Serum from Nipah Virus Patients Recognises Recombinant Viral Proteins Produced in *Escherichia coli*

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**SUMMARY:** The genes for Nipah virus (NiV) proteins were amplified from viral RNA, cloned into the plasmid pTriEx-3 Hygro, expressed, and purified using immobilized metal affinity chromatography. The recombinant N, F, and G NiV proteins (rNiV-N, rNiV-F, and rNiV-G), were successfully expressed in *Escherichia coli* and purified with a yield of 4, 16, and 4 mg/L, respectively. All 3 recombinant viral proteins reacted with all 19 samples of NiV-positive human sera. The rNiV-N and rNiV-G proteins were the most immunogenic. The recombinant viral proteins did not react with any of the 12 NiV-negative sera. However, serum from a patient with a late-onset relapsing NiV infection complication was found to be primarily reactive to rNiV-G only. Additionally, there is a distinctive variation in the profile of antigen-reactive bands between the sample from a case of relapsing NiV encephalitis and that of acute NiV infection. The overall findings of this study suggest that the recombinant viral proteins have the potential to be developed further for use in the detection of NiV infection, and continuous biosurveillance of NiV infection in resource-limited settings.

**INTRODUCTION**

Nipah virus (NiV) is a deadly zoonotic paramyxovirus with a broad host range. It first caused an outbreak of severe febrile encephalitis in Malaysia in 1998/1999 (1). Since then, outbreaks occur almost annually in India and Bangladesh (2–4). More recently, an outbreak was reported in the Philippines in 2014 (5). The average case fatality rate from the NiV outbreaks thus far is 74.5% (4). Bats are presumed to serve as a natural reservoir (6–8), as is evidenced by detection of NiV in bats in South East Asia (9–11), China (12), Madagascar (13), and Ghana (14). NiV’s wide geographical range puts many populations at risk of infection with NiV. Transmission routes may include intermediate hosts such as pigs (1,15), or may occur directly from horse to human (5), bat to human (2,16), or human to human, either from a live or deceased person (17,18). Current diagnostic procedures usually require the use of high containment facilities. The use of recombinantly produced NiV antigens for serological diagnosis via enzyme linked immunosorbent assay (ELISA) would provide a safer alternative. This study aims to produce NiV N, F, and G proteins using an *Escherichia coli* expression system. Here we report the evaluation of these recombinant viral proteins in immunoblot assays using sera of NiV-infected patients from the 1998 outbreak in Malaysia and also from a patient with a case of late-onset relapsing NiV complication.

**MATERIALS AND METHODS**

Virus, serum samples, bacteria, and plasmid: Genomic RNA from the NiV strain NV/My/99/VRI-2794 (GenBank accession no. AJ564621) was used. This strain, isolated from pigs, shares high sequence similarity (>99%) with human NiV isolates (15). Sera from healthy donors were obtained from individuals with no known previous exposure to NiV. NiV-positive sera were obtained from archived serum samples from NiV encephalitis patients known to be positive for anti-NiV IgM and IgG (data not shown). Serum was also
obtained from a patient with a late-onset relapsing NiV complication (21). Ethics clearance was obtained from the Medical Ethics Committee of the University Malaya Medical Centre MECID No. 20147-410. The E. coli strain, NovaBlue and RosettaBlue (DE3) pLacI, and plasmid pTriEx-3 Hygro were purchased from Novagen Inc (Madison, WI, USA).

Construction of recombinant plasmids: RNA was extracted from NiV using TRI Reagent® LS (Molecular Research Centre, Inc, Cincinnati, OH, USA) following the manufacturer's protocol and used as a template for cDNA synthesis using Superscript™ III reverse transcriptase (Invitrogen, Waltham, MA, USA). PCR amplification using Platinum™ Taq DNA polymerase high fidelity (Invitrogen Life Technologies, Waltham, MA, USA) and gene-specific primers were used to amplify the full length NiV nucleocapsid (N), and truncated rNiV-N, rNiV-F and rNiV-G. The amplified DNA fragments were digested using restriction enzymes outlined in Table 1, and cloned into the vector pTriEx-3 Hygro. The recombinant plasmids, encompassing the respective NiV gene and a vector-derived histidine tag (his-tag) at the C-terminus, were transformed into the E. coli cloning host NovaBlue, and the positive transformants were verified by DNA sequencing. The plasmids were then transformed into the E. coli expression host RosettaBlue (DE3) pLacI.

Expression of recombinant NiV proteins: Transformed E. coli were cultured in Luria-Bertani (LB) broth containing ampicillin (50 μg/mL), chloramphenicol (34 μg/mL), tetracycline (12.5 μg/mL), and 1% glucose, until OD600 > 0.6. Expression was induced by DNA sequencing. The plasmids were then transformed into the E. coli cloning host NovaBlue, and the positive transformants were verified by DNA sequencing. The plasmids were then transformed into the E. coli expression host RosettaBlue (DE3) pLacI.

Expression of recombinant NiV proteins: Transformed E. coli were cultured in Luria-Bertani (LB) broth containing ampicillin (50 μg/mL), chloramphenicol (34 μg/mL), tetracycline (12.5 μg/mL), and 1% glucose, until OD600 > 0.6. Expression was induced by the addition of isopropyl β-D-thiogalactosidase (IPTG) to a final concentration of 1 mM. After 4 hours, the bacterial pellet was harvested by centrifugation at 10,000 × g for 10 min. The pellet was resuspended in lysis buffer (300 mM NaCl, 20 mM NaH2PO4, 2 mg/mL lysozyme, pH 7.4), incubated on ice for 30 min, and then sonicated using a Branson (Danbury, CT, USA) Sonifier 250. The cell lysate was centrifuged at 10,000 × g for 10 min at 4°C. The clarified supernatant containing the soluble protein fraction was harvested for purification. The pellet (inclusion body) was washed with PBS and used for purification of insoluble protein. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analyses were performed to confirm the expression of the target recombinant proteins, designated rNiV-N, rNiV-F and rNiV-G.

Purification of recombinant NiV proteins: The histagged fused recombinant NiV proteins were purified by affinity chromatography using the HisTrap HP column attached to the AKTA® TM Purifier System (GE Healthcare, South East, England). For purification under native conditions, the clarified supernatant containing the soluble rNiV-N was loaded onto the column, which had been equilibrated with binding buffer (1 M NaCl, 20 mM NaH2PO4, 20 mM imidazole, pH 7.4). The column was then washed to remove unspecific proteins and the bound protein was eluted with elution buffer (1 M NaCl, 20 mM NaH2PO4, 500 mM imidazole, pH 7.4). The eluted fractions were collected, and analysed by SDS-PAGE and immunoblotting.

The inclusion bodies containing insoluble rNiV-F and rNiV-G were solubilised in 6 M and 8 M urea, respectively. The solubilised protein was applied to a HiTrap HP column, and purification was performed under native conditions with minor exceptions. The binding buffer (500 mM NaCl, 20 mM NaH2PO4, 20 mM imidazole, pH 7.4) and elution buffer (500 mM NaCl, 20 mM NaH2PO4, 500 mM imidazole, pH 7.4) contained 6 M urea for rNiV-F, and 8 M urea for rNiV-G to maintain denaturing conditions. The eluted fractions were collected, and analysed as mentioned above.

Peptide mass fingerprinting and mass spectrometry: The purified proteins were separated by SDS-PAGE and stained with colloidal Coomassie blue (Bio-Rad, Hercules, CA, USA). Protein spots were excised from the gel bands according to the expected molecular masses of the expressed recombinant proteins. In the SDS-PAGE of purified rNiV-G and rNiV-F, there were additional protein bands of lower molecular weight (MW) than predicted. These bands were also excised for identification. The gel plugs were destained, dried, and trypsin-digested (Sequencing Grade Modified Trypsin, Promega, Madison, WI, USA) for 2 hours at 37°C. The peptides were extracted from the gel plugs using solvent solution (0.1% trifluoroacetic acid and 50% acetonitrile) and transferred into new wells. The solvent was evaporated off, and the dried peptides were reconstituted with saturated matrix (α-cyano-4-hydroxycinnamic acid in 0.5% trifluoroacetic acid and 50% acetonitrile) and spotted onto sample slides. The sample slides were analysed using a 4800 Plus MALDI-TOF/TOF Analyzer (Applied Biosystems/SCIEX, Foster City, CA, USA). The proteins were then identified by a peptide mass fingerprinting search of the NCBIinr database using the Mascot web engine (http://www.matrixscience.com/search_form_select).

Table 1. Primers used in the amplification of NiV genes and expected product size

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer1)</th>
<th>PCR product size (bp)</th>
<th>Expected size (kDa)</th>
<th>Amino acid position</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>5' TTTTTTGGATCCGAGTGAGTTATCTTGGAGAACGGCC 3'</td>
<td>1,599</td>
<td>66</td>
<td>1-532</td>
</tr>
<tr>
<td>R</td>
<td>5' TTTTTTGGCCGCCGCCCACATCGCTGACGAAATC 3'</td>
<td>921</td>
<td>38.9</td>
<td>179-485</td>
</tr>
<tr>
<td>F</td>
<td>5' TCTACGAGATACATCAATTGACGACATGGCAGAC 3'</td>
<td>1,506</td>
<td>58.4</td>
<td>78-579</td>
</tr>
<tr>
<td>R</td>
<td>5' CTAATGCGCGCCCTTAAACAGATCTAAGGAGTCGTTG 3'</td>
<td>1,506</td>
<td>58.4</td>
<td>78-579</td>
</tr>
</tbody>
</table>

1: Restriction enzyme sites: (), BamHI; Bold, NorI; [], EcoRI; O, PstI; underlined, XhoI. F; forward, R; reverse.
Immunoblot analyses: Protein samples were mixed with loading buffer, boiled for 5 min and subjected to SDS-PAGE on denaturing 12.5% polyacrylamide gels. The separated proteins were then transferred to a nitrocellulose membrane and blocked with 3% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA). For detection of the his-tagged recombinant proteins, the membrane was incubated with HisDetector Nickel AP-conjugate (KPL, Gaithersburg, MD, USA) at a 1:5,000 dilution for 1 hour at room temperature, and subsequently developed using the substrate BCIP/NBT (KPL). For immunoblot analysis using human sera, the membrane was incubated with serum samples at a 1:100 dilution for 1 hour at room temperature. Sera from 12 healthy individuals, 19 patients previously confirmed with acute NiV infection, and 1 patient with a relapsing NiV complication were included in the analyses. After washing with TBS-T (50 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20, pH 7.4), anti-human IgG monoclonal antibody conjugated to alkaline phosphatase (Sigma-Aldrich) at a 1:5,000 dilution was added to the membrane, and incubated for 1 hour at room temperature. The membrane was then washed and developed using the substrate BCIP/NBT as previously described (22). Densitogram analysis of the immunoblot was performed using the image analysis software ImageJ (National Institutes of Health, Bethesda, MD, USA). To check for specificity, the recombinant proteins were also reacted with sera (1:100 dilution) from individuals confirmed to be previously infected with other related paramyxoviruses (mumps and measles), as well as other common infections, such as influenza A and dengue.

RESULTS

Expression and purification of the recombinant NiV proteins: The rNiV-N protein was recovered in its soluble form both from the bacterial cytoplasm, and as protein aggregates inside inclusion bodies (Fig. 1A), while rNiV-F and rNiV-G were expressed mainly in inclusion bodies (Fig. 1B and C). The recombinant his-tagged proteins were successfully purified from the cell lysate or inclusion bodies by means of affinity purification. After purification, immunoblot analysis revealed a protein band corresponding to the predicted molecular mass of ~66 kDa for rNiV-N, ~39 kDa for rNiV-F, and ~58 kDa for rNiV-G (Fig. 1). However, in the purified products, an additional band for rNiV-F and multiple bands for rNiV-G of lower molecular mass were also observed. The identities of all observed bands were confirmed by mass spectrometry (Table 2). The majority of the bands were removed after purification for rNiV-F protein, but not for rNiV-G protein. The yield of the purified protein was 4 mg/L for rNiV-N, 16 mg/L for rNiV-F, and 4 mg/L for rNiV-G.

Antigenicity of the recombinant NiV proteins: Sera from all 19 NiV-positive patients reacted with rNiV-N and rNiV-G proteins, while reactions involving the rNiV-F protein resulted in only faint bands (Fig. 2). However, the serum from the patient with the relapsing NiV encephalitis complication did not react as robustly to the rNiV proteins as compared to the other positive patients’ sera. This was especially notable for reactions

### Table 2. Identification of expressed NiV proteins by peptide mass fingerprinting and MALDI-TOF MS analysis

<table>
<thead>
<tr>
<th>Protein</th>
<th>Approx. band size (kDa)</th>
<th>Mowse score</th>
<th>Sequence coverage (%)</th>
<th>No. matched peptides</th>
<th>Calculated mass</th>
<th>Expect value</th>
</tr>
</thead>
<tbody>
<tr>
<td>rNiV-N</td>
<td>66</td>
<td>1,020</td>
<td>68</td>
<td>37</td>
<td>58,132</td>
<td>5.4e-097</td>
</tr>
<tr>
<td>rNiV-F</td>
<td>39</td>
<td>136</td>
<td>10</td>
<td>4</td>
<td>60,243</td>
<td>6.6e-009</td>
</tr>
<tr>
<td></td>
<td>251</td>
<td>245</td>
<td>10</td>
<td>4</td>
<td>8.3e-020</td>
<td></td>
</tr>
<tr>
<td>rNiV-G</td>
<td>58</td>
<td>582</td>
<td>35</td>
<td>15</td>
<td>66,996</td>
<td>3.4e-053</td>
</tr>
<tr>
<td></td>
<td>301</td>
<td>315</td>
<td>23</td>
<td>10</td>
<td>1.7e-026</td>
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<td></td>
<td>181</td>
<td>226</td>
<td>20</td>
<td>9</td>
<td>3.4e-034</td>
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<td></td>
<td>131</td>
<td>268</td>
<td>18</td>
<td>7</td>
<td>8.6e-022</td>
<td></td>
</tr>
</tbody>
</table>

1): denotes lower MW fragments of the respective recombinant proteins.
NiV Patient Sera Recognises Recombinant Viral Protein

Fig. 2. A representative of the immunoblot analysis of (A) rNiV-N, (B) rNiV-F, and (C) rNiV-G using sera from NiV-infected patients (lanes 1–10) and healthy donors (lanes 11–20). Lane M: molecular weight marker. Arrows indicate expected position of respective proteins.

NiV Patient Sera Recognises Recombinant Viral Protein

with rNiV-N. The serum from the relapsing complication case reacted more with rNiV-G compared to rNiV-N and rNiV-F (Fig. 3). None of the 12 healthy donors’ sera produced a signal in reaction to either of the 3 recombinant NiV proteins. There was also no signal when reacted with sera from individuals positive for measles, mumps, influenza A, or dengue viruses (data not shown).

DISCUSSION

This study aimed to produce recombinant NiV proteins, namely the N, F, and G proteins, for potential use in serological surveillance for NiV infection. After purification, rNiV-N with high purity was obtained and observed as a single band in SDS-PAGE analysis, while additional band(s) were observed for purified rNiV-F and rNiV-G. These consistently observed lower MW bands were likely truncated forms of the recombinant proteins that could have resulted from the presence of internal translation initiation or ribosome binding sites. It is also possible that these bands resulted from N-terminal degradation during the protein translation and/or purification processes. The presence of the C-terminus his-tag indicate that the lower MW bands observed in the immunoblot were not the result of premature termination during translation. However, this observation was not reported by Eshaghi et al. (23) who produced the G protein in baculovirus. The purification step greatly reduced the abundance of the lower MW fragments the final product for rNiV-F, but not for rNiV-G. Further modification steps will likely help to improve the purity of rNiV-G. Nonetheless, we have shown that the lower MW fragments of the rNiV-F and rNiV-G proteins did not affect the discrimination of negative and positive sera in the downstream assays.

Of the 3 recombinant viral proteins, rNiV-N and rNiV-G proteins were found to be highly immunogenic as observed in the immunoblot assays. All the NiV-positive patients’ sera reacted with the recombinant
NiV proteins, although to a lesser extent for rNiV-F. Interestingly, 4 of the positive sera reacted with the lower MW bands of rNiV-G (\( \sim 25 \) kDa or \( \sim 30 \) kDa band), suggesting that these bands are also immunogenic. From mass spectrometric analysis of the 2 lower MW bands, the peptide sequences matched regions that correspond to the top of the globular head of the G protein. These regions are known to be involved in receptor binding (24) and are found to be immunogenic in many paramyxoviruses (25). It is unclear, however, why the other patients’ sera did not react with these bands. This may suggest that there is differential antibody response during infection in certain individuals. The weaker reaction between the rNiV-F protein and positive sera suggest that rNiV-F is not as immunogenic as the rNiV-N and rNiV-G proteins. Perhaps, the anti-F antibody in the sera is directed against the biologically active (26) or glycosylated forms of the viral protein.

The recombinant NiV proteins were also probed with serum from a case of relapsing NiV encephalitis complication (21). In contrast to all the other NiV positive patients’ sera, this serum reacted mainly to the rNiV-G protein, followed by the rNiV-F and the rNiV-N proteins. The patient was known to be asymptomatic during the NiV outbreak in 1998/1999, during which the patient likely contracted the illness while being in contact with relatives who had NiV encephalitis. The patient was later diagnosed with an NiV infection primarily based on MRI imaging. In the report, serum from the patient was used to probe NiV-infected Vero cells. The patient was negative for anti-NiV IgM by immunofluorescence assay, indicating that it was not a primary infection at the time. However, the patient’s serum tested positive for anti-NiV IgG, although the specific protein targeted by the IgG response could not be determined. It is possible that the patient is persistently infected with an NiV that has altered viral nucleocapsids, resulting in defective or non-infectious virus, as has been previously observed in other paramyxovirus infections, including mumps (27) and measles (28). Thus, it is possible that the anti-N antibody produced against the mutated virus only weakly recognises rNiV-N in comparison to those in the acute positive patients’ sera. It would be of great interest to further evaluate the NiV immunological profiles during relapse or late-onset NiV, which are not well characterized. Wong et al. (29) reported a rise in anti-NiV IgG titers in a case of a 4-month late-onset infection. However, the ELISA was performed using an infected cell lysate, rendering it impossible to determine if the increased titer was associated with a specific viral protein.

Recombinant proteins expressed in prokaryotic systems are not glycosylated. While specific N-glycosylation is required for biological function of the NiV gly-
understanding about the immunopathology of the dis-
relapsing NiV infection complication to have a better
further studies need to be performed on the case of
particularly in Malaysia. Lastly, our findings suggest
further evaluated and developed for use in resource
limited settings for seroprevalence studies of NiV infec-
tion system. This process would enable production of
recombinant proteins, especially rNiV-N and rNiV-G, retain
their immunogenicity and are recognized by anti-NiV
antibodies in NiV patients’ sera. Additionally, this
study is the first to describe variation between the
profiles of antigen-reactive bands identified via
serodiagnostic immunoblots for a relapse patient’s
serum compared to those with acute infection. In line
with the outcome of this study, the proteins can now be
further evaluated and developed for use in resource
limited settings for seroprevalence studies of NiV infec-
tion and for NiV biosurveillance of at risk populations,
particularly in Malaysia. Lastly, our findings suggest
further studies need to be performed on the case of
relapsing NiV infection complication to have a better
understanding about the immunopathology of the dis-
ease.

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sity of Malaya-MOHE High Impact Research Fund (E000013-20001).

Conflict of interest None to declare.

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