

**IMMUNOGENICITY STUDY OF DNA VACCINE AND
DNA VACCINE CARRIER EXPRESSING VP1 OF ENTEROVIRUS
71 IN THE PRIME BOOST VACCINATION STRATEGY**

NUR AYUNI KADIR

**Thesis submitted in fulfillment of the
requirements for the degree
of Master of Science**

UNIVERSITI SAINS MALAYSIA

2008

DEDICATIONS

**This thesis is specially dedicated to my beloved husband,
Nik Muhammad Nasuha Nik Razin,
Also to my parents, brothers and sisters,
And finally My Heart, my lovely son,
Nik Amir Mursyid**

**Thank you for your love and support
I love u all.**

ACKNOWLEDGEMENTS

Bismillahirrahmanirrahim

In the name of Allah, the most Generous and the most Merciful. All praise is due to Allah and Syukur Alhamdulillah in this golden moment Allah has given me an opportunity to accomplish my effort to obtain the degree of Master of Science.

During this research project, there are several people involved directly and indirectly thereby I would like to acknowledge them and express my deepest appreciations.

I would like to thank my supervisor, Professor Zainul F. Zainuddin for giving me an opportunity to pursue my postgraduate study under his guidance and supervision. My appreciation also goes to my co-supervisor, Prof Madya Dr. Mustaffa Musa for his excellent guidance and helpful comments throughout this study. I have learned a lot from both of them especially their commitment and dedication which really inspire me to the career in academia and research. May Allah bless both of them.

My greatest appreciation goes to my beloved husband- Nik Muhammad Nasuha Nik Razin. Thank you for your love, thank you for always being there for me and supports me whenever I need it. My special gratitude also goes to my parents, parents' in-law and all my brothers and sisters. My lovely son Nik Amir Mursyid – you have given me strength and passions to be the best Umami and I love u, My Heart.

Finally I wish to express my special thanks to my friends and colleagues especially Nurul, Aniek, Azura, Tini, Bad, Eza, Abdah, Kak Wana, Kak Maryam , and NMN group. Thanks a lot for your help, guidance and friendship. I also would like to thank all lecturers and staff of Immunology Department and PPSK.

ACKNOWLEDGEMENTS

Bismillahirrahmanirrahim

In the name of Allah, the most Generous and the most Merciful. All praise is due to Allah and Syukur Alhamdulillah in this golden moment Allah has given me an opportunity to accomplish my effort to obtain the degree of Master of Science.

During this research project, there are several people involved directly and indirectly thereby I would like to acknowledge them and express my deepest appreciations.

I would like to thank my supervisor, Professor Zainul F. Zainuddin for giving me an opportunity to pursue my postgraduate study under his guidance and supervision. My appreciation also goes to my co-supervisor, Prof Madya Dr. Mustaffa Musa for his excellent guidance and helpful comments throughout this study. I have learned a lot from both of them especially their commitment and dedication which really inspire me to the career in academia and research. May Allah bless both of them.

My greatest appreciation goes to my beloved husband- Nik Muhammad Nasuha Nik Razin. Thank you for your love, thank you for always being there for me and supports me whenever I need it. My special gratitude also goes to my parents, parents' in-law and all my brothers and sisters. My lovely son Nik Amir Mursyid – you have given me strength and passions to be the best Ummi and I love u, My Heart.

Finally I wish to express my special thanks to my friends and colleagues especially Nurul, Aniek, Azura, Tini, Bad, Eza, Abdah, Kak Wana, Kak Maryam , and NMN group. Thanks a lot for your help, guidance and friendship. I also would like to thank all lecturers and staff of Immunology Department and PPSK.

ACKNOWLEDGEMENTS

Bismillahirrahmanirrahim

In the name of Allah, the most Generous and the most Merciful. All praise is due to Allah and Syukur Alhamdulillah in this golden moment Allah has given me an opportunity to accomplish my effort to obtain the degree of Master of Science.

During this research project, there are several people involved directly and indirectly thereby I would like to acknowledge them and express my deepest appreciations.

I would like to thank my supervisor, Professor Zainul F. Zainuddin for giving me an opportunity to pursue my postgraduate study under his guidance and supervision. My appreciation also goes to my co-supervisor, Prof Madya Dr. Mustaffa Musa for his excellent guidance and helpful comments throughout this study. I have learned a lot from both of them especially their commitment and dedication which really inspire me to the career in academia and research. May Allah bless both of them.

My greatest appreciation goes to my beloved husband- Nik Muhammad Nasuha Nik Razin. Thank you for your love, thank you for always being there for me and supports me whenever I need it. My special gratitude also goes to my parents, parents' in-law and all my brothers and sisters. My lovely son Nik Amir Mursyid – you have given me strength and passions to be the best Ummi and I love u, My Heart.

Finally I wish to express my special thanks to my friends and colleagues especially Nurul, Aniek, Azura, Tini, Bad, Eza, Abdah, Kak Wana, Kak Maryam , and NMN group. Thanks a lot for your help, guidance and friendship. I also would like to thank all lecturers and staff of Immunology Department and PPSK.

TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS	i
TABLE OF CONTENTS	ii
LIST OF TABLES	vi
LIST OF FIGURES	vii
LIST OF ABBREVIATIONS	viii
ABSTRAK	x
ABSTRACT	xii
CHAPTER ONE: INTRODUCTION	
1.1 Introduction to Enterovirus 71 (EV71)	1
1.2 Diseases due to EV71	3
1.2.1 HFMD and herpangina	3
1.2.2 Neurological disease	3
1.3 Epidemiology of EV71	4
1.4 Pathogenesis of EV71 infection	6
1.5 EV71 molecular genetics of virulence	7
1.5.1 5' UTR	7
1.5.2 VP1 gene and protein	8
1.6 Immunity to EV71 infection	10
1.6.1 Innate immunity	10
1.6.2 Adaptive immunity	11
1.6.2.1 Organs of the immune system	11
1.6.2.2 Cell-mediated immunity	13
1.6.2.3 Humoral immunity	16
1.6.2.4 Mucosal immunity	16
1.7 Control of EV71 infection by antiviral agents	17
1.8 Vaccine development against EV71 infection	18
1.8.1 Inactivated and live attenuated viral vaccine	19
1.8.2 Subunit vaccines	19
1.9 DNA vaccine	20
1.9.1 Mechanisms of DNA vaccination	22
1.9.2 Enhanced efficiency of DNA vaccine	26
1.9.2.1 Intracellular bacteria as delivery system for DNA vaccine	26

1.9.2.2	Ubiquitination	29
1.9.2.3	Prime boost vaccination approach	30
1.10	Rationale and objectives of the study	33

CHAPTER TWO: MATERIALS AND METHODS

2.1	Materials	
2.1.1	Bacterial strains	35
2.1.2	Plasmids and vectors	35
2.1.3	Chemicals and reagents	36
2.1.4	Kits and consumables	38
2.1.5	Antibodies, enzymes and laboratory equipments	39
2.1.6	Mice	43
2.1.7	Sterilised, deionised distilled water	43
2.1.8	Media for bacterial culture	43
2.1.8.1	Luria Bertani (LB) Broth	43
2.1.8.2	Luria Bertani Agar (LB agar)	43
2.1.8.3	Tryptic Soy Broth (TSB)	44
2.1.8.4	Tryptic Soy Agar (TSA)	44
2.1.9	Buffers and solutions	
2.1.9.1	2,2'-Azinodi 3-Ethyl Benzthiazoline Sulfonic (ABTS)	44
2.1.9.2	ACK lysis buffer (6 x)	45
2.1.9.3	Assay diluent	45
2.1.9.4	Calcium chloride (CaCl ₂ , 100 mM)	45
2.1.9.5	Coating buffer	45
2.1.9.6	Complete RPMI medium	45
2.1.9.7	Concanavalin A (Con A, 10 mg/ml)	46
2.1.9.8	Ethidium bromide (EtBr, 10 mg/ml)	46
2.1.9.9	Glycerol (80%)	46
2.1.9.10	Kanamycin (50 mg/ml)	46
2.1.9.11	Magnesium chloride (MgCl ₂ , 10 mM)	46
2.1.9.12	methyl-[³ H]-Thymidine solution	46
2.1.9.13	Perm/Wash Buffer	47
2.1.9.14	Phosphate Buffered Saline (PBS)	47
2.1.9.15	Phosphate Buffered Saline-Tween 20 (PBS-T20)	47
2.1.9.16	Sodium bicarbonate (NaHCO ₃ , 3%)	47

2.1.9.17	Staining buffer for flow cytometric analysis	47
2.1.9.18	Stop solution	48
2.2	Methods	
2.2.1	Agarose gel electrophoresis	48
2.2.1.1	Preparation of agarose gel	48
2.2.1.2	Loading samples in agarose gel	48
2.2.1.3	Gel running and DNA visualization	48
2.2.1.4	Estimation of the size of the DNA fragments	49
2.2.2	Preparation of competent cells by CaCl ₂ method	49
2.2.3	Transformation of DNA into competent cells	50
2.2.4	Extraction of plasmid DNA from bacteria	50
2.2.5	Determination of the DNA concentration	52
2.2.5.1	Determination of DNA concentration using UV spectroscopy	52
2.2.5.2	Determination of DNA concentration using agarose gel	52
2.2.6	Restriction enzyme (RE) digestion	52
2.2.7	Long term storage of DNA products	53
2.2.8	Immunogenicity study	
2.2.8.1	Preparation of vaccine candidates for mice immunization	53
2.2.8.1.1	VP1 plasmid DNA vaccine (pVaxUbVP1)	53
2.2.8.1.2	VP1 DNA vaccine carrier (StUbVP1)	54
2.2.8.2	Immunization of mice	55
2.2.8.3	Blood collection for the determination of total serum IgG and IgG subclasses	59
2.2.8.4	Mice sacrifice and splenocytes preparation	60
2.2.8.5	Lymphocyte transformation test (LTT)	61
2.2.8.6	Measurement of extracellular cytokines (IFN- γ and IL-4) production by ELISA	62
2.2.8.7	Multicolour cell surface antigens and intracellular cytokines staining by flow cytometry	64
2.2.9	Statistical analysis	66

CHAPTER THREE: RESULTS

3.1	Transformation of pVaxUbVP1 into <i>S. typhi</i> Ty21a to produce DNA vaccine carrier (StUbVP1)	68
3.2	Immunogenicity studies of the vaccine candidates in mice	70
3.2.1	Total IgG and IgG subclasses production after immunization of mice with the vaccine candidates	70
3.2.2	Proliferative response of splenocytes after immunization of mice with the vaccine candidates	72
3.2.3	Extracellular cytokines (IFN- γ and IL-4) production after immunization of mice with the vaccine candidates	75
3.2.4	Intracellular cytokines (IFN- γ , IL-2 and IL-4) production in CD4 ⁺ and CD8 ⁺ T-cells from mice immunized with the vaccine candidates	78

CHAPTER FOUR: DISCUSSION AND CONCLUSIONS 86

REFERENCES 97

APPENDICES 109

LIST OF TABLES

		Page
Table 1.1	The major cell-mediated and humoral immune responses to viral infections.	12
Table 2.1	List of bacterial strains.	35
Table 2.2	List of plasmids and vectors.	35
Table 2.3	List of general chemicals and reagents.	36
Table 2.4	List of kits and consumables.	38
Table 2.5	List of conjugated antibodies.	39
Table 2.6	List of enzymes.	40
Table 2.7	List of laboratory equipments.	40
Table 2.8	Immunization schedules of the mice in Format A-D.	58
Table 2.9	Classification of responses based on the fold increase in the percentage of cells expressing selected intracellular cytokines by flow cytometry	67
Table 3.1	The distribution of specific anti-rUbVP1 IgG subclasses in immunized mice.	73
Table 3.2	T cell responses of selected cytokines as determined by flow cytometry analysis.	85

LIST OF FIGURES

	Page
Figure 1.1	Genome structure of EV71. 2
Figure 1.2	Partial alignment of VP1 deduced amino acid sequences. 9
Figure 1.3	CD4 ⁺ T helper lymphocyte subsets. 15
Figure 1.4	Mechanisms of generation of antigen specific humoral and cell mediated immune response. 21
Figure 1.5	Mechanisms of APCs in the induction of immunity against plasmid-encoded antigen. 23
Figure 1.6	Mechanisms of DNA vaccine delivery by attenuated intracellular bacteria followed by antigen processing and presentation. 28
Figure 1.7	Prime-boost vaccination strategies synergistically amplify T cell immunity to specific antigens. 31
Figure 1.8	Research flow for the immunogenicity study of DNA vaccine (pVaxUbVPI) and live attenuated <i>S. typhi</i> Ty21a as a delivery vehicle for DNA vaccine (StUbVPI). 34
Figure 2.1	pVAX1 plasmid map. 41
Figure 2.2	Plasmid map of previously constructed DNA vaccine (pVaxUbVP1). 41
Figure 2.3	The sequence of UbVP1 gene. 42
Figure 3.1	1% agarose gel electrophoresis analysis of plasmids from <i>S. typhi</i> Ty21a transformants. 69
Figure 3.2	Analysis of total IgG antibody level against purified rUbVP1 protein. 71
Figure 3.3	Stimulation index of splenocytes of mice 74
Figure 3.4	The concentration of IFN- γ secretion in supernatants from cultured splenocytes of mice. 76
Figure 3.5	The concentration of IL-4 secretion in supernatants from cultured splenocytes of mice. 77
Figure 3.6	Percentage of intracellular IFN- γ expression in A) CD4 ⁺ T cells and B) CD8 ⁺ T cells. 79
Figure 3.7	Percentage of intracellular IL-2 expression in A) CD4 ⁺ T cells and B) CD8 ⁺ T cells. 81
Figure 3.8	Percentage of intracellular IL-4 expression in (A) CD4 ⁺ T cells and (B) CD8 ⁺ T cells. 83

LIST OF ABBREVIATIONS

3' UTR	3' untranslated region
5' UTR	5' untranslated region
Abs	Absorbance
AFP	Acute flaccid paralysis
A	Alanine
ADCC	Antibody-dependent cell mediated cytotoxicity
APC	Antigen presenting cells
CA16	Coxsackie A16
CNS	Central nervous system
Con A	Concanavalin A
CTL	Cytotoxic T-cells
ddH ₂ O	Deionised distilled water
DNA	Deoxyribonucleic acid
DC	Dendritic cells
EV71	Enterovirus 71
GFP	Green fluorescent protein
HFMD	Hand foot and mouth disease
HA	Herpangina
HMEC-1	Human endothelial cell line
HIV	Human immunodeficiency virus
Ig	Immunoglobulin
IFN	Interferon
IL	Interleukin
IRES	Internal ribosome entry site
i.m	Intramuscular
IVIG	Intravenous immunoglobulin
LC	Langerhans cell
LPS	Lipopolysaccharide
MCP-1	Macrophage chemo-attractant protein-1
MIF	Macrophage migration inhibition factor
MR	Magnetic resonance
MHC	Major histocompatibility complex
NK	Natural killer
O.D	Optical density

ORF	Open reading frame
RD	Rhamdomyosarcoma
PBS	Phosphate buffered saline
PE	Pulmonary edema
PMN	Polymorphonuclear neutrophils
RE	Restriction enzyme
RNA	Ribose nucleic acid
RT	Room temperature
SI	Stimulation index
Ub	Ubiquitin
UV	Ultra violet
V	Valine
Th	T-helper cells

KAJIAN IMUNOGENISITI VAKSIN DNA DAN VAKSIN DNA PEMBAWA YANG MENGEKSPRES VP1 DARI ENTEROVIRUS 71 DENGAN STRATEGI VAKSINASI 'PRIME-BOOST'

ABSTRAK

Enterovirus 71 (EV71) ialah agen penyebab bagi penyakit tangan, kaki dan mulut pada kanak-kanak dan boleh mendatangkan komplikasi saraf yang serius. Di Malaysia, wabak pertama jangkitan EV71 berlaku pada tahun 1997 di Sarawak dan menyebabkan 34 kes kematian berpunca daripada sindrom saraf. Sehingga kini masih tidak terdapat vaksin untuk pencegahan jangkitan EV71. Vaksinasi dianggap sebagai kaedah yang paling berkesan untuk mengawal jangkitan EV71. Oleh itu kumpulan penyelidikan kami telah merintis untuk membangunkan calon vaksin yang melibatkan pembangunan gen sintetik VP1 daripada EV71 yang berangkai dengan gen ubikuitin (UbGR) dan diklonkan dalam vektor DNA vaksin yang mempunyai promoter eukariotik yang kuat iaitu pVAX1, untuk menghasilkan calon DNA vaksin yang dinamai pVaxUbVP1. Keupayaan vaksin ini untuk menghasilkan tindak balas imun di kaji pada mencit BALB/c dengan menggunakan dua kaedah penyampai: sebagai vaksin DNA yang disuntik ke dalam otot atau secara oral melalui bakteria hidup yang dilemahkan iaitu *Salmonella typhi* Ty21a sebagai pembawa vaksin DNA dan strain yang membawa pVaxUbVP1 dikenali sebagai *StUbVP1*. Kedua-dua calon vaksin digunakan melalui pendekatan 'homologous' dan 'heterologous prime-boost': Format A (pVaxUbVP1 sahaja), Format B (*StUbVP1* sahaja), Format C (*StUbVP1* sebagai vaksin primer dan pVaxUbVP1 sebagai vaksin 'booster') dan Format D (pVaxUbVP1 sebagai vaksin primer dan *StUbVP1* sebagai vaksin 'booster'). Keputusan kajian menunjukkan bahawa Format A dan D menghasilkan tahap IgG serum yang signifikan sementara asai subkelas IgG menunjukkan paras IgG2a adalah lebih tinggi dibandingkan IgG1 dalam kedua-dua format imunisasi. Penghasilan sitokin IFN- γ secara *in vitro* adalah signifikan bagi Format A, Format B dan Format D sementara penghasilan IL-4 pula secara relatifnya adalah rendah pada semua format imunisasi melainkan

Format D. Penghasilan sitokin intrasel (IFN- γ , IL-2 dan IL-4) oleh sel T CD4⁺ dan CD8⁺ pula menunjukkan tindakbalas tahap sederhana ke tinggi pada imunisasi Format A dan Format D. Analisis juga menunjukkan bahawa pVaxUbVP1 dalam format 'homologous prime boost' (Format A) menghasilkan rangsangan imun sistem jenis Th1 sementara penggunaan Format D (pVaxUbVP1 sebagai vaksin primer dan SfUbVP1 sebagai vaksin 'booster') menghasilkan rangsangan campuran Th1-Th2. Kesimpulannya, vaksin DNA pVaxUbVP1, apabila digunakan bersendirian secara 'homologous prime boost' atau sebagai vaksin primer dalam kaedah 'heterologous prime boost' bersama SfUbVP1, menunjukkan potensi untuk dibangunkan sebagai vaksin terhadap EV71.

IMMUNOGENICITY STUDY OF DNA VACCINE AND DNA VACCINE CARRIER EXPRESSING VP1 OF ENTEROVIRUS 71 IN THE PRIME BOOST VACCINATION STRATEGY

ABSTRACT

Enterovirus 71 (EV71) is a highly infectious causative agent of hand, foot and mouth disease (HFMD) in children and which could lead to severe neurological complications. In Malaysia, the first epidemic occurred in 1997 in Sarawak and caused 34 deaths due to severe neurological syndrome. There is currently no vaccine available against EV71. Vaccination is considered the most effective means to control EV71 infection. Thus our group have exploring the development of a candidate vaccine involving the construction of a synthetic VP1 gene of EV71 fused to a ubiquitin (UbGR) gene and cloned into a DNA vaccine vector with a strong eukaryotic promoter known as pVAX1, to create the candidate DNA vaccine pVaxUbVP1. The immunogenicity of the constructed DNA vaccine was evaluated in BALB/c mice involving two methods of delivery: as a naked DNA vaccine delivered intramuscularly or delivered orally via the live attenuated bacteria *Salmonella typhi* Ty21a, of which the recombinant strain carrying pVaxUbVP1 was designated as StUbVP1. Both candidate vaccines were used in homologous and heterologous prime boost approaches: Formats A (pVaxUbVP1 alone), B (StUbVP1 alone), C (StUbVP1 as primer vaccine and pVaxUbVP1 as booster), and D (pVaxUbVP1 as primer vaccine and StUbVP1 as booster). The results indicated that total IgG levels in serum was significant in Formats A and D whereas IgG subclasses assay showed that IgG2a levels were higher than IgG1 levels in both immunization formats. Production of *in vitro* IFN- γ was significant in mice vaccinated using Formats A, B and D, whereas IL-4 production was relatively low in all groups of immunization but shows a significant increase in Format D. The percentage of intracellular cytokine (IFN- γ , IL-2 and IL-4) production by CD4⁺ and CD8⁺ population of T cells showed a moderate to high response in Formats A and D. The analyses also showed that the use of pVaxUbVP1 in a

homologous prime boost format (Format A) resulted in a Th1 type of immune response whereas using Format D (pVaxUbVP1 as primer vaccine and StUbVP1 as booster) gave a mixed Th1-Th2 types immune response. In conclusion, pVaxUbVP1 used alone in a homologous prime boost approach or as the primer vaccine in a heterologous prime boost immunization format together with StUbVP1, show potential for further development as a vaccine against EV71.

CHAPTER 1

INTRODUCTION

1.1 Enterovirus 71

Enterovirus 71 (EV71) belongs to the human Enterovirus A species of the *Enterovirus* genus within the family *Picornaviridae*. EV71 was first isolated and characterized from neurological cases occurred in California from 1969-1973 (Schmidt *et al.*, 1974). Virions of EV71 consists of a non-enveloped capsid surrounding a core of single stranded, positive polarity RNA approximately 7.5 kb in size (Brown & Pallansch, 1995). The viral capsid is icosahedral in symmetry and composed of 60 identical units (protomers) each consisting of four structural proteins VP1-VP4.

The single open reading frame (ORF) encodes a polyprotein of 2194 amino acids and is flanked by untranslated regions (UTRs) at the 5' and 3' end. A variable length poly-A tract is located at the terminus of the 3' UTR. The polyprotein is subdivided into three regions, P1, P2 and P3. P1 encodes four viral structural proteins 1A-1D (VP1-VP4), whereas P2 and P3 encode seven non-structural proteins designated 2A-C and 3A-D respectively (Brown & Pallansch, 1995). The genome structure of EV71 is shown in Figure 1.1.

The clinical manifestations caused by EV71 infection vary from mild hand, foot and mouth disease (HFMD) or herpangina to neurological diseases such as aseptic meningitis, encephalitis, pulmonary edema and death (McMinn, 2002). Children less than 5 years of age are particularly susceptible to the most severe forms of EV71 associated neurological disease. Historically, series of EV71 associated outbreak of HFMD and encephalitis have been reported from the United States (Alexander *et al.*, 1994), Australia (Gilbert *et al.*, 1988), Europe (Chumakov *et al.*, 1979, Nagy *et al.*, 1982 & Melnick, 1984), Japan (Fujimoto *et al.*, 2002), Brazil (da Silva *et al.*, 1996) and Malaysia (AbuBakar *et al.*, 1999a, Cardosa *et al.*, 1999 & Chan *et al.*, 2000).

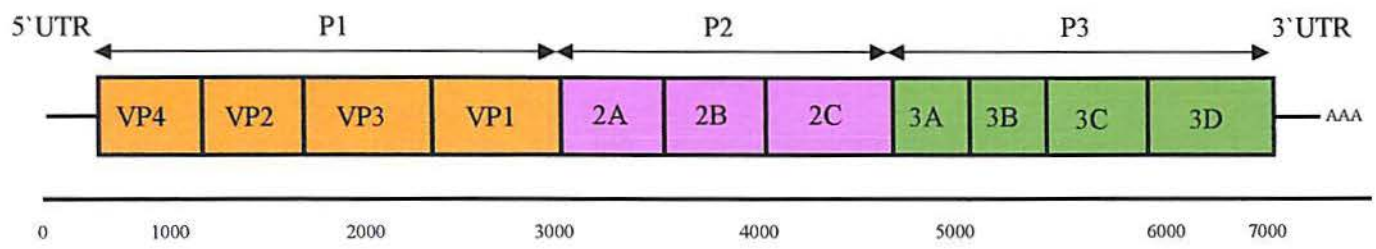


Figure 1.1: Genome structure of EV71. The single ORF is flanked by UTRs at the 5' and 3' ends and a variable length poly-A tail is found at the 3' UTR (Adapted from Brown & Pallansch, 1995).

1.2 Diseases due to EV71

1.2.1 HFMD and herpangina

HFMD is the illness characterized by three to four days of fever and the development of vesicular anathema which is an ulcer or eruption occurring on mucous secreting surfaces such as on the buccal mucosa, tongue, gums and palate. Papulovesicular exanthema also appears on the hands, foots and buttocks. EV71 has been increasingly recognized as the cause of this disease (AbuBakar *et al.*, 1999a, Abubakar *et al.*, 1999b, Cardosa *et al.*, 1999, Chang *et al.*, 1999a, Chan *et al.*, 2000 & Chang *et al.*, 2002) while Coxsackie A16 (CA16) (Chang *et al.*, 1999b & Shah *et al.*, 2003) is also frequently associated with HFMD epidemics. EV71 associated HFMD is considered clinically indistinguishable from HFMD caused by CA16. However infection with CA16 rarely results in neurological disease or death (Mc Minn, 2002).

In addition to HFMD, EV71 was identified as a causative agent of herpangina during epidemics in Hong Kong (Samuda *et al.*, 1987), Japan (Komatsu *et al.*, 1999) and Taiwan (Ho *et al.*, 1999 & Wang *et al.*, 1999). Herpangina is an illness characterized by an abrupt onset of fever and sore throat, which are associated with the development of raised popular lesions on the mucosa of the anterior pillars of Fauces, soft palate and uvula. EV71 associated herpangina was very prevalent during the 1998 Taiwanese epidemic (Chang *et al.*, 1999b, Ho *et al.*, 1999 & Wang *et al.*, 1999) and the second most diagnosed after HFMD.

1.2.2 Neurological diseases

EV71 have been recognized as a highly neutropic virus and associated with a diverse range of neurological diseases such as aseptic meningitis, brainstem or cerebellar encephalitis, acute flaccid paralysis (AFP) and several post-infectious neurological syndromes (Alexander *et al.*, 1994).

AFP associated with EV71 have been established by numerous studies and it appears to be milder and associated with higher rates of complete recovery than that occurring due to poliovirus infection (Samuda *et al.*, 1987 & McMinn *et al.*, 2001). It was demonstrated that many clinically diagnosed cases of EV71 associated AFP are not due to cytopathic damage to the anterior horn motor neurons which likely occurs in cases of poliomyelitis, but are rather due to other neuropathological mechanisms (Ramos-Alvares *et al.*, 1969). In addition, clinical and magnetic resonance (MR) imaging study of EV71- associated AFP showed the presence of transient MR changes in the anterior horns and ventral roots of the spinal cord in six of seven cases studied (Chen *et al.*, 2001). Nevertheless, it is clear that EV71 may induce AFP by several mechanisms other than virus-mediated destruction of the anterior horn motor neurons, and this is reflected in the more varied clinical presentation of EV71 associated AFP than that seen during poliovirus infection.

EV71 infection can also cause brainstem encephalitis which is the most severe neurological manifestation. This disease occurs most frequently as an extension of spinal cord disease (Lum *et al.*, 1998, Hsueh *et al.*, 2000 & McMinn *et al.*, 2001) but may occur in isolation (Huang *et al.*, 1999 & Wang *et al.*, 1999). MR imaging and post mortem studies indicated that the medulla oblongata, reticular formation, pons and midbrain structures are those most frequently involved (Lum *et al.*, 1998, Huang *et al.*, 1999 & McMinn *et al.*, 2001).

1.3 Epidemiology of EV71

Since the initial description of EV71 in 1974, outbreaks of EV71 infection have occurred periodically throughout the world. In the Asia Pacific region, occurrence of EV71 associated HFMD outbreaks was first identified in Japan in 1973 and later in 1978 (Miwa *et al.*, 1980 & Tagaya *et al.*, 1981). Both epidemics showed the same characteristic features of low prevalence of acute neurological disease.

Increased occurrence of large scale outbreaks and high level of endemic circulation of EV71 have been reported in Sarawak in 1997 (Cardosa *et al.*, 1999), followed by smaller outbreaks in Japan (Komatsu *et al.*, 1999), Peninsular Malaysia (Lum *et al.*, 1998) and Singapore in 1998 (Singh *et al.*, 2000). In 1997, a cluster of paediatric death due to encephalitis and cardiac failure was observed in Sarawak (Cardosa *et al.*, 1999). Thirty-four deaths from this syndrome were reported (Cardosa *et al.*, 1999) and four fatal cases were detected in peninsular Malaysia (Lum *et al.*, 1998).

The largest and most severe EV71 associated outbreak reported to date was in 1998 which occurred in Taiwan (Ho *et al.*, 1999, Hsiung and Wang, 2000 & Liu *et al.*, 2000). A total of 129106 cases of HFMD and herpangina (HA) were reported over a period of 8 months (Ho, 2000). Previous virological studies have identified the cause of the HFMD/HA epidemics as due to both EV71 and Coxsackievirus A16 (CA16) (Ho *et al.*, 1999 & Ho, 2000), with EV71 isolated from 2/3 of the cases. Severe neurological diseases due to EV71 were identified in 405 cases and 78 children died mainly due to the development of neurogenic pulmonary edema (Ho *et al.*, 1999, Hsiung and Wang, 2000 & Liao and Hung, 2001).

A large scale outbreak of HFMD was reported in Singapore and southern peninsular Malaysia in 2000 during which several fatal encephalomyelitis were reported in both countries (Lum *et al.*, 2002). The sole cause of the epidemic in Singapore was identified as due to EV71, whereas both EV71 and echovirus 7 were isolated from cases of HFMD and encephalomyelitis in peninsular Malaysia (Chua *et al.*, 2001 & Lum *et al.*, 2002).

1.4 Pathogenesis of EV71 infection

The pathogenesis of EV71 is still not completely understood. However, several mechanisms have been proposed suggesting that central nervous system (CNS) dysfunction and acute respiratory distress syndromes led to the acute fatality of EV71 infection (Lin *et al.*, 2003 & Wang *et al.*, 2003).

EV71 is a neutropic virus and is similar to other enteroviruses. The infection is transmitted mainly by the fecal oral route, but oral-oral route transmission is also possible (Lin *et al.*, 2002). The initial replication site of EV71 probably occur in the intestine as the virus could be detected in feces for weeks after an acute infection (Li *et al.*, 2002).

However, it is unclear how EV71 is disseminated from the initial replication site to the CNS. One possibility is that EV71 may spread to the CNS through the neural route (Lin *et al.*, 2002). Another possibility is this virus may enter the blood circulation after replication in the gut mucosa.

Liang *et al.* (2004) hypothesized that endothelial cells are the extraneural sites for replication of EV71. A study has been carried out to test the susceptibility of human endothelium to EV71 infection using a clinical isolate of EV71 to infect a human endothelial cell line (HMEC-1) and the results demonstrated that human HMEC-1 cells is permissive for EV71 infection *in vitro* (Liang *et al.*, 2004). The same studies also suggested that EV71 not only infect HMEC-1 cells but could render them to undergo several cytopathic and pro-inflammatory changes including the secretion of Interleukin-6 (IL-6), and chemokines which are macrophage migration inhibition factor (MIF) and macrophage chemo-attractant protein-1 (MCP-1). Collectively, local secretion of IL-6, MIF and MCP-1 by EV71 infected endothelial cells may augment the recruitment of leukocytes and amplify the inflammatory responses, which may play an important role in the pathogenesis of EV71 infection.

1.5 EV71 molecular genetics of virulence

In order to identify EV71 neurovirulence determinants, several studies have been carried out to compare genomic regions known to influence the neurovirulence of poliovirus, which are the 5' UTR (AbuBakar *et al.*, 1999a & Shih *et al.*, 2000) and VP1 gene (Brown *et al.*, 1999 & Shih *et al.*, 2000) of EV71 strains isolated from mild and severe clinical cases. However, these studies have all failed to identify neurovirulence determinants. The reason which has been suggested by McMinn (2001), is that enterovirus neurovirulence is a complex phenotypic characteristic and it is likely to be determined by more than one region of the virus genome. Other factors such as age-related resistance to enterovirus infection, host factors, the presence of cross protective immunity or specific major histocompatibility complex (MHC) haplotypes, are also likely to play significant roles in limiting the severity of most EV71 infection.

1.5.1 5' UTR

The enterovirus 5' UTR contains a group of conserved secondary structural elements that collectively form the internal ribosome entry site (IRES). The IRES regulates enterovirus replication through the control of cap-independent translation of polyprotein (Gromeier *et al.*, 1999). The enterovirus IRES is composed of a variable number of stem-loop structures that depend on the virus serotype, but is remarkably uniform in function. IRES-like stem-loop structures have been identified within the 5' UTR of EV71 (AbuBakar *et al.*, 1999a).

Single nucleotide changes within the poliovirus IRES have been found to result in large alterations in neurovirulence (Evans *et al.*, 1985). Although it seems likely that the EV71 IRES is an important neurovirulence determinant, nucleotide variation linked to neurovirulence in the EV71 IRES has not been identified to date, and the role of this structure in the control of EV71 neurovirulence remains unclear (McMinn, 2002).