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ORIGINAL RESEARCH PAPER

Establishment of a neutralization assay for Nipah virus using a high-titer pseudovirus system

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Abstract

Objective To construct a high-titer Nipah pseudovirus packaging system using the HIV lentivirus backbone vector and establish a safe neutralization assay for Nipah pseudovirus in biosafety level 2 facilities.

Methods Nipah virus (NiV) fusion protein (F) and glycoprotein (G) recombinant expression plasmids, psPAX2, and pLenti CMV Puro LUC (w168-1) were transiently transfected into 293T cells for 72 h for the generation of a NiV pseudovirus. The neutralization ability of Nipah virus F and G protein antibodies was assessed using the pseudovirus.

Results A NiV pseudovirus was constructed using 293T cells. The ideal mass ratio of plasmid psPAX2: w168-1: F: G for transfection was determined to be 4:4:1:1. The specificity of recombinant F and G protein expression was indicated by indirect immunofluorescence and western blotting. The pseudovirus particles showed obvious spikes under a transmission electron microscope. The NiV pseudovirus titer was 4.73×10^5 median tissue culture infective dose per mL, and the pseudovirus could be effectively neutralized by polyclonal antibodies specifically targeting the F and G proteins respectively.

Conclusions A NiV pseudovirus was successfully generated using HIV vector systems, and was used as a platform for a safe and reliable pseudovirus-based neutralizing assay that can be performed in biosafety level 2 facilities.

Keywords Fusion protein · Glycoprotein · Neutralization assay · Nipah virus · Pseudovirus

Introduction

Nipah virus (NiV) is an enveloped, single-stranded, non-segmental, negative-stranded RNA virus belonging to the genus *Henipavirus*, in the *Paramyxoviridae* family, which causes Nipah virus disease (NVD). NVD is a virulent zoonotic disease that causes acute respiratory illness and fatal encephalitis (Yob et al. 2001). The primary carrier of NiV is fruit bats, but NiV exhibits a broad host tropism, and can infect humans, pigs, horses, dogs, and other animals (Ang et al. 2018; Looi et al. 2007). Studies have shown that close contact with pigs was the primary source of human Nipah infections. NiV outbreaks have occurred mainly in Southeast Asia, with almost annual occurrences in Bangladesh, and

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human-to-human transmission has been observed, raising concerns about a worldwide pandemic (Chakraborty et al. 2016; Epstein et al. 2006; Gurley et al. 2007; Nikolay et al. 2019).

An outbreak of NiV started in Malaysia in 1998–1999 and 663 laboratory-confirmed cases of NVD were diagnosed as of September 2021 according to World Health Organization (WHO) data. The outbreak resulted in approximately 388 deaths (the case fatality rate was 58.52%), with an average case fatality rate of between 40 and 90% (https://www.who.int/emergencies/disease-outbreak-news/item/nipah-virus-disease---india). The total number of people at risk of NiV infection exceeds two billion including all countries in which NiV outbreaks and Pteropus bats live naturally (Satterfield et al. 2016). NVD has been listed by the WHO as a priority disease for research, development, control and prevention (Mehand et al. 2018).

The NiV genome is approximately 18.2 kb in length, consisting of two families, NiV-M (18246nt) and NiV-B (18252nt). The genome of the NiV encodes six structural proteins: nucleocapsid (N); phosphoprotein (P); matrix protein (M); fusion protein (F); glycoprotein (G); and RNA polymerase (L). The F and G proteins are responsible for cellular attachment of the virus and subsequent host cell entry (Bae et al. 2019), and play an important role in the virus crossing the blood–brain barrier. The NiV envelope G protein binds to the host cell receptors ephrin-B2 and/or -B3 receptors inducing a conformational change, which in turn triggers the refolding of the F protein (Liu et al. 2015; Wong et al. 2017), prompting the activation of the F glycoprotein and viral membrane fusion (Steffen et al. 2012). The conformational change of the F protein starts with the synthesis of an inactive precursor of F protein (F0) that is cleaved by a host protease into two subunits, F1 and F2, which are linked by disulfide bonds. The fusion peptide of the virus contained in the F1 subunit mediates the fusion of the virus and the host cell membrane, which enables the viral genome to enter the cells (Wong et al. 2021).

There are no effective drugs or treatments available for NiV, therefore, it is desirable to establish a safe and reliable method to detect NiV as early as possible. However, NiV is a biosafety level 4 pathogen that should be handled in biosafety level (BSL) 4 facilities, which has restricted the development of anti-NiV vaccines and drugs. Various platforms have investigated the feasibility of employing one or two outer membrane proteins, such as the F protein and/or G protein, as targets to design immunogens to induce protective immunity. For example, a glycoprotein from the related Henipavirus hendra virus (HeV), has been approved to protect horses from lethal HeV infection and has been licensed for use by the equine industry in Australia (Khusro et al. 2020; Middleton et al. 2014). Therefore, antibodies and inhibitors against F and G proteins could be potential therapeutic agents to block NiV entry into cells. Pseudoviruses expressing NiV-F and -G proteins on the particle surface largely mimic infectious NiV, therefore, a neutralizing assay using such pseudoviruses may be useful to detect NiV in BSL-2 laboratories.

A NiV pseudovirus was constructed from pCDNA3.1(+)-F and pCDNA3.1(+)-G recombinant plasmids using the HIV lentivirus packaging system under BSL-2 conditions, and the pseudovirus packaging conditions were optimized to generate a high-titer pseudovirus. Then, the protein expressions were identified and the morphology of the pseudovirus was determined. In brief, a pseudovirus-based neutralization assay was developed, laying the foundation for developing a pseudovirus-based neutralizing antibody detection platform.

Materials and methods

Cell cultures

Hep2, A549, 293T, Huh7, MDCK, LLC-MK2, Vero E6, and HeLa cells were kept in our laboratory and maintained in high glucose Dulbeco's modified essential medium (DMEM, Thermo), which was supplemented with 10% Fetal bovine serum (FBS, Thermo), penicillin, streptomycin, and HEPES (Thermo). The cells were incubated in a 5% CO_2 environment at 37 °C and subcultured at 80% confluence.

Recombinant NiV-F and NiV-G expression, purification and immunization

NiV F and G genes derived from the National Center for Biotechnology Information (NCBI) nucleotide database (GenBank access No. AF212302 and AJ564621) were synthesized by Tsingke

Biotechnology (Inc., Ltd, Beijing, China), and then cloned into the prokaryotic expression vector pET-30a(+) (Novagen, 69909) using BamH I and Not I restriction enzyme, respectively. The recombinant NiV-F and NiV-G plasmids were transformed into *Escherichia coli* (*E. coli*) *BL21* (*DE3*, Takara) competent cells which were cultured in lysogeny broth medium supplemented with kanamycin (100 μg/mL) at 180 rpm at 37 °C until the OD600 was about 0.6–0.8, respectively. Then 0.1 mM Isopropyl β-D-Thiogalactoside (IPTG) was added into the medium and then was harvested after 4 h at 6,000 × g for 20 min at 4 °C. The supernatant was removed and washed twice with phosphate-buffered saline (PBS). Then the precipitate was resuspended in buffer (20 mM sodium phosphate, 500 mM NaCl) and sonicated in ice-water bath. The lysate was added to NI–NTA agarose (Qiagen), and the adsorbed proteins were eluted with a linear gradient of imidazole. Rabbits were inoculated by the intramuscular route at days 0, 14 and 28 with 100 μg of the NiV-F and NiV-G protein with adjuvant (Sigma), respectively, and sacrificed at day 35. Serum against the recombinant NiV-F and NiV-G protein were collected and centrifuged at 1500 × *g* for 10 min at 4 °C.

Construction of the NiV F and G expression vectors and detection of protein expression by indirect immunofluorescence

The codon-optimized NiV F and NiV G were downloaded from NCBI nucleotide database (Genbank access No. AY816748 and AY816745) and synthesized, and then cloned into the mammalian expression plasmid pcDNA3.1(+) (Invitrogen, V790-20) to construct the envelope plasmids pcDNA3.1-F and pcDNA3.1-G, respectively, using restriction endonuclease digestion with EcoRI and XbaI, at the 5′ and 3′ ends, respectively. The recombinant plasmids were sequenced with primer pairs and the results were compared with reference sequences. Sanger sequencing was performed with the following primers: forward, 5′-GGAATTCATGGTGGTGATCCTGGACAAGCGG-3′; reverse, 5′-GATCTAGATTAGATGTACCGGTAGGTGTCGGTG-3′ (NiV-F), forward, 5′-GGAATTCATGCCTGCCGAGAACAAGAAAGTGCGGTTCGAGAAC-3′; reverse, 5′-GATCTAGATCAGGCGTAGTCGGGCACGTCGTAGGGGTAGC-3′ (NiV-G).

293T cells were cultured in the 24-well plates (1.5×10^5/well) overnight, then protein expression plasmids pcDNA3.1-F, psPAX2 (Addgene, 12260), and pLenti CMV Puro LUC (w168-1) (Addgene, 17477) were cotransfected into 293T cells reaching 60% confluence, using the transfection agent Polyethylenimine Linear (PEI, Sigma) in a 5% CO_2 environment for 48 h at 37 °C, then psPAX2, w168-1, and recombinant plasmid pcDNA3.1-G were subjected to the same method. psPAX2, w168-1, and vector pcDNA3.1 were also cotransfected as a control group. Immunofluorescence identification was conducted by the following protocol: the three groups were fixed in 4% (w/v) paraformaldehyde at 4 °C for 30 min and then blocked with Phosphate buffered saline (PBS, Thermo) with 5% (w/v) bovine serum albumin (BSA) for 1 h at 37 °C. For F and G proteins, the primary antibodies were 1:2,000 dilutions of rabbit anti-NiV F and rabbit anti-NiV G, respectively, and the rabbit antibodies were obtained by immunizing rabbits with protein in our laboratory. For the HIV p24 protein, the primary antibody was mouse anti-p24 (1:20,000 dilution, TaiWei Technology, China), and the cells were incubated with primary antibodies for 2 h at 37 °C and then washed three times. The secondary antibody for the F and G proteins was DyLight 594 goat anti-rabbit IgG (1:5,000 dilution, Thermo), and for the p24 protein was DyLight 488 goat anti-mouse IgG (1:5,000 dilution, Thermo). The cells were incubated with secondary antibodies for 1 h at 37 °C. Finally, the cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) at room temperature for 30 min. The antigen–antibody results were observed by fluorescence microscopy.

Optimization of pseudovirus production in cells

Hep2, A549, 293T, Huh7, MDCK, LLC-MK2, Vero, and HeLa cells were cultured in T75 flasks at a density of 3×10^5 cells/mL, and 60–70% confluent cells were treated with the three different groups. w168-1, psPAX2, pcDNA3.1-F, and pcDNA3.1-G plasmids (3.75 μg each) were cotransfected into the above cells to generate the NiV pseudovirus. The pMD2.G plasmid was used as the positive control and non-transfected cells were used as the negative control. The luciferase values of the cell lysates were measured using the Bright-Glo luciferase assay system kit and

then, the target cell lines were selected to generate the NiV pseudovirus.

Optimization of transfection ratio for NiV pseudovirus

After the packaging cells were identified, the transfection quality ratios of pcDNA3.1-F, and pcDNA3.1-G were optimized. W168-1 and psPAX2 were used at a ratio of 1:1, and pcDNA3.1-F and pcDNA3.1-G were transfected into 293T cells at ratios of 4:1, 2:1, 1:1, 1:2, and 1:4, as described previously. After transfection for 48 h, the luciferase values were detected as described previously. When the optimal ratio of the outer membrane protein plasmid (pcDNA3.1-F and pcDNA3.1-G) was determined, we investigated the ideal ratio of the backbone plasmid psPAX2 and including w168-1, which were transfected in the optimal cells at ratios of 1.5:1, 4:1, 2:1, 1:1, 1:2, and 1:4 with the outer membrane protein plasmids. The luciferase values were detected after transfection for 48 h in a 5% CO_2 environment at 37 °C according to the manufacturer's recommendations. Under the optimal conditions, NiV pseudoviruses were harvested after 72 h incubation. For concentration and purification, the supernatants were filtered with filters (0.22 μm, Millipore) and centrifuged at 30,000 × *g* using 20% (m/v) sucrose for 6 h. Then, the pseudoviruses were collected by resuspension in 500ul PBS and stored at − 80 °C in 1-mL aliquots until use. To maintain the best performance a frozen pseudovirus should be thawed once to prevent repeated freeze–thaw cycles.

Western blot analysis

Approximately 40 *μL* of NiV pseudovirus was mixed with 6 × loading buffer and heated for 5 min at 100 °C to perform SDS-PAGE (Solarbio) using 5% (v/v) stacking gel and 10% (v/v) separating gel. The gels were blotted onto a 0.4 μm Polyvinylidene Fluoride (PVDF, Solarbio) membrane and incubated in a blocking buffer containing 5% (w/v) BSA and PBS with 0.5% v/v Tween 20 (PBS-T) for 3 h at 4 °C. Washing was performed between the steps using PBS-T. The primary antibodies were anti-F rabbit serum, anti-F rabbit serum (1:2,000 dilution), and anti-p24 mouse serum (1:20,000 dilution), which were incubated for 2 h at 37 °C. Horseradish peroxidase (HRP)-conjugated rabbit and mouse IgG antibodies (1:5,000 dilution) were used as secondary antibodies for 1 h at 37 °C. Additionally, 3,3′,5,5′-tetramethylbenzidine (TMB) was used as a chromogenic substrate for HRP.

Morphological observations

NiV pseudovirus (5 μL) was adsorbed on the electron microscope carrier net, and conventional negative staining was performed using 1% phosphotungstic acid. After the carrier net was dried, the particle morphology and size were observed using transmission electron microscopy (FEI, Tecnai12).

Pseudovirus target cells

Hep2, 293T, Huh7, MDCK, LLC-MK2, Vero, and HeLa were selected as candidate cells, and the cells were diluted to a concentration of 5×10^5 cells/mL in 96-well plates and cultured at 37 °C in a 5% CO_2 environment. The above cells were inoculated with the pseudovirus (1:100 dilution), in 96-well culture plates and the experiments were repeated six times, along with a control cell for every group, and the luciferase values were detected 36 h after infection.

Titration of pseudoviruses

For the NiV pseudovirus, the 50% tissue culture infectious dose ($TCID_{50}$) values were determined by the following method: NiV pseudovirus was diluted by serial fivefold dilutions (9 dilutions in total) with PBS, and a control cell was used as a negative control. Then, the target cells previously described (3×10^4 cells/100 μL) were seeded into the pseudoviruses in 96-well plates. After 48-h incubation in a 5% CO_2 environment at 37 °C, the culture supernatants were aspirated gently to leave 100 μL in each well; then, 100 μL of luciferase substrate (Promega, USA) was added to each well. After incubation for 2 min at room temperature, 150 μL of lysate was transferred to white solid 96-well plates for the detection of luminescence using a Glomax microplate luminometer (Promega, USA).

Pseudovirus neutralization assay

Rabbit polyclonal antibodies against the F and G proteins were serially diluted twofold (7 dilutions in total), mixed with 200 TCID50 NiV pseudovirus, and

incubated at 37 °C for 1.5 h. The NiV pseudovirus control and 293T cells control were used as the positive and negative controls, respectively. 293T cells were seeded 100 uL on 96-well plates at the density of 5×10^5 cells/mL and incubated at 37 °C in a 5% CO_2 environment, then the 100 uL pseudovirus-antibody mix was added to the cells to incubate for 48 h, relative luciferase activity (%) was detected. The relative luciferase activity (%) could be calculated according to the following equation:

$$\left(1-\frac{\text{mean Luc from each sample (virus + antibody)} - \text{mean Luc from cell control}}{\text{mean maximum Luc from virus control} - \text{mean Luc from cell control}}\right)\times 100\%$$

The results of the neutralization assays were plotted by normalization to samples where no antibody was used.

Statistical analysis

The data are shown as the mean with standard deviation for every condition. Statistical analysis and graphs were generated using GraphPad Prism 8.0 software. One-way analysis of variance was used to compare differences between groups, and the least significant difference test was used for multiple comparisons between two groups. A p-value <0.05 was considered to represent statistical significance between groups. Each experiment was repeated at least three times.

Results

Determination of NiV F and G protein expression by indirect immunofluorescence

The experimental groups consisting of pcDNA3.1-F, w168-1, and psPAX2 and pcDNA3.1-G, w168-1, and psPAX2 were transfected into 293T cells, and specific red fluorescence (F and G proteins) and green fluorescence (p24 protein) were observed under a fluorescence microscope after 48 h, while in the control groups, consisting of pCDNA3.1, w168-1, and psPAX2, only specific green fluorescence (p24 protein) was observed, indicating that F protein, G protein, and p24 protein were successfully expressed (Fig. 1).

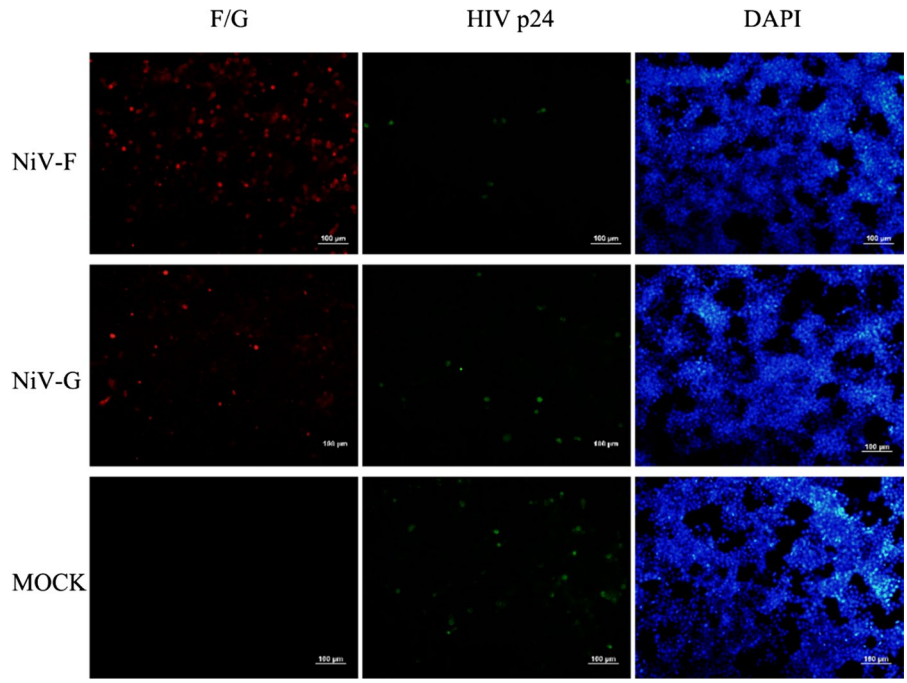

Fig. 1 F and G protein expression in 293T cells by indirect immunofluorescence. In the first column, NiV F and G proteins were incubated with the primary antibodies, rabbit antiserum to NiV F and NiV G, respectively, the secondary antibody was DyLight 594 anti-rabbit IgG; in the second column, Gag-p24 protein was incubated with anti-p24 mouse serum, the secondary antibody was DyLight 488 anti-mouse IgG; in the third column, 293T cell nuclei were stained with 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI, Thermo) Scale bars:100 μm.

Optimization of pseudovirus production

The plasmids pcDNA3.1-F, pcDNA3.1-G, w168-1, and psPAX2 were transfected into Hep2, A549, 293T, Huh7, MDCK, LLC-MK2, Vero, and HeLa cells, and the Luc values were measured 48 h after transfection. The pMD2.G plasmid as the positive control was able to be successfully transfected into all the above cells (Fig. 2a). NiV pseudovirus-transfected 293T cells showed significantly higher luciferase values than the other six cells ($P < 0.05$); therefore, 293T cells were determined to be the best packaging cells for the NiV pseudovirus. The luciferase values at a 1:1 ratio of pcDNA3.1-F: pcDNA3.1-G compared with 4:1, 2:1, 1:2, and 1:4 ratios demonstrated significant increment ($P < 0.05$, Fig. 2b). The luciferase values were also significantly higher ($P < 0.05$) for a 4:1 ratio of backbone plasmid to membrane plasmid (pcDNA3.1-F and pcDNA3.1-G) compared with 2:1, 1:1, 1:2, and 1:4 ratios (Fig. 2c). Thus, NiV pseudovirus packaging plasmid psPAX2: w168-1: pcDNA3.1-F: pcDNA3.1-G showed the highest packaging efficiency when the ratio was 4:4:1:1.

Western blot identification of protein expression

The western blot of the NiV pseudovirus showed distinct bands for the individual proteins, with the F protein at approximately 48 kDa (Fig. 3a), the G protein at 67 kDa (Fig. 3b), and the HIV P24 protein

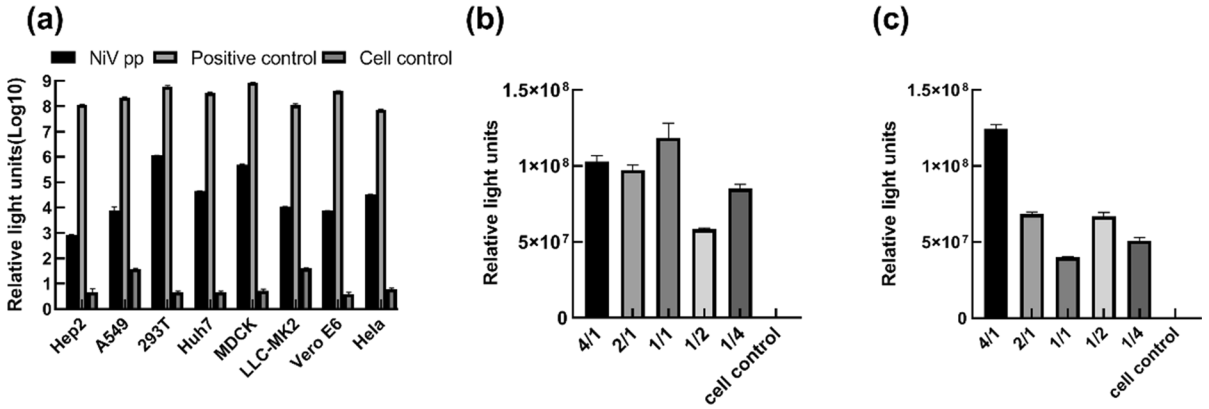

Fig. 2 NiV pseudovirus packaging system optimization. **a** Optimization of the cell type, **b** optimization of transfection quality ratio of plasmid pcDNA3.1-F to pcDNA3.1-G; and **c** optimization of transfection quality ratio of backbone plasmid and envelope plasmid. All data are expressed as mean values ± standard deviations

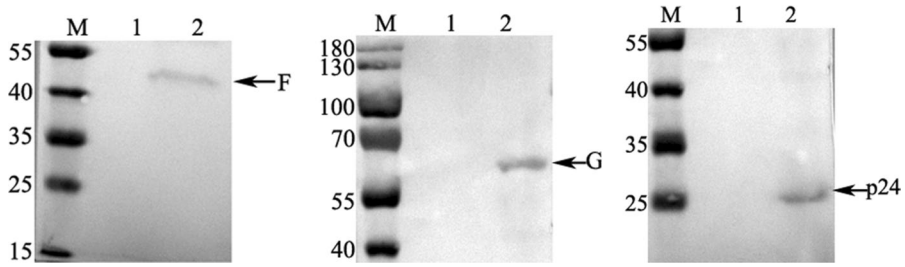

Fig. 3 Western blot identification of NiV pseudovirus F protein, G protein, and HIV P24 protein. **a** Identification of F protein using rabbit polyclonal antibody to F protein. **b** Identification of G protein using rabbit polyclonal antibody to G protein. **c** Identification of p24 protein using mouse monoclonal antibody to p24 protein. In **a**, **b**, and **c** lane 1 is the cell control and lane 2 is the NiV pseudovirus

band at 24 kDa (Fig. 3c), which corresponded to the expected sizes, indicating the specific expression of NiV F and G proteins and HIV P24 protein in the pseudovirus particles.

Transmission electron microscopy analysis of pseudovirus morphology

The NiV pseudovirus was centrifuged at 30,000 × *g* for 6 h and negatively stained by conventional methods. The morphology was observed by transmission electron microscopy, which indicated some round or oval particles with an envelope and spikes on the membrane, with diameters between 100 and 200 nm (Fig. 4).

Sensitive cell screening for NiV pseudovirus infection

Hep2, 293T, Huh7, MDCK, LLC-MK2, Vero, and HeLa cells were infected with NiV pseudovirus, and Luc was detected after incubation at 37 °C for 36 h. The highest Luc value was observed when 293T cells were infected, which was significantly higher than the value in other cells ($P < 0.05$, Fig. 5), indicating that 293T cells were the most sensitive cells for NiV pseudovirus infection of the tested cells. Thus, the neutralization of NiV pseudovirus was established using 293T cells, and the NiV pseudovirus titer was calculated to be 4.73×10^5 $TCID_{50}$ per mL.

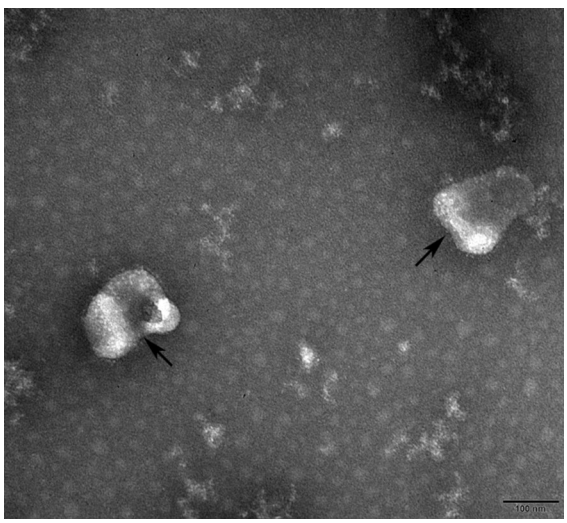

Fig. 4 Morphological observation of NiV pseudovirus under an electron microscope. Pseudovirus was negatively stained with 1% uranyl acetate, scale bars:100 μm

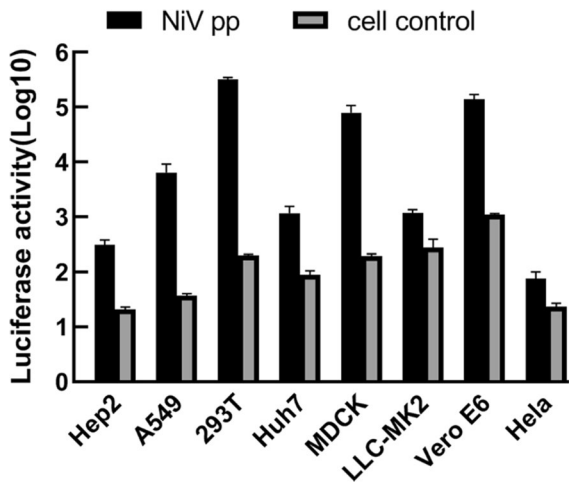

Fig. 5 NiV pseudovirus infection in different cells. Pseudoviruses were used to infect Hep2, 293T, Huh7, MDCK, LLC-MK2, Vero, and HeLa cells at equal doses, and Luc was detected after incubation at 37 °C for 36 h. Data from six replicates are shown as the mean ± standard deviation

NiV pseudovirus neutralization activity

Rabbit polyclonal antibodies against F protein and G proteins were co-incubated with NiV pseudovirus (200 TCID50) and inoculated in 293T cells, showing a dose-dependent neutralizing activity, with 51.82% anti-serum inhibition of F protein at a serum dilution of 1:800 and 74.54% anti-serum inhibition of G protein at a serum dilution of 1:1600 (Fig. 6). These results indicated that a pseudovirus neutralization assay was successfully established, which could be applied to a serum neutralizing antibody assay.

Discussion

NiV has been recognized as a worldwide threat since 1999 because of its extremely high fatality rate and widespread nature. However, currently there are no available drugs or treatments for NiV, and no clinical trials have been initiated for candidate NiV vaccines. Live NiV virus research must be carried out in a BSL-4 laboratory; therefore, it is desirable to establish NiV detection methods that can be performed in

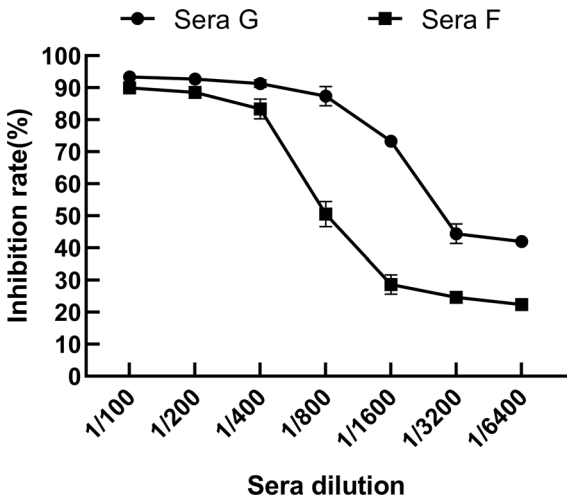

Fig. 6 NiV pseudovirus neutralization assay. NiV pseudovirus (200 $TCID_{50}$) was coincubated with serial dilutions of rabbit anti-F serum and rabbit anti-G serum, and the mixtures were added to 293T cells and incubated at 37 °C for 48 h. Experiments were repeated three times and the data are shown as the mean ± standard deviation. Rabbit anti-F polyclonal antibody and rabbit anti-G polyclonal antibody were used in these experiments

a BSL-2 laboratory. *Paramyxoviridae* viruses enter cells by fusion of the viral envelope with the host cell membrane, a process that requires the involvement of two membrane proteins, namely the attachment glycoprotein and the fusion glycoprotein (Jardetzky et al. 2014). Viral glycoprotein-mediated entry into cells may be pH-dependent. Chikungunya virus (CHIKV) and vesicular stomatitis viruses (VSV) infect cells in a pH-dependent manner, while other viruses, such as amphoteric murine leukemia retrovirus (MLV), infect cells in a pH-independent manner (Akahata et al. 2010). NiV invades host cells mainly through a pH-independent, membrane-fusion mechanism mediated by F and G proteins.

A pseudovirus was obtained by constructing the HIV genome, which lacks the gene encoding the envelope protein, in a plasmid vector, then constructing a viral envelope protein sequence to be expressed in another plasmid, and finally co-transfecting all plasmid vectors into cells to assemble a recombinant viral particle. Because the pseudovirus particle does not possess a complete genome, this virus particle cannot replicate continuously, and research can be carried out in non-BSL-4 biocontainment facilities.

We proposed to develop a NiV pseudovirus using a HIV lentiviral vector with a luciferase reporter gene and the membrane proteins F protein and G protein and perform a serum neutralization assay. The advantages of this approach are that the assay can be implemented at the BSL-2 level without exposure to the highly pathogenic NiV and the luciferase activity will directly indicate the ability of the pseudovirus to neutralize antibodies. NiV F and G gene sequences have been optimized to construct pseudovirus according to eukaryotic codon usage bias. The pseudovirus could be obtained using the most common HIV-type packaging system and a two-plasmid system, which were easy to construct, however, the titers were low (Nie et al. 2017). We selected a three-plasmid packaging system (Suzuki et al. 2020), with psPAX2 as the packaging plasmid containing the cytomegalovirus (CMV) promoter that controls the expression of the HIV structural protein gene sequence. The vector plasmid w168-1 contains the cis-acting elements and luciferase reporter genes required for HIV reverse transcription, integration and packaging. The envelope plasmid was optimized for the synthesis of NiV F and G genes, which are responsible for binding and fusion with the receptor during the viral invasion of a cell.

To obtain high titers of pseudovirus, we optimized the packaging cell type and plasmid ratios (Ou et al. 2020). Then 293T cells were selected as the most suitable packaging cells with an optimized plasmid psPAX2: w168-1: F: G transfection ratio of 4:4:1:1, which was consistent with the results of Nie (Nie et al. 2017). Both the luciferase reporter gene assay and protein expression identification indicated the successful assembly of the pseudovirus in the cells. We selected NiV-sensitive cells for titration to evaluate the packaging effect. For neutralization assessment, we co-incubated NiV F and G protein serum antibodies with 200 $TCID_{50}$ pseudovirus, which showed varying degrees of dose-dependent neutralizing activity, which was similar to the results of Kishishita (Kishishita et al. 2013), and our generated titer of NiV was higher than that generated by Kishishita. At a serum dilution of 1:100, the anti-serum inhibition of the F and G proteins was 90.09% and 96.17%, respectively, the luciferase activity was reduced to a minimum, and the pseudovirus was almost completely neutralized. The anti-serum inhibition rate of the F protein was 51.82% at a serum dilution of 1:800

and the inhibition rate of the G protein was 74.54% at a serum dilution of 1:1600. The inhibition of the NiV pseudovirus by F and G immunized sera could indicate the presence of the F and G proteins in the pseudovirus. The inhibition curves of the F and G proteins showed the same trends, but the effect of the G serum in neutralizing the pseudovirus was greater than that of the F serum, which was related to the higher titer of G serum than F serum. Therefore, the pseudovirus may replace the live virus in neutralization assays to a certain extent, overcoming the limitations of the live virus, including the difficulties in culturing the live virus in vitro and the highly infective and lethal nature of the live virus.

The pseudovirus neutralizing antibody assay can be used for serum neutralizing antibody detection and evaluation of the effect of a vaccine because of its safety, rapidity, high specificity, and the fact that the reporter gene is proportional to the serum neutralizing antibody titer, which can enable the objective quantification of the serum neutralizing antibody levels. Weissman has constructed SARS-CoV-2 D614 and G614 strains of pseudoviruses using HIV lentiviral vectors to evaluate the neutralization effect of serum antibodies from vaccinated people (Weissman et al. 2021). Both strains showed 100% neutralization activities, and pseudovirus detection methods have been shown to correlate well with live virus detection methods. Qiu constructed pseudovirus-neutralizing antibodies for H7N9 avian influenza virus, which had a sensitivity of 85.7% and specificity of 100% (Qiu et al. 2013). Similarly, Zhao has established a Middle East Respiratory Syndrome coronavirus (MERS-CoV) inhibition assay using pseudoviruses as alternatives to live viruses, which circumvented the BSL-3 assay limitations and allowed the rapid detection of MERS-CoV entry inhibitors and was also used to assess the neutralization of vaccine-induced MERS-CoV antibodies (Zhao et al. 2013). Pseudoviral technology is also widely used in receptor studies (Hu et al. 2020), animal models (Wong et al. 2017), vaccine development (Folegatti et al. 2020; Tan et al. 2020), and international standard products (Mattiuzzo et al. 2015), as it can replace BSL-3 and BSL-4 level pathogens, greatly facilitating the study of virulent pathogens.

Conclusion

F and G proteins for NiV are important components of the vesicle proteins involved in cellular adsorption and viral entry, and it has been shown that both proteins contain neutralizing epitopes (Jardetzky et al. 2014) that can be studied as targets for viral entry modes. In this work, we constructed a NiV pseudovirus packaging system using HIV lentiviral vectors and high-titer pseudoviruses that were obtained through condition optimization. This system was applied to neutralization assays, laying the foundation for the establishment of a safe and reliable neutralizing antibody method. Pseudovirus assays require a shorter experimental time compared with traditional assays, which is convenient for drug and vaccine studies. Although both immunofluorescence and western blot analysis showed that the NiV membrane proteins F and G were expressed successfully, and the NiV pseudovirus exhibited dose-dependent neutralizing activity of anti-F and G rabbit polyclonal antibodies, there are limitations to the present study that should be noted. Serum samples from naturally infected pigs or fruit bats are required for future neutralization assays, which could further indicate the neutralizing activity of the constructed pseudovirus.

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Declarations

Conflict of interest The authors declare no conflict of interests.

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