UNIVERSITI PUTRA MALAYSIA

IMMUNOGENICITY OF SOLUBLE ENTEROVIRUS 71 RECOMBINANT VP1 PROTEIN IN MICE MODEL

SUHAILI MUSTAFA

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IMMUNOGENICITY OF SOLUBLE ENTEROVIRUS 71 RECOMBINANT VP1 PROTEIN IN MICE MODEL

By

SUHAILI MUSTAFA

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IMMUNOGENICITY OF SOLUBLE ENTEROVIRUS 71 RECOMBINANT VP1 PROTEIN IN MICE MODEL.

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SUHAILI MUSTAFA

January 2017

Chairman: Norazizah Shafee, PhD
Faculty: Biotechnology and Biomolecular Sciences

Enterovirus 71 (EV71) is one of the causative agents of the hand, foot and mouth disease (HFMD) in human. Outbreaks of HFMD may lead to severe pathogenesis in young children and infants worldwide. Children below 5 years of age are more susceptible to severe forms of HFMD which are associated with neurological complications. In adults, EV71 infections are mild and uneventful. Despite the threat to children worldwide, there is still no effective vaccine available that can really prevent this disease. Development of an effective vaccine towards EV71 infection is obstructed by the lack of suitable candidates. To date, various type of candidate vaccines are being studied and evaluated. They include inactivated whole-viruses, attenuated viruses, virus-like particles, subunit proteins, and DNA vaccines. However, the most promising of them are in the form of inactivated whole virus. The risk of using the inactivated whole virus as a vaccine is a potential for incomplete inactivation, resulting in the vaccinee receiving live virulent virus. To address this issue, alternative vaccine candidates have been developed. They include subunit viral proteins that served as the immunogens. Viral protein 1 (VP1), the capsid protein of EV71, has a higher degree of antigenicity compared to its other structural proteins. It also serves as a major viral neutralization determinant. These properties of VP1 have made it an ideal target in the development of EV71 vaccine. It is located in its C-terminal region. Therefore, peptides corresponding to these regions will be ideal candidates for EV71 vaccine development. In the present study a recombinant construct of *NPt-VP1*$_{198-297}$ *(NPt-VP1t)* were cloned and expressed in *E. coli* systems. The protein was expressed as a fusion with the Nucleocapsid protein of Newcastle disease virus (NDV). Fusion to the carrier molecule was done to ensure that the protein construcs can serve as strong immunogens when it is tested in subsequent *in vivo* studies. Despite the hydrophilic properties of the VP1 peptide, as well as their carrier molecule, the recombinant proteins were expressed mostly in the insoluble fractions of bacterial lysates. The soluble form of NPt-VP1t was only around 7% of the total protein. Despite its insoluble property, moderate

immunogenicity in animal studies was seen. Temperature adaptation was found to only minimally affect their solubility. In the present study, it was hypothesized that a soluble form of NPt-VP1t recombinant protein will increase its immunogenicity. Therefore, the main objective of the current study was to vaccinate the mice with the soluble fraction of the recombinant protein and evaluate the immune response produced. Following vigorous parameter testing, the NPt-VP1t was successfully produced in a soluble form. From the immunization study, this protein was found to have an increased immunogenicity in adult mice. In adult mice, the protein induced high levels of anti-VP1 IgG production. Purified VP1 antigen also stimulated activation, proliferation and differentiation of splenocytes harvested from the immunized mice. They also produced high levels of IFN-γ and IL-6 cytokines. Results obtained from this study contribute towards a better understanding of the NPt-VP1t recombinant protein as a candidate vaccine in EV71 infections.

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KEIMUNOGENAN PROTEIN TERLARUT ENTEROVIRUS 71 VP1 PROTEIN DALAM MODEL TIKUS

Oleh

SUHAILI MUSTAFA

Januari 2017

Pengerusi: Norazizah Shafee, PhD
Fakulti: Bioteknologi dan Sains Biomolekul

Enterovirus 71 (EV71) adalah salah satu agen penyebab kepada penyakit tangan, kaki dan mulut (HFMD) pada kebiasaanya yang menjangkiti manusia. Wabak HFMD boleh mengakibatkan patogenesis yang teruk kepada kanak-kanak dan bayi di seluruh dunia. Kanak-kanak yang berumur 5 tahun dan ke bawah adalah lebih mudah dijangkiti dengan penyakit tangan kaki dan mulut ini dan kebiasaanya sering dikaitkan dengan komplikasi neurologi. Manakala dalam kalangan orang dewasa pula, jangkitan EV71 hanya menyebabkan jangkitan yang ringan dan tidak memudaratkan. Walaupun penyakit ini mengancam kesihatan kanak-kanak di seluruh dunia, malangnya sehingga kini masih belum ada vaksin yang betul-beul berkesan untuk mencegah penyakit ini. Pembangunan vaksin yang berkesan terhadap jangkitan EV71 dikekang oleh kekurangan calon yang sesuai. Sehingga kini, pelbagai jenis vaksin sedang dikaji dan dinilai. Ia termasuk seluruh-virus yang dilemahkan, virus teratenuat, zarah seperti virus (VLP), protein subunit, dan DNA vaksin. Walau bagaimanapun, kebanyakannya adalah dalam bentuk virus yang dilemahkan ataupun dinyahaktifkan. Risiko menggunakan seluruh virus yang dinyahaktif sebagai vaksin mempunyai potensi di mana virus tersebut tidak dinyahaktif sepenuhya, keadaan ini boleh menyebabkan orang yang menerima pemvaksinan akan menerima virus yang masih aktif. Untuk menangani isu ini, alternatif vaksin telah dibangunkan. Ia termasuk subunit virus protein yang bertindak sebagai imunogen. Protein Virus 1 (VP1), ialah kapsid protein daripada EV71, mempunyai tahap keantigenan yang lebih tinggi berbanding struktur protein yang lain. Ia juga berfungsi sebagai penentu peneutralan utama virus. Ciri-ciri daripada VP1 ini telah menjadikannya sebagai sasaran yang ideal dalam pembangunan vaksin EV71. Ia terletak di kawasan terminal-C. Oleh itu, peptida yang sepadan dengan kawasan-kawasan ini akan menjadi calon yang ideal untuk pembangunan vaksin EV71. Dalam kajian sebelum ini, 100 asid amino daripada VP1fl telah dipendekkan dan dicantumkan dengan nukleokapsid protein daripada virus penyakit Newcastle (NDV). Pencantuman kepada molekul pengangkut dilakukan untuk memastikan bahawa konstrak protein boleh berfungsi sebagai imunogen yang kuat apabila diuji dalam

kajian *in-vivo*. Konstruk rekombinan daripada *NPt-VP1*$_{198-297}$ *(NPt-VP1t)* telah diklon dan diekspres dalam sistem *E. coli*. Walaupun peptida VP1 mempunyai sifat-sifat hidrofilik, sama juga dengan molekul pembawa, protein rekombinan telah mengekspres kebanyakannya dalam pecahan tidak larut lisat bakteria. Hanya sekitar 7% daripada jumlah protein adalah dalam bentuk larut NPt-VP1t. Walaupun tidak larut, keimunogenan sederhana telah dilihat dalam kajian haiwan. Penyesuaian suhu didapati hanya mempengaruhi kelarutan protien pada kadar yang minimum sahaja. Dalam kajian ini, ia telah hipotesiskan bahawa bentuk larut NPt-VP1t protein rekombinan akan meningkatkan kadar keimunogenan. Oleh itu, objektif utama dalam kajian semasa adalah untuk memvaksin tikus dengan pecahan larut protein rekombinan dan menilai tindak balas imun yang dihasilkan. Selepas beberapa ujian parameter, NPt-VP1t telah berjaya dihasilkan dalam bentuk larut. Daripada kajian imunisasi, protein ini didapati telah meningkatkan kadar keimunogenan dalam tikus dewasa. Dalam tikus dewasa, protein ini telah mengaruhkan tahap pengeluaran IgG anti-VP1 yang tinggi. Antigen VP1 tulen juga merangsang pengaktifan, percambahan dan pembezaan splenosit yang dituai dari tikus yang telah diimunisasi. Mereka juga menghasilkan IFN-γ dan IL-6 yang tinggi jika dibandingkan dengan tikus yang belum menerima sebarang imunisasi. Kesimpulannya, keputusan yang diperolehi daripada kajian ini menyumbang ke arah pemahaman yang lebih baik mengenai protein rekombinan NPt-VP1t sebagai calon vaksin dalam jangkitan EV71.

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I certify that a Thesis Examination Committee has met on 12 January 2017 to conduct the final examination of Suhaili bin Mustafa on his thesis entitled "Immunogenicity of Soluble Enterovirus 71 Recombinant VP1 Protein in Mice Model" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

Members of the Thesis Examination Committee were as follows:

Janna Ong binti Abdullah, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Mohd. Yunus bin Abd. Shukor, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Siti Aqlima binti Ahmad, PhD
Senior Lecturer
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Mali Wirotesangthong, PhD
Associate Professor
Chulalongkorn University
Thailand
(External Examiner)

NOR AINI AB. SHUKOR, PhD
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 28 February 2017

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

Norazizah Shafee, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Science
Universiti Putra Malaysia
(Chairman)

Khatijah Yusoff, PhD
Professor
Faculty of Biotechnology and Biomolecular Science
Universiti Putra Malaysia
(Member)

Muhajir Hamid, PhD
Associated Professor
Faculty of Biotechnology and Biomolecular Science
Universiti Putra Malaysia
(Member)

ROBIAH BINTI YUNUS, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

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Signature: _____
Name of Chairman of Supervisory Committee: Norazizah Shafee

Signature: _____
Name of Member of Supervisory Committee: Khatijah Yusoff

Signature: _____
Name of Member of Supervisory Committee: Muhajir Hamid

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LIST OF ABBREVIATIONS

AFP	Acute flaccid paralysis
Anti-His	Anti-Histidine antibody
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
bp	Base pair
BSA	Bovine serum albumin
CA16	Coxsackievirus A16
ConA	Concanavalin A
dNTP	Deoxyribonucleoside triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EV71	Enterovirus 71
F	Fusion protein
GSH	Reduced glutathione
GSSG	Glutathione disulfide
h	Hour
HBcAg	Hepatitis B core antigen
HFMD	Hand, foot and mouth disease
His	Histidine
HN	Heamagglutinin neuramiridase protein
IPTG	Isopropyl-β-D-thiogalactoside

IP	Insoluble Protein of NPt-VP1t
kb	Kilo base pair
kDa	Kilo Dalton
LB	Luria-Bertaini
min	Minute
MWCO	Molecular Weight Cut Off
NBT	Nitro Blue Tetrazolium
NDV	Newcastle disease virus
Ni^{2+}	Nickel ion
NP	Nucleocapsid protein
NPfl	Full length nucleocapsid protein
NPt	Truncated nucleocapsid protein
NPt-VP1t	Truncated NP fused with truncated VP1
O.D.	Optical density
PBS	Phosphate buffer saline
PCR	Polymerase Chain Reaction
PMSF	Phenylmethylsulfonyl fluoride
PVDF	Polyvinyl difluoride
rpm	Revolutions per minute
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec	Second
SP	Soluble expressed NPt-VP1t

TAE	Tris-acetate-EDTA buffer
TP	Total protein of NPt-VP1t
TBS	Tris-buffered saline
TEMED	N,N,N',N'-Tetramethyl-ethane-1,2-diamine
UTR	Untranslated region
VLP	Virus-like particle
VP1	Viral protein 1
$VP1_{198-297}$	Amino acid 198 to 297 of VP1
VP1fl	Full length Viral protein 1
x g	Relative centrifugal force

CHAPTER 1

INTRODUCTION

Human enterovirus 71 (EV71) belongs to the *Picornaviridae* family and has been noted as one of the causative agents of hand, foot and mouth disease (Chumakov *et al.,* 1979; Nagy *et al.,* 1982; Samuda *et al.,* 1987). EV71 infection is associated with a higher death rate and is primarily responsible for fatalities and outbreaks in Southeast Asia. The Enterovirus 71 (EV71) is a single-stranded and positive-sense RNA virus. The virus enters the host via fecal-oral route through contaminated food or water. The virus infection may lead to severe pathogenesis predominantly in children below five years old and immunocompetent adult. It may also cause mild symptom in the adult. In children the severe pathogenesis, such neurological complications including meningitis, encephalitis and acute flaccid paralysis maybe found. Due to its increased threat to humans, EV71 has emerged as an important enterovirus to be studied (Da Silva *et al.*, 1996). Since its first identification in 1969 (Schmidt *et al.,* 1974), EV71 epidemics have been reported periodically worldwide, with an increased incidence of fatal cases in the Asia-pacific regions. The outbreak in Cambodia in 2012 reported that almost 60 children died during the infection (Ministry of Health, Cambodia press release, 8 July 2012). A number of recurrence infections were detected throughout Asia-pacific region, but no large outbreak was reported. Based on the statistics issued by the National Health and Family Planning Commission of China in 2008 until 2013, more than 9 million cases of HFMD were reported, and 2,700 numbers of deaths was reported. From 2008 to 2012, around 80% of the severe cases were also reported and more than EV71 was caused 90% of fatal cases. (Li *et al.,* 2014; Liu *et al.,* 2015). In Malaysia, the largest incident of EV71 outbreak was in 1997, in the state of Sarawak (Chan *et al.,* 2000) where 29 children below the age of six years old died. From 1997 to 2000 the same incident was occurred in peninsular Malaysia just few deaths was reported (Lum *et al.,* 1998; Herrero *et al.,* 2003). According to the Outbreak News Today June, 18 2016, during the first full week of June, health officials note (computer translated) that the number of HFMD cases reported nationwide that week was 1,379 cases, an increase of 83 cases (6.4%) compared to the previous week (1,296 cases). In May, the Ministry of Health reported the number of cases exceeded the "threshold" of 644 cases per week, prompting an alert to be issued. Selangor contributed the highest number of cases to date compared to other states that 4,441 cases (32.6%), followed by Johor 1,393 cases (10.2%), Kuala Lumpur (WPKL) 1,317 cases (9.7%), Sabah 1,299 cases (9.5%) and Sarawak 1,108 cases (8.1%).

Unfortunately to this date, there is still no effective vaccine or drugs available against EV71 infection (Shih *et al.,* 2000; Racaniello, 2001; McMinn, 2002; Knowles *et al.,* 2011). Just a few preventive measures have been implemented to reduce and control the number of infections. Personal and environmental hygiene as well as avoidance of contact between infected and uninfected individuals are the only means to control the transmission and spread of the virus. The high threat and number of the infection in young children was made the vaccine development has become the main focus of the current EV71-related research. Many types of candidate vaccines are being evaluated.

They include inactivated whole-virus, attenuated virus, virus-like particles, subunit EV71 proteins, and DNA vaccines (Zhang *et al.,* 2010). In December 2015, the China Food and Drug Administration (CFDA) approved the 1st vaccine against EV71, an inactivated (killed) vaccine made by the Institute of Medical Biology at the Chinese Academy of Medical Sciences (Wang *et al.,* 2016). The vaccine developed by the institute showed vaccine efficacy of 97.4% (95% confidence interval, 92.9% - 99.0%) (Zhou *et al.,* 2016). A 2nd vaccine, also an inactivated vaccine, developed by Sinovac Biotech Ltd was approved January 2016. The vaccine developed by Sinovac showed a 97.5% vaccine efficacy (6 month) and a 94.8% vaccine efficacy (12 month) against EV71-associated HFMD cases and 89.3% (6 month) and 88.0% (12 month) for EV71-associated cases (Mao *et al.,* 2016). The potential of having the virulent revertant virus of live attenuated vaccines (Lin *et al.,* 2002) has made protein-based subunit EV71 vaccine a more favorable choice for vaccine development. Subunit vaccines are much safer than the inactivated and attenuated vaccines as they cause fewer adverse effects. The isolated subunit protein in a proper conformation can possess neutralizing epitopes. Therefore, viral protein 1 (VP1), the capsid protein of EV71, has a higher degree of antigenicity compared to its other structural proteins (Oberste *et al.,* 1999). It also serves as a major viral neutralization determinant. All of these properties made VP1 of EV71 an ideal target in the development of vaccines.

In a previous study, a few recombinant vaccines construct was developed. They are including first 100 amino acid of the N-terminal of VPI ($VP1_{1-100}$) (Ch'ng *et al.,* 2011a; Ch'ng *et al.,* 2011b; Sivasamugham *et al.,* 2006) and few construct from C-terminal of VP1. All of the constructs was fused into truncated Nucleocapsid protein (NPt), derived from the Newcastle disease virus as a carrier molecule. Out of these constructs one of the construct was gave a promising result. The construct was labeled as NPt-$VP1_{198-297}$ (NPt-VP1t). Despite it hydrophilic properties, the recombinant vaccine shows mostly expressed in insoluble form. Even though insoluble, results obtained shows that the recombinant protein exhibited strong immunogenic in mice. In this current study, it was hypothesized that soluble fraction of the recombinant protein will induced higher immune response in mice.

Therefore, the main objective of the current study is to investigate the immunogenicity of soluble recombinant NPt-VP1t protein in mice model. To achieve the main objective several specific aims were designed.

Specific aims of the study are:

1. To confirm the integrity of the recombinant *NPt-VP1t* gene in the pTrcHis2 expression plasmid.

2. To optimize nutritional and environmental parameters for NPt-VP1t protein expression.

3. To evaluate the different lysis methods for NPt-VP1t solubility.

4. To examine the immunogenicity of the soluble NPt-VP1t protein in adult mice.

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