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Production of the matrix protein of Nipah virus in *Escherichia coli*: Virus-like particles and possible application for diagnosis

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A B S T R A C T

The broad species tropism of Nipah virus (NiV) coupled with its high pathogenicity demand a rapid search for a new biomarker candidate for diagnosis. The matrix (M) protein was expressed in *Escherichia coli* and purified using a Ni-NTA affinity column chromatography and sucrose density gradient centrifugation. The recombinant M protein with the molecular mass (M_r) of about 43 kDa was detected by anti-NiV serum and anti-*myc* antibody. About 50% of the M protein was found to be soluble and localized in cytoplasm when the cells were grown at 30 °C. Electron microscopic analysis showed that the purified M protein assembled into spherical particles of different sizes with diameters ranging from 20 to 50 nm. The purified M protein showed significant reactivity with the swine sera collected during the NiV outbreak, demonstrating its potential as a diagnostic reagent.

1. Introduction

Nipah virus (NiV) is a zoonotic paramyxovirus that causes fatal encephalitic and respiratory illness in humans and livestock (Chua et al., 2000; Paton et al., 1999). The outbreak in Peninsular Malaysia in 1998 claimed 105 human lives and resulted in massive culling of about 1.1 million infected swine with encephalitis and respiratory diseases (Chua et al., 2000; Paton et al., 1999). Fruit bats (flying foxes) are believed to be the natural reservoir for NiV and may be introduced into pig farms through their secretions (Chua et al., 2002; Field et al., 2001). Other animals such as dogs, cats and horses can also be infected by the virus when they come in close contact with infected pigs (Chua et al., 1999, 2000, 2002). NiV outbreaks have occurred in Malaysia, Singapore, India and Bangladesh following various chains of transmission including intermediate host species (Chua et al., 2000), vehicle borne transmission (Luby et al., 2006), bat to human transmission (Hsu et al., 2004) and human-to-human transmission (ICDDRB, 2004). Identification of the spillover into human population has now been extended to Indonesia, India and Bangladesh (Chua et al., 2000; Hsu et al., 2004; ICDDRB, 2004; Luby et al., 2006). It is probably much more extensive due to undiagnosed cases in many countries. The ability of NiV to infect a variety of species along with its mode of transmission coupled with its high pathogenicity demand a rapid search for possible tools for diagnosis of early infection.

NiV has pleomorphic structure ranging from 50 nm to greater than 600 nm in diameter (Hyatt et al., 2001). The virus contains two envelope glycoproteins: the G protein is responsible for binding to the cellular receptors, Ephrin B2 and B3 (Bonaparte et al., 2005; Negrete et al., 2005) and the F protein mediates membrane fusion (Bossart et al., 2002). Lying beneath the viral envelope is the matrix (M) protein, which interacts with both the glycoproteins and the nucleocapsid (N) or ribonucleoprotein (RNP) complex (Lamb and Parks, 2007; Schmitt and Lamb, 2004).

The M protein is one of the abundant proteins in the virion and it is important in determining the virion architecture. The *M* gene is predicted to be 1359 nucleotides (nt) in length, with an ORF of 1059 nt, encoding the M protein (352 amino acids) with a predicted molecular mass (M_r) about 39.93 kDa. The first available AUG codon is predicted to have more probabilities to be the initiator rather than the other in-frame initiation codon at nucleotide 36 downstream of the first codon. Its high hydrophobic nature coupled with high net positive charge attribute to its property of association with membranes (Harcourt et al., 2000; Takimoto and Portner, 2004). The M protein is localized in the cytoplasm, predominantly at the plasma membrane when it was expressed in mammalian cells (Ciancanelli

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and Basler, 2006). However, there is no information available on the production of the M protein in bacteria. Therefore, the objectives of the study were: (i) to express the M protein in *Escherichia coli*; (ii) to purify and characterize the M protein and; (iii) to develop an ELISA for detecting anti-M antibody in swine serum samples.

2. Materials and methods

2.1. Serum samples

Swine anti-NiV serum samples, with known serum neutralization titer (SNT), were obtained from the Veterinary Research Institute, Ipoh, Malaysia. The serum samples were collected during the 1998–1999 NiV outbreaks in Malaysia.

2.2. Construction of recombinant plasmids

Total RNA was extracted from NiV infected cell culture medium (250 μl) using the TRI-REAGENT (Sigma, Missouri, USA) as recommended by the manufacturer. The extracted total RNA was used as a template for cDNA synthesis using the M-MLV Reverse Transcriptase (Promega, Madison, USA). The NiV *M* gene was amplified by using primers NiV-M-6 FD (CCATGGCCATGGAGCCGGACATC) and NiV-M-5 RV (GTAAGCTTCGCCCTTTAGAATTCTCCCTGT). The underlined nucleotides represent *Nco*I and *Hind*III restriction sites, respectively. The PCR products were digested with *Nco*I and *Hind*III and subsequently cloned into the corresponding restriction sites of the pTrcHis2 vector (Invitrogen, Carlsbad, USA) to produce recombinant plasmid, pTrcNiVM. The insert of the recombinant plasmid was confirmed to be in frame by DNA sequencing.

2.3. Expression of the M protein in E. coli

Shake flask cultures (50 ml) of transformed *E. coli* BL21(DE3) cells were grown in Luria Bertani (LB) medium containing ampicillin (50 μg/ml) at 25, 30 and 37 °C to an A_{600} of about 0.6–0.8 and protein expression was induced with IPTG (0.5 mM). The cultures (1 ml) were centrifuged at 11,500 × *g* for 30 s and cells were lysed using lysis buffer [50 mM Tris–HCl, pH7.4, 100 μg/ml lysozyme, 5 mM EDTA, pH 8, 1 mM phenyl methane sulfonyl fluoride (PMSF)]. Protein concentration was determined with the Bradford assay (Bradford, 1976).

2.4. Localization and solubility analyses

Localization and solubility analyses of the recombinant M protein produced in *E. coli* cells were carried out according to Coligan et al. (2000). The percentage of soluble M protein was measured with the Quantity One Quantitation Software (Bio-Rad, Hercules, USA) as described by Tan et al. (2004).

2.5. SDS-PAGE and Western blotting

Proteins were separated by SDS-PAGE and were either stained with Commassie Brilliant Blue or transferred onto nitrocellulose membranes using a semidry transfer cell (Bio-Rad, Hercules, USA) for Western blotting. The membranes were blocked with 5% skimmed milk in TBS (50 mM Tris–HCl, 150 mM NaCl; pH 7.5) for 1 h at room temperature (RT). Swine anti-NiV sera (1:200 dilution) or anti-His monoclonal antibody (GE healthcare, Pittsburg, USA) or anti-*myc* monoclonal antibody (1:5000 dilution; Invitrogen, Carlsbad, USA) was added to the membranes and shaken for overnight. The membranes were then washed with TBS-T (TBS + 0.01% Tween 20). Secondary antibody either anti-swine or anti-mouse antibody conjugated to alkaline phosphatase (1:5000 dilution; Kirkegard and Perry Laboratories, Gaithersburg, USA) was then added and incubated for another 1 h. After washing, the colour development was performed by adding 5-bromo-4-chloro-3′-indolyl phosphate *p*-toluidine salt (BCIP; Fermentas, Glen Burnie, USA) and nitro-blue tetrazolium chloride (NBT; Fermentas, Glen Burnie, USA) substrate.

2.6. Purification of NiV M protein and VLPs

Protein synthesis in *E. coli* was induced with IPTG (0.5 mM) for 2 h at 37 °C. The cells were centrifuged at 3440 × *g* for 10 min and the pellets were resuspended in lysis buffer (20 mM Na_3PO_4, 150 mM NaCl; pH 7.5) containing lysozyme (100 μg/ml) and incubated on ice for 30 min. The cell suspension was then lysed by sonication after adding PMSF (1 mM) and DNase (7 μg/ml) and incubated on ice for 15 min. The lysate, obtained after centrifugation at 39,200 × *g* for 30 min, was loaded onto a pre-equilibrated Ni-NTA agarose (Amersham biosciences, Pittsburg, USA) column and was incubated for 1 h at room temperature. The protein-bound resin was first washed with buffer A (20 mM Na_3PO_4, 150 mM NaCl; pH 7.5) followed by washing with buffer B (20 mM Na_3PO_4, 500 mM NaCl; pH 6). The bound recombinant M protein was eluted with elution buffer (20 mM Na_3PO_4, 500 mM NaCl, 500 mM imidazole; pH 7.4) and elute was analysed by SDS-PAGE and Western blotting.

The purified recombinant protein was dialyzed against dialysis buffer (50 mM Tris–HCl; pH 7.5, 150 mM NaCl). The dialyzed protein was concentrated with a 30 kDa cut-off polyethersulfone membrane (VIVASPIN6; Vivascience, Stonehouse, UK) at 4500 × *g*, 4 °C. The concentrated protein was layered on a step sucrose gradient 10, 20, 30, 40 and 60% (w/v) and centrifuged (rotor SW40Ti, at 36,000 rpm) for 5 h at 4 °C. Fractions (0.5 ml) were collected and analysed on SDS-PAGE. Positive fractions were then pooled and dialyzed against dialysis buffer.

2.7. Electron microscopy

The purified M protein (15 μl) was absorbed to carbon-coated grids (200 meshes) and stained with uranyl acetate (2%). The grids were viewed under a TEM (HITACHI-T-700) and micrographs were taken at appropriate magnifications (Tan et al., 2004).

2.8. ELISA

All washing steps were carried out five times with TBS-T buffer (TBS + 0.05% Tween 20). All antigens were diluted in TBS whereas antibodies were diluted in TBS-T buffer. U-shape polysterene microtiter plates were used as the solid-phase adsorbents. Sucrose gradient fractions (50 μl) or the purified recombinant M protein (100 ng/well; 100 μl) was added to the wells. After incubating for 18 h at 4 °C, the plates were washed and then blocked with 10% BSA (200 μl) in TBS and incubated for 2 h at RT. Subsequently, the plates were washed and incubated for 1 h at RT with either anti-*myc* monoclonal antibody (1:5000) or with the appropriate dilution (1:20) of the swine sera from infected and non-infected animals. After washing with TBS-T, either anti-mouse antibody (1:5000 dilution) or anti-swine immunoglobin IgG (1:3000 dilution) conjugated to alkaline phosphatase (KPL, Gaithersburg, USA) was added and the plates were incubated further for 1 h at RT. Following another washing step, the enzyme substrate solution containing *p*-nitrophenyl phosphate (0.1%; Sigma) in diethanolamine (1 M; Sigma), pH 9.5, was added. The reaction was stopped after 30 min incubation at RT, and the A_{405} values were measured with a microtiter plate reader (Bio-Tek, ELX 800, Winooski, USA). The significance of the readings between positive and negative sera was calculated using the *T*-Test statistical analysis.