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Immunoglobulin G enzyme-linked immunosorbent assay using truncated nucleoproteins of Reston Ebola virus

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SUMMARY

We developed an immunoglobulin G (IgG) enzyme-linked immunosorbent assay (ELISA), using partial recombinant nucleoproteins (rNP) of Reston Ebola virus (EBO-R) and Zaire Ebola virus (EBO-Z). We examined the reaction of 10 sera from cynomolgus macaques naturally infected with EBO-R to each of the partial rNP in the IgG ELISA. All the sera reacted to the C-terminal halves of the rNP of both EBO-R and EBO-Z. Most of the sera reacted to the RΔC (amino acid (aa) 360–739), and RΔ6 (aa 451–551) and/or RΔ8 (aa 631–739) at a higher dilution than to the corresponding truncated rNPs of EBO-Z. The results indicate that this IgG ELISA is useful for detecting EBO-R specific antibody, and may have a potential to discriminate EBO-R infection from other subtypes.

INTRODUCTION

Ebola virus, which belongs to the family *Filoviridae*, order *Mononegavirales*, is divided into four subtypes: Zaire Ebola virus (EBO-Z), Sudan Ebola virus (EBO-S), Côte d'Ivoire Ebola virus (EBO-CI), and Reston Ebola virus (EBO-R) [1]. Ebola virus has a negative-stranded RNA genome which encodes nucleoprotein (NP), P protein (VP35), matrix protein (VP40), glycoprotein (GP), second nucleoprotein (VP30), protein associated with the membrane (VP24), and RNA-dependent RNA polymerase (L) [2, 3]. EBO-Z, EBO-S, and EBO-CI emerged in equatorial Africa, and are known to cause haemorrhagic fevers in humans [4–6]. Experimental infection has also demonstrated that EBO-Z causes a similar fatal disease in guinea-pigs and non-human primates [7–9]. EBO-R emerged in a monkey export and breeding facility in the Philippines and caused fatal illness among non-human primates [10, 11]. EBO-R-infected monkeys were exported to the United States in 1989, 1990 and 1996 [12–16], and to Italy in 1992 [17]. No symptomatic infection has been recorded in humans infected with EBO-R [11, 13, 14, 17].

The epidemiological situation concerning EBO-R in the Philippines and the other Asian countries is not known. This is partly due to the lack of an EBO-R antibody-detection test kit that can be applied to epidemiological studies [18, 19]. Recently enzyme-linked immunosorbent assay (ELISA) for detecting immunoglobulin G (IgG) to EBO-Z using the recombinant NP (rNP) has been developed [20, 21]. The use of recombinant proteins has the great advantage of preparing the antigens without any specified facility, and in modification of the antigens suitable for the assay. In the present study, we prepared a panel of the truncated rNPs of EBO-R and EBO-Z and developed

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Table 1. *Primers for the amplification of DNA encoding EBO-R NP and EBO-Z NP*

Primers for EBO-R NP	Sequences
RES-N5F	5′-GCT GGA TCC* AGA GAA CTC GAC AGC CT-3′
RES-N5R	5′-ACC GAA TTC† GGG GTC AAT TGC ACT AT-3′
RES N6F	5′-GAC GGA TCC* GAC ACT ATC ATT CCT AAT AGT GC-3′
RES-N6R	5′-TTC GAA TTC† TCG GTG CCT GTT GTA TT-3′
RES-N7F	5′-GCA GGA TCC* GAG GAA CAA GAA GGT CA-3′
RES-N7R	5′-CTT GAA TTC† ACC GAT ATC AGG GTC TT-3′
RES-N8F	5′-GCT GGA TCC* TCA CAA TTG AAT GAA GAC C-3′
RES-N8R	5′-GTG GAA TTC† TTA CTG ATG GTG CTG CAA-3′

* *Bam*HI recognition site.
† *Eco*RI recognition site.

an IgG ELISA using the rNPs. This new IgG ELISA demonstrated high specificity and sensitivity to detect EBO-R antibodies.

METHODS

Sera

Two and four rabbits were immunized four times with the histidine-tagged entire EBO-R rNP (His-EBO-R-NP) and the entire EBO-Z rNP (His-EBO-Z-NP), respectively, using Imject-Alum (Pierce, Rockford, USA). The His-EBO-R-NP and His-EBO-Z-NP were prepared and purified as described previously [21, 22]. One cynomolgus monkey was immunized four times at 2-week intervals with the His-EBO-Z-NP using Imject-Alum. The sera were collected at 7, 30 and 73 days post immunization and used in the present study.

Ten serum samples collected from cynomolgus macaques at a monkey export and breeding facility in the Philippines (Facility A) were used. This facility had experienced an EBO-R outbreak in 1996 [11]. These sera were determined to be EBO-R antibody-positive by indirect immunofluorescence assay (IFA) [22]. Three of these 10 macaques were demonstrated to have EBO-R antigens in the sera by antigen-capture ELISA [23]. Seventy-two sera were also collected from cynomolgus monkeys at another breeding facility in the Philippines (Facility B) where no EBO-R outbreak had ever occurred. These 72 sera were found to be negative for EBO-R antibodies by IFA [22].

Preparation of the glutathione S-transferase (GST)-tagged truncated Ebola NPs

The DNAs encoding the truncated NP of EBO-R were amplified by polymerase chain reaction (PCR) from the cDNA of EBO-R (DDBJ accession no. AB050936) using the primers shown in Table 1. The PCR fragments were digested with both *Bam*HI and *Eco*RI, purified and subcloned into a pGEX-2T vector (Amersham Pharmacia Biotech, Little Chalfont, UK). The sequences of the inserts were confirmed to be identical to the originals. The GST-tagged truncated NPs were expressed in *E. coli* (BL-21 strain) and purified using glutathione Sepharose 4B column chromatography, according to the manufacturer's instructions (Amersham Pharmacia Biotech). The GST-tagged truncated EBO-R rNPs included RΔC (amino acids (aa) 360–739), RΔ5 (aa 360–461), RΔ6 (aa 451–551), RΔ7 (aa 541–640) and RΔ8 (aa 631–739). The truncated EBO-Z rNPs, ZΔC (aa 361–739), ZΔ5 (aa 361–460), ZΔ6 (aa 451–552), ZΔ7 (aa 541–640) and ZΔ8 (aa 631–739), were as previously reported [21]. The GST alone was expressed and used as the negative control antigen in the IgG ELISA.

IgG ELISA using GST-tagged truncated Ebola NPs

Wells of microtitre plates (Becton Dickinson, NJ, USA) were coated with the unified amount of RΔC, RΔ5, RΔ6, RΔ7, RΔ8, ZΔC, ZΔ5, ZΔ6, ZΔ7, ZΔ8 and GST in 100 μl of PBS, and incubated overnight at 4 °C. The amounts of the antigens were determined as described below. The plates were washed three times with phosphate-buffered saline (PBS) containing 0·05% Tween 20 (PBS-T); 200 μl of PBS-T containing 0·5% bovine serum albumin (PBS-T-BSA) was added, and incubated for 1 h at 37 °C. The wild monkey sera were diluted at 1 in 100, 1 in 400, and 1 in 1600 in PBS-T-BSA, and the hyper-immune rabbits and monkey sera were twofold serially diluted from

Table 2. *Optimization of GST-Ebola rNPs* on the ELISA plate*

	RΔC	RΔ5	RΔ6	RΔ7	RΔ8	ZΔC	ZΔ5	ZΔ6	ZΔ7	ZΔ8	GST
The amount of coated antigen (ng/well)†	82	43	39	26	28	90	47	39	18	37	22
Mean OD values‡ plus 3 standard deviation of 72 sera from Ebola uninfected monkeys§	0·04	0·03	0·04	0·04	0·02	0·09	0·04	0·04	0·02	0·03	ND¶

* RΔC (aa 360–739), RΔ5 (aa 360–461), RΔ6 (aa 451–551), RΔ7 (aa 541–640), RΔ8 (aa 631–739), ZΔC (aa 361–739), ZΔ5 (aa 361–460), ZΔ6 (aa 451–552), ZΔ7 (aa 541–640), ZΔ8 (aa 631–739).

† The amount of each GST-Ebola rNP used for IgG ELISA was determined according to the antigenicity of GST-tag using an anti-GST goat polyclonal antibody at a dilution of 1 in 500.

‡ OD value for GST was subtracted from that for each GST-Ebola rNP.

§ Cynomolgus monkeys derived from a monkey breeding facility (B) in the Philippines that have not experienced any Ebola outbreaks.

¶ Not done.

1 in 100 to 1 in 6400 in PBS-T-BSA; 100 μl of each serum dilution was added to the antigen-coated wells, and incubated for 1 h at 37 °C. After washing three times with PBS-T, the wells were reacted with 100 μl of horseradish peroxidase (HRP)-conjugated goat anti-human IgG (Zymed Laboratories Inc., CA) for monkey sera, or HRP-conjugated goat anti-rabbit IgG (Zymed Laboratories Inc.) for rabbit sera, at a dilution of 1 in 1000 in PBS-T-BSA. The plates were then incubated for 1 h at 37 °C. After washing three times with PBS-T, ABTS substrate (ABTS tablet and ABTS buffer; Roche Diagnostics, Mannheim, Germany) was added to the wells. The plates were then incubated for 30 min at room temperature and optical density (OD) at 405 nm were recorded. For each sample, the adjusted OD value was calculated by subtracting the OD of GST-coated well from that of GST-fusion antigen-coated well. The mean plus three standard deviation of the adjusted OD value of 72 serum samples from Ebola virus uninfected cynomolgus monkeys to each GST-tagged, truncated Ebola rNPs was lower than 0·1 (Table 2). Therefore, the cut-off value of the IgG ELISA was determined to be 0·1. The antibody titres of serum samples were defined as the reciprocals of the highest dilution yielding a positive value.

The optimization of GST-tagged proteins on ELISA plate

The amount of coated antigens on an ELISA plate was standardized according to the antigenicity of GST-tag. Briefly, several dilutions of the GST-tagged truncated Ebola rNPs were coated on a microtitre plate (Becton Dickinson, NJ, USA.). Then, the goat anti-GST polyclonal antibody (Amersham Pharmacia Biotech) and the HRP-conjugated anti-goat IgG rabbit polyclonal antibody (Zymed Laboratories Inc.) were added as the primary and secondary antibodies at dilutions of 1 in 500 and 1 in 1000, respectively. An OD value of 0·2 was taken as the cut-off value to determine the end point dilution of each GST-tagged antigen. The dilution of eight times lower than the each end point dilution of each GST-tagged antigen was defined as the amount of antigen coating. The amount of each GST-tagged antigen used for the IgG ELISA in this study is shown in Table 2.

Indirect immunofluorescence assay (IFA)

The entire NP of EBO-R or EBO-Z was stably expressed in HeLa cells as reported previously [22, 24].

The HeLa cells were trypsinized, washed with PBS, spotted on 14-well Teflon-coated slide glasses (AR Brown Co., Ltd., Tokyo, Japan), air dried and fixed with acetone at room temperature for 5 min. The slides were stored at −80 °C until use. The slides were thawed and dried just before use; 20 μl of diluted serum was spotted on the well of the slide, and incubated under humidified conditions at 37 °C for 1 h. After washing with PBS, the slides were reacted with fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgG antibody (Zymed Laboratories Inc.) at a dilution of 1 in 100 or with FITC-conjugated goat anti-rabbit IgG (Zymed Laboratories Inc.) at a dilution of 1 in 100. The slides were washed with PBS and examined for staining pattern under a fluorescent microscope. The antibody titre in the IFA was defined as the reciprocal of the highest dilution showing positive staining.

RESULTS

Reaction of hyper-immune sera to each truncated rNP of EBO-R and EBO-Z in the IgG ELISA

Reaction of EBO-R or EBO-Z hyper-immune rabbit sera and the EBO-Z hyper-immune monkey sera were examined by the IgG ELISAs with truncated EBO-R rNPs or EBO-Z rNPs (Fig. 1*a*, Fig. 1*b*, Table 3). The sera from EBO-R rNP-immunized rabbits (nos. 1 and 2) and those from EBO-Z rNP-immunized rabbits (nos. 3–6) reacted to RΔC, RΔ8, ZΔC and ZΔ8 at the titre of 6400 (Table 3). All the sera from EBO-R rNP-immunized rabbits reacted to RΔ5, RΔ6, RΔ7 and RΔ8, while the sera did not react to ZΔ6 and ZΔ7. On the other hand, all the sera from EBO-Z rNP-immunized rabbits reacted to ZΔ5, ZΔ6 and ZΔ8, while two of them did not react to RΔ6 and RΔ7.

The sera serially collected from the monkey immunized with the EBO-Z rNP were also examined (Fig. 1*b*, Table 3). The day 7 serum reacted to ZΔC, ZΔ5 and ZΔ6. The day 30 serum reacted to ZΔC, ZΔ5, ZΔ6 and ZΔ7, and the day 73 serum reacted to ZΔC, ZΔ5 and ZΔ6 at higher titres. Furthermore, the day 73 serum also reacted to ZΔ8, RΔC, RΔ5 and RΔ6.

Reaction of the sera from EBO-R infected monkeys to each truncated rNP of EBO-R and EBO-Z in the IgG ELISA

Ten IFA antibody positive monkey sera collected at the Facility A in the Philippines were examined for the reaction in the IgG ELISA (Fig. 1*c*, Table 3). All the sera reacted to RΔC and ZΔC. Seven, 9, 5 and 7 of the 10 sera reacted to RΔ5, RΔ6, RΔ7 and RΔ8,

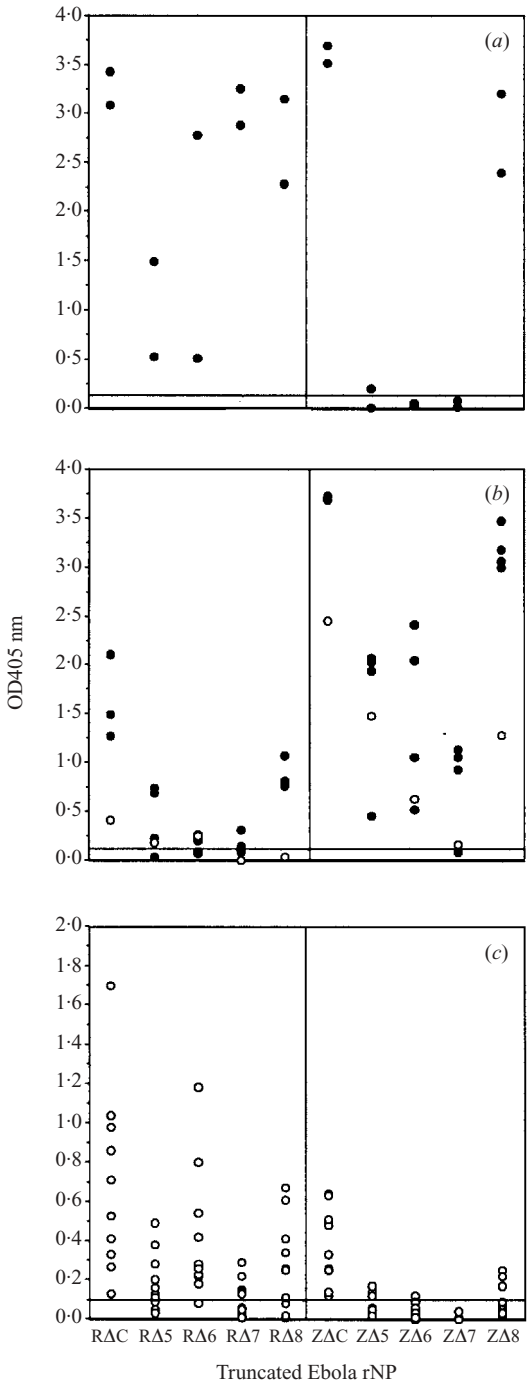

Fig. 1. The OD values at 405 nm in the IgG ELISA using truncated Ebola rNPs. (*a*) sera collected from two rabbits immunized with EBO-R rNP, (*b*) sera collected from four rabbits immunized with EBO-Z rNP (●) and from one monkey immunized with EBO-Z rNP at day 73 (○), (*c*) sera from 10 EBO-R infected monkeys. Each serum was tested at 1 in 100 dilution.

Table 3. *The reactivities of hyper-immune sera and the sera from cynomolgus monkeys naturally infected with EBO-R to the truncated rNPs* of EBO-R and EBO-Z in the IgG-ELISA*

	Titre with EBO-R rNP					Titre with EBO-Z rNP					IFA titre	
Serum samples	RΔC	RΔ5	RΔ6	RΔ7	RΔ8	ZΔC	ZΔ5	ZΔ6	ZΔ7	ZΔ8	EBO-R	EBO-Z
I. Rabbits immunized with EBO-R rNP												
No. 1	>6400†	>6400	>6400	>6400	>6400	>6400	400	—	—	>6400	2560	320
No. 2	>6400	800	400	>6400	>6400	>6400	—	—	—	>6400	1280	1280
II. Rabbits immunized with EBP-Z rNP												
No. 3	>6400	200	200	100	>6400	>6400	>6400	>6400	3200	>6400	2560	2560
No. 4	>6400	—	—	—	>6400	>6400	800	800	—	>6400	1280	5120
No. 5	>6400	1600	—	—	>6400	>6400	>6400	3200	>6400	>6400	2560	5120
No. 6	>6400	3200	200	400	>6400	>6400	>6400	>6400	>6400	>6400	40 960	81 920
III. A monkey immunized with EBO-Z rNP												
Day 7	—	—	—	—	—	200	100	800	—	—	<20	<20
Day 30	—	—	—	—	—	800	400	800	100	—	160	1280
Day 73	400	100	200	—	—	>6400	>6400	3200	100	3200	1280	5120
IV. EBO-R infected monkeys in Facility A‡												
No. 2728§	>1600	—	>1600	—	—	100	—	—	—	—	1280	320
No. 2669§	400	100	400	—	—	100	—	100	—	—	2560	640
No. 2921	>1600	—	400	400	—	100	—	—	—	—	1280	320
No. 2194	>1600	400	>1600	100	400	400	100	—	—	—	5120	2560
No. 2739§	100	—	—	100	100	100	—	—	—	—	1280	1280
No. 2408	>1600	100	>1600	400	>1600	400	—	—	—	100	10 240	1280
No. 2190	400	100	100	—	100	100	100	—	—	—	160	160
No. 2191	400	100	100	—	400	400	100	—	—	100	640	640
No. 2195	>1600	400	>1600	100	>1600	400	—	—	—	100	2560	1280
No. 2180	>1600	400	100	—	400	400	—	—	—	100	1280	160

* RΔC (aa 360–739), RΔ5 (aa 360–461), RΔ6 (aa 451–551), RΔ7 (aa 541–640), RΔ8 (aa 631–739), ZΔC (aa 361–739), ZΔ5 (aa 361–460), ZΔ6 (aa 451–552), ZΔ7 (aa 541–640), ZΔ8 (aa 631–739).

† OD of GST-Ebola rNPs was subtracted by that of GST, and the cut off value was determined to be 0·1 on the basis of the results of 72 Ebola virus uninfected sera.

‡ Several EBO-R outbreaks have occurred in Facility A in the Philippines.

§ EBO-R NP antigens were detected from the sera of Nos. 2728, 2669 and 2739 by antigen-capture ELISA [23] at the dilution of 1 in 20, 1 in 640 and 1 in 320, respectively. The (—) means negative at a dilution of 1 in 100.

respectively. Three, 1 and 4 of the 10 sera reacted to ZΔ5, ZΔ6 and ZΔ8, respectively, while none reacted to ZΔ7. The titres were at least 4 times higher for RΔC than for ZΔC in 8 of the 10 sera in the IgG. However, only 5 sera reacted to EBO-R rNP at least 4 times higher titre than to EBO-Z rNP in IFA.

DISCUSSION

In the present study, we developed the IgG ELISAs using the truncated rNPs of Ebola viruses. The reactions of Ebola antibody positive sera to the truncated rNPs of EBO-R and EBO-Z were analysed by the IgG ELISA. The truncated rNPs used in the IgG ELISAs covered the C-terminal halves of the NPs of EBO-R and EBO-Z. It has been reported that the C-terminal halves of the NPs are hydrophilic and antigenic, while the N-terminal halves are hydrophobic and far less antigenic [19, 21, 25].

All the hyper-immune rabbit sera reacted strongly to RΔC, RΔ8, ZΔC and ZΔ8 in the IgG ELISA. The EBO-Z rNP-immune monkey serum collected on day 73 after immunization reacted to ZΔC and RΔC. Ten sera from EBO-R infected monkeys that died or were sacrificed during the EBO-R outbreak in the Philippines in 1996 were also examined by the IgG ELISA. These 10 sera were confirmed to be EBO-R antibody positive by IFA. All the 10 sera reacted to RΔC and ZΔC in the IgG ELISA. Seven of the 10 sera also reacted to RΔ8, and four (nos. 2408, 2191, 2195 and 2180) of them further cross-reacted to ZΔ8. Similar reaction pattern was demonstrated by Western blotting (data not shown). The results suggest that ΔC and Δ8 contains cross-reactive epitopes between EBO-R and EBO-Z, and that the IgG ELISA using RΔC has a suitable degree of sensitivity compared with IFA using HeLa cells expressing EBO-R rNP. Eight of the 10 sera from EBO-R infected monkeys reacted to RΔC at least 4 times higher titre than to ZΔC in the IgG ELISA, while only 5 sera reacted to EBO-R rNP at least 4 times higher titre than to EBO-Z rNP in IFA. Recent reports demonstrated that humoral immune responses were mainly directed against the NP and the VP40 in Ebola virus infected humans [26, 27]. Therefore, the IgG ELISA using RΔC and ZΔC would be useful for detecting subtype-specific antibodies. Furthermore, 6 and 5 of the 10 sera reacted to RΔ6 and RΔ8 at a dilution of 1 in 400 or greater, respectively. The results suggest that the reaction to RΔC, RΔ6 and/or RΔ8 can be considered as a clue for truly positive reaction.

Several diagnostic methods have been developed to detect Ebola-specific antibodies. It has been reported that many of these methods lack the specificity in detecting past filovirus infections. Thus, previous serological surveys could not illustrate the epidemiology of the filoviruses [18, 19]. In this regard, the newly developed IgG ELISA using the truncated rNPs might be more useful for seroepidemiological studies, especially in combination with IFA using HeLa cells expressing Ebola rNP [22, 24].

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