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Detection of Antibodies against the Four Subtypes of Ebola Virus in Sera from Any Species Using a Novel Antibody-Phage Indicator Assay

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The natural host for Ebola virus, presumed to be an animal, has not yet been identified despite an extensive search following several major outbreaks in Africa. A straightforward approach used to determine animal contact with Ebola virus is by assessing the presence of specific antibodies in serum. This approach however has been made very difficult by the absence of specific reagents required for the detection of antibodies from the majority of wild animal species. In this study, we isolated a human monoclonal antibody Fab fragment, KZ51, that reacts with an immunodominant epitope on Ebola virus nucleoprotein (NP) that is conserved on all four Ebola virus subtypes. The antibody KZ51 represents a major specificity as sera from all convalescent patients tested (10/10) and sera from guinea pigs infected with each of the four Ebola virus subtypes competed strongly with KZ51 for binding to radiation-inactivated Ebola virus. These features allowed us to develop a novel assay for the detection of seroconversion irrespective of Ebola virus subtype or animal species. In this assay, the binding of KZ51 Fab-phage particles is used as an indicator assay and the presence of specific antibodies against Ebola virus in sera is indicated by binding competition. A prominent feature of the assay is that the Fab-phage particles may be prestained with a dye so that detection of binding can be directly determined by visual inspection. The assay is designed to be both simple and economical to enable its use in the field.

INTRODUCTION

Infection with Ebola virus produces a hemorrhagic fever in humans and certain nonhuman primates that is associated with very high case-fatality rates (80–90% in some outbreaks). Four subtypes of Ebola virus have been described. Three subtypes, Ebola Zaire, Sudan, and Ivory Coast, have been linked to infections in rainforests on the African continent. Ebola Zaire and Sudan viruses are the most widespread and have been responsible for all ~1000 known lethal cases of Ebola virus hemorrhagic fever in humans (Burton and Parren, 2000; Sanchez *et al.,* 2001; Wilson *et al.,* 2001). Ebola Ivory Coast virus was linked to a deadly epidemic among a chimpanzee community in the rainforest of Ivory Coast. The virus was transmitted to a researcher performing a necropsy, who became severely ill but survived (Formenty *et al.,* 1999a,b). The fourth subtype, Ebola Reston virus, has been found in monkeys imported into the United States and Italy from an export facility in the Philippines. The source of Ebola Reston virus infection of the monkeys in this facility however remains unknown. A number of animal care workers in the United States and the Philippines seroconverted but did not become ill. In contrast to the African Ebola viruses, Ebola Reston therefore does not appear to be pathogenic for humans (Rollin *et al.,* 1999; Miranda *et al.,* 1999). The four Ebola virus subtypes can be distinguished from each other on the basis of serological, protein chemical, and molecular biological techniques (Buchmeier *et al.,* 1983; Feldmann and Klenk, 1996; Sanchez *et al.,* 1996). The glycoprotein (GP) is the least conserved Ebola virus protein, differing at 34–43% of amino acid positions between heterologous subtypes (Sanchez *et al.,* 1993; Sanchez *et al.,* 1996; Feldmann and Kiley, 1999). Comparisons of Ebola viruses isolated from individuals at different time points in an outbreak as well as viruses from the same subtype isolated from distinct outbreaks have demonstrated a remarkable sequence conservation. Thus, the Ebola Zaire viral strain isolated in Yambuku, Democratic Republic of Congo (DRC) in 1976 differed at only 1.5% of GP amino acids from the strain isolated in Kikwit, DRC in 1995, although almost two decades and a geographical distance in excess of 600 miles separated these two outbreaks (Sanchez *et al.,* 1996, 1999).

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Although Ebola virus hemorrhagic fever outbreaks have been sporadic and relatively few people have died from the disease compared to other more widespread infectious diseases, the unpredictable, explosive, and highly lethal nature of the disease has been disturbing. A particular reason for concern has been the elusive nature of the natural Ebola virus reservoir and possible intermediate hosts. Small mammals, particularly bats, are often considered the most likely reservoir based on (1) *in vitro* and *in vivo* challenge studies (Swanepoel *et al.,* 1996; Turell *et al.,* 1996); (2) theoretical considerations such as similarities in proposed Ebola GP structure with certain vertebrate RNA viruses (Gallaher, 1996; Weissenhorn *et al.,* 1998; Gallaher *et al.,* 2001); and (3) the very high level of GP glycosylation (Sanchez *et al.,* 1998; Feldmann *et al.,* 1994) often linked to the evasion of neutralizing antibody responses in higher animals. A number of extensive field studies have been carried out following outbreaks in the DRC and Sudan in 1976 and again in the DRC in 1995 (Breman *et al.,* 1999; Reiter *et al.,* 1999; Leirs *et al.,* 1999). Literally thousands of animals were collected and analyzed. Vertebrates were assessed for the presence or contact history with Ebola virus by virus isolation attempts and/or serological analyses to detect the presence of Ebola virus antigens or specific antibodies. All these tests have come up negative (Breman *et al.,* 1999; Reiter *et al.,* 1999; Leirs *et al.,* 1999). It is possible that the Ebola virus host or even infection of the host is extremely rare and therefore, despite all effort, infected animals have not been collected. Alternatively, however, limitations of the assays used may have made detection difficult. First, virus isolation and antigen detection assays are unreliable for the exclusion of certain species as possible hosts as the virus may be sequestered or cleared from the circulation and/or viral titers may be too low to detect with these techniques. Second, serological assays suffer from the paucity of specific reagents to detect antibodies in sera from a wide range of wild animal species (Breman *et al.,* 1999).

To aid in the identification of anti-Ebola virus antibodies in human and animal sera, we set out to design an assay that would enable us to detect serum antibodies against all four subtypes of Ebola virus independent of the availability of reagents reactive with immunoglobulins from a wide range of animals. The assay described here is based on the human antibody KZ51, which reacts with a conserved immunodominant epitope on NP.

RESULTS

Isolation of Ebola virus-specific Fab fragments

Antibody Fab fragment phage display libraries prepared from bone marrows of two convalescent donors (designated K and L) and from pooled PBMC from 10 convalescent donors (designated E10), who were infected with Ebola Zaire virus during the 1995 outbreak in Kikwit, DRC, have been described previously (Maruyama *et al.,* 1999a,b). Selection of these libraries against a γ-irradiation-inactivated preparation of Ebola Zaire (Mayinga) virions yielded a panel of Fab fragments against Ebola virus: two against NP, one against GP, and four against sGP (Maruyama *et al.,* 1999b). To obtain more antibodies against Ebola virus, we prepared an additional library from the bone marrow of a rhesus macaque (designated J3P) who survived a high-dose laboratory challenge with Ebola Zaire virus. This library contained a diversity of 1×10^6 clones and was also selected against inactivated Ebola Zaire virus. Three novel Fab fragments against Ebola virus were identified. One Fab (J3P-K14) reacted strongly in ELISA with purified Ebola virions but weakly with a crude Ebola virus-infected cell supernatant; the other two Fab fragments (J3P-K9 and -K11) reacted relatively weakly with both these antigens (not shown). These patterns of reactivity are characteristic for antibodies against NP and GP, respectively (Maruyama *et al.,* 1999a). The reactivity of J3P-K14 with Ebola virus NP was confirmed in Western blot (see below).

The deduced amino acid sequences for the variable regions of light and heavy chains of the human and monkey antibodies against NP have been submitted to GenBank under Accession Nos. AY099259 through AY099264. We aligned the antibodies with a human immunoglobulin gene database (http://imgt.cnusc.fr:8104/) to determine the human germ-line genes most homologous to the sequences of these antibodies (Lefranc and Lefranc, 2001). Comprehensive sequencing of immunoglobulin variable region germ-line genes has not been performed for rhesus macaques. However as there is extensive sequence homology between humans and monkeys (Lewis *et al.,* 1993; Ermert *et al.,* 1995; Glamann *et al.,* 1998), we have indicated the closest human germline genes for comparison (Table 1). KZ51 and J3P-K14 are both IgG1,κ and ELZ510 is an IgG1,λ.

Identification of a broadly cross-reactive antibody against Ebola NP

We have previously reported that Fab KZ51 and ELZ510 reacted with NP of Ebola Zaire (Maruyama *et al.,* 1999b). To investigate whether the antibodies against NP reacted with a conserved epitope, we now performed Western blots with radiation-inactivated whole-virion preparations of all four Ebola virus subtypes: Zaire, Ivory Coast, Reston, and Sudan, as shown in Fig. 1. Interestingly, Fab KZ51 reacted with a protein of ~100 kDa MW, corresponding to Ebola virus NP, from all four subtypes. Staining of NP in Western blot was observed both with purified KZ51 Fab fragment and with KZ51-phage (Fab KZ51 displayed on the surface of filamentous phage linked to the minor bacteriophage coat protein III) (Fig. 1; Lanes 1 through 8). The monkey Fab J3P-K14, in contrast,

TABLE 1

Determination of Germ-Line Gene Usage[a]

Antibody	Isotype	VH	D	JH	VL	JL
KZ51	IgG1, κ	IGHV1-2	cnd[b]	IGHJ6	IGKV3-11	IGKJ4
ELZ510	IgG1, λ	IGHV3-23	IGHD2-2	IGHJ4	IGLV1-47	IGLJ3
J3P-K14 (monkey)[c]	IgG1, κ	IGHV4-59	cnd	IGHJ4	IGKV1-39	IGKJ3

[a] The closest germ-line gene found in a database search (http://imgt.cnusc.fr:8104/) is shown.

[b] cnd, could not be determined.

[c] Ig germ-line genes have not been comprehensively sequenced for rhesus macaques. The genes with highest homology found in alignments with the human repertoire are shown.

was specific for NP of Ebola Zaire virus and did not bind to NP from the remaining three subtypes (Fig. 1; Lanes 9 through 12). Radioimmunoprecipitation assays using lysates of Vero E6 cells infected with each of the four Ebola virus subtypes and metabolically labeled with [^{35}S]Cys-[^{35}S]Met, confirmed the Western blot analysis. In these assays, KZ51 immunoprecipitated a protein of ~100 kDa MW for all Ebola virus-infected cells irrespective of subtype, but not from mock-infected cells. Fab ELZ510 only precipitated NP from Ebola Zaire- and Ebola Ivory Coast-infected cells (data not shown).

Detection of seroconversion against Ebola Zaire virus in human sera using a Fab-phage competition ELISA

Our goal was to design an immunoassay which could detect the presence of antibodies against Ebola virus without the requirement for specific anti-antibody conjugates. We therefore set up a competition ELISA, in which the binding of Fab KZ51-phage or Fab J3P-K14-phage to immobilized Ebola Zaire virus NP was blocked by the additional sera from Ebola Zaire virus-seropositive individuals. Binding of the Fab-phage particles was detected with an HRPO-labeled sheep antibody against the M13 bacteriophage. The sera used in this analysis were derived from 10 individuals who survived infection with Ebola Zaire virus during the outbreak in Kikwit, DRC in 1995. The results shown in Fig. 2 demonstrate that all 10 sera tested competed for binding in ELISA with both KZ51-phage and J3P-K14-phage and that competition with KZ51-phage was superior to that of J3P-K14-phage.

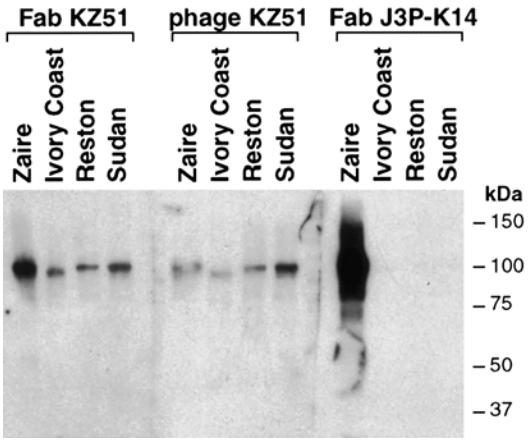

FIG. 1. Western blot of virions from the four Ebola virus subtypes (Ebola Zaire, Ivory Coast, Reston, and Sudan) stained with purified Fab KZ51 (Lanes 1–4), KZ51-phage (Lanes 5–8), and Fab J3P-K14 (Lanes 9–12). The relative molecular weights of marker proteins are shown in kDa. The Fab fragments (KZ51 and J3P-K14) and the KZ51-phage were stained with a goat anti-human F(ab′)$_2$-HRPO conjugate and a sheep anti-M13-HRPO conjugate, respectively, followed by chemiluminescence substrate and exposure to photographic film.

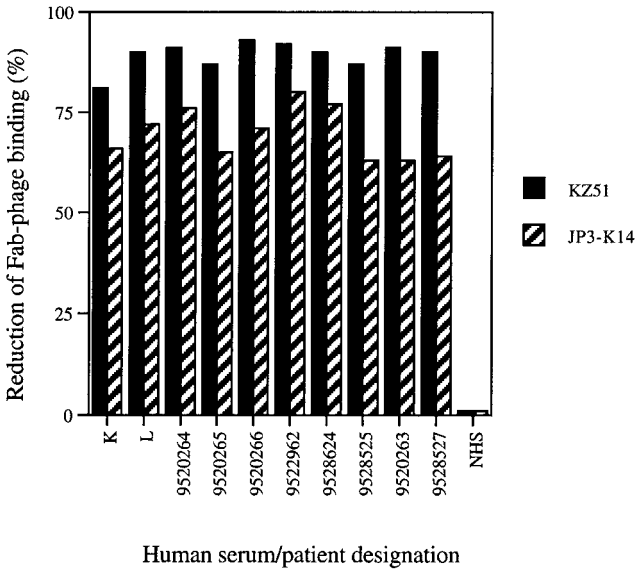

FIG. 2. Competition ELISA of KZ51- and J3P-K14-phage with 10 human sera from persons who survived infection with Ebola Zaire virus. Radiation-inactivated Ebola Zaire virions were coated on to the plate and incubated with the human sera diluted 1:10 followed by KZ51-phage of J3P-K14-phage. Following washing, bound Fab-phage was detected using a sheep anti-M13-HRPO conjugate. Binding reduction was determined as a percentage from wells incubated with Fab-phage in the absence of human anti-Ebola virus antibody. K and L indicate sera from the two individuals from whom antibody phage-display libraries have been prepared. The sera coded 95xxxxx are from individuals who also survived infection with Ebola Zaire virus during the outbreak in Kikwit, DRC. NHS indicates a representative normal human control serum.

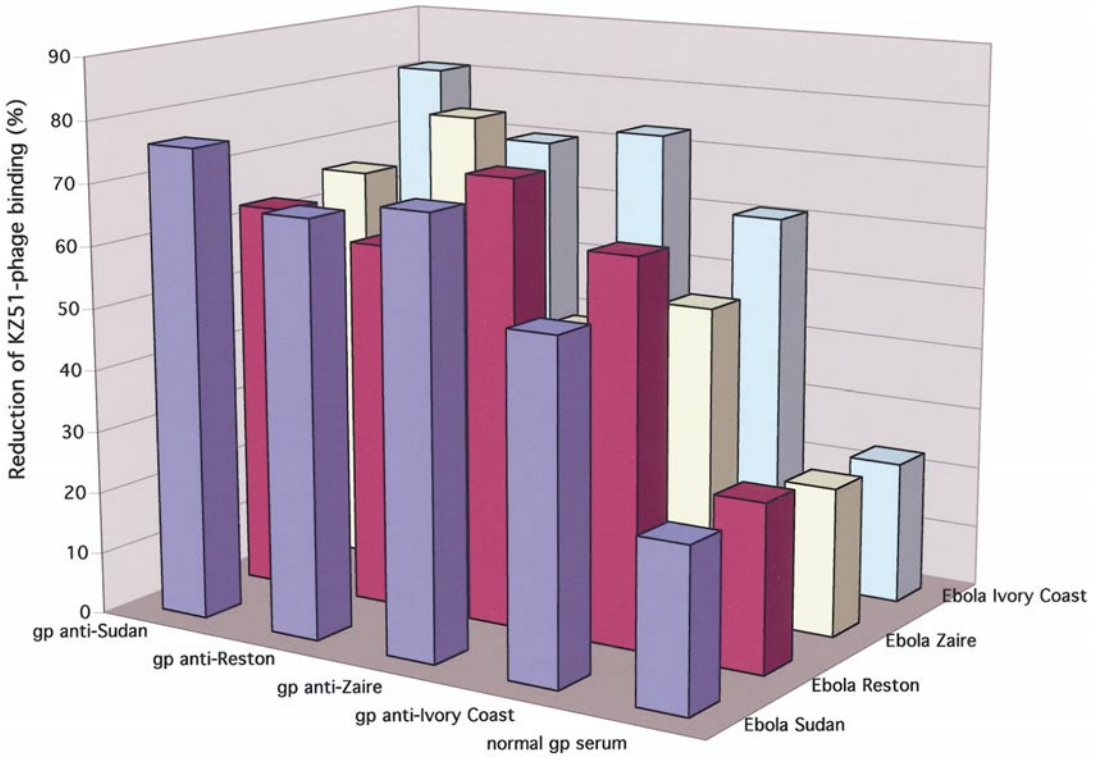

FIG. 3. Competition ELISA of KZ51-phage with sera from guinea pigs infected with Ebola virus subtypes. Radiation-inactivated Ebola virions (Sudan (dark blue), Reston (red), Zaire (yellow), and Ivory coast (light blue) subtypes) were coated to the plate and incubated with the guinea pig sera diluted 1:10 followed by KZ51-phage. Following washing, bound KZ51-phage was detected using a sheep anti-M13-HRPO conjugate. Binding reduction was determined as a percentage from wells incubated with KZ51-phage in the absence of guinea pig anti-Ebola virus antibody (shown on the vertical axis). The specificity of the guinea pig antisera used is indicated on the front horizontal axis and the virions immobilized on the plate are indicated on the right-hand-side horizontal axis. The data shown are representative for three independent competition experiments.

Detection of seroconversion against Ebola virus in animal sera using KZ51-phage competition ELISA

As the KZ51-phage behaved best in competition ELISA with convalescent human sera (Fig. 2), and KZ51-phage bound to NP from all four Ebola virus subtypes (Fig. 1), we continued working with this phage exclusively. Figure 3 shows the results of a series of KZ51-phage competition ELISAs with sera derived from guinea pigs that each survived infection with one of the four Ebola virus subtypes. The sera from the guinea pigs infected with Ebola Zaire, Reston, and Sudan all had high serum titers (as defined as the serum dilution at which an ELISA signal $\geq 2 \times$ background was achieved) against the homologous virus in ELISA ($\geq$1:50,000), whereas the serum titer of the guinea pig infected with Ebola Ivory Coast was lower ($\sim$1: 2000). Competition ELISAs were carried out for binding of KZ51-phage to immobilized inactivated Ebola virus for all four subtypes with each serum (Fig. 3). Figure 3 shows that sera from Ebola Zaire, Sudan, Ivory Coast, and Reston virus-infected guinea pigs generally compete well for KZ51-phage binding to Ebola virus irrespective of subtype immobilized on the plate. The only exception is the antiserum against Ebola Reston virus which reproducibly competes relatively weakly for binding of KZ51-phage to immobilized Ebola Zaire virus (44% reduction in Fig. 3). Some reduction of KZ51-phage binding was observed in the presence of normal guinea pig serum (23–27% in Fig. 3). To investigate whether this low level of inhibition by nonimmune sera was a general phenomenon, we performed competition experiments with control sera from a number of species, including guinea pig, goat, rabbit, and human. Some inhibition of

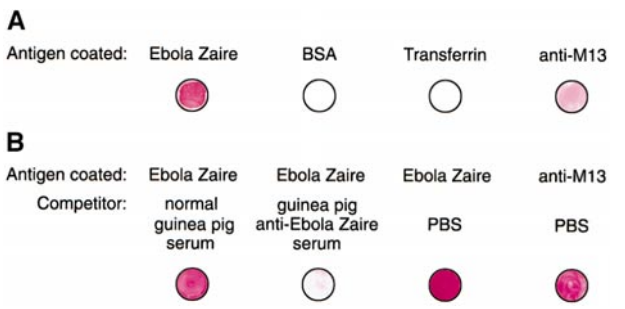

FIG. 4. PhAST immunoassay using red-colored KZ51-phage. (A) Binding of red-stained KZ51 phage to inactivated Ebola Zaire virus, BSA, transferrin, and sheep anti-M13 immobilized on nitrocellulose circles. (B) Competition PhAST immunoassay in which the binding of red KZ51-phage to immobilized Ebola Zaire virus was inhibited with a normal guinea pig serum, a guinea pig antiserum against Ebola Zaire, and PBS. The fourth spot shows the binding of red KZ51-phage to sheep anti-M13 as a positive control.

KZ51-phage binding occurred with all these sera ranging from a minimum of 7% for the human serum to a maximum of 27% for the guinea pig serum (data not shown).

Design of a competition assay using a phage-antibody spot test (PhAST)

We set out to design an assay based on the Fab-phage competition format described above that would be economical and easy to use, so that it could be performed in small laboratories or even in the field. As the Fab-phage contain the genetic information for the Fab in their genome, they already provide for an unlimited and economical supply of the competition reagent. The detection system used however provided a greater challenge. We investigated a number methods of which an assay based on Fab-phage particles directly stained with a dye appeared the most promising. KZ51-phage were amplified in *Escherichia coli*, PEG-precipitated, and then stained red with the industrial textile dye Disperse Red 60. Competition assays were then performed in a spot-test format in which we spotted inactivated Ebola Zaire virus on small pieces of nitrocellulose paper, which were then sequentially incubated with specific antibodies and the red-colored KZ51-phage particles. The results are shown in Fig. 4. Direct red KZ51-phage staining of control antigens immobilized on nitrocellulose is shown in Fig. 4A. The red phage strongly stained the nitrocellulose filter coated with inactivated Ebola Zaire virus and sheep anti-M13 antibody, but not filters coated with control antigens BSA or transferrin. Figure 4B shows the competition PhAST assay with the red KZ51-phage again strongly staining the immobilized radiation-inactivated Ebola Zaire virus. This staining was inhibited with serum from an Ebola Zaire virus-infected guinea pig, but not with serum from a noninfected control animal. This method should provide a rapid and low-cost procedure to assess the presence of antibodies against Ebola virus in the serum of any animal.

DISCUSSION

The search for the natural host of the filoviruses Ebola virus and Marburg virus has been long and extensive but unsuccessful (Breman *et al.,* 1999; Leirs *et al.,* 1999; Reiter *et al.,* 1999; Monath, 1999). This is of great concern as prevention of filovirus infection by surveillance or control of infected animals is thereby not feasible. There have been a limited number of outbreaks of Ebola or Marburg virus hemorrhagic fever over the years and only a small number of people (compared with other major pathogens such as HIV-1 and malaria) have died from the disease (Burton and Parren, 2000). Some have argued that this class of viruses and disease associated with infection have therefore gathered an undeserved high level of attention. We believe however that it would be prudent to be prepared for the possible occurrence of larger and increased frequency of outbreaks. It should be noted that the number of outbreaks has been on the rise since the mid-1990s when outbreaks of Ebola Zaire virus infections occurred in Gabon and the DRC followed by two simultaneous outbreaks of filovirus hemorrhagic fever, one with Marburg virus in the DRC and one with Ebola Sudan virus in Uganda in the year 2000 (Burton and Parren, 2000). A novel outbreak of Ebola virus hemorrhagic fever was confirmed in December 2001 in the Ogooue Ivindo province of Gabon and neighboring DRC (Leroy *et al.,* 2002). It may be that humans encroach more frequently on the habitat of the natural Ebola and Marburg virus hosts. Such an increased sampling of filoviruses from the wild may raise the concern that an outbreak would occur with a virus that is spread even more easily between humans (for example, through aerosols). Filoviruses may pose an additional threat, as there has been concern that they might be employed in a bioterrorist attack. Identification of the natural host for these viruses is therefore critical.

A problem with filovirus research is that all procedures with specimens that potentially contain live virus, and virus isolation procedures in particular, need to be performed in biosafety level 4 facilities. The detection of antibodies against filoviruses however can be performed on inactivated sera in regular laboratory facilities and therefore provide a much higher through-put assay for the identification of animals that have been in contact with a filovirus. It is highly problematic however to detect the presence of specific antibodies in sera from a wide range of wild animals. Protein A and G conjugates for example are highly variable in their affinity for IgGs from different species. Specific anti-antibody conjugates required for detection furthermore are not available for many species (Breman *et al.,* 1999). An additional complicating factor is that, in particular for many small mammal species, identification is difficult and not possible in the field and may require sophisticated techniques (Leirs *et al.,* 1999).

In this study, we focused on Ebola virus which has been responsible for most outbreaks and deaths due to filovirus hemorrhagic fever. We designed an immunoassay that can detect antibodies against the conserved Ebola virus protein NP in animal sera and determine seroconversion irrespective of Ebola virus subtype or animal species. The epitope recognized by the antibody against NP, mAb KZ51, recognizes a highly conserved epitope which is expressed on all four known subtypes of Ebola virus. The epitope also appears to be immunodominant as strong competition for binding to immobilized NP was observed with sera from 10/10 human donors who survived infection with Ebola Zaire and sera from guinea pigs infected with each of the four Ebola virus subtypes. We can of course not be certain that the natural Ebola virus host would also mount a strong response against this epitope, but the strong competition

observed with sera from both infected humans and infected guinea pigs at least makes this likely.

The competition assay described in this study was designed as a diagnostic rather than a quantitative tool. Particularly the PhAST assay may be useful to test large numbers of animals directly in the field or in local African laboratories. The production of the KZ51-phage and staining with the red dye is straightforward and economical. A low level of background inhibition was observed when sera from uninfected animals were tested. It would therefore appear prudent to establish a cutoff value of 50% or greater inhibition before a sample is considered positive. Positive samples should furthermore be sent to reference laboratories for confirmation. A caveat of the assay described is that we used an irradiation-inactivated preparation of Ebola virions as a source for NP. However, recombinant Ebola NP has recently been successfully produced in *E. coli* (Prehaud *et al.,* 1998) and baculovirus (Niikura *et al.,* 2001), which may provide good, and probably superior, alternatives to the inactivated virion preparations used here.

MATERIALS AND METHODS

Phage libraries and antibodies

Antibody phage-display libraries prepared from bone marrow from donors K and L who recovered from infection with Ebola Zaire virus during the 1995 outbreak in Kikwit, DRC have been described previously. A third phage-display library was prepared from pooled RNA prepared from PBMC from 10 survivors from this outbreak (Maruyama *et al.,* 1999a,b). The isolation of anti-Ebola virus Zaire NP Fabs KZ51 and ELZ510 has been described in Maruyama *et al.* (1999a,b).

Sera

Sera from 10 convalescent individuals from Kikwit, DRC, who recovered from Ebola hemorrhagic fever, were drawn between 5 and 8 July 1995. Sera from guinea pigs that survived infection with Ebola Zaire virus, Ebola Sudan virus, Ebola Ivory Coast virus, and Ebola Reston virus were prepared in the BSL4 facility at USAMRIID. The animals were challenged i.m. with 1000 PFU of each virus and serum was drawn 1 month later.

Library construction

A phage-display library was prepared from the bone marrow of a rhesus macaque (designated J3P) that survived challenge with Ebola Zaire virus after treatment with a Factor XIIa antagonist and developed a relatively high neutralizing serum titer against Ebola Zaire virus (IC_{80} = 1:80). Library construction in the phagemid vector pComb3H was performed as detailed previously (Glamann *et al.,* 1998; Maruyama *et al.,* 1999b).

Affinity selection of phage-display libraries

A γ-irradiated Ebola Zaire 1976 whole-virion preparation (inactivated by applying 2×10^6 rad of γ-radiation to a frozen sample) was used for selection as described previously (Maruyama *et al.,* 1999b) or Ebola virus GP was captured on immobilized wheat germ agglutinin (Sigma). The library was panned for four consecutive rounds of selection with increasing washing stringency (2, 5, and 10 wash steps thereafter, each consisting of a 5-min incubation and vigorous pipetting). After the last round of panning, colonies were picked, expanded, and phage isolated. Specific binders were identified in phage ELISA. To prepare soluble Fab, phagemid DNA was isolated, digested with *Nhe*I and *Spe*I restriction endonucleases to excise the cpIII gene, re-ligated, and used to transform *E. coli*.

Preparation of Fab-phage

Pcomb3H phagemid DNA containing the genetic information for the desired Fab fragment were used to transform *E. coli* XL1 blue (Stratagene, La Jolla, CA) and plated on LB plates containing carbenicillin (100 μg/ml) (for media recipes, see Barbas *et al.,* 2001). Starting from a single colony, 10 ml cultures were grown in superbroth (SB) medium supplemented with tetracycline (10 μg/ml) and carbenicillin (50 μg/ml) for about 6 h at 37°C while shaking (250 rpm) to an OD_{600} of ~0.8. VCS M13 helper phage (10^{12}) (Stratagene) was added to the culture, and the culture volume was increased to 100 ml. IPTG (Sigma, St. Louis, MO) was added to a final concentration of 10 μM, and the culture grown for 2 h at 37°C while shaking (300 rpm). Kanamycin was added to a final concentration of 70 μg/ml and the culture grown overnight at 30°C (300 rpm). The next day, the cells were pelleted by a 30-min centrifugation at 4000 *g* at 4°C. The supernatant was immediately aliquoted and frozen at −80°C. These Fab-phage preparations were used for competition ELISAs with sheep anti-M13-HRPO conjugate detection (described below).

Western blot

Western blotting was performed as described previously (Hansen *et al.,* 2001) except that blots were incubated with Fab KZ51 (10 μg/ml), Fab J3P-K14 (10 μg/ml), or KZ51-phage (1:12.5) (each diluted in PBS/0.05% Tween 20) and detection was performed using goat anti-human IgG $F(ab')_2$-HRPO conjugate (Pierce) diluted 1:5000 in PBS/0.05% Tween 20 or anti-M13 phage-HRPO conjugate; Amersham Pharmacia, Uppsala, Sweden) diluted 1:3000 in PBS/0.05% Tween 20.

Competition ELISA

Competition ELISA was essentially performed as described by Ditzel *et al.* (1995).

Preparation of red-colored Fab-phage

Fab-phage was prepared as described above, after which the phage was concentrated by PEG precipitation. After clarifying the bacterial supernatant by centrifugation at 4000 *g*, phage were precipitated by addition of polyethylene glycol 8000 to a concentration of 4% (w/v) and NaCl to a final concentration of 0.5 M. The mixture was incubated on ice for 30 min, followed by centrifugation at 8000 *g*. Phage was dissolved in 1 ml PBS and mixed with an equal volume of Disperse Red dye, which was prepared as described below, and incubated for 1 h at room temperature with occasional shaking. A 1/5 volume of a 30% BSA solution in PBS (pH 7.4) was added to stabilize the dye-particle surfaces and incubated for another 30 min at room temperature. The mixture was then centrifuged at 5000 *g* for 30 min and the pellet resuspended in 1 ml PBS containing 1% BSA and 0.02% Tween 20. The red-colored phage particles were used for the dip-stick competition assays.

The red dye was prepared by dispersing 5% (w/v) of the dye Disperse Red 60 (200%) (Organic Dyestuffs Corp., East Providence, RI) in PBS, followed by centrifugation at 20,000 *g* for 30 min. The pellet was resuspended in PBS and again centrifuged. After repeating this procedure a total of four times, the dye was spun one last time at low speed (125 *g*) for 1 h to remove aggregates. The supernatant was used for staining of Fab-phage particles as described above.

Phage-antibody spot test

Antigen assay. Nitrocellulose circles were prepared by punching 7-mm-diameter circles from a strip of nitrocellulose membrane (Bio-Rad, Hercules, CA) using a hole puncher. A 3-μl spot of each antigen was added directly to the membrane and the membrane was allowed to air dry at room temperature (RT) for 10 min. The antigens included the following: inactivated Ebola Zaire (1:10 in PBS), BSA (30 mg/ml in PBS), transferrin (200 μg/ml in PBS), and sheep anti-M13 (1:500 in PBS) (Amersham Pharmacia). The membrane was then immersed in 4% nonfat dry milk for blocking at RT for 20 min followed by air drying for 10 min. The membranes were transferred into a solution of red-colored Fab KZ51-phage for 1 h at RT, washed with PBS/0.05% Tween 20, allowed to dry, and observed for red staining.

Competition assay. Nitrocellulose membrane circles as prepared above and a 10 μl spot of inactivated Ebola Zaire virus was spotted and allowed to air dry for 10 min at RT. The nitrocellulose circles were placed into the wells of a 48-well microtiter plate. Serum from a noninfected and an Ebola Zaire virus-infected guinea pig were diluted 1:2 in PBS and 10 μl was added to the membranes. A control membrane coated with Ebola Zaire antigen was incubated with PBS. A second control membrane coated with anti-M13 antibody (diluted 1:500 in PBS) was also incubated with PBS. All membranes were incubated for 1 h at RT after which 50 μl of red-colored Fab-phage particles were added and incubated overnight at RT with gentle shaking. The membranes were then washed with PBS/0.05% Tween, allowed to air dry, and observed for staining.

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