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Reproducible generation of Nipah virus pseudovirions with uniform incorporation of F and G surface glycoproteins for high-throughput neutralization assays

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Abstract

Nipah virus (NiV) is a pathogen to be handled in BSL-4 facilities. Multiple surrogate systems such as virus-like particles (VLPs) and pseudoviruses enable carrying out NiV neutralization assays and study of virus entry pathways in biosafety level-2 (BSL-2) facilities. These are dual protein expression and surface display systems comprising NiV structural glycoproteins F and G as the key components. During generation of NiV VLPs or pseudovirions, ensuring batch to batch uniformity is a major concern due to the lack of proportionate incorporation of these proteins in the producer cells as well as their consistent incorporation in the particles. We established HEK293 pseudovirion producer cells that stably co-incorporated NiV F and G proteins to address the issue. Fluorescence-activated cell sorting (FACS) analysis of clonally selected cells for high and uniform level F and G protein co-incorporation; and their further expansion were carried out to further refine the system. High titer vesicular stomatitis virus (VSV)-based pseudoviruses which showed consistent incorporation of both the glycoprotein were reproducibly generated from these producer cells. In functional assays, these Nipah pseudovirions (NiV) exhibited a dose-dependent neutralization by commercial anti-NiV F and G antibodies as well as by convalescent serum from Nipah recovered patients. A pseudovirus neutralization test (PVNT) with a secreted alkaline phosphatase (SEAP) as the reporter was established in the study. The assay supports high-throughput adaptability with a quick turn-around time. It will aid large-scale human and animal serosurveillance studies in Nipah endemic regions as well as screening of virus entry inhibitors and monoclonal antibodies.

Keywords Nipah virus, Pseudovirion, Glycoprotein, G418, Hygromycin, Clonal selection, Serosurveillance, Spill over

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Introduction

Nipah virus (NiV) is a highly lethal zoonotic paramyxovirus causing fatal encephalitis in humans [1]. Fruit bats of the *Pteropus* genus serve as a natural reservoir of the virus [2]. The initial Nipah cases identified in 1998 in Malaysia and Singapore were caused by spill over infections from pigs to humans [3–5]. On the other hand, a human-to-human transmission was evident in the re-emerging outbreaks of the disease in Bangladesh and neighbouring regions in India [6, 7]. In southern peninsular India the first outbreak occurred in 2018 in Kerala with 18 laboratory-confirmed cases [8]. Phylogenetic analysis of the NiV strain causing this outbreak revealed a 96.15% similarity to the Bangladesh strain [9]. Since the first report from India in 2001, the state of Kerala witnessed multiple Nipah outbreaks since 2018 [10, 11]. Sequential outbreaks of the disease in the Indian subcontinent have raised concerns of a re-emerging trend of this highly transmissible virus posing a major public health threat. It is one among the WHO priority pathogens that require urgent research attention for effective containment measures.

The high fatality rate (around 90% in recent outbreaks) [12] as well as the lack of effective antiviral treatment and preventive vaccines impose any studies on infectious NiV strictly restricted to BSL-4 bio-containment facilities. The absence of easily employable surveillance systems poses hindrance to prevalence studies in both humans and animals, and also in the effective and early detection of subclinical cases that may help in prevention of disease outbreaks. To circumvent this difficulty and facilitate research, several surrogate systems employing virus-like particles and pseudovirions that support studies in a BSL-2 facility have been developed.

The 18.2 kb negative-sense RNA genome of NiV codes six structural proteins and three non-structural proteins [13, 14]. The structural proteins G and F are the key envelope glycoproteins facilitating NiV binding to its cellular receptor. The glycoprotein G binds to the Ephrin B2 or B3 receptor triggering a conformational change in the fusion protein F, supporting the viral envelope fusion with the host cell membrane [15, 16]. Earlier studies with surrogate viral systems involved the use of NiV-like particles (VLPs) and pseudotyped viruses using lentivirus or Vesicular Stomatitis virus (VSV)- based systems [13, 17, 18]. The NiV VLPs that co-express the surface glycoproteins G, F, and the matrix protein M have been used in neutralization assays [3]. A pseudovirus production system for henipavirus using the MuLV packaging cell line expressing the LACZ reporter gene has been reported [13]. Exploiting the broad host range and robust replication properties of VSV, a recombinant VSV (rVSV-G*ΔG)-based system with glycoprotein G coding region deleted has been used to generate pseudotypes of several heterologous viruses including NiV [19–22]. Antibody neutralization assay using the pseudotyped NiV showed a good correlation with the gold standard PRNT assay that uses infectious NiV indicating their functional resemblance [23]. In addition to the replication defective VLP and pseudovirus-based systems, a recombinant, replication competent NiV F and G chimeric Cedar virus platform has also been generated recently as a surrogate neutralization system [24].

During their generation, all these surrogate systems need multiple transfections of plasmids encoding NiV F/G proteins or matrix protein M into the producer cells. There is a critical need for optimization of plasmid concentrations used in the transfection in order to avoid inconsistency in the surface incorporation of these proteins in the producer cells. This has remained challenging [25]. As a consequence, there can be variable incorporation rates of these proteins in the VLPs or pseudovirions generated and batch-to-batch variability in the infectious virus titre. There have been no major attempts made to address this issue and generate systems that can produce high titer NiV VLPs or pseudovirions. In most of the currently available surrogate systems, the reporters used as indicators of infection are fluorescence or luminescence-based, making them less amenable to high throughput automation for large scale use in surveillance or screening studies. In the present study, we address these gaps by reproducibly generating high titer VSV-based NiV pseudoviruses from F and G stably-transfected and clonally selected producer cells. A secreted alkaline phosphatase (SEAP) was incorporated as a reporter for easy readout. We also show the use of these pseudovirions in neutralization assays employing commercial antibodies as well as convalescent serum from Nipah recovered individuals.

Materials and methods

Generation of NiV F and G expression vectors

Complete coding region sequences of NiV F (1640 bp; position 6650–8290) and G (1808 bp; position 8939–10747) genes derived from the National Center for Biotechnology Information (NCBI) database (GenBank Accession No.MH523642; NiV strain from the outbreak in Kerala in 2018 [9]) were used to synthesize gene constructs (BR Biochem, India). Plasmid vector backbones with a CMV promoter, pLXSN and pcDNA3.1 Hygro, were derived from the mammalian expression plasmids pLXSN-Axl (Addgene # 65222) and pHMC122 (a kind gift from Dr. Erica Ollman Saphire, La Jolla Institute of Immunology, USA). NiV F coding region was cloned to the *EcoRI-BamHI* linearized pLXSN vector backbone and the NiV G coding region was cloned to the *KpnI-HindIII* linearized pcDNA3.1 Hygro vector backbone using standard cloning procedures. Chemically competent *Escherichia coli (E. coli)* DH5α strains were transformed with

the ligated products and the plasmid DNA isolated from NiV F and G positive clones were used for transfection of mammalian cells.

Cell lines

BHK-21 baby hamster kidney cells (ATCC; CCL-10) and HEK293 human embryonic kidney cells (ATCC; CRL-1573), were originally purchased from the American Type Culture Collection (ATCC) and were cultured in high glucose DMEM (Gibco, USA).The culture media were supplemented with 2% or 10% Foetal Bovine Serum (FBS; Gibco, USA) and 1X antimycotic antibiotic solution (Cat No. 15240062; Sigma, USA) and cells were maintained at 37 °C in a 5% CO_2 atmosphere.

Plasmid transfections and generation of NiV F & G stable HEK293 producer cells

HEK293 cells (3×10^5) used for the production of pseudovirions were seeded in 6-well plates for 18 h and co-transfected with the protein expression plasmids pLXSN-NiV F and pcDNA-NiVG (2 µg/ml each) using polyethyleneimine (PEI Branched; Cat No.408727; MW 25000; Sigma, USA) (8 µg/ml). The selection of transfected cells stably expressing both proteins was carried out by growing the cells in the presence of geneticin (625 µg/ml; for the pLXSN-NiVF) and hygromycin (50 µg/ml; for the pcDNA-NiVG) for 60 days.

Clonal selection of NiV F and G co-expressing HEK293 producer cells

HEK293 producer cells stably expressing NiV F and NiV G proteins were subjected to limiting dilution to separate single cells in individual wells of a 96-well plate to allow clonal expansion. After two rounds of selection, individual colonies were expanded to large-scale cultures and cryopreserved. Three, well-growing clones were selected (293 FG-5F6, 293 FG-7C5, and 293 FG-8C7) for further characterization and pseudovirion production.

Flow cytometry analysis of membrane expression of F and G proteins in monoclonal HEK293 producer cells

293 FG-5F6, 293 FG-7C5, and 293 FG-8C7 cells were trypsinized from T25 flasks and 1×10^6 cells were stained with anti-NiV F (12B2; Cat. No Ab02792-1, Absolute antibody; USA) and anti-NiV G (48D3; Cat. No Ab2865-23.0, Absolute antibody; USA) antibodies in flow cytometry staining buffer (2% FBS in Phosphate Buffered Saline (PBS), pH 7.2) for 1 h in ice. After incubation, the cells were centrifuged at 1500 rpm for 5 min followed by washing the pellet in FACS washing buffer (0.5% BSA in PBS) to remove the unbound antibodies. The cell pellet was then treated with mouse (Anti-mouse IgG Fab_2 AlexaFluor; CST # 4408 S; Cell Signalling Technology (CST), MA, USA) and rabbit (Anti-rabbit IgG Fab_2 Alexa Fluor ® 594; CST # 8889 S; Cell Signalling Technology, (CST), MA, USA) secondary antibodies to anti-F and anti-G antibodies conjugated with Alexafluor 488 and AlexaFluor 597 respectively for 30 min in ice. Following washing, the cell pellet was resuspended in FACS staining buffer and the percentage of incorporation of F and G proteins was analyzed in a flow cytometer (BD FACS Aria Fusion, BD Biosciences, USA).

Immunofluorescence detection of F & G proteins in monoclonal HEK293 producer cells

3×10^4 cells from expanded 293FG-5F6 monoclonal cells were cultured in 24-well plates overnight, washed with 1X phosphate-buffered saline (PBS), and fixed with paraformaldehyde or pre-chilled acetone-methanol (1:1) at −20 °C for 5 min. The fixative was removed by washing the cells twice with 1X PBS and the cells were blocked using 5% BSA in PBS, with 0.05% Tween-20 for 30 min at room temperature. Subsequently, the cells were stained with anti-NiV F (12B2; Cat. No Ab02792-1, Absolute antibody; USA) and anti-NiV G (48D3; Cat. No Ab2865-23.0, Absolute antibody; USA) primary antibodies (1:500 dilution) overnight at 4°C. Followed by washing twice with 1X PBS, the cells were incubated with Alexafluor 488 conjugated anti-mouse (CST, Cat. # 4408 S, CST, USA) and AlexaFluor 597 conjugated anti-rabbit secondary antibodies (CST, Cat. # 8889 S, CST, USA) respectively at 1:1000 dilution. DAPI (Cell Signalling Technology, Cat. # 4083 S, CST, MA, USA) (1 µg/ml) was used as the nuclear stain in all experiments.

Production of NiV pseudovirions

NiV pseudovirus production was carried out as described earlier [26] using the Delta-G-VSV Pseudotyping system (Kerafast Inc. CA, USA). All assays were conducted as per the Institutional Biosafety Committee (IBSC) approved protocols in a BSL-2 laboratory. Initial rVSV G*ΔG-SEAP stocks were generated in the laboratory by plasmid transfections as per manufacturer's protocol. These stock viruses were titrated by a spot forming unit Assay As described below, and were found to have a titre of approx. 2.5×10^6 SFU per ml. These stocks were diluted in OptiMEM medium to infect the producer HEK293 cells (NiV F and G protein expressing polyclonal or the 293 FG-5F6 monoclonal stable cells) at an MOI 1 for 2 h at 37 °C. After infection, the inoculum was removed, and the cells were washed with PBS at least 3 to 4 times to ensure the removal of trace quantities of any residual VSV-ΔG- SEAP virus. The cells were cultured in complete culture medium for further 18–24 h before the pseudovirus harvest. On the day of harvest, the culture supernatants containing the pseudotyped virions were collected, centrifuged at 3000 rpm for 20 min and stored at −80 °C until use. The pseudovirions produced from

NiV F and G protein expressing polyclonal HEK293 cells were labelled as N-(PV)FG and those produced from the monoclone 293FG-5F6 cells were labelled as N-(PV) FG-5F6.

Titration of the pseudovirion stocks

Quantification of the viral particles in rVSV G*ΔG-SEAP or NiV pseudovirion stocks were carried out using a colorimetric spot forming unit assay. For this an alkaline phosphatase staining method was established using BCIP/NBT reagent (Cat.No.N1113; Tokyo Chemical Industry, TCI, Japan) to count the actual number of cells infected with pseudovirus and expressing the thermostable SEAP. Single-use aliquots of stock viruses were used to avoid inconsistencies in titer that may result from repeated freeze-thawing. Serial dilutions of the pseudovirus stocks were used to infect the target BHK-21 cells in 24-well plates for 2 h at 37 °C; followed by the removal of the inoculum and washing of the monolayer with 1X PBS. After 24 h incubation in fresh DMEM with 2% FCS containing culture medium, the cell culture plates were heated by placing in a water-bath at 65 °C to inactivate the endogenous alkaline phosphatase. For colorimetric spot counting of the infected cells, supernatants were removed and the monolayers were washed twice with 1X PBS followed by addition of 200 µl/well of BCIP/NBT reagent (1X solution in 20mM Tris-HCl pH 9.5, 2mM MgCl2 and 100mM NaCl) for staining the cells. Chromogenic development in the cells were monitored at room temperature and the reaction was stopped after 2 h by rinsing the wells twice with 1X PBS. After the removal of PBS, the plates were scanned by a ELISpot reader (ImmunoSpot by CTL., USA) and was subjected to automated focus counting by BioSpot 5.0 Professional software. Average number of spots from two corresponding dilutions in three independent experiments were used to calculate the pseudovirus virus titre (SFU per ml) of the inoculum stock.

Target cell infection and SEAP assay in culture supernatants

BHK21 target cells cultured in 96-well plates were infected with the N-(PV)FG or N-(PV)FG-5F6 pseudovirions by incubating the cells with diluted pseudovirus stocks containing the required number of spot forming units (SFU) for 2 h at 37 °C, As described above. 24 h post-infection, the plates were placed in a water bath at 65 °C and were incubated for 15 min to inactivate the endogenous alkaline phosphatase activity. The culture supernatants were analyzed for SEAP activity by mixing 50 µl of the supernatant with 150 µl of pNPP substrate solution (TCI, Japan; Cat. # N1109). After incubation for 10 min, absorbance at 405 nm (OD_{405}) was measured using GloMax® Discover Microplate Reader (Promega Inc; USA).

Detection of F & G protein incorporation in NiV pseudovirions by immunostaining

BHK-21 target cells were cultured in 24-well plates and the cell monolayer was infected with 500 SFU N-PV(FG)–5F6-SEAP pseudovirions. BHK-21 cells, infected with N-PV (FG) or rVSV G*ΔG-SEAP virus, were used As controls. Pseudovirions were allowed to adsorb to cell surface for 30 min at 25 °C (for fixation with acetone-methanol (1:1)) or for 30 min at 4 °C (for fixation with 4% paraformaldehyde in 1XPBS). After the duration of infection, the cell monolayer was washed twice with PBS and subjected to fixation. Immunostaining was done with specific antibodies As described above. The incorporated F and G proteins in the pseudovirions were microscopically visualized by imaging at 60X magnification in a Leica Stellaris5 confocal microscope (Leica Microsystems, Germany).

NiV pseudovirus-based neutralization assays

BHK-21 target cells (1×10^4) were seeded in 96-well plates in DMEM containing 10% FBS and 1X antimycotic antibiotic solution and incubated at 37 °C in a CO_2 incubator. N-PV(FG)–5F6- SEAP pseudovirions (120 SFU) were mixed with serial dilutions of commercial anti-NiV F and G antibodies or convalescent human sera from Nipah recovered patients [27] and incubated at 25 °C for 1 h. Anti-rabies human monoclonal antibody 8889 S (17C7); Rabishield-100 (40IU/ml), a NiV non-neutralizing antibody, purchased from the Serum Institute of India Pvt Ltd or serum from two healthy individuals were used As the negative controls in the neutralization Assays. Serum samples were heat inactivated at 56 °C for 30 min before using in the assays to inactivate the complement. The pseudovirus-sera/antibody mixture was added to the monolayer of BHK-21 cells. After 2 h infection at 37 °C, the mixture was removed from the cells, replenished with DMEM containing 2% FBS and 1X antimycotic antibiotic solution, and incubation was continued for 24 h. The cell culture supernatants were harvested; and incubated at 65 °C for 15 min to inactivate the endogenous alkaline phosphatase activity and the secreted alkaline phosphatase (SEAP) was measured as described above. Percentage neutralization was calculated with respect to non-neutralized pseudovirus infected controls using the formula:

$$\%\ \text{Neutralization} = \left[1 - \frac{(OD\ Test\ Sample - OD\ Cell\ control)}{(OD\ Virus\ control - OD\ Cell\ control)} \times 100\right]$$

Statistical analysis

Statistical analyses were performed using Graphpad prism 8.0 software. Pearson's correlation analysis was used to quantify the pixel-to-pixel proportionality in the signal levels of the two channels in immunofluorescence experiments and to analyze the correlation between Spot Forming Units and SEAP absorbances. In experiments with control and test groups, Student's t-test (unpaired) or Wilcoxon signed-rank test was used to compare the significance between the groups. P value < 0.05 was considered statistically significant.

Results

The general scheme of experiments followed to generate and characterize the NiV pseudovirions is shown in Fig. 1.

Generation of pseudovirions from HEK293 producer cells stably incorporating NiV F and G protein

HEK293 producer cells were co-transfected with the pLXSN-NiVF and pcDNA3.1-NiVG clone plasmids and selected with the antibiotics hygromycin and geneticin (G418) to generate stably transfected polyclonal cells.

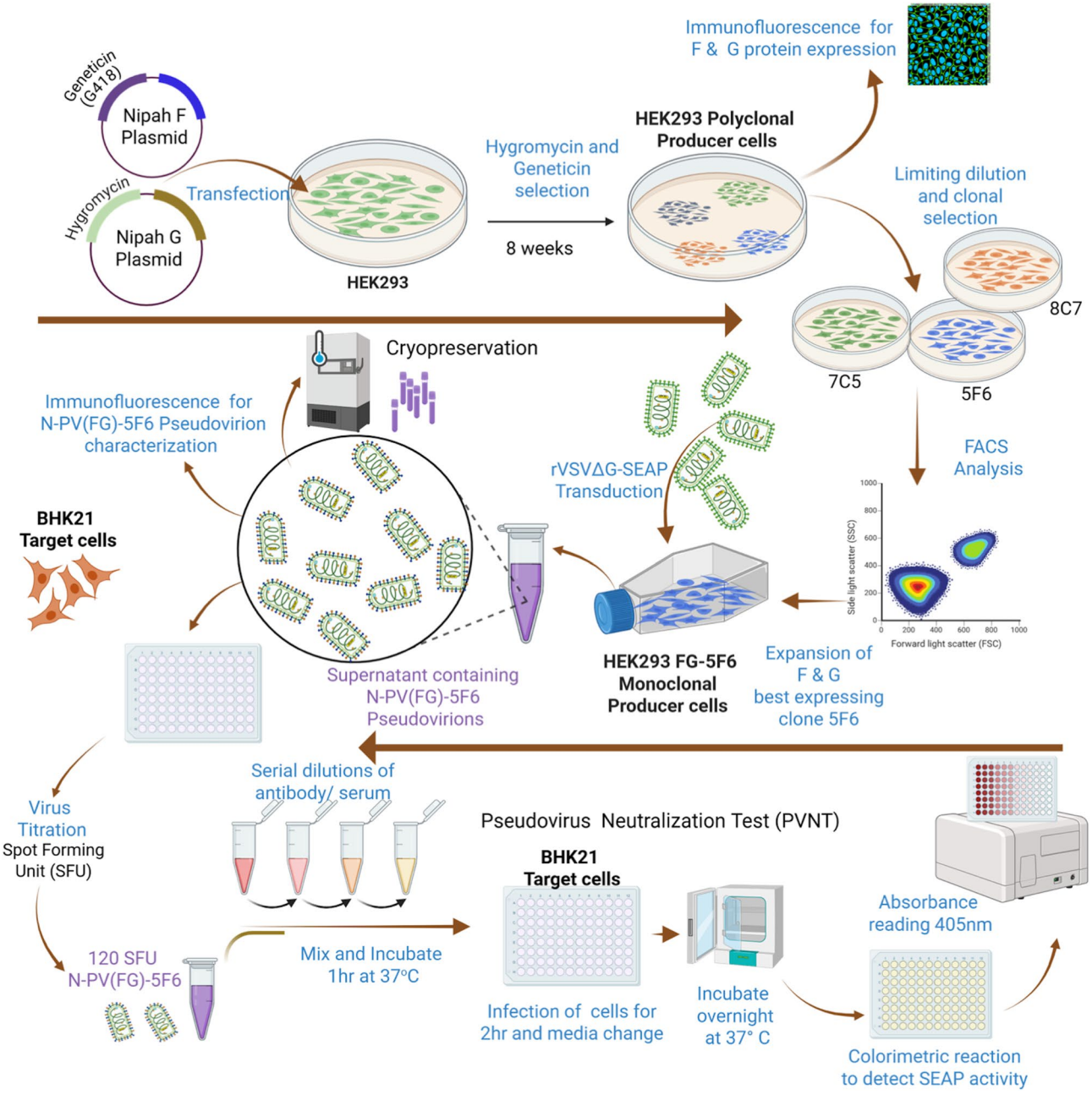

Fig. 1 Scheme of experiments for generation and characterization of NiV pseudovirions

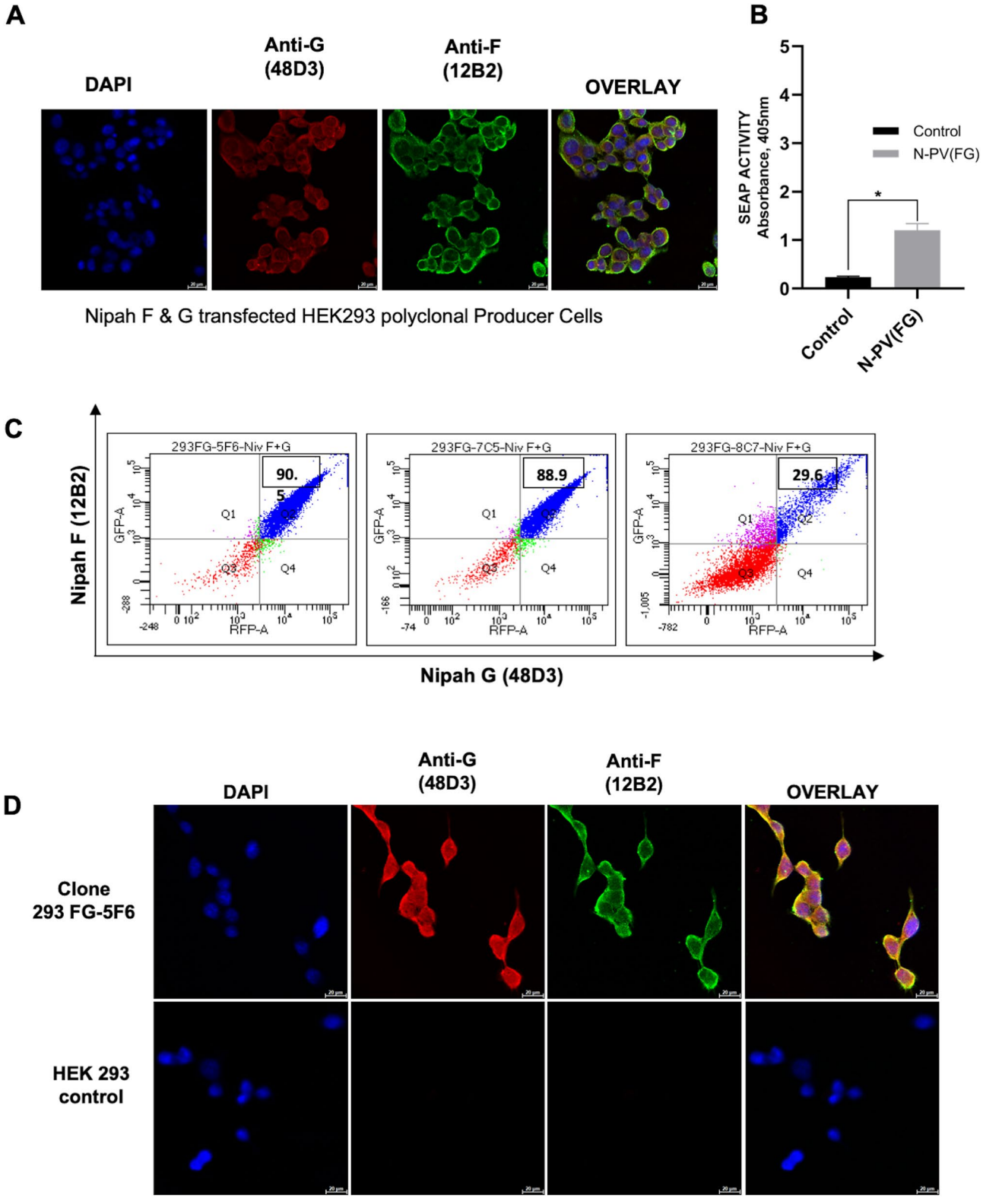

Fig. 2 (See legend on next page.)

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Fig. 2 Analysis of co-expression of NiV surface glycoproteins F and G in HEK293 polyclonal producer cells and clonally expanded stable HEK293 cell lines.**A** Confocal microscopy image of immunofluorescence analysis of polyclonal HEK293 producer cells stably expressing NiV F and G proteins. Cells were stained with primary antibodies to Nipah G (48D3) and Nipah F (12B2) proteins, respectively (Magnification, 60X; Scale bar, 20 µm). **B** Testing pseudovirion production by transfected HEK293 polyclonal producer cells. N-PV(FG) pseudovirions were generated by infecting the NiV F & G stably expressing producer HEK293cells with rVSV-G*ΔG-SEAP virus stock. The harvested virus was used to infect BHK21 target cells and the SEAP activity measurements were done in the culture supernatants after 24 h of infection. Supernatants from un-transfected HEK293 producer cells were used for control infection of target BHK21 cells; and the SEAP activity was compared. Experiments were done in duplicates and data presented as Mean ± SD of three independent experiments (N = 6); '*' indicates $P < 0.05$. Un-paired Student t-test was used for statistical analysis comparing the two groups. **C** NiV F and G protein expression in transfected, clonally selected HEK293 cells. Suspensions of cells from expanded monoclones 293FG-5F6, 293FG-7C5 and 293FG-8C7 were stained with NiV anti-G and anti-F antibodies As described in the methods; and analysed by FACS. The percentage incorporation of G and F proteins was found to be 90.5%, 88.9% and 29.6%, respectively. **D** Immunofluorescence analysis of NiV G and F protein co-expression in 293FG-5F6 monoclonal cells. The fixed cells were stained with primary antibodies 48D3 and 12B2; and with secondary antibodies conjugated with Alexafluor 597 and Alexafluor 488, respectively for G and F proteins. The membrane expression of the proteins were viewed under a confocal microscope. Un-transfected HEK293 cells served as mock control. DAPI was used as the nuclear stain (Magnification, 60X; Scale bar, 20 µm)

The co-incorporation of F and G proteins in the transfected cells was confirmed by immunofluorescence analysis using specific antibodies (Fig. 2A). The formation of syncytium cells with multiple nuclei due to the fusion of adjacent cells were visible in the transiently transfected HEK293 cells expressing F and G proteins. However, in cells selected for stable expression of these proteins, there were predominantly aggregation of cells with partial fusions of the membrane, rather than formation of larger syncytial cells (Supplementary Fig. 1 A and B).

Infection of these cells with 2.5×10^6 SFU/ml of the rVSV-G*ΔG-SEAP virus stock generated N-PV(FG)-SEAP pseudovirions 24 h post-transfection. This was evidenced by enhanced SEAP activity in the supernatants of BHK21 target cells infected with this virus compared to mock infection control (Fig. 2B).

Clonal selection and characterization of NiV F and G co-expressing monoclonal HEK293 producer cells

Stably co-transfected HEK293 cells with NiVF and G expressing plasmids (i.e. polyclonal producer cells that may contain clones with varying levels of NiV F and G expression) were further subjected to two successive rounds of selection under the selection pressure of hygromycin and geneticin. These cells were subjected to limiting dilution for the selection of single cell clones. Three well-defined and rapidly growing monoclones selected from 96-well plates, named as 293FG-5F6, 293FG-7C5, and 293FG-8C7, were expanded in 24-well plates and subjected to FACS analysis to determine the individual As well As the co-incorporation levels of F and G proteins. These three monoclones had respectively 90.5%, 88.9%, and 29.6% cells with equal expression of NiV F and G proteins (Fig. 2C). The best among them, 293FG-5F6 monoclonal cells, when subjected to immunofluorescence analysis, indicated uniform incorporation levels and membrane co-localization of the NiV F and G proteins confirming the observation in FACS analysis (Fig. 2D). Hence, 293FG-5F6 clone was further expanded and selected as producer cells for large-scale N-PV(FG)–5F6 pseudovirus production.

Reproducible generation of high-titre NiV N-PV(FG)–5F6 pseudovirions

BHK-21 monolayer cells infected with two- fold serial dilutions of N-PV(FG)–5F6 pseudovirions were subjected to alkaline phosphatase staining as described in the methods section. The infected cells revealed purple coloured spots indicating the production of SEAP with their counts decreasing in proportion to the dilutions (Fig. 3A). The virus titre, calculated based on the spot forming units (SFU), was found to be 1×10^4 SFU/ml (Fig. 3B). Using the same approach, we also estimated the lower limit of pseudovirion detection by SEAP assay. Serial dilutions of the virus inoculum with varying SFUs were used to infect BHK-21 cells and the SEAP activity was measured after 24 h. The SEAP activity correlated well with the virus quantity determined by the Spot forming assay (Fig. 3C). A perceptible difference in OD_{405} was observed in cells infected with pseudovirions as Low as 4–8 SFU; and an inoculum with 120 SFU of virus was found to give an approximate OD_{405} value of 1.0 which was selected for subsequent assays.

N-PV(FG) pseudovirions from the polyclonal producer cells and N-PV(FG)–5F6 pseudovirions from the 293FG-5F6 monoclonal cells were generated by infecting these cells with 2.5×10^6 SFU/ml of rVSV-G*ΔG-SEAP virus stock. Equal volumes (100 µl) of each of the produced pseudovirions were used to infect the BHK-21 target cells to compare the SEAP activity in the culture supernatants. The results indicated a significantly high titer pseudovirion production by the monoclonal cells (Fig. 3D). Kinetic analysis of the SEAP activity in culture supernatants from these cells revealed that the activity reaches maximum by 24 h post-infection and thereafter remains stable (Fig. 3E). So this time-point was chosen as the endpoint in subsequent biological assays.

In order to check the reproducibility of pseudovirion production, three batches of N-PV(FG)–5F6-SEAP viruses were generated from 293FG-5F6 clonal cells under identical conditions of rVSV-G*ΔG-SEAP infection. These three stock viruses were found to have consistent infectivity and similar virus titre as indicated by the

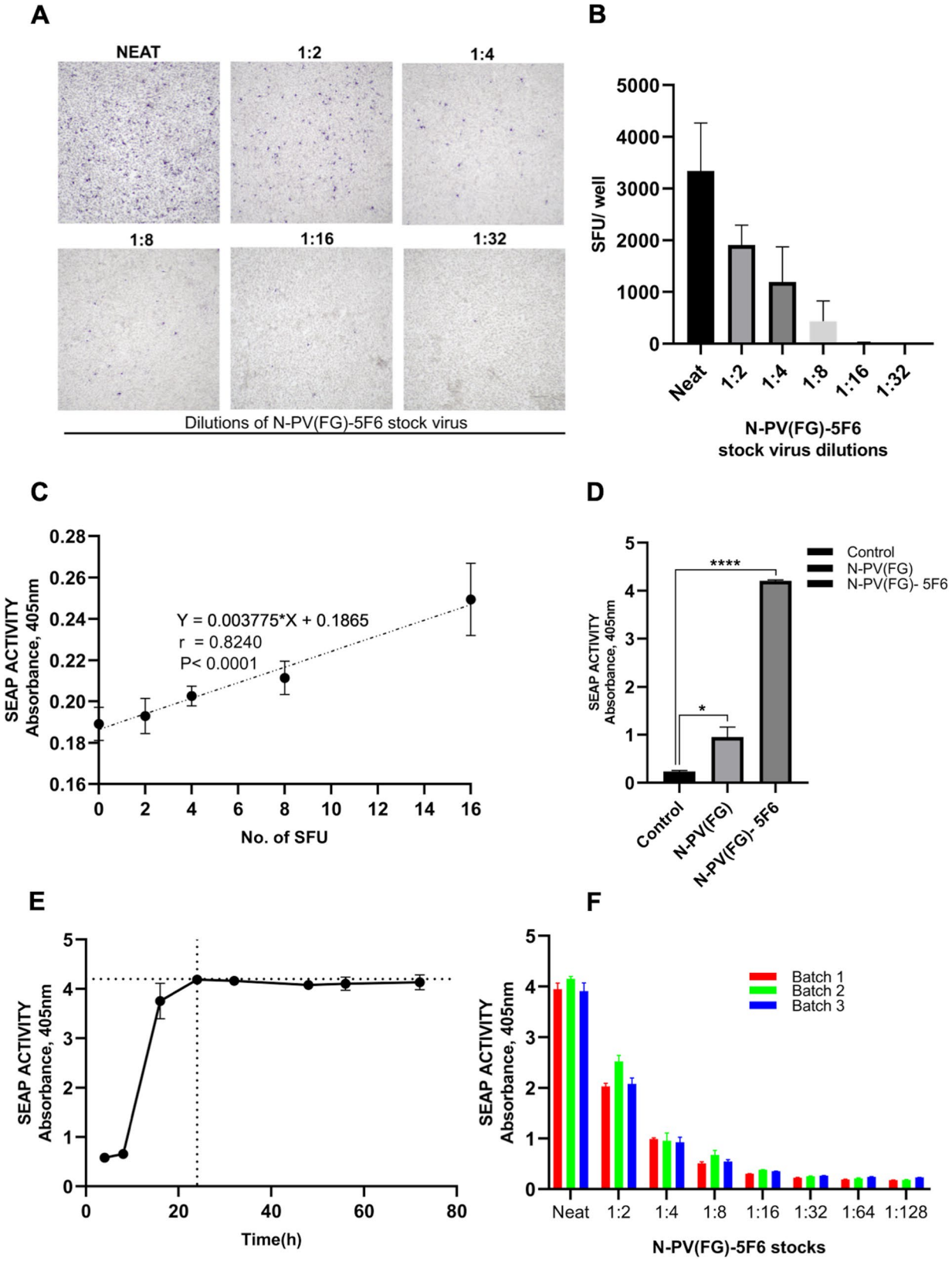

Fig. 3 (See legend on next page.)

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Fig. 3 Production of N-PV(FG)–5F6 pseudovirions from monoclonal HEK293 cells **A**. Titration of N-PV(FG)–5F6 pseudovirions. A colorimetric spot forming assay was used to stain the thermostable-SEAP producing, pseudovirus-infected cells as described in the methods. Bright purple coloured spots indicate the infected BHK-21 target cells, detected by alkaline phosphatase staining. Representative magnified fields from the ELISPOT reader scanned image of an assay plate infected with serial dilutions of the stock virus are shown. **B** Quantification of the number of spots forming units (SFU) per well. Spots were counted by automated focus counting using BioSpot 5.0 Professional software in the ELISPOT reader. Data is Mean ± SD of values of two virus dilutions from three independent experiments ($N = 6$). **C** Determination of lower limit of virus detection by SEAP assay. Virus inoculums with known amount of viral particles (SFU) were used to infect target BHK-21 cells and SEAP activity was measured for each dilution after 24 h. The values are Mean ± SD of two independent experiments done in triplicates ($N = 6$). Linear regression analysis was used to compute the R square values. **D** Comparison of SEAP activity in culture supernatants of target BHK-21 target cells infected with equal volumes of N-PV(FG) and N-PV(FG)–5F6 pseudovirions. The pseudovirion harvests were obtained from polyclonal and monoclonal HEK293 producer cells, respectively. The producer cells were initially infected with identical amounts of rVSV G*(△G- SEAP) for pseudovirion production. Supernatant from un-transfected HEK293 cells, infected with rVSV G*(△G- SEAP) was used for infecting the control cells. The Mean ± SD of duplicate values from three experiments are shown. '*' indicates $P < 0.05$; '****' indicates $P < 0.0001$; Unpaired Student t-test was used for statistical analysis for comparison between the two groups **E.** Kinetics of SEAP production in BHK-21 target cell supernatants post-N-PV(FG)–5F6 infection. Data points indicate the time points 4, 8, 16, 24, 32, 48, 56 and 72 h post-infection. The 24 h time-point fixed for subsequent experiments is indicated by the dotted line. Each value is a Mean ± SD of two independent experiments each done in triplicates. **F** Validation of the reproducibility of N-PV(FG)–5F6 pseudovirion production under identical conditions. Three batches of pseudovirions harvested from 293(FG)–5F6 monoclonal producer cells were used to infect BHK-21 target cells in various dilutions. SEAP activity in the culture supernatant was measured at 405 nm and plotted. Each value indicates Mean ± SD of triplicate values from three independent experiments ($N = 9$). Wilcoxon signed- rank test was used for statistical analysis for comparing the three batches of pseudovirions harvested ($P < 0.001$); and the values among the three batches did not show any statistical significance in any of the dilutions

SEAP activity in the target BHK-21 cell culture supernatants infected at different dilutions (Fig. 3F).

Detection of uniform incorporation NiV F and G proteins on N-PV(FG)–5F6 pseudovirions by immunostaining with specific antibodies

To identify whether NiV F and NiV G proteins co-incorporated on the N-PV(FG)–5F6 pseudovirions, immunostaining with specific antibodies was carried out on BHK-21 target cells post-infection. Two approaches were tested in the experiments. First, a 30 min infection at 25 °C followed by fixation with acetone-methanol which permitted membrane permeabilization and staining of even pseudovirions that were already internalized. Second, a 30 min infection at 4 °C followed by fixation with 4% paraformaldehyde, which permitted staining of only the cell surface-adsorbed pseudovirions. In acetone-methanol fixed cells, analysis by confocal microscopy at 60X magnification clearly visualized the immunostained viral particles, predominantly in the cytoplasm (Fig. 4A). Superimposition of the green and red fluorescence on them clearly indicated a uniform co-distribution of F and G proteins on the N-PV(FG)–5F6 pseudovirions. This was well apparent in the yellow colour staining in the overlay images of N-PV(FG)–5F6 pseudovirion infected cells (Fig. 4A). However, on the N-PV(FG) pseudovirions, as shown in the figure, the incorporation of the G protein was significantly low compared to the incorporation of the F protein. Also, in the control cells infected with rVSV-G*ΔG-SEAP virus, there was no fluorescent signal indicating the specificity of the detection of the F and G proteins by the anti-NiVF and anti-NiV G antibodies (Fig. 4A).

Scatter plot analysis of the F and G fluorescence detection on pseudovirions revealed that the pixel points had a distribution around a central straight line passing through the origin in the case of N-PV(FG)–5F6 infected cells, as against a skewed distribution on N-PV (FG) infected cells. This also indicated that these proteins had a uniform incorporation on N-PV(FG)–5F6 pseudovirions (Fig. 4B&C). Quantification of the mean fluorescence intensity of F & G proteins re-confirmed the equal levels of incorporation of both proteins in N-PV(FG)–5F6 pseudovirions, compared to that in N-PV(FG) (Fig. 4D&E). F and G protein detection levels in N-PV(FG)–5F6 pseudovirions had a Pearson's coefficient of 0.93 compared to 0.83 in N-PV(FG), indicating a significantly uniform incorporation of both the proteins in the former.

Comparative evaluation of NiV pseudovirion production from F and G plasmids transiently transfected cells and (FG)–5F6 cells

We compared the batch to batch consistency as well as F & G protein incorporation in the pseudovirions produced from HEK293 cells transiently transfected with NiV F & G plasmids as well as from the clone (FG)–5F6 cells. The schematic of the experimental method used is shown in Fig. 5A. Four batches of pseudoviruses, produced under identical conditions, were evaluated.

As shown in Fig. 5B, SEAP evaluation in the culture supernatant from target cell infection indicated that there is a significant variability in the production levels of the NiV pseudovirions among the batches from transiently transfected cells whereas the levels were consistent when stably transfected cells were used. Also, with equal volume of virus used for target cell infection, the amount SEAP activity was almost three-times higher (mean OD_{405} of 1.31 vs. mean OD_{405} of 3.96) for pseudovirions produced from F&G stably expressing (FG)–5F6 cells, indicating a significantly higher amount of virus production.

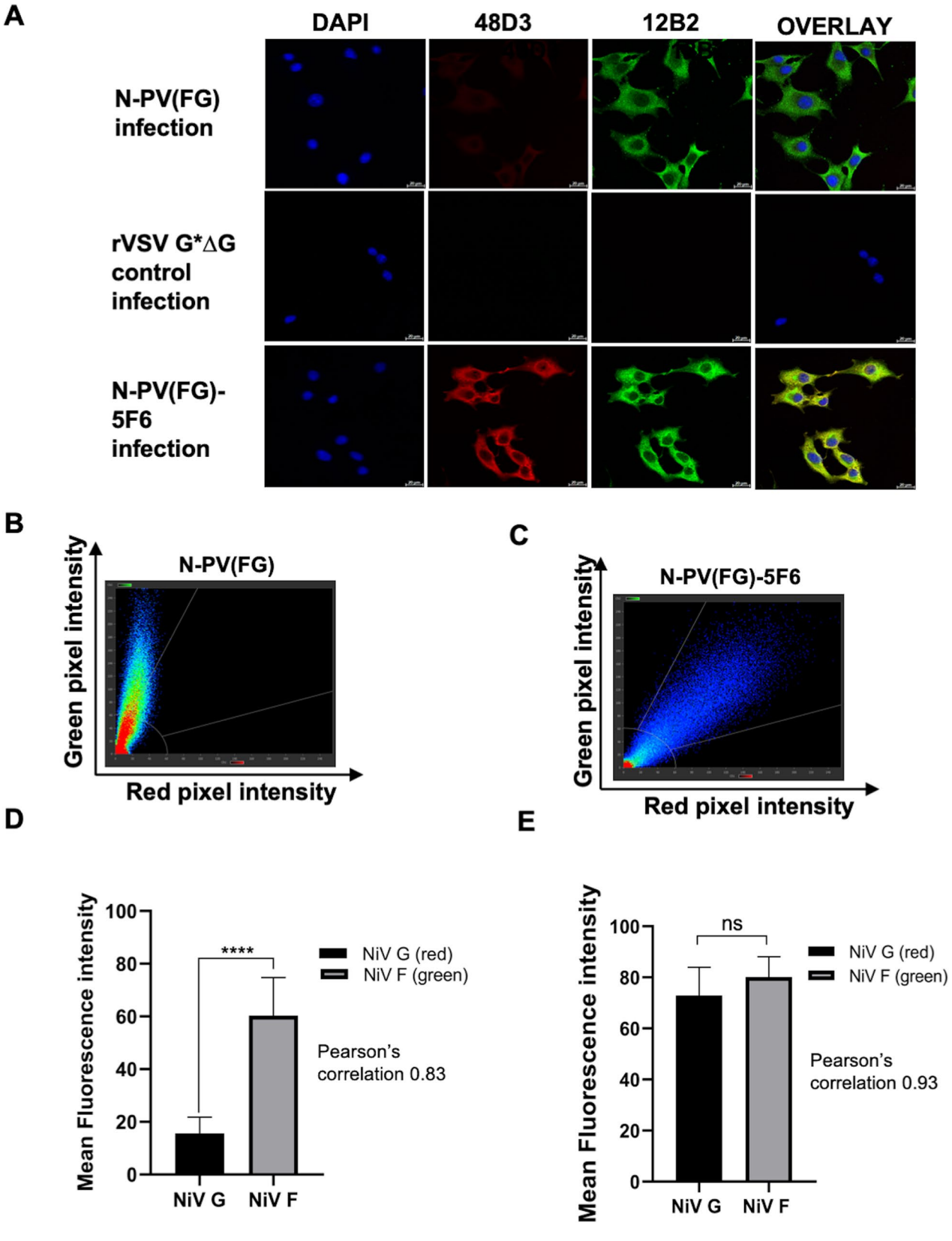

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Fig. 4 Evaluation of uniform incorporation of F &G proteins on NiV pseudovirions.**A** Localization of N-PV(FG)–5F6 pseudovirions in target cells post-infection by immunostaining with anti-NiV F & G antibodies and confocal microscopy. Target BHK-21 cells were infected with 500 SFU pseudovirions for 30 min at 25 °C. Cells were fixed in acetone: methanol (1:1) and stained with primary antibodies 48D3 and 12B2; and secondary antibodies Alexafluor 597 and Alexafluor 488. The pseudovirion infection was confirmed by the colocalization of green and red fluorescence as yellow fluorescence on the surface and within the cells **B & C.** Scatterplot of green and red pixel intensities corresponding to NiV F and G staining respectively on N-PV(FG) and N-PV(FG)–5F6 pseudovirions. In N-PV(FG) infection, the points of the scatterplot were distributed more towards the green fluorescence probe. On the other hand, in N-PV(FG)–5F6 infection, a proportional co-distribution, where the points of scatterplot clustered around a central straight line, was observed **D& E.** Quantification of F& G protein co-localization using Pearson's correlation coefficient (PCC). A PCC value of 0.83 in N-PV(FG) showed that the probes did not overlap in a fixed proportion whereas a high PCC value of 0.93 for N-PV(FG)–5F6 probes reflected proportional co-distribution. The values are the Mean ± SD of 14 region-of-interests (ROIs) fields from two independent experiments. **** $P < 0.0001$; ns- Not significant; Unpaired Student t-test was used for statistical analysis comparing the two groups

We also immunostained the F & G proteins incorporated onto pseudovirions after allowing them to adsorb onto BHK21 target cells and imaged them under a confocal microscope. In the target cells fixed with 4% paraformaldehyde, the membrane integrity was well preserved; and upon immunostaining, the pseudovirion particles were clearly visualized as distinct dots on the cell surface. As shown in Fig. 5C, the colocalization of the red and green fluorescent signals yielding the yellow coloured speckles and dots in the overlay image confirmed the uniform incorporation of both the proteins. There was significantly more incorporation of the F& G proteins in the NiV pseudovirions produced from (FG)–5F6 stably transfected cells than those produced after transient transfection. As with the low titre of virus, the number of fluorescent spots indicating the infectious particles were also low in the virus preparation from transiently transfected cells. These experiments clearly confirmed that the use of (FG)–5F6 clone has a distinct advantage on NiV pseudovirion production.

Pseudovirus neutralization assays using commercial anti-NiV antibodies and convalescent serum from Nipah infection recovered individuals

The usefulness of the N-PV(FG)–5F6 pseudovirions in functional Assays were confirmed by neutralization Assays. Commercial anti-NiV F and G antibodies gave 100% neutralization of the pseudovirions (Fig. 6A). The neutralization titer decreased with decreasing antibody concentration, confirming a dose-dependent neutralizing activity and its specificity. The 17C7 non-neutralizing antibody used as negative control did not neutralize the N-PV(FG)–5F6 pseudovirions, further confirming the specificity of the observations.

The susceptibility of the N-PV(FG)–5F6 pseudovirions to neutralization by human antibodies were evaluated using convalescent serum samples from three Nipah recovered subjects (collected during the Nipah outbreak that occurred in the Kozhikode district of Kerala, India in the year 2023). Serum samples (NiV patient 01, 02, and 03) were diluted to 1:10 followed by 2-fold serial dilutions; and used in the Assays. All the three samples showed neutralizing activity against NiV pseudovirions. While NiV serum 01 and 02 could completely neutralize the pseudoviruses, NiV03 gave more than 80% neutralization at a serum dilution of 1:10 (Fig. 6B). The percentage of neutralization showed a declining trend with increasing serum dilutions. Sera from two normal individuals used as controls in the assay did not neutralize the N-PV(FG)- 5F6 pseudovirions (< 20%). From these results, it could be concluded that the established high throughput system is sensitive and specific and could be used for serosurveillance in Nipah endemic regions.

Discussion

NiV spill over and human disease outbreaks are sporadic and unpredictable. The lack of commercial rapid diagnostic or serological tests prevent early detection and large-scale epidemiological investigations. Like for other BSL-4 pathogens such as Ebola virus (EBOV), surrogate systems, such as virus-like particles and pseudoviruses, have been developed for NiV. However, unlike EBOV and similar viruses that have only one surface glycoprotein (GP) that facilitate infection and to be included in these systems [28], NiV has two surface glycoproteins F and G that are to be incorporated. In all the previous reports on NiV VLP-based or pseudovirus-based systems, the authors attempted transient co-transfections of F and G expressing plasmids with multiple optimization attempts of outer membrane and backbone plasmid concentration ratios to obtain their co-expression. This resulted in the unreliable incorporation of these proteins in the progeny particles, making the approach challenging and less reproducible [17, 18, 29, 30]. In a recent study, a cell line stably expressing the NiV F protein was infected with a recombinant VSV expressing NiV G to produce high-titre pseudovirions [31]. Though the study had confirmed expression of the F & G proteins in the transfected and rVSV infected cell lines, it did not analyse the efficiency of incorporation of both the proteins in the generated pseudovirions.

In the present study, to overcome the above difficulties, we generated producer cells that stably incorporated the F and G proteins after transfection. Initial cloning of the F and G genes was carried out in mammalian expression vectors that have different antibiotic selection

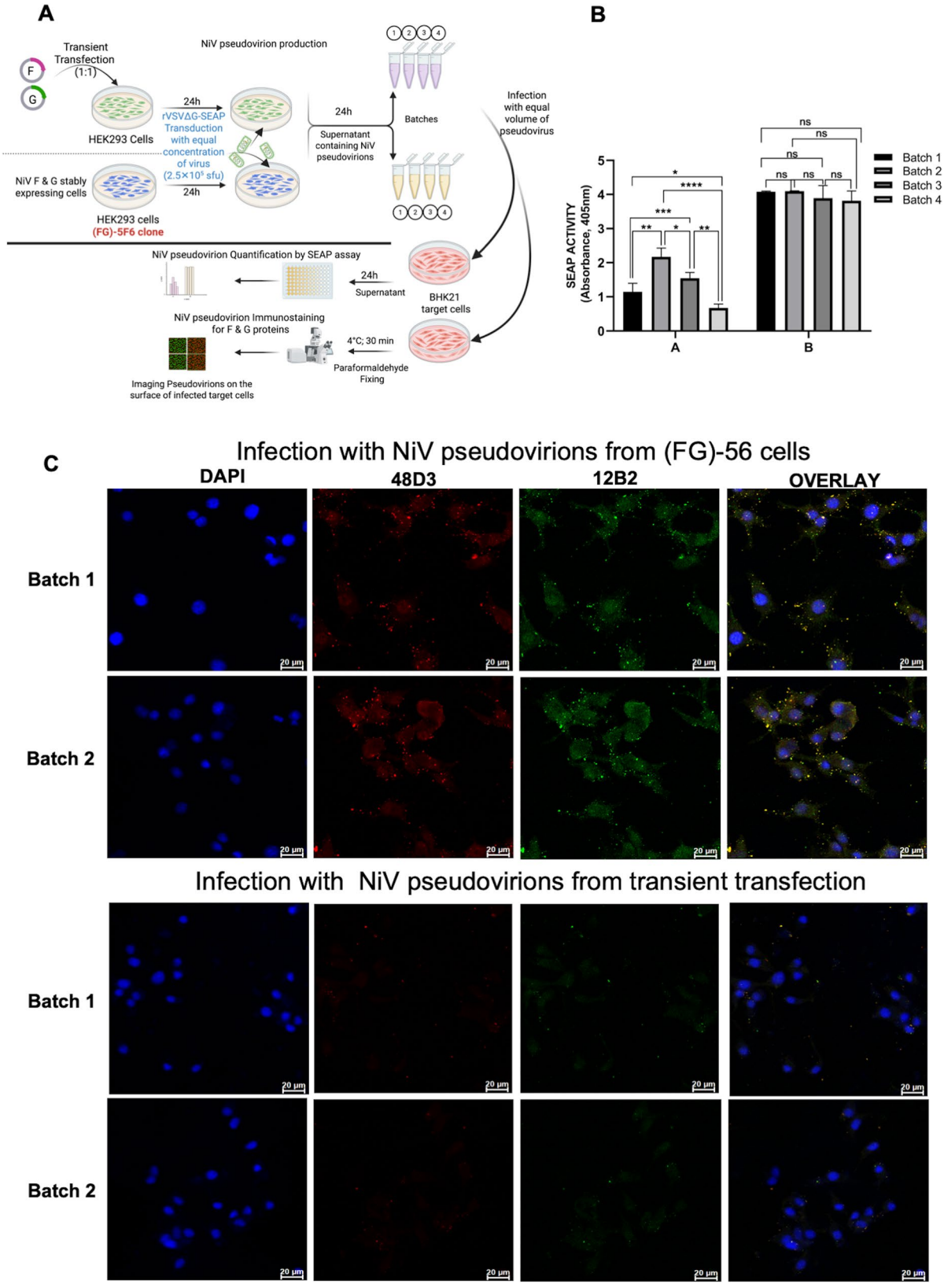

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Fig. 5 Comparative evaluation of NiV pseudovirion production from F and G plasmids transiently transfected cells and (FG)–5F6 cells**A**. Scheme of the experimental protocol followed. **B** Batch to batch reproducibility in NiV pseudovirion production from (A) HEK293 cells transiently transfected with NiV F & G plasmids and (B) HEK293 (FG)–5F6 clones stably expressing these proteins. As indicated in the scheme, the target BHK21 cells hexaplicate wells of a 96-well plate were infected with equal volume of the supernatants containing the NiV pseudovirions from these producer cells. The SEAP activity was measured in the BHK21 cell culture supernatants after 24 h as described in the protocols; and mean±SD was plotted. The statistical analysis was carried out with paired t-test. * $P<0.05$; ** $P<0.0$, *** $P<0.001$, **** $P<0.0001$; ns- Not significant. **C** Immunostaining of F and G proteins of NiV pseudovirions on the surface of target BHK-21 cells. Cells were infected for 30 min at 4 °C with 2.5×10^5SFU pseudovirions and were fixed for immunofluorescence with 4% paraformaldehyde, without membrane permeabilization. The viral particles adsorbed on the surface were immunostained with primary antibodies (48D3 and 12B2) followed by secondary antibodies conjugated with Alexafluor 597 and Alexafluor 488, respectively. The G and F proteins on the pseudovirions attached to the target cell membrane were visualised at 60X magnification in a confocal microscope. The yellow speckles represent colocalized green and red fluorescence of the NiV F and G proteins on the surface on the infected target cell membrane

markers, G418 and hygromycin; and stable polyclonal cells were generated after multiple rounds of selection for two months. As shown in Fig. 2A, immunofluorescence analysis confirmed that the NiV F and G proteins are incorporated on these producer HEK293 cells. Several previous studies have described the formation of large multinucleated syncytial cells in multiple cell lines, including HEK293 cells, when infected with NiV or when the viral glycoproteins F and G are co-expressed [32, 33]. In our studies also, during initial transient transfection with plasmids encoding the NiV F and G proteins, many syncytial cells could be observed (Supplementary Fig. 1 A). However, when these cells were selected under antibiotics for stable expression, the cells did not form large syncytium, but remained as clumps of cells with partial fusions (Supplementary Fig. 1B). As observed in previous studies, during viral infections or transient transfections, the cell fusion events happen between a donor cell expressing the viral glycoproteins and the adjacent target cells expressing the receptors for these glycoproteins. This involves cell signaling events, e.g. Rho GTPase pathway, in some viruses such as respiratory syncytial virus (RSV) facilitating the fusion between the cells [34]. However, in the HEK293 cells selected for prolonged stable expression of F and G proteins, all the cells in culture uniformly express both the viral glycoproteins as well as the Ephrin B2/B3 receptor proteins. We presume that this may lead to a two-way cross talk and reciprocal signaling among adjacent cells preventing larger cell fusion events. However, this hypothesis needs further detailed experimental validation, especially in the context that some studies have already shown the dispensability of cytoplasmic regions of Ephrin B2 receptors for facilitating NiV induced cell fusion events [35].

When pseudovirions generated from these cells were used to infect the target BHK-21 cells and the viral particles were visualized by immunostaining, both F and G proteins could be detected. However, when these images were overlayed and analysed, only a poor protein co-localization could be observed (Fig. 2A). This indicated that the N-PV(FG) pseudoviral particles generated from these polyclonal cells were still heterogenous even after prolonged selection for stable expression of both the F and G proteins.

To further improve the pseudovirion generation and make our assay more reproducible, the stable producer cells were subjected to clonal selection by limiting dilution to ensure single cell per well, which were further expanded and analyzed. Analysis of three clones (293FG-5F6, 293FG-7C5, and 293FG-8C7) from the clonal selection demonstrated a significant variation in the rate of expression of both the proteins; and only the clone 293FG-5F6 that showed more than 90% expression of both the proteins were chosen for the establishment of Nipah pseudovirus system. These clonal cells had an almost equal levels of F and G protein membrane incorporation as seen in the immunostaining (Fig. 2D). Pseudovirion particles from these producer cells had a conspicuous co-localization of both the proteins (Fig. 4A&F) and these cells showed consistency in production of pseudovirions with uniform infectivity (Fig. 3F). Co-localization of the fluorescence probes of NiV F and G have been seldom quantified in any of the previous studies, thus making this assay system novel and reliable.

Pseudovirus-based systems are finding increased use in antibody neutralization assays and antiviral screening assays for identifying virus entry inhibitors [36]; and simpler and high throughput-adaptable assays are a prerequisite for their large-scale use. The present study used secreted alkaline phosphatase (SEAP) as a reporter in the NiV pseudovirions generated. Earlier studies have used SEAP-reporter systems for the development of pseudovirus systems for other viruses such as human papillomavirus [37, 38]. Except for one report that used SEAP [20], all other previous studies on NiV surrogate systems, including the recent study [31] used either fluorescence or luminescence-based reporter Assays for monitoring infection. SEAP-based Assays are non-destructive and provide about 10-fold higher sensitivity than conventional luciferase-based systems [20, 39]. In our Assays, we could see that pseudovirions even As little as 4–8 SFU give a detectable, though small, expression of SEAP activity making the system sufficiently sensitive for neutralization assays with minimum residual undetected

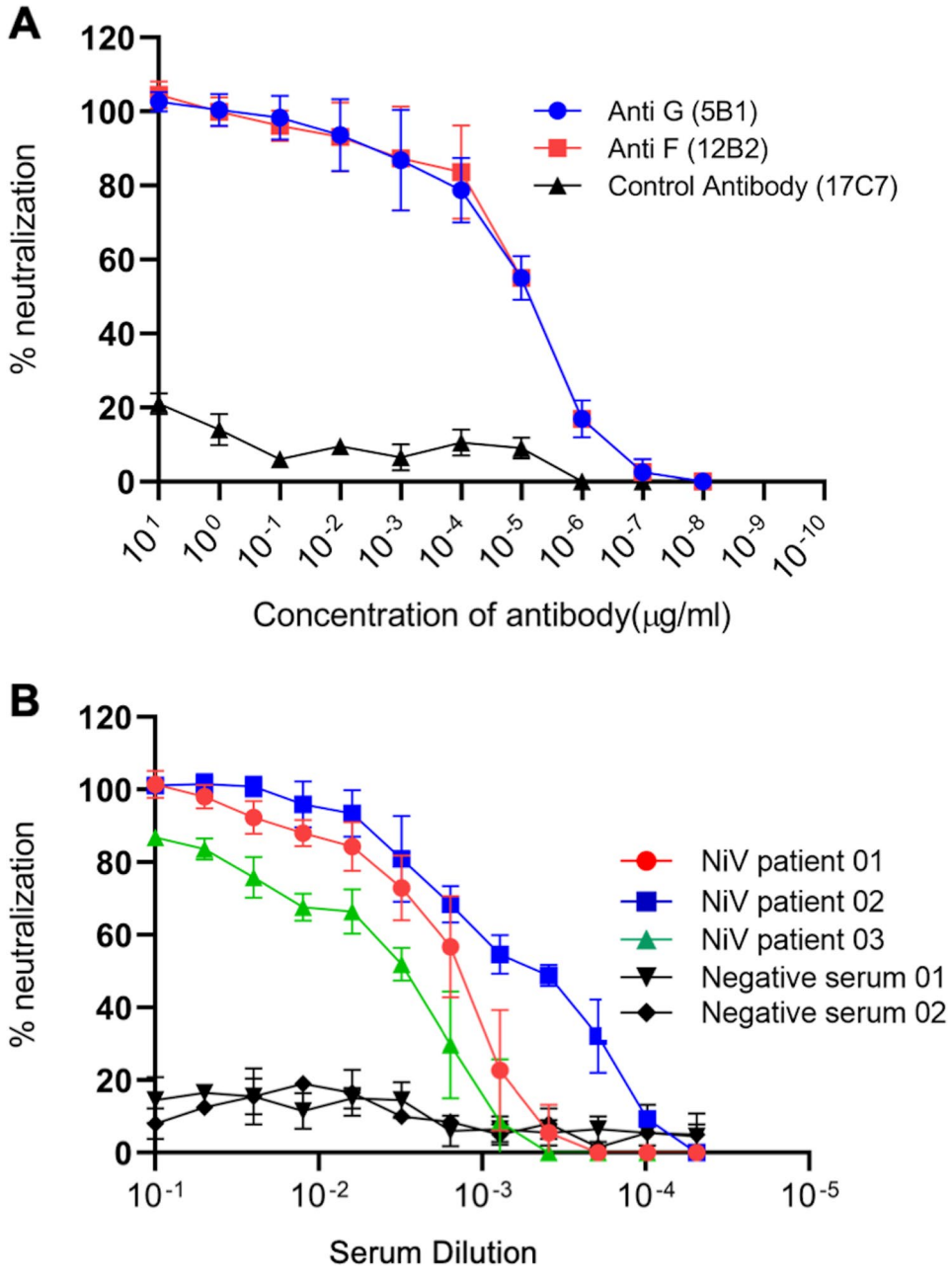

Fig. 6 N-PV(FG)–5F6 pseudovirion neutralization by commercial anti-Nipah antibodies and convalescent serum from Nipah recovered patients.**A** Neutralization curves of N-PV(FG)–5F6 pseudovirions with commercial Anti-G and Anti-F antibodies. 120 SFU pseudotyped virions were incubated with increasing concentrations of neutralizing antibodies to Nipah F and G structural proteins as described in the methods and used to infect target BHK-21 cells. A non-neutralizing antibody mAb 17C7 (Rabishield, Serum Institue, India) was used a negative control in the experiments. The infected cell supernatant was collected 24 h post-infection and SEAP assay was done. Percentage neutralization was calculated as detailed in the methods. Experiment was done in duplicates and each data point presented is a Mean+/- SD of three independent experiments ($N=6$). **B** Assay of NiV positive patient sera using N-PV(FG)–5F6 pseudovirions. Neutralization curves of N-PV(FG)–5F6 pseudovirions with NiV positive sera named As NiV patient 01, NiV serum 02 and NiV serum 03. 120 SFU pseudotyped virions were pre-incubated with increasing dilutions (1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280, 1:2560, 1:5120, 1:10,240, and 1:20,480) of patient sera. Experiment was done in triplicates and each data point is a Mean+/- SD of three independent experiments ($N=9$)

virus (Fig. 3C). It also offers high throughput adaptability As well As easy monitoring of the pseudovirion infection using conventional ELISA readers providing easier implementation in large-scale surveillance programs. We found that the SEAP activity becomes detectable by 18 h post-infection and reaches a peak by 24 h post-infection in NiV pseudovirion infected target cells (Fig. 3E), facilitating an early readout in biological assays using this system.

As surrogate systems may not necessarily mimic the structural organization and functional properties of the surface glycoproteins of natural viruses in all aspects, it is imperative to test the functionality of the pseudovirus particles generated. Neutralization by specific antibodies is a key parameter that indicates their functionality and is a useful property that helps to assess virus-specific immunity in a population. The use of pseudoviruses for evaluating antibody-mediated neutralization has been described in previous studies [40]; and also, earlier reports have indicated a good correlation of the results from these assays with conventional PRNT assays using natural, infectious viruses [23]. However, the use of too excess pseudoviruses can give false negative results in neutralization Assays and there is a need to use optimal virus concentration to obtain reliable and reproducible results. We found that a dilution of pseudovirion stock that gives a titer of approx. 120 SFU gave a dynamic working range OD_{405} of 1.00-0.1 in our PVNT assays starting with virus controls to negative/background controls. Accordingly, this dilution of the stock N-PV(FG)–5F6 pseudovirions was used in all the neutralization assays.

Antibodies generated in a mouse system (commercial anti-NiVF and G mAbs) as well as human convalescent serum were used to evaluate the pseudovirion functionality in neutralization assays. Both antibodies showed a dose-dependent neutralization of the NiV pseudovirions while the negative control antibodies did not neutralize the virus as expected (Fig. 6A & B). The Assay detect the neutralizing antibodies in 1:10 diluted serum samples indicating its comparable performance with conventional virus neutralization Assays. NiV pseudovirions were designed using the sequences of the Bangladesh strain of virus that caused the Nipah outbreak in Kerala in 2018 [9]; and recent studies have pointed out that the 2023 Nipah outbreak, from where the serum samples were collected, was also caused by the same strain of NiV. There were differences in the level of neutralization of the three patient samples used pointing out that the assay detects quantitative differences in the specific antibody titers in each of these patients. Further validation of the PVNT assay using serum samples against other Nipah virus variants need to be done to evaluate the broader utility of the VSV- based NiV pseudovirions developed in this study.

Conclusions

The present study developed a robust and reproducible system for generating VSV-based NiV pseudovirions. Compared to the transient transfection-based systems, the 293(FG)–5F6 cell line stably expressing NiV F & G proteins gave consistent and enhanced production of NiV pseudovirions (Fig. 5). These pseudovirions uniformly incorporated F and G proteins and can be effectively used in virus neutralization assays. One limitation of the current study is that it has used only a smaller number of clinical samples. We had only limited sample access as Nipah infections are not frequent in the state and all recent outbreaks are effectively contained. Further validation of the assay in a larger set of serum samples will make this rapid and high throughput-adaptable assay for cost-effective use in large scale epidemiological screening in simple laboratory settings. The assay will be particularly useful in serosurveillance studies as a generic assay when species-specific reagents are not available. This assay will also help in therapeutic monoclonal antibody and vaccine response evaluation as well as in drug discovery studies against Nipah.

Abbreviations

NiV	Nipah Virus
VLP	Virus-like particle
PVNT	Pseudovirus Neutralization Test
VSV	Vesicular Stomatitis Virus
FACS	Fluorescence Activated Cell Sorting
SEAP	Secreted Alkaline Phosphatase
BSL	Biosafety Level
TCID	Tissue Culture Infective Dose
BHK	Baby Hamster Kidney
HEK	Human Embryo Kidney
ATCC	American Type Culture Collection
MOI	Multiplicity of Infection

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12866-025-04375-9.

Supplementary Material 1. Supplementary fig. 1. Immunofluorescence analysis of syncytia formation in HEK 293 cells upon transfection with NiV F and G proteins. A HEK 293 cells transiently transfected with NiV F and G proteins depicting the formation of syncytial cells. Top panel: HEK293 control cells, Bottom panel: NiV F and G protein expressing plasmid transfected cells with large multinucleated syncytium. HEK293 cells expressing the plasmid- encoded NiV F and G proteins were fixed for immunofluorescence and stained with primary antibodies 12B2 and 48D3 respectively. Confocal microscopy revealed fused cells as indicated by the arrows. Supplementary fig. 2. Titration of N-PV(FG)-5F6 pseudovirions by colorimetric spot forming assay Correlation analysis of Spot forming unit with SEAP assay. Serial dilutions of the virus inoculum with varying SFUs were used to infect BHK-21 cells and the SEAP activity was measured after 24 h. The values are Mean ± SD of two independent experiments done in triplicates (***N*** = 6).

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Authors' contributions

GRV: Pseudovirion generation, clonal selection, virus neutralization assays, confocal and flow cytometry experiments; and writing the first draft of the manuscript. VV: Pseudovirion assays, ultracentrifugation experiments. SS : Generation of the NiV F & G clones. SSL : Cell culture experiments and virus titration. PP, SA and NKP: Convalescent serum sample collection. APM : Convalescent and healthy serum sample collection. ES: Conceived the study,

carried out overall supervision, arranged funding and edited and finalised the manuscript.

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Data availability
Data has not been deposited in any repositories; and all the data generated or analysed during this study are included in the manuscript.

Declarations

Ethics approval and consent to participate
The study protocol adhered to the Declaration of Helsinki; and was approved by the Institutional Human Ethics Committees (IHECs) of the Government Medical College, Kozhikode, Kerala (Approval No.GMCKKD/RP/2023/IEC/354), and the Institute of Advanced Virology, Thiruvananthapuram, Kerala (Approval No.IHEC/IAV/2023/02). Human serum samples were collected from Nipah recovered as well as healthy individuals, strictly adhering to the protocols approved by the IHEC. Informed, written consents were obtained from all the participants.

Consent for publication
All authors approved the final version of the manuscript and consented for publication.

Competing interests
The authors declare no competing interests.

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