

# Establishment of a Nipah virus rescue system

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**Nipah virus (NiV), a paramyxovirus, was first discovered in Malaysia in 1998 in an outbreak of infection in pigs and humans and incurred a high fatality rate in humans. Fruit bats, living in vast areas extending from India to the western Pacific, were identified as the natural reservoir of the virus. However, the mechanisms that resulted in severe pathogenicity in humans (up to 70% mortality) and that enabled crossing the species barrier were not known. In this study, we established a system that enabled the rescue of replicating NiVs from a cloned DNA by cotransfection of a constructed full-length cDNA clone and supporting plasmids coding virus nucleoprotein, phosphoprotein, and polymerase with the infection of the recombinant vaccinia virus, MVAGKT7, expressing T7 RNA polymerase. The rescued NiV (rNiV), by using the newly developed reverse genetics system, showed properties *in vitro* that were similar to the parent virus and retained the severe pathogenicity in a previously established animal model by experimental infection. A recombinant NiV was also developed, expressing enhanced green fluorescent protein (rNiV-EGFP). Using the virus, permissibility of NiV was compared with the presence of a known cellular receptor, ephrin B2, in a number of cell lines of different origins. Interestingly, two cell lines expressing ephrin B2 were not susceptible for rNiV-EGFP, indicating that additional factors are clearly required for full NiV replication. The reverse genetics for NiV will provide a powerful tool for the analysis of the molecular mechanisms of pathogenicity and cross-species infection.**

henipavirus | infectious clone | recombinant virus | reverse genetics

**N**ipah virus (NiV) was isolated in 1998 after an outbreak of fatal encephalitis with >40% mortality among pig farmers in Malaysia and Singapore (1). It was found to be antigenically and genomically related to Hendra virus (HeV), which was isolated in Australia in 1994 (2–4). In 1998, the virus spread from fruit bats to pigs and then to humans. In more recent outbreaks in Bangladesh with up to 70% mortality, NiV was probably transmitted directly from bats to humans, and it has been suggested that human-to-human transmission may occur (5–8). NiV has a broad host range, and high mortality rates are associated with human infections. In humans, NiV causes severe encephalitis with high fatality rates in both the Malaysian outbreak in 1998–1999 (1) and the Bangladesh outbreak in 2004 (9, 10). In contrast, the virus was responsible for a highly infectious respiratory disease with low mortality in pigs. Because of the broad host range and the high mortality rates associated with these infections, NiV has been classified as a biosafety level 4 (BSL-4) agent.

NiV has been assigned to a new genus, *Henipavirus*, within the subfamily Paramyxovirinae, family Paramyxoviridae (11). Molecular characterization of NiV showed that it is very closely related to another zoonotic paramyxovirus, HeV (70–85% sequence homology). In contrast to all other paramyxoviruses, NiV and HeV infect a wide range of species, including pigs, cats, and humans (12). The NiV genome is a single-stranded, negative-sense and nonsegmented RNA that is 18,246 nt (Malaysia isolate) or 18,252 nt (Bangladesh isolate) (3) in length, which is much larger than the other viruses in family Paramyxoviridae (~15,100–15,900 nt for rubraviruses, avulaviruses, respiroviruses, morbilliviruses, and pneumoviruses; 13,000 nt for meta-

pneumoviruses). The predicted gene-start, gene-end, RNA editing site and intergenic sequences are conserved, when compared with the morbilliviruses (4, 13–16). The number and order of genes are identical to those found in the respiroviruses and morbilliviruses. NiV has six transcription units encoding six structural proteins, the nucleocapsid (N), phosphoprotein (P), matrix protein (M), fusion protein (F), glycoprotein (G), and polymerase (L). The P gene is predicted to encode accessory proteins, namely V, W, and C proteins, as is the case for other paramyxoviruses. It was also shown that NiV conforms to the rule of six and uses a replication strategy that is similar to those of other paramyxoviruses (17).

To examine the role played by the different virus proteins in the pathology of NiV infections, it is necessary to be able to manipulate the virus genes within the complete virus genome. The technology, called reverse genetics, has revolutionized the field of negative-stranded RNA viruses. Recent fundamental observations from the studies based on the reverse genetics on various negative-stranded RNA viruses have provided a basis for the prevention of these viruses and for the development of novel therapies (18). In the present study, to establish this powerful technology for NiV, a full-length cDNA of the NiV genome was constructed and a reverse genetics system developed, using helper plasmids encoding N, P, and L proteins. To facilitate the study of NiV infections, a recombinant NiV that expresses enhanced green fluorescent protein (EGFP) was also constructed. These recombinant viruses were compared with the original NiV.

## Results

**Construction and Rescue of Recombinant NiVs.** A cDNA representing the full length of the NiV genome [NiV isolate from Malaysia (2)] was constructed. This was inserted in the plasmid pMDB1. Unique restriction sites were included at the end of each gene (pNiV6+), and, in a further construction, an extra restriction site was included at the end of the leader sequence [pNiV(7+)]. A third construction expressed the EGFP gene, which was inserted downstream of the N gene. The different constructions are shown in Fig. 1A.

To rescue infectious virus from the full-length clones, CV-1 cells were infected with MVAGKT7, a highly host-restricted strain of vaccinia virus MVA that expresses the T7 polymerase (19). A mixture of the genome plasmid [pNiV(6+)], N, P, and L supporting plasmids were transfected to the infected CV-1 cells. The ratio of the plasmids used was that determined by Halpin *et al.* (17) using a minigenome system for NiV.

The transfected CV-1 cells were cultured for 7 days with the

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The authors declare no conflict of interest.

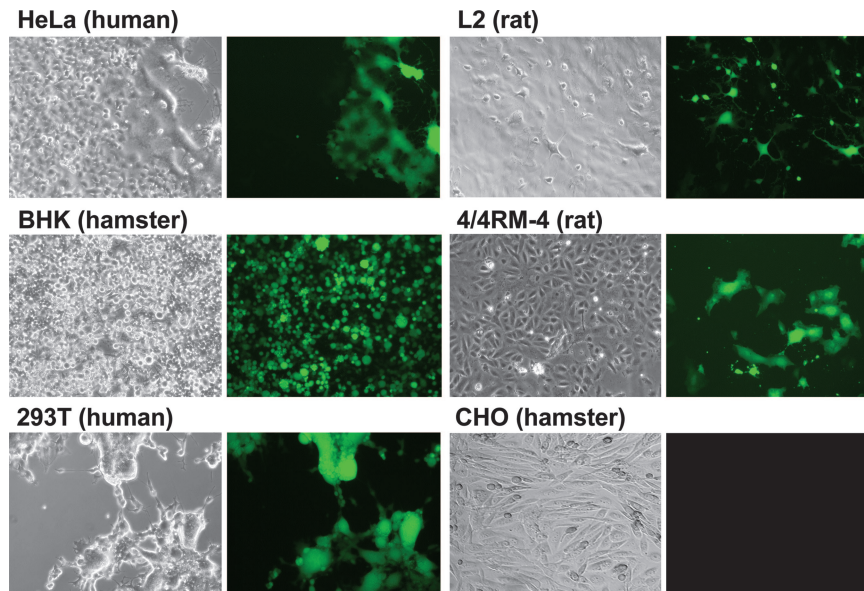
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Abbreviations: CPE, cytopathic effect; HeV, Hendra virus; NiV, Nipah virus; rNiV, rescued NiV.

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**Fig. 4.** Infection of different cell lines with rNiV-EGFP. Cells were infected with rNiV-EGFP at a multiplicity of infection of 0.01 pfu per cell and observed 48 h later, either by light microscopy or by fluorescence. The rNiV-GFP produced large multinucleate giant cells in HeLa and 293T cells and a moderate size of the CPE in 4/4RM-4 cells, but the size of the CPE was much smaller in BHK, and the size and number were significantly reduced in L2 cells. The expression of the EGFP, observed by fluorescence, appeared simultaneously and paralleled the CPE. Using the above CPE and fluorescence criteria, no infection was observed in CHO cells.

cells. However, the size of CPE was much smaller in BHK21 cells, and both size and number of CPE were markedly reduced in L2 cells. CPE was apparent after 1 day in most of these cells but was delayed by 24 h in L2 and 4/4RM-4 cells. The expression of the GFP, observed by fluorescence, appeared simultaneously and paralleled the CPE, including the delay in L2 and 4/4RM-4 cells. Using the above CPE and fluorescence criteria, no infection was observed in CHO, 208f, or P815 cells. Typical examples of CPE and fluorescence in the different cells are shown in Fig. 4.

Recently, ephrin B2 was identified as a cellular receptor for Nipah and Hendra viruses (23, 24). To examine whether the presence of this receptor correlated with our observations of permissibility with our rNiV-EGFP, we tested by RT-PCR the presence of ephrin B2 mRNA. Oligo(dT) was used for reverse transcription priming. The primers except for P815 cells were designed to amplify the region nucleotides 82–572 of human ephrin B2 gene, and primers for P815 were designed to amplify the region nucleotides 534–952 of mouse ephrin B2. The expected size of the amplified fragments was  $\approx$ 490 and 420 bp, respectively. With the exception of two cell lines, 208f and P815, there was a correlation between the expression of the mRNA and rNiV-EGFP replication (Fig. 5A). To confirm surface expression of ephrin B2 on the different cells, we also performed flow cytometry analysis. From the results, it was clearly shown that the 208f and P815 cells also express ephrin B2 on the cell surfaces (Fig. 5B). CHO cells did not express ephrin B2. These results are summarized in Table 1.

Previous studies by the Lee laboratory have shown that NiV-F/G pseudotyped vesicular stomatitis viruses could infect nonpermissive cells such as CHO after they were transfected with a human ephrin B2 plasmid (24). We therefore examined whether such transfected CHO cells would be permissive to NiV infection. One day after transfection with a plasmid that expresses the human ephrin B2 (OriGene Technologies, Rockville, MD), CHO cells were inoculated with the rNiV-EGFP. Expression of the EGFP as well as large CPE in the transfected CHO cells was well observed 24 h after the infection (Fig. 6). Thus, in CHO cells, the presence of human ephrin B2 renders them permissive for NiV.

## Discussion

In 1994, HeV was the first member of the newly classified *Henipavirus* genus to be isolated (25). Although a large proportion of certain fruit bats were shown to be seropositive for this virus (26, 27), there was little cross-species contamination. However, epidemics of the closely related NiV in Malaysia in 1998 showed the virus to have greater repercussions on the economic and health situation (28). Subsequent studies have shown that NiV is widespread throughout Asia and may even produce annual epidemics in countries such as Bangladesh (6, 10). The viruses involved in the outbreaks incurred mortality rates in humans varying from 40% to 70% and can be distinguished by nucleic acid sequencing (29, 30). To analyze the different parameters involved in the disease induced by the henipaviruses, we developed an animal model in hamsters (22). The disease induced in these animals is similar to that observed in man. To determine the contribution of the individual viral proteins to the pathogenesis of the disease, it was necessary to develop the reverse genetics technology for NiV. Although many clinically significant viral pathogens including newly emerged viruses, such as Ebola, Marburg, Hendra, and Nipah viruses, belong to the negative and single-stranded nonsegmented RNA viruses, the mechanisms for the severe pathogenicity and for crossing species barrier are not known. The studies based on the new technol-

**Table 1. Permissibility of different cell lines to NiV infection and the presence of ephrin B2**

Cells	Species tissue	Ephrin B2	Virus growth
BHK	Hamster kidney	+	+
293T	Human endothelial	+	+
HeLa	Human uterus	+	+
CHO	Hamster ovary	–	–
P815	Mouse mast cells	+	–
208f	Rat embryonic fibroblasts	+	–
L2	Rat lung epithelial	+	+
4/4RM4	Rat epithelial	+	+



plete nucleotide sequence of this virus is deposited as GenBank entry AY029767. Virus stocks were grown in Vero cells, and the virus used was the fifth passage on Vero cells after isolation. The virus titer of the stock was  $3.2 \times 10^7$  TCID<sub>50</sub>/ml.

**Construction of Plasmids.** NiV RNA was extracted from infected cells as described in ref. 32. RT-PCR was performed by using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). All cloning procedures were conducted by following protocols. To clone the full genome of the NiV, we designed a large number of primer pairs based on the NiV sequence in GenBank (accession no. AY029767). PCR amplifications were performed by using *LA Taq* DNA polymerase (Takara) or Pfu DNA polymerase (Stratagene, La Jolla, CA). PCR products were first cloned into pGEM-T easy (Promega, Madison, WI) and then subcloned into pKS, pGEM, or pMDB1. We obtained at least four clones from each amplification and confirmed that the sequence in each was identical. The fragments were joined by using internal enzyme sites, and the sequence of the joined fragments was confirmed. The leader and trailer sequences were amplified by using primers including *EagI* and *EcoRI* sites (leader) and *EcoRI* and *BsmI* sites (trailer). Each fragment was digested by *EcoRI* and *EagI* or *BsmI*, and cloned into pMDB1 by using the *EcoRI* and *EagI* or *BsmI* sites, respectively. Amplification of the six NiV genes was performed with primers containing unique restriction sites: N (NotI–FseI), P (FseI–PmeI), M (PmeI–MluI), F (MluI–SgfI), G (SgfI–AscI), and L (AscI–AgeI) (Fig. 1A). These six unique restriction enzyme sites were designed for future use. All amplified fragments were cloned into pGEM-T Easy (Invitrogen). The NiV genes were excised with the two appropriate enzymes within the pMDB1-MV plasmid. Finally, the assembled genes were excised with NotI and AgeI and were inserted into the plasmid pMDB1 with the leader and trailer sequences. The resulting plasmid was designated pNiV(7+). A second construction, pNiV(6+), was also made without the restriction enzyme recognition site in its leader sequence containing the original viral sequence. The complete cloned genome was sequenced. Three nucleotide changes (3022 C-T, 9248 C-T, and 11564 G-A) found compared with the parent virus were mutated back to the original published sequence.

For the construction of the full-length genome plasmid to express the EGFP, the EGFP gene was amplified from pEGFP-N1 (Clontech) by using the following primers. EGFP-F, 5'-GAGTCATGGTGAGCAAGGGCGAGGA-3' (SacI site in italics); and reverse primer EGFP-R, 5'-GGCCGGCCTATATCTACTTGTACAGCTCGTCCATGCC-3' (FseI site in italics; additional five nucleotides for rule of six in boldface). The intergenic region between the N and P junction was amplified by using the following primers. NP-F, GGCCGGCCTCCAATATTCTA (FseI site in italics); and reverse primer NP-R, GAGCTCCATTGGATGAATTGT-TATTA (SacI site in italics). The PCR products were cloned into pGEM-T Easy. The EGFP fragment was inserted downstream of the N-P intergenic region, followed by digestion by SacI. Finally, the fragment of EGFP connected to the N-P intergenic region was cloned into the FseI site of the pNiV(6+) (pNiV-EGFP).

To construct supporting plasmids for virus rescue, the sequence coding for the N protein was amplified by PCR with primers containing NotI and KpnI, and that for the P protein was amplified with primers containing BamHI and XhoI. Both PCR products were digested with NotI and KpnI or BamHI and XhoI, respectively, and cloned into a pKS(+) vector. The coding sequence for the L protein, to which NcoI and NotI were added, was generated in four cloning steps by using a pGEM-T Easy vector and was then cloned into pGEM5zf(+).

**Recovery of Viruses from cDNA.** CV-1 cells were seeded into six-well plates ( $2 \times 10^5$  cells per well) 1 day before infection

and transfection. The cells were infected with replication-deficient MVAGKT7 vaccinia virus in DMEM supplemented with 2% FBS 1 h before transfection. Before transfection, 28.4  $\mu$ l of FuGENE 6 (Roche, Basel, Switzerland) was mixed and incubated with 300  $\mu$ l of serum-free DMEM for 5 min. Plasmids (7  $\mu$ g of pNiV and the plasmids encoding transacting proteins, 1.25  $\mu$ g of pKS-N, 0.8  $\mu$ g of pKS-P, and 0.4  $\mu$ g of pGEM-L) were mixed into 100  $\mu$ l of serum-free DMEM. MVAGKT7 was removed from the cells and replaced with 2 ml of maintenance medium containing the plasmids and FuGene 6. Three days later,  $1.5 \times 10^5$  CV-1 cells per well were added. After 7 days, 500  $\mu$ l of the supernatant was removed and added to a confluent monolayer of CV-1 or Vero-E6 cells in a 75-cm<sup>2</sup> flask. When an advanced CPE was observed (after 1–2 days), the cells and medium were harvested and stored at  $-80^\circ\text{C}$ .

**Virus Growth.** Vero cells ( $1 \times 10^6$  per six-well plate) were infected with NiV, rNiV, or recombinant NiV expressing EGFP (rNiV-EGFP) at a multiplicity of infection of 0.01 for 1 h. The inoculum was removed, and the cells were washed once with medium and then incubated in DMEM plus 2% FBS. Cells and supernatants of wells infected with these viruses were harvested immediately, 24, 48, and 72 h later, centrifuged, and stored at  $-80^\circ\text{C}$ . The TCID<sub>50</sub>/ml of samples was measured by standard methods. The experiment was repeated three times.

**Infection of Hamsters.** Eight-week-old golden hamsters (*Mesocricetus auratus*) were injected i.p. with NiV after anesthetization with isoflurane (Aerrane; Baxter Healthcare, Old Toongabbie, New South Wales, Australia). The animals were housed in ventilated containers equipped with HEPA filters in the BSL-4 laboratory and were individually identified by using microchips.

**Expression of Ephrin B2 in Various Cell Types.** Total RNA was extracted from HeLa, BHK, 293, L2, 4/4RM-4, 208f, CHO, and P815 cells by using the commercial reagent ISOGEN (Nippon Gene, Tokyo, Japan). Subsequently, single-stranded DNA was synthesized by reverse transcription, using SuperScript II (Invitrogen GIBCO) with oligo(dT) primer. The cDNA corresponding to the selected region of ephrin B2 was amplified by RT-PCR. The primers for the cells, except for P815, were designed to amplify the region nucleotides 82–572 of human ephrin B2 gene, and primers for P815 were designed to amplify the region nucleotides 534–952 of mouse ephrin B2.

HeLa, 293, BHK, CHO, L2, 208f, and P815 cells were stained with mouse anti-human ephrin B2 polyclonal antibody and analyzed by using flow cytometry. Cells were also stained with an anti-ephrin B2 antibody (P-20 from Santa Cruz Biotechnology, Santa Cruz, CA) followed by an Alexa Fluor 488-conjugated goat anti-rabbit IgG secondary antibody (Molecular Probes, Eugene, OR). FACS analysis was carried out by using a FAC-SCalibur machine (Becton Dickinson, San Jose, CA). The data were acquired and analyzed with CellQuest software (Becton Dickinson). Cells that were not stained with antibody were used as the negative control.

The plasmid expressing human ephrin B2 (OriGene Technologies) was transfected into CHO cells, a line that is not susceptible to NiV infection. One day after transfection, the cells were infected with rNiV-EGFP.

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