The application of a safe neutralization assay for Ebolavirus using lentivirus-based pseudotyped virus

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Abstract

Ebolavirus (EBOV) is responsible for several EBOV disease (EVD) outbreaks in Africa, with a fatality rate of up to 90%. During 2014-2016, An epidemic of EVD spread throughout Sierra Leone, Guinea and Liberia, and killed over 11,000 people. EBOV began to circulate again in the Democratic Republic of Congo in 2018. Due to the need for a BSL-4 facility to manipulate this virus, the development and improvement of specific therapeutics has been hindered. As a result, it is imperative to perform reliable research on EBOV under lowered BSL restrictions. In this study, we developed a safe neutralization assay based on pseudotyped EBOV, which incorporates the glycoprotein of the 2014 EBOV epidemic strain into a lentivirus vector. Our results demonstrated that the tropism of pseudotyped EBOV was similar to that of authentic EBOV, but with only one infection cycle. And neutralizing activity of both authentic EBOV and pseudotyped EBOV were compared in neutralization assay using three different samples of antibody-based reagents against EBOV, similar results were obtained. In addition, an indirect ELISA was performed to show the relationship between IgG and neutralizing antibody against EBOV detected by our pseudotyped EBOV-based neutralization assay. As expected, the neutralizing antibody titers varied with the IgG titers detected by indirect ELISA, and a correlation between the results of the two assays was identified. By comparison with two different assays, the reliability of the results detected by the pseudotyped EBOV-based neutralization assay was confirmed. Collectively, in the absence of BSL-4 restrictions, pseudotyped EBOV production and neutralizing activity evaluation can be performed safely and in a manner that is neither labor- nor time-consuming, providing a simple and safe method for EBOV-neutralizing antibody detection and the assessment of immunogenicity of EBOV vaccines. All these remarkable advantages of the newly established assay highlight its potential to further application in assessment of immunogenicity of EBOV vaccine candidates.

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Short Running Title: A safe pseudotyped EBOV used in neutralization assay

Summary

Ebolavirus (EBOV) is responsible for several EBOV disease (EVD) outbreaks in Africa, with a fatality rate of up to 90%. During 2014-2016, An epidemic of EVD spread throughout Sierra Leone, Guinea and Liberia, and killed over 11,000 people. EBOV began to circulate again in the Democratic Republic of Congo in 2018. Due to the need for a BSL-4 facility to manipulate this virus, the development and improvement of specific therapeutics has been hindered. As a result, it is imperative to perform reliable research on EBOV under lowered BSL restrictions. In this study, we developed a safe neutralization assay based on pseudotyped EBOV, which incorporates the glycoprotein of the 2014 EBOV epidemic strain into a lentivirus vector. Our results demonstrated that the tropism of pseudotyped EBOV was similar to that of authentic EBOV, but with only one infection cycle. And neutralizing activity of both authentic EBOV and pseudotyped EBOV were compared in neutralization assay using three different samples of antibody-based reagents against EBOV, similar results were obtained. In addition, an indirect ELISA was performed to show the relationship between IgG and neutralizing antibody against EBOV detected by our pseudotyped EBOV-based neutralization assay. As expected, the neutralizing antibody titers varied with the IgG titers detected by indirect ELISA, and a correlation between the results of the two assays was identified. By comparison with two different assays, the reliability of the results detected by the pseudotyped EBOV-based neutralization assay was confirmed. Collectively, in the absence of BSL-4 restrictions, pseudotyped EBOV production and neutralizing activity evaluation can be performed safely and in a manner that is neither labor- nor time-consuming, providing a simple and safe method for EBOV-neutralizing antibody detection and the assessment of immunogenicity of EBOV vaccines. All these remarkable advantages of the newly established assay highlight its potential to further application in assessment of immunogenicity of EBOV vaccine candidates.

Keywords: EBOV, lentivirus-based, pseudotyped virus, neutralization assay, neutralizing activity evaluation, absence of BSL-4

1. Introduction

EBOV is a negative-sense single RNA virus capable of causing acute hemorrhagic fever with a frightening fatality rate that can reach up to 90%(Cantoni and Rossman, 2018; Harrod, 2015). EBOV is responsible for several outbreaks in Central and West Africa (Awini et al., 2017; Baize et al., 2014; Subissi et al., 2018). Due to its high lethality and frequent recurrence, EBOV is a substantial threat to public health.

Neutralizing antibodies play a crucial role in the race and balance between pathogens and host protection, and thus, neutralizing antibodies are considered essential to assess immune function, immunogenicity evaluation of vaccines, and antiviral drug research. For some lethal viruses, including EBOV, research on live virus is restricted to BSL-4 laboratories; therefore, conventional serological detection methods cannot provide a

safe way to evaluate neutralizing activity, resulting in a bottleneck of the antibody-based detection of such viruses.

Currently, pseudotyped viruses are widely used as a powerful tool in studying multiple aspects of the infection progress of various viruses. With advantage of safety, many groups have produced diverse pseudotyped viruses bearing glycoproteins (GPs) of different viruses to achieve their research goals (Cheresiz et al., 2014; Lennemann et al., 2017; Zhao et al., 2013). Here, based on the luciferase-expressing human immunodeficiency virus type 1 (HIV-1) backbone, we aim to develop and apply a neutralization assay for EBOV using the generated lentivirus-based pseudotyped EBOV bearing GP on the surface, which may reduce the risk and threat of this virus due to its single infection cycle.

2. Materials and Methods

2.1 Ethics statement

All animal studies in this work were approved by the Animal Care and Use Committee of Chinese People's Liberation Army (No. SYXK2009-045). All efforts were made to minimize animal suffering.

2.2 Recombinant plasmid construction and generation of EBOV pseudotyped virus

The complete genome of EBOV is shown in Fig. 1a, and the full-length G gene of EBOV (GenBank accession no. KJ660346.2) was synthesized by Sangon Biotech Biotechnology Co., Ltd. (Shanghai, China) and cloned into the pcDNA4.0 vector. The recombinant plasmid was confirmed by enzyme digestion analysis and by DNA sequencing, and the details of the recombinant plasmid are shown in Fig. 1b. The pseudotyped EBOVs was produced by the transfection of HEK293T cells with a two-plasmid system, which consists of the GP-encoding plasmid (pcDNA4.0-G) and luciferase-expressing plasmid (pNL4-3.luc.RE).

2.3 Infectivity and titer determination of pseudotyped EBOV

For infectivity detection, various target cells from different species (Huh-7, HEK293T, A549, C6/36, Vero, BHK, BSR) were infected with pseudotyped EBOV serially diluted in DMEM without FBS. Then DMEM containing 10% FBS was added to replace DMEM without FBS at 4 h post-infection, and relative luciferase units (RLUs) were measured 48 h later. After normalized by OD value to negative control, positive result was recognized as more than 3.0 under a definitive dilution.

2.4 Western blot analysis

Cells (HEK293T) were transfected with the two-plasmid system and incubated at 37 °C until the pseudotyped EBOV was harvested. The cells were harvested and lysed. After centrifugation, the lysates were mixed with 4×LDS sample buffer (ThermoFisher Scientific) and denaturing at 90for 10 min. Samples were loaded onto to a 12% SDS-PAGE gel. After electrophoresis, proteins were transferred onto nitrocellulose (NC) membranes. The NC membranes were then blocked using SuperBlocking Buffer (Thermo Fisher, TX, USA) at room temperature for 2 h and subsequently incubated with anti-p24 antibody (Sino Biological, China) and anti-EBOV GP antibody (rabbit serum immunized with purified, truncated EBOV GP in*Escherichia coli*) for 1 h at room temperature. After three washes with phosphate-buffered saline containing tween 20 (PBST), the NC membranes were incubated with HRP-conjugated goat anti-rabbit IgG (Bios, China) at room temperature for 1 h. The signals were visualized on a Fujiflm LAS-4000 image reader (Fujiflm, Tokyo, Japan) with SuperSignal West Dura Extended Duration Substrate Kit (Thermo Fisher).

2.5 Microscopy

After staining with 1% sodium phosphotungstate, the grids loaded with EBOV pseudotyped virus were observed using transmission electron microscopy (TEM). For immunoelectron microscopy (IEM), the EBOV pseudotyped virus was also bound to formvar-coated grids, and then incubated with murine anti-EBOV GP monoclonal antibody and gold-labeled goat anti-mouse IgG (Sigma-Aldrich, Saint Louis, MO, USA). After, the formvar-coated grids were stained with 1% sodium phosphotungstate and observed.

2.6 Neutralization assays

A pseudotyped EBOV-based neutralization assay was developed to evaluate the neutralizing activity of different samples. Briefly, 50 μL pseudotyped EBOV -containing supernatants were incubated with 50 μL five-fold dilution series of samples at 37 for 1 h. The pseudotyped EBOV-sample mixtures were then added onto Huh-7 cells seeded in 96-well culture plates at an 80-90% monolayer density. Four hours later, the Huh-7 cells were washed with phosphate-buffered saline (PBS) and incubated for another 48 h. The Huh-7 cells were lysed using 30 μL/well of cell lysis buffer (Promega, WI, USA), and 20 μL lysates were used to determine luciferase activity by adding luciferase substrate (Promega) into the 96-well luminometer plates. Live EBOV was also applied to neutralization assay for comparison with the pseudotyped EBOV-based neutralization assay, which was described previously (Qiu et al., 2014).

2.7 Indirect ELISA

The 96-well microtiter plates (Corning Costar, USA) were coated with recombinant truncated EBOV GP (1 μg/well) at 4 °C overnight, then blocked with 1% BSA at 37 °C for 2 h. 100 μL serially diluted serum samples were added into the wells, and incubated at 37 for 90 min. After five washes, HRP-conjugated goat anti-horse IgG (Bios, China) was added and incubated for an additional hour at 37 °C. Afterwards, 100 μL of the substrate 3,3',3,5'-tetramethyl benzidine (TMB) (Sigma, USA) was added and incubated for 10 min at room temperature. After five washes, and 50 μL of 0.5 M H_2SO_4 was added to stop the reaction. The optical density (OD) values were measured at a wavelength of 450 nm. Positive result was recognized as over 2.1 after normalized by OD value to negative control.

3. Results

3.1 Production of pseudotyped EBOV

A diagram of pseudotyped EBOV production is shown in Fig. 1c. Briefly, the two-plasmid system (2 μg of each of plasmids) was transfected into HEK293T cells in a 6-well plate, pseudotyped EBOV-containing supernatants were harvested at 24-36 h-post-transfection, followed by titer determination.

3.2 Incorporation of EBOV GP into pseudotyped virus particles

To identify whether EBOV GP was expressed in the pseudotyped EBOV system, western blotting was performed. As shown in Fig. 1d, both EBOV GP and HIV-1 p24 protein were identified in the pseudotyped EBOV. TEM was performed to analyze the morphology of pseudotyped EBOV particles. As shown in Fig. 1e, pseudotyped EBOV particles with diameters of approximately 100-120 nm were observed in pseudotyped virus-containing supernatants, outer envelop protein, EBOV GP, were observed on the surface of pseudotyped EBOV, which revealed efficient packaging and secretion of pseudotyped EBOV. The result of IEM observation was shown in Fig. 1f, multiple gold particles were found to bind to EBOV GP on the surface of pseudotyped virus. Altogether, the data suggests that EBOV GP was effectively incorporated onto the surface of HIV-1 backbone, and pseudotyped EBOV were packaged and rescued.

3.3 Infectivity screening and kinetics of pseudotyped EBOV

To figure out a susceptible cell line of pseudotyped EBOV for subsequent research, we screened several cell lines from different species. As shown in Fig. 2a. All tested cells infected with the pseudotyped EBOV were found to have relatively high RLU values, except for C6/36 cells. These findings suggested that the rescued pseudotyped EBOV could infect different target cells in this research, except C3/36 cells from mosquitoes, which coincides with the characteristics of EBOV (EBOV has no infectivity in C6/36 cells), indicating that the infective ability of pseudotyped EBOV was higher in Huh-7 cells than in any other cell lines, as a result, Huh-7 cells were selected as a tool to study kinetics of pseudotyped EBOV production.

The RLU values of pseudotyped EBOV rescued at different time points are shown in Fig. 2b, pseudotyped EBOV could reach a peak RLU value at 24 h post-transfection. The titers of pseudotyped EBOV that were rescued at different times are shown in Fig. 2c, the pseudotyped EBOV had the highest titer when rescued between 24-36 h post- transfection. To confirm the single-cycle infectivity of the pseudotyped EBOV, we harvested the supernatant from Huh-7 cells infected with pseudotyped EBOV, which was used to reinfect

Huh-7 cells. Two days later, RLU activity and titer of the supernatant were detected, representing the second-cycle characteristics of pseudotyped EBOV. As shown in Fig. 2d and 2e, the RLU activity of the second life cycle was substantially lower than that of the first life cycle, and the titer of pseudotyped EBOV in its second life cycle could not be detected. These findings showed that there was only one life cycle of pseudotyped EBOV, implying that this pseudotyped EBOV should be safe for research.

3.4 Comparison between pseudotyped EBOV-based and authentic EBOV-based neutralization assay

Equine immunoglobulin fragments against EBOV, which were produced previously (Zheng et al., 2016), were used as a positive control for the optimization of the pseudotyped EBOV-based neutralization assay. After optimization, the pseudotyped EBOV-based neutralization assay was used to measure the neutralization activity of the immunoglobulin fragments, and the neutralization assay based on authentic EBOV was also performed. As expected similar results were acquired between the two different assays (Fig. 3a). Both NT_{50} values were higher than 1:20,000 (1:20,480 for pseudotyped EBOV; 1:21,333 for live EBOV), suggesting that the pseudotyped EBOV could be used in neutralization assay with similar infectivity compared to authentic and live EBOV, but with a reduced health risk.

3.5 Application of pseudotyped EBOV-based neutralization assay

Three samples of antibody-based reagents against EBOV (immunoglobulin fragments, lyophilized IgG, equine antisera) were evaluated *in vitro* using the pseudotyped EBOV-based neutralization assay. As shown in Fig. 3b, the neutralization efficiency of all samples correlated with serially diluted concentrations, and a linear relationship was confirmed. The NT_{50} and NT_{90} of the three samples are shown in Fig. 3c and 3d. The results revealed that all samples showed high neutralization activity to the EBOV pseudotyped virus. We concluded that the pseudotyped EBOV-based neutralization assay was sufficiently reliable to evaluate neutralizing antibody titers of different samples.

To show the relationship between IgG and neutralizing antibody against EBOV, we further compared the results of the pseudotyped EBOV-based neutralization assay and an indirect ELISA. As shown in Table 1, the neutralizing antibody titers varied with the IgG titers detected by indirect ELISA, and a correlation between the results of the two assays was identified, suggesting the validity and reliability of the results detected by the pseudotyped EBOV-based neutralization assay.

4. Discussion

The absence of basic research and translational technology has been highlighted since the 2014-2016 EBOV epidemic in West Africa, the reason for which is BSL-4 facility restrictions. It is a priority to develop a safe method for EBOV research with low BSL restrictions and definite applicability.

Although the result of the neutralization assay is a gold standard for antibody detection, BSL-4 laboratories are not available for many research groups. In this situation, many groups developed an increasing number of agents, which are single replication cycle pseudotyped viruses, for hazardous pathogens based on the lentivirus system (Chen et al., 2018; Qiu et al., 2013; Zhao et al., 2013). In addition, the efficient expression of the luciferase reporter gene in infected cells was demonstrated after GP-mediated infection (Fig. 2b, 2c), indicating the infection efficiency. These features make pseudotyped EBOV a safe and quantifiable tool for antiviral drug discovery and neutralizing activity evaluation.

Among the viral proteins, GPs are considered the major pathogenicity factor (Panina et al., 2017). EBOV entry into target cells is initiated by the interaction between the viral GP and receptors on the surface of target cells. As a result, the GP of EBOV was selected to construct the pseudotyped EBOV in this study. As expected, GP endowed the pseudotyped EBOV with similar infectivity of authentic EBOV, as indicated by the result that the pseudotyped EBOV could infect different target cells in this study, except C3/36 cells from mosquitoes (Fig. 2a).

All assays involving live EBOV have to be performed under BSL-4 conditions because of the high lethality

of EBOV, which limits the application of these assays. However, the pseudotyped EBOV generated from our two-plasmid system has only a single infection cycle (Fig. 2d, 2e), so that it cannot cause mass infection and death. In addition, based on the pseudotyped EBOV, we developed a neutralization assay to evaluate the neutralizing activity of the antibody products. As our data show (Fig. 3a), the pseudotyped EBOV neutralization assay can successfully be used for neutralizing activity evaluation, having the consistent results with an authentic EBOV-based neutralization assay in neutralizing antibody detection, confirming its potential application value in antiviral drug discovery and neutralizing antibody evaluation.

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Conflict of Interest Statement

The authors declare that they have no conflict of interests regarding the publication of this study.

Date Availability Statement

The authors confirm that the data supporting the findings of this study are available within the article [and/or] its supplementary materials.

References

Awini, E.A., Bonney, J.H.K., Frimpong, J.A., Ampofo, W.K., Koram, K.A., 2017. Information gaps in surveillance data and effects on the Ghanaian response to the Ebola outbreak in West Africa. Ghana medical journal 51, 115-119.

Baize, S., Pannetier, D., Oestereich, L., Rieger, T., Koivogui, L., Magassouba, N., Soropogui, B., Sow, M.S., Keita, S., De Clerck, H., Tiffany, A., Dominguez, G., Loua, M., Traore, A., Kolie, M., Malano, E.R., Heleze, E., Bocquin, A., Mely, S., Raoul, H., Caro, V., Cadar, D., Gabriel, M., Pahlmann, M., Tappe, D., Schmidt-Chanasit, J., Impouma, B., Diallo, A.K., Formenty, P., Van Herp, M., Gunther, S., 2014. Emergence of Zaire Ebola virus disease in Guinea. N Engl J Med 371, 1418-1425.

Cantoni, D., Rossman, J.S., 2018. Ebolaviruses: New roles for old proteins. PLoS Negl Trop Dis 12, e0006349.

Chen, Q., Tang, K., Zhang, X., Chen, P., Guo, Y., 2018. Establishment of pseudovirus infection mouse models for in vivo pharmacodynamics evaluation of filovirus entry inhibitors. Acta pharmaceutica Sinica. B 8, 200-208.

Cheresiz, S.V., Kononova, A.A., Razumova, Y.V., Dubich, T.S., Chepurnov, A.A., Kushch, A.A., Davey, R., Pokrovsky, A.G., 2014. A vesicular stomatitis pseudovirus expressing the surface glycoproteins of influenza A virus. Archives of Virology 159, 2651-2658.

Gire, S.K., Goba, A., Andersen, K.G., Sealfon, R.S., Park, D.J., Kanneh, L., Jalloh, S., Momoh, M., Fullah, M., Dudas, G., Wohl, S., Moses, L.M., Yozwiak, N.L., Winnicki, S., Matranga, C.B., Malboeuf, C.M., Qu, J., Gladden, A.D., Schaffner, S.F., Yang, X., Jiang, P.P., Nekoui, M., Colubri, A., Coomber, M.R., Fonnie, M., Moigboi, A., Gbakie, M., Kamara, F.K., Tucker, V., Konuwa, E., Saffa, S., Sellu, J., Jalloh, A.A., Kovoma, A., Koninga, J., Mustapha, I., Kargbo, K., Foday, M., Yillah, M., Kanneh, F., Robert, W., Massally, J.L., Chapman, S.B., Bochicchio, J., Murphy, C., Nusbaum, C., Young, S., Birren, B.W., Grant, D.S., Scheiffelin, J.S., Lander, E.S., Happi, C., Gevao, S.M., Gnirke, A., Rambaut, A., Garry, R.F., Khan, S.H., Sabeti, P.C., 2014. Genomic surveillance elucidates Ebola virus origin and transmission during the 2014 outbreak. Science 345, 1369-1372.

Harrod, K.S., 2015. Ebola: history, treatment, and lessons from a new emerging pathogen. Am J Physiol Lung Cell Mol Physiol 308, L307-313.

Konduru, K., Shurtleff, A.C., Bavari, S., Kaplan, G., 2018. High degree of correlation between Ebola virus BSL-4 neutralization assays and pseudotyped VSV BSL-2 fluorescence reduction neutralization test. J Virol Methods 254, 1-7.

Lee, J.E., Fusco, M.L., Hessell, A.J., Oswald, W.B., Burton, D.R., Saphire, E.O., 2008. Structure of the Ebola virus glycoprotein bound to an antibody from a human survivor. Nature 454, 177-182.

Lee, S.S., Phy, K., Peden, K., Sheng-Fowler, L., 2017. Development of a micro-neutralization assay for ebolaviruses using a replication-competent vesicular stomatitis hybrid virus and a quantitative PCR readout. Vaccine 35, 5481-5486.

Lennemann, N.J., Herbert, A.S., Brouillette, R., Rhein, B., Bakken, R.A., Perschbacher, K.J., Cooney, A.L., Miller-Hunt, C.L., Ten Eyck, P., Biggins, J., Olinger, G., Dye, J.M., Maury, W., 2017. Vesicular Stomatitis Virus Pseudotyped with Ebola Virus Glycoprotein Serves as a Protective, Noninfectious Vaccine against Ebola Virus Challenge in Mice. Journal of Virology 91.

Maruyama, T., Rodriguez, L.L., Jahrling, P.B., Sanchez, A., Khan, A.S., Nichol, S.T., Peters, C.J., Parren, P.W., Burton, D.R., 1999. Ebola virus can be effectively neutralized by antibody produced in natural human infection. J Virol 73, 6024-6030.

Mbala Kingebeni, P., Villabona-Arenas, C.J., Vidal, N., Likofata, J., Nsio-Mbeta, J., Makiala-Mandanda, S., Mukadi, D., Mukadi, P., Kumakamba, C., Djokolo, B., Ayouba, A., Delaporte, E., Peeters, M., Muyembe Tamfum, J.J., Ahuka Mundeke, S., 2018. Rapid confirmation of the Zaire Ebola Virus in the outbreak of the Equateur province in the Democratic Republic of Congo: implications for public health interventions. Clin Infect Dis.

Mohan, G.S., Li, W., Ye, L., Compans, R.W., Yang, C., 2012. Antigenic subversion: a novel mechanism of host immune evasion by Ebola virus. PLoS Pathog 8, e1003065.

Nkengasong, J.N., Onyebujoh, P., 2018. Response to the Ebola virus disease outbreak in the Democratic Republic of the Congo. Lancet 391, 2395-2398.

Panina, A.A., Dementieva, I.G., Aliev, T.K., Toporova, V.A., Balabashin, D.S., Bokov, M.N., Pozdnyakova, L.P., Shchemchukova, O.B., Dolgikh, D.A., Sveshnikov, P.G., Kirpichnikov, M.P., 2017. Recombinant Antibodies to the Ebola Virus Glycoprotein. Acta Naturae 9, 84-91.

Qiu, C., Huang, Y., Zhang, A., Tian, D., Wan, Y., Zhang, X., Zhang, W., Zhang, Z., Yuan, Z., Hu, Y., 2013. Safe Pseudovirus-based Assay for Neutralization Antibodies against Influenza A(H7N9) Virus. Emerging Infectious Diseases 19, 1685-1687.

Qiu, X., Wong, G., Audet, J., Bello, A., Fernando, L., Alimonti, J.B., Fausther-Bovendo, H., Wei, H., Aviles, J., Hiatt, E., Johnson, A., Morton, J., Swope, K., Bohorov, O., Bohorova, N., Goodman, C., Kim, D., Pauly, M.H., Velasco, J., Pettitt, J., Olinger, G.G., Whaley, K., Xu, B., Strong, J.E., Zeitlin, L., Kobinger, G.P., 2014. Reversion of advanced Ebola virus disease in nonhuman primates with ZMapp. Nature 514, 47-53.

Shrivastava-Ranjan, P., Flint, M., Bergeron, E., McElroy, A.K., Chatterjee, P., Albarino, C.G., Nichol, S.T., Spiropoulou, C.F., 2018. Statins Suppress Ebola Virus Infectivity by Interfering with Glycoprotein Processing. mBio 9.

Subissi, L., Keita, M., Mesfin, S., Rezza, G., Diallo, B., Van Gucht, S., Musa, E.O., Yoti, Z., Keita, S., Djingarey, M.H., Diallo, A.B., Fall, I.S., 2018. Ebola Virus Transmission Caused by Persistently Infected Survivors of the 2014-2016 Outbreak in West Africa. J Infect Dis.

Sullivan, N.J., Hensley, L., Asiedu, C., Geisbert, T.W., Stanley, D., Johnson, J., Honko, A., Olinger, G., Bailey, M., Geisbert, J.B., Reimann, K.A., Bao, S., Rao, S., Roederer, M., Jahrling, P.B., Koup, R.A.,

Nabel, G.J., 2011. CD8+ cellular immunity mediates rAd5 vaccine protection against Ebola virus infection of nonhuman primates. Nat Med 17, 1128-1131.

Tong, Y.G., Shi, W.F., Liu, D., Qian, J., Liang, L., Bo, X.C., Liu, J., Ren, H.G., Fan, H., Ni, M., Sun, Y., Jin, Y., Teng, Y., Li, Z., Kargbo, D., Dafae, F., Kanu, A., Chen, C.C., Lan, Z.H., Jiang, H., Luo, Y., Lu, H.J., Zhang, X.G., Yang, F., Hu, Y., Cao, Y.X., Deng, Y.Q., Su, H.X., Sun, Y., Liu, W.S., Wang, Z., Wang, C.Y., Bu, Z.Y., Guo, Z.D., Zhang, L.B., Nie, W.M., Bai, C.Q., Sun, C.H., An, X.P., Xu, P.S., Zhang, X.L., Huang, Y., Mi, Z.Q., Yu, D., Yao, H.W., Feng, Y., Xia, Z.P., Zheng, X.X., Yang, S.T., Lu, B., Jiang, J.F., Kargbo, B., He, F.C., Gao, G.F., Cao, W.C., China Mobile Laboratory Testing Team in Sierra, L., 2015. Genetic diversity and evolutionary dynamics of Ebola virus in Sierra Leone. Nature 524, 93-96.

Volchkov, V.E., Volchkova, V.A., Slenczka, W., Klenk, H.D., Feldmann, H., 1998. Release of viral glycoproteins during Ebola virus infection. Virology 245, 110-119.

Yu, D.S., Weng, T.H., Wu, X.X., Wang, F.X.C., Lu, X.Y., Wu, H.B., Wu, N.P., Li, L.J., Yao, H.P., 2017. The lifecycle of the Ebola virus in host cells. Oncotarget 8, 55750-55759.

Zhao, G.Y., Du, L.Y., Ma, C.Q., Li, Y., Li, L., Poon, V.K.M., Wang, L.L., Yu, F., Zheng, B.J., Jiang, S.B., Zhou, Y.S., 2013. A safe and convenient pseudovirus-based inhibition assay to detect neutralizing antibodies and screen for viral entry inhibitors against the novel human coronavirus MERS-CoV. Virology Journal 10.

Zheng, X., Wong, G., Zhao, Y., Wang, H., He, S., Bi, Y., Chen, W., Jin, H., Gai, W., Chu, D., Cao, Z., Wang, C., Fan, Q., Chi, H., Gao, Y., Wang, T., Feng, N., Yan, F., Huang, G., Zheng, Y., Li, N., Li, Y., Qian, J., Zou, Y., Kobinger, G., Gao, G.F., Qiu, X., Yang, S., Xia, X., 2016. Treatment with hyperimmune equine immunoglobulin or immunoglobulin fragments completely protects rodents from Ebola virus infection. Sci Rep 6, 24179.

Table 1. Results of antibody evaluation with anti-ZEBOV reagents [a]

	Neutralization assay	**Neutralization assay**	**Indirect ELISA**
	NT_{50}	**NT_{90}**	
Lyophilized IgG	10,240	2,560	20,480
horse serum	10,240	1,280	5,120

[a] Three replications were performed for each trial.

Figure legends

Figure 1. Production and identification of pseudo-EBOV. (a) The complete genome of ZEBOV. (b) Detail of the ZEBOV G gene insertion in pcDNA4.0-G. The ZEBOV G gene was inserted through the BamH I site at the 5' end and the Xba I site at the 3' end, and the expression of the EBOV G gene was driven by the CMV immediate-early promoter (arrow). (c) Production strategy of pseudo-EBOV. After co-transfection with pcDNA4.0-G and pNL4-3.luc.RE, HEK293T cells were cultured at 37 with 5% CO_2 for 24-36h, finally the pseudo-EBOV in supernatant was harvested. (d) Western blot analysis of pseudo-EBOV. HEK293T cells were transfected with two-plasmid system, or indicated plasmid, respectively. At 30h p.t., the transfected cells were harvested and lysed. Western blot was performed for indicated proteins by using corresponding antibodies. (e) Electron microscopy of pseudo-EBOV. The harvested pseudo-EBOV were applied to grids, stained with 1% sodium phosphotungstate, followed by observed and imaged using transmission electron microscopy. Pseudo-EBOV particles are indicated by red arrows. (f) Immunoelectron microscopy of pseudo-EBOV. The harvested pseudo-EBOV were applied to grids, followed by incubation with murine anti-EBOV GP monoclonal antibody for 1h at room temperature. After three washes, gold-labeled goat anti-mouse IgG was used as secondary antibody. Subsequently, the formvar-coated grids were stained with 1% sodium phosphotungstate after three washes. Images were acquired with transmission electron microscopy.

Figure 2. Infectivity and growth kinetics of pseudo-EBOV. (a) Cell tropism of pseudo-EBOV in a

variety of cell lines was detected. As the positive control, pseudotyped vesicular stomatitis virus (VSV) was generated by the transfection of HEK293T cells with the VSV GP-encoding plasmid and pNL4-3.luc.RE. Data represent the mean relative luciferase units (RLUs) ± standard deviation (SD) from four parallel wells in 96-well culture plates. (b and c) Huh-7 cells were infected with pseudo-EBOV harvested at different post transfection time points. RLUs and titers of pseudo-EBOV were measured 48h later. Data represent the mean relative luciferase units (RLUs) ± standard deviation (SD) from four parallel wells in 96-well culture plates. (d) The pseudo-EBOV harvested at 36 h post transfection represents the pseudo-EBOV in first cycle, while the pseudo-EBOV harvested after 2 days after the first cycle of infection in Huh-7 cells represents the pseudo-EBOV in the second cycle. RLUs of pseudo-EBOV in the first and second cycle were measured. Data represent the mean relative luciferase units (RLUs) ± standard deviation (SD) from four parallel wells in 96-well culture plates. P-value was determined by unpaired Student's t-test, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, or no significance (n.s) (e) The titers of pseudo-EBOV in the first and second cycle were determined.

Figure 3. Neutralization assay for the evaluation of the neutralizing activity against EBOV. (a) Equine immunoglobulin fragments were subjected to pseudo-EBOV-based neutralization assay and live EBOV-based neutralization assay, respectively. The neutralizing titers of equine immunoglobulin fragment determined by the two assays are indicated. The average results of three independent experiments are presented. (b) Neutralizing activities of three different antibody-based samples were evaluated by pseudo-EBOV-based neutralization assay, negative horse IgG was used as negative control. The correlation between the neutralization efficiency and serial dilution of all samples is shown. The data represent three independent experiments.(c, d) NT_{50} and NT_{90} of the samples derived from pseudo-EBOV-based neutralization assay are shown. L.O.D., limit of detection. The mean ± standard deviations from three independent experiments are shown.

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