | 1.1 (1/95) | 0 (0/95) |
| 2012 | 0 (0/111) | 1.8 (2/111) | 2.7 (3/111) | 0 (0/111) | 0.9 (1/111) | 0 (0/111) |
| 2013 | 1.8 (2/110) | 5.5 (6/110) | 0.9 (1/110) | 0.9 (1/110) | 0.9 (1/110) | 0.9 (1/110) |
| Total | 2.5 (19/748) | 2.5 (19/748) | 1.2 (9/748) | 1.1 (8/748) | 1.2 (9/748) | 0.9 (7/748) |

[a] The filovirus species for which each positive sample had the highest optical density value in the glycoprotein (GP)-based enzyme-linked immunosorbent assay was selected when a sample showed cross-reactivity to GPs of multiple species.

**Table 2. Comparison of immunoglobulin G positive rates to filovirus antigens**

| | Positive rates (no. positive/no. total) to the respective antigens | | |
|---|---|---|---|
| Year | Ebola | Marburg | Total |
| 2006 | 11.2 (12/107) | 0.9 (1/107) | 12.1 (13/107) |
| 2007 | 9.1 (9/99) | 1.0 (1/99) | 10.1 (10/99) |
| 2008 | 6.8 (7/103) | 1.0 (1/103) | 7.8 (8/103) |
| 2009 | 12.5 (9/72) | 4.2 (3/72) | 16.7 (12/72) |
| 2010 | 7.8 (4/51) | 0 (0/51) | 7.8 (4/51) |
| 2011 | 6.3 (6/95) | 0 (0/95) | 6.3 (6/95) |
| 2012 | 5.4 (6/111) | 0 (0/111) | 5.4 (6/111) |
| 2013 | 10.0 (11/110) | 0.9 (1/110) | 10.9 (12/110) |
| Total | 8.6 (64/748) | 0.9 (7/748) | 9.5 (71/748) |

## Figure legends

**Figure 1. Immunoglobulin G (IgG) antibodies detected in the sera collected from *Eidolon helvum* in Zambia.**

Serum samples were tested (1:100 dilution) for IgG antibodies specific to Zaire, Sudan, Tai 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 (i.e. significantly higher OD values) from the negative population (Supplementary Figure 1).

**Figure 2. Filovirus species-specificity of immunoglobulin G (IgG) 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 for each filovirus antigen are shown. Sample IDs are shown on the horizontal axis.

**Figure 3. Reactivity of filovirus GP antibody-positive samples in western blotting.**

Representative positive sera diluted at 1:100 were tested for the reactivity to Zaire (ZFB06-21), Sudan (ZFB11-63), Tai forest (ZFB11-14), Bundibugyo (ZFB11-16), Reston (ZFB06-41) and Angola (ZFB13-56) GPs in western blotting. Mouse

monoclonal antibodies ZGP42/3.7 and AGP127-8 were used as positive controls for Ebola and Marburg viruses, respectively. Z, Zaire; S, Sudan; T, Tai Forest; B, Bundibugyo; R, Reston; A, Angola; N, negative control.

**Figure 4. Seroprevalence of each filovirus species and reported outbreaks in Central and West Africa since 2005.**

Relative percentages of the immunoglobulin G positive samples for each filovirus species are shown in the stacked bar chart (left). The reported filovirus outbreaks in humans in the Central and West African countries since 2005 are summarized (right). DRC, Democratic Republic of the Congo.

Figure clean

Click here to download Figure: Ogawa_Anti-filovirus_IgG_in_Zambian_fruit_bats_Figures_revised_clean.pptx

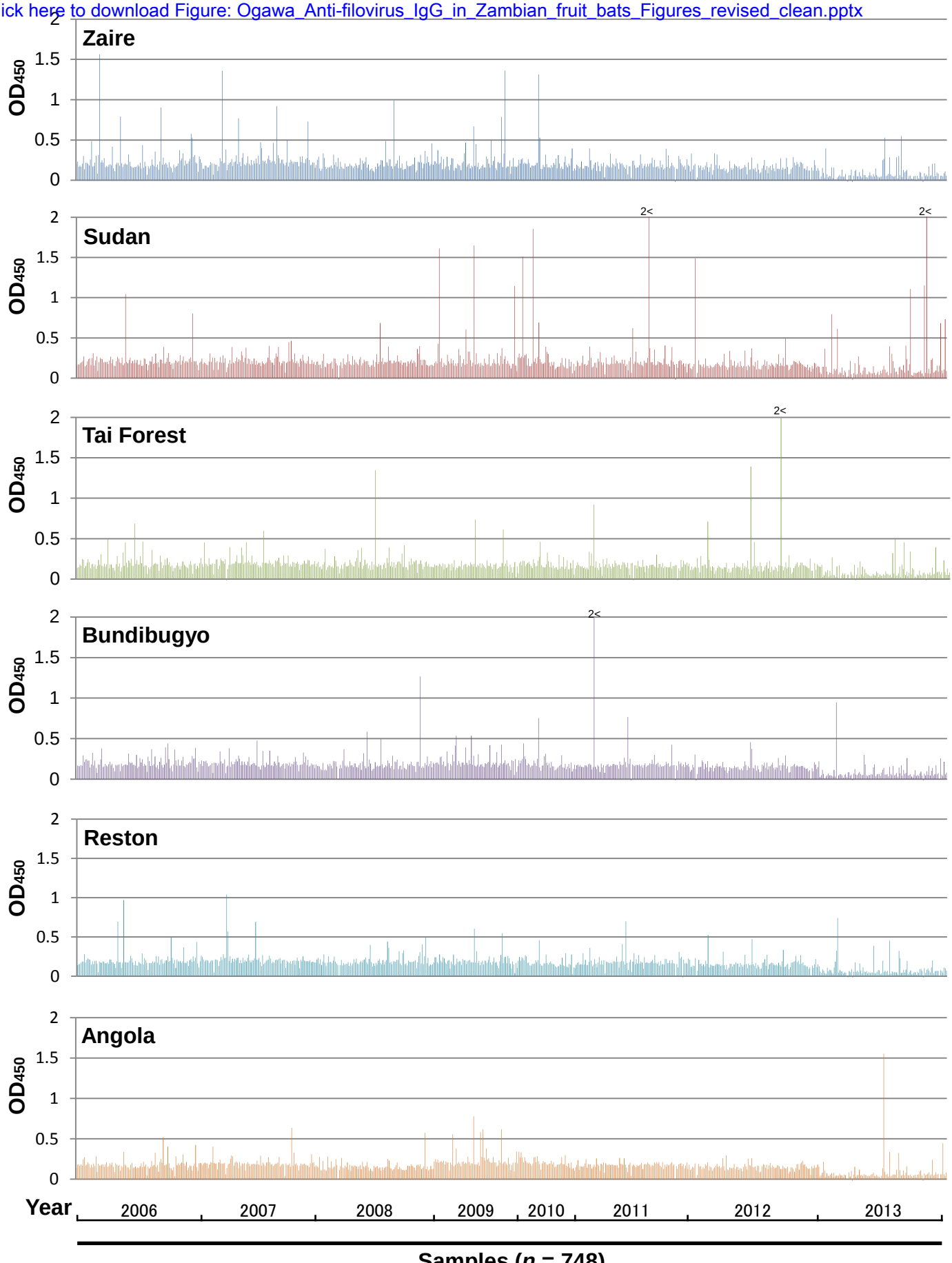



Figure 1

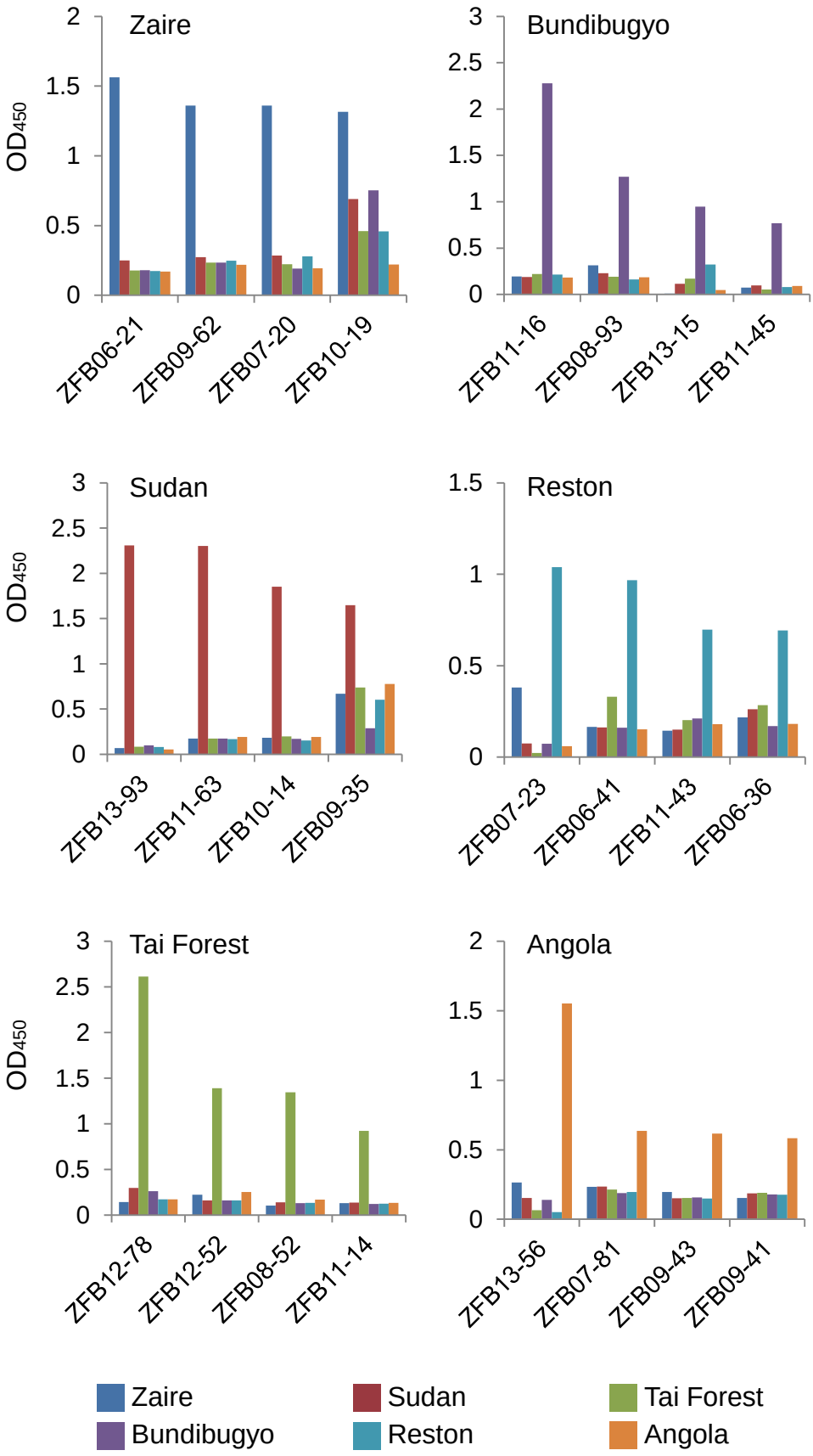

Zaire
Bundibugyo
Sudan
Reston
Tai Forest
Angola
OD450
ZFB06-21
ZFB09-62
ZFB07-20
ZFB10-19
ZFB11-16
ZFB08-93
ZFB13-15
ZFB11-45
ZFB13-93
ZFB11-63
ZFB10-14
ZFB09-35
ZFB07-23
ZFB06-41
ZFB11-43
ZFB06-36
ZFB12-78
ZFB12-52
ZFB08-52
ZFB11-14
ZFB13-56
ZFB07-81
ZFB09-43
ZFB09-41
Zaire
Bundibugyo
Sudan
Reston
Tai Forest
Angola

**Figure 2**

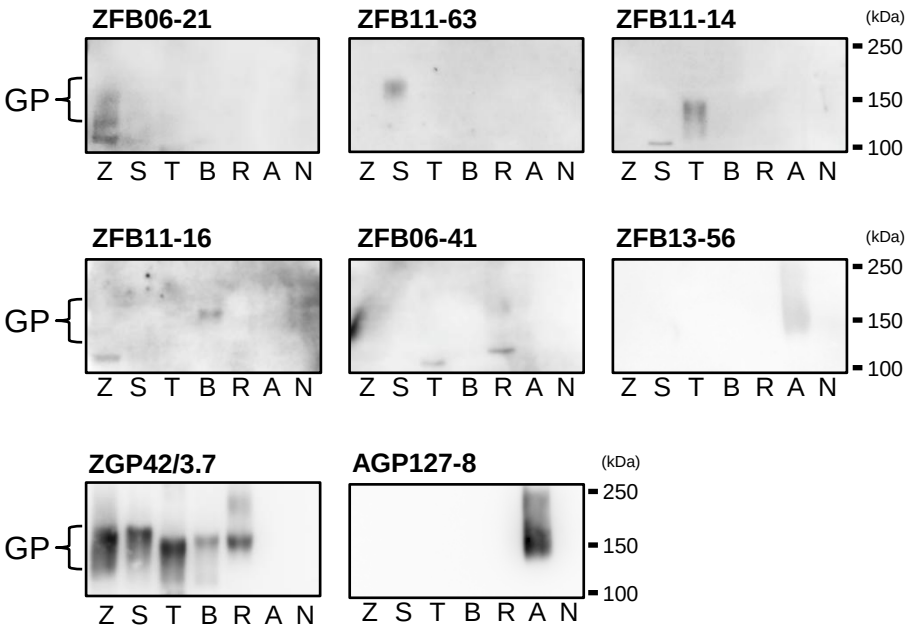

ZFB06-21
ZFB11-63
ZFB11-14
(kDa)
250
150
100
GP
Z S T B R A N
ZFB11-16
ZFB06-41
ZFB13-56
ZGP42/3.7
AGP127-8

**Figure 3**

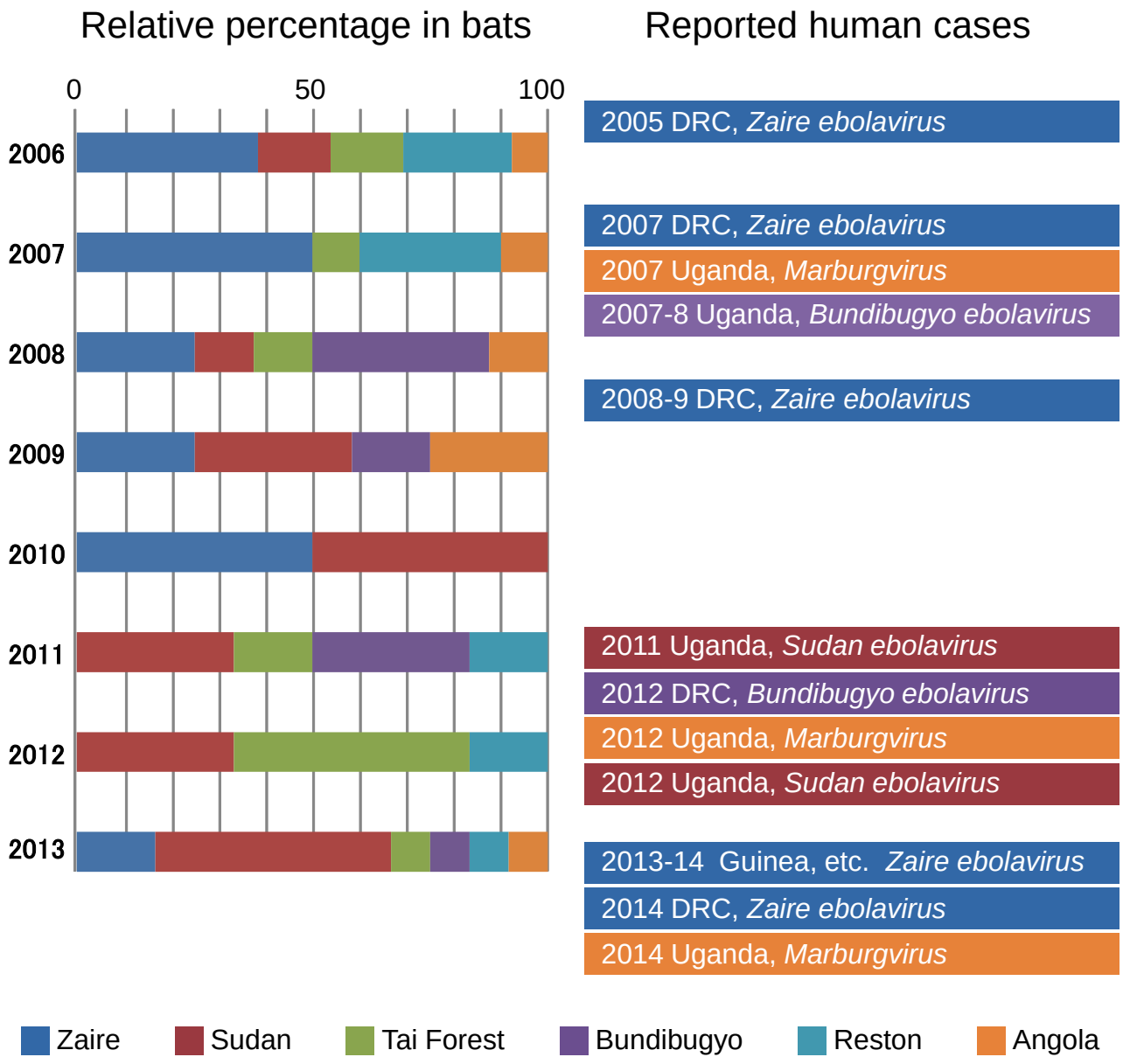

Relative percentage in bats
Reported human cases
0
50
100
2006
2007
2008
2009
2010
2011
2012
2013
2005 DRC, Zaire ebolavirus
2007 DRC, Zaire ebolavirus
2007 Uganda, Marburgvirus
2007-8 Uganda, Bundibugyo ebolavirus
2008-9 DRC, Zaire ebolavirus
2011 Uganda, Sudan ebolavirus
2012 DRC, Bundibugyo ebolavirus
2012 Uganda, Marburgvirus
2012 Uganda, Sudan ebolavirus
2013-14 Guinea, etc. Zaire ebolavirus
2014 DRC, Zaire ebolavirus
2014 Uganda, Marburgvirus
Zaire
Sudan
Tai Forest
Bundibugyo
Reston
Angola

**Figure 4**

**Supplementary Table 1.** Summary of the fruit bat serum samples analyzed

| | | | No. of fruit bats | | |
|---|---|---|---|---|---|
| Date | Province | District | M | F | Total |
| December 2, 2006 | Central Province | Serenje District | 31 | 16 | 47 |
| December 9, 2006 | Central Province | Serenje District | 20 | 40 | 60 |
| November 30, 2007 | Central Province | Serenje District | 7 | 58 | 65 |
| December 7, 2007 | Central Province | Serenje District | 19 | 15 | 34 |
| November 28, 2008 | Central Province | Serenje District | 28 | 52 | 80 |
| December 13, 2008 | Central Province | Serenje District | 10 | 13 | 23 |
| December 1, 2009 | Central Province | Serenje District | 19 | 53 | 72 |
| December 6, 2010 | Central Province | Serenje District | 13 | 34 | 47 |
| December 10, 2010 | Copperbelt Province | Ndola District | 3 | 1 | 4 |
| December 2, 2011 | Copperbelt Province | Ndola District | 18 | 20 | 38 |
| December 5, 2011 | Central Province | Serenje District | 24 | 33 | 57 |
| November 30, 2012 | Copperbelt Province | Ndola District | 22 | 38 | 60 |
| December 7, 2012 | Central Province | Serenje District | 16 | 35 | 51 |
| December 5, 2013 | Copperbelt Province | Ndola District | 23 | 53 | 76 |
| December 10, 2013 | Central Province | Serenje District | 10 | 24 | 34 |
| Total | | | 263 | 485 | 748 |

**Supplementary Table 2. Serum immunoglobulin G antibody titers of the positive sera**

| | ELISA endpoint titers[a] | | | |
|---|---|---|---|---|
| Antigen | 100 | 400 | 1600 | 6400 |
| Zaire | 0[b] | 16 | 2 | 1 |
| Sudan | 0 | 16 | 3 | 0 |
| Tai Forest | 0 | 6 | 3 | 0 |
| Bundibugyo | 0 | 5 | 2 | 1 |
| Reston | 1 | 7 | 1 | 0 |
| Angola | 1 | 4 | 1 | 1 |

[a] Titers were expressed as the reciprocal of the highest dilution which gave an optical density value above background. ELISA, enzyme-linked immunosorbent assay.

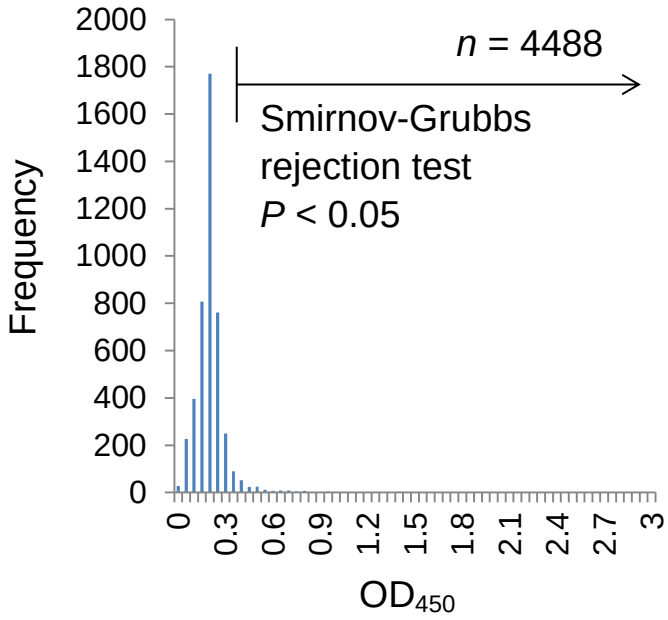
[b] Number of the samples with indicated titers are shown.

**Supplementary Table 3. The epidemics of filovirus diseases in humans in Central and West Africa since 2000**

| Filovirus disease | Species | Year | Country[a] | No. of cases (No. of deaths) | Fatality (%) |
|---|---|---|---|---|---|
| Marburg hemorrhagic fever | *Marburg marburgvirus* | 2004-2005 | Angola | 252 (227) | 90.1 |
| | | 2007 | Uganda | 2 (2) | 100 |
| | | 2012 | Uganda | 23 (15) | 65.2 |
| | | 2014 | Uganda | 1 (1) | 100 |
| Ebola hemorrhagic fever | *Zaire ebolavirus* | 2001-2002 | Gabon, RC | 122 (96) | 78.7 |
| | | 2002-2003 | RC | 178 (157) | 88.2 |
| | | 2005 | RC | 12 (9) | 75.0 |
| | | 2007 | DRC | 264 (187) | 70.8 |
| | | 2008-2009 | DRC | 32 (15) | 46.9 |
| | | 2013-2014 | Guinea, Liberia, Sierra Leone, Nigeria, Senegal, Mali | 21826 (8689)[b] | 39.8 |
| | | 2014 | DRC | 66 (49) | 74.2 |
| | *Sudan ebolavirus* | 2000-2001 | Uganda | 425 (224) | 52.7 |
| | | 2004 | South Sudan | 17 (7) | 41.2 |
| | | 2011 | Uganda | 1 (1) | 100 |
| | | 2012a | Uganda | 24 (17) | 70.8 |
| | | 2012b | Uganda | 7 (4) | 57.1 |
| | *Bundibugyo ebolavirus* | 2007-2008 | Uganda | 149 (37) | 24.8 |
| | | 2012 | DRC | 77 (36) | 46.8 |

[a] RC and DRC indicate Republic of the Congo and Democratic Republic of the Congo, respectively.

[b] As of January 20, 2015.




**Supplementary Figure 1. The frequency distribution of the fruit bat sera according to optical density (OD) values obtained by glycoprotein (GP)-based enzyme-linked immunosorbent assay (ELISA).**

All OD values obtained by ELISA with GP antigens from filovirus strains (Zaire, Sudan, Tai Forest, Bundibugyo, Reston, and Angola) representing the respective filovirus species were analyzed concurrently ($n$ = 4488). The frequency distribution chart reveals that the sample population consists of a single major peak with low OD values and outliers with high OD values. Smirnov-Grubbs rejection tests were employed to evaluate the statistical significance of each OD value ($P < 0.05$), and statistical outlier samples (more than 0.485) were considered positive.