Characterization of the envelope glycoprotein of a novel filovirus, Lloviu virus

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Abstract

Lloviu virus (LLOV), a novel filovirus detected in bats, is phylogenetically distinct from viruses in the genus *Ebolavirus* and *Marburgvirus* in the family *Filoviridae*. While filoviruses are known to cause severe hemorrhagic fever in humans and/or nonhuman primates, LLOV is biologically uncharacterized since infectious LLOV has never been isolated. To examine the properties of LLOV, we characterized its envelope glycoprotein (GP), which likely plays a key role in viral tropism and pathogenicity. We first found that LLOV GP principally shares the primary structure with the other filovirus GPs. Similarly to the other filoviruses, virus-like particles (VLPs) produced by transient expression of LLOV GP, matrix protein, and nucleoprotein in 293T cells had densely arrayed GP spikes on a filamentous particle. Mouse antiserum to LLOV VLP was little cross-reactive to viruses of the other genera, indicating that LLOV is a serologically distinct from the other known filoviruses. For functional study of LLOV GP, we utilized a vesicular stomatitis virus (VSV) pseudotype system and found that LLOV GP requires low endosomal pH and cathepsin L, and that human C-type lectins act as attachment factors for LLOV entry into cells. Interestingly, LLOV GP-pseudotyped VSV infected particular bat cell lines more efficiently than viruses bearing other filovirus GPs. These results suggest that LLOV GP mediates cellular entry in a manner similar to the other filoviruses while showing preferential tropism for some bat cells.

INTRODUCTION

Filoviruses are nonsegmented, negative-stranded RNA viruses grouped into two genera, *Marburgvirus* and *Ebolavirus*. These filoviruses are known to cause severe hemorrhagic fever in human and/or nonhuman primates with case mortality rates of up to 90% (1). There is one known species of *Marburgvirus*, *Marburg marburgvirus*, consisting of two viruses, Marburg virus (MARV) and Ravn virus. On the other hand, five distinct species are known in the genus *Ebolavirus*; *Zaire ebolavirus*, *Sudan ebolavirus*, *Taï forest ebolavirus*, *Bundibugyo ebolavirus,* and *Reston ebolavirus*, represented by Ebola virus (EBOV), Sudan virus (SUDV), Taï forest virus (TAFV), Bundibugyo virus (BDBV), and Reston virus (RESTV), respectively. Among ebolaviruses, a difference in pathogenicity was suggested. EBOV is thought to be the most pathogenic, killing up to approximately 90% of patients, whereas RESTV has never caused lethal infection in humans (2) and is less pathogenic in experimentally infected nonhuman primates than EBOV (3).

Recently, a filovirus-like RNA genome was detected in the lungs, livers, rectal swabs, and/or spleens of bat (*Miniopterus schreibersii*) carcasses found in Cueva del Lloviu, Asturias, Spain. This novel filovirus was designated Lloviu virus (LLOV), whose name was derived from the cave in which it was first found (4). LLOV is phylogenetically distinct from other filoviruses and thus proposed to belong to the new genus *Cuevavirus*, species *Lloviu cuevavirus*, in the family *Filoviridae* (4). However, the biological properties of this novel virus are uncharacterized since infectious LLOV has not been isolated yet.

Filovirus particles consist of at least seven structural proteins, including the nucleoprotein (NP), viral protein (VP) 35, VP40, glycoprotein (GP), VP30, VP24, and

71 polymerase (L) genes (5). The envelope GP is responsible for both receptor binding and
72 fusion of the virus envelope with the host cell membrane (6, 7). GP undergoes
73 proteolytic cleavage by host proteases such as furin, resulting in the two subunits, GP_1
74 and GP_2, which are linked by a disulfide bond (7, 8). GP is highly glycosylated with
75 large amounts of N- and O-linked glycans, most of which are located in its middle
76 one-third, designated the mucin-like region (MLR), which plays an important role in
77 attachment to the preferred target cells (9, 10). Although MLR is found in all known
78 filovirus GPs, its highly variable amino acid sequences and sugar chain structures
79 suggest different GP properties among filovirus species. Membrane-anchored cellular
80 C-type lectins have been found to facilitate filovirus infection *in vitro* through binding
81 to glycans on the MLR (11-13). It was also shown that MLR contains epitopes for
82 antibody-dependent enhancement (ADE) of filovirus infection *in vitro* (14, 15).
83 To provide information for estimation of the infectivity and potential
84 pathogenicity of LLOV, this study focused on GP, which likely plays major role in the
85 replication cycle and the pathogenicity of filoviruses (10, 16). In this study, we
86 investigated the morphology of virus-like particles consisting of LLOV GP, VP40, and
87 NP, compared the antigenicity of GP among filoviruses, and analyzed the ability of GP
88 to mediate virus entry into cells. Here we show that LLOV GP has the potential to
89 mediate viral entry into cells of various animal species, including primates, in a manner
90 similar to the other filoviruses while showing preferential tropism for some particular
91 bat cells.
92

MATERIALS AND METHODS

Cells. Human embryonic kidney (HEK) 293, HEK293T, and African green monkey kidney Vero E6 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fatal calf serum (FCS) and penicillin-streptomycin. Bat cell lines ZFB11-97 and SuBK12-08 were established by transfecting an expression plasmid encoding the Simian virus 40 large T antigen (pCXN2-Flag-SV40LT kindly provided by Drs. H. Sawa and Y. Orba, Hokkaido University Research Center for Zoonosis Control) into primary kidney cells of bats captured in Zambia. The transfected cells were selected by culturing in the presence of G418 (200 μg/ml). ZFB11-97, SuBK12-08, and Madin-Darby canine kidney (MDCK) cells were grown in minimal essential medium (MEM) with 10% FCS, L-glutamine, and penicillin-streptomycin. SK-L cells (17) were cultured in MEM with 10% FCS, L-glutamine, and penicillin-streptomycin, and 0.3% tryptose phosphate broth (GIBCO). Bat cell lines BKT1, FBKT1, YubFKT1, IndFSPT1, and DemKT1, were established as described previously (18). Bat species were identified by morphology, habitat, and BLAST searches using the sequences of their cytochrome b genes (nucleotide positions 1-400). BKT1, FBKT1, YubFKT1, IndFSPT1, DemKT1, human chronic myelogenous leukemia (K562), and K562 clones expressing human macrophage galactose-type C-type lectin (hMGL) or dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN) (19, 20) were grown in RPMI-1640 medium with 10% FSC, L-glutamine and penicillin-streptomycin.

Construction of plasmids expressing GP, NP, and VP40. Coding regions of the GP, NP, and VP40 genes of LLOV were synthesized in pBS II SK vector (FASMAC) based on the nucleotide sequence of LLOV (GenBank accession number: JF828358). The NP

117 and VP40 genes were synthesized according to the coding regions reported in the
118 database. Since the ebolavirus envelope GP is expressed through transcriptional editing
119 (21, 22), the coding region of the GP gene was synthesized with an additional adenosine
120 at the putative editing site. After digestion by restriction enzymes, each gene was cloned
121 into mammalian expression vector pCAGGS (23). The expression plasmids for EBOV
122 (strain Mayinga), SUDV (strain Boniface), TAFV (strain Cote d'Ivoire), BDBV (strain
123 Bundibugyo), RESTV (strain Pennsylvania) and MARV (strains Angola and Musoke)
124 were constructed as described previously (24).
125
126 **Purification of virus-like particles (VLPs).** HEK293T cells were transfected with
127 plasmids encoding GP, VP40, and NP of LLOV, EBOV, SUDV, TAFV, BDBV,
128 RESTV or MARV (strain Angola) using TransIT LT-1 regent (Mirus) according to the
129 manufacturer's instructions. Forty-eight hours later, VLPs were purified from culture
130 supernatants by ultracentrifugation at 28,000 g at 4°C for 1.5 hours with a 25% sucrose
131 cushion. VLP pellets were resuspended in phosphate-buffered saline (PBS).
132
133 **SDS-PAGE and western blotting.** HEK293T cells were transfected with plasmids
134 encoding filovirus GPs and lysed 48 hours after transfection with a lysis buffer (10 mM
135 Tris·HCl [pH 7.8], 0.15 M NaCl, 1 mM EDTA, 0.1% Nonidet P-40 and protease
136 inhibitor mixture)(Roche). Cell lysates were mixed with SDS-PAGE sample buffer with
137 or without 5% 2-mercaptoethanol. After electrophoresis on 5–20% SuperSep (Wako),
138 separated proteins were blotted on a polyvinylidene difluoride membrane (Millipore).
139 The membrane was incubated with 1:2000-diluted mouse antisera to filovirus VLPs (see
140 below), followed by incubation with peroxidase-conjugated goat anti-mouse IgG (H+L)

(Jackson ImmunoResearch). The bound antibodies were visualized with Immobilon Western (Millipore).

Mouse antisera and enzyme-linked immunosorbent assay (ELISA). Five-week-old female BALB/c mice were immunized twice intraperitoneally with purified VLPs (100 μg/mouse) at 3-week intervals. Antisera were collected 7 days after the second immunization. Animal studies were carried out in strict accordance with the Guidelines for Proper Conduct of Animal Experiments of the Science Council of Japan. The protocol was approved by the Hokkaido University Animal Care and Use Committee. The GP-based ELISA was performed as described previously (25). Serum samples were serially diluted with PBS containing 0.05% Tween 20, 0.5% bovine serum albumin, and 2% FCS. Bound antibodies were visualized by adding peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch) and 3,3',5,5'-tetramethylbenzidine (Sigma). The reaction was stopped by adding 1 N phosphate acid to the mixture, and the optical density (OD) at 450 nm was measured.

Electron microscopy. Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) were carried out as described previously (26, 27). Purified VLPs fixed with 0.25% glutalaldehyde were adsorbed to a collodion-carbon-coated copper grids and negatively stained with 2% phosphotungstic acid solution (pH=5.8). For immuno-TEM, we used an anti-LLOV GP monoclonal antibody (LGP14-2) produced in this study as described previously (15), and an immunogold-conjugated goat anti-mouse IgG (H + L) polyclonal antibody (BB International). Samples were examined with an H-7650 electron microscope (Hitachi) at 80kV. For SEM, cells transfected with

plasmids expressing LLOV GP, VP40, and NP were fixed with 2.5% glutalaldehyde in 0.1 M phosphate buffer (pH 7.4) and postfixed with 1% osmium tetroxide in the same buffer. The fixed samples were dehydrated with a series of ethanol gradients, substituted with t-butanol, and dried in an ES-2030 freeze dryer (Hitachi). Dried specimens were coated with platinum by using Mild spatter E-1046 (Hitachi). The samples were observed with an S-4700 electron microscope (Hitachi) at 15 kV.

Vesicular stomatitis virus (VSV) pseudotyped with filovirus GPs. Using VSV containing the green fluorescent protein (GFP) gene instead of the receptor-binding VSV G protein gene (VSVΔG*-G)(6), pseudotyped viruses with GPs of EBOV, RESTV, MARV strains Angola and Musoke, and LLOV (VSVΔG*-Zaire, VSVΔG*-Reston, VSVΔG*-Angola, VSVΔG*-Musoke, and VSVΔG*-Lloviu, respectively), were generated and the infectious units (IUs) of stock viruses were determined in Vero E6 cells, according to a previous study (6). The genome copy number of each pseudotyped VSV preparation was quantified by real-time RT-PCR. Real-time RT-PCR was performed using One Step SYBR PrimeScript RT-PCR Kit II (TaKaRa Bio) and a CFX96 Real Time System (BIO RAD) with primers (GFP498-F: CAAGATCCGCCACAACATCG and GFP-617R: GACTGGGTGCTCAGGTAGTG) to detect the GFP gene in the VSV genome.

Virus titration. To determine infectivities of VSVs pseudotyped with filovirus GPs, appropriately diluted virus stocks were pretreated with an anti-VSV G monoclonal antibody, VSV-G(N)1-9, to abolish the background infectivity of parental VSVΔG*-G (15). K562 clones expressing hMGL and DC-SIGN grown on 96-well plates were

189 infected with VSV pseudotyped with filovirus GPs (50-150 IU determined in K562
190 cells), and infectivities were determined by counting the number of GFP-positive cells
191 using flow cytometry as described previously (19, 20). For the assays of
192 antibody-dependent enhancement (ADE) of infection, 10-fold serially diluted mouse
193 antisera were mixed with equal volumes of pseudotyped VSVs (50-150 IU determined
194 in K562 cells). After 1-hour incubation at room temperature, the mixture was inoculated
195 into K562 cells grown on 96-well plates. At 20 hours post-inoculation, GFP-positive
196 cells were counted with an IN Cell Analyzer 2000 (GE Healthcare). To determine the
197 infectivities in adherent cells of different animal origins, cell monolayers grown on
198 96-well plates were infected with VSVs pseudotyped with filovirus GPs. Twenty hours
199 later, the virus infectivity in each cell line was determined by counting the number of
200 GFP-expressing cells under a fluorescent microscope, and IUs per 10^6 genome copies
201 were calculated.
202
203 **Inhibitor treatments.** Vero E6 cells were pretreated with ammonium chloride (Wako),
204 monensin (Sigma), cathepsin B, or L inhibitors (CA-074Me and FY-dmk, respectively,
205 Calbiochem) for 30 min at 37°C. Treated cells were then infected with VSVΔG*-Zaire,
206 VSVΔG*-Angola, VSVΔG*-Lloviu, and VSVΔG*-G appropriately diluted to yield
207 200–2000 IUs/10^6 cells. At 20 hours post-inoculation, GFP-positive cells were counted
208 with the IN Cell Analyzer 2000 (GE Healthcare).
209
210

RESULTS

Characterization of the primary structure of LLOV GP. Though the envelope GPs of ebolaviruses are expressed through transcriptional editing (21, 22), similar characteristics were not shown for LLOV GP in a previous study, and the reported LLOV GP gene did not have an open reading frame of the single transmembrane GP (4). Thus, we first analyzed the nucleotide and deduced amino acid sequences of LLOV GP, and found that the nucleotide sequence of LLOV GP had 7 adenosines at positions 910-916. Based on sequence comparison with the EBOV genome, which has the editing site at positions 880-816, we assumed that this stretch of 7 adenosines could be the editing site of the LLOV GP gene. Accordingly, an open reading frame of the full length transmembrane GP gene was produced by adding an adenosine at this putative editing site (Fig.1A). We found that LLOV envelope GP had a potential cleavage site (i.e., RRRR recognized by host ubiquitous proteases such as furin) and MLR, similarly to the other filoviruses. The predicted MLR of LLOV GP differed from that of EBOV GP in length and location (i.e., LLOV MLR was located over the cleavage site and was a little shorter than EBOV MLR) (Fig. 1A and B). We confirmed the approximate molecular size of LLOV GP ($GP_{1,2}$, approximately 120-130 kD) and its cleavage product, the GP_1 subunit (approximately 100 kD), by western blotting (Fig. 1C). These results suggested that LLOV GP principally might share biological characteristics with the other filovirus GPs.

Morphology of VLPs consisting of LLOV GP, VP40, and NP. Filoviruses are characterized by their filamentous forms. However, LLOV particles have never been verified morphologically. To determine the possible shape of LLOV particles, we

235 investigated the morphology of VLPs consisting of LLOV GP, VP40, and NP. TEM
236 analyses revealed that LLOV VLPs were filamentous (Fig. 2A and E), like EBOV (Fig.
237 2C and G) and MARV VLPs (Fig. 2D and H). Numerous spikes were observed on the
238 VLP surface and immuno-TEM with an anti-LLOV GP monoclonal antibody confirmed
239 the presence of LLOV GPs on the surface (Fig. 2B and F). Similarly to the other
240 filoviruses (28), VLPs with a uniform diameter of approximately 70 nm and varied
241 lengths were observed. This was consistent with a previous study showing that the
242 diameters of VLPs were narrower than reported for actual EBOV particles (80-nm
243 diameter) (1, 29). By SEM, numerous filamentous structures were observed on the
244 surfaces of cells transfected with plasmids expressing LLOV GP, VP40, and NP (Fig. 3),
245 which has a similarity to EBOV budding (27). These results suggested that LLOV
246 shared morphological characteristics with the other known filoviruses.
247
248 **Antigenic comparison among filovirus GPs.** While LLOV is shown to be
249 phylogenically distinct from the other filovirus species, serological information is
250 lacking. The amino acid sequence of LLOV GP has 35% and 28% similarity with
251 EBOV and MARV, respectively. To compare the antigenic relationships among
252 filovirus GPs, we produced antisera to each GP by immunizing mice with VLPs, and
253 performed GP-based ELISA (Fig. 4). We found that anti-LLOV GP sera showed
254 exclusive reactivity to the LLOV GP antigen (Fig. 4A). Similarly, anti-MARV GP sera
255 only reacted with the MARV GP antigen (Fig. 4G). Antisera to EBOV, SUDV, TAFV,
256 BDBV, and RESTV GPs showed slight cross-reactivity with ebolavirus antigens at the
257 lowest dilution of the sera but not with the LLOV and MARV GP antigens (Fig. 4B to

F). These results indicated that LLOV was distinct not only phylogenically but also serologically from the other known filoviruses.

Functional study of LLOV GP with chemical inhibitors. For functional study of LLOV GP, we produced VSV pseudotyped with LLOV GP, which could infect Vero E6 cells, and thus confirmed that the full length GP produced with 8 adenosines at the putative editing sites was fully functional as a single transmembrane GP. It has been shown that endosomal acidification and proteolytic processing with the cellular cysteine proteases cathepsin L and/or B are required for filovirus GP-mediated entry *in vitro* (6, 7, 30). To investigate the requirement of these factors for the LLOV GP function, we examined the infectivities of pseudotyped VSV in Vero E6 cells pretreated with chemical inhibitors (Fig. 5). Pretreatment of cells with ammonium chloride and monensin markedly reduced the infectivity of VSVΔG*-Lloviu, as was the case with VSVΔG*-Zaire, VSVΔG*-Angola, and VSVΔG*-G, suggesting that LLOV GP requires a low pH for cellular entry. We found that pretreatments with a cathepsin L inhibitor at concentrations of 2.5 and 10 μM significantly reduced the infectivities of VSVΔG*-Lloviu, -Zaire, and -Angola. However, treatments at 0.625 μM did not show any inhibitory effects on the VSVΔG*-Lloviu infectivity, and rather enhanced the infectivity. Interestingly, when cells were treated with a cathepsin B inhibitor, the infectivity of VSVΔG*-Zaire was reduced significantly in a dose-dependent manner, whereas much less inhibition were observed in the VSVΔG*-Lloviu, -Zaire, and -Angola infectivities. These cathepsin inhibitors did not affect the infectivity of VSVΔG*-G.

Human C-type lectin-mediated entry of pseudotyped VSVs. C-type lectins expressed on the host cell surface are thought to serve as an attachment factor for filovirus GP, and C-type lectin-mediated entry is believed to be one of the important factors responsible for filovirus tropism and pathogenicity (11, 19, 20). Thus, we investigated the potential of LLOV GP to use the human C-type lectins hMGL and DC-SIGN, both of which are known to enhance filovirus infectivity (Fig. 6). VSVΔG*-Lloviu infected DC-SIGN-expressing cells more efficiently than hMGL-expressing cells, which was similar to VSVΔG*-Zaire and VSVΔG*-Reston. VSVΔG*-Lloviu infected hMGL-expressing cells more efficiently than VSVΔG*-Musoke ($P < 0.05$), but less than VSVΔG*-Angola ($P < 0.05$), but there were no significant differences among VSVΔG*-Lloviu, VSVΔG*-Zaire, and VSVΔG*-Reston. On the other hand, VSVΔG*-Lloviu infected DC-SIGN-expressing cells more efficiently than VSVΔG*-Angola ($P < 0.05$) and VSVΔG*-Musoke ($P < 0.01$), and less efficiently than VSVΔG*-Zaire ($P < 0.05$), but there was no significant difference between VSVΔG*-Lloviu and VSVΔG*-Reston.

Difference in ADE activity between anti-EBOV and anti-LLOVGP antisera. Antibody-dependent enhancement of infection is also a known *in vitro* phenomenon observed for comparatively highly lethal filoviruses (e.g., Zaire and Angola) (15, 31, 32). To investigate the potential of LLOV GP to induce ADE antibodies, K562 cells were infected with VSVΔG*-Lloviu or VSVΔG*-Zaire in the presence of mouse antisera specific to the respective viruses (Fig. 7). We confirmed the ADE activity of the anti-Zaire serum as indicated by markedly enhanced infectivities of VSVΔG*-Zaire at the serum dilutions of 1:10 and 1:100 (Fig 7A). By contrast, only minimal ADE activity

306 was seen in the anti-LLOV serum, although similar amounts of specific IgG antibodies
307 were detected in both antisera by ELISA (Fig. 4). Consist with the absence of
308 cross-reactive IgG in ELISA, little cross-ADE activity was observed in the ADE assay.
309
310 **Cellular tropism of LLOV GP.** To estimate the GP-dependent tropism that is likely
311 reflected by the prevalence of LLOV receptors, we infected various cell lines of
312 different animal origins (Table 1) with pseudotyped VSVs and their infectivities were
313 compared (Fig. 8A). VSVΔG*-Lloviu infected cells that were derived from the human,
314 African green monkey, pig, dog, and bat in a similar manner to VSVΔG*-Zaire and
315 VSVΔG*-Reston (Fig. 8A). VSVΔG*-Angola and VSVΔG*-Musoke had higher IUs in
316 these cells than VSVΔG*-Lloviu, VSVΔG*-Zaire, and VSVΔG*-Reston, except in a
317 cell line from the Yaeyama flying fox (*Pteropus dasymallus yayeyamae*; FBKT1) (Fig
318 8A). Since some species of bats are suspected to be natural reservoirs of filoviruses
319 (33-37), we focused on these bat cells and relative infectivities were determined (Fig.
320 8B). Interestingly, VSVΔG*-Lloviu infected IndFSPT1 and SuBK12-08 more
321 efficiently than the other viruses tested.
322
323

DISCUSSION

This study provides fundamental information on the properties of LLOV GP. The RNA editing that is required to produce the full length transmembrane GP is a common characteristic of viruses belonging to the genus *Ebolavirus* (21, 22). The presence of the editing site in the LLOV GP gene supports the notion that this virus is more related to ebolaviruses than marburgviruses. Phylogenic analyses also suggested that LLOV might have the same ancestor as ebolaviruses (4). On the other hand, LLOV GP has MLR and a furin cleavage site, both of which are common features shared by previously known filoviruses (38, 39). By TEM and SEM of VLPs, we further found morphological similarity between LLOV and other filoviruses, which have numerous GP spikes located on the filamentous VLP surface. Viral entry assays with chemical inhibitors also suggest that LLOV GP, as well as GPs of the other filoviruses, requires low endosomal pH and proteolytic processing for virus entry into cells. Taken together, these results indicate that the structure and function of LLOV GP are primarily similar to those of the other filovirus GPs.

However, the requirement of cathepsin L might be controversial since a high concentration (> 1 μM) of FY-dmk was suggested to inhibit not only cathepsin L but also cathepsins B and likely other endosomal cysteine proteases (40). FY-dmk at the lowest concentration tested in this study (0.625 μM) did not reduce infectivities of neither VSVΔG*-Lloviu, VSVΔG*-Zaire, nor VSVΔG*-Angola, suggesting that cathepsin L is not essential for LLOV infection similarly to the other filoviruses (40, 41). It was also shown that Cathepsin B and Cathepsin L activities are not required for EBOV replication in a mouse model (41). Thus, further studies are needed to clarify the

347 in vivo importance of the GP cleavage by cathpsins and some other host proteases for
348 filovirus infection.
349 Hepatocytes, dendritic cells, monocytes, and macrophages, all of which express
350 cell surface C-type lectins, are thought to be the preferred target cells of filoviruses and
351 increased infection of these cells might be directly involved in the pathogenesis of
352 filovirus infection (11, 19, 42-44). We demonstrated that LLOV GP utilized human
353 C-type lectins, hMGL and DC-SIGN, most likely as attachment factors, as reported with
354 the other filovirus GPs (13, 19, 20, 45). These C-type lectins have different glycan
355 specificities (i.e. hMGL and DC-SIGN preferentially react with O- and high-mannose
356 type N-glycans, respectively) (46-48). Like EBOV and RESTV GPs, LLOV GP showed
357 greater preference for DC-SIGN than hMGL compared with MARV GPs, suggesting
358 that LLOV GP, particularly its MLR, might have a carbohydrate structure comparable
359 to that of ebolaviruses. Our data suggest that LLOV GP has a tropism to cells
360 expressing C-type lectins and might potentially infect human immune cells such as
361 dendritic cells and macrophages.
362 Like C-type lectins, ADE antibodies mostly recognize epitopes on MLR of
363 filovirus GPs, leading to enhanced infectivity (11, 13, 42). Thus, MLR is thought to
364 play important roles for these two attachment functions of GP. In addition to the
365 primary structure of MLR (i.e., the presence of different epitopes and sugar chains
366 among filoviruses), the GP2 region seems to have key amino acid residues (e.g., the
367 amino acid at position 547 in MARV GP) contributing to the efficiency of ADE- and
368 C-type lectin-mediated entry (15, 20). Although detailed functional mapping and
369 structural analysis are still needed, our data indicate that the overall properties of LLOV

370 GP for ADE- and C-type lectin-mediated entry are comparable to those of RESTV GP
371 rather than highly virulent EBOV and MARV (strain Angola) (11, 13, 42).
372 VSVΔG*-Lloviu infected all cell lines tested as well as VSVΔG*-Zaire and
373 VSVΔG*-Reston (Fig. 5A). Recently, RESTV was detected in pigs in the Philippines
374 and China, and more recent studies have revealed that EBOV causes respiratory disease
375 in pigs (49-53), suggesting a potential role of this animal in filovirus ecology. In this
376 study, VSVΔG*-Lloviu infected pig cells (SK-L cells) in a similar manner to
377 VSVΔG*-Zaire and VSVΔG*-Reston. Although GP is not the only determinant
378 controlling filovirus pathogenicity, our data suggest that LLOV, at least, meets the
379 minimum requirements to infect pig cells.
380 Interestingly, neither VSVΔG*-Angola nor VSVΔG*-Musoke infected FBKT1
381 cells derived from the Yaeyama flying fox (*Pteropus dasymallus yayeyamae*), although
382 the infectivities of these viruses in the other cell lines tested were uniformly higher than
383 those of the other viruses (Fig.5A). This finding suggests the existence of cellular
384 receptors/coreceptors that interacts with EBOV, RESTV, and LLOV GPs but not
385 MARV GP. Although several cellular molecules were reported to be involved in
386 filovirus entry (e.g., T-cell immunoglobulin domain and mucin domain 1, Tyro 3 family,
387 C-type lectin, or Niemann-Pick C1) (11-13, 42-47, 54-57), there is only limited
388 information on these molecules in bats. It would be of interest to clarify whether these
389 cellular molecules play critical roles in tissue tropism and/or host range restriction of
390 filoviruses. Alternatively, there might be a new receptor of LLOV in bats.
391 VSVΔG*-Lloviu infected SuBK12-08 and IndFSPT1 more efficiently than the
392 other viruses (Fig. 5B). It should be noted that SuBK12-08 was derived from the same
393 insectivorous bat species, Schreiber's bat (*Miniopterus schreibersii*), in which LLOV

394 was first detected in Europe. However, since LLOV likely causes lethal infection of
395 Schreiber's bat, this bat species may not serve as the natural host that can maintain this
396 virus in nature. On the other hand, IndFSPT1 was derived from fruit bats. Considering
397 that some species of fruit bats are suspected to be natural hosts of filoviruses (33, 58),
398 strong tropism to this fruit bat species may suggest that fruit bats also play some roles in
399 the ecology of LLOV.
400 While LLOV seems to be highly pathogenic for some species of bats (e.g.,
401 Schreiber's bat), its ability to infect human and nonhuman primates and the pathogenic
402 potential for these hosts can only be hypothesized, since infectious LLOV has never
403 been isolated. In this study, we used a replication-incompetent VSV pseudotype system
404 that enabled us to investigate the cellular tropism mediated by simple interaction
405 between LLOV GP and its cellular ligands. Although a reverse genetics approach and *in*
406 *vivo* experiments for infectious LLOV are needed to provide direct evidence of the viral
407 pathogenicity and host specificity, our data suggest that the overall properties of LLOV
408 GP are similar to those of the other filoviruses, and that LLOV has the potential, at least
409 from the aspect of GP-receptor/coreceptor interaction, to infect many mammalian cells,
410 including those of the human, monkey, and pig, with preferential tropism for some bat
411 cells.
412

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622 **FIGURE LEGENDS**

623

624 **FIG. 1 Primary structure of LLOV envelope GP.** Nucleotide and deduced amino
625 acid sequences of LLOV GP (A). Deduced amino acid sequences are shown in bold
626 letters. Blue, green, and yellow lines represent the signal peptide, MLR, and
627 transmembrane domain predicted by GENETYX Ver.10, NetOGlyc, and TopPred 0.01,
628 respectively. Purple and red letters represent the putative editing and cleavage sites,
629 respectively. Comparison of EBOV and LLOV MLRs (B). Potential O-glycosylation
630 sites were predicted by NetOGlyc, and MLRs were defined as the regions between the
631 first and last amino acid residues showing the score over the threshold (0.5). Arrows
632 indicate the cleavage sites. Western blotting of filovirus GPs (C). Proteins in the lysate
633 of HEK293T cells transfected with the plasmid expressing LLOV GP (lanes 1 and 2),
634 EBOV GP (lanes 3 and 4), MARV GP (lanes 5 and 6), or empty vector (lanes 7 and 8)
635 were separated by SDS-PAGE under non-reducing (lanes 1, 3, 5, and 7) or reducing
636 (lanes 2, 4, 6, and 8) conditions.

637

638 **FIG. 2 TEM of filovirus VLPs.** Purified VLPs produced from 293T cells transfected
639 with plasmids expressing LLOV (A, B, E, and F), EBOV (Zaire) (C and G), and MARV
640 (Angola) (D and H) proteins were fixed and stained as described in Materials and
641 Methods. For immuno-TEM (B and F), an anti-LLOV GP monoclonal antibody was
642 used. Scale bars represent 500 nm (A to D) and 200 nm (E to H). Arrowheads indicate
643 gold particles.

644

645 **FIG. 3 SEM of LLOV VLPs.** HEK293T cells transfected with pCAGGS expressing
646 LLOV GP, VP40, and NP (A and B) or pCAGGS alone (C and D) were fixed at 48
647 hours after transfection. Samples were observed with an S-4700 scanning electron
648 microscope (Hitachi). Scale bars represent 5 μm (A and C) and 2 μm (B and D).
649
650 **FIG. 4 Cross-reactivities of anti-GP sera among filoviruses in ELISA.** Ten-fold
651 serial dilutions of mouse antisera to EBOV (anti-Zaire), SUDV (anti-Sudan), TAFV
652 (anti-Tai forest), BDBV (anti-Bundibugyo), RESTV (anti-Reston), LLOV (anti-Lloviu),
653 and MARV (anti-Marburg) were tested for IgG reactivities to LLOV (A), EBOV (B),
654 SUDV (C), TAFV (D), BDBV (E), RESTV (F), and MARV (G) GP antigens. Three
655 mice were used for each virus and averages and standard deviations are shown.
656
657 **FIG. 5 Effects of chemical inhibitors on infectivities of pseudotyped VSVs.** Vero E6
658 cells were pretreated with ammonium chloride (AMC; A), monensin (MON; B), and
659 cathepsin B and L inhibitors (Cat B; C, and Cat L; D) for 30 min at 37°C. The treated
660 cells were then infected with VSVΔG*-Zaire (Zaire), VSVΔG*-Angola (Angola),
661 VSVΔG*-Lloviu (Lloviu), and VSVΔG*-G (VSV) appropriately diluted to yield 200–
662 2000 IUs/10^6 cells. At 20 hours post-inoculation, GFP-positive cells were counted using
663 an IN Cell Analyzer 2000 (GE Healthcare). The percentages of infectivity were
664 determined by setting the number of the untreated cells to 100%. Each experiment was
665 performed three times, and averages and standard deviations are shown.
666
667 **FIG. 6 Infectivities of pseudotyped VSVs in C-type lectin-expressing cells.** K562
668 cells expressing hMGL or DC-SIGN were infected with VSVΔG*-Zaire,

669 VSVΔG*-Reston, VSVΔG*-Lloviu, VSVΔG*-Angola, and VSVΔG*-Musoke. The
670 infectivity of each pseudotyped VSV on K562 clones was determined by counting the
671 number of GFP-positive cells using flow cytometry, and the percentages of infectivity
672 in K562-hMGL and -DC-SIGN were determined by setting the number of the infected
673 K562 to 100%. Each experiment was performed three times, and averages and standard
674 deviations are shown. Statistical significance of the differences was determined by
675 Student's t-test (see P values in the text).
676
677 **FIG. 7 ADE activities of antisera to EBOV and LLOV GPs.** Ten-fold serially diluted
678 mouse anti-EBOV (A) and anti-LLOV (B) sera ware mixed with equal volumes of the
679 VSVΔG*-Zaire and VSVΔG*-Lloviu. Relative infectivity was determined by setting
680 the number of infected K562 cells without antisera to 100%. Each experiment was
681 performed three times, and averages and standard deviations are shown.
682
683 **FIG. 8 Infectivities of pseudotyped VSVs in mammalian cell lines.** VSVΔG*-Zaire,
684 VSVΔG*-Reston, VSVΔG*-Lloviu, VSVΔG*-Angola, and VSVΔG*-Musoke were
685 inoculated into several mammalian cell lines. Infectious units (IUs) of each virus in
686 different cell lines were determined by counting the number of GFP-expressing cells
687 and each IU was then standardized based on 10^6 copies of the VSV genome determined
688 by real-time RT-PCR (A). Relative infectivities in bat cell lines are given by setting
689 each IU in Vero E6 to 1.0 (i.e., [IU in bat cells]/[IU in Vero E6 cells] (B). Each
690 experiment was performed three times, and averages and standard deviations are shown.
691 Infectivities of VSVΔG*-Angola, and VSVΔG*-Musoke were under the limit of
692 detection (*).

TABLE 1 Origins of cell lines used in this study

Cell line	Species	Zoological name	Organ
Vero E6	African green monkey	*Chlorocebus* sp.	Kidney
HEK293	Human	*Homo sapiens*	Kidney
SK-L	Pig	*Sus scrofa domesticus*	Kidney
MDCK	Dog	*Canis lupus familiaris*	Kidney
BKT1	Greater horseshoe bat[a]	*Rhinolophus ferrumequinum*	Kidney
FBKT1	Yaeyama flying fox[b]	*Pteropus dasymallus yayeyamae*	Kidney
YubFKT1	Eastern bent-winged bat[c]	*Miniopterus fuliginosus*	Kidney
IndFSPT1	Indian flying fox[d]	*Pteropus giganteus*	Spleen
DemKT1	Leschenault's rousette[e]	*Rousettus leschenaulti*	Kidney
ZFB11-97	Gambian epauletted fruit bat[f]	*Epomophorus gambianus*	Kidney
SuBK12-08	Schreiber's bat[g]	*Miniopterus schreibersii*	Kidney

[a] Nucleotide sequence identity is 98% (manuscript in preparation).

[b] Previously described (18).

[c] Nucleotide sequence identity is 99%.

[d] Nucleotide sequence identity is 98%.

[e] Nucleotide sequence identity is 100%.

[f] Nucleotide sequence identity is 89%.

[g] Nucleotide sequence identity is 98%.

A

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M  V  P  T  Y  P  Y  S  S  L  L  D  W  R  P  P  P  N  T  L  P  W  I  L  N  L  V  V  F  Y  T  I  A  W  L  P  G  G  V  S  40
ATGGTGCCCA CCTACCCGTA CAGCAGCCTA TTAGATTGGA GACCACCACC AAACACCCTA CCATGGATCC TCAACCTTGT GGTCTTTTAT ACCATAGCCT GGCTGCCCGG GGGAGTCTCA 120
G  I  P  L  G  L  L  G  N  N  S  I  T  Q  T  V  V  D  N  V  V  C  K  E  H  L  A  T  T  D  Q  L  Q  A  I  G  L  G  L  E  80
GGAATTCCAC TCGGTTTGTT GGGAAACAAC AGCATCACCC AAACTGTCGT GGACAATGTA GTGTGCAAGG AACACCTTGC CACAACAGAT CAGCTACAGG CTATTGGATT GGGACTAGAG 240
G  L  G  E  H  A  D  L  P  T  A  T  K  R  W  G  F  R  S  D  V  I  P  K  I  V  G  Y  T  A  G  E  W  V  E  N  C  Y  N  L  120
GGGCTTGGTG AACATGCTGA CCTCCCGACT GCCACCAAGC GATGGGGTTT TCGATCTGAT GTCATCCCAA AAATCGTGGG ATACACCGCT GGGGAATGGG TGGAAAACTG CTACAATCTT 360
E  I  T  K  K  D  G  H  P  C  L  P  S  P  P  T  G  L  L  G  Y  P  R  C  R  Y  V  H  R  A  K  G  A  G  P  C  P  G  G  N  160
GAAATCACCA AGAAAGATGG TCATCCTTGC CTCCCCAGCC CGCCAACTGG CTTACTTGGC TATCCCCGAT GCCGCTATGT CCACAGAGCC AAAGGAGCAG GCCCTTGCCC AGGTGGGAAT 480
A  F  H  K  H  G  S  F  F  L  Y  H  G  M  A  S  T  V  I  Y  H  G  V  T  F  T  E  G  T  I  A  F  L  I  V  P  K  D  A  P  200
GCTTTCCACA AACATGGTTC TTTCTTTCTG TACCACGGTA TGGCTTCTAC AGTAATTTAT CATGGTGTAA CCTTTACGGA AGGCACAATT GCTTTCCTAA TTGTCCCGAA GGATGCACCC 600
R  L  K  A  G  L  G  T  G  F  S  H  Q  A  E  N  Q  N  P  N  N  Q  F  R  T  T  T  L  D  Y  D  V  M  S  P  W  M  D  N  A  240
CGTCTCAAGG CAGGGCTTGG AACAGGATTC AGTCATCAAG CAGAGAACCA AAACCCAAAC AACCAATTTC GAACAACAAC TTTAGATTAT GATGTAATGA GTCCTTGGAT GGACAATGCT 720
T  F  F  F  R  A  R  E  D  T  S  M  L  I  Q  T  R  Y  P  P  A  N  L  E  L  V  Q  E  R  L  A  N  L  T  G  D  Q  A  D  P  280
ACCTTCTTCT TTCGAGCGAG GGAAGACACA TCAATGCTAA TCCAAACAAG GTACCCTCCA GCAAATCTAG AGCTTGTTCA AGAAAGATTG GCTAATCTTA CCGGAGATCA AGCTGATCCA 840
S  K  M  E  E  I  V  A  E  V  L  T  L  E  L  G  D  W  S  G  W  T  T  K  K  N  R  S  T  N  H  T  A  K  K  P  F  T  S  I  320
TCAAAGATGG AAGAGATTGT CGCTGAGGTT TTGACATTGG AGCTCGGTGA TTGGTCCGGT TGGACAACTA AAAAAAACCG CAGTACAAAC CATACGGCTA AGAAACCCTT CACCAGCATC 960
W  F  N  Q  G  Q  D  W  P  E  A  H  D  G  S  S  G  V  H  P  P  T  S  F  C  C  W  T  T  L  P  L  E  H  S  S  N  S  G  A  360
TGGTTCAACC AAGGACAAGA CTGGCCAGAA GCCCATGACG GATCATCAGG AGTTCATCCT CCAACCTCAT TCTGCTGTTG GACAACCCTG CCTCTGGAAC ATTCTTCGAA CTCCGGGGCG 1080
E  P  C  T  K  A  P  A  G  N  T  T  N  N  V  H  H  C  C  S  W  V  R  I  Q  A  V  H  P  G  N  T  S  G  E  I  S  M  P  L  400
GAACCCTGCA CGAAGGCACC GGCGGGAAAC ACCACCAACA ATGTCCATCA CTGCTGCTCC TGGGTCAGGA TACAAGCCGT ACATCCAGGC AATACCTCTG GTGAAATTTC GATGCCATTG 1200
G  G  S  S  A  C  V  S  S  I  P  L  L  G  S  V  S  N  N  S  S  I  Q  E  L  E  T  S  S  K  S  A  T  E  L  T  T  P  I  N  440
GGAGGGTCTT CGGCATGTGT GTCGTCGATA CCCCTCCTGG GTTCAGTGAG CAACAATAGT TCAATACAGG AGCTTGAGAC TTCATCTAAA AGTGCAACAG AATTGACAAC TCCCATCAAT 1320
H  S  Q  S  L  Q  L  A  S  V  T  N  T  P  T  P  T  T  Q  S  K  S  W  T  V  D  Y  N  N  T  T  P  T  M  D  P  T  T  I  L  480
CACTCCCAAT CACTACAGCT CGCATCCGTC ACAAACACCC CCACACCGAC AACACAGTCC AAGTCCTGGA CAGTTGACTA CAACAACACA ACGCCAACCA TGGATCCCAC AACAATACTG 1440
T  T  P  D  T  A  T  I  P  P  N  N  S  S  D  H  N  A  T  T  T  S  K  T  R  R  R  R  Q  V  N  P  V  P  P  T  I  T  Q  Q  520
ACGACACCCG ACACCGCAAC CATTCCCCCT AACAACTCAT CTGATCACAA CGCCACAACA ACAAGCAAAA CAAGACGAAG GAGACAGGTC AACCCAGTGC CCCCAACGAT CACCCAACAA 1560
T  S  T  S  I  N  T  S  H  H  P  N  M  T  T  Q  L  A  R  H  P  S  V  Q  T  R  M  Q  N  P  S  C  N  P  N  L  R  Y  W  T  560
ACCTCTACAA GCATCAATAC CTCCCACCAC CCCAATATGA CAACACAGTT AGCAAGACAT CCGAGTGTGC AAACAAGGAT GCAAAACCCC AGCTGTAATC CCAACCTTAG ATACTGGACA 1680
S  R  E  M  S  N  A  G  G  L  A  W  I  P  W  I  G  P  G  I  E  G  G  I  T  D  G  I  M  E  H  Q  N  T  I  V  C  Q  L  R  600
AGCCGGGAGA TGAGTAATGC TGGGGGGCTT GCATGGATTC CATGGATTGG ACCAGGGATT GAGGGAGGGA TCACAGACGG GATAATGGAG CATCAGAACA CAATTGTCTG TCAGTTACGG 1800
E  L  A  N  T  T  T  K  A  L  Q  L  F  L  R  A  T  T  E  L  R  T  Y  S  I  L  N  R  H  A  I  D  F  L  L  Q  R  W  G  G  640
GAGCTCGCGA ACACCACTAC TAAAGCCCTA CAGCTTTTCC TCCGGGCTAC CACTGAGCTC CGAACCTACT CTATCCTCAA CCGCCATGCG ATTGACTTTC TACTACAGCG TTGGGGTGGT 1920
T  C  R  I  L  G  P  N  C  C  I  E  P  H  D  W  S  A  N  I  T  A  E  I  N  H  I  R  E  D  I  L  N  H  H  E  I  Q  P  S  680
ACCTGCAGAA TCCTTGGCCC AAACTGCTGT ATCGAACCTC ATGATTGGTC TGCCAACATT ACGGCTGAGA TAAATCATAT TAGAGAAGAT ATCCTGAACC ATCATGAGAT CCAACCTTCT 2040
Q  D  P  S  F  W  T  G  W  Q  Q  W  I  P  T  G  A  S  A  L  G  I  I  L  A  I  L  A  L  I  C  L  C  R  I  T  R   717 amino acids
CAAGACCCCT CCTTTTGGAC TGGATGGCAA CAGTGGATCC CAACAGGAGC CAGTGCTCTC GGAATCATCC TGGCAATATT AGCCTTGATT TGTCTGTGCA GAATAACACG A 2151 nucleotides
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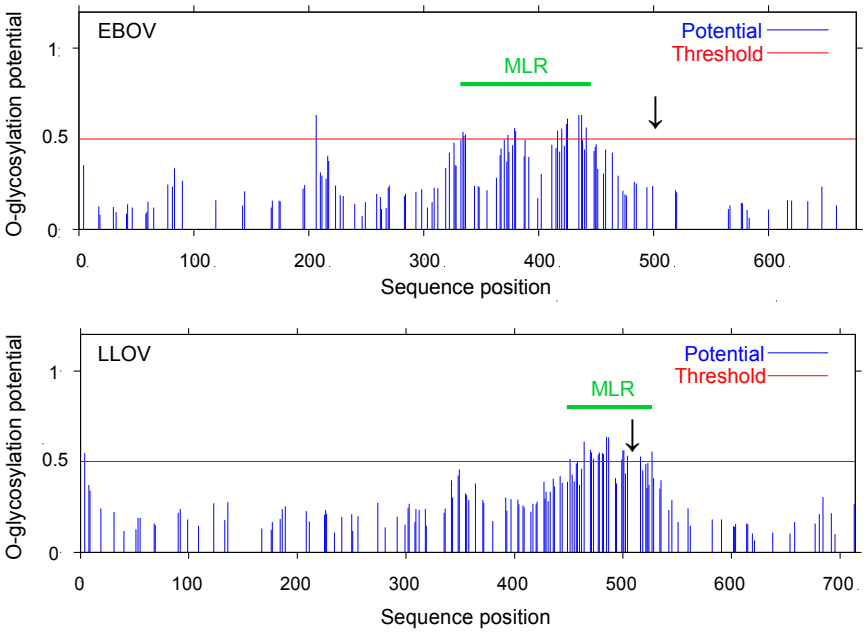
B



C

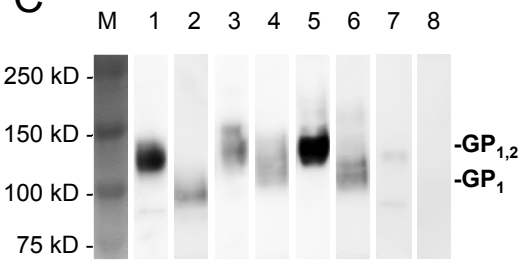


Fig. 1 Maruyama *et al.*

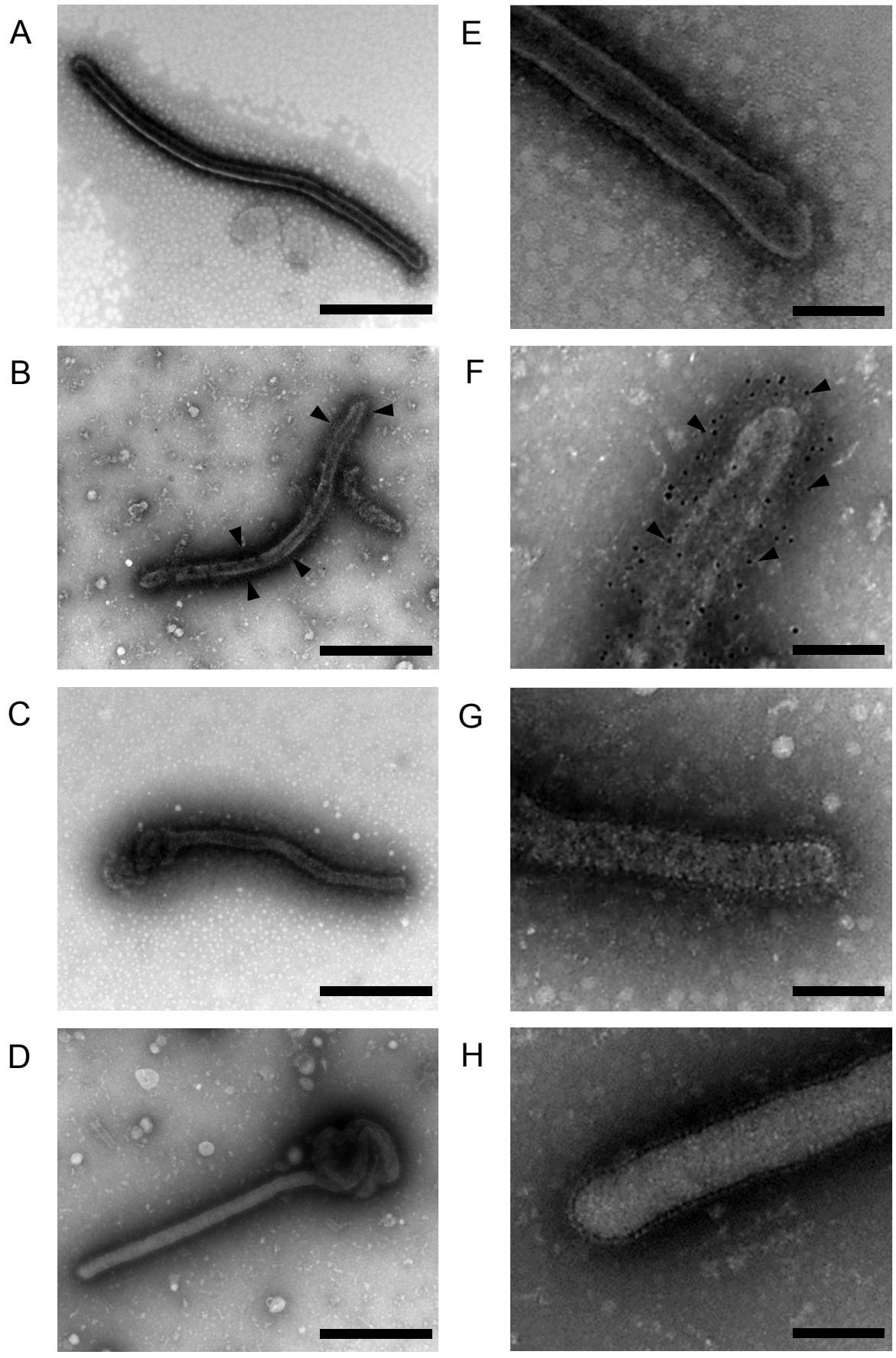


Fig. 2 Maruyama *et al.*

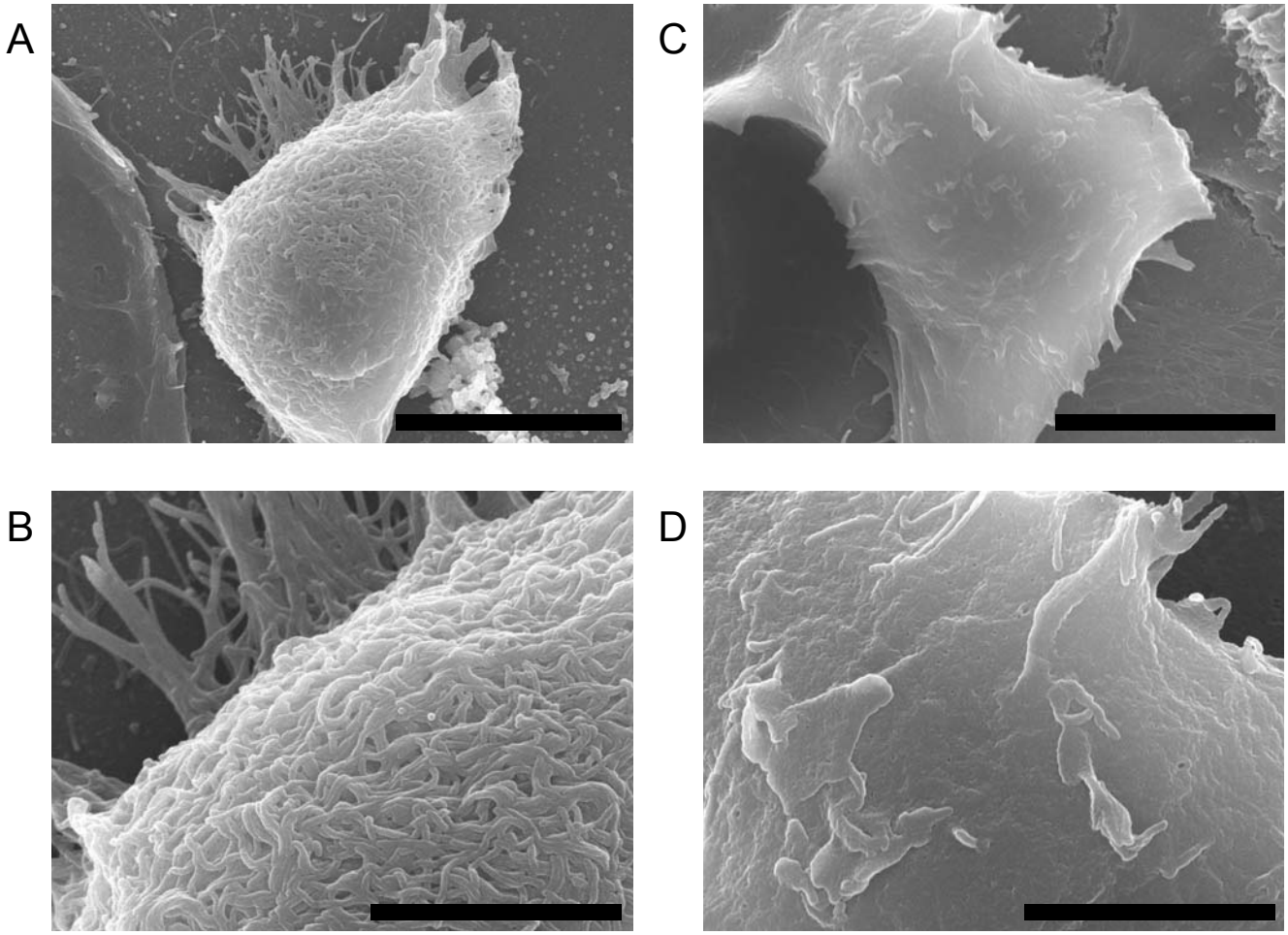


Fig. 3 Maruyama *et al.*

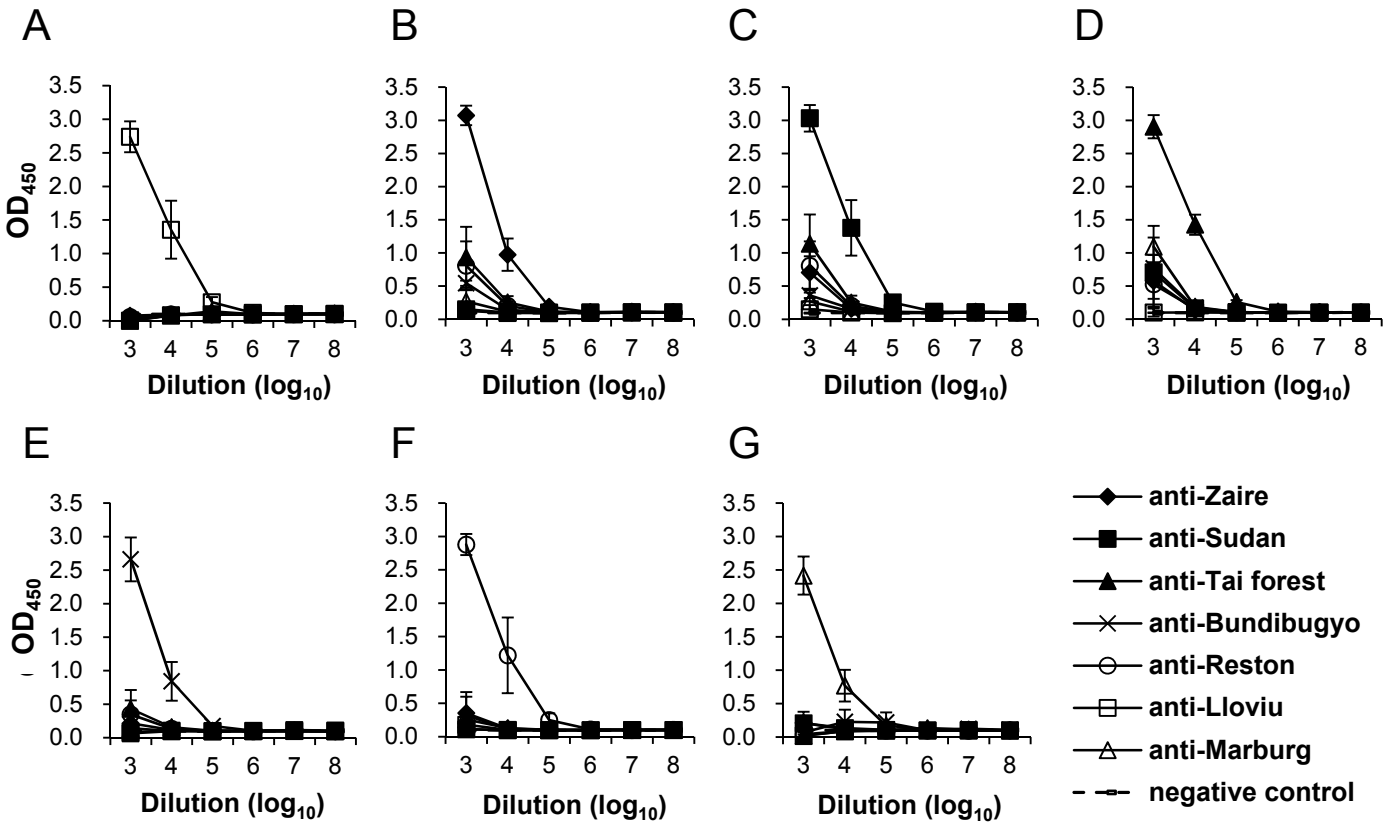
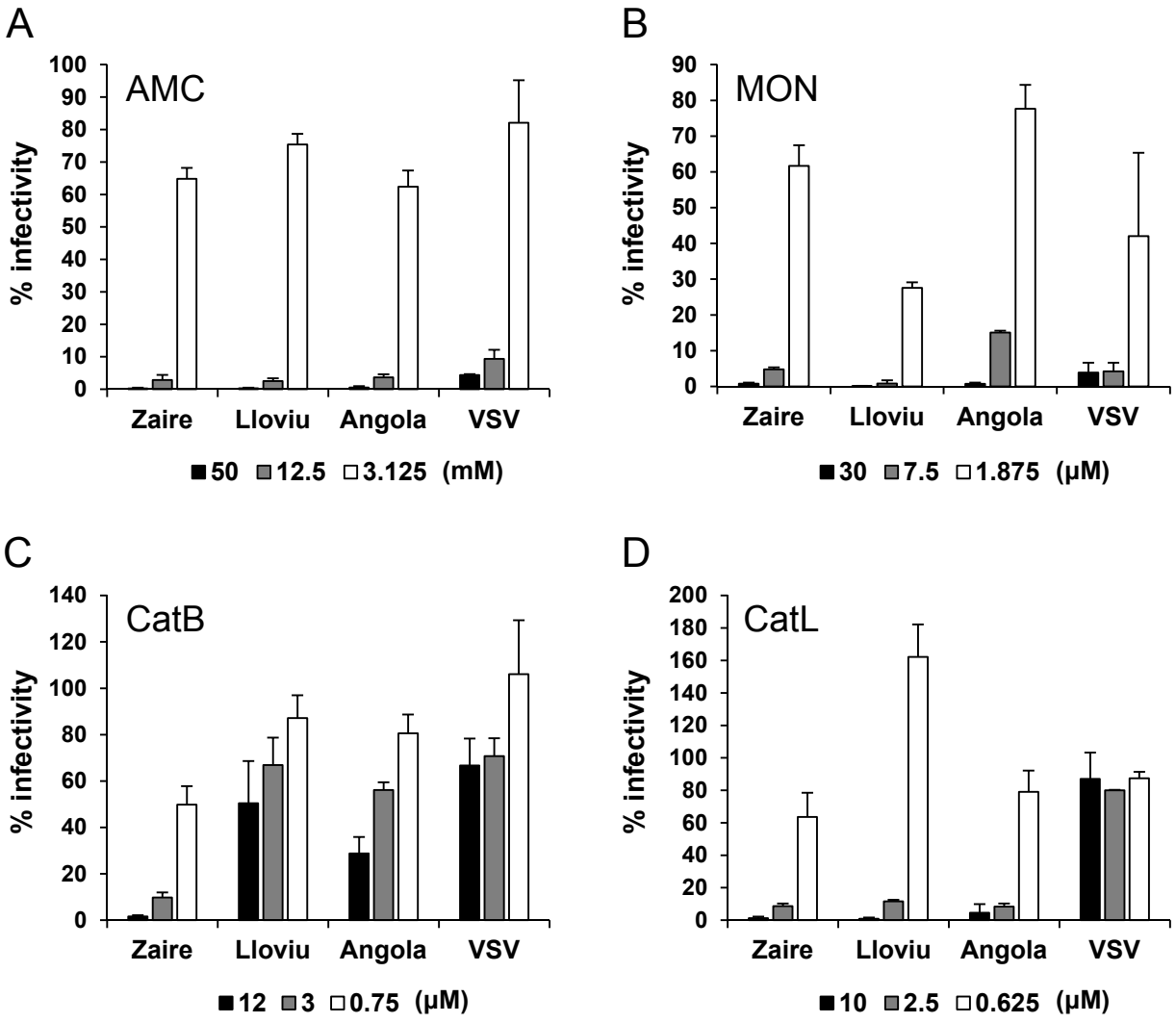


Fig. 4 Maruyama *et al.*



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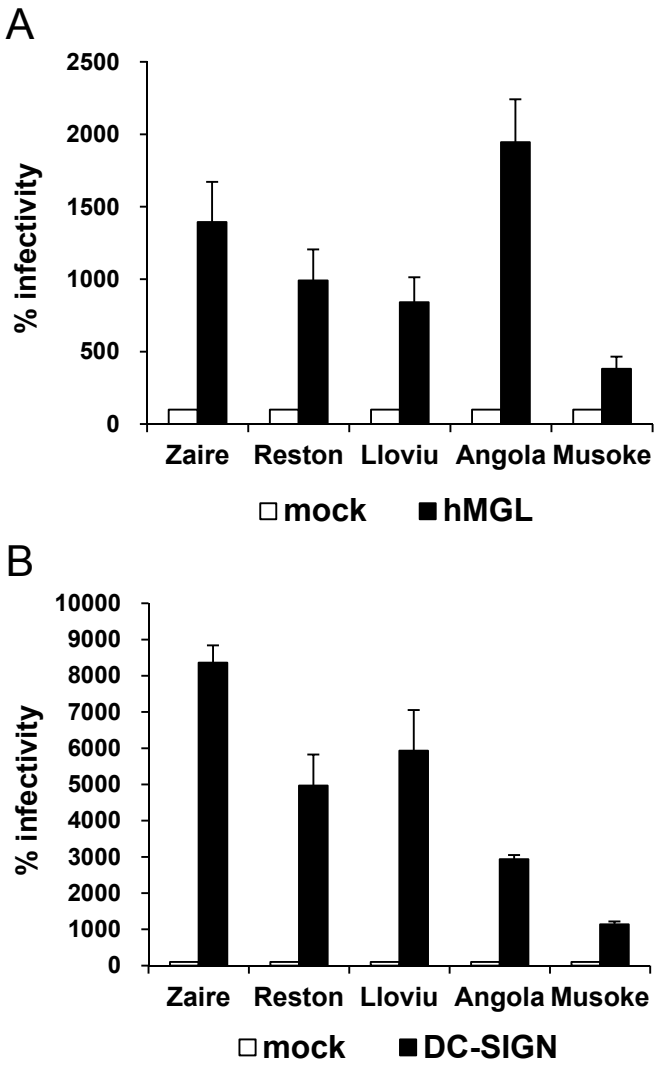

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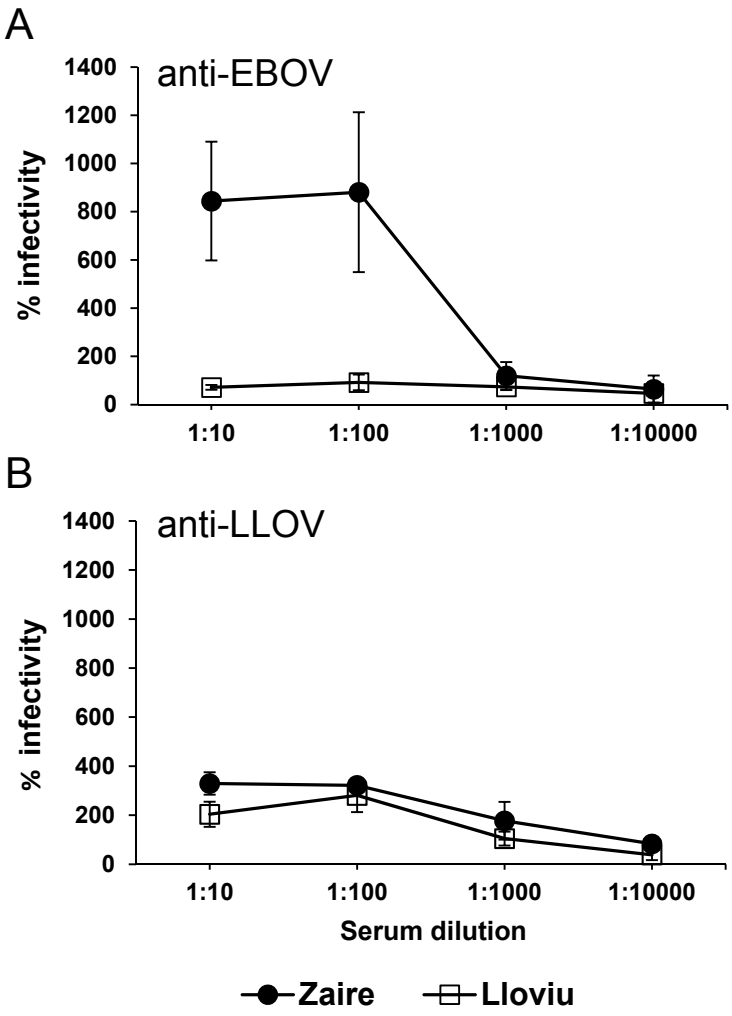

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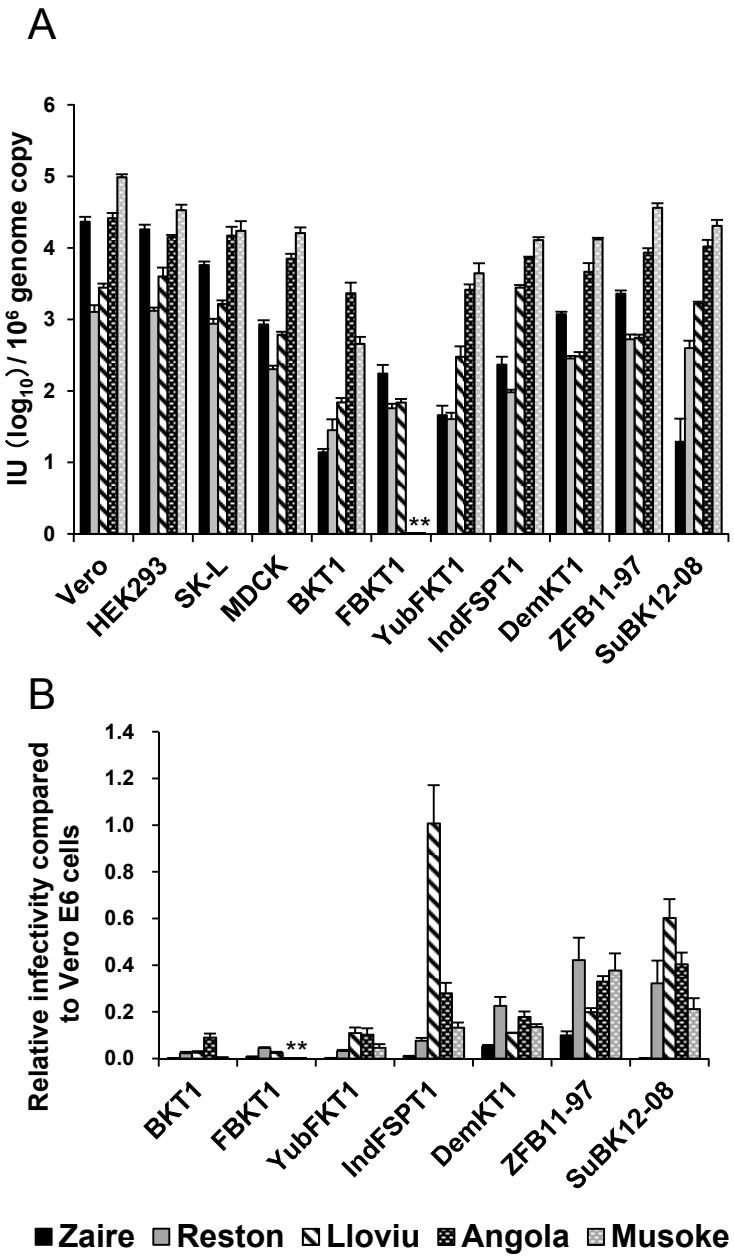

Fig. 8 Maruyama *et al.*