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SHORT COMMUNICATION

The importance of GDN motif in domain III and GXGXG motif in domain VI of L protein in replication of Nipah virus

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ABSTRACT

This study aims to determine the importance of conserved GDN motif in domain III and GXGXG motif in domain VI in Nipah virus (NiV) L protein. Four mutated L genes produced in an earlier study were inserted individually into plasmid pCITE. Optimised transfection protocol was successful in transfecting these plasmids, two helper plasmids (coding for N and P protein), NiV minigenome containing chloramphenicol acetyltransferase (CAT) reporter gene and T7 promoter. Successful *in vitro* transcription/translation in the NiV minireplicon system was monitored by CAT expression. In conclusion, GXGXG motif was important in the NiV minireplicon system but change of GDN motif does not affect L protein.

Keywords: GDN motif of domain III, GXGXG motif of domain VI, conserved domain, L gene Nipah virus minireplication system

INTRODUCTION

Nipah virus (NiV) is a zoonotic *Paramyxovirus* causing respiratory illness and fatal encephalitis in human. Outbreaks have occurred in Malaysia, Singapore, India and Bangladesh, and a putative NiV was also associated with human disease in the Philippines (Ching *et al*., 2015; Chua, 2003). NiV is a member of the *Henipavirus* genus within the family *Paramyxoviridae* due to the relatedness with Hendra virus (HeV) and was first isolated from a pig farmer who lived in a village called Kampung Nipah (Harcourt *et al.*, 2000).

Nipah virus (NIV) is a negative-sense single-stranded RNA virus of approximately 18.2 kb genome length. It codes for six major structural proteins including the nucleocapsid (N), phosphoprotein (P), matrix (M), fusion (F), glycoprotein (G) and large protein (L) (Harcourt *et al*., 2000). Alignment of amino acid sequence of L proteins of non-segmented negative strand (NNS) RNA viruses, showed six highly conserved domains that are joined by variable length of spacer regions with relatively low sequence homology (Poch *et al*., 1990). L protein subunit of NiV consists of 2244 amino acids which make up a multi-functional complex with RNA dependent RNA polymerase (RdRp) which has enzymatic activities required for mRNA synthesis and genome production such as nucleotide polymerisation, mRNA capping, polyadenylation and methylation (Harcourt *et al*. 2001; Cox and Plemper, 2017). For other *Paramyxoviruses*, conserved domain III (the invariant GDNQ sequence) has been assigned to be essential for phosphodiester bond formation (Chattopadhyay *et al*., 2004; Magoffin *et al*., 2007; Fearns *et al.*, 2017) and conserved domain VI (GXGXG motif) was linked to cap methylation activities (Li *et al*., 2006).

Each conserved domain is predicted to carry out specific biochemical functions related to RNA synthesis. As for the L protein of NiV, the importance of these conserved domains has not been fully defined. Hence, in this study we will determine the effect of mutations in these conserved domains of the L protein to the Nipah virus replication by using a minireplicon system. The effect of mutations to the L protein conserved motifs will also be determined and compared with other viruses among the Paramyxoviruses.

MATERIALS AND METHODS

Plamid construction

Replacement of single base NiV-L (Genbank accession number, AF 212302) gene mutation in Domain III and Domain VI generated by PCR based site-directed mutagenesis has been previously validated in Jalani and Ibrahim (2016). Each mutation in Domain III (N2497D and E2500G) and Domain VI (G5528A and Q5532A) has

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been designed and assumed to retain polymerase function and cap methylation. Individual genes were ligated to plasmid pCITE producing clones bearing wild-type L gene (pCITE-L) and four clones carrying the mutated L gene plasmids (pCITE-L mutated: N2497D, E2500G, G5528A and Q5532A) were verified by sequencing.

Plasmid transfection

Plasmid with wild type or mutated L genes from this study and helper plasmids encoding for N and P protein, plasmid pUC18-mNiV and T7 promoter (all kindly provided by Mr. Shahreza M. Sheriff (Universiti Kebangsaan Malaysia) were transfected in BHK-21 cell. Minigenome and supporting plasmids were mixed in OPTI-MEM and transfected in semiconfluent BHK-21 cell in 75cm² flask using Lipofectamine LTX (Invitrogen) (Table 1). Post transfection was allowed for 5 h followed by change with fresh DMEM medium with 10% Fetal Bovine Serum (FBS). Incubation was continued for 48 h at 37 °C with 5% carbon dioxide. The ability to express protein was confirmed using TnT Quick Coupled (Promega Corporation, USA) system. Transfected cells were collected, lysed using 1.2 mL CAT lysis buffer provided in the kit, subjected to centrifugation at 12,000 ×*g* for 15 min at 4 °C and supernatants stored in –80 °C before further analysis.

Table 1: Transfection components per T-75 flasks.

Component	Plasmid/ Solution	Concentration/ Volume
N gene	pCITE-N	4.5 µg
P gene	pCITE-P	3 µg
L /mutated L gene	pCITE-L/pCITE- L mutated	1.5 µg
T7 promoter	pTRIEX-T7	7.5 µg
Minigenome	pUC18-mNiV	13.5 µg
Reduced serum medium	OPTI-MEM	3.75 mL
Transfection reagent	Lipofectamine LTX	46.87 µL

Protein detection and CAT assay

Supernatants were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970) using 10% (w/v) separating gel. Following transfer to nitro cellulose membrane, recombinant protein was detected by monoclonal antibody to anti-polihitidine-alkaline phosphatase (Sigma-Aldrich Inc., USA). Chemiluminescent CDP-Star™ (GE Health Care, UK) was added for detection.

CAT protein presence was determined by CAT ELISA kit (Cat. No. 11 363 727 001, Roche) following the manufacturer's instructions. ELISA was performed using 800 µg protein for each sample and the CAT activity was measured by value of the samples absorbance at 405 nm. The amount of CAT protein (ng/mL) was estimated from the CAT protein standard curve.

RESULTS

We have constructed four plasmids containing mutated L genes. Plasmid N2497D has a mutation in Domain III at nucleotide position 2497 with codon change from adenosine to guanine and the predicted changed to motif GDDE from GDNE. E2500G mutant has a single nucleotide change at 2500 position from guanine to cytosine producing a GDNQ motif from wild type GDNE motif. At conserved Domain VI, nucleotide mutation on the position 5528 in G5528A, changed wild type GEGSG motif from guanine to adenosine producing AEGSG motif. Whereas Q5532N mutant has a change at position 5532 that changed the amino acid from adenosine to cytosine producing GNGSG motif (Jalani and Ibrahim, 2016).

All four constructs were able to express the respective proteins in the *in vitro* transcription/translation experiment (Figure 1) with N protein producing ~58 kDa, P protein 78 kDa. Mutated L proteins with approximate band size of ~250 kDa were detected in Figure 1B albeit with high background staining.

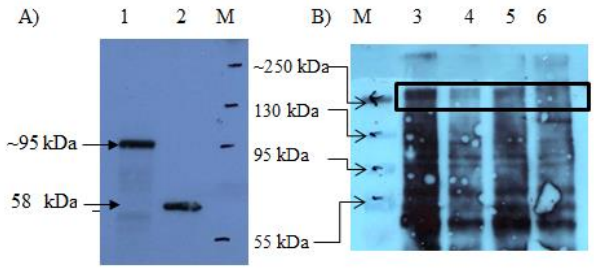

Figure 1: Western blot profile of *in vitro* transcription/translation of contructs. A) Protein band translated from plasmid with P and N genes and B) Bands from mutated L genes. M, Protein marker; 1, P protein; 2, N protein. 3,4, 5 and 6, mutated L protein (N2497D); 4, E2500G; 5, G5528A; 6, Q5532A.

The transfection protocol utilised in this study was optimised for the minireplicon system to express CAT successfully. Comparison of the CAT expression levels of gene mutants with GDDE, GDNQ and GNGSG motifs showed higher expression compared to wild type (Figure 2). Mutant G5528A with AEGSG motif however showed lower CAT expression at 32.4% compared to wild type. No CAT activity was observed in mock transfected sample without minigenome insert.

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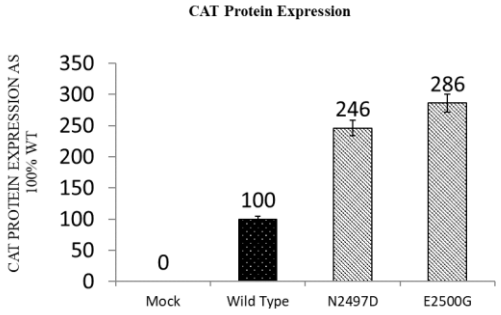
(a)



(b)

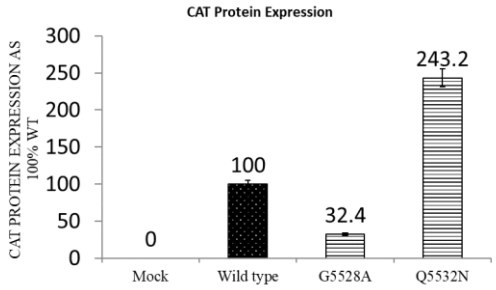


Figure 2: CAT protein expression in transfected cells containing minigenome pUC-NiV, plasmids N, P and wild type or mutated L plasmids. The level of CAT expression for each mutant in Domain III (a) and Domain VI (b) was calculated as a percentage of the wild-type expression.

DISCUSSION

In this study, N and P proteins were successfully expressed using NiV minireplicon system. Plasmid pUC18-mNiV is a minigenome of NiV carrying the leader region with 5' regulatory sequence (leader region), transcription/replication start regions, the 3' trailer sequences of chloramphenicol acetyltransferase (CAT) reporter gene open reading frame (ORF) which was driven by the T7 promoter, a T7 terminator and Hepatitis Delta virus (HDV) antigenome ribozyme (Sheriff, 2008). The minigenome T7 transcript is in the antigenomic sense which can be replicated to genomic sense RNA by viral proteins N, P, and L when co-transfected in BHK-21 cells. The newly synthesized genomic RNA is transcribed into mRNA followed by translation of CAT.

The molecular weight of protein N in this study corroborates with the size previously reported in Wang *et al.* (2000) of 57 993 Da. P protein was estimated to be 78 301 Da with isoelectric point (pI) of 4.44. A protein band of 95 kDa was indicated in this study due to the acidic amino acid residues at the N terminal molecule of protein P causing slow migration during separation by SDS-PAGE. This phenomenon can frequently be observed in P protein among the members of subfamily *Paramyxovirinae* (Wang *et al.*, 2000). As for the mutated L protein, the size was similar to the estimated molecular weight of ~257 kDa, comprising 2,244 amino acids (Harcourt *et al*., 2001).

The minireplicon assay system of NNS RNA viruses was based on the transcription/replication system initially developed by Freiberg *et al.* (2008). In the minireplicon system, T7 RNA polymerase promoter directed the transcription of NiV minigenome in transfected cell line producing negative-sense primary transcripts which were analogous to the negative-sense genome RNA of NiV. The synthesised RNA transcript was self-cleaved by a Hepatitis Delta virus ribozyme sequence located adjacent to the leader region of the minigenome. N, P and L proteins co-expression in the transfected cell allows formation of ribonucleoprotein (RNP) complex which in turn acts as a functional template for 'viral' replication. The resulting RNP will then replicate naturally in the cell which is indicated by the expression of CAT.

In this study, the effect of mutations in L gene can be indirectly analysed in this replication/transcription system by measuring the production of CAT protein. CAT expression level in cells with mutations in domain III (N2497D and E2500G) and domain VI (G5528A) motifs were higher than in wild type L gene (Figure 2). Neverthelesss, all the motif changes caused by the single nucleotide change in conservative amino acid substitution showed that nucleotides changes at 2497, 2500 and 5528 still allowed the successful transcription and replication of the minireplicon system. This indicates that the nucleotide change in conserved domains III (N2497D and E2500G) and VI (G5528A) of L gene in Nipah virus does not affect CAT expression hence the L protein activity. This observation is unique observation from this study if compared to other studies in other Paramyxoviruses.

Previous study on vesicular stomatitis virus (VSV) showed that mutation from GDN motif to GDD exhibited 27% wild type activity using an *in vitro* transcription assay (Sleat *et al.*, 1993). A study in Rinderpest virus (RPV) RNA polymerase showed that GDD motif was not functional implying that in RPV residue, GDNQ motif was an absolute requirement in retaining the polymerase function (Chattopadhyay *et al*., 2004). In that study, E2500G mutant had a mutation of the Q residue in GDNQ motif that decreased the CAT activity to 33% lower than the wild type. This indicates that the amino acid changes resulting in reduction of polymerase ability that is dependent on the GDNQ motif is unique in *Paramyxovirus* among NNS RNA viruses.

Mutational analysis of the amino acid at position E in GDNE motif in Domain III was most likely not important for the catalytic activity but for maintaining polymerase structure in *Henipavirus* (Magoffin *et al*., 2007). That observation may be true as proven in this study. Motif change in domain III from GDNE to GDDE (N2497D) or production of GDNQ motif (E2500G) both showed increased CAT expression. This implies that GDN motif within the conserved domain III in Nipah virus L protein observed in this study is not important in retaining the polymerase activity and different from other NNS RNA viruses.

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A substitution in domain VI in G5528A mutant changed the glycine rich motif to AEGSG from GNGSG and Q5532N mutant from GEGSG to GNGSG. Glycine-rich motif (GXGXG) for G5528A mutant was lost but was retained in Q5532N mutant. This study proved that the GXGXG motif is very important for retaining normal RNA polymerase activity as previously described in other studies. A mutation study in domain VI of Sendai virus (SeV) showed that RNA polymerase activity was normal in most mutants except for two that became inactive completely (Murphy *et al*., 2010). Deletion within the GXGXG motif (glycine-rich) caused loss of activity in RNA synthesis by the mutant. The mutant loss the ability to form P-L complex required for normal L RNA activity. The glycine-rich motif is likely to be the L protein binding site for SeV and possibly other paramyxoviruses from the observation showing reduced methylation. This motif is far more flexible to amino acid changes in this motif than VSV.

Both VSV and SeV are members of Mononegavirales G(D/E)G(S/A)G motif in the L protein (Cortese *et al.*, 2000; Gopinath *et al.*, 2008). Glycine plays an important role in cap methylation in VSV whilst SeV mutation or substitution in VSV mutant within this glycine-rich motif resulted in inactivation. In addition, removal of cap methylation has no effect on VSV mRNA synthesis. It is likely that the mutant in domain VI negatively affected L protein folding causing complete inactivation of this protein (Murphy *et al*., 2010).

CONCLUSION

GDN motif in domain III is not important to retain L protein activity for NiV as shown in this study when nucleotide change were introduced in the conserved domains III and VI. However, GXGXG motif in domain VI is very important in retaining L protein activity indicated in the NiV minireplication system.

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