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Enzyme-linked immunosorbent assay for the detection of filovirus species-specific antibodies

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Running title: **Filovirus species-specific ELISA**

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Abstract

Several enzyme-linked immunosorbent assays (ELISA) for the detection of filovirus-specific antibodies have been developed. However, diagnostic methods to distinguish antibodies specific to the respective species of filoviruses, which provide the basis for serological classification, are not readily available. We established an ELISA using His-tagged secreted forms of the transmembrane glycoproteins (GPs) of five different Ebola viruses (EBOV) species and one Marburg virus (MARV) strain as antigens for the detection of filovirus species-specific antibodies. The GP-based ELISA was evaluated by testing antisera collected from mice immunized with virus-like particles, as well as humans and nonhuman primates infected with EBOV or MARV. In our ELISA, little cross-reactivity of IgG antibodies was observed in most of the mouse antisera. Although sera and plasma from some patients and monkeys showed notable cross-reactivity with the GPs from multiple filovirus species, the highest reactions of IgG were uniformly detected against the GP antigen homologous to the virus species that infected individuals. We further confirmed that MARV-specific IgM antibodies were specifically detected in specimens collected from patients during the acute phase of infection. These results demonstrate the usefulness of our ELISA for diagnostics as well as ecological and serosurvey studies.

Introduction

Ebola virus (EBOV) and Marburg virus (MARV) belong to the family *Filoviridae,* and cause severe hemorrhagic fever in primates (18). While MARV consists of a single species, *Lake Victoria marburgvirus*, four distinct EBOV species are known: *Zaire ebolavirus* (ZEBOV), *Sudan ebolavirus* (SEBOV), *Côte d'Ivoire ebolavirus* (CIEBOV), and *Reston ebolavirus* (REBOV). The phylogenetically distinct *Bundibugyo ebolavirus* (BEBOV) was recently identified in Uganda and was proposed as a new species of EBOV (Fig. 1) (29).

EBOV and MARV are filamentous, enveloped, single-stranded, negative-sense RNA viruses. The virus genome encodes seven structural proteins, nucleoprotein (NP), polymerase cofactor (VP35), matrix protein (VP40), glycoprotein (GP), replication-transcription protein (VP30), minor matrix protein (VP24), and RNA-dependent RNA polymerase (L). EBOV also express at least one secreted non-structural glycoprotein (sGP) (18). GP is responsible for receptor binding and fusion of the viral envelope with host cell membranes (9, 20, 32) and has an important role in the pathogenesis of filovirus infection (3, 21, 33). GP is the main target of neutralizing antibodies, and most of the known ZEBOV-specific monoclonal antibodies (MAbs) show little cross-reactivity to other filovirus species (22, 25, 31).

Serological diagnostic methods based on enzyme-linked immunosorbent assays (ELISA) using the recombinant EBOV and MARV NP antigens have been developed to detect filovirus-specific antibodies (4, 15). Using ZEBOV NP antigen, NP-specific antibodies were broadly detected in animals infected with ZEBOV, SEBOV, CIEBOV, or REBOV (15) indicating strong cross-reactivity among EBOV species. It is predicted, however, that the antibody response to GP is more species-specific due to the

larger genetic variability with this protein which is supposed to be the main target of the host humoral immune response. Therefore, in this study we developed a filovirus species-specific ELISA using recombinant GP antigens to serologically distinguish filovirus species.

Materials and Methods

Plasmids. Viral RNA extracted from the supernatant of Vero E6 cells infected with ZEBOV, SEBOV, CIEBOV, BEBOV, REBOV, or MARV strain Angola, was used for cloning of the respective GP cDNAs lacking the transmembrane domain and cytoplasmic tail. The cDNAs of truncated EBOV and MARV GPs with a C-terminal histidine (His) tag (His-EBOV-GP, His-MARV-GP) were cloned into a pATX vector. Finally, the cDNA fragments of His-EBOV-GP and His-MARV-GP were inserted into the mammalian expression vector pCAGGS/MCS, which contains the chicken β-actin promoter (11). All clones were sequence confirmed prior to expression.

Monoclonal antibodies (MAb). The hybridoma cells producing EBOV GP-specific MAb ZGP42/3.7 (IgG1) (22, 24), which recognizes a linear epitope on GP comprising the sequence GEWAFWENKKN, and MARV GP-specific MAb AGP127-8 (IgG1) were grown in DMEM (Sigma) and RPMI (Sigma), respectively, supplemented with fetal calf serum (FCS) and antibiotics. Mouse ascites were obtained by a standard procedure and MAbs were purified from ascites fluid using protein A agarose columns (Bio-Rad). The S139/1 monoclonal antibody (IgG2a), which binds to the hemagglutinin of influenza A viruses (34), was used as a negative control.

Sera and plasma. Five-week-old female BALB/c mice were twice immunized intraperitoneally with 100 μg virus-like particles (VLPs) (12, 19) in a 3-week interval, and the serum samples were collected 7-10 days after the second immunization. Convalescent phase plasma samples were collected from cynomolgus macaques vaccinated and/or infected with EBOV as described previously (25). ZEBOV convalescent human plasma (patients 2 to 7) and serum (patients 1 and 8) samples were

obtained 51 to 135 days after the onset of ZEBOV infection during the 1995 outbreak in Kikwit, Democratic Republic of the Congo (23). SEBOV convalescent patient serum samples (patients 9 and 10) were collected about two months after onset during the Ebola hemorrhagic fever 2000 outbreaks in Uganda associated with SEBOV in 2000 (2). These EBOV-infected human samples were kindly provided by Dr. T.G. Ksiazek (Centers for Disease Control and Prevention). MARV-infected human blood samples (patients 11 to 21) were collected within a few days after the onset of symptoms from patients admitted of the 2004/05 outbreak in Angola (27). Blood collection during outbreak investigations were approved under special response protocol established between the World Health Organization and national authorities.

Expression and purification of His-EBOV-GP and His-MARV-GP. Human epithelial kidney 293T cells cultured in high glucose DMEM containing 10% FCS and antibiotics were transfected with pCAGGS expressing His-EBOV-GP (pCHis-ZEBOV-GP, pCHis-SEBOV-GP, pCHis-CIEBOV-GP, pCHis-BEBOV-GP, or pCHis-REBOV-GP) or His-MARV-GP (pCHis-MARV-GP) using TransIT LT1 (Mirus). Forty-eight hours after transfection, the supernatants were collected and the recombinant GPs were purified using the Ni-NTA Purification System (Invitrogen) according to the manufacturer's instructions. The majority of contaminant protein was removed with wash buffer containing 15mM imidazole. Finally, bound proteins were collected with elution buffer containing 250mM imidazole. To monitor inevitable nonspecific reactions (i.e., nonspecific antibodies) to FCS-derived impurities in each GP preparation, control antigens (FCS-derived proteins non-specifically bound to the Ni-beads) were prepared using the Ni-NTA column under the same conditions. The eluted protein was concentrated using Amicon Ultra 4 spin colums (Millipore) and

dialyzed against PBS at 4°C overnight. Purified His-EBOV-GP and His-MARV-GP were analyzed by sodium dodecyl sulfate-polyacylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie brilliant blue. Western blotting was performed using ZGP42/3.7, AGP127-8, and anti-His MAbs (COVANCE).

ELISA. ELISA plates (Nunc Maxisorp) were coated with the purified GPs or control antigens (100ng/50μl/well) in PBS at 4°C overnight, and then washed with PBS containing 0.05% Tween 20 (PBST). Unspecific binding of the antibodies was avoided by blocking with 3% skim milk (150μl/well) for 2 hours at room temperature. Monkey plasma samples were pre-incubated with 2% FCS to absorb antibodies to FCS components, since they were exposed to FCS by injection of the vaccines or viruses diluted in DMEM containing FCS. After washing three times with PBST, 50μl of appropriately diluted serum/plasma samples or the GP-specific MAb in PBST containing 1% skim milk was added and incubated for 1 hour at room temperature. After washing three times with PBST, the bound antibodies were detected using the following secondary antibodies conjugated with horseradish peroxidase diluted in 1% skim milk in PBST: goat anti-mouse IgG (Jackson ImmunoResearch), goat anti-monkey IgG (ROCKLAND), goat anti-human IgG (Jackson ImmunoResearch), or donkey anti-human IgM (Jackson Immuno Research). After incubation for 1 hour at room temperature and three PBST washes, 50 μl of 3,3,5,5- tetramethylbenzidine were added to each well and incubated for 15 minutes at room temperature. Reaction was stopped by adding 1N sulfuric acid and the optical density (OD) at 450 nm was measured.

Phylogenetic analysis. Phylogenetic analysis was based on whole amino acid sequences of filovirus GPs. The sequences were analyzed using GENETYX (Genetyx Corp., Japan) for Windows Version 7 software. A phylogenetic tree was constructed

using the neighbor-joining bootstrap method (1,000 replicates) in MEGA 4.0 software (26). Amino acid sequences of ZEBOV (strain Mayinga-76), ZEBOV (strain Kikwit-95), SEBOV (strain Boniface-76), SEBOV (strain Maleo-79), CIEBOV (strain Côte d'Ivoire-94), BEBOV, REBOV (strain Reston-89), REBOV (strain Siena Philippine-92), MARV (strain Musoke-80), and MARV (strain Angola/2005) used in phylogenetic analyses were obtained from GenBank under accession numbers Q05320, P87666, Q66814, Q66798, Q66810, ACI28624, Q66799, Q89853, P35253, and Q1PD50, respectively.

151 **Results**

152 **Expression and purification of recombinant EBOV and MARV GPs.**
153 Expression and secretion of His-EBOV-GP and His-MARV-GP in the supernatants of
154 293T cells transfected with a plasmid encoding His-GP was confirmed by
155 immunoblotting using anti-GP and anti-His MAbs (data not shown). These
156 recombinant GPs were purified as described in Materials and Methods. All purified
157 His-GPs were detected by SDS-PAGE and immunoblotting, using anti-GP and anti-His
158 MAbs as prominent protein bands of the predicted size of the transmembrane
159 anchor-minus EBOV and MARV GPs (Fig. 2). These purified GPs were used as
160 antigens for the ELISA described in the following experiments.

161 **Sensitivity of the GP-based ELISA.** The sensitivity of the purified GP-based
162 ELISA was tested using anti-EBOV-GP MAb ZGP42/3.7 and anti-MARV-GP MAb
163 AGP127-8. Serial 10-fold dilutions of the antibodies (10^{-5} to 10^{2} μg/ml) were
164 prepared and the reactivity to each GP antigen was examined (Fig. 3). The negative
165 control MAb, S139/1, did not bind to any His-GPs in the ELISA. At concentrations
166 ranging from 0.1μg/ml to 100μg/ml, ZGP42/3.7 reacted with all His-EBOV-GPs but not
167 His-MARV-GP, whereas AGP127-8 reacted specifically with His-MARV-GP but not
168 any of the His-EBOV-GP. The detection limit for specific antibodies using this assay
169 was approximately 0.01-0.1μg/ml.

170 **Specificity of the GP-based ELISA.** Next, the species-specificity of the ELISA
171 was assessed by testing the antisera of mice immunized with VLP containing the
172 respective EBOV and MARV GPs. We found that species-specific IgG antibodies
173 were clearly detected in these mouse antisera (Fig. 4). All the anti-EBOV IgG
174 antibodies in the sera showed low reactivity to heterologous EBOV GPs, and no

cross-reactivity to MARV GP was found (Figs. 4a-e). Similarly, anti-MARV VLP serum antibodies reacted to MARV GP but not to EBOV GPs (Fig. 4f). These results indicated that this GP-based ELISA sufficiently detected filovirus species-specific antibodies.

Analysis of clinical samples in GP-based ELISA. To further confirm the specificity of our ELISA, we used convalescent-phase plasma samples obtained from monkeys experimentally infected with ZEBOV or SEBOV (Fig. 5). We detected IgG antibodies in the ZEBOV-infected monkey plasma with higher reactivity against His-ZEBOV-GP than against any heterologous GP antigens. Although IgG antibodies in the SEBOV-infected monkey plasma showed binding to all His-EBOV-GPs, the highest reactivity was observed with the homologous antigen, His-SEBOV-GP. Neither of these plasma antibodies reacted with MARV GP.

We then examined IgG antibody levels in serum/plasma derived from ZEBOV-, SEBOV- and MARV-infected patients (Fig. 6a). In most of the samples tested, IgG antibodies to homologous GP antigens were detected with highest reactivity (Fig 5a). All of the samples derived from ZEBOV-infected patients cross-reacted with His-CIEBOV-GP and His-BEBOV-GP antigens, whereas only one of the SEBOV-infected human samples (no. 9) showed cross-reactivity with His-REBOV-GP. Overall, the level of cross-reactivity was consistent with the phylogenetic relationship among EBOV species (Fig. 1). On the other hand, in most of the samples from patients infected with Angola MARV, IgG antibodies to His-MARV-GP were specifically detected, except for specimens 17 and 21, which showed no IgG response to any GP. Interestingly, IgG antibodies detected in specimen 11 showed remarkable cross-reactivity with the heterologous antigens, His-CIEBOV-GP and His-BEBOV-GP.

We next evaluated whether GP-specific IgM antibodies could be detected in the patient serum/plasma samples using the GP-based ELISA (Fig. 6b). ZEBOV- or SEBOV-specific IgM antibodies were only detected in patients 2 and 10. In contrast, MARV-specific IgM antibodies were detected in 8 out of the 11 specimens derived from MARV Angola-infected patients. No obvious IgM cross-reactivity to heterologous GP antigens was found in these samples (data not shown).

205 **Discussion**

206 In this study, we established a GP-based ELISA to detect filovirus species-specific
207 antibodies. To date, lysates from Vero E6 cells infected with live EBOV and MARV
208 or recombinant EBOV and MARV NPs have been used as antigens in ELISA for
209 detection of filovirus-specific antibodies (4, 6, 15). Since the NPs of EBOV and
210 MARV contain similar amino acid sequences (16), common antibody epitopes seem to
211 be present (10). Indeed, cross-reactivity was to be expected among all EBOV species
212 (14, 15). Therefore, NP antigens may be useful for the detection of genus-specific
213 antibodies but not for the detection of species-specific humoral responses (6, 14, 15)

214 Heterogeneity of EBOV and MARV GPs has been demonstrated at the genetic
215 level through sequence analyses (15, 17). An ELISA using recombinant ZEBOV GP
216 expressed in a baculovirus-insect cell expression system was reported previously (14),
217 but it is known that the protein glycosylation pathways in insect cells differ from those
218 in mammalian cells (5). This may significantly affect the antigenic properties of
219 filovirus GPs, since large amounts of both N- and O-linked carbohydrate chains are
220 present in GP molecules. To overcome this difficulty, we used mammalian 293T cells
221 for the expression of GP antigens, and verified the sensitivity and specificity of
222 GP-based ELISAs. Our results were consistent with a previous study suggesting that
223 anti-EBOV GP antibodies were highly species-specific and showed little
224 cross-reactivity to GPs of other EBOV species (25). These findings indicated that
225 most antibodies induced against filovirus GPs recognized epitopes in the variable
226 regions of the protein. Expectedly, the serological classification mirrors the
227 phylogenetic relationship of the different GPs (Fig. 1). Interestingly, serological
228 characterization of anti-BEBOV antibodies clearly supports the molecular

investigations (29) suggesting that BEBOV represents a new species within the EBOV genus. (Fig. 7).

IgG antibodies in some of the serum and plasma samples collected from infected monkeys and humans showed appreciable cross-reactivity to heterologous antigens, whereas antibodies in the mouse sera produced by immunization with VLPs specifically reacted to the homologous antigens. This result led us to conjecture that VLP immunization and live virus infection induce a distinct antibody repertoire or that the antibody repertoire of mice differs from that of primates. Interestingly, the serum of patient 11 infected with Angola MARV contained IgG, but not IgM antibodies, cross-reactive to His-CIEBOV-GP and His-BEBOV-GP. It might be possible that prior to infection with Angola MARV, this patient was infected with CIEBOV, BEBOV, or another unknown filovirus whose GP has epitopes shared among CIEBOV and BEBOV. In the sera of patients 17 and 21, neither IgG nor IgM antibodies were readily detected. An explanation for this observation might be the difference of immunological conditions in individuals. Or, alternatively, the serum samples have been collected before a detectable antibody response was induced.

Notably, our GP-based ELISA detected Angola MARV-specific IgM antibodies in most of the sera collected during the acute or subacute phase of infection, although it has been reported that detection of antibodies is only of limited use to for acute case diagnosis due to a lack of detectable antibody response (7). The present study suggests that if proper antigen and sensitive assays is available IgM antibodies can be useful for the diagnosis of acute EBOV and MARV infections, and support the use of antigen capture ELISA and reverse transcription-PCR, the most commonly used technologies .

Despite the more recent discovery of REBOV in domestic pigs in the Philippines

(1) and the discovery of fruit bat species as potential reservoirs for EBOV and MARV (8, 13, 28, 30), the search for the reservoirs and potential amplifying hosts remains ongoing. Advanced diagnostic technologies are welcome here and our new GP-based species-specific antibody detection ELISA may be useful tools for future ecological and seroepidemiological studies in endemic areas of Central Africa and part of Asia.

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388

389 **Figure Legends**

390

391 **Fig. 1.** Phylogenetic analysis of filovirus GP amino acid sequences. The
392 phylogenetic tree was constructed using the neighbor-joining method. For
393 construction of this tree, we used 10 GP amino acid sequences, each comprising a
394 whole GP amino acid sequence. Numbers at branch points indicate bootstrap values
395 (1,000 replicates).

396

397 **Fig. 2.** Identification and characterization of purified His-GPs. His-EBOV-GP and
398 His-MARV-GP were analyzed on 8% SDS-PAGE and stained with Coomassie brilliant
399 blue (a). Immunoblotting of purified His-GPs were performed using MAbs to EBOV
400 (ZGP42/3.7), MARV GPs (AGP127-8) (b) and His-tag (c). Arrows indicate the
401 location of the His-GPs. The protein bands represent His-ZEBOV-GP (lane 1),
402 His-SEBOV-GP (lane 2), His-CIEBOV-GP (lane 3), His-BEBOV-GP (lane 4),
403 His-REBOV-GP (lane 5), and His-MARV-GP (lane 6). Lane 7 shows FCS-derived
404 proteins used as a control antigen (see Materials and Methods).

405

406 **Fig. 3.** Sensitivity of GP-based ELISA. Serial 10-fold dilutions of MAbs to EBOV
407 (a) and MARV (b) were prepared and tested. S139/1 (specific to influenza virus
408 hemagglutinin) was used as a negative control antibody (c).

409

410 **Fig. 4.** IgG antibodies detected in mouse antisera. Serial 10-fold dilutions of the
411 anti-ZEBOV (a), anti-SEBOV (b), anti-CIEBOV (c), anti-BEBOV (d), anti-REBOV (e),

412 and anti-MARV (f) sera obtained from mice immunized with EBOV and MARV VLPs
413 were tested for IgG antibodies reacting with His-GPs.
414
415 **Fig. 5.** IgG antibodies detected in experimentally infected monkey sera. C105, C332,
416 C508, and C725 were infected with ZEBOV, whereas C0287 and C0436 were infected
417 with SEBOV. All sera were diluted 1:1,000. Naïve monkey serum was used as a
418 negative control.
419
420 **Fig. 6.** IgG and IgM antibodies detected in human sera. OD values for specific IgG
421 (a) and IgM (b) antibodies in the patient sera are shown. Sera from 21 individuals
422 were analyzed in 1:1,000 dilutions. Naïve human serum (no. 22) was used as a
423 negative control.
424

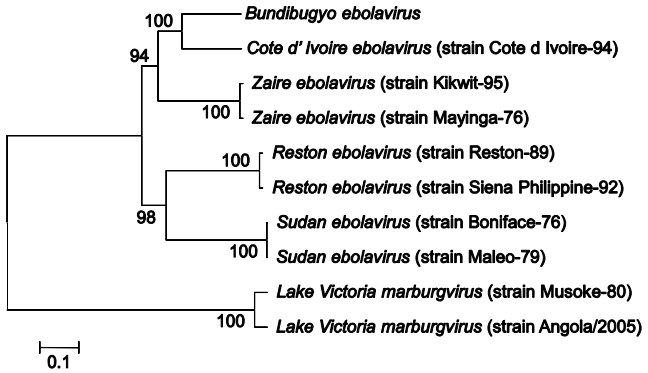


Fig. 1. Phylogenetic analysis of filovirus GP amino acid sequences. The phylogenetic tree was constructed using the neighbor-joining method. For construction of this tree, we used 10 GP amino acid sequences, each comprising a whole GP amino acid sequence. Numbers at branch points indicate bootstrap values (1,000 replicates).

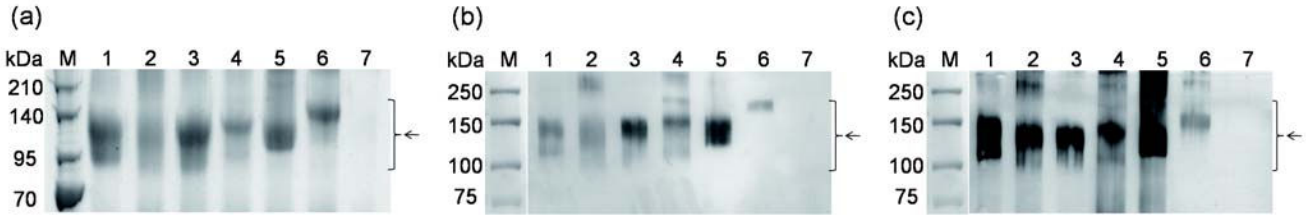


Fig. 2. Identification and characterization of purified His-GPs. His-EBOV-GP and His-MARV-GP were analyzed on 8% SDS-PAGE and stained with Coomassie brilliant blue (a). Immunoblotting of purified His-GPs were performed using MAbs to EBOV (ZGP42/3.7), MARV GPs (AGP127-8) (b) and His-tag (c). Arrows indicate the location of the His-GPs. The protein bands represent His-ZEBOV-GP (lane 1), His-SEBOV-GP (lane 2), His-CIEBOV-GP (lane 3), His-BEBOV-GP (lane 4), His-REBOV-GP (lane 5), and His-MARV-GP (lane 6). Lane 7 shows FCS-derived proteins used as a control antigen (see Materials and Methods).

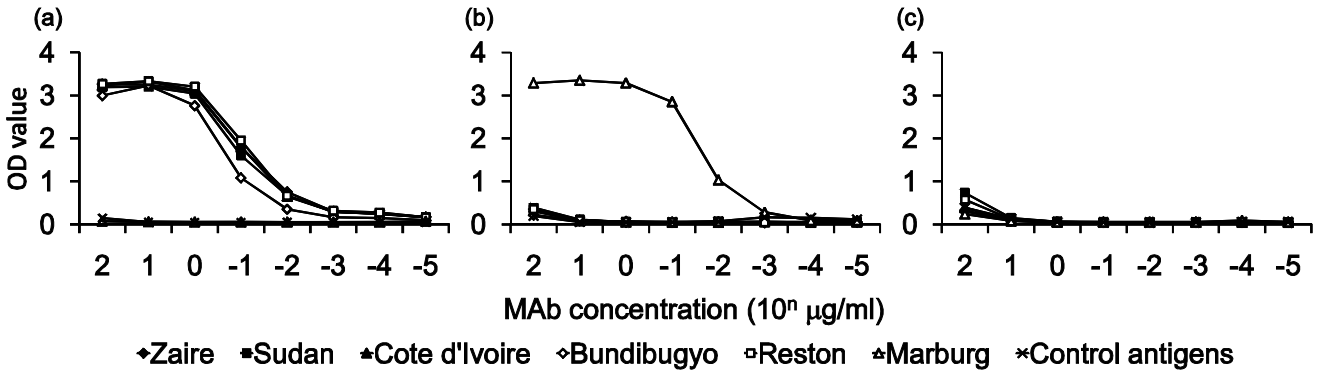


Fig. 3. Sensitivity of GP-based ELISA. Serial 10-fold dilutions of MAbs to EBOV (a) and MARV (b) were prepared and tested. S139/1 (specific to influenza virus hemagglutinin) was used as a negative control antibody (c).

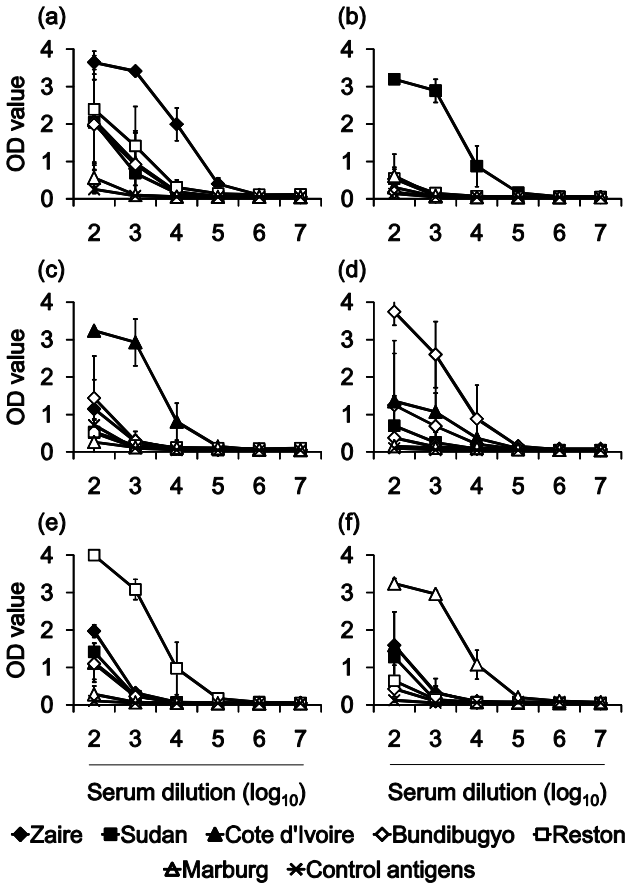


Fig. 4. IgG antibodies detected in mouse antisera. Serial 10-fold dilutions of the anti-ZEBOV (a), anti-SEBOV (b), anti-CIEBOV (c), anti-BEBOV (d), anti-REBOV (e), and anti-MARV (f) sera obtained from mice immunized with EBOV and MARV VLPs were tested for IgG antibodies reacting with His-GPs.

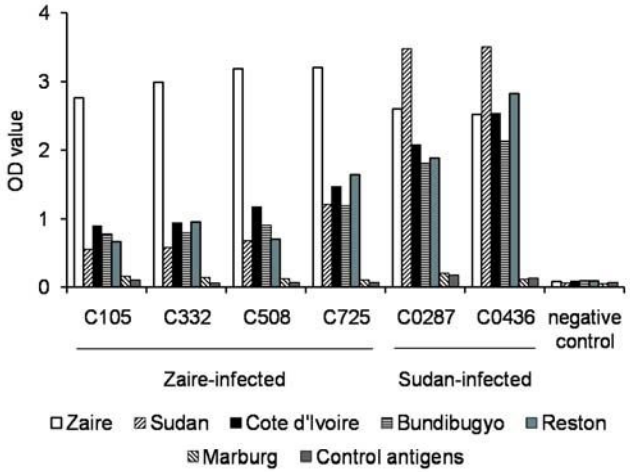


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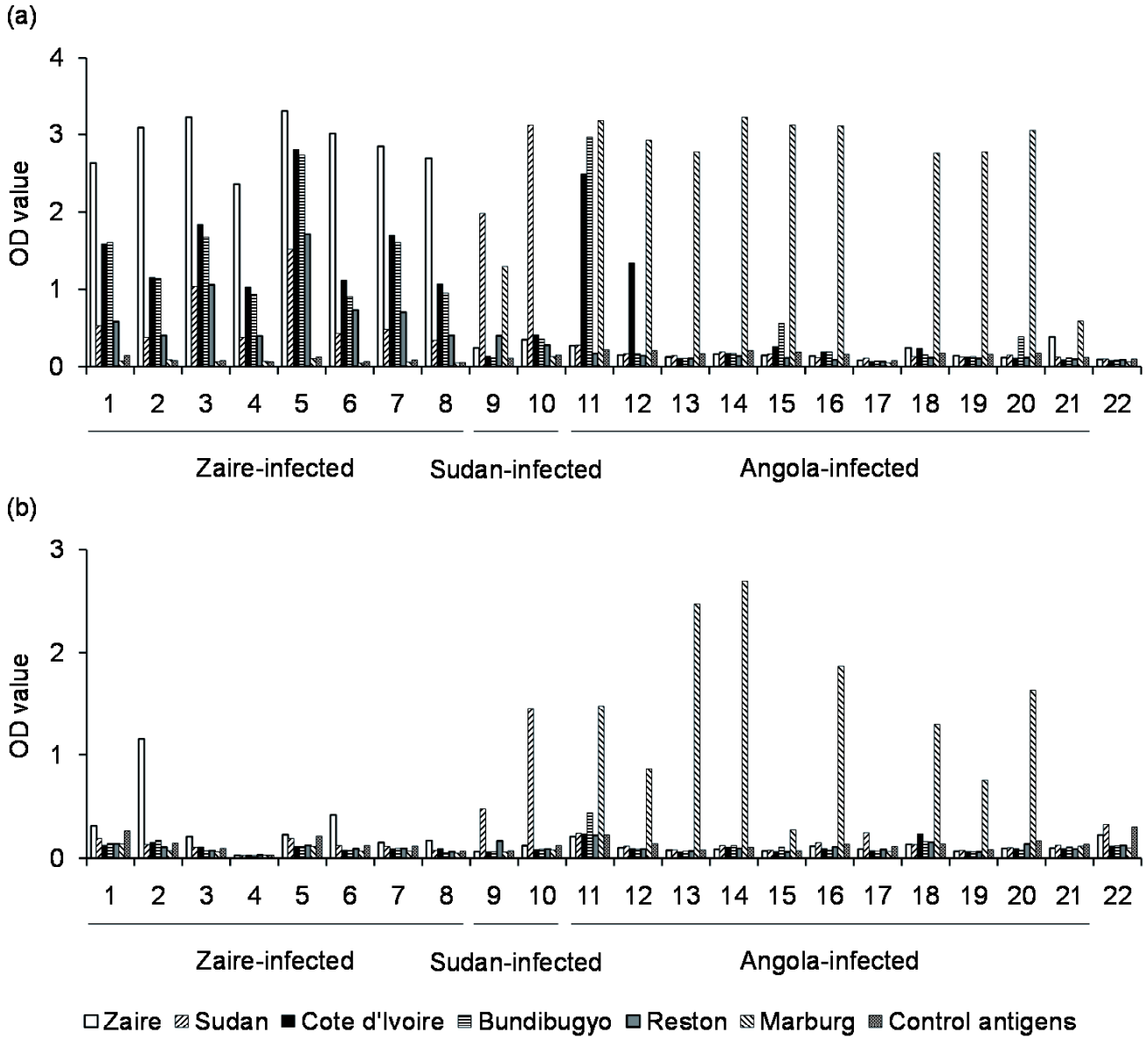


Fig. 6. IgG and IgM antibodies detected in human sera. OD values for specific IgG (a) and IgM (b) antibodies in the patient sera are shown. Sera from 21 individuals were analyzed in 1:1,000 dilutions. Naïve human serum (no. 22) was used as a negative control.