CLONING AND EXPRESSION OF GA PROTEIN OF NIPAH VIRUS IN YEAST EXPRESSION SYSTEM

by

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CERTIFICATE

This is to certify that the dissertation entitled

Cloning and expression of GA protein of Nipah virus in yeast expression system

is the bonafide record of research work done by

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during the period from July 2008 to October 2008

under my supervision.

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PENGKLONAN DAN PENGEKSPRESAN PROTEIN GA DALAM SISTEM YIS

Abstrak

Sedekad yang lepas, serangan jangkitan Nipah virus (NiV) di seluruh dunia telah menunjukkan kadar kematian setinggi 92%. NiV merupakan wabak yang amat bahaya kepada manusia and juga binatang. Oleh itu, pembangunan vaksin telah menjadi satu strategi yang penting untuk mencegah wabak ini. Menurut kajian sebelum ini, glikoprotein NiV (NiV-G) telah diklonkan dalam vektor pengekspresan yis *Pichia pastoris* (pZMF) yang dikawal oleh promoter AOX1. Walau bagaimanapun, pengekspresan rekombinan glikoprotein G dalam sistem ini didapati tidak stabil. Oleh itu, kestabilan rekombinan protein dikaji dengan mengklonkan sebahagian gen (gen GA) yang disyaki sebagai penyebab ketidakstabilan dalam sistem biologikal yang sama. Penghasilan rekombinan protein dalam yis dikesan melalui analisis Western blot. Kajian ini telah menunjukkan bahawa pengekspresan protein rekombinan adalah stabil and konsisten dengan berat molekul sebanyak 40 kDa. Berat molekul protein rekombinan sebanyak 40 kDa ini telah mencadangkan bahawa rekombinan gen GA adalah stabil dalam pengekspresan keseluruhan gene NiV-G. Tambahan pula, rekombinan protein yang dihasilkan boleh dijadikan sebagai vaksin sekiranya protein ini didapati potensi antigenik dan immuogenik yang tinggi.

CLONING AND EXPRESSION OF GA PROTEIN OF NIPAH VIRUS IN YEAST EXPRESSION SYSTEM

Abstract

Nipah virus (NiV) is a deadly zoonotic paramyxovirus that has emerged and re-emerged over the last 10 years. In human, the infection leads to encephalitis with 92% mortality rate. This has underlined the importance for the development of a vaccine candidate in preventing such zoonotic disease. In previous study, the gene encoding the NiV glycoprotein (NiV-G) was cloned into the yeast *Pichia Pastoris* expression vector (pZMF) under the control of AOX1 promoter. However, the recombinant G glycoprotein expression was found to be highly unstable in this system. Therefore, the stability of the recombinant protein was investigated by studying one of the truncated gene fragment (GA gene). The truncated gene encoding the gene sequence 1-867 was cloned and expressed in the same biological system. The detection of the truncated recombinant protein was performed through Western blot analysis. The result of this study indicated that the truncated fragment was stable and the expression was consistent with protein molecular weight of 40 kDa. The absent of degradation incident in this study had suggested that GA gene fragment was stable in full length G-glycoprotein expression. In addition, the truncated recombinant protein could be utilized as a promising vaccine candidate if the recombinant protein was found to be highly antigenic and immunogenic.

Chapter 1: Introduction

Nipah virus (NiV) is a deadly emerged virus found to be the etiology agent in an outbreak of severe febrile encephalitis in human. It had been classified under the family of Paramyxoviridae under a new genus of Henipavirus (1999). Its genome encodes six structural proteins: nucleocapsid (N), phosphor- (P), matrix- (M), fusion- (F), glyco- (G), and large- (L) protein (Harcourt *et al.*, 2000, Wong *et al.*, 2001)

The first outbreak of NiV occurred in 1998 and 1999 in Penisular Malaysia. It affected 265 patients nationwide with more than 105 mortalities. NiV has continue to reemerged, with outbreaks in Bangladesh in 2001 and 2003 (Hsu *et al.*, 2004). Further outbreak had occurred in Bangladesh in early 2004 and 2005. The most recent outbreak occurred in India and Bangladesh in 2007, and it was associated with a higher incidence of acute respiratory distress syndrome which happened concurrently with encephalitis, person-to-person transmission and with a fatality rate of 75% (Gurley *et al.*, 2007).

NiV had been classified as a zoonotic bio-safety level 4 (BSL-4) agent and was included as one of the pathogenic agent of bio-defense concern by the Centers for Disease Control and Prevention (CDC), and National Institute of Allergy and Infectious Diseases (NIAID). Therefore, the strategy of prevention (vaccination) for such a zoonotic disease has become a priority. The use of vaccine is the most efficient and cost-effective method in preventing infectious diseases.

In previous study, NiV glycoprotein (NiV-G) gene fragment was cloned into the *Pichia* expression vector (yeast system) for the production of a recombinant protein. However, the molecular weight of the recombinant protein expressed was found to be smaller than expected. Similarly, a smaller protein fragment was obtained in the bacteria expression system with an identical gene. These results had suggested that the translated recombinant NiV-G protein was highly unstable and susceptible to be degraded by the host machinery.

Therefore, the instability of the recombinant protein was investigated by studying one of the truncated gene fragments. The truncated gene encoding the gene sequence 1-870 was cloned and expressed in the same biological system. The detection of the truncated recombinant protein was performed through Western blot analysis. Besides identifying the root of degradation, the truncated recombinant protein could be utilized as a promising vaccine candidate if it was found to be highly antigenic and immunogenic.

NiV-G was chosen for vaccine development based on the study done by Guillaume *et al.* (2004a) and Tamin *et al.* (2002). These studies had proven that NiV-G protein was highly antigenic and immunogenic. The administration of subunit vaccine which expressed the NiV-G and NiV-F (Fusion protein) protein protected the animal model from lethal NiV infection. Additionally, NiV-G is the most important target for the production of neutralizing antibodies (Tamin *et al.*, 2002)

In this study, NiV-G was expressed in yeast *Pichia pastoris* expression system. Yeast, as an eukaryote it offered the major advantage of posttranslational modification (Liu *et al.*, 2004). Many proteins that end up as an inactive inclusion bodies in bacterial system are produced as biologically active molecules in *P. pastoris* (Juozapaitis *et al.*, 2006). Besides, yeast expression system is generally regarded as faster, easier, and less expensive compared to the expression systems derived form higher eukaryotes, such as insect and mammalian tissue culture cell systems.

Chapter 2: Review of Literature

2.1 Outbreak of Nipah virus

An outbreak of encephalitis primarily affecting pig farmers had occurred during 1998-1999 in Malaysia (Chua *et al.*, 1999). 265 cases of severe febrile encephalitis were reported in Perak, Negeri Sembilan, and Selangor with 40% of fatality rate. By the end of May 1999, it was spread to the neighboring country, Singapore. 11 cases with 1 death had been reported among abattoir workers who slaughtered pigs which were imported from affected areas of Malaysia (Paton *et al.*, 1999).

In 2001, an outbreak of acute encephalitis had emerged in Siliguri (West Bengal) town of India between January 31 and February 23. A total of 66 probable cases with 45 deaths were reported (Harit *et al.*, 2006).

In Bangladesh, a few outbreak of acute encephalitis had emerged with 9 deaths were reported in Mehepur district in 2001 (Hsu *et al.*, 2004), 8 deaths were reported in Naogoan district in 2003 (Hsu *et al.*, 2004), 1 death was reported in Rajbari districts in 2004 (Quddus *et al.*, 2004), 27 deaths with 75% fatality rate were reported in Faridpur district in 2004 (Gurley *et al.*, 2007), 11 deaths with 92% fatality rate were reported in Tangail district in 2005 (ICDDR, 2005), and 8 deaths were reported in Rajbari and Manikgonj in 2008 (ICDDR, 2008).

2.2 Transmission of the disease

Originally, NiV was transmitted from fruit bats to domestic animals (pigs). The most likely initiating event was the shedding of the NiV in their saliva on the partial eaten fruit (Yob *et al.*, 2001). Pigs were infected when they contacted with the infective saliva. This was supported by the evidence during the Malaysian outbreak occurred in 1999. The affected farm was found to be located in close proximity with the tropical forest, habitat of the fruit bats (Chua *et al.*, 1999).

In the Malaysian outbreak, NiV was found to be transmitted from infected pig to human. Most of the infected individual was occupationally associated with pigs either as the pig farmers or abattoir workers (Paton *et al.*, 1999; Sahani *et al.*, 2001; Chan *et al.*, 2002) . According to Chong *et al.* (2003), the direct transmission of NiV from bats to human was low. This was due to the inadvertent direct contact with bats or bats' secretion. During the Malaysian outbreak, a person-to-person transmission of NiV was not documented (Mounts *et al.*, 2001).

Conversely, a person-to-person transmission was demonstrated during the outbreak of NiV in Faridpur District, Bangladesh in year 2004. Detection of NiV RNA on hospital surfaces indicated that the infected patients shed virus into the environment which provided an opportunity to transmit NiV to the others. The health workers and hospital visitors were infected after exposure to hospitalized patients with NiV illness (Gurley *et al.*, 2007). In contrast, the absent of person-to-person transmission in Malaysia and Singapore could be due to differences in patient care practices (Gurley *et al.*, 2007).

During the subsequent outbreak in Tangail District, Bangladesh in 2005, a direct transmission of NiV from bat to human was reported. Bats were attracted by the agriculture of foods that available seasonally and this had brought the bats into proximity with humans. The infective saliva and urine from the bats had contaminated the date palm sap and human was infected by drinking the fresh, raw date palm sap (Luby *et al.*, 2006).

2.3 Isolation of aetiological agent

A novel paramyxovirus, named as Nipah Virus was firstly isolated from cerebrospinal fluid of a patient from the village of Sungai Nipah in March 1999 (Chua *et al.*, 1999, Chua *et al.*, 2000). Subsequently, NiV was isolated from Malaysian Island flying-foxes and the serological evidence had suggested that it was one of the natural hosts for the NiV (Chua *et al.*, 2002). In 2004, NiV from experimental infected pigs was isolated and sequenced. The findings showed two possible origins of NiV that contributed to the Malaysia outbreaks from 1998 to 1999 (AbuBakar *et al.*, 2004).

Continuously, NiV had been isolated in Cambodia (Reynes *et al.*, 2005), Bangladesh (Harcourt *et al.*, 2005), Thailand (Wacharapluesadee *et al.*, 2005), and India (Chadha *et al.*, 2006). The NiV isolated from four human cases in Bangladesh had shown a significant NiV genetic heterogeneity. These datas suggested that there were multiple introductions of NiV into the population. These datas also suggested that viruses circulating in different areas have unique genetic signatures and these strains may have co-evolved within local natural reservoir.

2.4 Molecular characterization of Nipah virus

NiV had been characterized under the subfamily of Paramyxovirinae within the family Paramyxoviridae and genus of Henipavirus. The phylogenetic analysis for NiV illustrated that it does not group under any of the established genera of the subfamily Paramyxovirinae. Furthermore, the unique features of the NiV genome, the broad host range and antigenic cross-reactivity clearly indicates that NiV should be considered as a new genus within the subfamily of Paramyxovirinae (Harcourt *et al.*, 2000).

2.5 The origin of Nipah virus

Pterious bats served as the natural reservoir of NiV. This was proven by a serological diagnostic survey in bats with the demonstration of neutralizing antibodies against NiV-infection (Yob *et al.*, 2001). The virus was later isolated from urine and partial eaten fruit of flying floxes (*Pterious hypomelanus*)(Chua *et al.*, 2002). As a consequence of recent ecological changes such as deforestation, the natural habitat of the fruit bats had been destructed. This caused the migration of bats to live closer to human and domestic animals which facilitated the transmission of virus to new species. As a result, the zoonoses had become more pathogenic (Guillaume *et al.*, 2004a).

2.6 Strategy of prevention and treatment of Nipah virus infection

The outbreak of NiV poses a major health problem worldwide, which would destroy the economies of many countries. Therefore, there is a demand for rapid detection as well as serological diagnostic for the monitoring of the disease. Besides, the development of vaccine candidate is rather important to protect the high risk population such as BSL-4 staff and the first responder who may encounter the virus.

2.6.1 Development of animal model

The developments of animal models are important especially for newly-emerged viruses, to understand the pathology and pathogenesis of the disease. Primarily, Wong *et al.* (2003) had demonstrated that golden hamster was a suitable model for NiV infection. It produced a similar pathogenesis as in NiV-infected human. Further study indicated that hamster model was able to develop a neutralizing antibody following the recombinant NiV subunit immunization. However, both relapsing and late-onset cases of infection had not been observed in this model (Guillaume *et al.*, 2004a; Guillaume *et al.*, 2006). Subsequently, experimental NiV infections in cats were performed. The findings had indicated that cats provide a consistent model for acute NiV infection and associated pathogenesis (Mungall *et al.*, 2006). Recently, the preliminary study of Bossart *et al.* (2007) had suggested that ferret might be a potential study model for NiV infection.

2.6.2 Development of rapid diagnostic test

The high mortality rate which established in NiV infection had underlined the importance of rapid diagnostic test in wild, domestic animal, and human. However, the diagnostic tests were restricted by the limitation of BSL-4 facilities in most of the developing countries. Therefore, most of the researchers had developed a diagnostic system without BSL-4 facilities such as the rapid immune plaque assay, real-time RT-PCR assay and Enzyme-Linked ImmunoSorbent Assay (ELISA).

Initially, a rapid immune plaque assay had been developed by Crameri *et al.* (2001) for rapid detection and quantification of the NiV and anti-viral antibody. It involved NiV infection on monolayer Vera cell line from the biological samples (if present). The antigen in the infected cell was detected by antibodies generated by a bacteria-expressed portion of HeV P protein. However, the test was time consuming. It required more than 24 hours for the detection of syncytia formation in order to perform antibody testing. Besides, the test was not specific to detect the NiV since both NiV and HeV infection could not be differentiated. However, this assay was able to be performed in laboratories that do not have the BSL-4 facilities (Crameri *et al.*, 2001).

Continuously, a TaqMan™ real time RT-PCR of NiV nucleoprotein had been developed by Guillaume *et al.* (2004b). It has advantage over plaque assays where it provides rapid (few hours), quantitative and specific results. Importantly, TaqMan™ RT-PCR can be utilized as a differential diagnostic test between NiV and HeV infection as it is unable to detect Hendra virus DNA. Besides, the assay had been proven valid for monitoring the NiV in serum samples from infected animals (Guillaume *et al.*, 2004b).

A duplex nested RT-PCR (nRT-PCR) of NiV nucleoprotein had been developed by Wacharapluesadee *et al.* (2007). The sensitivity of the nRT-PCR was found to be higher than Nipah TaqMan™ assay as reported previously (Guillaume *et al.*, 2004b). The findings from the study had found that nRT-PCR assay was suitable for NiV surveillance in the wild, domestic animals and for diagnostic of human disease.

In 2004, a solid-phase blocking ELISA utilizing monoclonal antibody (MAb) specific to NiV had been developed for detection of antibodies to NiV. It shows no cross-reaction with HeV. Therefore the test was specific. Furthermore, it involved quick and simple procedure compared to the existing indirect ELISA (Kashiwazaki *et al.*, 2004).

In advance, a recombinant protein for NiV-nucleoprotein (Eshaghi *et al.*, 2005; Juozapaitis *et al.*, 2006; Yu *et al.*, 2006), NiV-glycoprotein (Wang *et al.*, 2006b), and NiV-fusion protein (Wang *et al.*, 2006b) had been expressed in different systems for the development of a recombinant protein-based ELISA diagnostic test. The recombinant protein-based ELISA was found to be more sensitive to the inactivated-virus-based ELISA (Yu *et al.*, 2006). In addition, it is a safer method for diagnostic since it does not involve inactivation of a live virus. It eliminates the requirement of a BSL-4 laboratory during the diagnostic preparation. Furthermore, the production of recombinant protein is rapid and cost-effective. Therefore, this method is especially useful in cases of large-scale epidemiological investigations, as well as in developing countries where high-security laboratory are not available.

2.6.3 Development of vaccine (animal)

The development of vaccine is useful in preventing the transmission of disease in future NiV outbreak. Additionally, vaccination on the high risk groups is rather important to protect them from the lethal infection. Up to date, a vaccine candidate had been successfully developed in swine. Besides, some animal trial had been performed in hamster, mice and cat model.

A recombinant vaccinia virus-based vaccine encoding NiV-F (VV-NiV.F) and NiV-G (VV-NiV.G) glycoprotein was firstly developed in golden hamster model (Guillaume *et al.*, 2004a). In this study, co-infection of VV-NiV.F and VV-NiV.G was found to protect the animal model against NiV challenge. It obtained high antibody levels but relatively low neutralizing antibodies. In contrast, a higher titer of neutralizing antibodies was found in the mice which had been vaccinated with a different strain of vaccinia virus, wild-type (WT) vaccinia (WR strain) (Tamin *et al.*, 2002). However, WR virus was much more virulent and grew to high titers in several organs.

Vaccination with a combination of recombinant canarypox virus-based vaccine encoding NiV-G glycoprotein and F fusion protein (ALVAC-F/G) had successfully protected pigs from NiV challenge (Weingartl *et al.*, 2006). The sera from the ALVAC-F/G-vaccinated animals showed a moderate neutralizing activity against the related HeV. These findings had gained more information regarding the immunogenicity of the vaccine. It proved the possibility to protect the NiV infection with a single vaccine.

A subunit vaccine formulation containing recombinant, soluble, and attachment glycoprotein from HeV (sG_{HeV}) and CpG adjuvant was found to be able to protect the cat models from NiV lethal infection (McEachern *et al.*, 2008). A CpG adjuvant had been employed to stimulate mucosal and Th1 immunity. The combination of sGHeV and CpG had induced a sufficient level of antigen-specific plasma Ig, neutralizing antibodies and anti-specific mucosal IgA.

Recently, a NiV G and F gene had been constructed in the mammalian expression vector pCAGGS under chicken beta-actin promoter. The boosting of pCAGG-NiV-F and pCAGG-NiV-G expression vector into six-week-old female mice (BALB/c) had stimulated the production of a specific antibody. Furthermore, this antibody was able to neutralize the infection of VSVdeltaG*F/G (NiV G and F envelope glycoproteins pseudotyped recombinant vesicular stomatitis virus expressing green fluorescence protein). Thus, DNA vaccination of NiV-F/G could be an efficient vaccine strategy against NiV infection (Wang *et al.*, 2008).

Chapter 3: Objective

The main aims of works that had been carried out were:-

1. To amplify a truncated NiV-G gene named as GA gene.
2. To propagate the recombinant plasmid using conventional transformation method in bacterial system.
3. To express the GA gene in yeast expression system.
4. To demonstrate the presence of recombinant protein by mean of SDS-PAGE and Western Blot

Chapter 4: Experimental Overview

The flow chart of experimental overview in this study was shown as below.