



The Application of a Safe Neutralization Assay for Ebola Virus Using Lentivirus-Based Pseudotyped Virus

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Received: 28 July 2020 / Accepted: 19 April 2021
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Dear Editor,

Zaire Ebola virus (EBOV) is a negative-sense single-stranded RNA virus that is capable of causing acute hemorrhagic fever with a frightening fatality rate that can reach up to 90%. Due to its high lethality and frequent recurrence, EBOV is a substantial threat to public health. Research about live EBOV is restricted to BSL-4 laboratories; therefore, conventional serological detection methods do not provide a safe way to evaluate neutralizing activity, resulting in a bottleneck in antibody-based detection of viruses such as EBOV. Here, we aim to develop and apply a neutralization assay for EBOV using a generated lentivirus-based pseudotyped EBOV bearing GP on its surface.

The genome of the luciferase-expressing lentivirus plasmid pNL4-3.luc.R-E- is shown in Fig. 1A, and a diagram outlining the method used to produce pseudotyped

EBOV is shown in Fig. 1B. Briefly, HEK293T cells were transfected with the two-plasmid system (pcDNA4.0-GP & pNL4-3.luc.R-E-, 2 µg of each plasmid), and pseudotyped EBOV-containing supernatants were harvested at 24–36 h post-transfection (h p.t.). Titer determinations were subsequently performed (Supplementary Material). Among viral proteins, GPs are considered the major pathogenicity factor (Panina *et al.* 2017). Entry of EBOV into its target cells is initiated by interaction between the viral GP and receptors on the surfaces of the target cells. Therefore, GP of EBOV (GenBank accession no. KJ660346.2) was used to construct the pseudotyped EBOV in this study. To identify whether EBOV GP is expressed in the pseudotyped EBOV system, western blotting was performed. As shown in Supplementary Fig. S1, both EBOV GP and HIV-1 p24 protein were identified in pseudotyped virus-containing supernatants and transfected cell lysates. Transmission electron microscopy (TEM) was performed to analyze the morphology of pseudotyped EBOV particles. As shown in Fig. 1C, pseudotyped EBOV particles with diameters of approximately 100–120 nm were observed in pseudotyped virus-containing supernatants, and EBOV GPs were observed on the surface of the pseudotyped EBOV, indicating efficient packaging and secretion of the pseudotyped EBOV. Multiple gold particles were found to bind to EBOV GP on the surface of the pseudotyped virus by immunoelectron microscopy (IEM) observation (Fig. 1D). Altogether, the data suggest that EBOV GP was effectively incorporated on the surface of the HIV-1 backbone, and pseudotyped EBOV were packaged and rescued.

Due to broad tissue and cellular tropism of EBOV, we used three human cell lines from different tissues (Huh-7, liver; HEK293T, kidney; and A549, lung), one monkey cell line (Vero, kidney epithelial cells obtained from an African green monkey), two rodent cell lines (BHK-21, baby hamster kidney cells; BSR, BHK-21 cells constitutively

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s12250-021-00405-8>.

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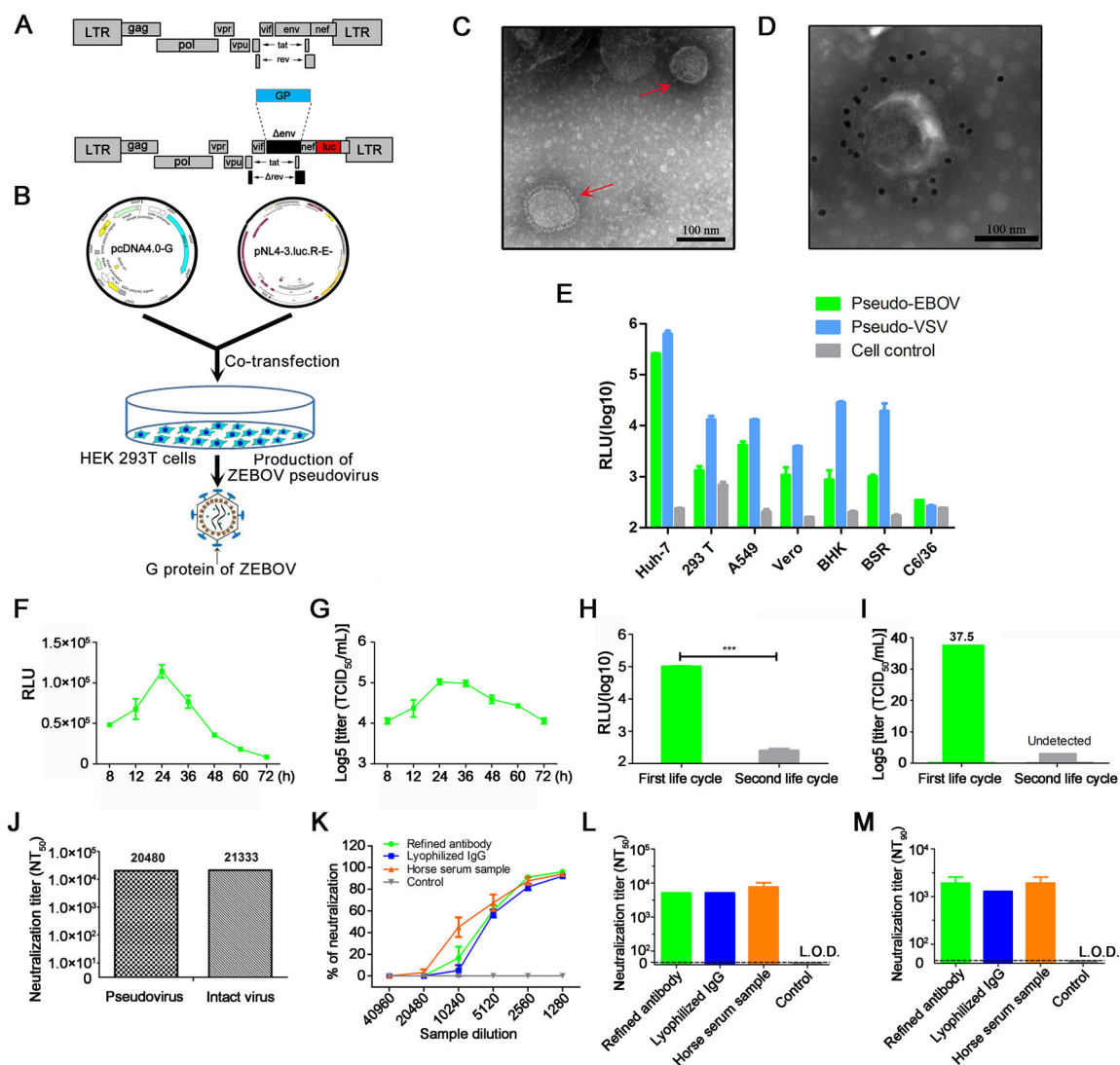


Fig. 1 Production and application of pseudo-EBOV. **A** Schematic diagrams of the HIV genome (top) and pNL4-3.luc.R-E- (bottom). **B** Strategy for the production of pseudo-EBOV. **C** Electron microscopy of pseudo-EBOV. The harvested pseudo-EBOV was applied to grids, stained with 1% sodium phosphotungstate, and observed and imaged using transmission electron microscopy. Pseudo-EBOV particles are indicated by red arrows. **D** Immunoelectron microscopy of pseudo-EBOV. The harvested pseudo-EBOV was applied to grids, followed by incubation with a murine anti-EBOV GP monoclonal antibody for 1 h at room temperature. After three washes, gold-labeled goat anti-mouse IgG was used as a secondary antibody. After three washes, the formvar-coated grids were stained with 1% sodium phosphotungstate, and transmission electron microscopic images were acquired. **E** Cell susceptibility of pseudo-EBOV was detected in a variety of cell lines. Pseudotyped VSV-G was used as a positive control. Data represent the mean relative luciferase units (RLUs) \pm standard deviation (SD) from four parallel wells in 96-well culture plates. **F** and **G** Huh-7 cells were infected with pseudo-EBOV harvested at various times after transfection. The RLUs and titers of pseudo-EBOV were measured 48 h later. RLUs were normalized to the RLU value of the negative control; a positive result was defined as the ratio more than 3.0. For titration, TCID₅₀ (50% tissue culture

infective dose) were calculated by the method of Reed and Muench. **H** and **I** The pseudo-EBOV harvested at 36 h p.t. represents the pseudo-EBOV in the first cycle, while the pseudo-EBOV harvested from Huh-7 cells 2 days after the first cycle of infection represents the pseudo-EBOV in the second cycle. The RLUs and titers of pseudo-EBOV in the first and second cycles were measured. P-value was determined using the unpaired Student's *t*-test; ****P* < 0.001. **J** Equine immunoglobulin fragments were subjected to the pseudo-EBOV-based neutralization assay and the live EBOV-based neutralization assay. The neutralizing titers of equine immunoglobulin fragments determined by the two assays are indicated. The averaged results of three independent experiments are presented. **K** Neutralizing activities of three different antibody-based samples were evaluated using the pseudo-EBOV-based neutralization assay; negative horse IgG was used as the negative control. The correlation between the neutralization efficiency and serial dilution of all samples is shown. **L** and **M** The NT₅₀ and NT₉₀ of the samples derived from pseudo-EBOV-based neutralization assay are shown. L.O.D., limit of detection. The means \pm standard deviations from three independent experiments are shown. The data represent three independent experiments.

expressing T7 RNA polymerase), and one mosquito cell line (C6/36 cells obtained from *Aedes albopictus* larvae) to analyze whether pseudotyped EBOV maintains the same susceptible cells as authentic EBOV. As a positive control, we constructed a pseudovirus expressing vesicular stomatitis virus glycoprotein (VSV-G) because of its capability of infecting various target cell lines (Zhao *et al.* 2013a). As expected, GP endowed the pseudotyped EBOV with same ability to infect target cells as authentic EBOV; the pseudotyped EBOV was able to infect all of the target cells tested in this study except C3/36 cells from mosquitoes (Fig. 1E). These findings suggest that the rescued pseudotyped EBOV is able to infect a variety of target cells but not mosquito C3/36 cells; this is consistent with the characteristics of EBOV (EBOV has no infectivity in C6/36 cells). Thus, it matches the features of EBOV, which is not mosquito-borne and cannot infect mosquito cells. The results also indicate that the infectivity of pseudotyped EBOV was higher in Huh-7 cells than in any of the other cell lines tested; as a result, Huh-7 cells were selected for study of the kinetics of pseudotyped EBOV production.

The RLU values of pseudotyped EBOV rescued at indicated time points post-transfection are shown in Fig. 1F. Pseudotyped EBOV reached a peak RLU value at 24 h p.t.. The titers of pseudotyped EBOV rescued at different times are shown in Fig. 1G; 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 supernatant from Huh-7 cells infected with pseudotyped EBOV and used it to reinfect other Huh-7 cells. Two days later, RLU activity and the viral titer of the supernatant, representing the second-cycle characteristics of pseudotyped EBOV were measured. As shown in Fig. 1H, the RLU activity of the second life cycle of the virus was substantially lower than that of the first life cycle, and the titer of pseudotyped EBOV (Fig. 1I) in its second life cycle could not be detected. These findings show that the pseudotyped EBOV has only one life cycle, demonstrating that this pseudotyped EBOV should be safe for use in research.

Equine immunoglobulin fragments against EBOV that had been produced previously (Zheng *et al.* 2016) were used as a positive control in the optimization of the pseudotyped EBOV-based neutralization assay. 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 (Supplementary Material). As expected, similar results were acquired using the two different assays (Fig. 1J). Both NT₅₀ 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 assays in a manner comparable to authentic, live EBOV but with a reduced health risk.

Three samples of antibody-based reagents against EBOV (immunoglobulin fragments, lyophilized IgG, and equine antisera) were evaluated *in vitro* using the pseudotyped EBOV-based neutralization assay. As shown in Fig. 1K, the neutralization efficiency of all samples correlated with the serially diluted sample concentration, and a linear relationship was confirmed. The NT₅₀ and NT₉₀ of the three samples are shown in Figs. 1L and 1M. The results reveal that all samples showed high neutralization activity against the EBOV pseudotyped virus. We concluded that the pseudotyped EBOV-based neutralization assay was sufficiently reliable for use in the evaluation of 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 with those of an indirect ELISA (Supplementary Material). 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 that the results obtained using the pseudotyped EBOV-based neutralization assay are valid and reliable.

The lack of basic research and translational technology has been highlighted since the 2014–2016 EBOV epidemic in West Africa. Due to BSL-4 facility restrictions, it is a priority to develop a safe method for EBOV research that can be used under low BSL restriction conditions and has further applicability. Although the result of the neutralization assay is gold standard for antibody detection, BSL-4 laboratories are not available to many research groups. Due to this situation, many groups have developed an increasing number of agents, especially, single replication cycle pseudotyped viruses, for hazardous pathogens based on the lentivirus system (Qiu *et al.* 2013; Zhao *et al.* 2013b; Chen *et al.* 2018). In addition, efficient expression of the luciferase reporter gene in infected cells after GP-mediated infection was demonstrated (Figs. 1F and 1G), indicating that infection by these pseudotyped EBOV is efficient. These features make pseudotyped EBOV a safe and quantifiable tool for antiviral drug discovery and for the evaluation of neutralizing activity.

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

	Neutralization assay		Indirect ELISA
	NT ₅₀	NT ₉₀	
Lyophilized IgG	10240	2560	20480
Horse serum	10240	1280	5120

^aThree replications were performed for each trial.

It is required that all assays involving live EBOV be performed under BSL-4 conditions because of the high lethality of EBOV, and this restriction limits the application of these assays. However, the pseudotyped EBOV generated using our two-plasmid system has only a single infection cycle (Figs. 1H and 1I) and therefore cannot cause exposure and death. In addition, we developed a neutralization assay based on the pseudotyped EBOV that can be used to evaluate the neutralizing activity of EBOV antibodies. As our data shown (Fig. 1J), the pseudotyped EBOV neutralization assay can be successfully used in the evaluation of neutralizing activity, and it produces results that are consistent with those obtained using an authentic EBOV-based neutralization assay for neutralizing antibody detection, thereby confirming its potential application value in antiviral drug discovery and neutralizing antibody evaluation.

Acknowledgements We thank Gary Wong for help with experiments involving authentic EBOV. We thank our lab members and colleagues at Jilin University for helpful discussions during the course of the study. This research was supported by the National Science and Technology Major Project of the Ministry of Science and Technology of China (Grant 2016ZX10004222).

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Animal and Human Rights Statement All animal studies in this work were approved by the Animal Care and Use Committee of the

Chinese People's Liberation Army (No. SYXK2009-045). All efforts were made to minimize animal suffering.

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