PROPHLACTIC AND THERAPEUTIC POTENTIAL OF SYNTHETIC PEPTIDES AGAINST ENTEROVIRUS 71 (EV71)

DAMIAN FOO GUANG WEI

NATIONAL UNIVERSITY OF SINGAPORE

2008

PROPHLACTIC AND THERAPEUTIC POTENTIAL OF SYNTHETIC PEPTIDES AGAINST ENTEROVIRUS 71 (EV71)

DAMIAN FOO GUANG WEI
B.Sc. (Hons), NUS

A THESIS SUBMITTED FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

DEPARTMENT OF MICROBIOLOGY

NATIONAL UNIVERSITY OF SINGAPORE

2008

ACKNOWLEDGEMENTS

This study would not have been possible without the following people. I would like to express my sincere thanks and utmost gratitude to:

Associate Professor Poh Chit Laa for the opportunity to pursue my postgraduate studies under her supervision and to undertake this project. Thank you for your invaluable guidance and support throughout the course of this study. The knowledge and experiences gained in your laboratory were truly invaluable.

Assistant Professor Sylvie Alonso for her unwavering guidance, sound advice and most importantly, for her friendship. Thank you for your constant encouragement, support and the opportunity to continue my postgraduate studies under your close supervision. I am indebted to you for providing me with the motivation to develop a passion towards science and sharing your research experiences with me.

Associate Professor Vincent Chow Tak Kwong for his co-supervision, advice and guidance throughout the course of this study.

Associate Professor Lu Jinhua, Assistant Professor Kevin Tan Shyong Wei and Assistant Professor Theresa Tan May Chin for being my PhD qualifying examiners and the opportunity for me to continue with my postgraduate studies.

Mr Ramachandran and **Mrs Phoon Meng Chee** for their help during the animal study and their technical advices in tissue culture work and *in vitro* microneutralization assay.

Mr Ramasamy Pallan, Mr Goh Ting Kiam, Paul, Jasmine, Li Li and Auntie Zainal for their friendships and help in all administrative and technical matters.

Boon King, Eng Lee, Adrian, Andrew, Boon Eng, Si Ying, Wen Wei, Grace, Li Rui, Wei Xin and Joo Lee for their help and encouragement. Thank you for all your invaluable advices and concern. Thank you for the joy and laughter we have shared.

Chew Ling ♥ for her patience and understanding throughout my postgraduate studies. Thank you so much for your endless encouragement, support and help especially with the bacterial work. Also, thank you for accompanying me in lab during my late night stints. Your love and sincerity has indeed changed me into a better person.

My parents for their unconditional love, concern and understanding in every possible ways. Thank you for your constant support throughout my studies. You have given me everything a child can possibly dream of. Without you, I won't be who I am today.

God for his eternal guidance, love and being there in my times of need. You have given me spiritual courage and strength to face new challenges and overcome all difficulties. Continue to guide me and strengthen my faith in you. Amen.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

ADCC	Antibody-Dependent Cellular Cytoxicity
AFP	Acute Flaccid Paralysis
ANS	Autonomic Nervous System
APC	Antigen Presenting Cell
bp	Base Pair
BSA	Bovine Serum Albumin
ºC	Degree Celsius
CA3	Coxsackievirus A3
CA5	Coxsackievirus A5
CA9	Coxsackievirus A9
CA11	Coxsackievirus A11
CA15	Coxsackievirus A15
CA16	Coxsackievirus A16
CB3	Coxsackievirus B3
CB6	Coxsackievirus B6
CD	Cluster of Differentiation
cDNA	Complementary Deoxyribonucleic Acid
cm	Centimeter
CNS	Central Nervous System
CO_2	Carbon Dioxide
CPE	Cytopathic Effect
cpm	Counts Per Minute
CSF	Cerebrospinal Fluid

Ct	Threshold Value
CTL	Cytotoxic T-Lymphocyte
DC	Dendritic Cell
ddH_2O	Double Distilled Water
DKP	Diphtheria Toxoid
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid
Echo 7	Echovirus 7
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-linked Immunosorbent Assay
EV68	Enterovirus 68
EV69	Enterovirus 69
EV70	Enterovirus 70
EV71	Enterovirus 71
FCS	Fetal Calf Serum
FMDV	Foot and Mouth Disease Virus
FITC	Fluorescent Isothiocyanate
FRET	Fluorescence Resonance Energy Transfer
g	Gravitational Force
GBS	Guillain-Barré Syndrome
GST	Glutathione *S*-transferase
H	Hour

HCV	Hepatitis C Virus
HFMD	Hand, foot and mouth Disease
HLA	Human Leukocyte Antigen
HRP	Horse Radish Peroxidase
HTLV	Human T-cell Lymphotropic Virus
H+L	Heavy and Light Chains
ICR	Institute of Cancer Research
IFA	Immunofluorescence Assay
IFN-γ	Interferon-gamma
imDC	Immature Dendritic Cell
IRES	Internal Ribosome Entry Site
Ig	Immunoglobulin
IL	Interleukin
IPTG	Isopropyl-ß-D-thiogalactopyranosid
IPV	Inactivated Polio Vaccine
IVIG	Intravenous Immunoglobulin
Kan	Kanamycin
kb	Kilobase
kDa	Kilodalton
L	Liter
LB	Luria-Bertani
LBM	Lim Benyesh-Melnick
µg	Microgram
µl	Microliter

M	Molar
MAR	Monoclonal Antibody-resistant
MCS	Multiple Cloning Site
MDCK	Monkey Kidney
MEM	Minimal Essential Medium
MHC	Major Histocompatibility Complex
min	Minute
ml	Milliliter
mm	Millimeter
mM	Millimolar
MoDC	Monocyte-derived Dendritic Cell
mRNA	Messenger Ribonucleic Acid
MRC-5	Human Lung Fibroblast
MW	Molecular Weight
NBC	Newborn Cotton
NBW	Newborn White
nm	Nanometer
nt	Nucleotide
OD	Optical Density
OPD	O-phenylenediamine Dihydrochloride
OPV	Oral Polio Vaccine
ORF	Open Reading Frame
%	Percentage
P	Polyprotein

PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PE	Polyethylene
pg	Picogram
RD	Rhabdomyosarcoma
RNA	Ribonucleic Acid
rpm	Revolution Per Min
RT-PCR	Reverse Transcription Polymerase Chain Reaction
s	Second
SDS-PAGE	Sodium Dodecyl Sulphate-polyacrylamide Gel Electrophoresis
SI	Stimulation Index
SP	Synthetic Peptide
$TCID_{50}$	Tissue Culture Infectious Dose 50%
TE	Tris-EDTA
TEMED	Tetramethylethylenediamine
Th	T-helper
Tm	Melting Temperature
TMB	Tetramethylbenzidine
TNF-α	Tumour Necrosis Factor-alpha
U	Unit
UTR	Untranslated Region
V	Voltage
Vero	African green monkey kidney

VP	Viral Capsid Protein
v/v	Volume/volume ratio
WHO	World Health Organization
w/v	Weight/volume ratio

SUMMARY

Enterovirus (EV71) is the agent of Hand, foot and mouth disease (HFMD), a common and mild illness amongst infants and young children. However, in recent years, this pathogen has posed a serious threat; large scale outbreaks of HFMD have been reported in the Asia-Pacific region with an increasing number of cases of neurological complications which resulted in high fatality rates.

In this study, characterization of the linear neutralizing epitopes on the VP1 capsid protein of the Enterovirus 71 strain 41 (5865/SIN/00009) (belonging to subgenogroup B4 and isolated from a fatal case in Singapore) was undertaken. Antisera were raised in adult Balb/c mice against 95 overlapping diphtheria toxoid-conjugated synthetic peptides of 15 amino acids in length spanning the entire VP1 capsid protein. Two synthetic peptides, designated SP55 (VP1 amino acid residues 162 to 177) and SP70 (VP1 amino acid residues 208 to 222) were capable of eliciting neutralizing antibodies against EV71.

Based on *in vitro* microneutralization assay, the synthetic peptide SP70 was able to elicit a higher EV71-neutralizing response with a neutralizing antibody titer of 1:32 in mice when compared to the synthetic peptide SP55, eliciting an EV71-neutralizing antibody titer of 1:8. The anti-SP70 antiserum was found to be almost as efficient as the immune serum raised against the heat-inactivated homologous EV71 whole virion with a neutralizing antibody titer of 1:64. In addition, the total IgG response specific to EV71 whole virion measured in the anti-SP55 or anti-SP70 antiserum was found to be as high as that measured in the immune serum raised

against the heat-inactivated homologous EV71 strain 41. Immunization of mice with either synthetic peptide predominantly enhanced IgG1 production, suggesting that the neutralizing antibodies elicited are likely belonging to the IgG1 subtype.

The amino acid sequences represented by SP55 and SP70 lie towards the C-terminal part of the VP1 capsid protein of EV71 strain 41. The hydrophobicity profiles showed that these regions are located within the major hydrophilic regions of VP1 and hence they are expected to be exposed at the surface of the protein. Alignment with databases showed that the amino acid residues represented by SP70 are highly conserved amongst the VP1 sequences of 25 representative EV71 strains from different subgenogroups. *In vitro* microneutralization assay has shown that the immune serum raised against SP70 was able to neutralize heterologous EV71 strains with similar efficiencies to that obtained with the homologous EV71 strain 41, thereby suggesting that SP70 might represent an interesting and promising peptide-based vaccine candidate for EV71.

In addition, when passively administered to one-day-old suckling Balb/c mice challenged with a lethal dose of 10^3 $TCID_{50}$ virus/mouse, the anti-SP70 antibodies were able to confer 80% *in vivo* protection comparable to antiserum raised against the homologous heat-inactivated EV71 whole virion. The level of protection conferred by the anti-SP70 antiserum against heterologous EV71 strains was almost similar to that obtained against the homologous strain, supporting that the VP1 amino acid sequences represented by SP70 contain a highly conserved neutralizing linear epitope.

Histological examination and real-time RT-PCR hybridization probe-based assay revealed viral infiltration in the small intestines of EV71-infected mice and the neutralizing anti-SP70 antibodies plays a major role in the inhibition of EV71 replication *in vivo* which significantly reduced the viral titer. The cytokine profiles for EV71-challenged mice showed elevated IL-6 and IFN-γ levels in unprotected mice whereas significant lower levels were observed for mice which were protected by passively-transferred immune sera. This observation suggests a correlation between pro-inflammatory cytokines and the severity of EV71 infection.

The use of synthetic peptide(s) as capture antigen(s) in immunoassays represents an interesting approach for the serodiagnostic of EV71 infection as it would avoid the need for propagating infectious viruses. Antigenic sites on VP1 protein of EV71 strain 41 (5865/SIN/00009) were mapped by Pepscan analysis using the 95 overlapping synthetic peptides spanning the entire VP1 amino acid sequence against EV71-neutralizing sera from pediatric patients. A major IgG-specific immunodominant linear epitope (VP1 amino acid residues 91 to 111), defined by the core amino acid sequence 'LEGTTNPNG', was identified.

Therefore, a 15 amino acid-based synthetic peptide SP32 (DLPLEGTTNPNGYAN) which contains the core sequence of the immunodominant VP1 linear epitope was over-expressed in *Escherichia coli* as a soluble recombinant GST-SP32 fusion protein. When used as a capture antigen in Western blot, the recombinant GST-SP32 fusion protein significantly reacted with human anti-EV71 IgG antibodies with high specificity when compared to both the recombinant GST-VP1 fusion protein and EV71 whole virion as capture antigen. In addition,

computational analysis also showed that the amino acid sequence represented by SP32 was highly specific for EV71 strains with no significant homology with other enteroviruses. Altogether, these data indicate that recombinant GST-SP32 fusion protein which harbored the immunodominant VP1 linear epitope of EV71 could be potentially used as a capture antigen in Western blot for detecting human anti-EV71 IgG antibodies.

The identification of human $CD4^+$ T-cell epitopes within a protein vaccine candidate is of great interest as it provides a better understanding of the mechanisms involved in protective immunity and may therefore help in the design of effective vaccines and diagnostic tools. The entire amino acid sequence representing the VP1 capsid protein of EV71 strain 41 was submitted to analysis using a virtual matrix-based prediction program (ProPred) for the identification of promiscuous HLA-DR ligands. Three regions spanning amino acids 66 to 77, 145 to 159 and 247 to 261 of VP1 were predicted to bind more than 25 different HLA-DR alleles.

The corresponding peptides (SP1 to SP3) were then tested for their abilities to induce proliferation of $CD4^+$ T cells isolated from peripheral blood mononuclear cells of five human volunteers screened positive for previous EV71 exposure and one EV71-negative volunteer. Upon stimulation with either peptide, $CD4^+$ T cell proliferative responses were observed for all EV71-positive volunteers, indicating the presence of EV71-specific memory $CD4^+$ T cells. The amplitude of the proliferative responses was peptide- and HLA-DR-dependent, and correlated well with the ProPred predicted binding efficiencies.

This study also showed that $CD4^+$ T cells from EV71-positive volunteers produced significant levels of IL-2 and IFN-γ upon stimulation, indicative of a T cell differentiation into Th1-type subset. Among the three peptides, SP2 induced the highest proliferative response and cytokine production. In addition, the SP2-induced proliferative response could be inhibited with anti-major histocompatibility complex (MHC) class II antibody, indicating that SP2 may represent a MHC class II-restricted $CD4^+$ T-cell epitope. Hence, this study demonstrates that the ProPred algorithm can accurately predict the presence of human $CD4^+$ T-cell epitopes within the VP1 capsid protein of EV71, and therefore represents a useful tool for the design of subunit vaccines against EV71. The identification of $CD4^+$ T-cell epitopes also provides a better understanding in protective immunity and may help in diagnostic tools against EV71.

CHAPTER 1 LITERATURE REVIEW

1.1 Picornaviruses

Picornaviruses are one of the oldest known viruses. At present, the Picornaviridae family is divided into five main genera which consist of rhinoviruses, enteroviruses, aphthoviruses, cardioviruses and heptaviruses (REACH). The enteroviruses mostly inhabit the alimentary tract and include polioviruses, coxsackieviruses, echoviruses, human enteroviruses 68 to 71 and human hepatitis A virus (Melnick, 1996).

Not until 1908 was poliovirus identified by two Austrian physicians, Karl Landsteiner and E. Popper. Following their discovery, polio became a reportable disease entity, and the state of Massachusetts began counting polio cases in 1909 (Bradshaw, 1989). The acid-sensitive rhinoviruses and aphthoviruses are known to cause common cold and the foot-and-mouth disease in cattle, respectively. The acid stability of enteroviruses enabled them to be well-ingested and reach the intestinal tracts of both animals and humans (Levy *et al*., 1994). The human enteroviruses were originally classified on the basis of human disease manifestations, replication and pathogenesis in newborn mice, and propagation using *in vitro* cell culture method. However, based mostly on molecular properties, they have recently been re-classified into five different species which includes human enteroviruses A through D and polioviruses (King *et al*., 2000).

Most enterovirus infections are either mild or asymptomatic. However, infections in neonates are frequently life-threatening. Study has shown that enteroviruses can also cause common chronic diseases such as dilated

cardiomyopathies, insulin dependent diabetes mellitus and chronic fatigue syndrome (Muir *et al*., 1998). Several enteroviruses have been shown to be associated with gastrointestinal disorders, meningitis/encephalitis and respiratory illness (Levy *et al*., 1994). Table 1.1 summarizes the various clinical symptoms associated with enteroviruses in infections.

Table 1.1 Clinical manifestations of enteroviruses. Each symptom may potentially be caused by more than one enterovirus (Melnick, 1996).

Clinical Manifestations	Enterovirus Serotypes
Paralysis	Poliovirus 1 to 3; Coxsackievirus A7, A9, B2 to B5; Echovirus 4, 6, 9, 11, 30; Enterovirus 70, 71
Aseptic Meningitis	Poliovirus 1 to 3; Coxsackievirus A2, A4, A7, A9, A10, B1 to B6; Echovirus 1 to 11, 13 to 23, 25, 27, 28, 30, 31; Enterovirus 70, 71
Hand, foot and mouth disease (HFMD)	Coxsackievirus A5, A10, A16, Enterovirus 71
Herpangina	Coxsackievirus A2 to A6, A8, A10
Acute hemorrhagic conjunctivitis	Coxsackievirus A24, Enterovirus 70
Encephalitis	Echovirus 2, 6, 9, 19
Meningoencephalitis	Coxsackievirus B1 to B5, Enterovirus 70, 71
Pericarditis, myocarditis	Coxsackievirus B1 to B5

1.2 Genomic and organization of enteroviruses

Enteroviruses possess a single positive-stranded RNA genome of approximately 7,500 nucleotides. The complete genomic sequences of several Enterovirus 71 (EV71) strains have been determined, including the prototype BrCr strain (Accession no. U22521), the neurovirulent MS/7423/87 strain (Accession no. U22522) (Brown and Pallansch, 1995), the fatal 5865/sin/000009 strain (Accession no. 316321) and the non-fatal 5666/sin/002209 strain (Accession no. 352027) isolated from two patients during the Hand, foot and mouth disease (HFMD) outbreak in Singapore in the year 2000. (Singh *et al.,* 2002).

The enteroviral genome comprises a 5' untranslated region (5'UTR), a long open reading frame (ORF) encoding a polyprotein of 2,194 amino acids, a short 3' untranslated region (UTR) and a poly-adenylated tail. The 5'UTR contains determinants for translation of the viral RNA by internal ribosome entry site (IRES) mechanism for the amplification of viral RNA and for its neurovirulence (Evans *et al*., 1985), and its end is modified by the presence of a covalently bound protein VPg. The polyprotein is sub-divided into three regions, namely P1, P2 and P3. The P1 region encodes four structural proteins (VP1 to VP4), the P2 and P3 regions encode seven non-structural proteins (2A to 2C and 3A to 3D, respectively) (Figure 1.1). The polyprotein is co- and post-translationally cleaved to give rise to four structural proteins, namely VP1, VP2, VP3 and VP4. The 3'UTR region contains a pseudo-knot like structure that is crucial for negative-stranded RNA synthesis (Brown and Pallansch, 1995).

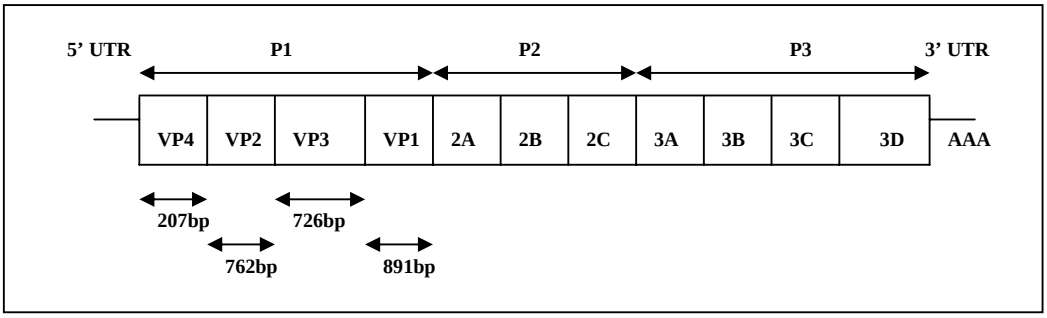


Figure 1.1 Genome structure of EV71. The single open reading frame (ORF) is flanked by UTRs at the 5' and 3' ends. A variable length poly-A tail is found at the 3' UTR. The ORF is divided into three regions: the P1 region encodes four structural proteins VP1 to VP4, the P2 and P3 regions encode seven non-structural proteins 2A to 2C and 3A to 3D, respectively.

1.2.1 The enteroviral capsid proteins

The single large open reading frame (ORF) encodes a polyprotein of approximately 250 kDa in size which is proteolytically processed by virus-encoded proteinases to yield structural and non-structural proteins. This is a full-length molecule on which RNAs are translated, while others are packaged as viral genomes by the newly synthesized coat proteins (Harper, 1998; Johnson and Sarnow, 1995). The viral capsid is icosahedra (T=1) in symmetry and is composed of sixty identical units (protomers). Each promoter is comprised of four structural proteins: VP1 to VP4. Among them, VP1, VP2 and VP3 are the main structural components of the viron, whereas VP4 is relatively unstructured and wholly internal (Hogle *et al.*, 1985). The VP1 capsid protein contains all three major neutralization sites that have been identified on the surface of poliovirus. However, VP2 and VP3 capsid proteins contain two and one neutralization site, respectively (Van der Marel *et al*., 1983; Van Wezel *et al*., 1983). Since X-ray crystallographic structures have been obtained for several picornaviruses, a consensus structural model for the EV71 coat protein was also developed to facilitate diagnostic immunoassays and vaccine development (Ranganathan *et al.,* 2002). The unique surface features of EV71 were characterized by homology modeling and comparing its structural model with experimental structures of other enteroviruses such as bovine enterovirus, rhinovirus and poliovirus. Although the antigenic determinants for EV71 have not been fully characterized but surface topography analysis and protein structure modeling have shown that the 3D structure of VP1 protein forms peaks separated by canyons and the immunogenic sites were surface-exposed and thus constituted a large immunogenic site for EV71 (Figure 1.2). Among all enteroviruses, a study has indicated that the VP1 region appears to be the most immunodominant amongst the other capsid proteins (Oberste *et al.*, 1999).

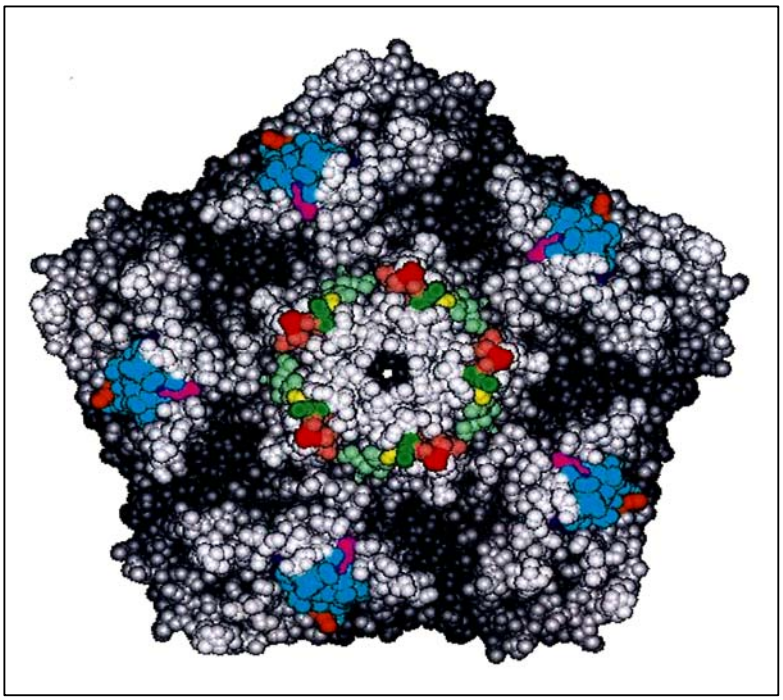


Figure 1.2 Diagrammatic representation of the EV71 icosahedra virus capsid. The capsid protein surface is assigned colors by depth, the deepest being dark grey and the outermost being white. The antigenic regions of VP1 are denoted by light green, red, green, yellow and salmon (Ranganathan *et al.*, 2002). (Permission granted by publisher, Adis)

1.2.2 Infection cycle

Typically, in an early event of any infection by enteroviruses, the initial uncoating of viral genome is the primary objective to gain entry into the host cell. To begin, endocytosis of viruses generally involves receptors, which is mediated by clathrin-coated pits and vesicles (Racaniello, 1995). Upon entry into the host cell, the virus genome is translated to produce the RNA polymerase and other enzymes. As shown in Figure 1.3, the entire replicative cycle of enteroviruses occur in the cytoplasm of infected cells. During viral replication, one of the products made is the viral RNA-dependent RNA polymerase which copies the genomic RNA to produce a negative-sense strand. This forms the template for positive-stranded genomic RNA synthesis which occurs via a multi-stranded replicative intermediate complex, some of which are translated whereas others are believed to be packaged as viral RNA into preformed capsids (Ansardi *et al.,* 1996; Whitton *et al.,* 2005). Once infected, cells typically displayed cytopathic effects (CPE) that comprise a series of cellular changes (Schlegel and Kirkegaard, 1995). The cell nucleus gradually alters in morphology until it acquires a characteristic crescent shape late in infection. This is followed by migration of the chromatin, in which chromosomal DNA is found in increasingly smaller regions of the nucleus that are often associated with the nuclear membrane (pkynosis). Ribosomes are aggregated in the cytoplasm and clusters of membranous vesicles form in great numbers. Eventually, the cells get rounded and lysed (Figure 1.3).

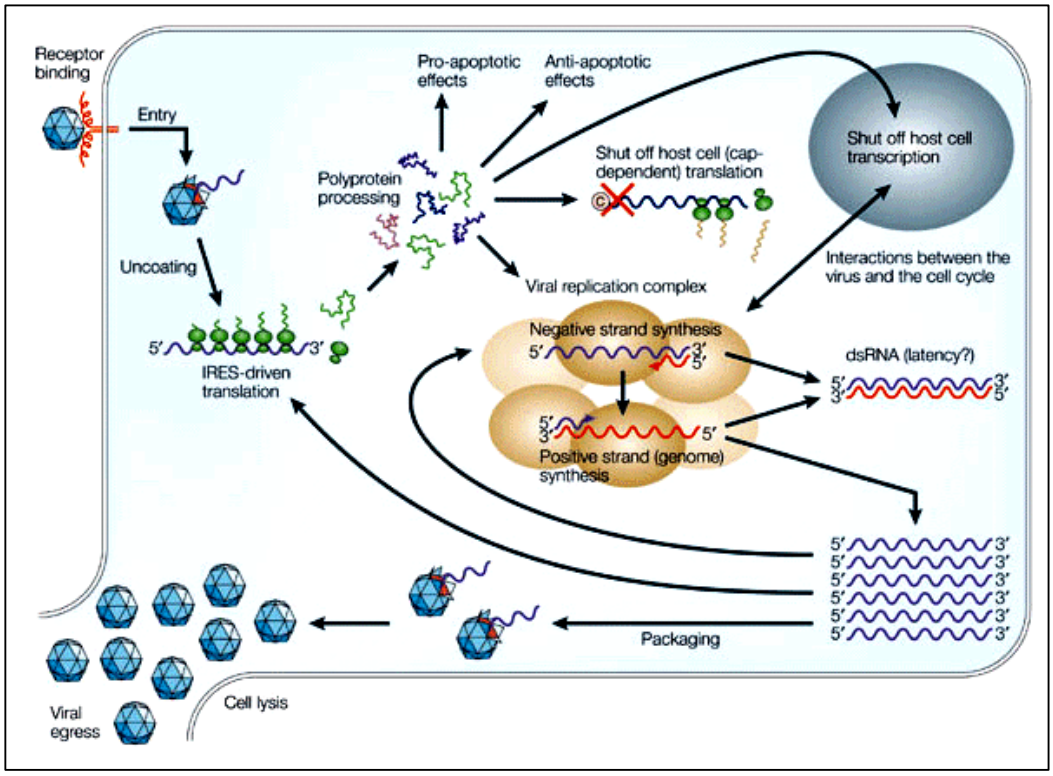


Figure 1.3 Life cycle of Picornaviruses (Whitton *et al.*, 2005). (Permission granted by Nature publishing group).

1.3 Enterovirus 71 (EV71) infection

1.3.1 Epidemiological studies

EV71 was first identified in the United States from the stool of an infected infant in the year 1969 (Schmidt *et al.,* 1974). Australia was the first country in which EV71 was isolated outside of the United States during an epidemic of aseptic meningitis between 1972 and 1973 (Kennett *et al.*, 1974). In the year 1975, the neurovirulence of EV71 was manifested during a large EV71 outbreak in Bulgaria which resulted in 44 fatalities (Chumakov *et al.*, 1979). However, an association of EV71 with the Hand, foot and mouth disease (HFMD) was not made until the year 1973, during the small epidemics in both Sweden and Japan (Blomberg *et al.*, 1974; Hagiwara *et al.*, 1978).

In the last 10 years, there was an increase of epidemics and neuropathogenicity of HFMD caused by EV71, particularly in the Asia-Pacific region. Before 1997, outbreaks caused by EV71 were of smaller scale and most were not associated with acute neurological disease. Since 1997, several large epidemics and high-level endemic circulations of EV71 strains have been reported. A summary of the main outbreaks from 1997 to present is presented in Table 1.2. Ever since the major HFMD outbreaks within the Asia-Pacific region in 2000, small scale outbreaks were also reported in countries such as Malaysia, Taiwan, China, Hong Kong, Brunei, India, Singapore, USA and Germany from year 2000 to 2006 (http://www.promedmail.org). Recently, in 2006, a large scale of HFMD outbreak occurred in Sarawak, Malaysia and 13 fatalities were reported. At the same time, approximately 3,000 of non-fatal HFMD cases were recorded in Singapore and 75% of the cases were caused by EV71 (Ministry of Health, Singapore).

Table 1.2 Summary of main HFMD outbreaks from 1997 to present.

Year	Location	Estimation of Total Cases	Agent Involved	Fatal Cases	References
1997	Sarawak Malaysia	2,628	EV71 CA16	29	Chan *et al.*, 2000; Cardosa *et al.*, 2003; McMinn *et al.*, 2002
1998	Taiwan	129,106	EV71 CA16	78	Chang *et al.*, 1999; Ho *et al.*, 1999
1999	Western Australia	6,000	EV71 CA16	29	Cardosa *et al.*, 2003; McMinn *et al.*, 2001b
2000	Singapore	3,790	EV71 CA16	4	Ahmad, 2000; Chan *et al.*, 2003
2006	Sarawak Malaysia	14,505	EV71	13	Sarawak State Health Department
2006	Singapore	3,000	EV71 CA16	0	Ministry of Health, Singapore
2007	Singapore	496	EV71 CA16	0	Ministry of Health, Singapore
2008	Anhui China	24,934	EV71	30	Fuyang Health Department
2008	Singapore	14,063	EV71 CA16	0	Ministry of Health, Singapore

1.3.2 Phylogenetic studies

The comparison of nucleotide and deduced amino acid sequences have identified four major phylogenetic groups within the enterovirus genus: coxsackievirus A16 (CA16)-like viruses (cluster A), a coxsackievirus B (CB)-like group containing all CB and echoviruses, as well as CA9 and enterovirus 69 (EV69) (cluster B), poliovirus-like viruses (cluster C), and EV68 and EV70 (cluster D) (Pöyry *et al*., 1996; Pringle, 1999). EV71 was also found to be very closely linked to CA16 where they share a nucleotide sequence homology of approximately 77% and 89% homology based on deduced amino acid sequences and hence falls within cluster A.

1.3.2.1 VP1-based classification

The understanding of the molecular epidemiology and evolution of EV71 took a major step forward when Brown *et al.* (1999) reported the phylogenetic analysis of EV71. To investigate the genetic variability of various EV71 strains and their associations with outbreaks, the complete cDNA sequence (891bp) encoding the VP1 capsid protein of EV71 strains isolated from various countries over a 30-year period was analyzed and the monophylogenetic serotype was further divided into three distinct genogroups A, B and C (Brown *et al*., 1999). Genogroup A contains a single member, the prototype EV71 strain BrCr-CA-70 whereas genogroup B consists of 65 strains isolated from 1972 to 1997 in the United States, Australia, Columbia and East Malaysia (Sarawak). Genogroup C is made up of 47 strains isolated from 1986 to 1988 from the United States, Australia, the Republic of China, Canada and mainland Malaysia (Brown *et al*., 1999) (Figure 1.4). Within each genogroup, EV71 strains were classified into subgenogroups (B1 to B5 and C1 to C4) mostly based on their VP1 or VP4 gene sequences. Within similar subgenogroup, EV71 strains share more

than 92% nucleotide sequence identity whereas the nucleotide sequence identity between the three genogroups ranges from 78% to 83%.

1.3.2.2 VP1- and VP4-based classification

Phylogenetic relationships of EV71 strains isolated from major outbreaks in the Asia-Pacific region were also established based on other parts of the EV71 genome such as the 5'UTR (Abubakar *et al.*, 1999; Wang *et al.*, 2000) and the VP4 (Shimizu *et al.*, 1999; Chu *et al*., 2001; Cardosa *et al.*, 2003; Lin *et al.*, 2006). Chu *et al.* (2001) examined a partial VP4 region (207 bp) of 23 EV71 Taiwanese strains from the 1998 epidemic and 21 other strains deposited in the GenBank, (the prototype BrCr strain, the neurovirulent 7423/MS/87 strain, three strains isolated during the 1986 outbreak in Taiwan, and 16 strains isolated from Japan). Cardosa *et al*. (2003) analyzed the partial VP1 and VP4 regions of 128 EV71 strains isolated from the year 1970 to 2002 from the United States, Japan, Taiwan, Malaysia, Singapore, China, Bulgaria, Hungary and the United Kingdom. Analysis of either the VP1 or VP4 gene sequences provided similar phylogenetic classification of the EV71 strains. However, higher bootstrap values were observed in the VP1 dendrograms, hence providing greater confidence when elucidating new genogroups (Cardosa *et al.*, 2003). Similar phylogenetic classification between the VP1 and VP4 regions was further shown in two recent separate studies which carried out genetic analysis of a 414 bp region within the VP1 sequence and a 207 bp region within the VP4 sequence on the EV71 strains isolated in Taiwan from the year 1988 to 2005 (Lin *et al.*, 2006; Kung *et al.*, 2007). Both studies demonstrated the predominance of the subgenogroup B1 before the year 1998 whilst the subgenogroup C2 was the major etiologic group in the 1998 outbreak. The subgenogroup B4, which was a minor etiologic group during the 1998

outbreak, became the major sub-genogroup isolated from 1999 to 2003. During the year 2004, the subgenogroup C4 emerged to be the predominant sub-genogroup in Taiwan.

1.3.2.3 Relationship between subgenogroups and outbreak occurrence

There seems to be no correlation between the severity of disease and the genetic lineage of the EV71 strains since viruses from all three genogroups are capable of causing disease (Brown *et al*., 1999). McMinn *et al.* (2001a) compared the complete VP1 gene sequence of 66 EV71 strains isolated from Malaysia, Singapore, Taiwan and Western Australia between 1997 and 2001 and established two sub-lineages within the genogroup B which circulated in Southeast Asia between 1997 and 2001. EV71 strains in the subgenogroup B3 were the predominant strains isolated from the epidemics in Sarawak and peninsular Malaysia in 1997 and in Western Australia in 1999 (Figure 1.5). Partial genetic analysis (500 bp) of the VP1 capsid protein from 18 EV71 strains involved in the 1998 outbreak in Taiwan has shown that majority of the viruses belonged to subgenogroup C2 (Shih *et al.*, 2000b). Based on the phylogenetic analysis of the VP1 region of 45 EV71 strains which were isolated over a 6-years period in Yamagata, Mizuta *et al.* (2005) found that outbreaks in Yamagata were mainly caused by EV71 strains belonging to the subgenogroups B4, C2 and C4. However, there were a few isolated EV71 strains that could not be classified into any of the three subgenogroups and they were classified under a new subgenogroup known as B5 (Figure 1.6). More EV71 strains were classified into the subgenogroup B5 when Ooi *et al*. (2007) analyzed the VP1 regions of EV71 isolated from 277 patients in Malaysia from 2000 to 2004. It was shown that 168 patients were infected with EV71 strains from the subgenogroup B4 whereas 68 patients were

infected with strains from the subgenogroup C1 and 41 patients were infected with the newly emerged EV71 strain belonging to the subgenogroup B5.

At present, a total of 10 subgenogroups have been identified. It was established that there is a great diversity of EV71 strains circulating in the Asia-Pacific region and other parts of the world. However, no significant differences in genome sequence were found between EV71 strains isolated from fatal and non-fatal cases. In the year 2000, EV71 strains belonging to the subgenogroup B4 were responsible for the HFMD outbreak in Singapore. Using a comparative sequence analysis, Singh *et al.* (2002) showed that the fatal Singapore strain 5865/sin/000009 which belongs to the subgenogroup B4 had 99% nucleotide and 100% amino acid homology with the non-fatal Singapore strain 5666/sin/002209 from similar subgenogroup. However, both strains displayed significant differences when compared to other EV71 strains including the prototype BrCr strain (genogroup A) and the neurovirulent strain MS/7423/87 (subgenogroup B2). Therefore, there is no particular subgenogroup that is associated with severe neurological complications (Singh *et al.*, 2002; Cardosa *et al.*, 2003). Due to its potential in causing severe neurological diseases, further studies on EV71 are necessary to understand the factors responsible for neurovirulence and epidemic potential.

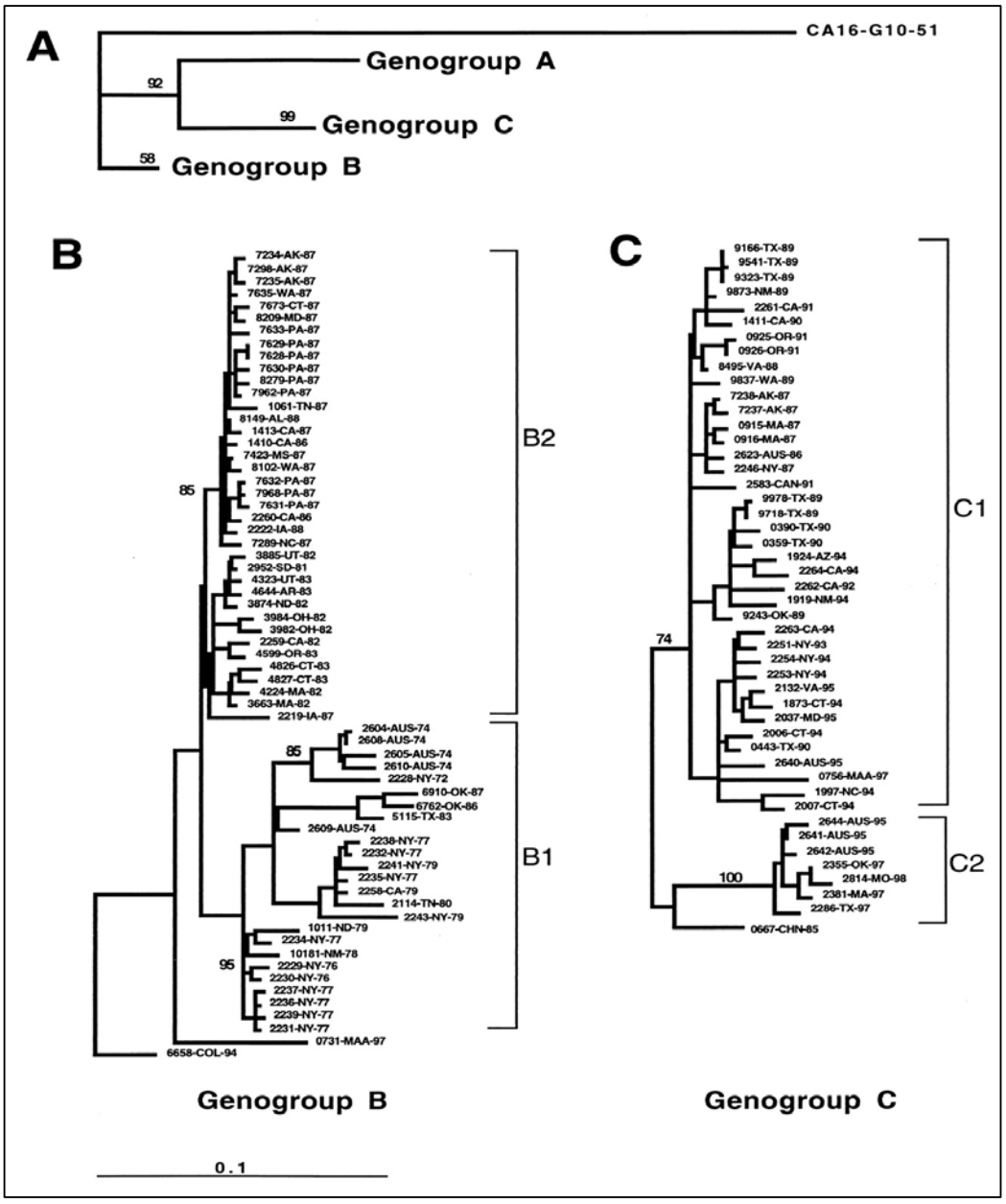


Figure 1.4 Classification of 113 EV71 strains into genogroups based on the VP1 gene (nucleotide position 2442 to 3332). The dendrogram was generated by the neighbor-joining method with the DNADIST distance measure program (PHYLIP, version 3.5) (Brown *et al*., 1999). (Permission granted by publisher, ASM Journals).

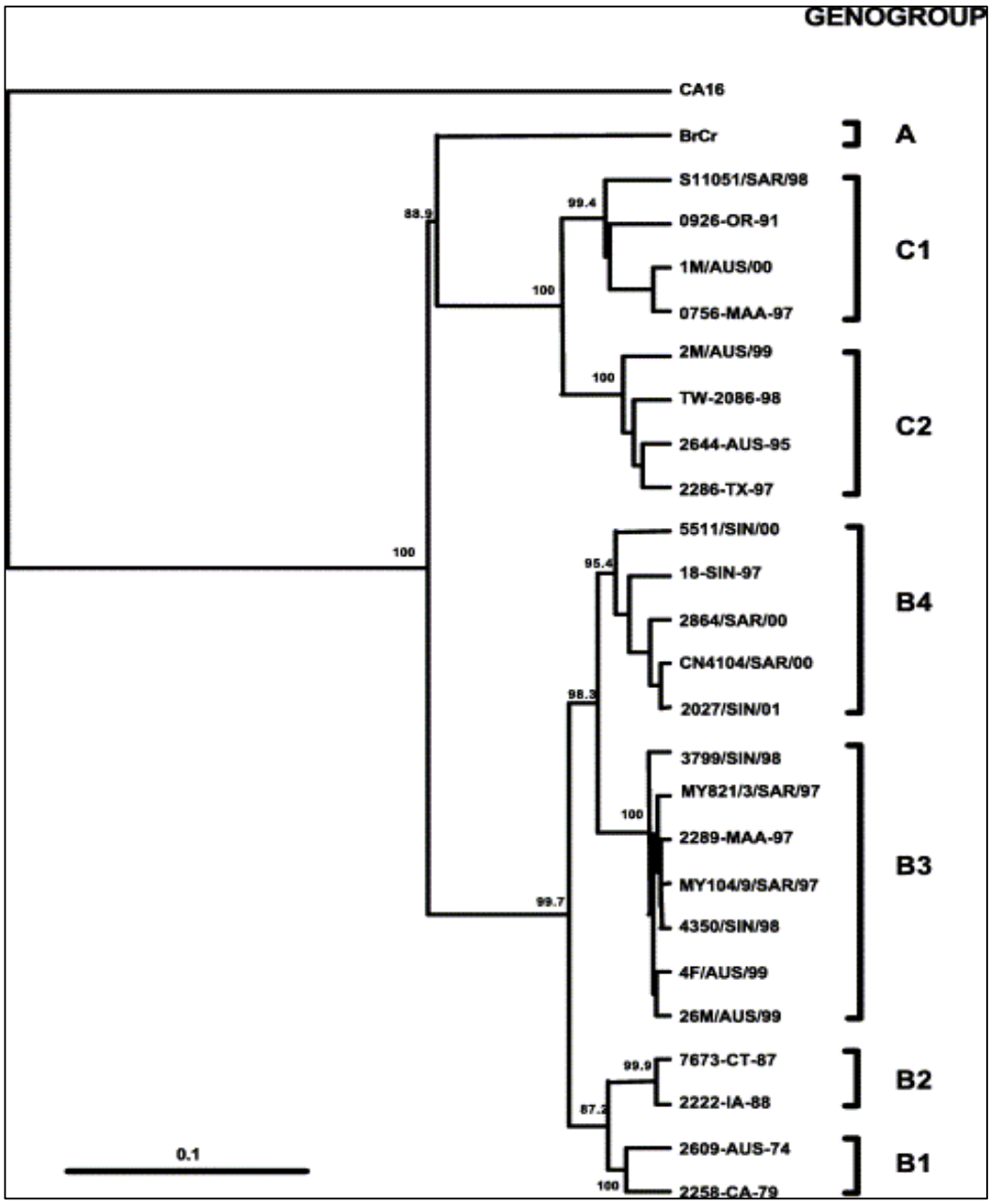


Figure 1.5 Phylogenetic tree showing classification of 25 EV71 field isolates into subgenogroups based on alignment of the complete VP1 sequence (nucleotide position 2442 to 3332). Branch lengths are proportional to the number of nucleotide differences. Strain names indicate a unique number/country or US state of isolation/year of isolation: AUS – Australia; CA – California, USA; CT – Connecticut, USA; IA – Indiana, USA; MAA – peninsular Malaysia; OR – Oregon, USA; SAR – Sarawak, Malaysia; SIN – Singapore; TW – Taiwan; TX – Texas, USA. The VP1 nucleotide sequence of CA16 was used as an out-group in the analysis (McMinn *et al*., 2001a). (Permission granted by publisher, ASM Journals).

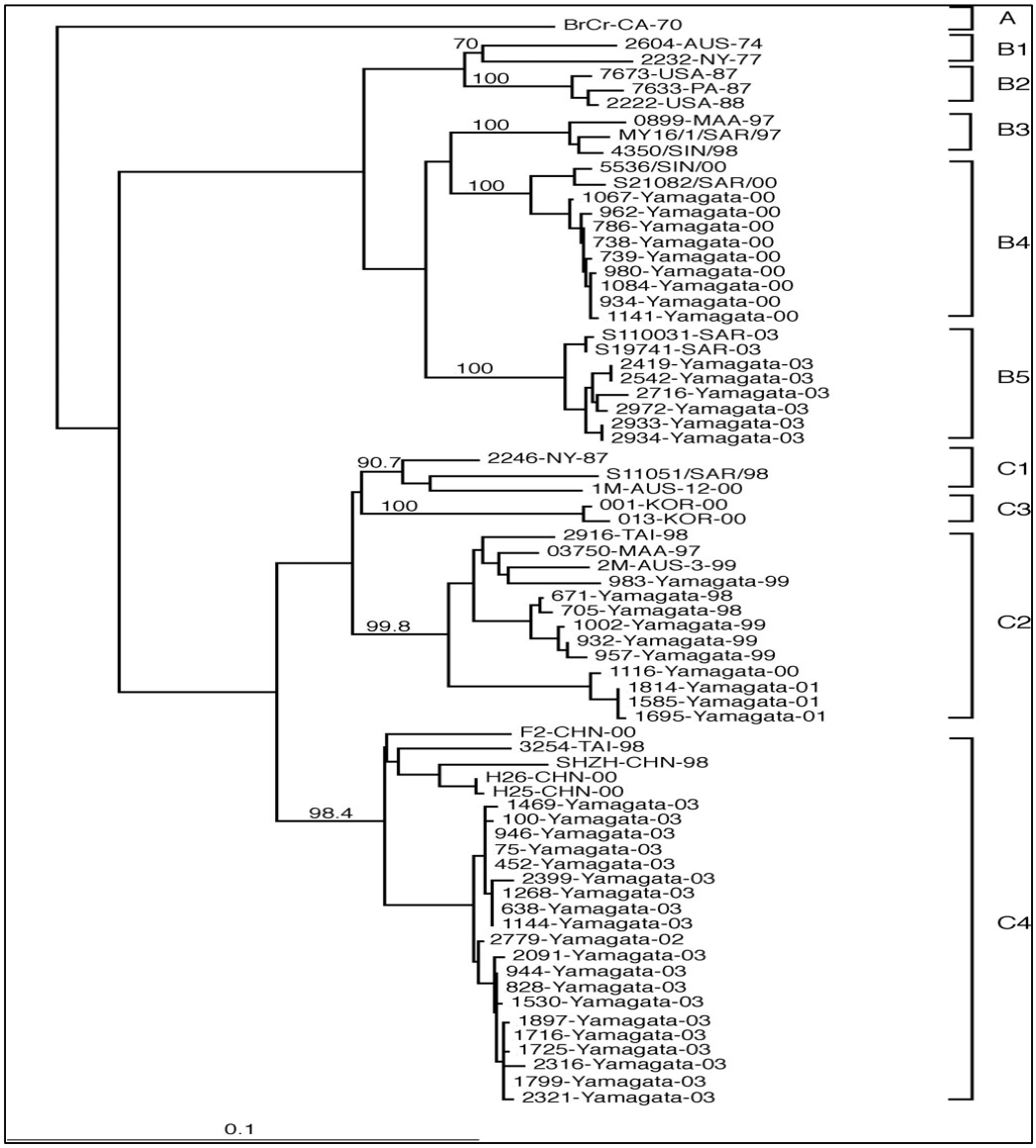


Figure 1.6 Phylogenetic classification based on the complete VP1 region (891 nucleotides). Representative EV71 strains were isolated in Yamagata, Japan, between 1998 and 2003, and reference strains (Mizuta *et al*., 2005). (Permission granted by publisher, ASM Journals).

1.3.3 Clinical features of diseases caused by enterovirus 71 (EV71)

A typical clinical course for all fatal EV71-associated cases has been observed: the symptoms are displayed for less than a week before hospitalization and subsequently the conditions of the patients rapidly deteriorates with a fatal outcome within hours to a few days of hospitalization (Liu *et al.*, 2000; Chan *et al.*, 2000). However, EV71 infections cause very diverse symptoms, ranging from none to fatality and it remains unknown why different hosts have such a range of clinical outcomes.

1.3.3.1 Hand, foot and mouth disease (HFMD)

EV71 has been increasingly recognized as the main etiological agent of HFMD which is described as a mild childhood disease usually characterized by three to four days of fever. This disease is commonly associated with the development of vesicular exanthem on the buccal mucosa, tongue, gums and palate, as well as papulovesicular exanthem on the hands, feet and buttocks (Figure 1.7). Other frequently encountered symptoms include poor appetite, vomiting as well as lethargy (Liu *et al.*, 2000). Other enterovirus serotypes that are commonly known to cause HFMD include CA16, CA5, CA9 and Echo 7 (Melnick, 1996). EV71 has been identified as the main causative agent of large scale HFMD outbreaks over the last few years in the Asia-Pacific region.

The HFMD clinical symptoms caused by EV71 are generally indistinguishable from those caused by CA16. Based on clinical features observed in patients suffering from HFMD in Japan (Komatsu *et al*, 1999), Malaysia (Chan *et al.*, 2000) and Western Australia (McMinn *et al.*, 2001b), larger vesicles were observed in children

suffering from CA16 infection as compared to those with EV71 infection. The rashes were more frequently papular and/or petechial with areas of diffuse erythema on the trunk and limbs.

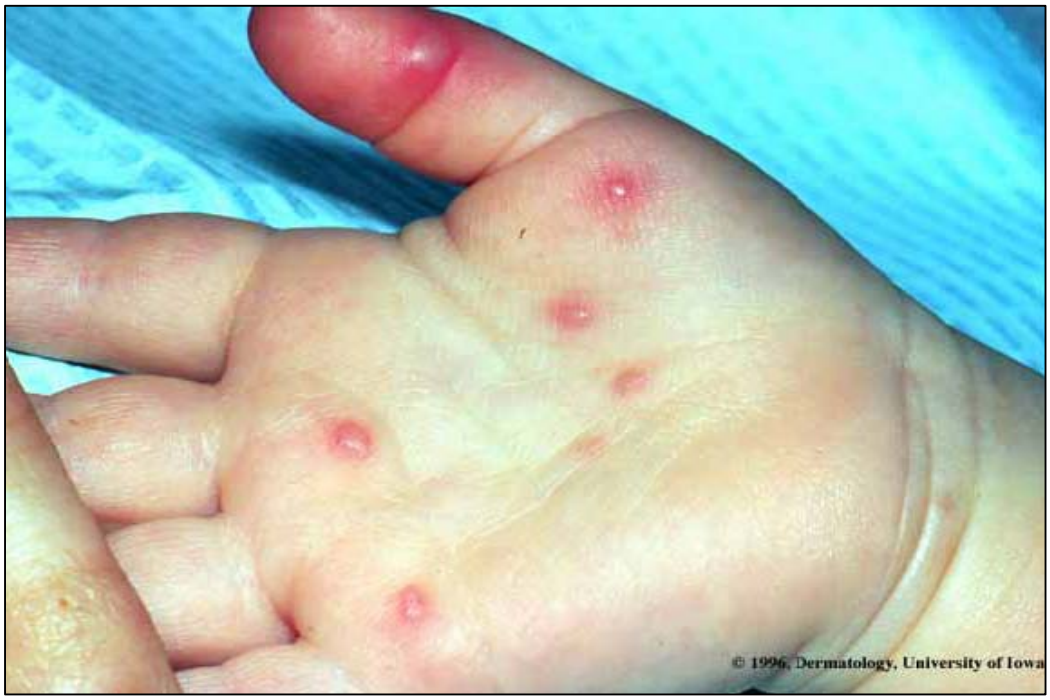


Figure 1.7 Vesicles on the palm of a child infected with hand, foot and mouth disease (HFMD). Adapted from the Dermatologic Image Database, Department of Dermatology, University of Iowa College of Medicine, USA, 1996 (http://tray.dermatology.uiowa.edu/ImageBase). (Permission granted by University of Iowa).

1.3.3.2 Other EV71-associated diseases

Apart from HFMD, EV71 also causes a variety of neurological diseases including aseptic meningitis, acute flaccid paralysis (AFP), encephalitis and poliomyelitis-like paralysis (Melnick, 1984; Lum *et al.,* 1998a; Liu *et al.,* 2000), which are frequently accompanied by complications such as herpangina, pulmonary edema and myocarditis (Chang *et al.,* 1999; Chan *et al.,* 2000). Children under five years of age were found to be highly susceptible to EV71-associated neurological diseases (Liu *et al.*, 2000; Chan *et al.*, 2000). These observations were recorded for children with acute EV71 infection during the large scale HFMD outbreaks in the Asia-Pacific region. However, these were not presented in children suffering from CA16 infection (Alexander *et al.*, 1994; Lum *et al.*, 1998a). In the 1999 Perth epidemic, only one of the five cases of acute flaccid paralysis had poliomyelitis-like disease, with two cases identified as transverse myelitis and two others as Guillain-Barré Syndrome (GBS) (McMinn *et al.,* 2001a). Acute cerebella ataxia has been linked to EV71 infection in many previous epidemics (Ishimaru *et al.,* 1980; Takimoto *et al.,* 1998; Komatsu *et al.,* 1999). Studies of neurogenic pulmonary edema due to EV71 infections were carried out (Lum *et al.,* 1998b; Cardosa *et al.,* 1999). In each case, the infections appeared to be confined to the brainstem, with histological evidence of acute inflammatory encephalitis, isolation of EV71 or identification of the EV71 antigen within the neurons (Lum *et al.,* 1998b; Wang *et al.,* 1999). These studies supported the hypothesis that pulmonary edema was of neurogenic origin and was secondary to autonomic dysfunction, resulting from infection of specific regulatory structures within the brainstem. Non-specific febrile illnesses are common clinical manifestations of enterovirus infections in young children and have been described in association with EV71 (Ho *et al.,* 1999; Merovitz *et al.,* 2000).

Respiratory diseases associated with EV71 infection include pharyngitis, croup, bronchiolitis and pneumonia (Gilbert *et al.,* 1988; Merovitz *et al.,* 2000). A single case of intrauterine infection with EV71 was reported by Chow *et al* (2000).

1.3.4 Immunopathogenesis of EV71 infection

A recent study has shown that EV71 patients suffering from brainstem encephalitis and pulmonary edema had a significantly lower phytohemagglutinin stimulation index, suggesting that lower EV71-specific cellular response may be associated with the immunopathogenesis of EV71-related pulmonary edema (Chang *et al.*, 2006). A previous study suggested that EV71 enters the host through the alimentary tract and uses the lymphatic circulatory system to reach target organs (Melnick, 1996). Pro-inflammatory cytokine profiles in association with EV71-associated pulmonary edema and brainstem encephalitis were studied and the levels of IL-6 and IFN-γ were found to be significantly higher in patients with severe EV71 infections (Lin *et al.,* 2003; Wang *et al.,* 2007). These findings suggest that both IL-6 and IFN-γ seems to play prominent roles in the overwhelming disease process caused by EV71.

1.4 Diagnosis of enterovirus 71 (EV71)

1.4.1 Tissue culture isolation and serotyping

EV71 infections are conventionally diagnosed on the basis of viral isolation and *in vitro* microneutralization tests. The standard procedure of enterovirus detection and isolation involves inoculation of cell line in cultures with patient throat swabs, vesicular fluid, cerebrospinal fluid or stool samples. The cell lines used are Vero (African green monkey kidney) cells, human Rhabdomyosarcoma (RD) cells, MRC-5

(human lung fibroblast) cells and MDCK (monkey kidney) cells (Ho *et al.*, 1999; Wang *et al.*, 2000). For some enteroviruses such as EV71, cytopathic effects (CPE) are often difficult to discern as it does not involve the entire cell monolayer on initial isolation and may be mistaken for a non-specific degeneration or toxic effect of the sample (Landry *et al.,* 1995). Therefore, several blind passages of the virus are required in certain cases before CPE becomes apparent and the presence of virus ascertained.

Although virus isolation has been regarded as the 'gold standard' for enterovirus identification, the procedures are poorly standardized and virus isolation data may vary considerably between laboratories. However, there are a number of problems associated with conventional diagnosis of EV71. This approach is very time-consuming (requiring at least three to four weeks for an accurate diagnosis) and it also faces issues regarding sensitivity and specificity of the test method. Sensitivity is largely dependent on the type and quality of the specimen, the timing of the specimen collection, and specimen storage before arriving in the laboratory (Morens *et al.,* 1991; Grandien *et al.,* 1995; Landry *et al.,* 1995). Upon isolation, enterovirus identification is determined using *in vitro* microneutralization assay with serotype-specific sera. However, typing by such approach using reference sera for all serotypes individually is clearly impractical. To overcome this problem, type-specific hyperimmune sera have been mixed to give intersecting pools containing different combinations of individual antisera (Schmidt *et al.,* 1961; Grandien *et al.,* 1995; Melnick, 1996). The Lim Benyesh-Melnick (LBM) pool scheme consists of eight pools (designated A to H) containing antisera raised against forty-two different enterovirus types (Lim & Benyesh-Melnick, 1960; Melnick, 1973). Alternative

intersecting pools of antisera developed by the National Institute of Public Health and Environment in the Netherlands allowed identification of enteroviruses 68 to 71 (Kapsenberg, 1998). Enterovirus isolates are incubated with each antiserum pool before re-inoculated onto susceptible cells. After several days of incubation, the neutralizing pattern is recorded and the serotype of the viral isolate can be inferred. Finally, serotypic identification of enteroviruses could be determined using serotype-specific antisera.

The use of intersecting pools for typing enterovirus isolates is time-consuming, labor-intensive and costly. Moreover, the supply of antisera is limited, which poses a significant problem in virus serotyping during a HFMD epidemic (Melnick & Wimberly, 1985). Finally, this approach does not permit classification of identified enterovirus into respective genogroup or subgenogroup and a large number of enterovirus isolates are frequently classified as 'untypeable' by *in vitro* microneutralization assay (Morens *et al.,* 1991). There may be several reasons: (i) 'untypeable' isolates may contain mixtures of enterovirus (especially in an outbreak situation) (Blomberg *et al.,* 1974; Schmidt *et al.,* 1974; Nagy *et al.,* 1982; Kok *et al.,* 1992); (ii) not all enterovirus serotypes can be identified with intersecting pools. For examples, EV71 along with CA3, CA11, CA15, CA17 and CA24 cannot be typed using LBM pools; (iii) enteroviruses may form aggregates which can be neutralized only after treatment with sodium deoxycholate or chloroform, or by ultrafiltration to dissociate the aggregates (Wallis & Melnick, 1967; Kapsenberg *et al.,* 1980); (iv) isolates may be antigenic variants of recognized serotypes which are neutralized poorly or may not be neutralized at all by antiserum to the homologous prototype strain; (v) and finally, isolates that are genuinely 'untypeable' after excluding the

above possible reasons may truly represent a new or previously unrecognized enterovirus type (Caro *et al.,* 2001; Palacios *et al.,* 2002).

When initial attempts to serotype viral isolates using intersecting sera pools are unsuccessful, the additional labor and expenses required to achieve proper identification deters further investigations and such isolates may be left untyped. A small-scale quality control scheme on enterovirus identification involving eleven laboratories and ten samples was carried out in 1999 but none of the participating laboratories managed to report the standardized results for enterovirus serotyping (van Loon *et al.,* 1999).

1.4.2 Immunofluorescence assay

Another approach for EV71 detection is the indirect immunofluorescence assay (IFA) which involves the use of serotype-specific monoclonal antibodies for virus identification (Manzara *et al.*, 2002). This method allows serotype-specific identification of EV71 grown in cell culture without using antisera pools. Vero (African green monkey kidney) cells, human Rhabdomyosarcoma (RD) cells and MRC-5 (human lung fibroblast) cells are commonly used for inoculations of clinical specimens (Ho *et al.*, 1999).

To identify the presence of EV71, immunohistological staining with a Pan-Enterovirus Blend of monoclonal anti-EV71 antibodies (Chemicon International, USA) can be performed. The serotype-specific monoclonal antibody will bind to EV71 isolated from the cell culture and the antigen-antibody complex can be detected with a capture FITC-labeled (fluorescent isothiocyanate) antibody. Such approach is

an alternative to the *in vitro* microneutralization test for the serotype-specific identification of EV71. However, this method still involves the isolation and propagation of EV71 in cell cultures which generally require one to two weeks of labor-intensive work. In addition, the Pan-Enterovirus Blend of monoclonal anti-EV71 antibodies (Chemicon International, USA) was also reported to cross-react with CA16 (Yan *et al.*, 2001) and the monoclonal antibodies were raised against the prototype BrCr strain. Therefore, it might not be able to detect newly evolved EV71 strains.

1.4.3 Enzyme-Linked Immunosorbent Assay (ELISA)

Diagnosis of EV71 infection by ELISA can generally be carried out efficiently with up to ninety-six clinical samples at a time. It is low in cost and has reduced the need for experienced personnel and dedicated laboratories. A recombinant protein-based ELISA has been developed as a commercially available diagnostic kit for EV71 infection. The concept involves interactions between specific anti-EV71 antibodies in patient sera with the polyhistidine-VP1 fusion protein (Shih *et al*., 2000a). However, studies on antibody cross-reactivity between enteroviruses showed that antisera targeted to the VP1 capsid protein cross-reacted widely with other enteroviruses (Hovi and Roivainen, 1993). Further analysis uncovered a region within VP1 (amino acids 42 to 58 of VP1) that is highly conserved throughout the enteroviruses. Based on these findings, it follows that the use of the entire VP1 capsid protein as a capture antigen for human anti-EV71 antibody detection may not be appropriate as the assay would lead to false positive results in cases where the infection is caused by enteroviruses other than EV71. In addition, a previous study has also shown that the VP1 protein of EV71 when produced as a recombinant His-VP1 fusion protein was

highly insoluble and hence required tedious denaturation and renaturation steps before it could be used as capture antigens in immunoassay (Shih *et al.,* 2000a).

The detection of IgM in sera from EV71-infected patients by ELISA was shown to be effective in diagnosing acute EV71 infection (Tano *et al.* 2002; Shih *et al.,* 2000a) with a high sensitivity and specificity of 97.7% and 93.3%, respectively (Wang *et al.*, 2004). However, in the IgM-capture ELISA, the whole EV71 viral particle was used as the capture antigen and the need to prepare large quantities of purified virion for interactions with anti-human IgM antibodies in the assay is an expensive, laborious and lengthy process. Moreover, cross-reactions with antibodies against other enteroviruses could result in false positives. Therefore, the specificity of the IgM-based ELISA could be compromised by the presence of common epitopes within enteroviruses.

The use of synthetic peptide as capture antigen in immunoassay has been explored as well. Using broad-reactive peptides from the VP1 capsid protein of enteroviruses as capture antigens in IgG-based ELISA, it was shown to be useful for serodiagnosis of enteroviral infections (Samuelson *et al.,* 1993). Although this may be useful for identifying enterovirus, it is not specific for the detection of EV71 infection. Moreover, this approach is limited by the high production cost of synthetic peptide.

1.4.4 Molecular detection methods

1.4.4.1 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

More recently, several research groups have attempted to develop assays for the identification of EV71 based on conventional reverse transcription polymerase chain reaction (RT-PCR) followed by nucleic acid hybridization or sequencing. PCR has been accepted as the "new" standard for the detection of viruses in diagnostic microbiology laboratories bypassing the need for the tedious and lengthy process of isolating the virus from clinical specimens. Most PCR primers designed for the detection of enteroviruses were targeted at the highly conserved 5′UTR region (Rotbart, 1990; Zoll *et al.*, 1992) as well as the VP2 capsid protein-coding region. The advantage of a detection strategy for enteroviruses based on the 5′UTR is that a single pair of primers could be used for identifying enteroviruses in general. However, no information could be obtained with regard to the identity of specific enterovirus without proceeding to sequence the amplicons. Due to a lack of correlation between the nucleotide sequence of these genomic regions and enterovirus serotypes, neither the 5′UTR (Rueckert, 1996), the VP2-coding region (Oberste *et al.,* 1998) nor the RNA polymerase-coding region (Casas *et al.,* 2001) can be used for enterovirus typing. Although there were phylogenetic studies targeting the VP4-VP2 junction which suggested that this region might be more suitable for developing serotype specific diagnostics, it appeared to correlate partially with serotypes (Arola *et al.*, 1998; Oberste *et al.*, 1998).

Since genomic analysis of the VP1 capsid protein was shown to correlate more closely with serotypes, conventional PCR was developed to target at the VP1-encoding DNA region (Brown *et al.*, 2000; Singh *et al.*, 2000, 2002; Yan *et al.*, 2001).

Brown *et al*. (2000) designed two pairs of primers that targeted at regions within and around the VP1 ORF and were successfully able to amplify EV71 genome specifically from 125 different EV71 tissue culture isolates. This was followed by Singh *et al.* (2000) who designed another pair of primers which could detect EV71 specifically from tissue cultures which were inoculated with clinical specimens. In a separate study, Yan *et al.* (2001) developed two sets of primers for the specific detection of EV71 and CA16, respectively. Both primer pairs were designed based on the VP1 regions of respective viruses and were evaluated against 57 isolates of EV71, 16 isolates of CA16 and several other enteroviruses. Although the EV71-specific primers were shown to be highly specific for the amplification of EV71 genome, the primers designed for CA16 detection were found to be non-specific as they were able to amplify EV71 genome as well (Yan *et al.*, 2001).

The study by Singh *et al.* (2002) was the first group to detect EV71 directly from clinical specimens using specific primers targeted at the VP1 region. In this study, it was demonstrated that the three pairs of primers which were designed previously by Brown *et al.* (2000) and Singh *et al.*, (2000) had low sensitivities (only 5%) in the detection of EV71 directly from clinical specimens. Therefore, a semi-nested RT-PCR strategy was used and this method achieved a higher sensitivity (53%) in detecting EV71 directly from clinical specimens in contrast to only 20% detection rate for the cell culture method. An improvement of the conventional RT-PCR, known as the miniature RT-PCR system was developed by Liao *et al.* (2005) for rapid diagnosis of RNA viruses, such as Dengue virus and EV71. The principle was based on conventional RT-PCR carried out in a miniature volume of 12cm by 21cm by 8.5cm and was operated by 9V batteries. Serum collected from a patient was

loaded into a valve and the one-step RT-PCR process was carried out in the automated system which reduced cross-contamination. Since a microchip was used to control the temperature, high efficiencies of PCR with detection limits as low as 6pg/μl can be achieved.

1.4.4.2 Combination of RT-PCR and microarray

In a recent study, a combination of both multiplex RT-PCR and microarray method was developed to specifically differentiate EV71 from CA16 (Chen *et al.*, 2006). Using specific primers, RT-PCR was first carried out to amplify both EV71 and CA16 viral RNAs. The amplicons are then labeled with fluorescent dyes and added to array slides which are spotted with 60-mers degenerate oligonucleotide probes specific for the detection of either EV71 or CA16. A diagnostic accuracy of 92% and 95.8% was achieved for specific detection of EV71 and CA16, respectively. In another study, a microchip method involving conventional RT-PCR, followed by hybridizing the biotinylated PCR products to specific probes of EV71 with 82% sensitivity was developed (Tsao *et al.*, 2006). However, the detection of EV71 from clinical specimens by this approach is laborious and the time for a diagnosis requires a minimum of ten hours which is too long in any outbreak situations.

1.4.4.3 PCR-ELISA

The detection of enteroviruses by PCR-ELISA approach was developed by Rotbart *et al.* (1994, 1997) based on their earlier work on the identification of enteroviruses using conventional RT-PCR involving the principle of amplification by RT-PCR and detection of amplified DNA products by ELISA. Sets of primers which are specific for highly conserved sequences within the 5′UTR as well as the VP2

capsid protein-coding regions have been successfully used to develop efficient methods for the rapid and sensitive detection of enteroviruses (Romero, 1999). Such approach is currently available commercially as a Chemicon Pan-Enterovirus OligoDetect™ kit (Chemicon International, USA). This detection kit is based on the amplification and detection of nucleic acid products produced by RT-PCR. Theoretically, this detection method can be used to detect enteroviruses in different sample types which may escape detection by traditional cell culture approach due to small quantities of virus. As the primers were designed to target at the 5′UTR region of the viral genome, the PCR-ELISA method was applicable for the detection of enteroviruses in general, but not the specific serotype.

1.4.4.4 Real-time PCR

Real-time PCR is an improvement of the classical PCR amplification process in which monitoring of the accumulating amplicons during the course of the PCR can be carried out in real time. It was first described by Higuchi *et al.* (1993), who proposed a kinetic model that the process of denaturation, primer annealing and template extension did not just occur at discrete temperatures as in the case of conventional PCR, but may be occurring simultaneously during temperature transitions. In real-time PCR, monitoring of the DNA amplification process in real-time was made possible by labeling of probes and amplicons with fluorogenic molecules (Mackay *et al.*, 2002; Espy *et al.*, 2006). Due to the relatively small sample volume and high sensitivity of the real-time PCR system, small changes in fluorescence emission can be monitored readily, thereby enable small initial amount of templates to be detected (Wittwer *et al.*, 2001). Quantitation of the amount of templates in any specimen can also be achieved based on standard curves generated

from real-time PCR (Rasmussen, 2000). There are three major chemistries available for amplicon detection based on real-time PCR. They can be generally classified into amplicon sequence specific methods or non-specific methods.

The sequence non-specific detection method is based on DNA-binding fluorogenic molecules. A dye known as SYBR Green I is widely used in real-time PCR these days. Upon exposure to a suitable wavelength of light, it will fluoresce when associated with dsDNA which will be detected by sensitive fluorimeters in the real-time PCR instrument. For the SYBR Green I approach, fluorescent melting curve analysis can be applied for detection of different amplification products based on differences in melting temperature (Tm) which depends on the total GC content and length of the amplified product (Wittwer *et al.*, 2001). However, formation of primer-dimers can confuse interpretation of results, as the fluorogenic dye can bind to any dsDNA product. Therefore, the melting curve analysis is useful here where the shorter primer-dimer can be discriminated by its reduced Tm compared to the actual DNA amplicon (Wittwer *et al.*, 2001). As SYBR Green I-based assays are not specific, they are often used for screening assays where further analysis of specimen is usually required for confirmation (Espy *et al.*, 2006).

For amplicon sequence specific method, real-time PCR uses a *Taq* polymerase that has a 5′exonuclease activity. The principle of this process was first described by Holland *et al.* (1991) and it was later utilized with fluorescent and quencher dye by Livak *et al.* (1995). In the TaqMan probe-based approach, the probe contains one fluorophore (6-carboxy-fluoroscein) at its 5’ terminus and a quencher (6-carboxytetramethyl-rhodamine; TAMRA) at its 3’ terminus. In close proximity, both

dyes form a quenched system and no fluorescence is observed. However, when the probe hybridizes to the template, the 5′exonuclease activity of the *Taq* polymerase will hydrolyze the probe during PCR amplification. Once the fluorophore is separated from the quencher, the emission of the fluorophore is no longer being quenched and hence fluorescence will be detected. TaqMan probe-based real-time PCR assay has been used in diagnosis of viruses such as Epstein-Barr virus and Cytomegalovirus (Jebbink *et al.*, 2003). However, since the 5′exonuclease activity is irreversible, analysis of the results is finalized once the PCR amplification is completed. Therefore, unlike SYBR Green I assay, no secondary confirmation like the melting curve analysis is available for TaqMan probe-based assays.

Another approach involves the use of a pair of adjacent, fluorogenic hybridization oligoprobes. The upstream oligoprobe is labeled with a 3′ donor fluorophore (Fluorescein) and the downstream probe is labeled with either LightCycler Red 640 or Red 705 acceptor fluorophore at the 5′ terminus. When both oligoprobes are hybridized to the target template, they are located within 10 bp of each other. This approach involves the principle of fluorescence resonance energy transfer (FRET), a method in which the donor dye transfers a quantum of energy to the acceptor dye upon excitation by ultraviolet, and the acceptor dye releases a photon of different wavelength upon return to rest. Since more fluorescence is emitted with increased number of amplicons, quantification can be performed (Bernard *et al.*, 2001). In addition, since the probes are not significantly hydrolyzed, the LightCycler is able to monitor changes in fluorescence emission when the oligoprobes are denatured from their amplicons (Mackay *et al.*, 2003; Espy *et al.*, 2006). When the temperature is slowly raised, the probes will not be able to anneal to the target PCR

product and the FRET signal will be lost. The temperature where half the FRET signal is lost is usually referred to as the Tm of the Hybridization probe system which depends on the GC content and length of the hybridization probes. This strategy has been successfully used for differentiating Varicella-Zoster virus (Espy *et al.*, 2000a) from herpes simplex virus (Espy *et al.*, 2000b) and Hepatitis C virus (Ratge *et al*., 2000).

Several studies have been carried out to detect enteroviruses based on the real-time PCR approach. Read *et al.* (2001) developed a real-time SYBR Green I assay to detect enteroviruses, differentiating them from other viruses such as HSV-1, HSV-2 and Varicella-Zoster virus that are able to cause central nervous system (CNS) infections. Using the ABI Prism 7700 PCR instrument (USA), Verstrepen *et al.* (2001) and Nijhuis *et al.* (2002) developed TaqMan probe-based assays for specific detection of enteroviruses. In the study published by Verstrepen *et al.* (2001), a 95% detection rate of enterovirus was achieved and the assay was successfully able to detect up to 11.8 genome equivalents of enterovirus per ml of cerebrospinal fluid (CSF). Similarly, in the study reported by Nijhuis *et al.* (2002), the TaqMan probe-based assay developed was able to detect 60 different enteroviruses and no cross-reactivity with other viruses was observed. Direct detection of enteroviruses from the clinical specimens by real-time TaqMan RT-PCR was developed by Watkins-Riedel *et al.* (2002) and Petitjean *et al.* (2006) using the LightCycler real-time detection system (Roche Molecular Biochemicals, Germany) and the ABI Prism 7700 PCR instrument (USA). In another study, Kares *et al.* (2004) developed a Hybridization probe-based assay which not only could simultaneously detect enteroviruses and rhinovirus but it was able to differentiate them.

1.5 Treatment of enterovirus 71 (EV71) infection

A number of therapeutic agents with significant anti-viral activities against enteroviruses have been developed but none has come close to commercialization. Several stages in enterovirus replication such as cell susceptibility, attachment to host cell, uncoating, synthesis of viral mRNA and maturation of new virus proteins could serve as potential targets for therapeutic intervention.

1.5.1 Antiviral drugs

The "WIN" group of compounds is the most promising drugs to date. The antiviral activities of such compounds were thought to be mediated by stabilizing the viral capsid and preventing virus uncoating upon binding to host cell (Rotbart *et al.*, 1998). One of the "WIN" compounds – pleconaril was found to have significant therapeutic effects in aseptic meningitis, acute flaccid paralysis and encephalitis as a result of enteroviral infections (Rotbart *et al.*, 1998; Pevear *et al.*, 1999). Pleconaril (VP-63843) is a novel, broad-spectrum anti-picornaviral agent. This characteristic is highly desirable for any antiviral agent which targets a virus causing CNS and respiratory tract infections. Currently, pleconaril is undergoing Phase III clinical trials in the US. Clinical studies have shown that there was a reduction in the duration and the intensity of symptoms in children and adults with enteroviral meningitis (Romero, 2001). However, despite having a very good side effect profile which was attributed to its metabolic stability, pleconaril was found to show limited effectiveness against EV71 (Pevear *et al.*, 1999), especially against EV71-associated neurological complications. Other drugs that showed therapeutic potentials include enviroxime which inhibited RNA replication by targeting at the 3A region of the viruses (Heinz and Vance, 1995). The 3C protease inhibitors were developed by the Agouron

Pharmaceuticals which resulted in inhibition of specific viral protein processing (Patick *et al.*, 1997). However, studies of these antiviral agents were mainly based on polioviruses and rhinoviruses which are the two most important members of the Piconarviridae family and their effects on EV71 are still unknown.

1.5.2 Other therapeutic approaches

1.5.2.1 Intravenous immunoglobulin (IVIG)

IVIG is a blood product which contains pooled IgG antibodies extracted from the plasma of over a thousand blood donors. IVIG was used extensively in the management of neurological cases during HFMD outbreaks in Taiwan (Huang, 2001) and Western Australia (McMinn *et al.,* 2001a). Recently, a study has shown that the administration of IVIG could effectively reduce the level of pro-inflammatory cytokines such as interleukin-6 (IL-6) and interleukin-8 (IL-8) in patients suffering from autonomic nervous system (ANS) dysfunction and pulmonary edema, which arise from EV71-associated brainstem encephalitis (Wang *et al.*, 2006). Moreover, milrinone was shown to display therapeutic effects in EV71-induced pulmonary edema and/or pulmonary hemorrhage (Wang *et al*., 2005; Wang *et al*., 2006), suggesting that both milrinone and IVIG when used together, might be good therapeutic agents for EV71 infections. The antiviral activities of *Salvia miltiorrhiz*a (Danshen) were also tested against EV71 in an *in vitro* system and were able to neutralize EV71 and hence preventing cytopathic effects (CPE) in Vero cells, RD cells or MRC-5 cells, suggesting that the chinese herb might have a potential to be used as an therapeutic agent against EV71 (Wu *et al.*, 2007).

1.5.2.2 Interferon

Interferons have also been shown to be a potent inhibitor of most enteroviral infections and synergistic protection was observed when used in conjunction with capsid-binding compounds (Kandolf *et al.*, 1985; Langford *et al.,* 1985). Interferon-γ (IFN-γ) is a cytokine secreted mainly by Th1 T-cells and it enhances specific T-cell mediated immune responses. Direct antiviral effects of interferons include degradation of the viral mRNA and inhibition of protein synthesis. A recent study indicated that type I IFNs may play an important role in the host defense of EV71 and that an earlier detection of viral involvement of the central nervous system followed by immediate type I IFN therapy should be considered an appropriate treatment regimen for EV71 (Liu *et al.,* 2005). However, another study has shown that this approach of antiviral treatment was found to have limited effectiveness for EV71 (Arya, 2000).

1.6 Vaccines

Jenner's cowpox vaccine represented the first use of a live-attenuated organism which became the first generation of vaccines introduced in science. In the years following 1770, EEnglish physician Edward Jenner (1749-1823) observed that milkmaids infected with a viral disease called cowpox rarely developed a similar disease, smallpox, which was prevalent during that period. On May 1796, Jenner tested his theory by inoculating James Phipps, a healthy young boy who had never had cowpox or smallpox, with material from the cowpox blisters of the hand of Sarah Nelmes, a milkmaid who had caught cowpox. Six weeks later, Jenner injected the boy with fluid from a smallpox pustule, which would have been the routine attempt to

produce immunity at that time. However, the boy remained free of the dreaded smallpox.

The development of novel antiviral agents and vaccines that are effective against microorganisms resistant to current therapies is of paramount importance in the global fight against infectious diseases. Efforts have been substantially facilitated by the progress in the fields of molecular biology, which have enabled the production of new generation vaccines. Vaccines have been an effective device for mankind to control and even eradicate human diseases. In spite of the undeniable success of using vaccines against a large number of infectious diseases, the resourceful strategies of virus to escape their host's immune defense still pose a tough challenge to scientists for the development of effective vaccines. The increasing knowledge of the molecular basis of interactions between the pathogen and its host enables better and smarter approaches to design effective vaccines.

The host's immune defense mechanisms against infectious agents can be divided into two main components which include innate (native) and adaptive (acquired) immunity. The adaptive immunity can be further divided into two general types, namely, humoral immunity and cell-mediated immunity. Humoral immunity involves B-cells, which recognize and bind circulating soluble antigen via surface antibody receptors and respond by secreting antigen-specific antibodies. These activated B-cells will subsequently become plasma cells that provide long-term memory (Davis and McCluskie, 1999). For cell-mediated immunity, T-cells are the effector cells which will recognize antigen(s) presented on cell surfaces in the context of the major histocompatibility complex (MHC) genes. The $CD8^+$ T-cells are usually

activated and differentiated into cytotoxic T-cells which can destroy the infected host cells, induce apoptosis, or reduce the ability of the pathogen to replicate inside cells. The $CD4^+$ T-cells are activated to become T-helper cells which will secrete lymphokines that stimulate the activation and function of both B- and T-cells. Therefore, an ideal vaccine candidate should be able to trigger both humoral and cell-mediated immune responses in the host. In addition, the delivery system of a vaccine may contribute to the orientation of the immune response elicited and hence this must be taken into consideration when assessing the immunogenicity and protective efficacy of the vaccine candidate.

1.6.1 Live-attenuated vaccines

Live-attenuated vaccines are live micro-organisms that have been cultivated under conditions which disable their virulent properties. They are highly effective in evoking a full range of immune responses. The advantage of live attenuated vaccines is that one or two doses are able to provide immunity that can last for a long period of time. The use of live-attenuated organisms has since proven effective for rabies, yellow fever, poliomyelitis, measles, mumps, rubella and chicken pox (Gordon, 1997). A good example is the polio vaccine where two approaches are currently used. The first (Salk vaccine) was developed by Jonas Salk in 1955 which consists of an injected dose of inactivated poliovirus. Thereafter, Albert Sabin produced an oral polio vaccine (OPV) using live-attenuated poliovirus and human trials of Sabin's vaccine started in 1957 and it was finally licensed in 1962. The live-attenuated poliovirus replicates very efficiently in the gut which is the primary site of viral infection and replication. However it is unable to replicate efficiently within nervous system tissue. The OPV also proved to be superior in administration and is able to

provide longer and lasting immunity as compared to the Salk vaccine (Sabin *et al*., 1960).

A recent study has shown that active immunization of 1-day-old mice with avirulent EV71 strain or CA16 virus via the oral route developed anti-EV71 antibodies with neutralizing activities and passive immunization with the CA16-immune serum prolonged the survival rate of mice upon lethal EV71 challenge (Wu *et al.,* 2007). However, live vaccine carries the risk of contamination with potentially dangerous viruses and has been reported to cause complications and an example of such occurrence is the smallpox vaccine. Because this vaccine comprises of live virus, even though it is a weakened virus, occasionally it can cause infections in susceptible vaccinated people or those in close contact with them. People with weakened immune systems or certain skin conditions are also susceptible to complications from such vaccine (Gordon, 1997). In addition, reversion to the original pathogenic form is also possible. Interference from circulating antibody of any source like immunoglobulins or blood products can also result in vaccine failure. In addition, numerous studies have shown that frequent mutations of the viral genome might give rise to evolving strains within the community (Brown *et al.,* 1999; Mizuta *et al.,* 2005; Wang *et al.,* 2002) and these mutated strains may not be neutralized by antisera raised against the selected vaccine strain.

1.6.2 Killed whole vaccines

Killed vaccines are known to be stable, safe and can be given as polyvalent vaccines. However, multiple injections are required and cell-mediated immunity may not be induced (Nathanson and Langmuir, 1995). A previous study has used 0.1%

(v/v) formaldehyde as a chemical formulation to kill live virus prior to immunization and has shown a significant level of immunogenicity elicited in ICR mice by such approach (Yu *et al.,* 2000). Another study by Wu *et al.* (2002) showed that heat-inactivation at 56^{o}C for 30 min was also able to kill and inactivate EV71 as well as to elicit a significant immune response in immunized mice. Hence, the immunogenicity of the heat-inactivated EV71 is comparable to that of the formaldehyde-inactivated virus. Jonas Salk developed formaldehyde-inactivation process to inactivate poliovirus (Nathanson and Langmuir, 1995; Minor, 1997). Being the first successful vaccine for enteroviruses, it was also known as the inactivated polio vaccine (IPV), which was first licensed for human use in the year 1955 (Plotkin and Mortimer, c1994). However, the most dramatic effects on the incidence of poliomyelitis worldwide are due to the use of Sabin polio vaccine which was first licensed for human use in the year 1962 (Plotkin and Mortimer, c1994). Inactivation can also be carried out by β-propionolactone treatment and γ-irradiation. Although inactivated viruses may not be potent inducers of CTL response (Gordon, 1997) but the laborious and costly procedures involved in virus cultivation and purification as well as safety issues are the major concerns.

1.6.3 DNA vaccines

An alternative approach for vaccination is through direct inoculation of the host with DNA encoding a protein vaccine candidate. The use of DNA or mRNA for immunization is a recent development in the vaccine field. The DNA vaccine consist of a plasmid bearing the ORF of interest as well as control elements such as terminators, promoters and polyadenylators that allow expression of the target gene in eukaryotic cells. DNA vaccines against foot and mouth disease virus (FMDV) have

been developed based on virus genome (Beard *et al.,* 1999) and DNA encoding viral capsid protein (Chinsangaram *et al.,* 1998). Unfortunately, the DNA-vaccinated animals were either partially protected or unprotected from viral challenge, suggesting the need for further improvements (Chinsangaram *et al.,* 1998; Beard *et al.,* 1999). However, in a recent study, the VP1 gene of EV71 was cloned and expressed in a cell-free *in vitro* expression system using a eukaryotic expression vector, pVAX1 (Tung *et al.,* 2007). Female Balb/c mice were immunized with the DNA vaccine constructs and the anti-VP1 IgG antibody exhibited neutralizing activity against EV71.

1.6.4 Subunit or purified component vaccines

These third generation vaccines comprise purified components from pathogenic organisms which sometimes mimic a small region of microbial protein; hence they are considerably stable and safe for usage. A recent study conducted by Wu *et al.* (2002) compared the potency of a heat-inactivated EV71 vaccine with two subunits vaccines (DNA-based vaccine and recombinant VP1 protein-based vaccine) to elicit immunity and provide protection against lethal EV71 challenge in susceptible newborn Institute of Cancer Research (ICR) mice. All vaccine candidates were observed to elicit high titers of anti-EV71 neutralizing antibody in the mice model. With a challenge dose of 2,300 LD_{50} virus/mouse, suckling mice born to dams immunized with the inactivated virus showed 80% survival. However, the subunit viral vaccine provided protection only at a lower challenge dose of 230 LD_{50} virus/mouse with 40% survival for DNA vaccine and 80% survival for recombinant protein vaccine (Wu *et al.,* 2002). This suggests that the VP1 capsid protein contains neutralizing epitopes independent of other proteins of EV71.

In another recent study, a vaccine strategy using attenuated *Salmonella enterica* serovar Typhimurium strain to express and deliver the VP1 capsid protein of EV71 was shown to protect ICR newborn mice against EV71 infection (Chiu *et al.*, 2006). Using transgenic tomatoes expressing VP1 capsid protein as an oral vaccine, Chen *et al.* (2006) showed that the antisera from immunized mice could neutralize EV71 infectivity and this further highlighted the potential use of VP1 as the antigen to develop vaccines against EV71.

1.6.5 Synthetic peptide vaccines

Synthetic peptide represents an interesting alternative candidate as subunit vaccine. Production of synthetic peptide is much easier and faster with lesser adverse effects when administered as a potential vaccine. However synthetic peptides are poorly immunogenic due to their small size. To increase the immunogenicity of a synthetic peptide, it can be linked to a larger carrier protein to enhance uptake and processing by antigen presenting cells (APCs) such as dendritic cells (DCs) and macrophages. The use of either natural or artificial adjuvants has been found to enhance the immunogenicity of synthetic peptide-based vaccines by promoting their uptake and activating DCs to initiate the immune response (Rosenthal and Zimmerman, 2006). The immunogenicity of synthetic peptides could also be enhanced by designing chimeric peptide constructs incorporating both B-cell epitope(s) and T-cell epitope(s), thereby activating B- and T-cell immune responses. This approach was used to develop a hepatitis C virus (HCV) vaccine where both B-cell epitopes and T-helper epitopes when presented as a chimeric synthetic peptide elicited high titers of neutralizing antibody against the virus (Torresi *et al.*, 2007).

1.7 Epitope mapping

1.7.1 Approaches to epitope mapping

Several methods used for identifying protein epitopes have been described in several reviews (Berzofsky, 1985; Jemmerson & Paterson, 1986; van Regenmortel, 1989). Among them, only X-ray crystallography is based on a structural analysis and can describe the spatial relationships found in an antigen-antibody complex. In contrast, the other methods rely on binding assays and are therefore based on functional analysis.

1.7.2 X-ray crystallography

Crystallographic analysis of several protein-antibody complexes has permitted the direct visualization of interacting epitope and paratope residues (Padlan, 1992). In all cases, the epitope identified was found to be discontinuous and comprised between 15 to 22 amino acid residues that were observed to be in contact with residues of the paratope. Using X-ray crystallography, a previous study has shown that the neuraminidase epitope of the influenza virus is characterized as a discontinuous epitope which is localized on four surface loops and made up about 15 residues that appeared to be in contact with the antibody (Colman *et al.,* 1987).

1.7.3 Viral mutants and monoclonal antibodies

A most common method used in the mapping of viral epitopes involves the analysis of interactions between monoclonal antibodies and viral mutants. Viral variants with amino acid substitutions in their structural proteins are tested against a panel of neutralizing monoclonal antibodies which are raised against the whole virus. Amino acid substitutions of the monoclonal antibody-resistant (MAR) mutants are

analyzed and compiled to yield a list of residues thought to form a particular viral epitope. Among the picornaviruses, several escape mutant studies have been carried out with polio and rhinoviruses (Hogle and Filman, 1989; Mosser *et al.,* 1989; Sherry *et al.,* 1986). Stanway *et al*. (1983) has described that polioviruses can be divided into three serotypes on the basis of their neutralization reactions with specific immune sera. The single, major antigenic site involved in the neutralization of poliovirus type 3 was reported to be located at the N-terminal region of VP1 and this was based on the isolation of mutant polioviruses resistant to neutralization by monoclonal antibodies which had amino acid substitutions clustered in this region (Minor *et al.,* 1983; Minor *et al.,* 1984; Minor *et al.,* 1985). However, in contrast to this, the neutralization of poliovirus type 1 involves multiple epitopes, some of which appeared to be independent, and hence involve independent antigenic sites (Emini *et al.,* 1982). However, the Sabin strain of poliovirus type 2 resembles poliovirus type 3 in so far as most of the antibodies examined selected for mutations within the region homologous to the principal site of poliovirus type 3 and Minor *et al*., 1986 showed that poliovirus type 2 resembles poliovirus type 3 in having a major immunodominant locus in VP1.

Picornaviral antigenic sites may appear to be mainly limited to the tips of some variable, solvent-exposed loops (Hogle *et al.,* 1985; Lea *et al.,* 1994; Page *et al.,* 1988; Rossmann *et al.,* 1985). However, residues which are part of contact epitopes but not of the corresponding functional epitopes, and residues that are involved in functional epitopes but are not amenable to substitutions, will not be detected. Although amino acid substitutions which affect recognition of a protein by an antibody are generally limited to the antigen surface area in contact with the antibody

(Colman *et al.,* 1987; Knossow *et al.,* 1984), conformational differences in a surface loop may be induced by sequence and conformational changes in a neighboring loop as shown in studies on VP1 and VP3 by Filman *et al*. (1989). Similarly, studies on MAR mutants of Foot-and-mouth disease virus (FMDV) showed that substitutions at residues outside the antibody-binding site could also have an epitope-disrupting effect by forcing the immunodominant G-H loop to a different position, away from the C-terminus of VP1 (Parry *et al.,* 1990). In addition, Diamond *et al.* (1985) and Blondel *et al*. (1986) have raised the possibility that mutants located outside the binding site may escape viral neutralization by preventing some structural alterations. Therefore, the current view on the location and features of antigenic sites of epitopes mapped by MAR mutant analysis may be biased toward regions which do not compose the functional epitope itself but which, upon amino acid substitution, affect its structural conformation and hence its reactivity with the neutralizing monoclonal antibodies.

1.7.4 Anti-peptide antibodies

This approach consists of using peptide fragments for immunization, followed by testing to determine if the resulting anti-peptide antibodies react with the intact viral protein. A significant reactivity is usually considered as an indication that the respective peptide may be an epitope within the protein. It should be noted that epitope mapping studies undertaken with this approach are highly controversial due to 'order-disorder' paradox (Dyson *et al.,* 1988). Antibodies against a highly disordered state (the peptide) are able to recognize the highly ordered state (the folded protein in its native conformation), while the reverse may not necessarily be the case. Another common approach to epitope mapping involves measuring the ability of antibodies raised against the intact molecule to cross-react with recombinant or synthetic peptide

fragments. If a peptide is able to bind to the antibodies, it can be assumed that it contains a linear epitope of the protein. A more precise localization of the epitope is achieved by testing overlapping peptides of decreasing size and determining the smallest peptide that still possesses a significant level of antigenic activity (Benjamin, 1977).

1.7.5 Mapping using recombinant peptides

Localization of epitopes can be carried out by expressing parts of the viral protein in a prokaryotic expression system and measuring the antigenic activity between the recombinant proteins and antibodies raised against the whole virus particle. A study has described that this approach is able to identify linear epitopes but may not be able to identify conformational epitopes because the conformation needed for antigenicity may not be retained in the fusion products (van Regenmortel, 1992). Using this method, Ketterlinus and Wiergers (1994) have managed to map antigenic domains within the VP1 capsid protein of poliovirus by introducing successive nested deletions into recombinant VP1 which are then subjected to immunoblotting with monoclonal antibodies raised against the entire VP1 protein. In studies carried out on the human T-cell lymphotropic virus (HTLV) by Hadlock *et al.* (1995), the immunodominant HTLV-specific epitope within the HTLV-1 transmembrane glycoprotein was delineated using neutralizing antibodies raised against the whole virus particles.

1.7.6 Mapping using synthetic peptides

Epitope mapping with synthetic peptides consists of synthesizing a large number of short peptides (usually via automated solid-phase synthesis on a polymeric

resin) and testing each of them for their capacity to be recognized by anti-protein antibodies. The pepscan method developed by Geysen *et al.* (1984, 1987) has been used extensively in the identification of viral epitopes. This method allows the concurrent synthesis of hundreds of peptides on polyethylene pins and hence suitable for systematic screening of all possible overlapping peptides of a protein. Antigenic sites on capsid proteins of poliovirus type 3 (Roivainen *et al.,* 1991) and CA9 (Pulli *et al.,* 1998) have been identified using the peptide-scanning technique. This approach has also enabled the identification of serotype-specific epitopes of coxsackievirus B3. By testing overlapping peptides spanning the entire VP1 capsid protein of CB3 with sera raised against the whole virus particle, several antigenic determinants were identified (Haarmann *et al.,* 1994). Type-specificity of the peptides were further tested by using antisera raised against other coxsackieviruses (CB1 to CB6) to eliminate the possibility of cross-reactivities between serotypes. However, the nature of pepscan analysis restricts epitope detection to linear epitopes only.

In addition, there is evidence that the antigenicity of peptides is highly dependent on the type of immunoassay used to determine their antigenic reactivity (van Regenmortel *et al.,* 1988). Peptides adsorbed onto a solid phase tend to have a significant proportion of their surface not favourable for binding to antibody and hence often found to possess lower reactivity than free peptides in solution. Furthermore, the activity of certain peptides is found to increase when conjugated to a carrier, presumably because the lower steric hindrance at the surface of the carrier protein allows the peptide to adopt a more suitable conformation for antibody recognition. The choice of conjugation procedure as well as the use of different

coupling reagents have also been shown to greatly influence the antibody-binding properties of the peptide (van Regenmortel *et al.,* 1988).

1.8 Animal models for Enterovirus 71 (EV71)

Although immune responses such as elevated antibody levels detected soon after vaccination may give some indication of the immunogenicity of a vaccine construct, they do not necessarily provide a reliable guide to the capability of the vaccine to prime an *in vivo* protective response. Successful protection against infection in an animal model remains the best mean to address the protective efficacy of a vaccine. Hoever, one of the major barriers to develop EV71 vaccine is the lack of a suitable universal animal model for the testing of vaccine immunogenicity and protective efficacy (McMinn *et al.,* 2002). Mice are susceptible to EV71 infection in the first few days of life before they become completely resistant to the virus by 6 days old (Roberts and Boyd, 1987; Yu *et al.,* 2000). In addition, EV71 infection has been found asymptomatic in most strains of adult mice which include Balb/c, C3H, ICR, CD28 knock-out and TNF-α receptor knock-out mice (Wu *et al.,* 2001). Hence, the efficacy of an EV71 vaccine candidate cannot be evaluated in actively immunized mice but can only be addressed via passive immunization whereby newborn mice are challenged with EV71 and subsequently injected with the immune serum from actively immunized adult mice (Foo *et al.,* 2007b; Yu *et al.,* 2000). Pathogenicity of enteroviruses had been studied in laboratory animals such as newborn mice (Koroleva and Gracheva, 1980) and *cynomolgus* monkeys (Hashimoto *et al.,* 1982). Such experimental studies can yield various information regarding the pathogenicity of EV71 in different hosts.

In 1975, a widespread outbreak of a severe neuroparalytic disease occurred in Bulgaria. The disease was manifested as poliomyelitis, meningoencephalitis and acute flaccid paralysis, mostly ending lethally. Koroleva and Gracheva (1980) conducted animal model experiments for EV71 using the virus-specific materials as inoculants for subcutaneous injection into Newborn Cotton (NBC) rats and Newborn White (NBW) mice. In addition to newborn animals, three to four weeks old adult cotton rats and white mice were infected intracerebrally, intramuscularly or with a combination of both. EV71 strains isolated from material obtained during the outbreak of poliomyelitis-like disease in Bulgaria were found to be pathogenic for NBW mice and NBC rats. The disease symptoms were similar to those caused by coxsackie A viruses. In NBW mice, the infection manifested as paresis, paralysis and ended lethally and in NBC rats, the lethal outcome was preceded by a sharp deterioration of general condition and paresis. Myositis induced in the NBC rats and NBW mice was a characteristic of enterovirus A. In addition, diffuse inflammatory degenerative changes were noted in the central nervous system (CNS) of adult cotton rats and the infection was found to be asymptomatic in adult white mice.

In contrast to the clinical lesions observed in various animals by the EV71 strains isolated from the Bulgarian outbreak, the prototype EV71 BrCr strain did not induce a clinically manifested disease in both NBC rats or NBW mice as well as in the adults (Chumakov *et al.,* 1979). Preliminary studies were also conducted on primates using rhesus monkeys and green marmosets as laboratory animals. Syrian hamsters were also used as an animal model for EV71 infection (Chumakov *et al.,* 1979). It is important to note that the EV71 strains isolated from Bulgarian outbreak possessed the ability to damage the CNS in monkeys and adult cotton rats.

Recently, experimental infections with EV71 were undertaken in Taiwan (Yu *et al.*, 2000). EV71 was found to be able to induce death in neonatal mice in an age- and dosage-dependent manner. The mortality rate was 100% following intraperitoneal inoculation of one-day old ICR mice. Mortality gradually decreased as the age of mice increased at the time of inoculation and no deaths occurred for mice older than six days of age. Lethargy, failure to gain weight, rare limb tremors and paralysis were other symptoms observed in the EV71-infected mice prior death (Yu *et al.*, 2000).

Nagata *et al.* (2002) studied the neurovirulence of EV71 by neuropathological analysis of adult *Cynomolgus* monkeys after experimental infections with five EV71 strains which were isolated from individual patients with fatal encephalitis, meningitis and HFMD. Upon intraspinal inoculation, the monkeys developed neurological manifestations within one to six days post inoculation, irrespective of the strains used. These manifestations included not only pyramidal tract signs such as flaccid paralysis but also signs such as tremors and ataxia. Histological and viral examinations confirmed virus replication in the spinal cord, brainstem, cerebella cortex and dentate nuclei and cerebrum. The EV71 strains isolated during the 1970s and 1990s showed no particular genetic differences with respect to neurotropism. Therefore, it is clear that EV71 has a wider neurotropism than polioviruses (Nagata *et al.,* 2002). Although *Cynomolgus* monkeys were susceptible to infection with low passage clinical isolates of EV71 who eventually developed encephalomyelitis and poliomyelitis-like paralysis after subcutaneous (Hashimoto *et al.,* 1978) or intraspinal inoculation (Hagiwara *et al.,* 1984), the high cost of purchase and maintenance of these animals is a major barrier to their usage in large scale pathogenesis studies. A more promising approach to understand the pathogenesis of enterovirus encephalitis and for testing candidate

vaccine is through the development of transgenic mice that may express the appropriate human receptor molecule (McMinn *et al.,* 2002).

1.9 Specific aims

Enterovirus 71 has been regarded as the most important neurotropic enterovirus after the eradication of the poliovirus (McMinn, 2002). Currently, there are neither specific antiviral therapies nor effective vaccines available for EV71. Although inactivated whole virion has been suggested as potential vaccine candidate, studies have also shown that subunit vaccines expressing the full-length VP1 capsid protein were able to elicit neutralizing antibodies and conferred protection to newborn mice upon lethal viral challenge (Wu *et al.,* 2002; Chiu *et al.,* 2006). However, the exact location of these neutralizing epitopes along VP1 has not been identified.

Therefore, this study aims to identify and characterize the linear neutralizing epitopes on the VP1 capsid protein of the Enterovirus 71 strain 41 (5865/SIN/00009) (belonging to subgenogroup B4). Immune sera raised against 95 diphtheria toxoid-conjugated synthetic peptides spanning the entire VP1 region were raised in adult Balb/c mice to identify regions that are able to elicit neutralizing antibodies against EV71 and hence confer *in vitro* protection to Rhabdomyosacroma (RD) cells as host. The *in vivo* protective efficacy of these antibodies via maternally- and passively-transferred routes was evaluated using suckling Balb/c mice challenged with a lethal viral dose.

Previous studies have shown that the immunogenicity of a synthetic peptide could also be enhanced by designing a chimeric peptide construct incorporating both

B-cell and T-cell epitopes (Haro and Gomara, 2004; Torresi *et al.*, 2007). Hence, this study also aims to identify human $CD4^+$ T-cell epitopes on the VP1 capsid protein using the ProPred prediction software to predict promiscuous epitopes. The ability of the predicted epitopes to induce human EV71-specific $CD4^+$ T-cell proliferation was tested experimentally. The identified $CD4^+$ T-cell epitopes when linked to neutralizing linear VP1 epitopes as chimeric peptides can be further explored to elicit a higher titer of protective antibodies against EV71.

Isolation of EV71 in cell cultures, followed by neutralization using serotype-specific antisera such as the Lim Benyesh-Melnick (LBM) pool has been the practice in most diagnostic laboratories as the gold standard (Lim *et al*., 1960).). However, the duration for the isolation and identification of EV71 may require approximately one to two weeks and require specialized personnel. As the viral titer present in clinical specimens may be low, there is a need to propagate the virus and this contributes to the lengthy period for virus isolation. Hence, to design a peptide-based diagnostic specifically for EV71, this study also aims to identify IgG-specific immunodominant linear epitope on the VP1 capsid protein based on Pepscan analysis by applying a series of overlapping synthetic peptides spanning the entire VP1 to screen against sera from pediatric patients containing specific anti-EV71 IgG antibodies. As an alternative to synthesize peptide, the identified epitope was over-expressed as a soluble recombinant GST-fusion protein and evaluated as a capture antigen in Western blot analysis for the specific detection of human anti-EV71 IgG antibodies.

CHAPTER 2 MATERIALS AND METHODS

2.1 Microbiology

2.1.1 Bacterial work

2.1.1.1 Bacterial strains and plasmids

Bacterial strains and plasmids used for this study are listed in Table 2.1. The restriction map of pGEX-6p-1 vector (GE Healthcare Life sciences, UK) is shown in Figure 2.1.

2.1.1.2 Culture and storage of bacterial cells

LB medium was prepared according to Miller (1972) (Appendix I). LB agar was prepared by adding 1.5% (w/v) Oxoid Agar Technical No. 3 to liquid LB medium. All media with or without agar, were autoclaved for 20 min at 121ºC. *Escherichia coli* (*E. coli*) strains transformed with recombinant plasmids were maintained on LB agar plates supplemented with the appropriate antibiotics (ampicillin at 100µg/ml and/or chloramphenicol at 34µg/ml). Strains in current use were stored at 4°C on relevant plates for approximately 2 weeks. Long term storage was carried out by re-suspending exponential bacterial cultures in LB media supplemented with 20% glycerol (v/v) and stored at -80°C.

2.1.1.3 Preparation of chemically competent *E. coli* cells

E. coli strains were inoculated into 10 ml of LB media supplemented with the appropriate antibiotics and grown at 37ºC. An aliquot (1 ml) from the overnight culture was transferred into 50 ml of LB broth. The cells were grown to early exponential phase (OD_{600}= 0.15 to 0.3) in an orbital shaker at 37ºC. Cells were

harvested via centrifugation at 8,000 x g for 20 min and washed with 10 ml of ice-cold 0.1M $CaCl_2$.

Table 2.1 Bacterial strains and plasmids used in this study.

***Escherichia coli* Strains**	**Relevant Characteristics**	**References**
TOP10	Str^r, F^-, *recA1*, *endA1*, *galU*, *galK*	Invitrogen
DH5α	ϕ80d*lacZΔ*M15 *recA1 endA1 gyrA96 thi-1 hsdR17*(r_K^- m_K^+) *supE44 relA1 deoR Δ* (*lacZYA-argF*)*U169*	Invitrogen
BL21 (DE3) pLysS	Cam^r, F^-, dcm omp T hsdS($r_B^-m_B^-$) gallI, (DE3) [pLysS Cam^r]	Novagen
Plasmids		
pGEX-6P-1	Amp^r; expression vector for N-terminal GST fusion constructs	GE Healthcare Life Sciences
pGEX-6P-1-VP1	pGEX-6P1 carrying EV71 VP1 gene cloned as a 891 bp *Bam*HI-*Sal*I fragment	This study
pGEX-6P-1-SP32	pGEX-6P1 harboring residues 94-108 of VP1 cloned as a 44 bp *Bam*HI-*Sal*I fragment	This study

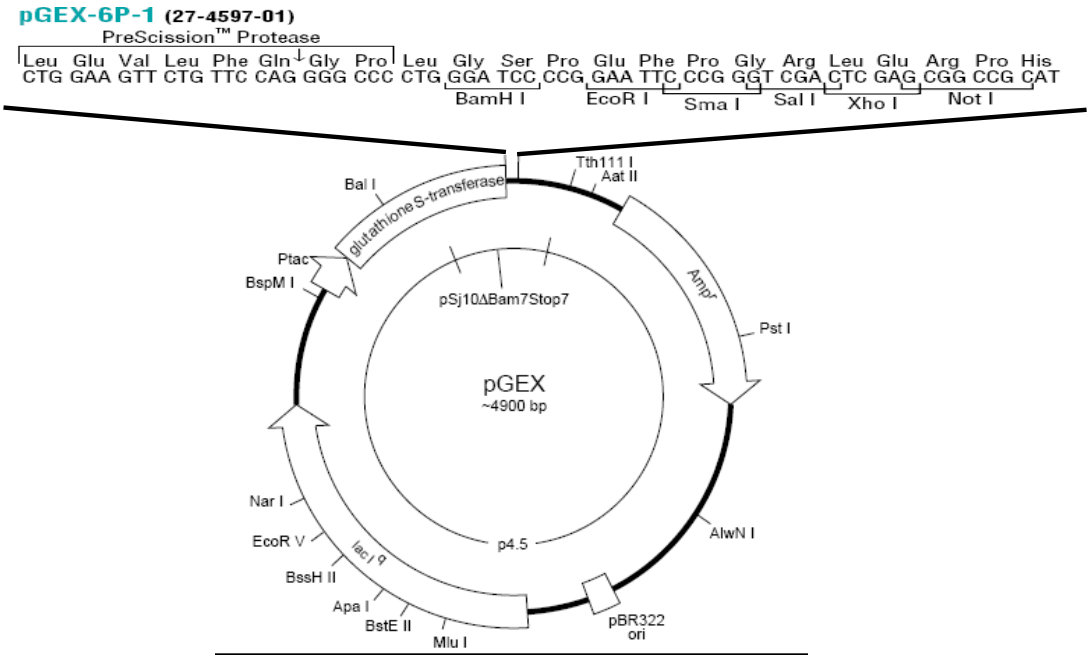


Figure 2.1 Vector map of the pGEX-6p-1 vector. The MCS is linked to the 3' end of the gene encoding for glutathione *S*-transferase (GST) and the nucleotide sequence encoding the recognition sequence for PreScission™ Protease. The GST domain can thus be cleaved from the fusion protein. A beta-lactamase gene that confers resistance to ampicillin is present in the vector to enable recombinant selection.

The supernatant was discarded and the cell pellet was resuspended in 10 ml of 0.1M $CaCl_2$ and incubated on ice for 30 min. The cells were subsequently centrifuged for 20 min at 8,000 x g. The supernatant was discarded and the cell pellet was resuspended in 5 ml of ice-cold 75mM $CaCl_2$ containing 15% (v/v) glycerol. The cell suspension was aliquoted into 200 µl aliquots and immediately stored at -80ºC immediately. Competent cells were capable of retaining their optimal competency if used within a month.

2.1.1.4 Transformation of chemically competent *E. coli* cells

The frozen chemically competent *E. coli* cells to be transformed were allowed to thaw on ice. Approximately 50 to 100 ng of plasmid DNA were added to 200 µl of thawed competent cells and incubated on ice for 30 min. The cells were subjected to heat shock for 1 min at 42ºC. LB broth (800 µl) was then added and incubated for 90 min at 37ºC in an orbital shaker rotating at 250 rpm. Aliquots of 100 µl of the transformed cells were plated on LB agar supplemented with the appropriate antibiotics and incubated overnight at 37ºC.

2.1.2 Virus work

2.1.2.1 EV71 strains

All virus strains used in this study are listed in Table 2.2.

Table 2.2 Virus strains used in this study.

EV71 Strains	Subgenogroups	GenBank Accession no.
7423/MS/87 (MS strain)	B2	U22522
5865/Sin/000009 (Strain 41)	B4	AF316321
2933-Yamagata-03	B5	AB213648
1585-Yamagata-01	C2	AB177812
75-Yamagata-03	C4	AB177813

2.1.2.2 Virus propagation

A 75cm^2 tissue culture flask (Nunc, USA) containing a 2 to 3 days old confluent Rhabdomyosarcoma (RD) cell monolayer was rinsed once with sterile phosphate buffered saline (PBS) and replaced with fresh minimal essential medium (MEM) growth media (Appendix II). A volume of 100 µl of virus suspension was added onto the monolayer. The flask was incubated at 37^{o}C and observed for cytopathic effect (CPE) as a result of viral infection. Infected cells were seen to round up, showed shrinkage and marked nuclear pyknosis, became refractile, eventually degenerated and fell off the surface (Lennette, 1969). The culture supernatants were harvested, transferred to a new vial and kept at -20^{o}C for short term storage and -80^{o}C for long term storage.

2.1.2.3 Purification and concentration of virus

Approximately 500 ml of culture supernatants containing viruses were obtained and clarified of the cell debris by centrifugation at 10,000 x g for 20 min at 4^{o}C. The virions were precipitated by adding 2.3% (w/v) sodium chloride (Sigma Aldrich, USA), followed by 7% (w/v) polyethylene glycol 8000 (PEG8000) (Sigma Aldrich, USA) with constant stirring for 1 h at 4^{o}C and stored overnight. The mixture was then centrifuged at 10,000 x g for 20 min at 4^{o}C to collect the PEG-virus pellet. The supernatant was gently discarded and the pellet was resuspended with 5 ml of TN buffer (Appendix II). The pellet was dislodged by gentle pipetting and the suspension was subjected to centrifugation at 3,000 x g for 30 min at 4^{o}C. The supernatant was collected and the virus was purified by loading the supernatant over 1 ml of 30% sucrose gradient cushion (Appendix II). The mixture was centrifuged at 25,000 x g for 4 h at 4^{o}C. The supernatant was gently discarded and the pellet was resuspended in

500 µl of PBS. The purified virus stock was kept at -20°C for short term storage or -80°C for long term storage. Before use as an immunogen, the virus was inactivated by heating at 56°C for 30 min. The amount of viral protein was quantified by the Bradford method using the commercially available Bradford assay kit (Bio-Rad Laboratories, USA) according to the manufacturer's instructions.

2.1.2.4 50% Tissue culture infective dose ($TCID_{50}$) assay

The assay was carried out in RD cells using the Reed and Muench formula (Reed and Muench, 1938) as outlined in Appendix III. To assay the number of infectious virion in a purified virus stock, serial 10-fold dilutions of the stock virus suspension were carried out in a 96-well microtiter plate using MEM growth media as diluent. A monolayer of RD cells from a $75cm^2$ tissue culture flask was harvested upon trypsinization with 3 ml of trypsin-EDTA (Gibco, USA) and the addition of MEM growth media to obtain a final concentration of 1 x 10^3cells/µl. A volume of 50 µl cell suspension was seeded into each well of a 96-well microtiter plate and 25 µl of each viral dilution were inoculated into appropriate wells in quadruplicates. The control wells contained RD cells without any virus. The plate was incubated at 37°C and observed daily for CPE up to 48 h.

2.1.2.5 *In vitro* microneutralization assay

The presence of neutralizing antibodies in human or mouse antisera was assayed in an *in vitro* microneutralization assay. Human or mouse immune serum samples were incubated for 30 min at 56°C to inactivate the complement. Briefly, 25 µl of two-fold serial serum dilutions were mixed with equal volumes of 10^3 $TCID_{50}$ of virus per well in triplicates in a 96-well microtiter plate. The virus-serum mixture was

incubated at 37°C for 2 h and 5 x 10^4 RD cells were then seeded in all wells. The plate was then incubated in a 37°C incubator with 5% CO_2 for 2 days. The control well contained virus and cell suspension with no mouse serum. The neutralizing antibody titer was read as the highest dilution of the mouse serum that inhibited virus growth.

2.2 Cell biology

2.2.1 Mammalian cell line

2.2.1.1 Regeneration and culture of Rhabdomyosarcoma (RD) cells

A vial of RD cells stored in liquid nitrogen was immediately transferred to a 37°C water bath. Once thawed, the cell suspension was transferred to a sterile 75cm^2 tissue culture flask containing MEM reviving media (Appendix II) in order to dilute the toxic effect of dimethylsulphoxide (DMSO) which was present in the preserving media. The cells were allowed to grow for about 1 to 2 days. When the cells were confluent, the reviving media was discarded and the cell monolayer was rinsed once with 10 ml PBS. Trypsin-EDTA (3 ml) was then added and incubated for 1 to 2 min at 37°C. The flask was tapped gently to detach the cell monolayer and 7 ml of the MEM growth media were added to dilute the trypsinized cells. The suspension of RD cells was split with a ratio of 1:4 to 1:6 for seeding in a 75cm^2 tissue culture flask followed by incubation in a 37°C incubator with 5% CO_2.

2.2.1.2 Storage of RD cells

A confluent monolayer of RD cells in a 75cm^2 tissue culture flask was washed with PBS. The cell monolayer was then treated with 3 ml of trypsin-EDTA to detach the RD cells from the flask, followed by the addition of 7 ml MEM growth media. After centrifugation for 10 min at 300 x g, the supernatant was gently discarded and

the cell pellet was resuspended in 2 ml of MEM freezing media (Appendix II). The cell suspension was transferred into a freezing vial and kept at -20^{o}C for a day before transferring to a -80^{o}C freezer to ensure gradual freezing. After 2 days, the vial was transferred to liquid nitrogen for long term storage.

2.2.2 T cell proliferation assay

2.2.2.1 Isolation of peripheral blood mononuclear cells (PBMCs)

Blood cells were subjected to separation by Ficoll-Hypaque (GE Healthcare Life Sciences, UK) gradient centrifugation for 40 min at 400 x g. The buffy coats separated by Ficoll were carefully transferred into a falcon tube and diluted up to a volume of 50 ml with PBS supplemented with 2mM EDTA (PBS/EDTA). The mixtures were centrifuged for 10 min at 300 x g. The supernatants were gently discarded and the cell pellets were resuspended with 50 ml of PBS/EDTA. The washing step was repeated with centrifugation for 10 min at 300 x g. For removal of platelets, the cell pellets were resuspended with 50 ml of PBS/EDTA and were centrifuged for 15 min at 200 x g. The washing step was repeated once. After washing, PBMCs were then counted using a haemocytometer.

2.2.2.2 *In vitro* culturing and activation of dendritic cells (DCs)

PBMCs were resuspended in 80 µl of PBS/EDTA per 10^7 total cells followed by incubation with 20 µl CD14 MicroBeads (Miltenyi Biotec Inc., USA) for 15 min at 4^{o}C. The suspension was centrifuged for 10 min at 300 x g and the pellet was resuspended up to 10^8 cells in 500 µl of PBS/EDTA, followed by magnetic separation using LS columns (Miltenyi Biotec Inc. USA) according to the manufacturer's instructions. Briefly, cell suspensions were applied on top of the column and the

unbound cells were washed three times with 3 x 3 ml of column buffer (PBS/EDTA supplemented with 0.5% BSA). $CD14^+$ cells were eluted with 5 ml of column buffer by firmly flushing out the positive fraction using the plunger supplied with the column. After elution, the cells were centrifuged for 10 min at 300 x g. The supernatant was then gently discarded and the cell pellet was resuspended with RPMI 1640 media (Invitrogen, USA) supplemented with human interleukin 4 (IL-4) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (each at 20ng/ml) to generate immature DCs (imDCs). The cells were counted using a haemocytometer, seeded in 6-well plates with 10^6 cells per well and cultured for 7 days in a 37°C incubator with 5% CO_2. To activate imDCs, the cells were stimulated with the indicated peptide at 5mM concentration in the presence of LPS (0.5μg/ml) for 1 day and incubated at 37°C with 5% CO_2 before being used as antigen-presenting cells (APCs) in proliferation assays. When indicated, monoclonal anti-human MHC class II (HLA-DP, DQ & DR) antibodies (Ancell Co. USA) were also added at a final concentration of 20μg/ml.

2.2.2.3 $CD4^+$ T cell selection and proliferation

The isolation of $CD4^+$ T cells using CD4 MicroBeads (Miltenyi Biotec Inc. USA) was carried out as described in section 2.2.2.2. $CD4^+$ T cells were resuspended in AIM-V® media (Invitrogen, USA) supplemented with 10% autologous serum and seeded in flat-bottomed 96-well plates with 10^5 cells per well in the presence or absence of 10^4 activated DCs. The cells were cultured for 7 days in an incubator at 37°C with 5% CO_2. The stimulator (DCs) to responder cells ($CD4^+$ T cells) ratio was 1:10. At 24 h prior to the end of incubation for the proliferation assay, the cells were pulsed with 0.5μCi of [methyl-^{3}H] thymidine per well and incubated for 24 h in an

incubator at 37°C with 5% CO_2. The cells were then harvested and counted on a liquid scintillation counter. The radioactivities of the proliferated $CD4^+$ T cells were expressed as the mean counts per min (cpm) from triplicate determinations. The stimulation index (SI) was calculated as the mean counts of cell-associated [^{3}H] thymidine per min recovered from wells containing activated DCs divided by the mean counts of the cell-associated [^{3}H] thymidine per min recovered from wells containing imDCs.

2.3 Molecular biology

2.3.1 Design and synthesis of EV71-specific primers and probes

The VP1 nucleotide sequences of EV71 strain 41 were analyzed. Using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST) together with the DNASTAR program, highly conserved nucleotide sequences of the VP1 capsid protein were defined. For the real-time hybridization probe-based RT-PCR, two primers (namely EvVP1F and EvVP1R) and two hybridization probes (namely EvVP1_FL and EvVP1_LC) were designed (Table 2.3). For cloning recombinant GST-VP1 fusion protein, two primers (namely GST-VP1F and GST-VP1R) were designed (Table 2.3). A pair of oligonucleotide encoding the synthetic peptide SP32 (nt280 to nt324 of VP1 ORF) were synthesized and they contain a pair of start and stop codons (TAA) at the 5′ and 3′ ends, respectively. A *Bam*HI and *Sal*I restriction site were designed at the 5′ and 3′ ends, respectively. For annealing, both oligonucleotides were heated for 3 min at 90°C, followed by incubation for 15 min at 37°C. The annealed product was stored at -80°C for long term storage.

Table 2.3 Nucleotide sequences of EV71-specific primers and hybridization probes.

Primers / Probes	Nucleotide Sequences (5′ - 3′)	Positions
EvVP1F	GAG AGT TCT ATA GGG GAC AGT	2466 to 2489
EvVP1R	AGC TGT GCT ATG TGA ATT AGG AA	2669 to 2647
EvVP1-FL[a]	GAT GAC TGC TCA CCT GTG TGT TTT GAC C-FL	2553 to 2526
EvVP1-LC[a]	LC640-GCT GGC AGG GCC TGG GTA AGT GCC-P	2518 to 2494
GST-VP1F[b]	TGG ATC CGG AGA TAG AGT GGC AG	2435 to 2457
GST-VP1R[b]	GCC GAA GTC GAC TCA AAG GGT AG	3347 to 3325

[a] EvVP1-FL was labeled with fluorescein at the 3' end.
[a] EvVP1-LC was labeled with LC Red 640 at the 5' end and phosphorylated at the 3' end.
[b]GST-VP1F was designed with the *Bam*H1 restriction site (underlined).
[b]GST-VP1R was designed with the *Sal*I restriction site (underlined).

2.3.2 Design and synthesis of conjugated and unconjugated synthetic peptides

A set of 95 overlapping synthetic peptides spanning the entire sequence of the VP1 capsid protein of enterovirus 71 strain 41 (5865/Sin/000009) was synthesized at Mimotopes Pty Ltd (Clayton Victoria, Australia). Each peptide contains 15 amino acid residues with 12 residues overlapping with the adjacent peptides. For the conjugated synthetic peptide, a cysteine residue was added to the C-terminal end which was chemically linked to the carrier protein, Diphtheria toxoid (DKP), through a linker, Maleimidocaproyl-N-Hydroxysuccinimide. Unconjugated synthetic peptides do not contain the cysteine residue at the C-terminal end and are not linked to DKP. For the human $CD4^+$ T-cell epitope study, four peptides (SP1, SP2, SP3 and SP4) within the VP1 capsid protein which consist of 15 amino acid residues each were synthesized by Mimotopes Pty Ltd (Clayton, Victoria, Australia). Peptides were >90% in purity as assessed by high-performance liquid chromatography (HPLC) and were used without further purification. Approximately 1.7 mg of peptide was dissolved in 340 µl DMSO (Sigma Aldrich, USA) to yield a 5mM working concentration.

2.3.3 RNA work

2.3.3.1 Total RNA extraction

Small intestines from mice were harvested and homogenized for total RNA extraction using the RNeasy extraction kit (Qiagen, USA) according to the manufacturer's instructions. The extracted RNA was then analyzed for the presence of EV71 using real-time hybridization probe Reverse Transcription-Polymerase Chain Reaction (RT-PCR) for the detection of EV71 RNA (Tan *et al.,* 2006).

2.3.3.2 Viral genomic RNA extraction

Viral genomic RNA extraction was carried out using the QIAamp® Viral RNA Mini Kit (Qiagen, USA) according to the manufacturer's instructions. Briefly, the extraction was based on the selective binding properties of a silica-gel based membrane together with microspin technology to extract RNA from the virus. The viral samples were first lysed with a lysis buffer which aids in denaturing RNases. The viral genomic RNA was then bound to the membrane in the presence of the carrier RNA and absolute ethanol, followed by two washes with wash buffer to remove any contaminating proteins and lipids. The RNA was then eluted in 80 µl of elution buffer (RNase-free water containing 0.04% sodium azide).

2.3.3.3 Conventional RT-PCR amplification

The EV71 genomic RNA was first denatured for 10 min at 70^{o}C in the presence of forward and reverse primers in the following mix:

Reagent	Quantity
Viral RNA	5.0 µl
ddH_2O	5.0 µl
Forward primer GST-VP1F (10pmol/µl)	1.0 µl
Reverse primer GST-VP1R (10pmol/µl)	1.0 µl
Total reaction volume	12.0 µl

To the reaction mix, 0.5mM dNTP mix, 0.01M dithiothreitol, 1x first strand buffer and 10 U of Superscript II™ RNase H-reverse transcriptase (Life Technologies, USA) was added and the total volume was adjusted to 20 µl with ddH_2O. Reverse transcription was carried out for 1 h at 42^{o}C following which the

enzyme was heat-inactivated for 15 min at 70°C. The VP1 cDNA was then amplified by PCR using 5 µl of the cDNA template in a total reaction volume of 50 µl:

Reagent	Quantity
First strand cDNA template	5.0 µl
Forward primer GST-VP1F (10pmol/µl)	1.0 µl
Reverse primer GST-VP1R (10pmol/µl)	1.0 µl
dNTP mix (2mM)	5.0 µl
10x Reaction buffer	5.0 µl
*Pfu*Turbo™ DNA Polymerase (2 U/µl) (Stratagene, USA)	1.0 µl
ddH_2O	32.0 µl
Total reaction volume	50.0 µl

The reaction was carried out in a thermal cycler (GeneAmp® PCR system 2400PE, Applied Biosystems, USA) for 35 cycles with the following parameters: denaturation for 1 min at 95°C; annealing for 30 s at 55°C; extension for 90 s at 72°C; followed by a final extension step for 10 min at 72°C. The double-stranded VP1 cDNA was stored at -80°C for long term storage.

2.3.3.4 Hybridization probe-based real-time RT-PCR

Real-time RT-PCR analysis was carried out in the LightCycler from Roche Molecular Biochemicals, Germany. The real-time RT-PCR assay for the specific detection of EV71 strain 41 was carried out using the LightCycler RNA Amplification Hybridization Probe kit according to manufacturer's instructions (Roche Molecular Biochemicals, Germany). Briefly, the test kit allows a one-step RT-PCR to be performed in glass capillaries using the LightCycler instrument. The enzyme mix

contained a mixture of reverse transcriptase and "Faststart" *Taq* Polymerase that allowed reverse transcription of RNA template and subsequent cDNA amplification. The reaction mix also contained reaction buffer, dNTP mix (dUTP instead of dTTP) and 30mM $MgCl_2$. Each reaction was performed in a reaction capillary by mixing the reagents, followed by spinning down the mixture briefly with the help of a special LightCycler Centrifuge Adapter. The assay was carried out in a final reaction volume of 10 µl that comprised the following constituents:

Reagent	**Quantity**
$MgCl_2$	0.8 µl
Forward Primer - EvVP1F (5µM)	1.0 µl
Reverse Primer - EvVP1R (5µM)	0.6 µl
Hybridization Probe - EvVP1_FL (2µM)	1.0 µl
Hybridization Probe - EvVP1_LC (2µM)	1.0 µl
LightCycler RNA Amplification Reaction Mix	2.0 µl
LightCycler RNA Amplification Enzyme Mix	0.2 µl
PCR grade H_2O	2.8 µl
RNA Template	1.0 µl
Final reaction volume	10.0 µl

cDNA was synthesized from the RNA for 10 min at 95°C and amplification was carried out for 40 cycles using the following parameters: denaturation for 30 s at 95°C; annealing for 15 s at 55°C; and extension for 9 s at 72°C. The temperature transition rates for all preceding steps were set at 20°C/sec for rapid thermal ramping. Melting curve analysis was also carried out after the PCR reaction. A rapid thermal ramping to 95°C was achieved for the complete denaturation of the PCR amplicons

following hybridization for 10 s at 60°C. A slow steady transition from 60°C to 95°C was then performed. Subsequently, a graph was plotted using –dF/dT against temperature which would show a curve peak, corresponding to the melting temperature (Tm) of the PCR amplicons.

2.3.4 DNA work

2.3.4.1 Isolation of plasmid DNA

The method employed for plasmid DNA isolation is governed by the nature of its subsequent manipulation. The modified alkaline lysis method of Birnbolm and Doly (1979) and the modified boiling method of Holmes and Quigley (1981) were routinely used for primary screening of recombinant clones. For DNA sequencing or molecular manipulations such as cloning, the plasmid DNA has to be of high purity as a template. Hence, a commercial plasmid mini-prep kit, Wizard™ SV Miniprep DNA purification Kit (Promega, USA) was used instead.

2.3.4.1.1 Preparation of plasmids by the modified alkaline lysis method of Birnbolm and Doly (1979)

A 1.5 ml overnight bacterial culture was harvested by centrifugation for 3 min at 8,000 x g and the cell pellet was resuspended in 200 µl of Solution I (50mM glucose, 50mM Tris-HCl and 10mM EDTA, pH 8.0 containing 5mg/ml lysozyme) in a clean eppendorf tube. For cell lysis, 400 µl of Solution II (0.2M NaOH containing 1% SDS) were added and the contents were gently mixed by inversion until a clear solution was obtained. The sample was then incubated for 10 min at room temperature before adding 300 µl of ice-cold 3M sodium acetate (pH 4.8). The sample was gently mixed by inversion, incubated for 10 min at 4°C and then subjected to

centrifugation for 10 min at 8,000 x g. The supernatant was transferred to a fresh eppendorf tube and one volume of phenol saturated with TE Buffer (10mM Tris-Cl and 1mM EDTA, pH 8.0) was added and mixed thoroughly. After centrifugation for 10 min at 8,000 x g, the upper aqueous phase was collected. DNA precipitation was carried out by adding 2 volumes of ice-cold absolute ethanol and incubating the mixture for 2 h at -20°C. The DNA pellet was recovered by centrifugation for 15 min at 8,000 x g, washed once with ice-cold 75% ethanol, air-dried and then resuspended in 50 µl of DNase-free TE Buffer containing 10µg/ml RNase.

2.3.4.1.2 Preparation of plasmids by the modified boiling method of Holmes and Quigley (1981)

A 1.5 ml overnight bacterial culture was harvested by centrifugation for 3 min at 8,000 x g and the cell pellet was resuspended in 100 µl of STET buffer containing 10 µl of a freshly-prepared lysozyme solution (10mM Tris-HCl containing 10mg/ml lysozyme, pH 8.0). The mixture was incubated for 5 min at room temperature and then boiled for 45 s. The cellular debris was removed by centrifugation for 15 min at 8,000 x g. The plasmid DNA in the supernatant was precipitated with 10 µl of ice-cold 3M sodium acetate and 200 µl of ice-cold absolute ethanol. The precipitated DNA was recovered by centrifugation for 15 min at 8,000 x g and the DNA pellet was washed once with ice-cold 70% ethanol and air-dried. The pellet was finally resuspended in 50 µl of DNase-free TE Buffer containing 10µg/ml RNase.

2.3.4.1.3 Plasmid purification using the Wizard™ SV Miniprep DNA Purification Kit (Promega, USA)

The Wizard ™ SV Miniprep DNA Purification Kit was routinely employed for small scale isolation of plasmid DNA. The method employed was essentially based on the alkaline lysis method with an additional column purification step. The column contains an anion exchange resin that allows the negatively-charged plasmid to bind to the resin whilst contaminating proteins are flushed away by the application of a wash buffer. The isolation of plasmid DNA was carried out according to the manufacturer's instructions. Briefly, a 5 ml overnight bacterial culture was centrifuged for 5 min at 8,000 x g and the cell pellet was resuspended in Cell Resuspension Solution™. The suspension was subsequently clarified by the addition of 250 µl of Lysis Solution™ and incubated for 5 min with 10 µl of alkaline protease. The cleared lysate was then neutralized with 350 µl of Neutralization Solution™. The resulting precipitate containing proteins and cellular debris were removed by centrifugation for 10 min at 8,000 x g and the supernatant applied to the DNA binding column. The resin-bound plasmid DNA was washed twice with an ethanol-based wash solution (750 and 250 µl, respectively). The purified plasmid DNA was eluted from the column by the addition of 100 µl of pre-warmed TE buffer and centrifugation for 2 min at 8,000 x g.

2.3.4.2 Restriction endonuclease digestion of DNA

Digestion of DNA was carried out with restriction enzymes (New England Biolabs, USA) according to the manufacturer's instructions. Briefly, the reaction was carried out in an eppendorf tube containing 1 to 2 µg of DNA, restriction enzyme(s) in the corresponding 1x digestion buffer. BSA was added according to the

recommendations of the manufacturer. All reactions were incubated for at least 1 h at optimal temperatures specified by the manufacturer.

2.3.4.3 Agarose gel electrophoresis of DNA

DNA fragments were screened by horizontal gel electrophoresis using varying percentage of agarose (Sigma Aldrich, USA). For screening of recombinant clones and restriction mapping, the agarose gels were prepared in TBE buffer (45mM Tris, 45mM Boric acid and 1.0mM EDTA, pH8.0). When the DNA restriction fragments or PCR products were needed for cloning, electrophoresis was performed in low melting point agarose (Sigma Aldrich, USA) prepared in TAE buffer (40mM Tris-acetate, 1mM EDTA, pH 7.9). DNA products were mixed with a gel loading buffer (0.25% bromophenol blue and 40% w/v sucrose in water) and loaded into the wells. The size of each DNA fragment in the gel was estimated by comparison with standard DNA ladders (MBI Fermentas GeneRuler®, UK). Electrophoresis was carried out at 100 V and the gels were stained with a 0.5µg/ml ethidium bromide for 20 min before visualizing the DNA bands under UV illumination.

2.3.4.4 Purification of DNA using the GFX™ Purification kit (GE Healthcare Life Sciences, UK)

Digested DNA fragments were purified by the GFX™ Purifcation kit according to the manufacturer's instructions. Briefly, the desired DNA fragments were carefully excised from the low melting point agarose gel. An aliquot (10 µl) of the Capture Buffer™ was added for every 10 mg of gel (up to a maximum 300 mg). The mixture was incubated for 15 min at 55ºC. For DNA purification from solution, a volume of approximately 50 to 100 µl PCR product was added to 500 µl of Capture

buffer™ instead. The mixture was passed through a column containing DNA-binding resin by centrifugation for 30 s at 8,000 x g. The column was then washed once with 500 µl of Wash Buffer™. DNA was eluted from the column using 40 µl of TE buffer.

2.3.4.5 DNA Ligation

Reactions were carried out using T4 DNA ligase (New England Biolabs, USA) and the LigaFast™ Rapid DNA Ligation System (Promega, USA) according to the manufacturer's instructions. Briefly, 1 µl of the digested plasmid vector and 3 µl of the digested insert DNA (in a molar ratio of 1:3) were added to 1 µl of T4 DNA ligase, 10 µl of 2x ligation buffer and 5 µl of ddH_2O to a total reaction volume of 20 µl. The ligation mixture was incubated for 5 min at room temperature before being used for transformation.

2.3.4.6 DNA automated cycle sequencing

Automated cycle sequencing was carried out using the Perkin Elmer Applied Biosystems BigDye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer Applied Biosystems, USA). The kit contained the BigDye Terminator Ready Reaction Mix which was consisted of the A,C,G,T-dye terminators, dITP, dATP, dTTP, dCTP, Tris-HCl (pH 9.0), $MgCl_2$ and AmpliTaq DNA polymerase FS. DNA Polymerase FS is a mutant form of *Taq* DNA polymerase which does not posses 5′→3′ nuclease activity and has a reduced discrimination for dideoxynucleotides, thereby permitting the use of lower level of dye-labeled terminators in the reaction. The dNTPs mix includes dITP in place of dGTP to minimize band compressions. Each sequencing reaction was carried out in a final reaction volume of 20 µl that comprised the following constituents:

Reagent	Quantity
BigDye Terminator Ready Reaction Mix	4.0 µl
Double stranded DNA template (~50-100ng/µl)	4.0 µl
5x Sample Buffer	2.0 µl
Primer (10pmol/µl)	1.0 µl
ddH_2O	9.0 µl
Total reaction volume	20.0 µl

The cycle sequencing reaction was carried out in the GeneAmp PCR system 2400 Thermal Cycler (Perkin Elmer Applied Biosystems, USA). The sequencing reaction was carried out for 25 cycles using the following parameters: denaturation for 30 s at 96ºC; annealing for 5 s at 50ºC; and extension for 4 min at 60ºC. Unincorporated dye terminators were removed by precipitating the DNA from the sequencing reaction with 2.5 volumes of ice-cold absolute ethanol and 0.1 volume of 3M sodium acetate for 2 h at -20ºC. The DNA was recovered via centrifugation for 20 min at 8,000 x g. The pellet was subsequently washed with 70% ice-cold ethanol and air dried. Automated sequencing of the DNA was carried out at the Clinical Research Centre Sequencing Laboratory, National University of Singapore.

2.4 Biochemistry

2.4.1 Denaturing PAGE

2.4.1.1 Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out using a vertical slab gel unit (Mini-Protean III Cell) (Bio-Rad Laboratories, USA) according to the manufacturer's instructions. The slab gel (8.3cm X 7.3cm X 0.75mm) was made up of a 12% (w/v) polyacrylamide

separating gel (Appendix IV) and a 5% (w/v) polyacrylamide stacking gel (Appendix IV). Briefly, the separating gel was casted between two grease-free glass plates. The gel mixture was poured up to 1.5 cm below the bottom of the comb. Ethanol (70%) was then carefully layered over the gel mixture to ensure the formation of a straight meniscus and to prevent inhibition of gel polymerization by oxygen present in the air. The gel was allowed to polymerize for 45 min at room temperature. Thereafter, the ethanol was discarded and with the comb in position, the stacking gel mixture was layered over the separating gel. This was allowed to polymerize for 30 min at room temperature before the comb was removed and the wells were flushed with the SDS running buffer (Appendix IV). An aliquot of 20 µl of the protein sample (10 µg total protein) was prepared for electrophoresis by boiling for 5 min with 6 µl of the SDS gel loading dye (Appendix IV). The Precision Plus™ protein standards (Bio-Rad Laboratories, USA) with defined molecular weights were used as markers. Electrophoresis was carried out using a constant current of 80 to 120 V for 2 to 3 h at room temperature until the loading dye reached the bottom of the gel and the markers were well separated.

2.4.1.2 Staining of polyacrylamide gels

After the protein samples were separated by electrophoresis, the gels were soaked in a SDS staining solution (Appendix IV) for 1 h with constant shaking. Stained gels were soaked in a SDS destaining solution (Appendix IV) for 2 h with constant shaking or until protein bands could be visualized as sharp blue bands against a clear background. This was followed by a 1 h of final destaining step in distilled water. The protein gel was dried by placing it in between an appropriately

sized sheet of 3M Whatman™ filter paper in a Bio-Rad Gel Dryer (Bio-Rad Laboratories, USA) for 2 h at 80ºC.

2.4.2 Western blot analysis

2.4.2.1 Electrophoretic transfer of proteins

The resolved protein bands in the SDS-PAGE gels were electro-blotted onto a pre-cut nitrocellulose membrane (Bio-Rad Laboratories, USA) using a Bio-Rad Mini Blot Cell (Bio-Rad Laboratories, USA). The polyacrylamide gel was equilibrated in the Western transfer buffer (Appendix V) for 30 min prior to the transfer. To facilitate the transfer, the nitrocellulose membrane was presoaked in the transfer buffer as well. After equilibration, the transblotting sandwich was assembled and the protein bands were transferred either overnight at a constant voltage of 30 V for 1 h at 4ºC or at a constant voltage of 100 V for 1 h at room temperature.

2.4.2.2 Immunogenic development of Western blots

The transferred nitrocellulose membrane was soaked in the Western blocking buffer (Appendix V) for 1 h at room temperature before incubation with the mouse or human immune sera at 1:50 dilution for 1 h at room temperature followed by three washes with the Western washing buffer (Appendix V). Detection was carried out using a alkaline phosphatase (AP)-conjugated goat anti-mouse IgG (H + L) secondary antibody from the WesternBreeze® chromogenic immunodetection system (Invitrogen, USA) or horseradish peroxidase (HRP)-conjugated goat anti-human IgG antibody (GE Healthcare Life Sciences, UK) according to the manufacturer's instructions. Briefly, the membrane was incubated with the secondary antibody for 1 h

at room temperature, followed by three washes with the washing buffer. The reaction was visualized with the addition of the chromogenic substrate.

2.4.3 Expression and analysis of recombinant GST-tagged fusion proteins

2.4.3.1 Growth and induction of bacteria

A freshly grown single colony of *E. coli* strain carrying the recombinant GST-fusion protein was used to inoculate 10 ml of LB broth and grown overnight at 37ºC. An aliquot of 10 ml of this pre-culture was used to inoculate a 100 ml flask containing LB broth supplemented with the appropriate antibiotics. The culture was allowed to grow under the same conditions until it reached an OD_{600} of 0.5 to 0.6. The inducer, Isopropylthio-β-D-galactoside (IPTG) was added to the culture to a final concentration of 1mM for expression studies. The culture was allowed to grow for another 4 h. The bacterial cultures were harvested by centrifugation for 20 min at 8,000 x g. The supernatants were gently discarded and the cell pellets resuspended in 2 ml of PBS. The cell pellets were used immediately or stored at -80ºC until used.

2.4.3.2 Preparation of cell extracts

The bacterial cell suspensions were sonicated using a 1/8 inch probe in a MSE-Soniprep-150 Sanyo GallenKamp ultrasonic disintegrator (Leicester, UK). Sonication was performed for 10 cycles comprising pulses for 15 s interspersed with 20 s of cooling intervals. Throughout the sonication process, cell suspensions were maintained at a low temperature by the use of ice-alcohol slurry. Bacterial cell wall debris as well as insoluble inclusion bodies were separated from the soluble fraction by centrifugation for 20 min at 8,000 x g. The supernatants which consisted of the soluble crude cell extracts were kept on ice until used.

2.4.3.3 Purification of recombinant GST-tagged fusion proteins

2.4.3.3.1 MicroSpin™ GST Purification kit (GE Healthcare Life Sciences, UK)

Fusion proteins are recovered from the matrix under mild elution conditions, incorporating 10mM glutathione in the elution buffer which preserves protein antigenicity and function according to the manufacturer's instructions. Briefly, the resin in the Glutathione Sepharose™ 4B MicroSpin column was gently mixed by vortexing. The columns were spun down to discard the storage buffer before use. Approximately 600 µl of protein samples was added to the column and incubated for 10 min at room temperature in order to ensure optimal binding of GST proteins to the Glutathione Sepharose matrix. The flow through was collected via centrifugation for 1 min at 800 x g. The column was washed with 250 to 600 µl of PBS and the spin column was centrifuged for 1 min at 800 x g after each wash. Glutathione elution buffer (150 µl) was added to the column and incubated at room temperature for 10 min. Eluents were collected by centrifugation for 1 min at 800 x g and stored at -80^{o}C until used.

2.4.3.3.2 GSTrap™ Fast Flow column kit (GE Healthcare Life Sciences, UK)

The GSTrap™ Fast Flow column (1 ml) provides rapid and mild purification of GST-tagged fusion proteins to >90% purity in a single affinity purification step. Glutathione Sepharose™ Fast Flow column is designed for the purification of glutathione S-transferases and glutathione binding proteins. The fusion proteins were eluted under mild, non-denaturing conditions. The glutathione ligand was coupled via a 10-carbon linker to highly cross-linked 4% agarose. The coupling was optimized to give high binding capacity for GST-tagged fusion proteins and other glutathione binding proteins. Briefly, the column was equilibrated with 5 column volumes of

binding buffer (140mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, and 1.8mM KH_2PO_4, pH 7.3) Samples were applied using a syringe fitted to the luer adaptor with a flow rate of 0.2 to 1 ml/min during sample application. Approximately 5 column volumes of the binding buffer were loaded into the column for washing. The proteins were then eluted with 5 column volumes of elution buffer (50mM Tris-HCl, 10mM reduced glutathione, pH 8.0) and stored at -80^{o}C until used.

2.4.3.4 Bradford assay

Protein concentrations were determined by the Bradford assay (Bio-Rad Laboratories, USA) according to the manufacturer's instructions. The absorbance of the reaction mixture was measured at 595nm after 8 min of incubation at 23ºC. Bovine serum albumin (BSA) (Sigma Aldrich, USA) was used as the standard within the range from 1 to 10µg/µl.

2.4.4 Enzyme-linked Immunosorbent Assay (ELISA)

2.4.4.1 Detection of specific mouse or human IgG antibodies

The 96-well microtiter plates were coated overnight at 4^{o}C with 50 µl of 0.1M carbonate buffer (pH 9.6) containing 10µg/ml of synthetic peptide or purified whole EV71 strain 41. After blocking with 2% BSA, plates were incubated with 50 µl of mouse or human immune sera at 1:50 dilution for 1 h at 37^{o}C and then washed three times with PBS containing 0.1% Tween 20 (PBS-T) (Sigma-Aldrich, USA). Detection was carried out using horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (H+L) (Bio-Rad Laboratories, USA) or HRP goat anti-human IgG (H+L) secondary antibodies (GE Healthcare Life Sciences, UK) at 1:3000 and 1:6000 dilutions, respectively. After 1 h incubation at 37^{o}C and three washes with PBS-T.

The reaction was revealed by the addition of the o-Phenylenediamine dihydrochloride (OPD) substrate (Sigma Aldrich, USA) and the reaction was stopped after 30 min using 1M H_2SO_4. The absorbance at 490nm was measured by an ELISA plate reader (Tecan Sunrise, USA).

2.4.4.2 IgG-subtying

The profiles of specific IgG subtypes in the mice hyperimmune sera were determined by a commercially available mouse sub-type isotyping kit (Zymed Laboratories Inc., USA) according to the manufacturer's instructions by using 10μg/ml of the purified virion to coat the 96-well microtiter plates as described in section 2.4.4.1. In brief, this assay contains a complete set of ELISA reagents primarily designed for screening and determining the class and subclass of mouse antibodies. It is based on strepavidin-biotin amplification (LAB-SA) system and is coupled to horseradish peroxidase to serve as the signal generating reagent.

2.4.5 Cytokine analysis

Cytokines present in mouse serum samples or $CD4^+$ T-cell culture supernatants were measured using the Bio-Plex™ suspension array system (Bio-Rad Laboratories, USA) according to the manufacturer's instructions. Briefly, the multiplex suspension bead array immunoassay was performed using the mouse 4-Plex kit to specifically evaluate IL-6, IL-10, TNF-α and IFN-γ or the human 6-Plex kit to specifically evaluate cytokines of Th1- (IL-2, TNF-α, IFN-γ) and Th2- (IL-4, IL-6, IL-10) type subsets. Briefly, the Bio-Plex™ cytokine assays are designed in a capture sandwich immunoassay format. Antibody specifically directed against the cytokine of interest is covalently coupled to color-coded 5.6 μm polystyrene beads and the

antibody-coupled beads are allowed to react with the sample. After performing a series of washes to remove unbound protein, a biotinylated detection antibody specific for a different epitope on the cytokine is added and the result is the formation of a sandwich of antibodies around the cytokine. The reaction mixture is then detected by the addition of streptavidin-phycoerythrin (streptavidin-PE), which binds to the biotinylated detection antibodies.

2.4.6 Immunohistochemical analysis

Cryosection of intestinal tissue with a thickness of 4 μm were made from the frozen tissue and fixed on Poly-L-lysine glass slide. Permeabilization of the fixed tissue section was carried out by incubation with 0.2% Triton (Sigma Aldrich, USA) for 10 min, followed by incubation with the mouse anti-EV71 monoclonal antibody (Chemicon International, USA) for 30 min at room temperature. The slide was washed three times with PBS, followed by incubation with biotinylated goat anti-mouse IgG secondary antibodies (Zymed Laboratories, USA) for 30 min at room temperature. The reaction was visualized by the addition of aminoethyl carbazole substrate (Zymed Laboratories, USA) to give a red colored peroxidase stain and the slide was counterstained with haematoxylin (Zymed Laboratories, USA) to generate a blue background according to the manufacturer's instructions.

2.4.7 HLA-DR typing

Blood samples from volunteers of both sexes, 25 to 31 years of age, were collected in tubes containing 4% sodium citrate as anti-coagulant. Genomic and serological typing was performed by WHO Immunology and Training Research Centre, Singapore as a kind gift from its director, Professor Chan SH.

2.5 *In vivo* work

2.5.1 Immunization of mice

Inbred Balb/c mice were purchased from the Centre for Animal Resources of the National University of Singapore. All institutional guidelines for animal care and use were strictly followed throughout this study. Groups of 5 adult (6 weeks old) female Balb/c mice were intraperitoneally immunized with either 50 μg of synthetic peptides conjugated to the Diphtheria toxoid or the heat-inactivated EV71 strain 41 (10 μg total protein) in a 50% emulsion of Freund's complete adjuvant (Sigma Aldrich, USA). Two booster doses in 50% emulsion with Freund's incomplete adjuvant (Sigma Aldrich, USA) were given at 3 weeks intervals. The volume of each vaccine suspension administered was 400 μl. The immune sera were collected 7 days after the last immunization. Sera containing EV71-neutralizing antibodies were pooled and stored at -80^{o}C until use.

2.5.2 EV71 lethal challenges

Groups of 6 mice at different age groups, ranging from one-day-old to two-weeks-old were inoculated intraperitoneally with 100 μl of EV71 strain 41 (10^3 $TCID_{50}$). In another experiment, groups of 6 mice at day 1 after birth were given different doses of EV71 strain 41 (ranging from 1 $TCID_{50}$ to 10^3 $TCID_{50}$ per mouse) via the intraperitoneal route. Mice in the control group were given 100 μl of PBS. Mice were monitored daily for body weight gain/loss and the occurrence of mortality until 3 weeks post inoculation.

2.5.3 Protection studies

2.5.3.1 Protection afforded by maternal-transferred antibodies

The immunized female adult mice as described in section 2.1.4 were allowed to breed at 2 weeks after the last immunization. One-day-old suckling mice born to immunized dams were inoculated with 100 µl of virus (10^3 $TCID_{50}$) per mouse via the intraperitoneal route. Suckling mice in the control group were injected intraperitoneally with 100 µl of PBS. All suckling mice were monitored daily for body weight gain/loss and the occurrence of mortality up to day 21 post infection as the experimental endpoint.

2.5.3.2 Passive protection afforded by mice immune sera

Groups of 6 mice at day 1 after birth were injected intraperitoneally with a lethal dose of EV71 (10^3 $TCID_{50}$ per mouse). One day after the viral challenge, suckling mice were injected intraperitoneally with mice immune sera containing different titers of EV71-neutralizing antibodies. Suckling mice in the control groups were either injected intraperitoneally with the naive sera containing no EV71-neutralizing antibody or not injected with any antisera. All suckling mice were monitored daily for body weight gain/loss and the occurrence of mortality up to day 21 post infection as the experimental endpoint.

2.5.4 Harvesting of mouse organs

The dead mice were cleaned with 70% ethanol, secured and bellied down onto a dissecting board. The abdomen was dissected open and the small intestine was aseptically harvested in MEM for total RNA extraction using the RNeasy extraction kit (section 2.3.3.1) or subjected to immunohistochemical analysis (section 2.4.6).

2.5.5 Human serum specimens

Serum samples were collected from children attending a pediatric outpatient clinic at the National University Hospital of Singapore. All sera were screened for the presence of neutralizing antibodies against EV71 strain 41 using the *in vitro* microneutralization assay (section 2.1.2.6). A serum neutralizing antibody titer of 1:8 and above was used as an indicator of previous EV71 exposure.

2.6 Computational analysis

2.6.1 Hydrophobic profile and BLAST search

The protein sequence of VP1 was analyzed for hydrophobic and hydrophilic regions using the Kyte and Doolittle method. The amino acid sequences of the synthetic peptides were used as query sequences in a protein-protein BLAST (blastp 2.2.17) search using the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome). Alignment of the amino acid sequences was also undertaken by using the Clustal method of DNASTAR MegAlign.

2.6.2 T-cell epitope prediction

The VP1 amino acid sequence of EV71 strain 41 was used as a protein candidate to identify promiscuous human $CD4^+$ T-cell epitope(s) based on the ProPred prediction software (http://www.imtech.res.in/raghava/propred). This server is useful in identifying promiscuous binding regions which are able to bind to a total of 51 alleles belonging to 9 serologically defined HLA-DR molecules that are encoded by DRB1 and DRB5 genes. The quantitative matrices used in this prediction method include HLA-DR1 (2 alleles), HLA-DR3 (7 alleles), HLA-DR4 (9 alleles),

HLA-DR7 (2 alleles), HLA-DR8 (6 alleles), HLA-DR11 (9 alleles), HLA-DR13 (11 alleles), HLA-DR15 (3 alleles) and HLA-DR51 (2 alleles). The server analyzed the VP1 sequence using each of the alleles independently and computed the binding strength of all the peptides. The prediction threshold was set at 3% and peptides predicted to bind at least 50% of the major histocompatibility complex (MHC) class II molecules were considered promiscuous for binding.

2.7 Statistical analysis

The results were analyzed using the unpaired Student *t* test. Differences were considered significant at a *P* value of <0.05.

CHAPTER 3 IDENTIFICATION OF NEUTRALIZING LINEAR EPITOPES FROM THE VP1 CAPSID PROTEIN OF ENTEROVIRUS 71 USING SYNTHETIC PEPTIDES

3.1 Introduction

EV71 is one of the main etiological agents of Hand, foot, and mouth disease (HFMD) and has been associated to neurological complications with various clinical severities (Hagiwara *et al.,* 1978). Currently, the only means of preventing and controlling the spread of EV71 during outbreaks is by public health surveillance and quarantine. A number of promising antiviral agents with activity against enteroviruses are still undergoing clinical trials. However, one major obstacle to the successful use of any antiviral agents in EV71-associated encephalitis is that patients may have already suffered irreversible brain damage due to the high viral load at the time of treatment. Therefore, although the antiviral agent may have high level of activity against EV71, it is unlikely that much clinical improvement can be expected after treatment. Consequently, major research efforts are needed to emphasize on the prevention of EV71 infection instead of treatment and this can be carried out via the development of effective vaccines.

Previous studies have focused and shown that VP1 to be highly immunogenic and contained neutralizing epitope(s) as compared to the other capsid proteins of EV71 (Chen *et al.,* 2006; Chiu *et al.,* 2006; Wu *et al.,* 2001). Although there may also be regions along VP2, VP3 or VP4 that are able to elicit neutralizing antibodies against EV71 but these are yet to be identified. Hence, this study focused on the identification and characterization of linear neutralizing epitopes within VP1. Synthetic peptides are considered promising candidates for new-generation vaccines as large quantities of peptides can be chemically synthesized readily and safely.

Peptide vaccines containing neutralizing epitope(s) are capable of inducing specific antibodies that can inhibit the interaction between the viral capsid protein and its cellular receptor thereby preventing virus entry. In this study, characterization of the linear neutralizing epitopes on the VP1 capsid protein of the Enterovirus 71 strain 41 (5865/SIN/00009) (belonging to subgenogroup B4 as the homologous EV71 strain and isolated from a fatal case) was undertaken. Balb/c mice antisera were raised against 95 diphtheria toxoid-conjugated synthetic peptides spanning the entire VP1 region and their neutralizing abilities of the antisera were tested in *in vitro* microneutralization assay. Synthetic peptides, designated SP55 and SP70, were successfully reported to elicit high titers of neutralizing antibody against EV71. The *in vivo* protective potential of SP70 was compared to that of the heat-inactivated homologous EV71 whole virion and to synthetic peptide SP12 which represents an immunogenic but non-neutralizing VP1 linear epitope. Finally, the ability of anti-SP70 antibodies to confer *in vivo* passive protection against heterologous EV71 strains was studied as well.

3.2 Results

3.2.1 Identification of EV71-neutralizing antisera from mice immunized with synthetic peptides (preliminary study)

A set of 95 overlapping diphtheria toxoid-conjugated synthetic peptides spanning the entire VP1 capsid protein of EV71 strain 41 were individually injected into groups of 2 female Balb/c mice via intraperitoneal route. Synthetic peptides were administered over 3 doses with Freund's adjuvant at 3 weeks-interval between each dose. Each peptide consists of 15 amino acids in length with 12 residues overlapping with the adjacent peptides. The specific IgG antibody titers were determined by ELISA for all antisera raised against individual synthetic peptide. At the same time, the antisera were analyzed in *in vitro* microneutralization assay using Rhabdomyosarcoma (RD) cells infected with a viral dose of 10^3 $TCID_{50}$ of EV71 strain 41. Two distinct antisera, obtained from mice immunized with synthetic peptides SP55 (VP1 amino acid residues 162 to 177) and SP70 (VP1 amino acid residues 208 to 222), showed significant neutralizing activities against the homologous EV71 strain.

3.2.2 EV71-neutralizing antisera from mice immunized with SP55, SP70 or heat-inactivated homologous EV71 whole virion

To confirm the preliminary results obtained from the screening described in section 3.2.1, groups of 5 female Balb/c mice were immunized intraperitoneally with either the conjugated synthetic peptides SP55, SP70, SP12 or the heat-inactivated homologous EV71 strain 41, where SP12 (VP1 amino acid residues 34 to 48) and the EV71 whole virion represented negative and positive control groups, respectively. The neutralizing activities of individual antiserum obtained from the SP55-, SP70-,

SP12- or heat-inactivated EV71-immunized mice were determined by *in vitro* microneutralization assay. The infectivity of the homologous EV71 strain 41 was observed over a period of 48 hours (Figure 3.1). The anti-EV71 immune serum allowed a complete protection from cytopathic effect (CPE) at serum dilutions up to 1:64 (Figure 3.2). As expected, antisera from the naive and SP12-immunized mice did not confer any protection to RD cells from CPE. Complete protection of RD cells from CPE was observed with dilutions ranging from neat to 1:32 of the antiserum from the SP70-immunized mice (Figure 3.3). The antiserum from the SP55-immunized mice also showed a neutralizing activity with complete protection from CPE at serum dilutions from neat to 1:8 (Figure 3.4). These data demonstrate that the immune sera obtained from the SP70- and, to a lesser extent, the SP55-immunized mice contain neutralizing antibodies against the homologous EV71 strain 41 which suggests that neutralizing VP1 linear epitopes are present in both synthetic peptides SP70 and SP55.

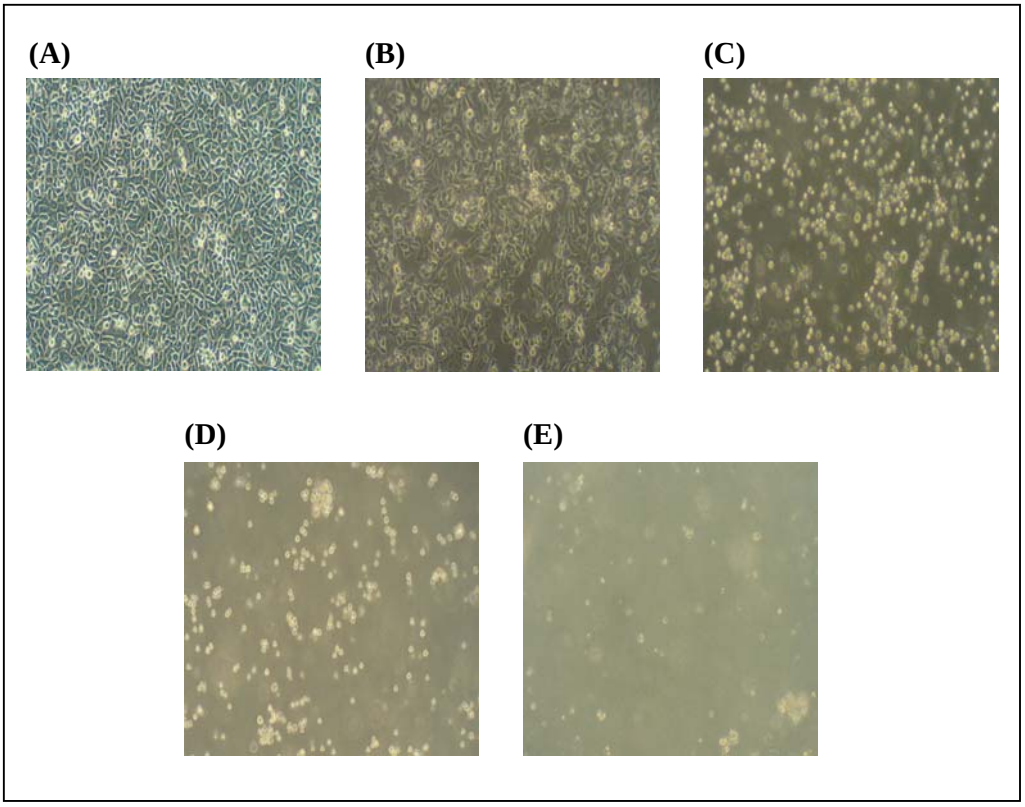


Figure 3.1 Infection of RD cells with a viral dose of 10^3 $TCID_{50}$ EV71 strain 41. **(A)** Monolayer of RD cells at 4 h post infection. **(B)** RD cells showing signs of CPE at 16 h post infection. **(C)** 50% of RD cells rounded up, showed shrinkage and marked nuclear pyknosis at 28 h post infection. **(D)** RD cells became refractile and started to lift off the adherent surface at 40 h post infection. **(E)** Complete CPE at 48 h post infection.

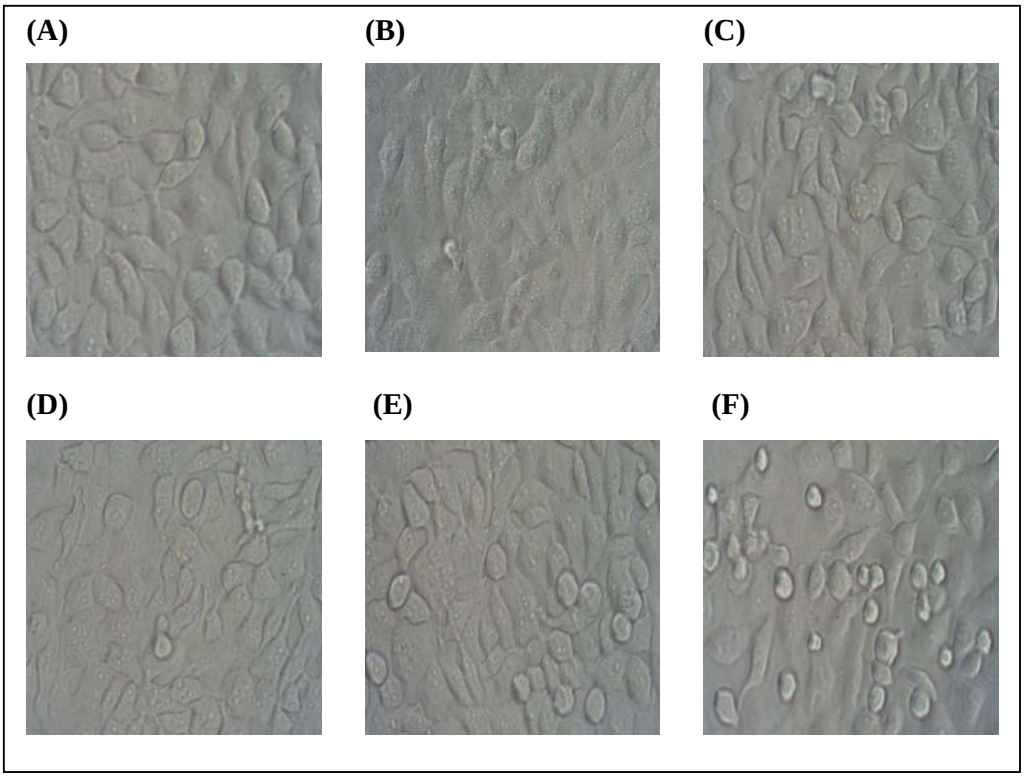


Figure 3.2 *In vitro* microneutralization assay using a representative serum sample from mice (n=5) immunized with the heat-inactivated homologous EV71 whole virion. RD cells were infected with 10^3 $TCID_{50}$ EV71 pre-incubated with various dilutions of the anti-EV71 immune serum. Survival of cells was observed with neat serum **(A)**; serum dilution of 1:8 **(B)**; serum dilution of 1:16 **(C)**; serum dilution of 1:32 **(D)**; serum dilution of 1:64 **(E)**. Moderate CPE of approximately 50% was observed with serum dilution of 1:128 **(F)**.

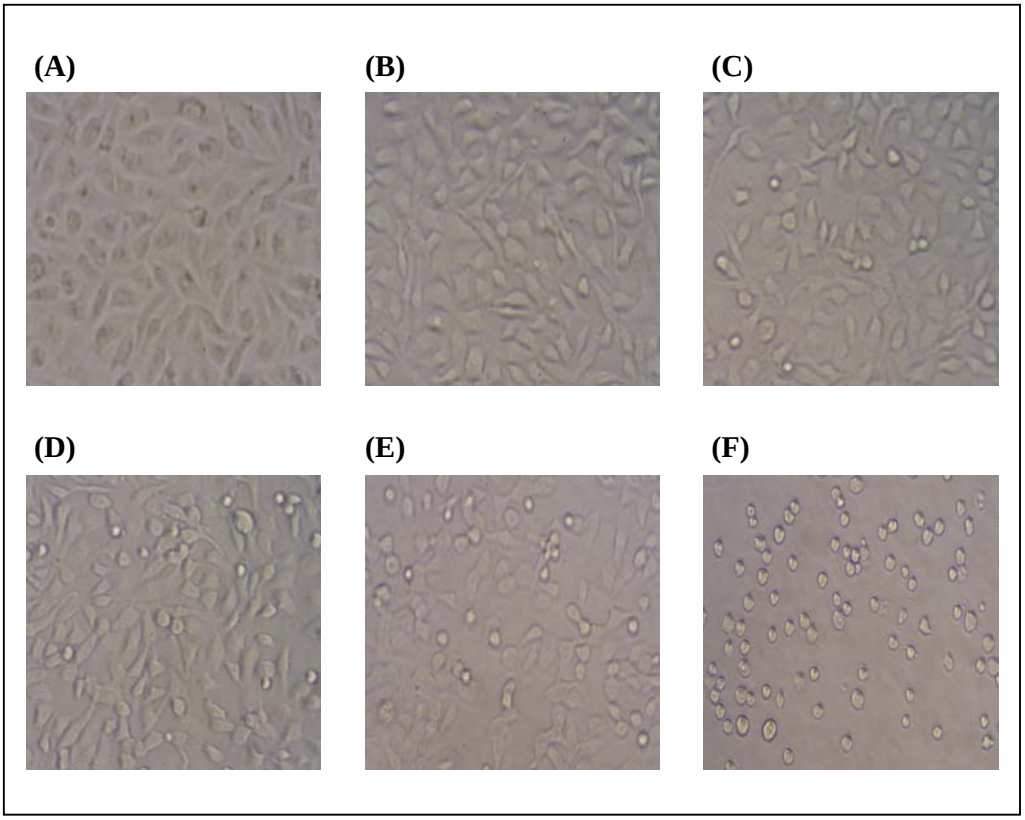


Figure 3.3 *In vitro* microneutralization assay using a representative serum sample from mice (n=5) immunized with the synthetic peptide SP70. RD cells were infected with 10^3 $TCID_{50}$ EV71 pre-incubated with various dilutions of the anti-SP70 immune serum. Survival of cells was observed with neat serum **(A)**; serum dilution of 1:8 **(B)**; serum dilution of 1:16 **(C)**; serum dilution of 1:32 **(D)**. Moderate CPE of approximately 50% was observed with serum dilution of 1:64 **(E)** whereas complete CPE was observed with serum dilution of 1:128 **(F)**.

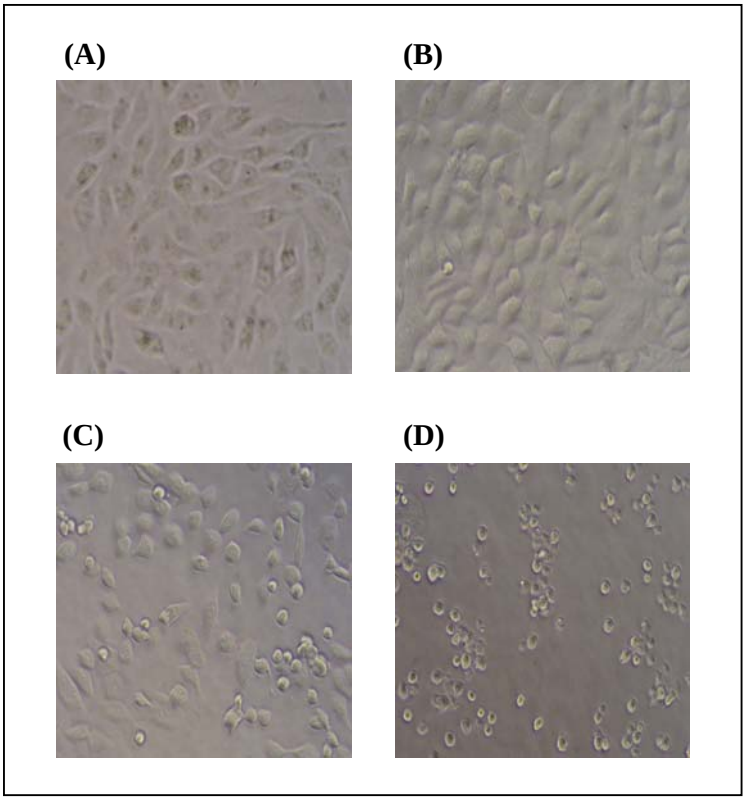


Figure 3.4 *In vitro* microneutralization assay using a representative serum sample from mice (n=5) immunized with the synthetic peptide SP55. RD cells were infected with 10^3 $TCID_{50}$ EV71 pre-incubated with various dilutions of the anti-SP55 immune serum. Survival of cells was observed with neat serum **(A)**; serum dilution of 1:8 **(B)**; serum dilution of 1:16 **(C)**. CPE was observed with serum dilution of 1:32 **(D)**.

3.2.3 Immunoreactivity of antisera from mice immunized with SP12, SP55 or SP70

Based on *in vitro* microneutralization assay, it was described in section 3.2.2 that individual immune serum obtained from groups of SP55-, SP70- or SP12-immunized mice had similar neutralizing antibody titer against the homologous EV71 strain within respective groups and hence the immune serum samples from individual mouse within each group were pooled into anti-SP55 antisera, anti-SP70 antisera or anti-SP12 antisera, respectively. The immunoreactivity of the pooled antisera was assayed against EV71 total proteins by Western blot analysis. For each set of pooled antisera tested, a single band was revealed at an apparent molecular weight (MW) of 32kDa (Figure 3.5). This band very likely corresponds to the VP1 monomer whose apparent MW is 32.7kDa, indicating that the mice antisera raised against synthetic peptides SP12, SP55 and SP70 contained specific anti-VP1 antibodies that do not cross-react with other viral proteins. The immuno-specificity of all three sets of pooled antisera towards the set of 95 synthetic peptides was also assayed by ELISA where the peptides were individually used as coating antigens. A strong positive signal was obtained when the pooled antisera were incubated with the corresponding peptide used for immunization (homologous peptide), demonstrating the effectiveness of intraperitoneal immunization with each synthetic peptide. Furthermore, a positive signal was also obtained when the pooled antisera were incubated with synthetic peptides whose amino acid sequences overlap the sequence of the homologous peptide (overlapping peptides). Interestingly, both the anti-SP12 and anti-SP55 antisera showed reactivity against the synthetic peptides SP19, SP20, SP21 and SP40, suggesting that both SP12 and SP55 share common linear epitopes with SP19, SP20, SP21 and SP40 (Table 3.1).

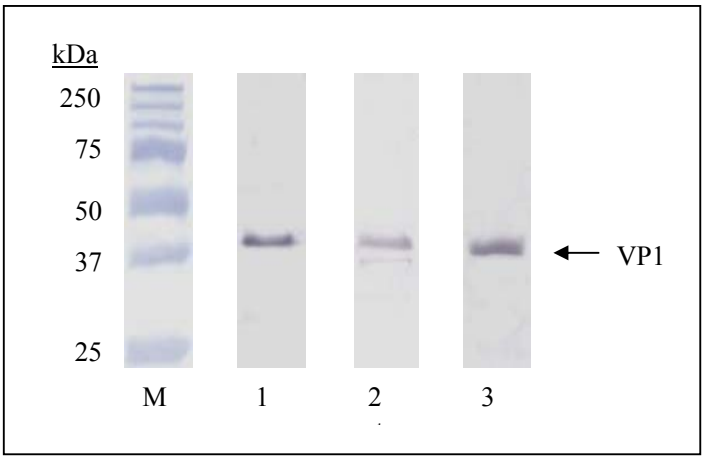


Figure 3.5 Western blot analysis using the synthetic peptide-antisera as primary antibodies. The amount of EV71 total proteins loaded in each lane was 10 μg. The lanes are as follows: lane M, molecular weight marker, lane 1, pooled antisera raised against synthetic peptide SP12; lane 2, pooled antisera raised against synthetic peptide SP55; lane 3, pooled antisera raised against synthetic peptide SP70. Arrow indicates the presence of VP1 in respective lanes.

Table 3.1 Immunospecificity of SP12-, SP55- and SP70-immune sera.

Peptides	VP1 Amino Acid Positions	Peptide Sequences[a]	Antiserum Specificity		
			Reactivity with homologous peptide[b]	Reactivity with overlapping peptides[b]	Reactivity with other peptides[b]
SP12	34-48	AC - VSSHRLDTGEVPALQC - DKP	+++	SP10, SP11, SP13, SP14, SP15	SP19, SP20, SP21, SP40
SP55	163-177	AC - PESRESLAWQTATNPC - DKP	+++	SP54, SP56	SP19, SP20, SP21, SP40
SP70	208-222	AC - YPTFGEHKQEKDLEYC - DKP	+++	SP68, SP69, SP71, SP72	-

[a] AC denotes an acetyl group attached to the amino (N)-terminal end and DKP denotes the carrier protein (Diphtheria toxoid) conjugated at the carboxy (C)-terminal end.

[b] Peptide specificities were based on ELISA readings at 450nm. The data represents the average of three independent experiments.

3.2.4 Analysis of IgG responses elicited by SP55, SP70 and SP12

To further characterize the antibody response raised upon immunization with the synthetic peptides SP12, SP55 or SP70, the total IgG response against EV71 whole virion was measured by ELISA. The antibody level elicited by the individual respective synthetic peptide was found to be as high as those obtained upon immunization with the heat-inactivated EV71 whole virion (Table 3.2A). In addition, examination of the IgG subtypes revealed that IgG1 antibody was predominantly produced in pooled immune sera raised against the three respective synthetic peptides individually, and at levels comparable to that measured in the pooled immune sera obtained from mice immunized with the heat-inactivated homologous EV71 whole virion (Table 3.2B). In contrast, the levels of IgG2a, IgG2b and IgG3 measured in pooled immune sera from mice immunized with the respective synthetic peptide were not significantly different from the levels measured in naive mice whereas those measured in the pooled immune sera from mice immunized with the heat-inactivated homologous EV71 whole virion were significantly higher (Table 3.2B). Altogether, these data indicate that the intraperitoneal administration of conjugated synthetic peptides SP12, SP55 and SP70 elicited mainly a Th2 immune response whereas the heat-inactivated homologous EV71 whole virion triggered a mixed Th1/Th2 response.

Table 3.2 Total and IgG sub-type responses in SP12-, SP55- and SP70-immune sera.

(A) Total IgG response

Immunogen	OD_{450nm}
SP55	0.87 (0.02)
SP70	0.88 (0.04)
SP12	0.87 (0.03)
Heat-inactivated virus	0.98 (0.01)
Naive	0.17 (0.02)

(B) IgG sub-type response

Immunogen	OD_{450nm}			
	IgG1	IgG2a	IgG2b	IgG3
SP55	0.47 (0.04)	0.10 (0.02)	0.09 (0.02)	0.11 (0.04)
SP70	0.80 (0.02)	0.18 (0.01)	0.14 (0.03)	0.12 (0.01)
SP12	0.72(0.02)	0.11 (0.01)	0.10 (0.01)	0.17 (0.03)
Heat-inactivated virus	0.83 (0.03)	0.73 (0.01)	0.78 (0.01)	0.80 (0.02)
Naive	0.08 (0.02)	0.09 (0.02)	0.08 (0.01)	0.07 (0.01)

(A) IgG-based ELISA was carried out using individual sera at a dilution of 1:50. The 96-well microtiter plates were coated with purified virions (10μg/ml). OD_{450nm} readings are expressed as the average of 5 antisera per group. Standard deviation of OD readings is in parenthesis.

(B) IgG-based ELISA was carried out using pooled sera (n=5) at a dilution of 1:50. Wells of the 96-well microtiter plates were coated with the corresponding unconjugated synthetic peptide (10μg/ml). The data represents the average of three separate experiments. Standard deviation of OD readings at 450nm is in parenthesis.

3.2.5 *In silico* analysis of VP1 amino acid sequences represented by SP55 and SP70

The Kyte and Doolittle hydrophobicity profile of the VP1 capsid protein of EV71 strain 41 indicated that both synthetic peptides SP55 and SP70 contained amino acid sequences which are located within the major hydrophilic regions of the VP1 protein (Figure 3.6), therefore in regions likely to be exposed on the surface of the capsid protein. A protein-protein BLAST (blastp 2.2.17) search demonstrated that the VP1 amino acid sequences represented by SP55 and SP70 were highly specific for EV71 strains and no significant homology with other enteroviruses such as coxsackieviruses and echoviruses was found. Alignment using the Clustal method of MegAlign (DNASTAR program) also indicated that the amino acid sequence represented by SP70 was found to be totally conserved amongst 25 other EV71 strains from subgenogroups A to C4 whereas the amino acid sequence represented by SP55 showed 80% identity with the genogroup A, BrCr-CA-70 strain, from US and 85 to 100% homology with the other representative strains from subgenogroups B1 to C4 (Figure 3.7).

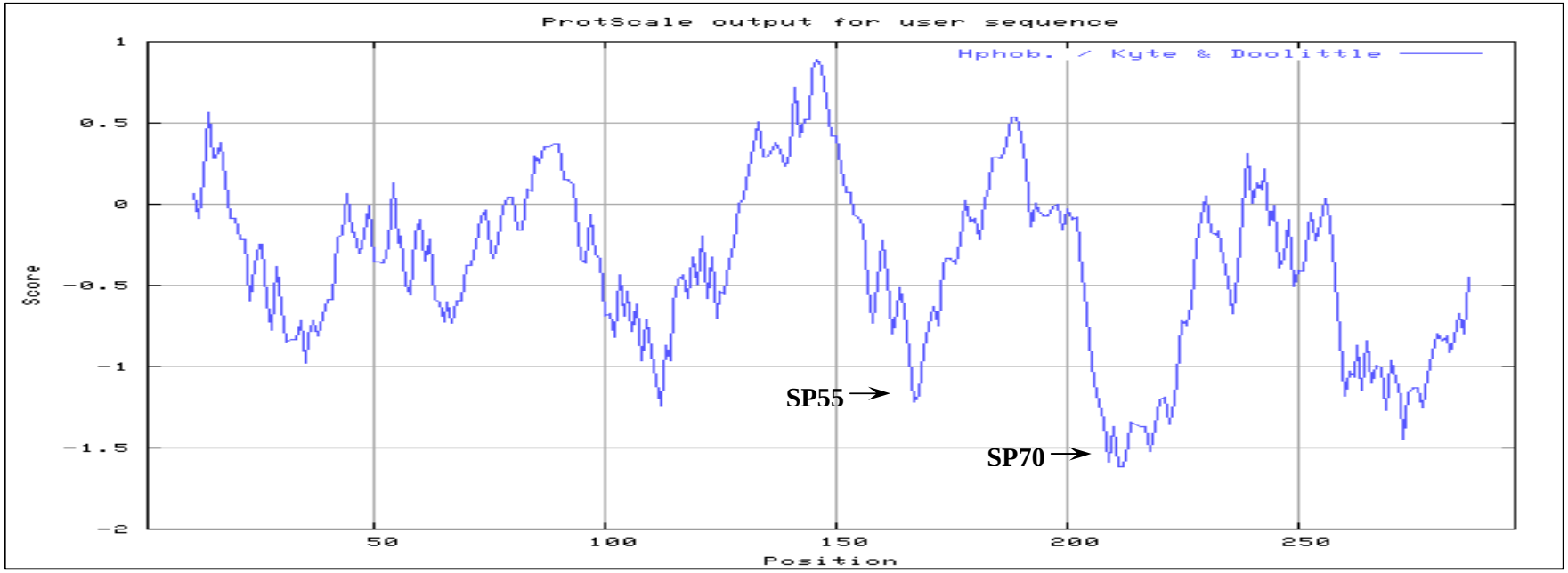


Figure 3.6 Kyte and Doolittle hydrophobicity profiles of the VP1 capsid protein of EV71 strain 41. The horizontal axis represents the amino acid position in VP1 and the vertical axis represents the hydropathy scores. Positive scores indicate hydrophobicity while negative scores indicate hydrophilicity. SP70 (VP1 amino acid residues 208-222) and SP55 (VP1 amino acid residues 163-177) are indicated on the plot.

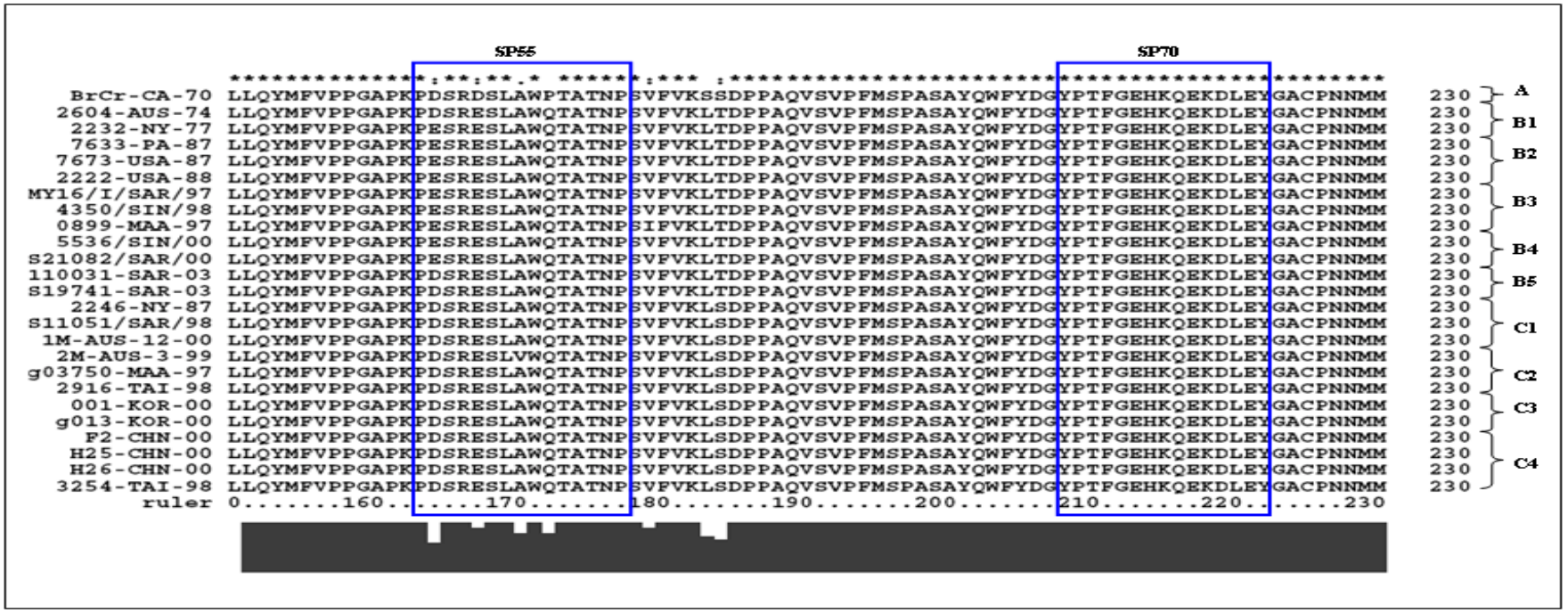


Figure 3.7 Alignment of amino acid sequences represented by the synthetic peptides SP55 and SP70 against heterologous EV71 strains from different subgenogroups based on the VP1 amino acid sequences.

3.2.6 In vitro protection afforded by antisera from mice immunized with SP55, SP70 or heat-inactivated homologous EV71 strain 41 against heterologous EV71 strains

The neutralizing potential of the immune sera raised against the synthetic peptides SP55 or SP70, against heterologous strains of EV71 was addressed. Representative EV71 strains from subgenogroups B2, B5, C2 or C4 were incubated individually with each set of pooled antisera and *in vitro* microneutralization assay was carried out as described in section 3.2.2. The pooled antisera from mice immunized with the heat-inactivated homologous EV71 strain 41 was also assayed and it was observed that this pooled antisera was able to protect RD cells from CPE against heterologous EV71 strains from subgenogroups B5, C2 and C4 as efficiently as against the homologous EV71 strain 41 (subgenogroup B4) (Table 3.3). However, it was found slightly less efficient to protect against heterologous EV71 strains from the subgenogroup B2 (Table 3.3).

The pooled antisera from mice immunized with SP70 showed neutralizing activities against heterologous EV71 strains from subgenogroups C2 and C4 as high as the one obtained against the homologous EV71 strain 41 with significant neutralizing antibody titer of 1:32. However, neutralizing activities against heterologous EV71 strains from subgenogroups B2 and B5 were slightly lower with neutralizing antibody titer of 1:16 (Table 3.3). In contrast, the pooled antiserum from mice immunized with SP55 displayed the same neutralizing activity against all EV71 strains tested, with a neutralizing antibody titer of 1:8 (Table 3.3). Therefore, the data demonstrated the protective potential of SP70 and, to a lesser extent, SP55 against heterologous EV71 strains. In addition, this further supports the in silico analysis

(section 3.2.5) which indicated that the neutralizing VP1 linear epitopes present in both SP70 and SP55 are highly conserved amongst representative EV71 strains from different subgenogroups.

Table 3.3 Neutralizing antibody titers elicited by SP55, SP70 and heat-inactivated homologous EV71 whole virion in mice against heterologous EV71 strains.

	Subgenogroups of EV71				
Immunogen	**B2[c]**	**B4[b]**	**B5[c]**	**C2[c]**	**C4[c]**
Whole virion[a]	1:32	1:64	1:64	1:64	1:64
SP55	1:8	1:8	1:8	1:8	1:8
SP70	1:16	1:32	1:16	1:32	1:32

[a] Heat-inactivated EV71 strain 41 (5865/SIN/00009).

[b] Homologous EV71 strain.

[c] Heterologous EV71 strains.

3.2.7 EV71 infection in suckling mice

Using suckling Balb/c mice as an infection model for EV71, the infection was characterized by hairless skin lesions and paralysis of limbs, and both clinical manifestations persisted throughout the observation period (Figure 3.8). Suckling Balb/c mice from different age groups were infected intraperitoneally with the homologous EV71 strain 41 at a lethal dose of 10^3 $TCID_{50}$ per mouse. Mice which were infected at day 1 after birth died by day 11 post infection. With mice infected at day 4 after birth, death was delayed but all mice died by day 16 post infection. Mice which were infected at day 7 or day 10 after birth had higher survival rates of 60% and 90%, respectively by day 21 post infection. All mice which were infected at day 14 after birth survived throughout the experimental period.

To determine the lethal viral dosage, groups of mice at day 1 after birth were infected intraperitoneally with different doses of the homologous EV71 strain 41, ranging from 1 $TCID_{50}$ to 10^3 $TCID_{50}$ per mouse. A dose-dependent mortality was observed with a high viral dose resulting in mice death at an earlier time point. With an infective dose of 10^3 $TCID_{50}$ virus, all mice died by day 11 post infection whereas a 30% survival rate was observed for those which were infected with a lower viral dose at 1 $TCID_{50}$ per mouse (Figure 3.9). When the viral dose was reduced to 10^2 $TCID_{50}$ or 10 $TCID_{50}$ per mouse, the mice survival was delayed to day 14 or day 17 post infection, respectively (Figure 3.9). With a viral dose of 1 $TCID_{50}$, an overall 20% survival rate was observed up to day 21 post infection as the experiment endpoint and mice which were not infected did not show any sign of distress and all of them survived (Figure 3.9).

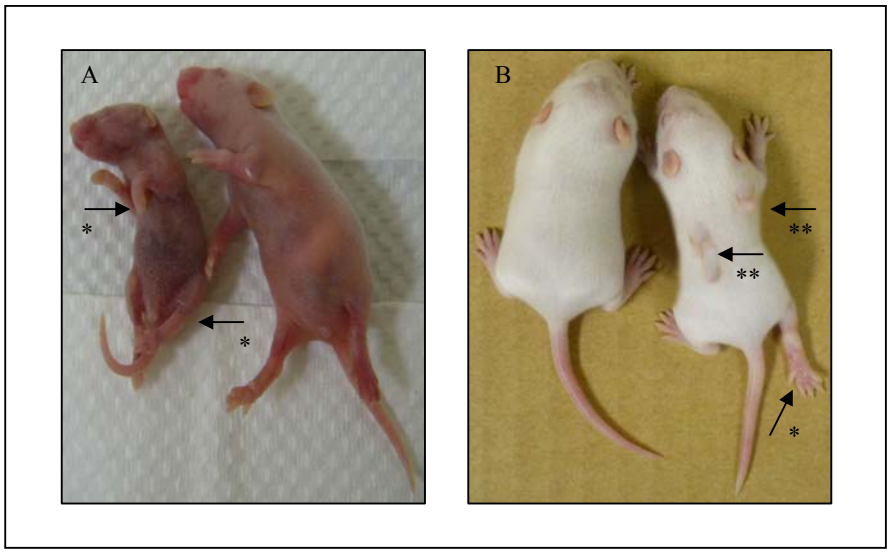


Figure 3.8 Viral infection of suckling Balb/c mice with the homologous EV71 strain 41 at a lethal dose (10^3 $TCID_{50}$ per mouse). (A) Mice at day 3 post infection are shown. The mouse on the right side is a non-infected age-matched control. **(B)** Mice at day 9 post infection are shown. The mouse on the left side is a non-infected age-matched control. Limb paralysis is represented by * and skin lesion is represented by **.

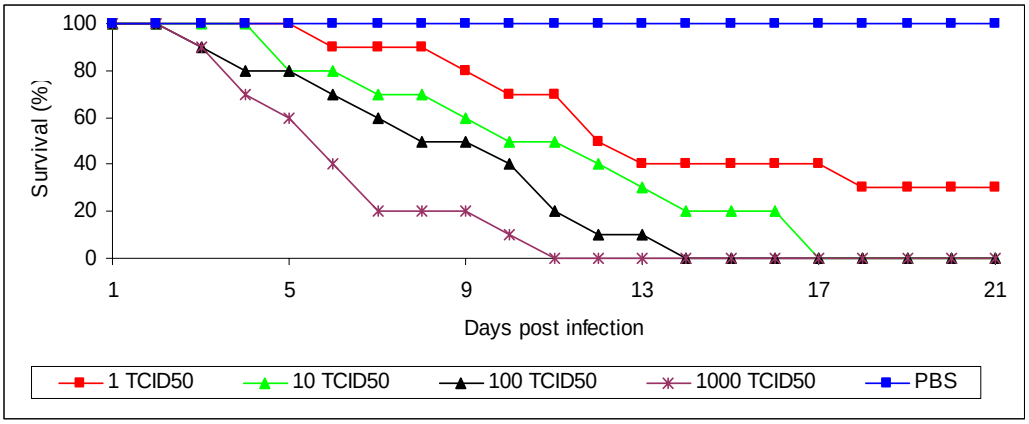
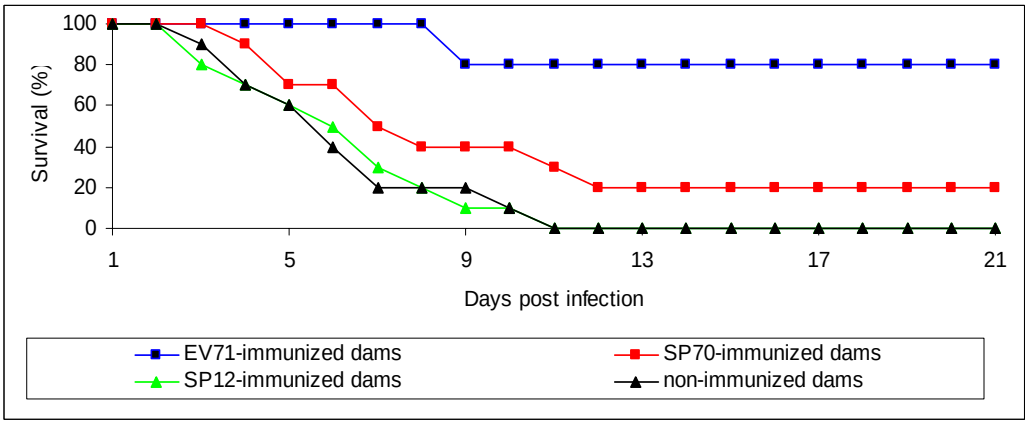


Figure 3.9 Dose dependency of EV71-induced death. Groups of Balb/c mice (n=6) at day 1 after birth were intraperitoneally infected with increasing doses of the homologous EV71 strain 41 (ranging from 1 $TCID_{50}$ to 10^3 $TCID_{50}$ per mouse). Mice in the control group were given 100 µl of PBS. Death was monitored daily until day 21 post infection.

3.2.8 *In vivo* protection against the lethal homologous EV71 strain 41 challenge in suckling Balb/c mice born to immunized dams

To evaluate the protective efficacy of maternal-transferred antibodies, suckling Balb/c mice born to immunized dams were challenged with the homologous EV71 strain 41 and their mortality rates were observed. As described in section 3.2.2, the synthetic peptide SP70 was able to elicit a higher anti-EV71 neutralizing antibody titer of 1:32 as compared to SP55 which elicited a lower titer of 1:16. Hence, SP70 was selected as a representative vaccine candidate for immunization. At a lethal virus challenge dose of 10^3 $TCID_{50}$ per mouse, the group of suckling mice born to dams which received the heat-inactivated homologous EV71 whole virion showed a protective efficacy of 80% whereas only 1 mouse among 6 suckling mice born to dams which received the synthetic peptide SP70 survived (Figure 3.10A). The control mice born to non-immunized dams including those born to dams immunized with the synthetic peptide SP12 did not survive by day 11 post infection (Figure 3.10A). To evaluate the severity of EV71 infection, body weights for all suckling mice were recorded every 3 days until day 21 post infection. The EV71-infected suckling mice did not gain much weight as compared to those who were protected against the viral infection. The protected mice had an average of approximately 7g in body weight as compared to those who were unprotected with a significant reduction in average body weight of 3g before they died by day 11 post infection (Figure 3.10B).

(A)



(B)

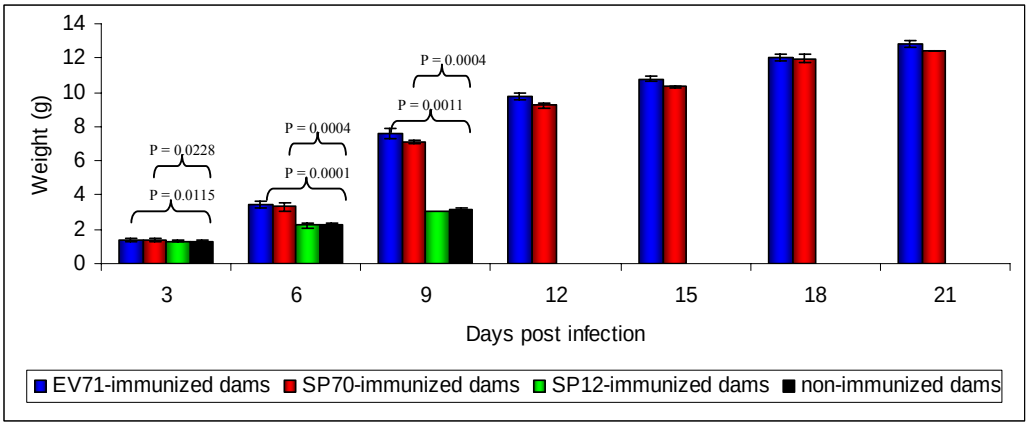


Figure 3.10 *In vivo* protection of suckling Balb/c mice with maternal-transferred antibodies upon challenged with the homologous EV71 strain 41. (A) One-day-old mice (n=6) born to dams immunized with heat-inactivated homologous EV71 whole virion, SP70 or SP12 were infected with 100 µl of virus (10^3 $TCID_{50}$ per mouse) via intraperitoneal route. Control mice born to non-immunized dams were infected with similar viral dose. Death was monitored until day 21 post infection. **(B)** Monitoring of body weight gain/loss in suckling mice upon EV71 challenge. On day 11 after infection, control mice were dead. Body weights were recorded for all surviving mice every 3 days until day 21 post infection.

3.2.9 *In vivo* passive protection against lethal EV71 challenge in suckling Balb/c mice

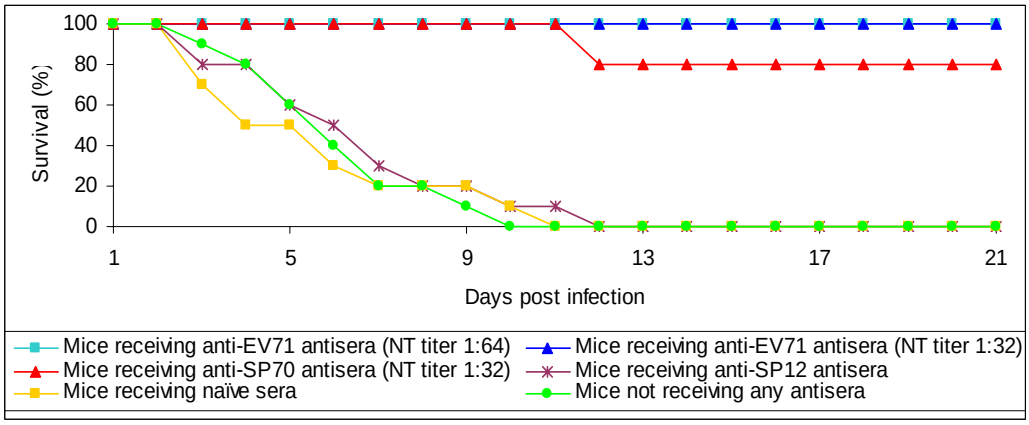
3.2.9.1 Homologous EV71 strain 41 challenge

To assess the efficacy of *in vivo* passive protection by immune sera raised against the heat-inactivated homologous EV71 whole virion (anti-EV71) or synthetic peptide SP70 (anti-SP70), suckling Balb/c mice born to naive dams were administered with the respective antisera one day after challenge with the homologous EV71 strain 41 at a lethal dose of 10^3 $TCID_{50}$ per mouse. Groups of mice that received the pooled anti-EV71 antisera with neutralizing antibody titers of 1:64 or 1:32 survived throughout the experimental period (Figure 3.11A). The pooled anti-SP70 antisera with a neutralizing antibody titer of 1:32 provided 80% protection whereas EV71-infected suckling mice which did not receive any antisera and those which received either pooled naive sera or pooled immune sera from mice immunized with the synthetic peptide SP12 (anti-SP12) did not survive by day 12 post infection (Figure 3.11A). The body weight of mice that were protected against EV71 infectivity rose steadily up to an average of approximately 12g at day 21 post infection (Figure 3.11B). At day 9 post infection, they weighed approximately 8g on average when compared to unprotected mice that had significant reduction in body weight to approximately 3g in average before they died from EV71 infection (Figure 3.11B). When the neutralizing antibody titer of anti-EV71 antisera was reduced to 1:16, the survival rate of EV71-infected suckling mice dropped to 70% and subsequently to 50% when the neutralizing antibody titer was further reduced to 1:8 (Figure 3.12A). A 50% and 20% survival rate were observed with the anti-SP70 antisera at reduced neutralizing titer of 1:16 and 1:8, respectively (Figure 3.12B).

3.2.9.2 Heterologous EV71 strains challenge

Groups of suckling Balb/c mice born to naive dams were challenged with heterologous EV71 strains at a lethal dose of 10^3 $TCID_{50}$ per mouse and immune sera raised against the heat-inactivated homologous EV71 whole virion (anti-EV71) or synthetic peptide SP70 (anti-SP70) were administered one day after challenge. When suckling mice were infected with any of the heterologous EV71 strains followed by administering with the pooled anti-EV71 antisera at a neutralizing antibody titer of 1:32, all of them survived up to day 21 post infection (Table 3.4). Mice infected with heterologous EV71 strains from the subgenogroup B2 or B5 had 80% survival rates when they were administered with the pooled anti-SP70 antisera with a neutralizing antibody titer of 1:32 (Table 3.4). Upon lethal challenge with heterologous EV71 strains from the subgenogroup C2 or C4, the pooled anti-SP70 antisera protected 70% of infected suckling mice (Table 3.4).

(A)



(B)

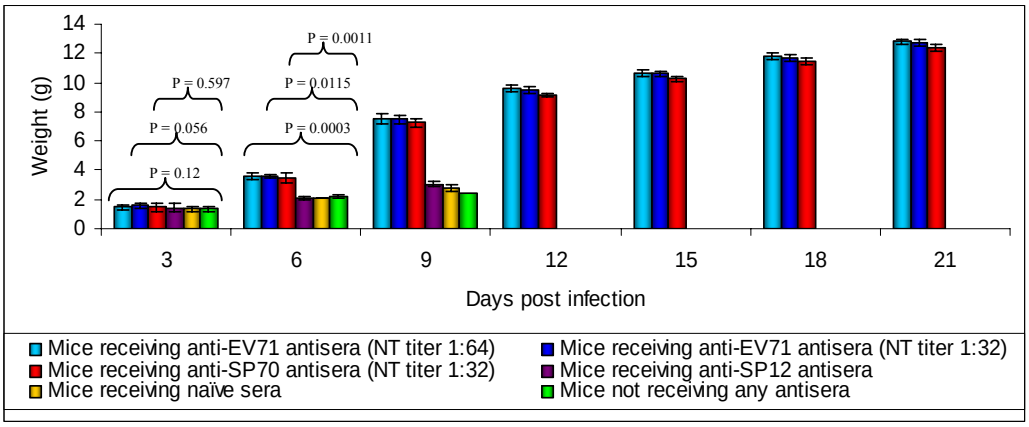
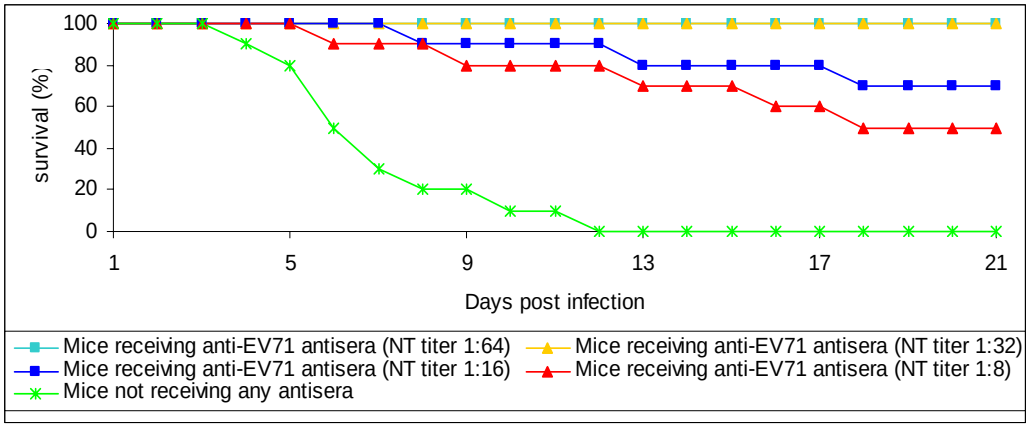


Figure 3.11 *In vivo* passive protection of suckling Balb/c mice upon challenged with the homologous EV71 strain 41. (A) Groups of one-day-old mice (n=6) were intraperitoneally infected with a lethal viral dose of 10^3 $TCID_{50}$ per mouse. One day post infection, suckling mice were administered with pooled immune sera from EV71-, SP70- or SP12-immunized mice. Mice in control groups were given naive sera or not administered with any sera. Death was monitored daily until day 21 post infection. **(B)** Monitoring of body weight gain/loss in suckling mice upon EV71 challenge. Body weights were recorded for all surviving mice every 3 days until day 21 post infection.

(A)



(B)

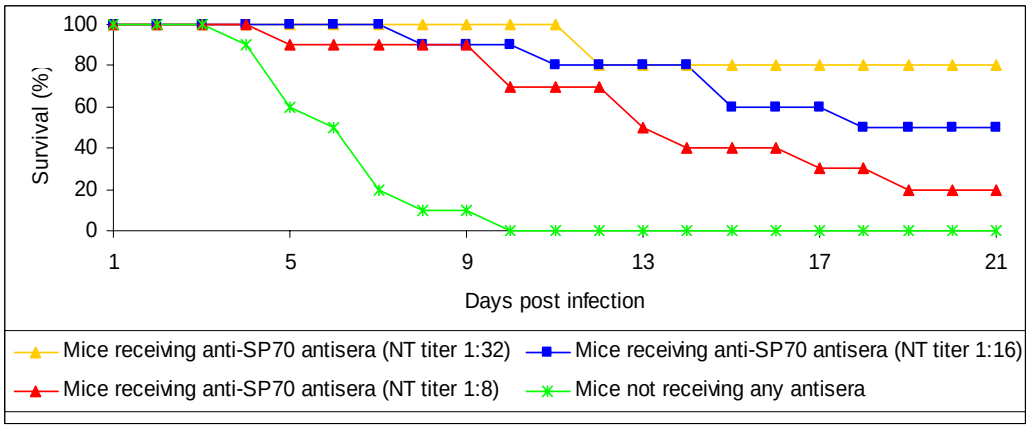


Figure 3.12 *In vivo* passive protection study conferred by different anti-EV71 neutralizing antibody titers. Groups of one-day-old Balb/c mice (n=6) were infected with the homologous EV71 strain 41 at a lethal dose of 10^3 $TCID_{50}$ per mouse. One day after infection, suckling mice were administered with serially diluted pooled immune sera from **(A)** EV71-immunized mice or **(B)** SP70-immunized mice. Mice in control groups were not administered with any sera. Death was monitored until day 21 post infection.

Table 3.4 Survival rates of suckling Balb/c mice upon challenged with the homologous or heterologous EV71 strains.

EV71 Subgenogroups	Mouse Antisera[a]	Survival Rate
B2[c]	anti-EV71	100%
	anti-SP70	80%
B4[b]	anti-EV71	100%
	anti-SP70	80%
B5[c]	anti-EV71	100%
	anti-SP70	80%
C2[c]	anti-EV71	100%
	anti-SP70	70%
C4[c]	anti-EV71	100%
	anti-SP70	70%

[a] One day upon EV71 challenge at a lethal dose of 10^3 $TCID_{50}$ per mouse, suckling mice were administered with a 1:32 dilution of immune sera from mice immunized with the homologous heat-inactivated EV71 strain 41 (anti-EV71) or synthetic peptide SP70 (anti-SP70) and monitored until day 21 post infection. Each group contained 6 mice.

[b] Homologous EV71 strain.

[c] Heterologous EV71 strains.

3.2.10 Histological examination in EV71-infected suckling Balb/c mice

To evaluate the severity of virus infection and its route of infectivity, histological examinations revealed EV71 infiltration in small intestines of suckling Balb/c mice infected with the homologous EV71 strain 41 at a lethal dose of 10^3 $TCID_{50}$ per mouse. The presence of EV71 was detected with the commercially available mouse anti-EV71 monoclonal antibody. From micrographs of representative cryosections, infected mice displayed damaged intestinal structures indicating extensive viral replications within the gut. Mice which received either pooled anti-EV71 or anti-SP70 antisera upon lethal EV71 challenge were protected against virus infection and they presented intact intestinal structures (Figure 3.13C & D). However, non-protected mice which either received pooled anti-SP12 antisera or did not receive any sera upon lethal EV71 challenge showed signs of tissue damage in their small intestines (Figure 3.13B & E). The red colored peroxidase stain indicated EV71 infiltration of intestinal tissues (Figure 3.13).

3.2.11 Detection of EV71 by real-time RT-PCR hybridization probe-based assay

In order to quantify the viral load in infected animals, intestinal tissues were harvested from suckling Balb/c mice upon challenge with the homologous EV71 strain 41 at a lethal dose of 10^3 $TCID_{50}$ per mouse and viral RNA was extracted from homogenized tissues for the detection of EV71. Upon real-time RT-PCR assay, a 204-base pairs PCR product mapping within the VP1 region was obtained. Quantification analysis showed that the EV71 complete RNA transcript as the positive control for the real-time RT-PCR assay has a Ct value of 13 which represents 5×10^8 EV71 copies and every 3.3 Ct value difference represents a ten-fold difference in viral copies (Tan *et al*., 2006). Viral RNA sample from a representative mouse belonging to the group

which did not receive any sera indicated a Ct value of 33. In addition, viral RNA samples from representative mice belonging to groups which received either pooled anti-SP12 antisera or naive sera also indicated a Ct value of 33 (Figure 3.14B). This represents the presence of 50 EV71 copies in the three respective samples. However, representative mice belonging to groups which received either pooled anti-EV71 or anti-SP70 antisera had EV71 RNA transcripts with a higher Ct value of 37, representing approximately 5 EV71 copies (Figure 3.14C).

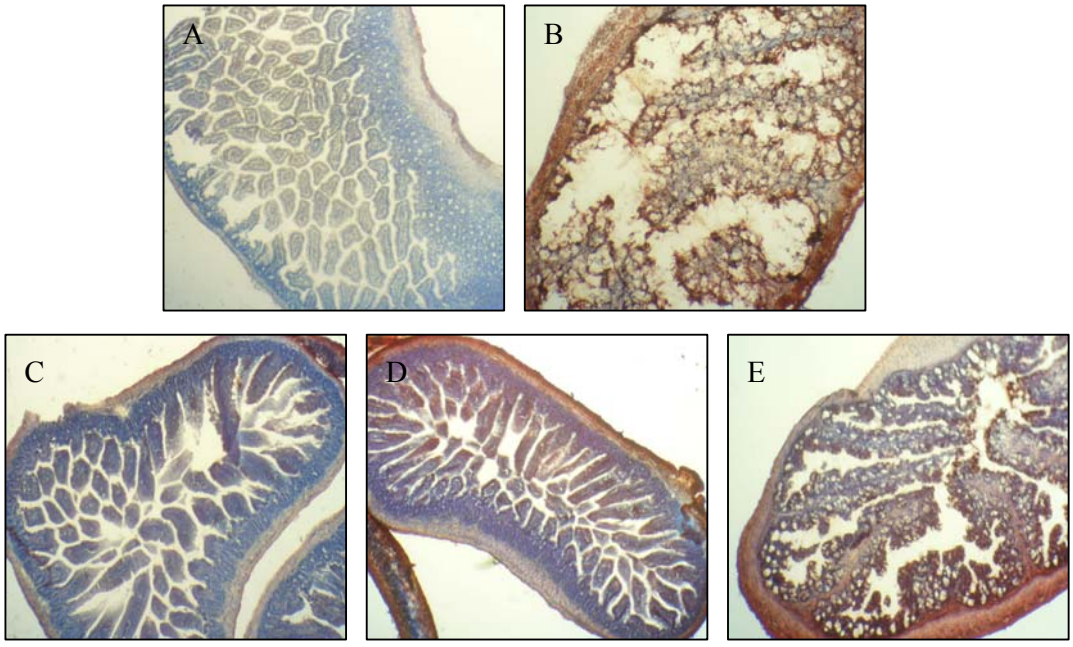


Figure 3.13 Detection of EV71 infection in small intestines of suckling Balb/c mice upon challenged with the homologous EV71 strain 41 at a lethal dose of 10^3 $TCID_{50}$ per mouse. Representative intestinal structures from: **(A)** Non-infected mouse as the negative control. **(B)** EV71-infected mouse as the positive control. **(C)** Mouse receiving the pooled anti-EV71 antisera. **(D)** Mouse receiving the pooled anti-SP70 antisera. **(E)** Mouse receiving the pooled anti-SP12 antisera. Cryosections of intestinal tissues were prepared and EV71 was detected with mouse anti-EV71 monoclonal antibody and biotinylated anti-mouse IgG before visualizing with aminoethyl carbazole substrate to give a red colored peroxidase stain as positive detection and haematoxylin to give a blue background.

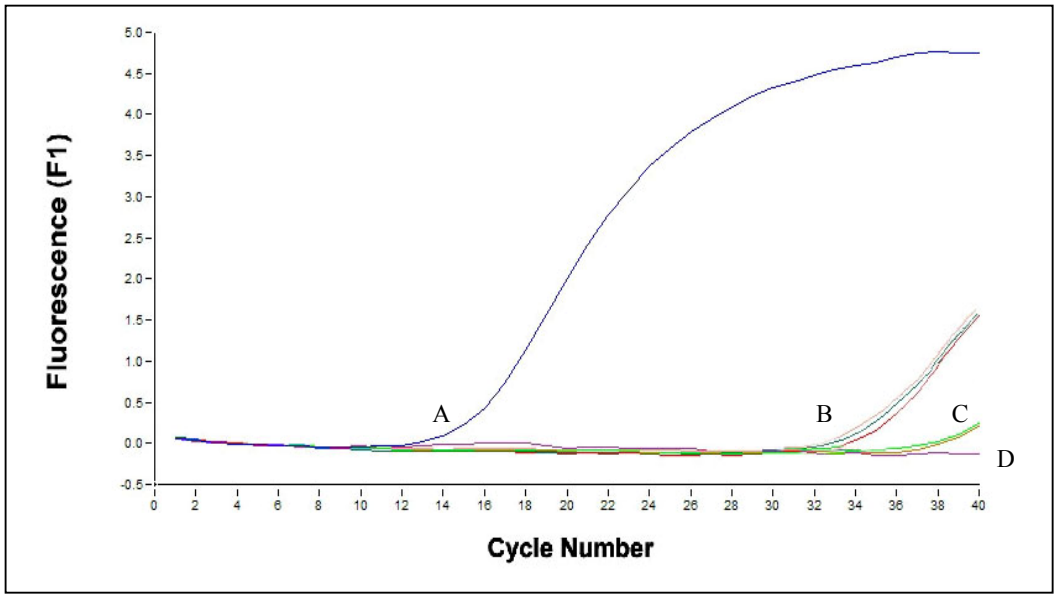
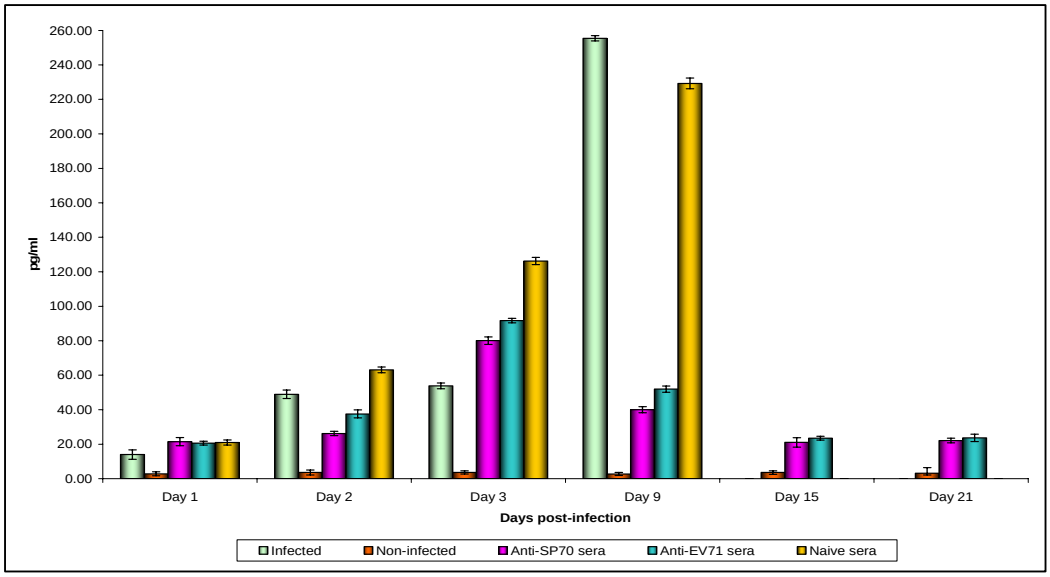


Figure 3.14 Detection of EV71 by real-time RT-PCR hybridization probe-based assay in suckling Balb/c mice upon challenge studies. (A) EV71 complete RNA transcript as the positive control with a Ct value of 13 representing 5 x 10^8 viral copies (Tan *et al.*, 2006). **(B)** RNA transcripts from representative mice belonging to groups that received pooled immune sera from SP12-immunized mice (n=5), pooled sera from non-immunized mice (n=5) or no sera with a Ct value of 33 representing 50 viral copies. **(C)** RNA transcripts from representative mice belonging to groups that received pooled immune sera from the homologous EV71 whole virion- or SP70-immunized mice (n=5) with a higher Ct value of 37 representing 5 viral copies. **(D)** The negative control includes all reagents without any RNA template.

3.2.12 Cytokine profiles in suckling Balb/c mice protected against lethal homologous EV71 strain 41 challenge

To assess the role of pro-inflammatory and anti-inflammatory cytokines during EV71 infection, one-day-old suckling Balb/c mice were infected with the homologous EV71 strain 41 at a lethal dose of 10^3 $TCID_{50}$ per mouse followed by administering pooled naive sera or immune sera from mice immunized with the heat-inactivated homologous EV71 whole virion (anti-EV71) or synthetic peptide SP70 (anti-SP70) at day 1 post infection. With a lethal viral dose, the levels of IL-6, IL-10, IFN-γ and TNF-α in mice were measured at different time points. However, at day 12 post infection, the unprotected mice from groups which received either pooled naive sera or no sera died from EV71 infection and hence it was not possible to obtain any data beyond this time point for these groups of mice. For IL-6, the unprotected mice displayed significant level throughout the viral infection and peaked at day 10 post infection with a concentration of approximately 260pg/ml (Figure 3.15A). However, the mice died at day 12 post infection whereas the protected mice survived up to day 22 post infection as the experimental endpoint. For the protected mice who received either pooled anti-SP70 or anti-EV71 antisera, the IL-6 level at day 10 post infection was significantly lower than that observed for the unprotected mice (Figure 3.15A). Similar pattern was observed for the IFN-γ production with a peak concentration of approximately 400pg/ml at day 10 post infection in the unprotected mice whereas the protected mice produced IFN-γ at a peak concentration of approximately 100pg/ml (Figure 3.15B). For IL-10, the level in protected mice increased up to day 10 post infection and remained high up to day 22 post infection as compared to the early onset of the viral infection (Figure 3.15C). The levels of TNF-α in unprotected and protected mice were elevated throughout the viral infection (Figure 3.15D).

(A)



(B)

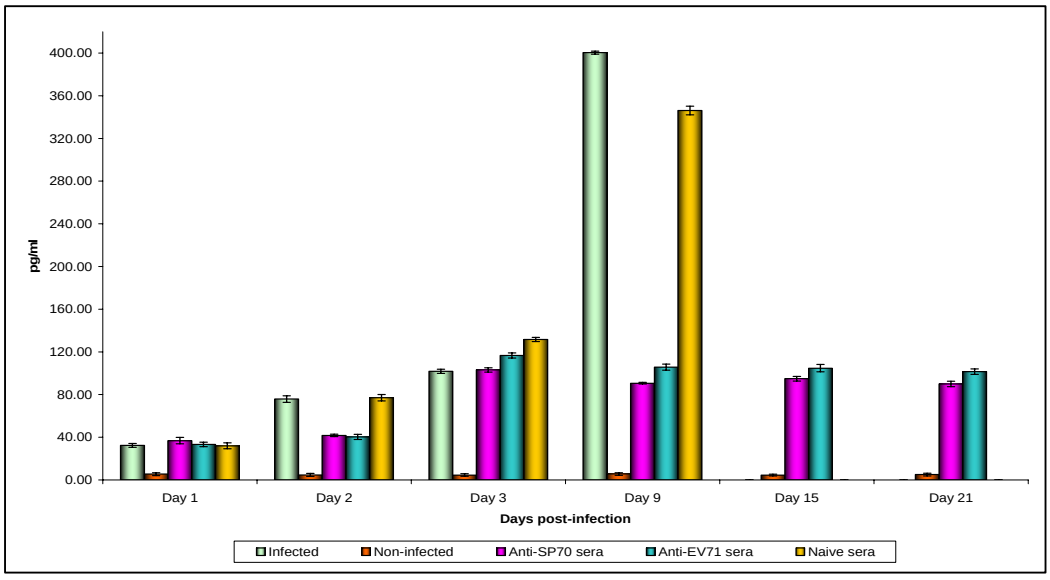
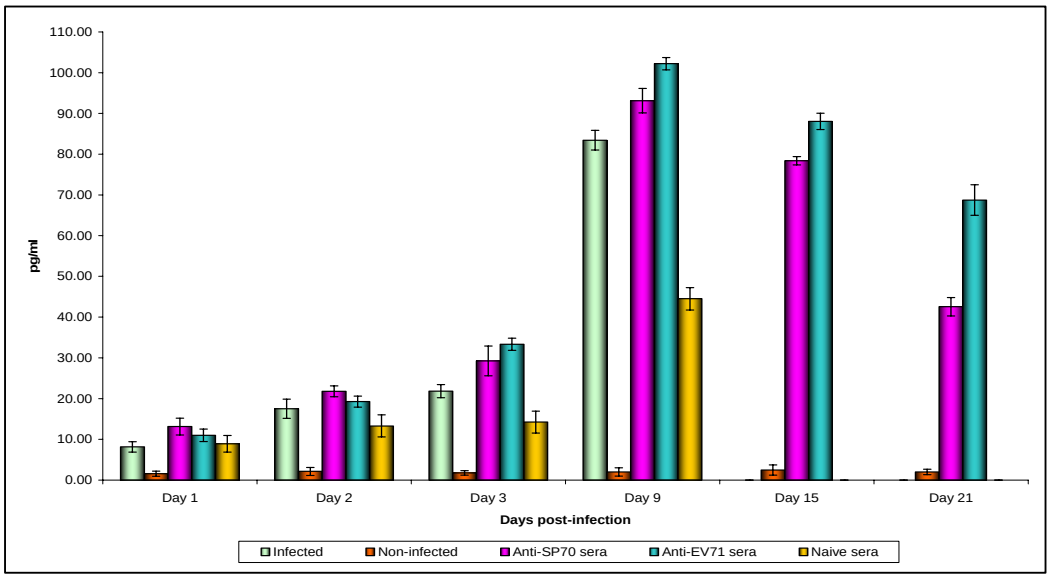


Figure 3.15 Cytokine profile in suckling Balb/c mice upon EV71 challenge. Groups of one-day-old suckling mice were challenged with the homologous EV71 strain 41 at a lethal dose of 10^3 $TCID_{50}$ per mouse followed by the administration of pooled naive sera or immune sera from mice immunized with the heat-inactivated homologous EV71 whole virion (anti-EV71) or synthetic peptide SP70 (anti-SP70) at day 1 post infection. At day 2, 3, 4, 10, 16 and 22 post infection, one mouse per group was sacrificed and serum samples were collected for cytokine analysis. **(A)** IL-6 measurement. **(B)** IFN-γ measurement. The data represent the mean of triplicate assays.

(C)



(D)

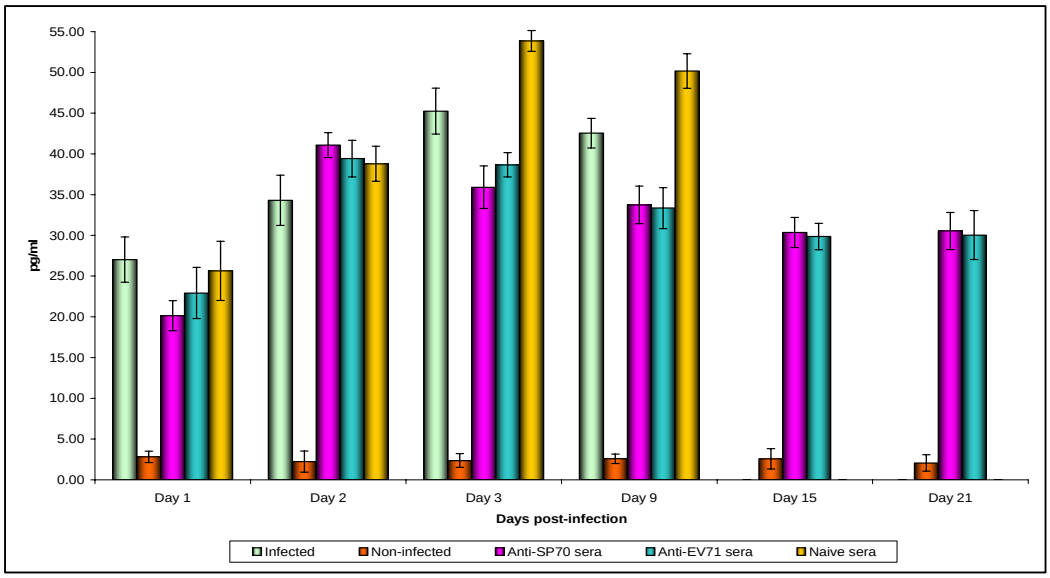


Figure 3.15 Cytokine profile in suckling Balb/c mice upon EV71 challenge. Groups of one-day-old suckling mice were challenged with the homologous EV71 strain 41 at a lethal dose of 10^3 $TCID_{50}$ per mouse followed by the administration of pooled naive sera or immune sera from mice immunized with the heat-inactivated homologous EV71 whole virion (anti-EV71) or synthetic peptide SP70 (anti-SP70) at day 1 post infection. At day 2, 3, 4, 10, 16 and 22 post infection, one mouse per group was sacrificed and serum samples were collected for cytokine analysis. **(C)** IL-10 measurement **(D)** TNF-α measurement. The data represent the mean of triplicate assays.

3.2.13 Immunogenicity of SP55 and SP70

To address whether VP1 regions represented by synthetic peptides SP55 or SP70 are potential peptide vaccine candidates for human, serum samples were collected from children attending a pediatric outpatient clinic at the National University Hospital (NUH) of Singapore and screened for the presence of anti-EV71 neutralizing antibodies by *in vitro* microneutralization assay. A serum neutralizing antibody titer of 1:8 and above was considered positive for EV71 exposure. In total, 40 individuals were found to be positive and their sera were designated as EV71-positive sera. The 95 overlapping unconjugated synthetic peptides spanning the entire VP1 sequence of EV71 strain 41 were used individually as coating antigens in an IgG-based ELISA. In this study, the cut-off absorbance for identifying reactive peptides is set as the mean optical density obtained from EV71-negative sera plus two standard deviation of the respective mean. With the EV71-positive or EV71-negative human sera as primary antibodies, no significant IgG-specific immunoreactivity was observed with SP55 or SP70 as coating antigens (Figure 3.16). Instead, two regions designated I and II, represented by synthetic peptides SP14, SP15 and SP31, SP32, SP33, respectively, were found to be immunoreactive with human sera (Figure 3.16). Region I reacted with EV71-positive and EV71-negative human sera whereas region II reacted specifically with EV71-positive human sera. In contrast, using mice immune sera raised the heat-inactivated EV71 whole virion, significant IgG-specific immunoreactivities were observed with SP55 or SP70 as coating antigens (Figure 3.17). Hence, the absence of human anti-SP55 and anti-SP70 IgG antibodies among patients who were exposed to EV71 suggests that the amino acid sequences represented by both SP55 and SP70 may be immunodominant in mice but not in human.

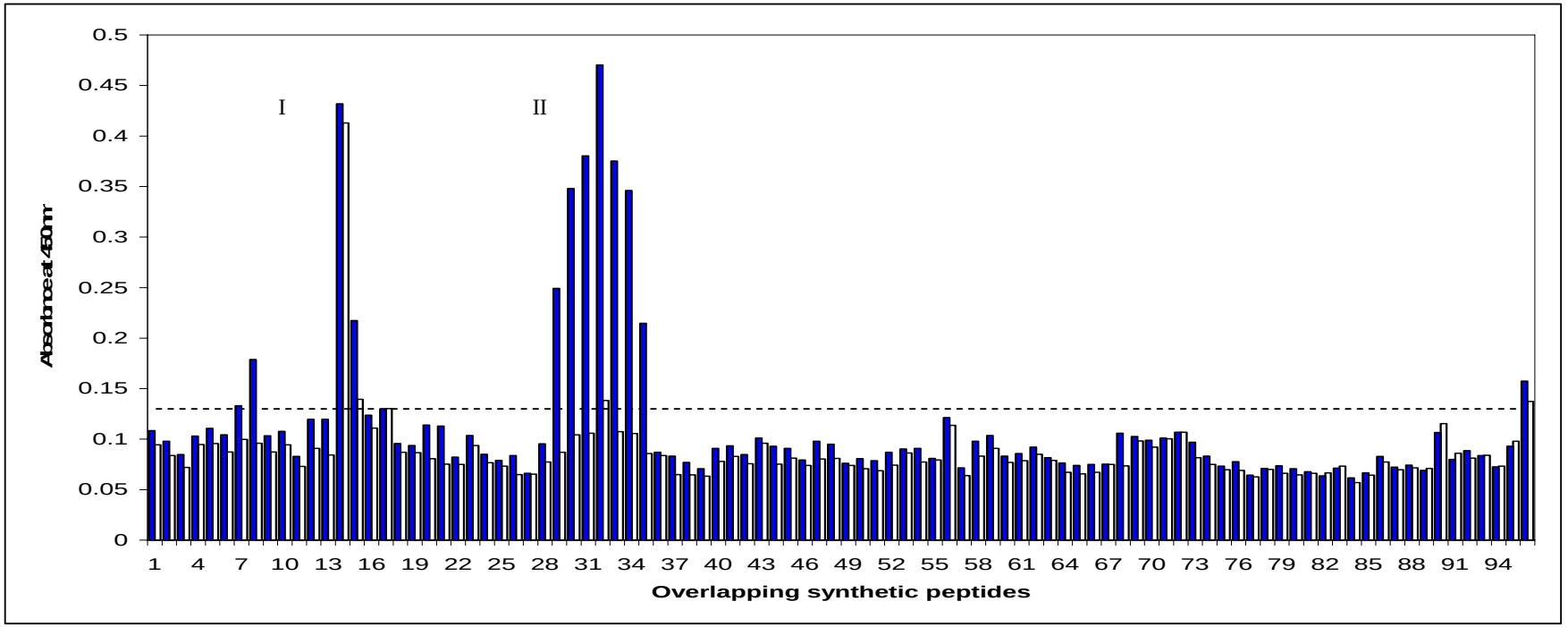


Figure 3.16 Immunoreactivity of VP1 capsid protein against human sera (at 1:50 dilution) by Pepscan analysis. The IgG-based ELISA was carried out with EV71-negative sera (open bars) and EV71-positive sera (black bars). The vertical axis denotes the absorbance readings measured at 450nm and the horizontal axis denotes the synthetic peptide number. Two linear immunoreactive sites were designated as sites I and II. The cut-off absorbance for identifying reactive peptides is represented by the dotted line. The data represent the mean of triplicate assays.

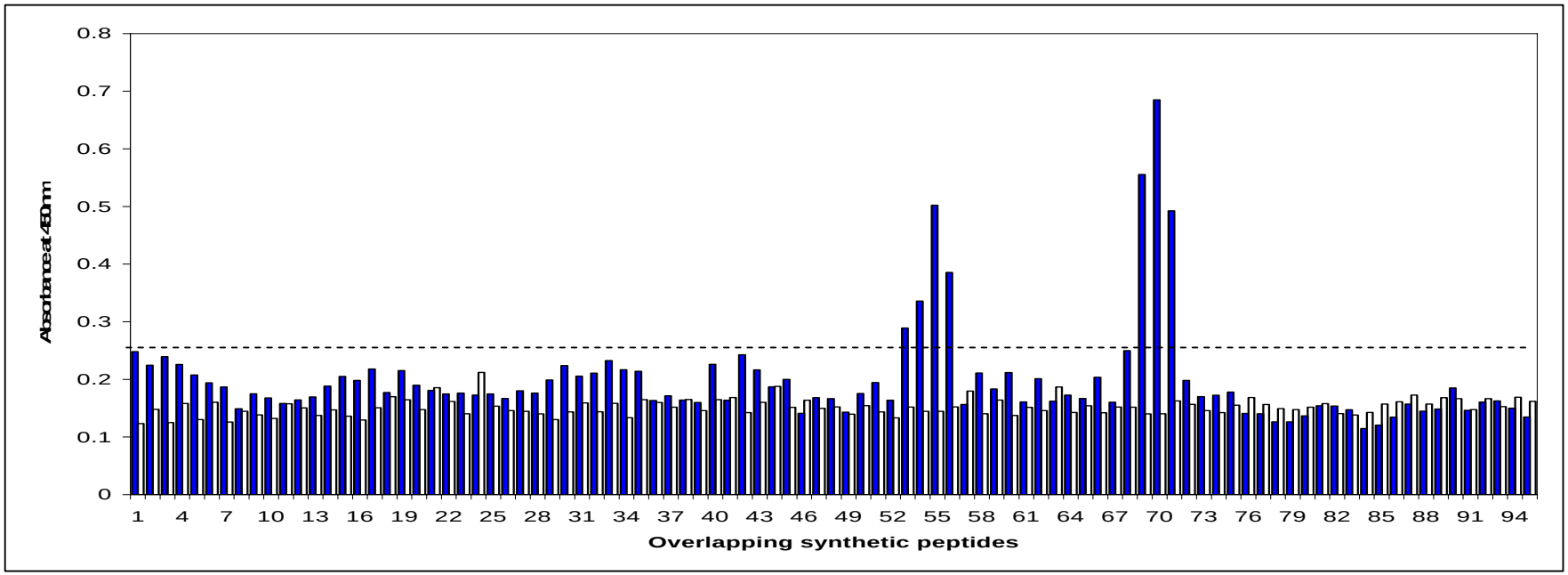


Figure 3.17 Immunoreactivity of VP1 capsid protein against mice immune sera (at 1:50 dilution) by Pepscan analysis. The IgG-based ELISA was carried out with naïve sera (open bars) and anti-EV1 immune sera (black bars). The vertical axis denotes the absorbance readings measured at 450nm and the horizontal axis denotes the synthetic peptide number. The cut-off absorbance for identifying reactive peptides is represented by the dotted line. The data represent the mean of triplicate assays.

3.3 Discussions

Previous studies have shown that the VP1 capsid protein of EV71 constitutes a potential subunit vaccine candidate by triggering the production of protective anti-EV71 neutralizing antibodies in a murine model of infection (Chen *et al.,* 2006; Chiu *et al.,* 2006; Wu *et al.,* 2001). However, these studies focused on the entire VP1 protein without mapping the exact location of the neutralizing epitope(s). In this study, a Pepscan strategy was employed in which 95 overlapping synthetic peptides were designed according to the primary sequence of the VP1 capsid protein of EV71 strain 41 (subgenogroup B4). The diphtheria toxoid-conjugated synthetic peptides were injected into adult Balb/b mice and the anti-EV71 neutralizing activities of the antisera were determined. This strategy allows the identification of neutralizing linear epitope(s) that can thus be used in various peptide vaccine designs. Two synthetic peptides, designated SP55 (VP1 amino acid residues 162 to 177) and SP70 (VP1 amino acid residues 208 to 222) were shown to elicit good production of neutralizing antibodies against EV71. These regions are different as compared to that previously identified along VP1 of the prototype enterovirus (poliovirus) which suggested that the antigenic region (VP1 amino acid residues 89 to 100 of poliovirus type 3) possessed virus-neutralizing activity (Minor *et al.,* 1983; Minor *et al.,* 1985; Minor *et al.,* 1984). Ferguson *et al.* (1985) has also shown that single amino acid substitutions within the VP1 amino acid residues 93 to 89 of poliovirus type 3 can reduce the virus-neutralizing activity.

In this study, the anti-SP70 antiserum was found to be almost as efficient as the immune serum raised against the heat-inactivated homologous EV71 whole virion (highest dilutions preventing cytopathic effect at 1:32 and 1:64, respectively), in

neutralizing EV71 in an *in vitro* microneutralization assay. In addition, the total IgG response specific to EV71 whole virion measured in the anti-SP55 or anti-SP70 antiserum was found to be as high as that measured in the immune serum raised against the heat-inactivated homologous EV71 strain 41.

The IgG subtypes analysis of both anti-SP55 and anti-SP70 antisera showed a strong IgG1 specific antibody response and a very low IgG2a, IgG2b and IgG3 antibody response which indicates a predominant Th2 immune response. These observations suggest that (i) both synthetic peptides SP55 and SP70 contain mainly B-cell epitopes and (ii) the neutralizing antibodies elicited by SP55 and SP70 belong to the IgG1 subtype.

The amino acid sequences presented by both synthetic peptides SP55 and SP70 lie towards the C-terminal part of the VP1 capsid protein of EV71 strain 41. The hydrophobicity profiles also showed that SP55 and SP70 are located within the major hydrophilic regions of VP1 and hence they are expected to be exposed at the surface of the protein. In this study, the hydrophobicity profile of VP1 using the Kyte and Doolittle plot was performed to further strengthen/enhance the speculations that regions represented by both SP55 and SP70 are hydrophilic and may be located on the surface of the capsid protein and hence highly immunogenic. However, it would have been useful to perform this profiling and identify the hydrophilic regions prior to the mice immunization study. This can greatly reduce the duration and the number of mice used for this study. In addition, both synthetic peptides map in the region (VP1 amino acid residues 66-132) recently shown to be involved in the dimerization of VP1 capsid protein (Lal *et al.,* 2006). The finding of neutralizing epitopes within this

region further demonstrates their involvement in virus binding and entry into host cells.

Several genogroups and subgenogroups of EV71 have evolved since its first discovery in 1969, and frequent mutations of the viral genome constitute a major difficulty in vaccine development when using the whole viral particle as a potential vaccine candidate (McMinn, 2002). Many fatality cases due to EV71 were caused by strains from different subgenogroups and studies have shown that the EV71 subgenogroups are constantly evolving within the community (Brown *et al.,* 1999; Mizuta *et al.,* 2005; Wang *et al.,* 2002) which emphasizes the importance of identifying the location of any conserved neutralizing epitope(s). The amino acid sequence of synthetic peptide SP70 appears to be totally conserved amongst 25 representative EV71 strains from different subgenogroups and this study has shown that the immune serum raised against SP70 was also able to neutralize heterologous EV71 strains comparable but lower for strains belonging to subgenogroups B2 and B5 to that obtained with the homologous EV71 strain 41. Due to the conserved amino acid sequence of SP70, one would expect the neutralizing antibody titer of the anti-SP70 immune serum to be similar when tested against both homologous and heterologous strains however this assumption was not observed here. Although it is known that VP1 contains neutralizing epitopes, which are represented by SP55 and SP70 in this study, but McMinn *et al.* (2002) discussed the possibility of numerous neutralizing epitopes localized among other regions of EV71 especially on capsid protein such as VP4. Hence, EV71 infection could still occur via different binding sites to host cell even though regions represented by SP55 or SP70 might be blocked by anti-SP55 or anti-SP70 neutralizing antibodies.

Although immune responses such as elevated antibody level detected soon after immunization may demonstrate the immunogenicity of a vaccine but they do not necessarily provide a reliable guide to its efficacy in priming an *in vivo* protective response. In addition, *in vitro* microneutralization data may not necessary correlate with *in vivo* protective potential as well. Successful protection against viral infectivity in animal models has long been considered as the best test to predict the efficacy of a vaccine. Benefits of vaccination could be derived either through active immunization or passive protection. In this study, EV71 infection was asymptomatic in adult Balb/c mice but suckling mice were susceptible to infection and hence the protective efficacy of the synthetic peptide SP70 was evaluated via passive immunization in newborn mice. Passive transfer of specific antibodies has been shown to reduce the severity of viral infections, including Japanese encephalitis infection (Kimura-Kuroda and Yasui, 1988), varicella infection (Huang *et al.,* 2001) and coxsackievirus infection (Geller and Condie, 1995). Here, the *in vivo* protective potential of EV71-neutralizing antibodies elicited by SP70 was tested in mice by passive transfer of immune sera and it was found that the anti-SP70 antisera was able to confer *in vivo* passive protection of up to 80% survival rate in suckling Balb/c mice which have been challenged with a lethal dose of the homologous EV71 strain 41. The level of protection conferred by the anti-SP70 antiserum against heterologous EV71 strains was almost similar but lower for strains belonging to subgenogroups C2 and C4 to that obtained against the homologous strain, supporting that the VP1 amino acid sequences represented by SP70 contain a highly conserved neutralizing linear epitope. In addition, significant reduction in the survival rate correlated with suckling mice receiving immune sera with progressively lower anti-EV71 neutralizing antibody titers indicating that the neutralizing titer determined *in vitro* correlates with the *in vivo* protective efficacy.

The cytokine profiles for EV71-challenged mice showed elevated IL-6 and IFN-γ levels in unprotected mice whereas significant lower levels were observed for mice which were protected by passively-transferred immune sera. This observation suggests a correlation between pro-inflammatory cytokines and the severity of EV71 infection.

Although a number of immunogens with good *in vivo* passive protective potential have been identified, the majority of EV71 vaccine candidates that have been studied relied mainly on whole viral particles (Wu *et al*., 2001; Yu *et al*., 2000). In this study, the mouse immune sera which were raised against EV71 whole virion did provide higher *in vivo* passive protection to suckling Balb/c mice against lethal EV71 challenge when compared with the anti-SP70 antisera. This is likely due to higher titers of neutralizing antibody elicited by several neutralizing epitopes located on the virus other than that represented by the synthetic peptide SP70 alone. The passively administered immune sera raised against EV71 whole virion might also contain antibodies which could induce lysis of the virus-infected host cells through activation of complement cascades or by mediating antibody-dependent cellular cytoxicity (ADCC) activities. Conventional vaccine preparations using the whole viral particle as an inactivated vaccine might be ideal since it offered good protection against viral infection. However, the laborious and costly procedures involved in EV71 production and purification as well as safety issues are of major concerns. Although this study has shown that the whole virion was able to elicit a high neutralizing antibody titer and hence conferred significant *in vivo* passive protection to suckling Balb/c mice against EV71, a mixture of both SP55 and SP70 as immunogen might trigger a greater neutralizing antibody response than either peptide

alone. Therefore, the level of *in vivo* protection might increase as comparable as those obtained with the whole virion as immunogen.

In conclusion, two independent amino acid sequences within the VP1 capsid protein of EV71 strain 41 which contain neutralizing linear epitopes were identified. These epitopes lie in the C-terminal moiety of the protein, in a hydrophilic region involved in VP1 dimerization. These sequences are highly conserved within the different subgenogroups, making them promising and attractive candidates for synthetic peptide-based EV71 vaccines. With high titers of anti-EV71 neutralizing antibody measured in human serum samples, no specific anti-SP55 or anti-SP70 IgG antibody was identified among those who were exposed to EV71, although anti-EV71 immune sera raised in Balb/c mice were able to react with SP55 or SP70. This suggests that the VP1 amino acid sequences represented by SP55 and SP70 are not immunodominant linear epitope in humans. Therefore, when administered as peptide vaccines with appropriate adjuvant, SP70 may be able to trigger a significant antibody response, capable of protecting efficiently against EV71 infection. In addition, anti-SP70 antibodies, and to a lesser extent, anti-SP55 antibodies may represent promising candidates for the development of therapeutic antibodies against EV71 infections.

CHAPTER 4 IDENTIFICATION OF IMMUNODOMINANT VP1 LINEAR EPITOPE OF ENTEROVIRUS 71 USING SYNTHETIC PEPTIDES FOR DETECTING HUMAN ANTI-EV71 IgG ANTIBODIES IN WESTERN BLOT

4.1 Introduction

Since 1997, several large epidemics of EV71 strains have been reported and these have caused significant mortality in the Asia-Pacific region. The common practice for detecting EV71 in any clinical specimen is by conventional isolation of the whole virus, followed by subsequent serotyping with serotype-specific antibodies and indirect immunofluorescence assay (IFA). This has been the practice in most diagnostic laboratories as the 'gold standard' for the identification of viral infections, although this method is labor-intensive and time-consuming. Another approach involving conventional RT-PCR is available for routine viral diagnosis. While direct detection of EV71 from clinical specimen is possible when the viral load is high, this approach is not sensitive enough to detect the virus from specimens with low viral load. Therefore, cell cultures were required to increase the quantity of viral particles (Tsao *et al.,* 2002a) for detection. In addition, the requirement for specific and expensive reagents as well as equipment further restricts its use for the detection of EV71 infection.

ELISA involves the detection of serotype-specific IgM or IgG which can indicate whether the patient is in the acute or convalescent phase of an infection, respectively. Studies on IgM-based ELISA for the diagnosis of EV71 infection were reported to have high sensitivity and specificity. However, this approach was too laborious as it involved the use of purified whole EV71 viral particle for the detection of EV71-specific antibody (Tano *et al.,* 2002; Tsao *et al.,* 2002a; Wang *et al.*, 2004).

In addition, transient production of rheumatoid factor is common during the acute phase of many infections. Hence, with the presence of rheumatoid or antinuclear factor, the detection of human IgM antibodies could result in non-specific identification of EV71 infection (Tano *et al.*, 2002; Tsao *et al.*, 2002a; Wang *et al.*, 2004). Another study has developed a serodiagnostic tool based on Western blot by using a re-natured whole VP1 protein which was presented as a recombinant His-VP1 fusion protein (Shih *et al.*, 2000a). The VP1 antigen specifically reacted with anti-EV71 antibodies and it was reported to show no cross immunoreactivity with anti-CA16 antibodies.

In all studies reported so far, either the whole VP1 capsid protein or the EV71 whole virion was used as capture antigen and it poses danger to laboratory workers if large quantities of pathogenic viruses were cultivated and further purified. In addition, this study has observed that the purification of recombinant VP1 fusion protein was carried out with much difficulties and the final yield was low. This could be due to the presence of several hydrophobicity regions within VP1. Therefore, it would be an advantage to identify an immunodominant region within VP1 which would be easier to either synthesize chemically or produce efficiently as a recombinant fusion protein. To map the immunodominant epitope(s), an overlapping synthetic peptide library can be used to screen against specific antibodies in an ELISA.

In this study, an IgG-specific immunodominant linear epitope on the VP1 capsid protein of EV71 strain 41 (5865/SIN/00009) was identified by Pepscan analysis using overlapping synthetic peptides spanning the entire amino acid sequence of VP1 to screen against sera from pediatric patients containing specific anti-EV71

IgG antibodies. The immunodominant VP1 linear epitope represented by the synthetic peptide SP32 was over-expressed as a soluble recombinant GST-SP32 fusion protein and used as a capture antigen in Western blot analysis for the detection of human anti-EV71 IgG antibodies. The efficacy of the purified recombinant GST-SP32 fusion protein, the EV71 whole virion and the recombinant GST-VP1 fusion protein used as capture antigens in Western blot analysis was evaluated.

4.2 Results

4.2.1 Mapping of the immunodominant linear epitope of VP1 capsid protein

To identify VP1 immunodominant linear epitope in human, serum samples were collected from children attending a pediatric outpatient clinic at the National University Hospital of Singapore and screened for the presence of anti-EV71 neutralizing antibodies by *in vitro* microneutralization assay. A serum neutralizing antibody titer of 1:8 and above was considered positive for EV71 exposure and designated as EV71-positive. Serum samples which failed to inhibit the virus from causing cytopathic effect (CPE) were designated as EV71-negative. In total, 40 EV71-positive serum samples were pooled and tested against the 95 overlapping synthetic peptides in the IgG-based ELISA. These peptides were also evaluated in parallel against pooled EV71-negative sera from 40 serum samples. The data revealed two immunoreactive regions designated as regions I and II as described in chapter 3 (Figure 3.20). The first region was represented by two overlapping synthetic peptides (SP14 and SP15), corresponding to VP1 amino acid residues 40 to 57 which mapped within or close to the N-terminal region of the VP1 capsid protein. However, the pooled EV71-negative sera also displayed immunoreactivities towards the same region which strongly suggests serological cross-reactivities. The second region spans VP1 amino acid residues 91 to 111 (represented by SP31 to SP33) which correspond to the middle region of the VP1 capsid protein (Figure 3.20). Interestingly, the pooled EV71-negative sera did not react with region II indicating that this region, defined by the core sequence ‘LEGTTNPNG’, represents the major immunodominant linear epitope on the VP1 capsid protein of EV71.

4.2.2 Expression of recombinant GST-VP1 and GST-SP32 fusion proteins

The synthetic peptide SP32 (DLPLEGTTNPNGYAN) which contained the core sequence of the immunodominant VP1 linear epitope was selected as a potential capture antigen for human anti-EV71 IgG antibodies. Over-expression in *E. coli* of both recombinant GST-VP1 and GST-SP32 fusion proteins was achieved by IPTG induction and they were produced as recombinant GST-tagged fusion proteins. SDS-PAGE analysis showed purified proteins with estimated molecular weights of 58kDa and 28kDa, which correspond to the sizes of the expected 58.7kDa recombinant GST-VP1 (Figure 4.1A, lane 2) and 27.8kDa recombinant GST-SP32 fusion protein (Figure 4.1B, lane 2), respectively. Using commercially available mouse anti-EV71 monoclonal antibody and anti-GST antibody, their immunoreactivities with purified recombinant GST-VP1 and GST-SP32 fusion proteins were evaluated in Western blot. A strong immunoreactivity was observed for both fusion proteins when using the commercially available mouse anti-EV71 monoclonal antibody as primary antibody (Figure 4.1C, lanes 2 and 4, respectively). In addition, using the commercially available mouse anti-GST antibody, similar immunoreactivity was also observed for both recombinant GST-VP1 and GST-SP32 fusion proteins (Figure 4.1C, lanes 1 and 3, respectively).

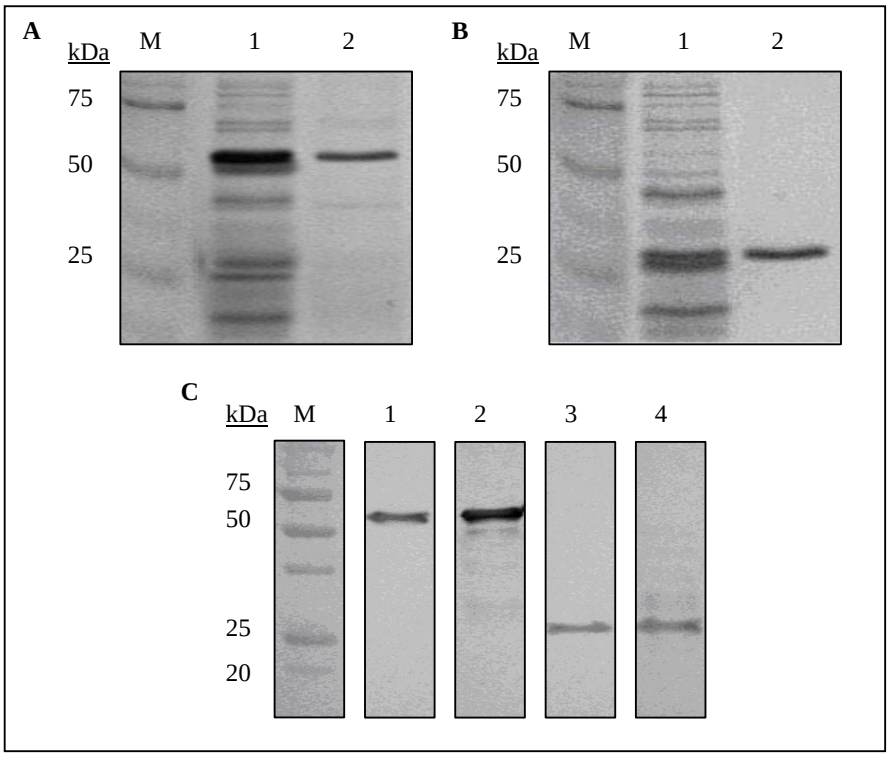


Figure 4.1 Expression of purified recombinant GST-VP1 and GST-SP32 fusion proteins. (**A)** SDS-PAGE of GST-VP1 crude and purified fractions. The lanes are as follows: lane M, molecular weight marker; lane 1, IPTG-induced crude cell lysate; lane 2, purified GST-VP1 fusion protein. **(B)** SDS-PAGE of GST-SP32 crude and purified fractions. The lanes are as follows: lane M, molecular weight marker; lane 1, IPTG-induced crude cell lysate; lane 2, purified GST-SP32 fusion protein. **(C)** Western blot analysis of the purified recombinant GST-VP1 and GST-SP32 fusion proteins. The amount of proteins loaded in each lane was 10 µg. The lanes are as follows: lane M, molecular weight marker; lane 1, anti-GST antibody against GST-VP1; lane 2, anti-EV71 antibody against GST-VP1; lane 3, anti-GST antibody against GST-SP32; lane 4, anti-EV71 antibody against GST-SP32. Bound antibodies were detected using secondary HRP-conjugated goat anti-mouse antibody.

4.2.3 Detecting human anti-EV71 IgG antibodies using purified recombinant GST-VP1 and GST-SP32 fusion proteins in IgG-based ELISA and Western blot

To investigate the potential of using the purified recombinant GST-SP32 fusion protein as a capture antigen in IgG-based ELISA, its efficacy was compared to that of the purified recombinant GST-VP1 fusion protein and EV71 whole virion as capture antigens. When tested against pooled EV71-positive sera, the human anti-EV71 IgG antibodies had strong immunoreactivities with the recombinant GST-VP1 fusion protein and EV71 whole virion. Surprisingly, similar immunoreactivities were observed with both capture antigens when tested against pooled EV71-negative sera, suggesting cross-reactivity with human non-EV71-specific antibodies. However, there was no immunoreactivity for the recombinant GST-SP32 fusion protein as a capture antigen when tested against pooled EV71-positive or EV71-negative sera. This suggests that the immunodominant VP1 linear epitope represented by the synthetic peptide SP32 may be hidden by the larger GST protein and hence not available to interact with human EV71-specific IgG antibodies. The immunoreactivities of human anti-EV71 IgG antibodies against all three capture antigens were then tested in Western blot using denaturing SDS-PAGE which ensures a totally unfolded conformation of the proteins. As already observed in ELISA, positive immunoreactivities were obtained with the pooled EV71-positive and EV71-negative sera when using the EV71 whole virion or the recombinant GST-VP1 fusion protein as capture antigens (Figure 4.2). In contrast, the recombinant GST-SP32 fusion protein was able to react specifically with the pooled EV71-positive sera but not with the pooled EV71-negative sera (Figure 4.2). These results strongly indicate that the

purified recombinant GST-SP32 fusion protein represents an interesting capture antigen candidate to detect human specific anti-EV71 IgG antibodies by Western blot.

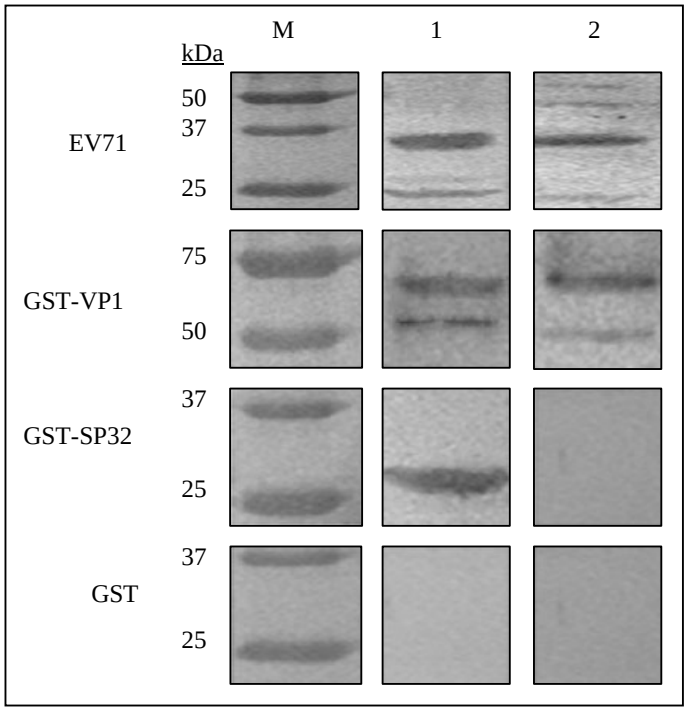


Figure 4.2 Western blot analysis of antigen reactivity with pooled EV71-positive or EV71-negative sera. Capture antigens used in this assay include the EV71 whole virion, recombinant GST-VP1 and GST-SP32 fusion proteins, and the native GST protein. The amount of proteins loaded in each lane was 10 μg. The lanes are as follows: lane M, molecular weight marker; lane 1, immunoblotted with pooled EV71-positive sera; lane 2, immunoblotted with pooled EV71-negative sera. Bound antibodies were detected using secondary HRP-conjugated goat anti-human IgG antibody.

4.2.4 Specificity of the purified recombinant GST-SP32 fusion protein as a capture antigen in Western blot

The purified recombinant GST-SP32 fusion protein was tested against commercially available monoclonal antibodies raised against other HFMD-causing enteroviruses such as Coxsackie A9 and Echovirus 6. The results only showed significant immunoreactivity against the commercially available mouse anti-EV71 monoclonal antibody but no cross-immunoreactivities were observed against the other two monoclonal antibodies. A protein–protein BLAST (blastp 2.2.17) search demonstrated that the amino acid sequence of the immunodominant VP1 linear epitope represented by the synthetic peptide SP32 was highly specific for EV71 strains and no significant homology with other enteroviruses such as coxsackieviruses and echoviruses was found. The SP32 amino acid sequence was also identified to be conserved amongst representative EV71 strains from different genogroups and subgenogroups (Figure 4.3).

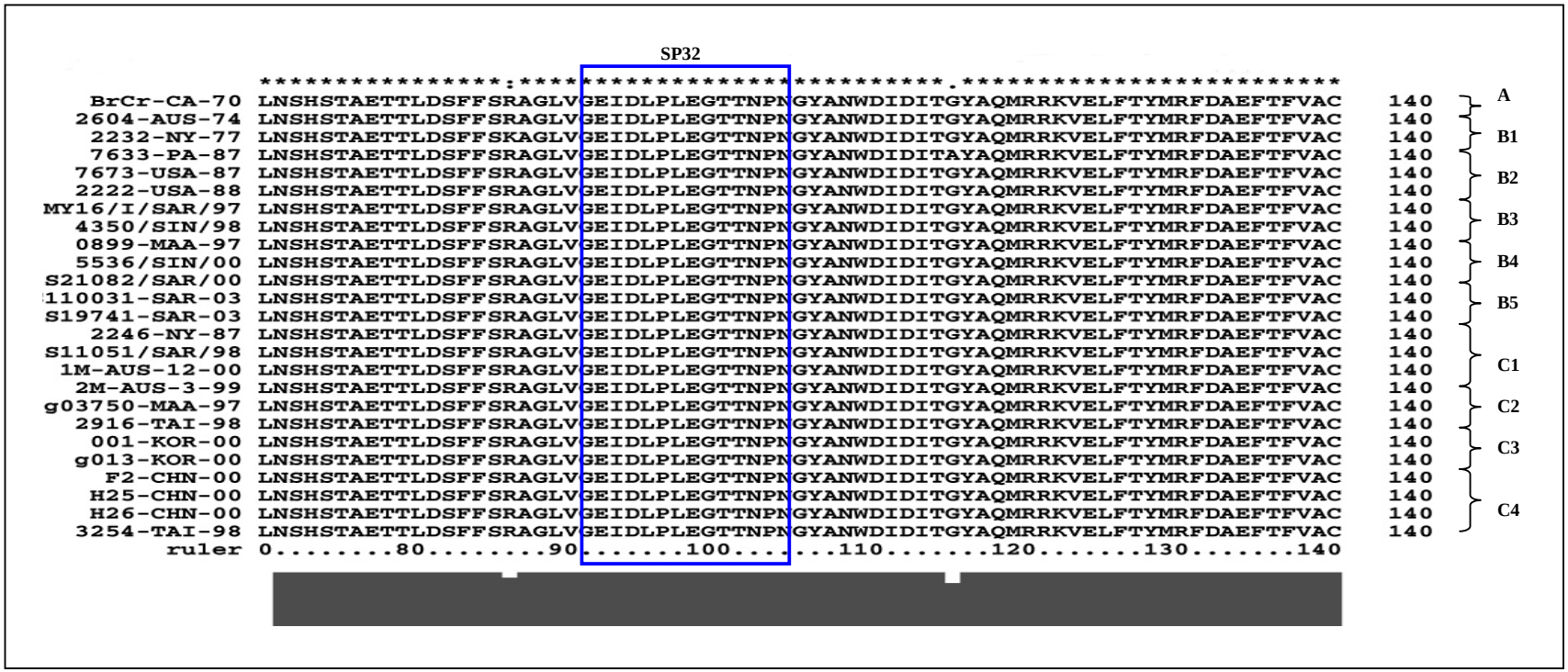


Figure 4.3 Alignment of amino acid sequences represented by the synthetic peptide SP32 against heterologous EV71 strains from different subgenogroups based on the VP1 amino acid sequences.

4.3 Discussions

The use of synthetic peptide(s) as capture antigen(s) in immunoassays represents an interesting approach for the serodiagnostic of EV71 infection as it would avoid the need for propagating infectious viruses. A synthetic peptide representing amino acid residues 42 to 55 of the VP1 capsid protein of enteroviruses when used as a capture antigen in an indirect IgG-based ELISA was found to be useful for serodiagnose a broad range of enteroviruses (Samuelson *et al.*, 1994). However, this immunodiagnostic method is not specific for the detection of human anti-EV71 IgG antibodies and is limited by high production cost and availability of synthetic peptide(s). A previous study has shown that the VP1 capsid protein of EV71 is immunogenic (Shih *et al.*, 2000a). However, very little information is available regarding the location of the immunodominant epitope(s) on VP1. By expressing the whole VP1 protein itself and non-overlapping fragments of VP1 as recombinant His-tagged fusion proteins in their native forms, a recent study has shown that human sera containing neutralizing antibodies against EV71 strain MS/7423/87 (GenBank accession number U22522) reacted strongly towards the N-terminal region of VP1 which suggests the presence of immunodominant epitope(s) (Tan and Cardosa, 2007).

Pepscan analysis using overlapping synthetic peptides has been widely used for identifying antigenic epitope(s) of viruses (Frank, 1992; Gao and Esnouf, 1996; He *et al.*, 2004a, 2004b; He *et al.*, 2005; Kramer *et al.*, 1994; Reineke, 1996). Generally, epitopes can be divided into 2 broad categories defined by linear and discontinuous (conformational) structures (Appel *et al.*, 1990; Barlow *et al.*, 1986; Laver *et al.*, 1990). In the present study, two IgG-specific immunoreactive linear regions within VP1 of EV71 strain 41 (5865/SIN/00009) have been identified using

the Pepscan approach. When used as capture antigen in ELISA, region I represented by overlapping synthetic peptides SP14 and SP15 was unable to specifically detect human anti-EV71 IgG antibodies as it reacted with the pooled EV71-negative sera as well. The second immunoreactive region was represented by overlapping synthetic peptides SP31, SP32 and SP33. This region in contrast, was highly specific for the detection of human anti-EV71 IgG antibodies and hence was identified as an IgG-specific immunodominant linear epitope on the VP1 capsid protein of EV71. When tested against human sera containing neutralizing antibodies against EV71 strain 41, there were significant immunoreactivities towards the synthetic peptide SP32 which contains the core amino acid sequence 'LEGTTNPNG'. In addition, the blast analysis showed that the VP1 amino acid sequence represented by SP32 was highly specific and conserved among EV71 strains indicating its high sensitivity as a capture antigen in detecting human anti-EV71 IgG antibodies.

The VP1 capsid protein of prototype EV71 BrCr strain (GenBank accession number U00871) when produced as a recombinant His-VP1 fusion protein was highly insoluble which required denaturation and renaturation before it could be used as a capture antigen in Western blot (Shih *et al.,* 2000a). The insolubility could be attributed to several hydrophobic transmembrane regions within VP1. In this study, the 15-amino acid immunodominant VP1 linear epitope represented by the synthetic peptide SP32 was produced as a soluble recombinant GST-SP32 fusion protein at high yields. Based on the Kyte and Doolittle hydrophobicity profile of the entire VP1 capsid protein of EV71 strain 41, this region (SP32) was identified to be mainly hydrophilic (Foo *et al.,* 2007a). The efficacy of the purified recombinant GST-SP32 fusion protein as a capture antigen for human anti-EV71 IgG antibodies was carried

out in both IgG-based ELISA and Western blot analysis. No significant immunoreactivity with human anti-EV71 IgG antibodies was obtained by ELISA and hence it was hypothesized that the antigenic site represented by SP32 may be masked by the much larger GST moiety, thereby inhibiting antigen-antibody interactions. Alternatively, the GST protein can be cleaved from the recombinant GST-SP32 fusion protein and the SP32 protein by itself can be used as capture antigen in ELISA. When the recombinant GST-SP32 fusion protein was used as a capture antigen in Western blot, a strong and specific immunoreactivity was obtained with human anti-EV71 IgG antibodies. This is likely due to the denatured state of the recombinant fusion protein where the steric hindrance caused by the GST protein was removed, thereby exposing the immunogenic region represented by SP32.

When used as capture antigens, both the recombinant GST-VP1 fusion protein and EV71 whole virion in contrast, were not able to specifically detect human anti-EV71 IgG antibodies. This could be attributed to the presence of numerous common epitopes between enteroviruses which have been shown to be present in capsid proteins (Cello *et al.,* 1993; Mertens *et al.,* 1983; Samuelson *et al.,* 1994; Samuelson *et al.,* 1993). Hence, human IgG antibodies elicited against common epitopes found on different viruses other than enteroviruses may display cross-reactivity when tested against the recombinant GST-VP1 fusion protein and EV71 whole virion. For example, cross-reactions were observed when using human anti-rhinovirus antisera or anti-cytomegalovirus antisera in immunoassays for the detection of enteroviruses (Couch and Knipe, 1990; Samuelson *et al.,* 1993). Cross-reactivity may also be attributed to human anti-poliovirus IgG antibodies which are commonly present in most of the pediatric patients as a result of the National childhood immunization

program. Consequently, human antibodies elicited against poliovirus may cross-react with EV71 whole virion (Mertens *et al.,* 1983; Tsao *et al.,* 2002a; Wang *et al.,* 2004).

In conclusion, this study clearly showed that the purified recombinant GST-SP32 fusion protein represents an interesting capture antigen in Western blot to detect specifically human anti-EV71 IgG antibodies. Therefore, the recombinant GST-SP32 fusion protein could be further developed as a serologic reagent in the detection of human specific anti-EV71 IgG antibodies for the identification of EV71 infection as well as a confirmatory assay for previous EV71 exposure. Since Western blot is a multi-step process, a simpler approach such as dot blot assay may be considered as a detection method instead. Alternatively, the recombinant SP32 protein may be cleaved from the GST-SP32 fusion protein to be used as capture antigens in ELISA.

CHAPTER 5 IDENTIFICATION OF HUMAN CD4+ T-CELL EPITOPES ON THE VP1 CAPSID PROTEIN OF ENTEROVIRUS 71

5.1 Introduction

EV71 epidemics with neurological, cardiac and/or pulmonary complications occurred worldwide with a particular surge in the Asia-Pacific region during the last ten years (Sanders *et al.,* 2006). The current strategy of preventing EV71 infection relies on public health surveillance and good hygiene practices. Presently, there is no specific drug against EV71 and treatments only provide relief for fever and pain. Natural viral infections usually lead to the production of protective neutralizing antibodies. In a recent study, the cord sera from 205 individuals were tested for the presence of anti-EV71 neutralizing antibodies and the authors described that the N-terminal moiety of the VP1 capsid protein is likely to harbor EV71-neutralizing epitopes (Tan and Cardosa, 2007). However, these epitopes have yet to be identified. In this study, we have identified three regions on the VP1 capsid protein of enterovirus 71 strain 41 (5865/SIN/00009) capable of inducing human EV71-specific $CD4^+$ T cell proliferation. $CD4^+$ T cells play a crucial and pivotal role in adaptive immunity, for the development of an antibody-based immunity (Collen *et al.,* 1989; Collen and Doel, 1990) as well as cytotoxic T cell responses (Gao *et al.,* 2002; Katrak *et al.,* 1991; Ossendorp *et al.,* 1998). Therefore, the identification of $CD4^+$ T-cell epitopes is of great interest and might help in the designing of more effective subunit vaccines.

5.2 Results

5.2.1 Prediction of HLA-DR-restricted epitopes

Computer-generated programs which predict MHC class II binding peptides are based on the identification of HLA-DR allele-specific motifs in antigenic peptides and on the structural definition of HLA-peptide complexes. The primary amino acid sequence of the VP1 capsid protein of EV71 strain 41 was analyzed using the T-cell epitope prediction ProPred algorithm which led to the identification of promiscuous regions potentially binding to HLA-DR molecules. Three regions (I to III) were predicted to bind more than 50% of the 51 HLA-DR alleles available in the ProPred database and were therefore considered as promiscuous CD4$^+$ T-cell epitopes (Figure 5.1). These regions map at amino acids 66 to 77, 145 to 159 and 247 to 261 of the VP1 protein (Table 5.1). The corresponding peptides, namely SP1, SP2 and SP3, respectively, were synthesized as well as a fourth peptide SP4 (scrambled), representing a VP1 region with poor prediction for promiscuous binding. A protein-protein BLAST (blastp 2.2.17) search indicated that the amino acid sequences of all four peptides were highly specific for EV71 strains with no significant homology with other enteroviruses.

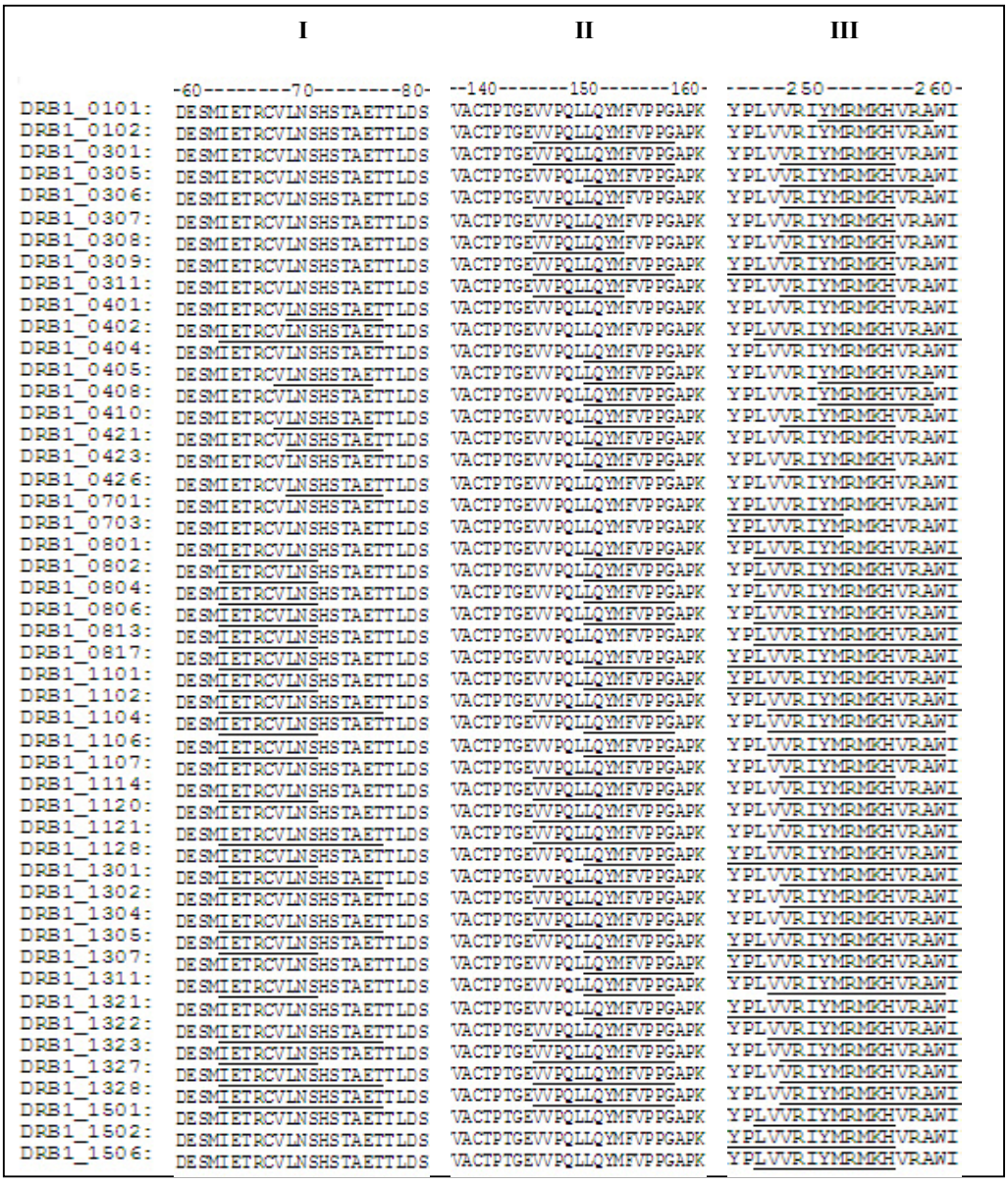


Figure 5.1 An output of ProPred analysis of the VP1 amino acid sequence in HTML view II for binding to 51 HLA-DR alleles. The sequence was analyzed for binding to 51 HLA-DR alleles using the ProPred program at the default setting (threshold value of 3). The peptide sequences predicted to bind more than 50% of the HLA-DR alleles available in the database are underlined.

Table 5.1 Sequences and locations of predicted promiscuous regions.

Peptides[a]	Predicted Regions	VP1 Amino Acid Sequences	Positions
SP1	I	H - IETRCVLNSHSTAET - OH	63 – 77
SP2	II	H - EVVPQLLQYMFVPPG - OH	145 – 159
SP3	III	H - LVVRIYMRMKHVRAW - OH	247 – 261
SP4	NA	H - PTGQNTQVSSHRLDT - OH	27 – 41

[a] peptides (SP1 to SP3) representing VP1 regions considered promiscuous for binding more than 25 different HLA-DR alleles. Peptide SP4 represents a scrambled peptide with poor prediction of promiscuous binding (NA: Not applicable).

5.2.2 HLA-DR typing and detection of anti-EV71 antibodies in human volunteers

A total of twenty human volunteers were recruited and HLA-DR genotyped for this study. Only six displayed HLA-DR alleles which were represented in the ProPred database. The sera of these six volunteers were screened for the presence of anti-EV71 neutralizing antibodies by *in vitro* microneutralization assay. Among these six volunteers, five were sero-diagnosed positive for the presence of anti-EV71 neutralizing antibodies with a neutralizing titer of 1:8 and above and one was identified as EV71-negative (Table 5.2). All volunteers were found negative for EV71 by cultural analysis indicating that none of them were currently infected with the virus. Western blot analysis was subsequently performed using a recombinant GST-VP1 fusion protein as capture antigen to detect anti-VP1 IgG antibodies in the serum of each volunteer. As expected, a positive signal was observed when using serum samples from all EV71-positive volunteers whereas no signal was detected in the EV71-negative volunteer, confirming previous studies which described VP1 as a highly immunogenic antigen (Chen *et al.,* 2006; Chiu *et al.,* 2006; Foo *et al.,* 2007a; Wu *et al.,* 2001; Yu *et al.,* 2000).

Table 5.2 EV71 exposure of volunteers and predicted peptide binding efficiencies.

Volunteers	HLA genotypes	Serum anti-EV71 neutralizing antibody titer[a]	Anti-VP1 IgG antibody[b]	Predicted binding efficiency (%)[c]			
				SP1	SP2	SP3	SP4
1	DRB1*0301	1:128	+	0	93	60	0
2	DRB1*0301	1:64	+	0	93	60	0
3	DRB1*0301	> 1:256	+	0	93	60	0
4	DRB1*0405	> 1:256	+	60	60	60	0
5	DRB1*1301	1:64	+	93	100	86	0
6	DRB1*0301	-	-	0	93	60	0

[a] Serum anti-EV71 neutralizing titer was determined using an *in vitro* microneutralization assay.

[b] Immunoblots using recombinant GST-VP1 fusion protein as capture antigen and 1:50 diluted serum sample from each volunteer as primary antibody. (+) represents the presence of a 58.7kDa band corresponding to the GST-VP1 fusion protein indicating the presence of specific anti-VP1 IgG antibody.

[c] Predicted binding efficiency of peptides based on specific HLA-DR allele.

5.2.3 CD4+ T cell proliferative responses

CD4+ T cells isolated from EV71-positive and -negative volunteers were stimulated with VP1-derived peptides (SP1 to SP4) or heat-inactivated EV71 whole virions using autologous monocytes-derived dendritic cells (MoDCs) as professional antigen-presenting cells (APCs). The cellular proliferative responses were expressed as stimulation index (SI) which corresponds to the ratio of the counts per minute (cpm) in the presence of antigen over the cpm without any antigenic stimulus. As expected, for all EV71-positive volunteers, elevated CD4+ T cell proliferative responses were observed upon stimulation with EV71 whole virions (Figure 5.2). However, stimulation with SP4 (scrambled peptide) did not result in any significant proliferative response regardless of the HLA-DR alleles tested.

A weak but non-negligible CD4+ T-cell proliferative response was observed for the EV71-negative volunteer upon stimulation with EV71 whole virions. CD4+ T cells isolated from EV71-positive volunteers proliferated significantly and with similar amplitudes upon stimulation with peptide SP2. However, the SI obtained upon stimulation with peptide SP1 or SP3 differ from one HLA-DR allele to another and was either similar or lower than the SI obtained upon SP2 stimulation (Figure 5.2). This data correlate with the ProPred analysis which predicted for the five EV71-positive volunteers; a higher binding efficiency for the VP1 region II (represented by SP2) as compared to regions I and III (represented by SP1 or SP3) (Table 5.2).

Furthermore, stimulation with SP1 failed to induce a significant CD4+ T cell proliferation for EV71-positive volunteers with HLA genotype DRB1*0301 (volunteers 1 to 3) which again, correlates with the ProPred program's predictions

(Table 5.2). Similarly, the SI obtained upon SP3 stimulation reflected well on the ProPred analysis which predicted that region III (corresponding to SP3 peptide) displays the highest binding efficiency for HLA genotype DRB1*1301. In addition, a background SI value was consistently obtained upon stimulation of CD4$^+$ T cells isolated from the EV71-negative volunteer with any of the stimulating antigens, indicating that the proliferative responses observed for volunteers 1 to 5 resulted from EV71-specific CD4$^+$ T cells (Figure 5.2).

Altogether, these data indicate that the ProPred program accurately predicted the binding efficiency for each peptide depending on the HLA-DR allele and therefore represents a reliable prediction approach to identify potential CD4$^+$ T-cell epitopes. The results also suggest that SP2 contains a promiscuous epitope which is able to induce significant EV71-specific CD4$^+$ T-cell proliferation.

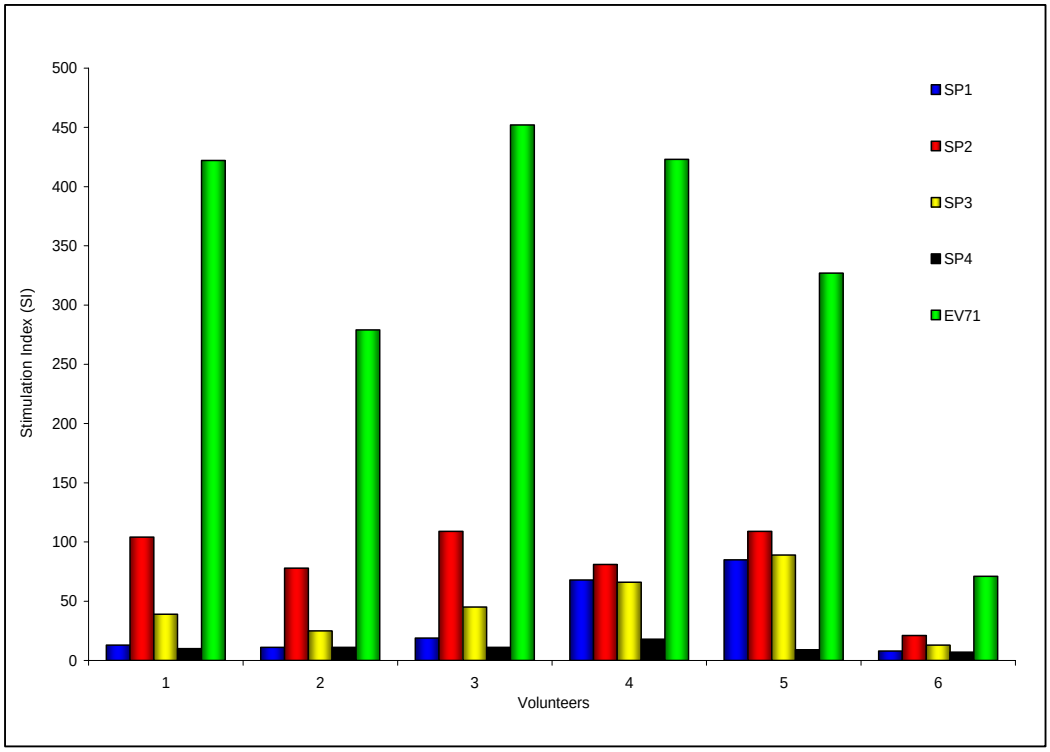


Figure 5.2 Proliferation of CD4$^+$ T cells upon stimulation with peptides or EV71 whole virions. Heat-inactivated EV71 whole virions, peptides (SP1 to SP4) or no antigen were first added to the monocyte-derived dendritic cells (MoDCs) prepared from each volunteer for activation before incubation with autologous CD4$^+$ T cells. [methyl-^{3}H] thymidine was added into the culture medium 24 h before counting. The stimulation index (SI) was calculated as the ratio of the counts per minute (cpm) in the presence of antigen over the cpm in the absence of antigen.

5.2.4 MHC class II-blocking experiment

To confirm that the observed CD4$^+$ T-cell proliferation is mediated via peptide presentation in association with MHC class II molecules, a MHC class II-blocking experiment was carried out. Peptide SP2 and anti-human MHC class II monoclonal antibodies were incubated with immature DCs (imDCs) from EV71-positive volunteers and CD4$^+$ T-cell proliferative responses were measured as described above. The co-incubation with anti-MHC class II antibodies resulted in a reduction of the SP2-induced CD4$^+$ T-cell proliferation by more than 80% (Figure 5.3). Since, no antibody-specificity controls such as anti-MHC class I antibodies or isotype-matched control antibodies were used in this assay, this data can only suggest that SP2 is presented by APCs in association with MHC class II molecules to effectively induce EV71-specific CD4$^+$ T cell proliferation and that SP2 may contain a MHC class II-restricted CD4$^+$ T-cell epitope.

5.2.5 Cytokine profile upon antigenic stimulation

To address whether antigens induce CD4$^+$ T cell differentiation into Th1- or Th2-type subsets, cytokine production by proliferating CD4$^+$ T cells was analyzed upon stimulation with either peptides (SP1 to SP4) or heat-inactivated EV71 whole virions. Significant levels of IL-2 and IFN-γ were detected in the supernatant of the SP1-, SP2-, SP3- and EV71-stimulated CD4$^+$ T cells from EV71-positive volunteers (Table 5.3). Similar to the proliferative responses, CD4$^+$ T cells from all EV71-positive volunteers secreted higher levels of IL-2 and IFN-γ in response to SP2 as compared to SP1 and SP3. No significant differences in the production of IL-4 and IL-10 were observed upon stimulation with SP1 to SP4 and those without any antigenic stimulus (Table 5.3). However, significant levels of IL-4 were detected

when using EV71 whole virions as stimulants for CD4$^+$ T cells from all EV71-positive and -negative volunteers. Instead, a significant level of IL-10 was measured in EV71-stimulated CD4$^+$ T cells from EV71-positive volunteers only. Altogether, these data indicate that the levels of cytokine production correlate well with the CD4$^+$ T-cell proliferative responses, with SP2 triggering the highest cytokine production regardless of HLA-DR alleles. Significant production of IL-2 and IFN-γ by proliferating CD4$^+$ T cells also suggests their differentiation into Th1-type subset.

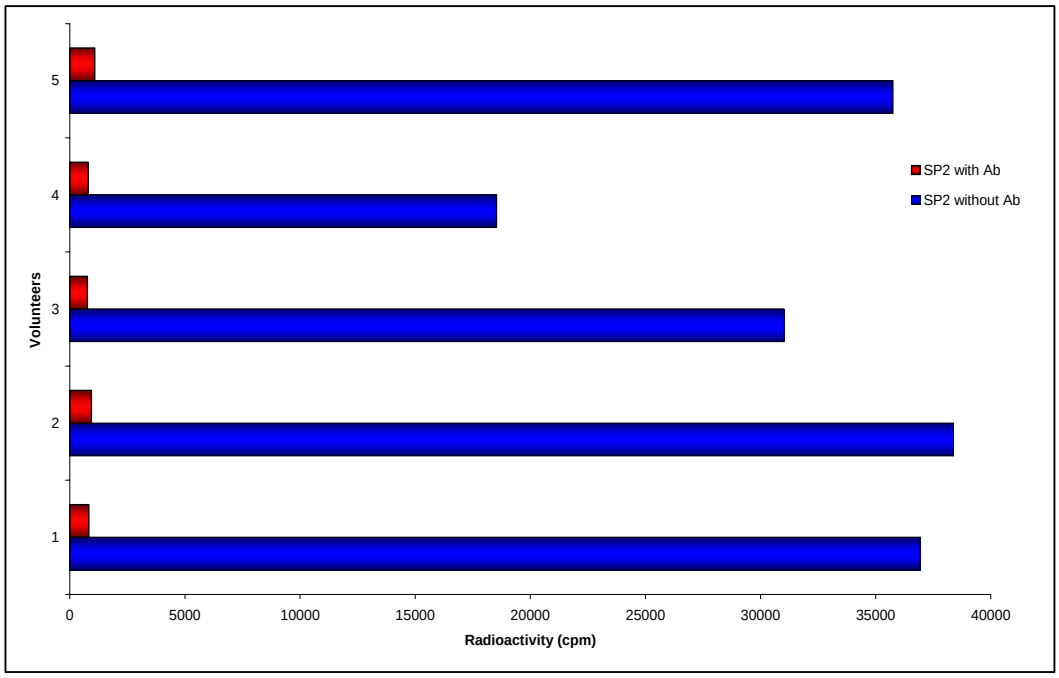


Figure 5.3 Proliferation of SP2-stimulated CD4$^+$ T cells in the presence of anti-human MHC class II monoclonal antibodies. Peptide SP2 (20µg/ml) was added to the monocyte-derived dendritic cells (MoDCs) from each EV71-positive volunteer with or without MHC class II blocking antibodies (20µg/ml). The mixtures were then incubated with autologous CD4$^+$ T cells and stimulation index (SI) was calculated as previously described.

Table 5.3 Antigen-specific cytokine[a] secretion by stimulated CD4$^+$ T cells.

				IL-2 (pg/ml)		
Volunteers	**SP1**	**SP2**	**SP3**	**SP4**	**EV71**	**No antigen**
1	4.8 (± 1.9)	42.0 (± 1.0)	13.9 (± 3.1)	4.3 (± 1.1)	102.5 (± 2.2)	2.0 (± 0.7)
2	6.2 (± 2.9)	26.9 (± 2.1)	20.3 (± 1.5)	3.8 (± 1.3)	107.7 (± 2.8)	2.6 (± 1.1)
3	3.3 (± 1.3)	35.4 (± 1.9)	19.8 (± 1.6)	5.4 (± 1.2)	106.2 (± 3.0)	2.7 (± 0.8)
4	28.1 (± 4.6)	22.6 (± 2.3)	23.0 (± 1.8)	5.4 (± 2.0)	108.2 (± 1.7)	2.1 (± 1.5)
5	38.3 (± 1.7)	33.0 (± 2.7)	34.2 (± 2.4)	6.2 (± 1.6)	106.4 (± 4.5)	2.6 (± 1.1)
6	4.1 (± 1.7)	9.3 (± 1.7)	4.8 (± 1.1)	5.1 (± 1.3)	79.4 (± 2.7)	2.7 (± 0.8)
				IFN-γ (ng/ml)		
Volunteers	**SP1**	**SP2**	**SP3**	**SP4**	**EV71**	**No antigen**
1	12.9 (± 1.8)	65.6 (± 3.8)	40.3 (± 3.8)	1.4 (± 1.5)	684.5 (± 3.4)	0.9 (± 0.3)
2	13.7 (± 1.7)	48.4 (± 1.3)	31.5 (± 3.3)	1.7 (± 0.2)	236.1 (± 2.3)	1.1 (± 0.7)
3	17.4 (± 1.2)	69.0 (± 1.2)	40.8 (± 2.2)	1.4 (± 0.6)	957.6 (± 2.1)	1.4 (± 1.1)
4	34.4 (± 2.1)	45.5 (± 1.6)	32.0 (± 1.3)	1.6 (± 0.5)	643.8 (± 3.6)	1.1 (± 0.6)
5	46.6 (± 2.9)	61.9 (± 3.6)	48.2 (± 1.2)	1.8 (± 0.4)	384.3 (± 2.8)	1.4 (± 1.1)
6	0.8 (± 0.5)	1.0 (± 0.5)	0.8 (± 0.3)	0.5 (± 0.4)	38.3 (± 1.2)	0.6 (± 0.3)
				IL-4 (pg/ml)		
Volunteers	**SP1**	**SP2**	**SP3**	**SP4**	**EV71**	**No antigen**
1	2.6 (± 1.5)	4.3 (± 4.5)	3.4 (± 0.8)	2.5 (± 1.0)	34.9 (± 1.3)	2.6 (± 1.2)
2	3.1 (± 1.7)	3.4 (± 2.7)	3.3 (± 0.4)	2.2 (± 1.9)	26.9 (± 4.8)	2.1 (± 1.3)
3	3.4 (± 1.2)	3.9 (± 3.3)	3.3 (± 2.0)	2.6 (± 1.4)	22.8 (± 2.1)	2.6 (± 1.3)
4	3.0 (± 1.6)	3.1 (± 0.6)	3.9 (± 1.4)	3.2 (± 1.6)	33.1 (± 0.5)	2.7 (± 1.6)
5	4.1 (± 4.6)	3.9 (± 3.3)	3.8 (± 4.3)	3.2 (± 1.5)	26.5 (± 2.2)	2.2 (± 0.9)
6	2.8 (± 1.7)	2.6 (± 1.6)	2.4 (± 1.1)	2.5 (± 0.8)	19.3 (± 0.4)	2.2 (± 0.8)
				IL-10 (ng/ml)		
Volunteers	**SP1**	**SP2**	**SP3**	**SP4**	**EV71**	**No antigen**
1	1.2 (± 0.1)	1.2 (± 0.2)	1.2 (± 0.2)	1.2 (± 0.2)	31.5 (± 0.2)	1.1 (± 0.2)
2	1.2 (± 0.2)	1.5 (± 0.2)	1.2 (± 0.2)	1.2 (± 0.2)	22.9 (± 0.3)	1.1 (± 0.3)
3	2.1 (± 0.2)	2.1 (± 0.2)	2.3 (± 0.3)	2.0 (± 0.3)	23.9 (± 0.8)	1.9 (± 0.1)
4	1.6 (± 0.2)	1.9 (± 0.1)	1.8 (± 0.1)	1.1 (± 0.4)	24.4 (± 0.2)	1.1 (± 0.4)
5	2.0 (± 0.3)	2.3 (± 0.3)	2.6 (± 0.2)	1.8 (± 0.1)	11.5 (± 0.3)	1.8 (± 0.2)
6	1.3 (± 0.4)	1.4 (± 0.4)	1.3 (± 0.3)	1.2 (± 0.1)	3.1 (± 0.1)	1.0 (± 0.4)

[a] Secretion of cytokines of Th1- (IL-2 and IFN-γ) and Th2- (IL-4 and IL-10) type subsets by CD4$^+$ T-cells upon stimulation by autologous MoDCs in response to peptides, EV71 whole virions (20μg/ml) or no antigen. Values represent average of data obtained from triplicate assays. Standard deviation is in parenthesis.

5.3 Discussions

The host immune response developed upon any viral infection is primarily CD4$^+$ T cell dependent including the induction of a cytotoxic cellular response and the development of an efficient antibody response. Several studies on B-cell epitopes led to the identification of serotype- and group-common specific B-cell epitopes on the VP1 capsid protein of enteroviruses (Cello *et al.,* 1993). The functional role of enterovirus-specific T-cells as well as the nature and specificity of their responses have been less well-characterized so far. Although animal studies have been carried out to identify T-cell epitopes on four structural proteins (VP1 to VP4) of enteroviruses (Beck and Tracy, 1989; Katrak *et al.,* 1991; Kutubuddin *et al.,* 1992; Mahon *et al.,* 1992; Wang *et al.,* 1989), only few studies based on the VP1 capsid protein were carried out in humans (Graham *et al.,* 1993; Simons *et al.,* 1993). Several approaches have been reported for the identification of T-cell epitopes. Overlapping synthetic peptides spanning the entire sequence of the protein candidate can be designed and assayed for their capabilities of activating CD4$^+$ T cells (Arend *et al.,* 2000; Ravn *et al.,* 1999; Vordermeier *et al.,* 2001). However, such systematic approach is costly, time-consuming and tedious.

Alternatively, prediction programs can be used to scan the protein of interest and predict regions likely to bind to MHC molecules. The discovery of MHC-binding motifs in proteins has led to the development of several computer algorithms based on the construction of a matrix of all possible amino acid side chain interactions for individual MHC-binding motifs (De Groot *et al.,* 1997; Hammer *et al.,* 1994). Such bioinformatics tools have been successfully employed to identify HLA-DR ligands derived from tumor antigens and endogenous proteins involved in autoimmune

diseases (Hammer *et al.,* 1997; Manici *et al.,* 1999). In addition, these programs have been reported to improve and accelerate the design of vaccines and diagnostic tests through the identification of promiscuous peptides from mycobacterial proteins (Panigada *et al.,* 2002; Vordermeier *et al.,* 2003). In this study, the ProPred program was employed to predict HLA-DR binding ligands within the VP1 capsid protein of EV71 strain 41 since HLA-DR constitutes the dominant isotype of human MHC class II molecules (Tsuji *et al.,* 1992). Activation of CD4$^+$ T cells is dependent upon the presentation of peptides by APCs in the context of MHC class II molecules. These peptides are generally of approximately 15 amino acids in length and derived from internalized proteins that entered the endocytotic pathway. The MHC genes are the most polymorphic present in the genome and the majority of the amino acid differences amongst the various alleles lie within the peptide-binding groove of the MHC class II molecules (Oftung *et al.,* 1997).

Upon analysis, numerous regions within VP1 were predicted to bind one or few HLA-DR alleles, but only three regions (I, II and III) were predicted to bind 50% or more HLA-DR alleles included in the ProPred database. These regions span amino acids 63 to 77, 145 to 159 and 247 to 261 of the VP1 protein, respectively. The corresponding peptides (SP1 to SP3) were synthesized and shown to be able to induce the proliferation of CD4$^+$ T cells from EV71-positive volunteers with different HLA-DR alleles but not from an EV71-negative volunteer, indicating that the peptides have stimulated EV71-specific memory CD4$^+$ T cells. The stimulation indexes (SI) obtained correlated well with the binding efficiencies predicted for each peptide and for each HLA-DR allele. Among the three peptides tested, SP2 was identified to be the most capable of inducing significant CD4$^+$ T-cell proliferative responses

regardless of HLA-DR alleles, and therefore corresponds to the definition of a promiscuous CD4$^+$ T-cell epitope. Studies have shown that although several T-cell epitopes are present within the entire protein antigen, T-cells tend to focus only on a few immunodominant epitopes whereas discrete cryptic epitopes remain unseen by the host immune system (Gao *et al.,* 1999; Novak *et al.,* 2001). Therefore, SP2 most likely contains an immunodominant CD4$^+$ T-cell epitope. In addition, using anti-MHC class II antibody, we have demonstrated that SP2 suggests to be a MHC class II-restricted CD4$^+$ T-cell epitope.

An ideal synthetic peptide-based vaccine should contain both B-cell epitope(s) and T-cell epitope(s) that are able to induce a protective antibody response as well as a cytotoxic T-cell response important for killing infected host cells. The first generation of peptide vaccines which includes both B- and T-cell epitopes has been reported and shown to have a very good protective efficacy (Tam, 1996). In addition, antigen-specific CD4$^+$ T-cells producing IFN-γ have been shown to be essential for activation and maintenance of CD8$^+$ T-cell-mediated immune responses and for B-cell differentiation (Gao *et al.,* 2002; Ossendorp *et al.,* 1998). A previous study on the identification of enterovirus cross-reactive T-cell epitopes suggested that IFN-γ release may be used as an indicator for specific T-cell activation (Cello *et al.,* 1996). However, a poor correlation between the degree of antigen-specific T-cell proliferation and IFN-γ production has been previously reported, suggesting that proliferating and IFN-γ-producing T cells may belong to functionally different subsets (Cello *et al.,* 1996). Instead, our results showed that the levels of IFN-γ secreted by peptide-stimulated CD4$^+$ T cells correlated well with the respective proliferative responses. In addition, the significant production of IL-2 and IFN-γ upon stimulation

with SP1 to SP3 clearly indicates a Th1-subtype differentiation. Instead, the cytokine production profile observed when stimulating with EV71 whole virions suggested a mixed Th1-Th2 response, as evidenced by the production of significant levels of IFN-γ, IL-2, IL-4 and IL-10. Interestingly, a weak but significant proliferative response and cytokine production were observed by EV71-stimulated CD4$^+$ T cells from the EV71-negative volunteer. However, neither anti-EV71 neutralizing activity nor anti-VP1 antibodies were detected in the serum. It is likely that this volunteer might have been exposed to cross-reactive enteroviruses such as coxsackievirus 16 (CA16) or poliovirus as a result of the National childhood immunization program.

In conclusion, we have identified three promiscuous CD4$^+$ T-cell epitopes within the VP1 capsid protein of EV71. We have shown that the ProPred program has accurately predicted the presence of these epitopes and their binding efficiencies to three different HLA-DR alleles. However, to confirm the promiscuous feature of these epitopes, more HLA-DR alleles should be tested. Among these three epitopes, one spanning amino acids 145 to 159 of VP1 appeared to be the most promiscuous, inducing high proliferation of, and cytokine production by, EV71-specific CD4$^+$ T cells. We believe that the identification of CD4$^+$ T-cell epitopes within VP1, the major immunogenic and protective antigen, will contribute to a better understanding of the immune correlates of protection against EV71 infections and will help in developing more effective immuno-therapeutic and immuno-prophylactic strategies.

CHAPTER 6 CONCLUSIONS

Enterovirus 71 (EV71) has been increasingly recognized as a major etiological agent for Hand, foot and mouth disease (HFMD) which has been associated with significant mortality in young children and infants during large scale outbreaks in the Asia-Pacific region over the last few years. Unlike CA16 which has limited pathogenicity, EV71 can give rise to major complications involving the central nervous system and this has raised public concern. At present, the only successful approach to prevent and control the spread of EV71 is by public health surveillance and quarantine. There are several promising therapeutic drugs with antiviral properties targeting enteroviruses but they are still undergoing clinical trials. However, a major obstacle to the successful use of these drugs in EV71-associated encephalitis is that most children may have already suffered irreversible brain damage due to the high viral load before diagnosis and treatment. Although the drugs may have successful antiviral activities against EV71, it is not likely that much clinical improvement can be expected after treatment. Hence, major research efforts are required to emphasize on the prevention of EV71 infection instead of therapy and this can be carried out via the development of effective vaccines. Vaccination is one of the most successful public health initiatives ever achieved. However, for some diseases, vaccine designs is a challenge. Several strategies have been devised to deliver specific and immunogenic vaccine components to the immune system in the hope of eliciting a therapeutic or prophylactic immune response. One such beneficial strategy is to select particular protein antigens from the microorganism for inclusion into recombinant vaccines. The most precise selection of vaccine component exists in epitope-based peptide vaccines.

6.1 Synthetic peptides as candidates to elicit protective antibodies

In this study, the characterization of linear neutralization epitopes on the VP1 capsid protein of the EV71 strain 5865/SIN/00009 (designated as Strain 41) was undertaken by analysis of Balb/c mice antisera raised against 95 diphtheria toxoid-conjugated synthetic peptides spanning the entire VP1 region. Two synthetic peptides, designated SP55 and SP70, successfully elicited high titers of neutralizing antibodies against EV71. As neutralizing response plays a protective role against viral infectivity, EV71-neutralizing antibodies elicited by synthetic peptides SP55 and SP70 were characterized based on their specificities and functional roles in the neutralization process. These peptide epitopes represent the minimal immunogenic region of VP1 protein antigen, and allow for precise direction of immune responses. The *in vivo* protective potential of EV71-neutralizing antibodies elicited by SP70 was also evaluated in mice by passive transfer of immune sera which demonstrated that the anti-SP70 antisera was able to confer *in vivo* passive protection of up to 80% survival rate in suckling Balb/c mice which have been challenged with either the homologous or heterologous EV71 strains. The protective efficacy induced by SP70 was comparable to the heat-inactivated EV71 whole virion as particulate antigen. Traditionally, vaccines have consisted of either live attenuated microorganisms or inactivated microorganisms delivered via injection. However, many pathogenic microorganisms are difficult to culture *in vitro*, and therefore production of live attenuated or inactivated vaccines of these pathogens is impractical. In addition, features of even attenuated microorganisms may result in detrimental immune responses, or the pathogen may contain material that initiates unwanted host responses. Hence, although the use of whole virion has been commonly

acknowledged as a good vaccine candidate, this study has shown that synthetic peptides can also be used to elicit good immune responses.

Compared with conventional vaccines, synthetic peptide offers several advantages over other forms of vaccines, particularly with regards to safety and ease of production. There is no risk of reversion or formation of adverse reassortants that can lead to virulence, which is a potential problem with live attenuated vaccine preparations. As compared to DNA vaccines, peptide-based vaccine has no risk of genetic integration or recombination. In addition, the advantage of a peptide-based strategy focus on the short amino acid sequence used to elicit protective immune responses which result in the production of antibodies against specific sites on the antigen. Therefore, considering the differences in genomic sequence between various EV71 strains due to mutations, only minor alteration to the synthetic peptide sequence is needed and hence newly synthesized peptides can be manufactured. However, easy as it may seems, peptide-based vaccine depends upon a short amino acid sequence to confer protection and antibodies raised against a specific region may not neutralize other EV71 strains especially if mutations occur within neutralizing epitopes. Different peptide candidates have to be designed according to identified neutralizing region(s) of different EV71 strains. Hence, due to several fatality cases caused by infections from EV71 strains belonging to different subgenogroups and the rapid evolution of the virus within the community, this emphasizes the importance of identifying the location of any conserved neutralizing epitope(s).

6.2 Improvements to synthetic peptide-based prophylactic strategy

Although previous studies have shown that the entire VP1 protein may elicit a significant immune response (Chen *et al.*, 2006; Wu *et al.*, 2001), the use of longer peptide precursors is not always desirable as tissue-specific processing and the frequent non-concordance of processing of longer exogenous peptides compared with endogenous antigens can result in unwanted immune responses. In this study, when all 95 synthetic peptides were screened against sera from human who have been exposed to EV71, SP70 was not identified to be reactive in the IgG-based ELISA. This suggests that during exposure to the whole virion, the neutralizing epitope represented by SP70 may not be presented by antigen-presenting cells (APCs) to elicit antibodies or the titer of anti-SP70 antibody present in the serum samples is lower than the detection limit. Nevertheless, this study has shown that SP70 contains the linear VP1 neutralizing epitope and anti-SP70 antibodies raised against the peptide are able to neutralize EV71 and hence confer good *in vitro* and *in vivo* protection against EV71 infectivity. Therefore, considering the use of SP70 as a potential peptide-based vaccine candidate, further improvement will be required especially on its mode of delivery. It is known that some drawbacks of peptide-based vaccine include the need to potently enhance the immunogenicity of the simple peptide, to stimulate T cells and to elicit immunological memory responses (Aguilar and Rodriguez, 2007; Brown and Jackson, 2005). Adjuvant science, lipopeptide conjugation and direct delivery to dendritic cells (DCs) are some of the approaches currently used to overcome these problems (Brown and Jackson, 2005). The covalent and non-covalent attachment of peptides to proteins that generally have some role in the innate immune response or target the proteins to receptors on APCs can be investigated as a mode of delivery and as an adjuvant for bound peptides (Aguilar and

Rodriguez, 2007; Brown and Jackson, 2005). Considering that peptide-based immunogens may be poor inducers of T-cell response, another approach to enhance the induction of T cells upon vaccination is to use protein transduction domains (PTDs) coupled to the protein immunogens to enhance their uptake by DCs and hence mediate their presentation to T cells. However, a recent study has demonstrated the successful use of human cytomegalovirus tegument protein pp65 as an alternative protein carrier to PTDs with a higher uptake efficiency by DCs and hence making pp65 a promising delivery system to induce significant immune responses for peptide-based vaccine (Scheller *et al.,* 2008).

One of the goals for vaccination is to induce immunity by selectively stimulating antigen-specific B cells and T cells, and hence a good vaccine should contain two antigenic epitopes which will induce both T-helper cell and B cell. However, the use of epitope-based vaccines is restricted to human of a given HLA haplotype, and as such need to be tailored to accommodate the natural variation in HLA genes. Epitope prediction remains a popular first-screening approach to identify candidate T cell determinants for subsequent biological validation. In this study, the identification focused on the use of a $CD4^+$ T-cell epitope prediction program, ProPred, to predict HLA-DR binding ligands within VP1. Since predictive algorithms are frequently combined with *in vitro* MHC-binding assays to confirm that the predicted ligands bind to the targeted MHC molecule (Buus, 1999), the predicted peptides in this study have shown to be capable of specifically inducing the proliferation of $CD4^+$ T cells from humans who have been exposed to EV71, indicating that the peptides have indeed stimulated EV71-specific memory $CD4^+$ T cells.

As the induction of B-cell response is generally dependent on the additional stimulation of $CD4^+$ T-helper cell, an alternative approach to peptide-epitope vaccine design is to covalently couple the B-cell epitope to one or two immunodominant $CD4^+$ T-cell epitope(s) that are selected from the numerous potential MHC class II ligands or to carrier protein which will provide a good source of T-cell epitopes. In this study, diphtheria toxoid was conjugated to the synthetic peptides as a carrier protein to enhance the immunogenicity of these peptides. However, in a clinical setting, the provision of T-cell help can be more readily achieved using defined T-cell epitopes that are matched to the haplotype of patients (Purcell *et al.,* 2003). In this study, the identification of human $CD4^+$ T-cell epitopes within VP1 will help in the development of a more effective immuno-prophylactic strategy when coupled with SP70 as the B-cell epitope, thereby enhancing the recruitment of T cells as well as the production of cytokines necessary for B-cell activation and differentiation into plasma cells.

6.3 Alternative diagnostic strategy

Beside vaccine development, the use of synthetic peptide instead of the whole virion as an alternative source of capture antigen in immunoassays may be an interesting approach for the serodiagnostic of EV71 infection. Pepscan analysis using overlapping synthetic peptides has been widely used for identifying antigenic epitope(s) of viruses and this study focused on the use of overlapping synthetic peptides to identify the major human immunodominant VP1 linear epitope of EV71. This epitope, represented by SP32, was produced as a soluble recombinant GST-SP32 fusion protein at high yields and when used as capture antigen in Western blot, a strong and specific immunoreactivity was obtained with human anti-EV71 IgG

antibodies. Compared with the use of EV71 whole virion, SP32 has a higher serological specificity and sensitivity as a capture antigen. Moreover, it has the additional advantage of reduced time of antigen production when compared with the laborious and expensive propagation of EV71 in the laboratory. However, it is noteworthy that such approach may be improved by the inclusion of other peptides, representing immunodominant linear epitopes identified within other capsid proteins. Nevertheless, this study suggests that SP32 could be further developed as a serologic reagent for the detection of human anti-EV71 IgG antibodies and the assay described here may constitute an important tool for the diagnosis of EV71 or a confirmatory assay for previous EV71 exposure.

REFERENCES

AbuBakar S., Chee H.Y., Shafee N., Chua K.B., Lam S.K. (1999). Molecular detection of enteroviruses from an outbreak of hands, foot and mouth disease in Malaysia in 1997. *Scand. J. Infect. Dis*. **31**:331-335.

Abbas A.K., Murphy K.M., Sher A. (1996). Functional diversity of helper T lymphocytes. *Nature*. **383**:787-793.

Aguilar J.C., Rodriguez E.G. (2007). Vaccine adjuvants revisited. *Vaccine.* **25**:3752-3762.

Ahmad K. (2000). Hand, foot and mouth disease outbreak reported in Singapore. *The Lancet*. **356**:1338.

Alexander J.P. Jr., Baden L., Pallansch M.A., Anderson L.J. (1994). Enterovirus 71 infections and neurologic disease--United States, 1977-1991. *J. Infect. Dis*. **169**:905-908.

Ansardi D.C., Porter D.C., Anderson M.J., Morrow C. (1996). Poliovirus assembly and encapsidation of genomic RNA. *Adv. Virus. Res*. **46**:1-68.

Appel J.R., Pinilla C., Niman H., Houghten R. (1990). Elucidation of discontinuous linear determinants in peptides. *J. Immunol*. **144**:976-983.

Arend S.M., Geluk A., Van Meijgaarden K.E., Van Dissel J.T., Theisen M., Andersen P., Ottenhoff T.H. (2000). Antigenic equivalence of human T-cell responses to Mycobacterium tuberculosis-specific RD1-encoded protein antigens ESAT-6 and culture filtrate protein 10 and to mixtures of synthetic peptides. *Infect. Immun.* **68**:3314-3321.

Arola A., Kallajoki M., Ruuskanen O., Hyypia T. (1998). Detection of enteroviral RNA in end-stage dilated cardiomyopathy in children and adolescents. *J. Med. Virol*. **56**:364-371.

Arya S.C. (2000). Antiviral therapy for neurological manifestations of enterovirus 71 infection. *Clin. Infect. Dis*. **30**:988.

Barlow D.J., Edwards M.S., Thornton J.M. (1986). Continuous and discontinuous protein antigenic determinants. *Nature*. **322**:747-748.

Barnard D.L. (2006). Current status of anti-picornavirus therapies. *Curr. Pharm. Des*. **12**:1379-1390.

Barnard D.L., Hubbard V.D., Smee D.F., Sidwell R.W., Watson K.G., Tucker S.P, Reece P.A. (2004). In vitro activity of expanded-spectrum pyridazinyl oxime ethers related to pirodavir: novel capsid-binding inhibitors with potent anti-picornavirus activity. *Antimicrob. Agents Chemother*. **48**:1766-1772.

Barnett P.V., Pullen L., Staple R.F., Lee L.J., Butcher R., Parkinson D., Doel T.R. (1996). A protection anti-peptide antibody against the immunodominant site of the A24 Cruzeiro strain of foot-and-mouth disease virus and its reactivity with other subtype viruses containing the same minimum binding sequences. *J. Gen. Virol*. **77**:1011-1018.

Beard C., Ward G., Rieder E., Chinsangaram J., Grubman M.J., Mason P.W. (1999). Development of DNA vaccines for foot-and-mouth disease, evaluation of vaccines encoding replicating and non-replicating nucleic acids in swine. *J. Biotechnol*. **73**:243-249.

Beck M.A., Tracy S.M. (1989). Murine cell-mediated immune response recognizes an enterovirus group-specific antigen(s). *J. Virol*. **63**:4148-4156.

Benjamin E. (1977). Immunohistochemistry of proteins Plenum Press. New York. USA. 2-265.

Bergelson J.M., Sepley M.P., Chan B.M.C., Heinler M.E., Finberg R.N. (1992). Identification of the antigen VLA-2 as a receptor for echovirus 1. *Science*. **255**:1718-1720.

Bernard A., Michel B., Delamarche E. (2001). Micromosaic immunoassays. *Anal. Chem*. **73**:8-12.

Berzofsky J.A. (1985). Intrinsic and extrinsic factors in protein antigenic structure. *Science*. **229**:932-940.

Bittle J.L., Houghten R.A., Alexander H., Shinnick T.M., Sutcliffe J.G., Lerner R.A., Rowlands D.J., Brown F. (1982). Protection against foot-and-mouth disease by immunization with a chemically synthesized peptide predicted from the viral nucleotide sequence. *Nature*. **298**:30-33.

Blomberg J., Lycke E., Ahlfors K., Johnsson T., Wolontis S., von Zeipel G. (1974). Letter: New enterovirus type associated with epidemic of aseptic meningitis and or hand, foot, and mouth disease. *Lancet*. **13**:112.

Blondel B., Crainic R., Fichot O., Dufraisse G., Candrea A., Diamond D., Girard M., Horaud F. (1986). Mutations conferring resistance to neutralization with monoclonal antibodies in type 1 poliovirus can be located outside or inside the antibody-binding site. *J. Virol*. **57**:81-90.

Bradshaw N. (1989). Polio in Kentucky-From Birthday Balls to the Breakthrough. *Register of the Kentucky Historical Society*. **87**:20-39.

Brown L.E., Jackson D.C. (2005). Lipid-based self-adjuvanting vaccines. *Curr. Drug Deliv*. **2**:383-393.

Brown B.A., Kilpatrick D.R., Oberste M.S., Pallansch M.A. (2000). Serotype-specific identification of Enterovirus 71 by PCR. *J. Clin. Virol*. **16**:107–112.

Brown B.A., Oberste M.S., Alexander J.P. Jr., Kennett M.L., Pallansch M.A. (1999). Molecular epidemiology and evolution of enterovirus 71 strains isolated from 1970 to 1998. *J. Virol*. **73**:9969-9975.

Brown B.A., Pallansch M.A. (1995). Complete nucleotide sequence of enterovirus 71 is distinct from poliovirus. *Virus Res*. **39**:195-205.

Buss S. (1999). Description and prediction of peptide-MHC binding: the 'human MHC project'. *Curr. Opin. Immunol*. **11**:209-213.

Cardosa M.J., Krishnan S., Tio P.H., Perera D., Wong S.C. (1999). Isolation of subgenus B adenovirus during a fatal outbreak of enterovirus 71-associated hand, foot, and mouth disease in Sibu, Sarawak. *Lancet*. **354**:987-991.

Cardosa M.J., Perera D., Brown B.A., Cheon D., Chan H.M., Chan K.P., Cho H., McMinn P. (2003). Molecular epidemiology of human Enterovirus 71 strains and recent outbreaks in the Asia-Pacific region: comparative analysis of the VP1 and VP4 genes. *Emerg. Infect. Dis*. **9**:461-468.

Caro V., Guillot S., Delpeyroux F., Crainic R. (2001). Molecular strategy for 'serotyping' of human enteroviruses. *J. Gen. Virol*. **82**:79-91.

Casas I., Palacios G.F., Trallero G., Cisterna D., Freire M.C., Tenorio A. (2001). Molecular characterization of human enteroviruses in clinical samples: comparison between VP2, VP1, and RNA polymerase regions using RT nested PCR assays and direct sequencing of products. *J. Med. Virol*. **65**:138-148.

Cello J., Samuelson A., Stalhandske P., Svennerholm B., Jeansson S., Forsgren M. (1993). Identification of group-common linear epitopes in structural and nonstructural proteins of enteroviruses by using synthetic peptides. *J. Clin. Micro*. **31**:911-916.

Cello J., Strannegård O., Svennerholm B. (1996). A study of the cellular immune response to enteroviruses in humans: identification of cross-reactive T-cell epitopes on the structural proteins of enteroviruses. *J. Gen. Virol*. **77**:2097-2108.

Chan K.P., Goh K.T., Chong C.Y., Teo E.S., Lau G., Ling A.E. (2003). Epidemic hand, foot and mouth disease caused by human Enterovirus 71, Singapore. *Emerg. Infect. Dis.* **9**:78-85.

Chan L.G., Parashar U.D., Lye M.S., Ong F.G., Zaki S.R., Alexander J.P., Ho K.K., Han L.L., Pallansch M.A., Suleiman A.B., Jegathesan M., Anderson L.J. (2000). Deaths of children during an outbreak of hand, foot and mouth disease in Sarawak, Malaysia: clinical and pathological characteristics of the disease. *Clin. Infect. Dis*. **31**:678–683.

Chang L.Y., Hsiung C.A., Lu C.Y., Lin T.Y., Huang F.Y., Lai Y.H., Chiang Y.P., Chiang B.L., Lee C.Y., Huang L.M. (2006). Status of cellular rather than humoral immunity is correlated with clinical outcome of enterovirus 71. *Pediatr. Res*. **60**:466-471.

Chang L.Y., Lee C.Y., Kao C.L., Fang T.Y., Lu C.Y., Lee P.I., Huang L.M. (2007). Hand, foot and mouth disease complicated with central nervous system involvement in Taiwan in 1980-1981. *J. Formos. Med. Assoc.* **106**:173-176.

Chang L.Y., Lin T.Y., Hsu K.H., Huang Y.C., Lin K.L., Hsueh C., Shih S.R., Ning H.C., Hwang M.S., Wang H.S., Lee C.Y. (1999). Clinical features and risk factors of pulmonary oedema after Enterovirus 71-related hand, foot, and mouth disease. *Lancet.* **354**:1682-1686.

Chang L.Y., Lin T.Y., Huang Y.C. (1998). Fulminant neurogenic pulmonary oedema with hand, foot and mouth disease. *Lancet.* **352**:367-368.

Chen H.F., Chang M.H., Chiang B.L., Jeng S.T. (2006). Oral immunization of mice using transgenic tomato fruit expressing VP1 protein from enterovirus 71. *Vaccine*. **24**:2944-2951.

Chen D., Duggan C., Texada D.E., Reden T.B., Kooragayala L.M., Langford M.P. (2005). Immunogenicity of Enterovirus 70 capsid protein VP1 and its non-overlapping N- and C-terminal fragments. *Antiviral Res.* **66**:11-17.

Chinsangaram J., Beard C., Mason P.W., Zellner M.K., Ward G., Grubman M.J. (1998). Antibody response in mice inoculated with DNA expressing foot-and-mouth disease virus capsid proteins. *J. Virol.* **72**:4454-4457.

Chiu C.H., Chu C., He C.C., Lin T.Y. (2006). Protection of neonatal mice from lethal enterovirus 71 infection by maternal immunization with attenuated Salmonella enterica serovar Typhimurium expressing VP1 of enterovirus 71. *Microbes Infect*. **8**:1671-1678.

Chow K.C., Lee C.C., Lin T.Y., Shen W.C., Wang J.H., Peng C.T., Lee C.C. (2000). Congenital enterovirus 71 infection: a case study with virology and immunohistochemistry. *Clin. Infect. Dis*. **31**:509-512.

Chow M., Yabrov R., Bittle J., Hogle J., Baltimore D. (1985). Synthetic peptides from four separate regions of the poliovirus type 1 capsid protein VP1 induce neutralizing antibodies. *Proc. Natl. Acad. Sci. USA.* **82**:910-914.

Chu P.Y., Lin K.H., Hwang K.P., Chou L.C., Wang C.F., Shih S.R., Wang J.R., Shimada Y., Ishiko H. (2001). Molecular epidemiology of enterovirus 71 in Taiwan. *Arch. Virol.* **146**:589-600.

Chumakov M., Voroshilova M., Shindarov L., Lavrova L., Gracheva L., Koroleva G., Vasilenko S., Brodvarova I., Nikolova M., Gyurova S., Gacheva M., Mitov G., Ninov N., Tsylka E., Robinson I., Frolova M., Bashkirtsev V., Martiyanova L., Rodin V. (1979). Enterovirus 71 isolated from cases of epidemic poliomyelitis-like disease in Bulgaria. *Arch. Virol*. 60:329-340.

Cohen A.D., Boyer J.D., Weiner D.B. (1998). Modulating the immune response to genetic immunization. *FASEB. J.* **12**:1611-1626.

Collen T., Doel T.R. (1990). Heterotypic recognition of foot-and-mouth disease virus by cattle lymphocytes. *J. Gen. Virol*. **71**:309-315.

Collen T., Pullen L., Doel T.R. (1989). T cell-dependent induction of antibody against foot-and-mouth disease virus in a mouse model. *J. Gen. Virol*. **70**:395-403.

Colman P.M., Laver W.G., Varghese J.N., Baker A.T., Tulloch P.A., Air G.M., Webster R.G. (1987). Three-dimensional structure of a complex of antibody with influenza virus neuraminidase. *Nature*. **326**:358-363.

Constant S., Sant' Angelo D., Pasqualini T., Taylor T., Levin D., Flavell R., Bottomly K. (1995). Peptide and protein antigens require distinct antigen-presenting cell subsets for the priming of $CD4^+$ T-cells. *J. Immunol*. **154**:4915-4923.

Couch R.B., Knipe D.M., eds. (1990). Rhinoviruses, Second edition. New York. USA.

Davis H.L, McCluskie M.J. (1999). DNA vaccines for viral diseases. *Microbes Infect*. **1**:7-21.

De Groot A.S., Jesdale B.M., Szu E., Schafer J.R., Chicz R.M., Deocampo G. (1997). An interactive Web site providing major histocompatibility ligand predictions: application to HIV research. *AIDS Res. Hum. Retroviruses*. **13**:529-531.

Diamond D.C., Jameson B.A., Bonin J., Kohara M., Abe S., Itoh H., Komatsu T., Arita M., Kuge S., Nomoto A., Osterhaus A.D.M.R., Crainic R., Wimmer E. (1985). Antigenic variation and resistance to neutralization in poliovirus type 1. *Science*. **229**:1090-1093.

Domingo E., Holland J.J. (1994). Mutation rates and rapid evolution of RNA viruses. The evolutionary biology of viruses. Raven Press. New York. USA. 161-184.

Domingo E., Holland J.J. (1998). High error rates, population equilibrium, and evolution of RNA replication systems. RNA genetics. Vol. III. Variability of RNA genomes. CRC Press. Boca Raton Florida. USA. 3-36.

Dyson H.J., Lerner R.A., Wright P.E. (1988). The physical basis for induction of protein-reactive antipeptide antibodies. *Annu. Rev. Biophys. Biophys. Chem*. **17**:305-324.

Emini E.A., Jameson B.A., Lewis A.J., Larsen G.R., Wimmer E. (1982). Poliovirus neutralization epitopes: analysis and localization with neutralizing monoclonal antibodies. *J. Virol.* **3**:997-1005.

Espy M.J., Teo R., Ross T.K., Svien K.A., Wold A.D., Uhl J.R., Smith T.F. (2000a). Diagnosis of varicella-zoster virus infections in the clinical laboratory by LightCycler PCR. *J. Clin. Microbiol*. **38**:3187-3189.

Espy M.J., Uhl J.R., Mitchell P.S., Thorvilson J.N., Svien K.A., Wold A.D., Smith T.F. (2000b). Diagnosis of herpes simplex virus infections in the clinical laboratory by LightCycler PCR. . *J. Clin. Microbiol*. **38**:795-799.

Espy M.J., Uhl J.R., Sloan L.M., Buckwalter S.P., Jones M.F., Vetter E.A., Yao J.D., Wengenack N.L., Rosenblatt J.E., Cockerill F.R. 3rd, Smith T.F. (2006). Real-time PCR in clinical microbiology: applications for routine laboratory testing. *Clin. Microbiol. Rev.* **19**:165-256.

Evans D.M., Dunn G., Minor P.D., Schild G.C., Cann A.J., Stanway G., Almond J.W., Currey K., Maizel J.V. Jr. (1985). Increased neurovirulence associated with a single nucleotide change in a noncoding region of the Sabin type 3 poliovirus genome. *Nature*. **314**:548-550.

Ferguson M., Evans D.M., Magrath D.I., Minor P.D., Almond J.W., Schild G.C. (1985). Induction by synthetic peptides of broadly reactive, type-specific neutralizing antibody to poliovirus type 3. *Virology*. **143**:505-515.

Filman D.J., Syed R., Chow M., Macadam A.J., Minor P.D., Hogle J.M. (1989). Structural factors that control conformational transitions and serotype specificity in type 3 poliovirus. *EMBO J*. **8**:1567-1579.

Foo D.G, Alonso S., Chow V.T., Poh C.L. (2007a). Passive protection against lethal enterovirus 71 infection in newborn mice by neutralizing antibodies elicited by a synthetic peptide. *Microbes Infect.* **11**:1299-1306.

Foo D.G, Alonso S., Phoon M.C., Ramachandran N.P., Chow V.T, Poh C.L. (2007b). Identification of neutralizing linear epitopes from the VP1 capsid protein of enterovirus 71 using synthetic peptides. *Virus Res.* **125**:61-68.

Frank R. (1992). Spot-synthesis: an easy technique for the positionally addressable, parallel chemical synthesis on a membrane support. *Tetrahedron.* **48**:9217-9232.

Fuller H., Del Mar C. (2006). Immunoglobulin treatment for respiratory syncytial virus infection. *Cochrane Database Syst. Rev.* **4**:CD004883.

Gao B., Esnouf M.P. (1996). Multiple interactive residues of recognition: elucidation of discontinuous epitopes with linear peptides. *J. Immunol.* **157**:183-188.

Gao F.G., Khammanivong V., Liu W.J., Leggatt G.R., Frazer I.H., Fernando G.J. (2002). Antigen-specific $CD4^+$ T-cell help is required to activate a memory $CD8^+$ T cell to a fully functional tumor killer cell. *Cancer Res*. **62**:6438-6441.

Gao Y., Wang C., Splitter G.A. (1999). Mapping T- and B-lymphocyte epitopes of bovine herpesvirus-1 glycoprotein B. *J. Gen. Virol*. **80**:2699-2704.

Geller T.J., Condie D. (1995). A case of protracted coxackie virus meningoencephalitis in a marginally immunodeficient child treated successfully with intravenous immunoglobulin. *J. Neurol. Sci.* **129**:131-133.

Geysen H.M., Meloen R.H., Barteling S.J. (1984). Use of peptide synthesis to probe viral antigens for epitopes to a resolution of a single amino acid. *Proc. Natl. Acad. Sci. USA*. **81**:3998-4002.

Geysen H.M., Rodda S.J., Mason T.J., Tribbick G., Schoofs P.G. (1987). Strategies for epitope analysis using peptide synthesis. *J. Immunol. Methods*. **102**:259-274.

Gilbert G.L., Dickson K.E., Waters M.J., Kennett M.L., Land S.A., Sneddon M. (1988). Outbreak of enterovirus 71 infection in Victoria, Australia, with a high incidence of neurologic involvement. *Pediatr. Infect. Dis. J*. **7**:484-488.

Gordon G. (1997). Viral vaccines. In: Viral Pathogenesis. Lippincott-Raven Publishers. PA. USA. 371-399.

Graham S., Wang E.C., Jenkins O., Borysiewicz L.K. (1993). Analysis of the human T-cell response to picornaviruses: identification of T-cell epitopes close to B-cell epitopes in poliovirus. *J. Virol*. **67**:1627-1637.

Grandien M., Forsgren M., Ehrnst A. (1995). Enteroviruses. Diagnostic procedures for viral, rickettsial, and chlamydial infections. Seventh edition. American Public Health Association. N.W. Washington D.C. USA. 279-297.

Greve J.M., Davis G., Meyer A.M., Forte C.P., Yost S.C., Marlor C.W., Kamarck M.E., McClelland A. (1989). The major human rhinovirus receptor is ICAM-1 *Cell*. **56**:839-847.

Haarmann C.M., Schwimmbeck P.L., Mertens T., Schultheiss H.P., Strauer B.E. (1994). Identification of serotype-specific and nonserotype-specific B-cell epitopes of coxsackie B virus using synthetic peptides. *Virology*. **200**:381-389.

Hadlock K.G., Goh C.J., Bradshaw P.A., Perkins S., Lo J., Kaplan J.E., Khabbaz R., Foung S.K. (1995). Delineation of an immunodominant and human T-cell lymphotropic virus (HTLV)-specific epitope within the HTLV-I transmembrane glycoprotein. *Blood*. **86**:1392-1399.

Hagiwara A., Tagaya I., Yoneyama T. (1978). Epidemic of hand, foot and mouth disease associated with enterovirus71 infection. *Intervirology*. **9**:60-63.

Hagiwara A., Yoneyama T., Takami S., Hashimoto I. (1984). Genetic and phenotypic characteristics of enterovirus 71 isolates from patients with encephalitis and with hand, foot and mouth disease. *Arch. Virol*. **79**:273-283.

Hammer J., Bono E., Gallazzi F., Belunis C., Nagy Z., Sinigaglia F. (1994). Precise prediction of major histocompatibility complex class II-peptide interaction based on peptide side chain scanning. *J. Exp. Med*. **180**:2353-2358.

Hammer J., Sturniolo T., Sinigaglia F. (1997). HLA class II-peptide binding specificity and autoimmunity. *Adv. Immunol*. **66**:67-100.

Hashimoto I., Hagiwara A. (1982). Pathogenicity of a poliomyelitis-like disease in monkeys infected orally with enterovirus 71: a model for human infection. *Neuropathol. Appl. Neurobiol*. **8**:149-156.

Hashimoto I., Hagiwara A., Kodama H. (1978). Neurovirulence in cynomolgus monkeys of enterovirus 71 isolated from a patient with hand, foot and mouth disease. *Arch. Virol*. **56**:257-261.

Haro I., Gomara M.J. (2004). Design of synthetic peptide constructs for the vaccine development against viral infections. *Curr. Protein Pept. Sci*. **5**:425-433.

Harper D.R. and Kinchington P.R. (1998). Vaccines and Immunotherapy. In: Molecular virology. Second edition. BIOS scientific publishers Ltd. Springer-Verlag Singapore Pte Ltd. 81-98.

He Y., Zhou Y., Siddiqui P., Niu J., Jiang S. (2005). Identification of immunodominant epitopes on the membrane protein of the severe acute respiratory syndrome-associated coronavirus. *J. Clin. Microbiol.* **43**:3718-3726.

He Y., Zhou Y., Wu H., Kou Z., Liu S., Jiang S. (2004a). Mapping of antigenic sites on the nucleocapsid protein of the severe acute respiratory syndrome coronavirus. *J. Clin. Microbiol.* **42**:5309-5314.

He Y., Zhou Y., Wu H., Luo B., Chen J., Li W., Jiang S. (2004b). Identification of immunodominant sites on the spike protein of severe acute respiratory syndrome (SARS) coronavirus: Implication for developing SARS diagnostics and vaccines. *J. Immunol.* **173**:4050-4057.

Heinz B.A., Vance L.M. (1995). The antiviral compound enviroxime targets the 3A coding region of rhinovirus and poliovirus. *J. Virol*. **69**:4189-4197.

Higuchi R., Fockler C., Dollinger G., Watson R. (1993). Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. *Biotechnology (N Y)*. **11**:1026-1030.

Ho M., Chen E.R., Hsu K.H., Twu S.J., Chen K.T., Tsai S.F., Wang J.R., Shih S.R. (1999). An epidemic of enterovirus infection in Taiwan. Taiwan Enterovirus Epidemic Working Group. *N. Engl. J. Med.* **341**:929-935.

Hogle J.M., Chow M., Filman D.J. (1985). Three dimensional structure of poliovirus at 2.9 Å resolution. *Science*. **229**:1358-1365.

Hogle J.M., Filman D.J. (1989). The antigenic structure of poliovirus. *Philos. Trans. R. Soc. Lond. B. Biol. Sci*. **323**:467-478.

Holland P.M., Abramson R.D., Watson R., Gelfand D.H. (1991). Detection of specific polymerase chain reaction product by utilizing the 5'----3' exonuclease activity of Thermus aquaticus DNA polymerase. *Proc. Natl. Acad. Sci. USA*. **88**:7276-7280.

Holland J., Spindler K., Horodyski F., Grabau E., Nichol S., VandePol S. (1982). Rapid evolution of RNA genomes. *Science*. **215**:1577-1585.

Hovi T., Roivainen M. (1993). Peptide antisera targeted to a conserved sequence in poliovirus capsid VP1 cross-react widely with members of the genus Enterovirus. *J. Clin. Microbiol*. **31**:1083-1087.

Huang Y.C., Lin T.Y., Lin Y.J., Lien R.I., Chou Y.H. (2001). Prophylaxis of intravenous immunoglobulin and acyclovir in perinatal varicella. *Eur. J. Pediatr*. **160**:91-94.

Hunt A.R., Frederickson S., Hinkel C., Bowdish K.S., Roehrig J.T. (2006). A humanized murine monoclonal antibody protects mice either before or after challenge with virulent venezuelan equine encephalomyelitis virus. *J. Gen. Virol*. **87**:2467-2476.

Ijaz M.K., Attah Poku S.K., Redmond M.J., Parker M.I., Babiuk L.A. (1991). Heterotypic passive protection induced by synthetic peptides corresponding to VP7 and VP4 of bovine rotavirus. *J. Virol*. **65**:3106-3113.

Ishimaru Y., Nakano S., Yamaoka K., Takami S. (1980). Outbreaks of hand, foot, and mouth disease by enterovirus 71. High incidence of complication disorders of central nervous system. *Arch. Dis. Child*. 55:583-588.

Jebbink J., Bai X., Rogers B.B., Dawson D.B., Scheuermann R.H., Domiati-Saad R. (2003). Development of real-time PCR assays for the quantitative detection of Epstein-Barr virus and cytomegalovirus, comparison of TaqMan probes, and molecular beacons. *J. Mol. Diagn*. **5**:15-20.

Jemmerson R., Paterson Y. (1986). Mapping epitopes on a protein antigen by the proteolysis of antigen-antibody complexes. *Science*. **232**:1001-1004.

Johnson K.L., Sarnow P. (1995). Viral RNA synthesis. In: Human enterovirus infections. American society for microbiology press. N.W. Washington D.C. USA.

Kandolf R., Canu A., Hofschneider P.H. (1985). Coxsackie B3 virus can replicate in cultured human foetal heart cells and is inhibited by interferon. *J. Mol. Cell. Cardiol*. **17**:167-181.

Kapsenberg J.G. (1998). Picornaviridae: the enteroviruses (polioviruses, coxsackieviruses, echoviruses). Laboratory diagnosis of infectious diseases. Principles and practice, Vol. 2. Viral, rickettsial and chlamydial disease. Springer-Verlag. New York. USA. 692-722.

Kapsenberg J.G., Ras A., Korte J. (1980). Improvement of enterovirus neutralization by treatment with sodium deoxycholate or chloroform. *Intervirology*. **12**:329-334.

Kares S., Lönnrot M., Vuorinen P., Oikarinen S., Taurianen S., Hyöty H. (2004). Real-time PCR for rapid diagnosis of entero- and rhinovirus infections using LightCycler. *J. Clin. Virol*. **29**:99-104.

Katrak K., Mahon B.P., Minor P.D., Mills K.H. (1991). Cellular and humoral immune responses to poliovirus in mice: a role for helper T cells in heterotypic immunity to poliovirus. *J. Gen. Virol*. **72**:1093-1098.

Kennett M.L., Birth C.J., Lewis F.A., Yung A.P., Locarnini S.A., Gust I.D. (1974). Enterovirus type 71 infection inMelbourne. *Bull WHO*. **51**:609-615.

Ketterlinus R., Wiegers K. (1994). Mapping of antigenic domains in poliovirus VP1 involved in structural rearrangements during virus morphogenesis and antigenic alterations of the virion. *Virology*. **204**:27-37.

Kimura-Kuroda J., Yasui K. (1988). Protection of mice against Japanese encephalitis virus by passive administration with monoclonal antibodies. *J. Immunol.* **141**:3606-3610.

King A.M.Q. (1988). Genetic recombination in positive strand RNA viruses. RNA genetics. Vol. II. Retroviruses, viroids and RNA recombination. CRC Press. Boca Raton. Florida. USA. 149-165.

King A.M.Q., Brown F., Christian P., et al. (2000). Picornaviridae. In: Virus taxonomy. Seventh report of the international committe on taxonomy of viruses. San Diego: Academic press. New York. USA. 657-678.

Knossow M., Daniels R.S., Douglas A.R., Skehel J.J., Wiley D.C. (1984). Three-dimensional structure of an antigenic mutant of the influenza virus haemagglutinin. *Nature*. **311**:678-680.

Kok P.W., Leeuwenburg J., Tukei P., Van Wezel A.L., Kapsenberg J.G., Van Steenis G., Galazka A., Robertson S.E., Robinson D. (1992). Serological and virological assessment of oral and inactivated poliovirus vaccines in a rural population in Kenya. *Bull. World Health Organ*. **70**:93-103.

Komatsu H., Shimizu Y., Takeuchi Y., Ishiko H., Takada H. (1999). Outbreak of severe neurologic involvement associated with Enterovirus 71 infection. *Pediatr. Neurol*. **20**:17-23.

Koroleva G.A., Gracheva L.A. (1980). Enterovirus type 71 pathogenicity for laboratory animals. *Vopr. Virusol*. **2**:185-190.

Kramer A., Schuster A., Reineke U., Malin R., Volkmer-Engert R., Landgraf C. Schneider-Mergener J. (1994). Combinatorial cellulose-bound peptide libraries: screening tools for the identification of peptides that bind ligands with predefined specificity. *Methods.* **6**:388-395.

Kung S.H., Wang S.F., Huang C.W., Hsu C.C., Liu H.F., Yang J.Y. (2007). Genetic and antigenic analyses of enterovirus 71 isolates in Taiwan during 1998-2005. *Clin. Microbiol. Infect*. **13**:782-787.

Kutubuddin M., Simons J., Chow M. (1992). Identification of T-helper epitopes in the VP1 capsid protein of poliovirus. *J. Virol*. **66**:3042-3047.

Lal S.K., Kumar P., Yeo W.M., Kar-Roy A., Chow V.T. (2006). The VP1 protein of human enterovirus 71 self-associates via an interaction domain spanning amino acids 66-297. *J. Med. Virol*. **78**:582-590.

Landry M.L., Fonseca S.N., Cohen S., Bogue C.W. (1995). Fatal enterovirus type 71 infection: rapid detection and diagnostic pitfalls. *Pediatr. Infect. Dis. J.* **14**:1095-1100.

Langford M.P., Barber J.C., Sklar V.E., Clark S.W. 3rd, Patriarca P.A., Onarato I.M., Yin-Murphy M., Stanton G.J. (1985). Virus-specific, early appearing neutralizing activity and interferon in tears of patients with acute hemorrhagic conjunctivitis. *Curr. Eye Res*. **4**:233-239.

Laver W.G., Air G.M., Webster R.G., Smith-Gill S.J. (1990). Epitopes on protein antigens: misconceptions and realities. *Cell*. **61**:553-556.

Lea S., Hernández J., Blakemore W., Brocchi E., Curry S., Domingo E., Fry E., Abu-Ghazaleh R., King A., Newman J., Stuart D., Mateu M.G. (1994). The structure and antigenicity of a type C foot-and-mouth disease virus. *Structure*. **2**:123-139.

Lennette E.H., Schmidt N.J., eds. (1969). Diagnostics procedures for viral and Rickettsial infections, Fourth edition. New York. USA.

Levy J.A., Conrat H.F., Owens R.A. (1994). Virology. Third edition. Imprints: Cliffs, Englewood N.J. Prentice Hall.

Liao C.S., Lee G.B., Liu H.S., Hsieh T.M., Luo C.H. (2005). Miniature RT-PCR system for diagnosis of RNA-based viruses. *Nucleic Acids Res*. **33**:e156.

Lim K.A., Benyesh-Melnick M. (1960). Typing of viruses by combinations of antiserum pools. Application to typing of enteroviruses (Coxsackie and ECHO). *J. Immunol*. **84**:309-317.

Lin T.Y., Chu C., Chiu C.H. (2002). Lactoferrin inhibits enterovirus 71 infection of human embryonal rhabdomyosarcoma cells in vitro. *J. Infect. Dis*. **186**:1161-1164.

Lin T.Y., Hsia S.H., Huang Y.C., Wu C.T., Chang L.Y. (2003). Proinflammatory cytokine reactions in enterovirus 71 infections of the central nervous system. *Clin. Infect. Dis*. **36**:269-274.

Lin K.H., Hwang K.P., Ke G.M., Wang C.F., Ke L.Y., Hsu Y.T., Tung Y.C., Chu P.Y., Chen B.H., Chen H.L., Kao C.L., Wang J.R., Eng H.L., Wang S.Y., Hsu L.C., Chen H.Y. (2006). Evolution of EV71 genogroup in Taiwan from 1998 to 2005: an emerging of subgenogroup C4 of EV71. *J. Med. Virol*. **78**:254-262.

Liu M.L., Lee Y.P., Wang Y.F., Lei H.Y., Liu C.C., Wang S.M., Su I.J., Wang J.R., Yeh T.M., Chen S.H., Yu C.K. (2005). Type I interferons protect mice against enterovirus 71 infection. *J. Gen. Virol*. **86**:3263-3269.

Liu C.C., Tseng H.W., Wang S.M., Wang J.R., Su I.J. (2000). An outbreak of enterovirus 71 infection in Taiwan, 1998; epidemiologic and clinical manifestations. *J. Clin. Virol*. **17**:23-30.

Livak K.J., Flood S.J., Marmaro J., Giusti W., Deetz K. (1995). Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. *PCR. Methods. Appl*. **4**:357-362.

Lu W.W., Hsu Y.Y., Yang J.Y., Kung S.H. (2004). Selective inhibition of enterovirus 71 replication by short hairpin RNAs. *Biochem. Biophys. Res. Commun*. **325**:494-499.

Lum L.C., Wong K.T., Lam S.K., Chua K.B., Goh A.Y., Lim W.L., Ong B.B., Paul G., AbuBakar S., Lambert M. (1998a). Fatal enterovirus 71 encephalomyelitis. *J. Pediatr*. **133**:795-798.

Lum L.C., Wong K.T., Lam S.K., Chua K.B., Goh A.Y. (1998b). Neurogenic pulmonary oedema and enterovirus 71 encephalomyelitis. *Lancet.* **352**:1391.

Mackay I.M., Arden K.E., Nitsche A. (2002). Real-time PCR in virology. *Nucleic Acids Res*. **30**:1292-1305.

Mackay I.M., Jacob K.C., Woolhouse D., Waller K., Syrmis M.W., Whiley D.M., Siebert D.J., Nissen M., Sloots T.P. (2003). Molecular assays for detection of human metapneumovirus. *J. Clin. Microbiol*. **41**:100-105.

Mahon B.P., Katrak K., Mills K.H. (1992). Antigenic sequences of poliovirus recognized by T cells: serotype-specific epitopes on VP1 and VP3 and cross-reactive epitopes on VP4 defined by using $CD4^+$ T-cell clones. *J. Virol*. **66**:7012-7020.

Manici S., Sturniolo T., Imro M.A., Hammer J., Sinigaglia F., Noppen C., Spagnoli G., Mazzi B., Bellone M., Dellabona P., Protti M.P. (1999). Melanoma cells present a MAGE-3 epitope to $CD4^+$ cytotoxic T cells in association with histocompatibility leukocyte antigen DR11. *J. Exp. Med*. **189**:871-876.

Manzara S., Muscillo M., La Rosa G., Marianelli C., Cattani P., Fadda G. (2002). Molecular identification and typing of enteroviruses isolated from clinical specimens. *J. Clin. Microbiol.* **40**:4554-4560.

Mateu M.G. (1995). Antibody recognition of picornaviruses and escape from neutralization: a structural view. *Virus Res.* **38**:1-24.

McMinn P.C. (2002). An overview of the evolution of enterovirus 71 and its clinical and public health significance. *FEMS Microbiology Reviews.* **26**:91-107.

McMinn P.C., Lindsay K., Perera D., Chan H.M., Chan K.P., Cardosa M.J. (2001a). Phylogenetic analysis of enterovirus 71 strains isolated during linked epidemics in Malaysia, Singapore and Western Australia. *J. Virol*. **75**:7732-7738.

McMinn P.C., Stratov I., Nagarajan L., Davis S. (2001b) Neurological manifestation of enterovirus 71 infection in children during hand, foot and mouth disease outbreak in Western Australia. *Clin. Infect. Dis*. **32**:236-242.

Melnick J.L. (1984). Enterovirus type 71 infections: a varied clinical pattern sometimes mimicking paralytic poliomyelitis. *Rev. Infect. Dis*. **6 Suppl 2**:S387-S389.

Melnick J.L. (1996). Enteroviruses: polioviruses, coxsackieviruses, echoviruses and new enteroviruses. Third edition. Lippincott-Raven publishers. Philadelphia, USA. 655-712.

Melnick J.L., Rennick V., Hampil B., Schmidt N.J., Ho H.H. (1973). Lyophilized combination pools of enterovirus equine antisera: preparation and test procedures for the identification of field strains of 42 enteroviruses. *Bull. World Health Organ*. **48**:263-268.

Melnick J.L., Wimberly I.L. (1985). Lyophilized combination pools of enterovirus equine antisera: new LBM pools prepared from reserves of antisera stored frozen for two decades. *Bull, World Health Organ*. **63**:543-550.

Mendelsohn C., Wimmer E., Racaniello V.R. (1989). Cellular receptors for poliovirus: molecular cloning, nucleotide sequence and expression of a new member of the immunoglobulin super family. *Cell.* **56**:855-865.

Merovitz L., Demers A.M., Newby D., McDonald J. (2000). Enterovirus 71 infections at a Canadian center. *Pediatr. Infect. Dis. J*. **19**:755-757.

Mertens T.H., Pika U., Eggers H.J. (1983). Cross antigenicity among enteroviruses as revealed by immunoblot technique. *Virology.* **129**:431-442.

Minor P.D. (1990). Antigenic structure of picornaviruses. *Curr. Top. Microbiol. Immunol*. **161**:121-154.

Minor P.D. (1997). Poliovirus. In: Viral Pathogenesis. Lippincott-Raven Publishers. PA. USA. 555-574.

Minor P.D., Evans D.M., Ferguson M., Schild G.C., Westrop G., Almond J.W. (1985). Principal and subsidiary antigenic sites of VP1 involved in the neutralization of poliovirus type 3. *J. Gen. Virol*. **66**:1159-1165.

Minor P.D., Evans D.M., Schild G.C., Ferguson M., Almond J.W. (1984). Identification of an antigenic site in the neutralization of type 3 poliovirus. *Rev. Infect. Dis*. **6**:S516-518.

Minor P.D., Ferguson M., Evans D.M., Almond J.W., Icenogle J.P. (1986). Antigenic structure of polioviruses of serotypes 1, 2 and 3. *J. Gen. Virol*. **67**:1283-1291.

Minor P.D., Schild G.C., Bootman J., Evans D.M., Ferguson M., Reeve P., Spitz M., Stanway G., Cann A.J., Hauptmann R., Clarke L.D., Mountford R.C., Almond J.W. (1983). Location and primary structure of a major antigenic site for poliovirus neutralization. *Nature*. **301**:674-679.

Mizuta K., Abiko C., Murata T., Matsuzaki Y., Itagaki T., Sanjoh K., Sakamoto M., Hongo S., Murayama S., Hayasaka K. (2005). Frequent importation of enterovirus 71 from surrounding countries into the local community of Yamagata, Japan, between 1998 and 2003. *J. Clin. Microbiol*. **43**:6171-6175.

Moore D.M., Cowan K.M. (1978). Effect of trypsin and chymotrypsin on the polypeptides of large and small plaque variants of foot-and-mouth disease virus: relationship to specific antigenicity and infectivity. *J. Gen. Virol*. **41**:549-562.

Morens D.M., Pallansch M.A., Moore M. (1991). Polioviruses and other enteroviruses. Textbook of human virology. Mosby Year Book. St. Louis. 427-297.

Mosser A.G., Leippe D.M., Rueckert R.R. (1989). Neutralization of picornaviruses: support for the pentamer bridging hypothesis. Molecular aspects of picornavirus infection and detection. American Society for Microbiology. Washington. D.C. USA. 155-168.

Muir P., Kammerrer U., Korn K., Mulders M.N., Poyri T., Weissbrich B., Kandolf R., Cleator G.M., Vanloon A.M. (1998). Molecular typing of enteroviruses: Current status and future requirements.

Nagata N., Shimizu H., Ami Y., Tano Y., Harashima A., Suzaki Y., Sato Y., Miyamura T., Sata T., Iwasaki T. (2002). Pyramidal and extrapyramidal involvement in experimental infection of cynomolgus monkeys with enterovirus 71. J. Med. Virol. **67**:207-216.

Nagy G., Takátsy S., Kukán E., Mihály I., Dömök I. (1982). Virological diagnosis of enterovirus type 71 infections: experiences gained during an epidemic of acute CNS diseases in Hungary in 1978. *Arch. Virol*. **71**:217-227.

Nathanson N., Langmuir A.D. (1995). The Cutter incident. Poliomyelitis following formaldehyde-inactivated poliovirus vaccination in the United States during the Spring of 1955. II. Relationship of poliomyelitis to Cutter vaccine. 1963. *Am. J. Epidemiol*. **142**:109-140.

Nijhuis M., Van Maarseveen N., Schuurman R., Verkuijlen S., De Vos M., Hendriksen K., Van Loon A.M. (2002). Rapid and sensitive routine detection of all members of the genus enterovirus in different clinical specimens by real-time PCR. *J. Clin. Microbiol*. **40**:3666-3670.

Novak E.J., Masewicz S.A., Liu A.W., Lernmark A., Kwok W.W., Nepom G.T. (2001). Activated human epitope-specific T cells identified by class II tetramers reside within a $CD4^+$ high, proliferating subset. *Int. Immunol*. **13**:799-806.

Oberste M.S., Maher K., Kilpatrick D.R., Pallansch M.A. (1999). Molecular evolution of the human enterovirus: Correlation of serotype with VP1 sequence and application to Picornavirus classification. *J. Virol*. **73**:1941-1948.

Oberste M.S., Maher K., Pallansch M.A. (1998). Molecular phylogeny of all human enterovirus serotypes based on comparison of sequences at the 5' end of the region encoding VP2. *Virus Res*. **58**:35-43.

Oftung F., Lundin K.E.A., Geluk A., Shinnick T.M., Meloen R., Mustafa A.S. (1997). Primary structure and MHC restriction of peptide defined T-cell epitopes from recombinantly expressed mycobacterial protein antigens. *Med. Principles Pract*. **6**:66-73.

Ooi M.H., Wong S.C., Podin Y., Akin W., Del Sel S., Mohan A., Chieng C.H., Perera D., Clear D., Wong D., Blake E., Cardosa J., Solomon T. (2007). Human enterovirus 71 disease in Sarawak, Malaysia: a prospective clinical, virological, and molecular epidemiological study. *Clin. Infect. Dis*. **44**:646-656.

Ossendorp F., Mengedé E., Camps M., Filius R., Melief C.J. (1998). Specific T helper cell requirement for optimal induction of cytotoxic T lymphocytes against major histocompatibility complex class II negative tumors. *J. Exp. Med*. **187**:693-702.

Padlan E.A. (1992). Structure of protein epitope deduced from X-ray crystallography. Structure of antigens. Volume 1. CRC Press, Boca Raton. Florida. USA. 29-42.

Page G.S., Mosser A.G., Hogle J.M., Filman D.J., Rueckert R.R., Chow M. (1988). Three-dimensional structure of poliovirus serotype 1 neutralizing determinants. *J. Virol*. **62**:1781-1794.

Palacios G., Casas I., Tenorio A., Freire C. (2002). Molecular identification of enterovirus by analyzing a partial VP1 genomic region with different methods. *J. Clin. Microbiol*. **40**:182-192.

Palacios G., Oberste M.S. (2005). Enterovirus as agents of emerging infectious diseases. *J Neuro Virol*. **11**:424-433.

Panigada M., Sturniolo T., Besozzi G, Boccieri M.G., Sinigaglia F., Grassi G.G., Grassi F. (2002). Identification of a promiscuous T-cell epitope in Mycobacterium tuberculosis Mce proteins. *Infect. Immun*. **70**:79-85.

Parry N., Fox G., Rowlands D., Brown F., Fry E., Acharya R., Logan D., Stuart D. (1990). Structural and serological evidence for a novel mechanism of antigenic variation in foot-and-mouth disease virus. *Nature*. **347**:569-572.

Patick A.K., Boritzki T.J., Bloom L.A. (1997). Activities of the human immunodeficiency virus type 1 (HIV-1) protease inhibitor nelfinavir mesylate in combination with reverse transcriptase and protease inhibitors against acute HIV-1 infection in vitro. *Antimicrob. Agents Chemother*. **41**:2159-2164.

Petitjean J., Vabret A., Dina J., Gouarin S., Freymuth F. (2006). Development and evaluation of a real-time RT-PCR assay on the LightCycler for the rapid detection of enterovirus in cerebrospinal fluid specimens. *J. Clin. Virol*. **35**:278-284.

Pevear D.C., Tull T.M., Seipel M.E., Groarke J.M. (1999). Activity of Pleconaril against enteroviruses. *Antimicrob. Agents Chemother*. **43**:2109-2115.

Pfaff E., Mussgay M., Bohm H.O., Schulz G.E., Schaller H. (1982). Antibodies against a preselected peptide recognize and neutralize foot and mouth disease virus. *EMBO. J.* **1**: 869-874.

Plotkin S.A., Mortimer E.A. (c1994). Vacines. Imprint: Philadelphia. W.B. Saunders Co.

Pöyry T., Kinnunrn L., Hyypiä T., Brown B., Horsnell C., Hovi T., Stanway G. (1996). Genetic and phylogenetic clustering of enteroviruses. *J. Gen. Virol.* **77**:1699-1717.

Pringle C.R. (1999). Virus taxanomy at the XIth International Congress of Virology. Sydney Australia. *Arch. Virol*. **144**:2065-2070.

Pulli T., Lankinen H., Roivainen M., Hyypiä T. (1998). Antigenic sites of coxsackievirus A9. *Virology*. **240**:202-212.

Purcell A.W., Zeng W., Mifsud N.A., Ely L.K., Macdonald W.A., Jackson D.C. (2003). Dissecting the role of peptides in the immune response: theory, practice and the application to vaccine design. J. *Pept. Sci*. **9**:255-281.

Racaniello V.R. (1995). Early events ininfection: Receptor binding and cell entry. In: Human enterovirus infections. American society for microbiology press. N.W. Washington D.C. USA. 73-93.

Ranganathan S., Singh S., Poh C.L., Chow V.T.K. (2002). The hand, foot and mouth disease virus capsid: sequence analysis and prediction of antigenic sites from homology modeling. *Appl. Bioinformatics*. **1**:43-52.

Rasmussen T., Poulsen T.S., Honoré L., Johnsen H.E. (2000). Quantitation of minimal residual disease in multiple myeloma using an allele-specific real-time PCR assay. *Exp. Hematol.* **28**:1039-1045.

Ratge D., Scheiblhuber B., Nitsche M., Knabbe C. (2000). High-speed detection of blood-borne hepatitis C virus RNA by single-tube real-time fluorescence reverse transcription-PCR with the LightCycler. *Clin. Chem.* **46**:1987-1989.

Ravn P., Demissie A., Eguale T., Wondwosson H., Lein D., Amoudy H.A., Mustafa A.S., Jensen A.K., Holm A., Rosenkrands I., Oftung F., Olobo J., von Reyn F., Andersen P. (1999). Human T cell responses to the ESAT-6 antigen from Mycobacterium tuberculosis. *J. Infect. Dis*. **179**:637-645.

Read S.J., Mitchell J.L., Fink C.G. (2001). LightCycler Multiplex PCR for the Laboratory Diagnosis of Common Viral Infections of the Central Nervous System. *J. Clin. Microbiol*. **39**:3056-3059.

Reed L.J., Muench H. (1938). A simple method of estimating fifty percent endpoints. *Am. J. Hyg*. **27**:4931-4937.

Reineke U., Sabat R., Kramer A., Stigler R.D., Seifert M., Michel T., Volk H.D., Schneider-Mergener J. (1996). Mapping protein-protein contact sites using cellulose-bound peptide scans. *Mol. Divers*. **1**:141-148.

Roberts G.B.S., Boyd J.F. (1987). The histopathology of enterovirus infections in newborn mice. *J. Infect*. **15**:45-56.

Roivainen M., Närvänen A., Korkolainen M., Huhtala M.L., Hovi T. (1991). Antigenic regions of poliovirus type 3/Sabin capsid proteins recognized by human sera in the peptide scanning technique. *Virology*. **180**:99-107.

Romero J.R. (1999). Reverse-transcription polymerase chain reaction detection of the enteroviruses. *Arch. Pathol. Lab. Med*. **123**:1161-1169.

Romero J.R. (2001). Pleconaril: a novel antipicornaviral drug. *Expert Opin. Investig. Drugs*. **10**:369-379.

Rosenthal K.S., Zimmerman D.H. (2006). Vaccines: all things considered. *Clin. Vaccine Immunol*. **13**:821-829.

Rossmann M.G., Arnold E., Erickson J.W., Frankenberger E.A., Griffith J.P., Hecht H.J., Johnson J.E., Kamer G., Luo M., Mosser A.G., Rueckert R.R., Sherry B., Vriend G. (1985). Structure of a human common cold virus and functional relationship to other picornaviruses. *Nature*. **317**:145-153.

Rotbart H.A. (1990). Diagnosis of enteroviral meningitis with the polymerase chain reaction. *J. Pediatr*. **117**:85-89.

Rotbart H.A., Ahmed A., Hickey S., Dagan R., McCracken G.H. Jr, Whitley R.J., Modlin J.F., Cascino M., O'Connell J.F., Menegus M.A., Blum D. (1997). Diagnosis of enterovirus infection by polymerase chain reaction of multiple specimen types. *Pediatr. Infect. Dis. J*. **16**:409-411.

Rotbart H.A., O'Connell J.F., McKinlay M.A. (1998). Treatment of human enterovirus infections. *Antiviral Res*. **38**:1-14.

Rotbart H.A., Sawyer M.H., Fast S., Lewinski C., Murphy N., Keyser E.F., Spadoro J., Kao S.Y., Loeffelholz M. (1994). Diagnosis of enteroviral meningitis by using PCR with a colorimetric microwell detection assay. *J. Clin. Microbiol*. **32**:2590-2592.

Ruekert R.R. (1996). Picornaviridae: The viruses and their replication. In Fields Virology. Third edition. Lippincott-Raven Publishers. Philadelphia, USA. 609-640.

Sabin A.B., Ramos-Alvarez M., Alvarez-Amezquita J., Pelon W., Michaels R.H., Spigland I., Koch M.A., Barnes J.M., Rhim J.S. (1960). Live, orally given poliovirus vaccine. Effects of rapid mass immunization on population under conditions of massive enteric infection with other viruses. *JAMA*. **173**:1521-1526.

Samuelson A., Cello J., Skoog E., Glimåker M., Jeansson S., Forsgren M. (1993). Enterovirus IgG ELISA using synthetic peptides as antigens. *Serodiagn. Immunother. Infect. Dis.* **5**:93-96.

Samuelson A., Forsgren M., Johansson B., Wahren B., Sallberg M. (1994). Molecular basis for serological cross-reactivity between enteroviruses. *Clin. Diagn. Lab. Immuno.* **1**:336-341.

Sanders S.A., Herrero L.J., McPhie K., Chow S.S., Craig M.E., Dwyer D.E., Rawlinson W., McMinn P.C. (2006). Molecular epidemiology of enterovirus 71 over two decades in an Australian urban community. *Arch. Virol*. **151**:1003-1013.

Sawyer M.H. (2001). Enterovirus infections: diagnosis and treatment. *Curr. Opin. Pediatr*. **13**:65-69.

Scheller N., Furtwängler R., Sester U., Maier R., Breinig T., Meyerhans A. (2008). Human cytomegalovirus protein pp65: an efficient protein carrier system into human dendritic cells. *Gene Ther*. **15**:318-325.

Schlegel A., Kirkegaard K. (1995). Cell biology of enterovirus infection. In: Human enterovirus infections. American society for microbiology press. N.W. Washington D.C. USA.

Schmidt N.J., Guenther R.W., Lennette E.H. (1961). Typing of ECHO virus isolates by immune serum pools. The "intersecting serum scheme". *J. Immunol*. **87**:623-626.

Schmidt N.J., Lennette E.H., Ho H.H. (1974). An apparently new enterovirus isolated from patients with disease of the central nervous system. *J. Infect. Dis*. **129**:304-309.

Sherry B., Mosser A.G., Colonno R.J., Rueckert R.R. (1986). Use of monoclonal antibodies to identify four neutralization immunogens on a common cold picornavirus, human rhinovirus 14. *J. Virol*. **57**:246-257.

Shimizu H., Utama A., Yoshii K., Yoshida H., Yoneyama T., Sinniah M., Yusof M.A., Okuno Y., Okabe N., Shih S.R., Chen H.Y., Wang G.R., Kao C.L., Chang K.S., Miyamura T., Hagiwara A. (1999). Enterovirus 71 from fatal and nonfatal cases of hand, foot and mouth disease epidemics in Malaysia, Japan and Taiwan in 1997-1998. *Jpn. J. Infect. Dis*. **52**:12-15.

Shih S.R., Li Y.S., Chiou C.C., Suen P.C., Lin T.Y., Chang L.Y., Huang Y.C., Tsao K.C., Ning H.C., Wu T.Z., Chan E.C. (2000a). Expression of capsid protein VP1 for use as antigen for the diagnosis of Enterovirus 71 infection. *J. Med. Virol.* **61**:228-234.

Shih S.R., Ho M.S., Lin K.H., Wu S.L., Chen Y.T., Wu C.N., Lin T.Y., Chang L.Y., Tsao K.C., Ning H.C., Chang P.Y., Jung S.M., Hsueh C., Chang K.S. (2000b). Genetic analysis of enterovirus 71 isolated from fatal and non-fatal cases of hand, foot and mouth disease during an epidemic in Taiwan, 1998. *Virus Res*. **68**:127-136.

Shih S.R., Tsai K.N., Li Y.S., Chueh C.C., Chan E.C. (2003). Inhibition of enterovirus 71-induced apoptosis by allophycocyanin isolated from a blue-green alga Spirulina platensis. *J. Med. Virol.* **70**:119-125.

Simons J., Kutubuddin M., Chow M. (1993). Characterization of poliovirus-specific T lymphocytes in the peripheral blood of Sabin-vaccinated humans. *J. Virol.* **67**:1262-1268.

Singh S., Chow V.T., Chan K.P., Ling A.E., Poh C.L. (2000). RT-PCR, nucleotide, amino acid and phylogenetic analyses of enterovirus type 71 strains from Asia. *J. Virol. Methods.* **88**:193-204.

Singh S., Chow V.T., Phoon M.C., Chan K.P., Poh C.L. (2002). Direct detection of Enterovirus 71 (EV71) in clinical specimens from a hand, foot and mouth disease outbreak in Singapore by reverse-transcription-PCR with universal enterovirus and EV71-specific primers. *J. Clin. Microbiol.* **40**:2823-2827.

Snapper C.M., Mond J.J. (1993). Towards a comprehensive view of immunoglobulin class switching. *Immunol. Today*. **14**:15-17.

Stanway G., Cann A.J., Hauptmann R., Hughes P., Clarke L.D., Mountford R.C., Minor P.D., Schild G.C., Almond J.W. (1983). The nucleotide sequence of poliovirus type 3 leon 12 a1b: comparison with poliovirus type 1. *Nucleic Acids Res.* **11**:5629-5643.

Street N.E., Mosmann T.R. (1991). Functional diversity of T-lymphocytes due to secretion of different cytokine patterns. *FASEB. J.* **5**:171-177.

Takimoto S., Waldman E.A., Moreira R.C., Kok F., Pinheiro Fde P., Saes S.G., Hatch M., De Souza D.F., Carmona Rde C., Shout D., De Moraes J.C., Costa A.M. (1998). Enterovirus 71 infection and acute neurological disease among children in Brazil (1988-1990). *Trans. R. Soc. Trop. Med. Hyg*. **92**:25-28.

Tam J.P. (1996). Recent advances in multiple antigen peptides. *J. Immunol. Methods.* **196**:17-32.

Tan C.S., Cardosa M.J. (2007). High-titred neutralizing antibodies to human enterovirus 71 preferentially bind to the N-terminal portion of the capsid protein VP1. *Arch. Virol.* **152**:1069-1073.

Tan E.L., Chow V.T., Kumarasinghe G., Tze R.P.L., MacKay I.M., Sloots T.P., Poh C.L. (2006). Specific detection of enterovirus 71 directly from clinical specimens using real-time RT-PCR hybridization probe assay. *Mol. Cell. Probes*. **20**:135-140.

Tano Y., Shimizu H., Shiomi M., Nakano T., Miyamura T. (2002). Rapid serological diagnosis of Enterovirus 71 infection by IgM ELISA. *Jpn. J. Infect. Dis*. **55**:133-135.

Torresi J., Stock O.M., Fischer A.E., Grollo L., Drummer H., Boo I., Zeng W., Earnest-Silveira L., Jackson D.C. (2007). A self-adjuvanting multiepitope immunogen that induces a broadly cross-reactive antibody to hepatitis C virus. *Hepatology*. **45**:911-920.

Tsao K.C., Chan E.C., Chang L.Y., Chang P.Y., Huang C.G., Chen Y.P., Chang S.C., Lin T.Y., Sun C.F., Chih S.R. (2002a). Response of IgM for Enterovirus 71 infection. *J. Med. Virol.* **68**:574-580.

Tsao K.C., Chang P.Y., Ning H.C., Sun C.F., Lin T.Y., Chang L.Y., Huang Y.C., Shih S.R. (2002b). Use of molecular assay in diagnosis of hand, foot and mouth disease caused by Enterovirus 71 or coxsackievirus A 16. *J. Virol. Methods.***102**:9–14.

Tsao L.Y., Lin C.Y., Yu Y.Y., Wang B.T. (2006). Microchip, reverse transcription-polymerase chain reaction and culture methods to detect enterovirus infection in pediatric patients. *Pediatr. Int.* **48**:5-10.

Tsuji K., Aizawa M., Sasazuki T. (1992). HLA 1991. Proceedings of the Eleventh Internaitonal Histocompatibility Workshop and Conference. Oxford University Press. New York. USA.

Tung WS, Bakar SA, Sekawi Z, Rosli R. (2007). DNA vaccine constructs against enterovirus 71 elicit immune response in mice. *Genet. Vaccines Ther*. **5**:6.

Van der Marel P., Hazendonk T.G., Henneke M.A., Van Wezel A.L. (1983). Induction of neutralizing antibodies by poliovirus capsid polypeptides VP1, VP2 and VP3. *Vaccine*. **1**:17-22.

Van Loon A.M., Cleator G.C., Ras A. (1999). External quality assessment of enterovirus detection and typing. European Union Concerted Action on Virus Meningitis and Encephalitis. *Bull. World Health Organ*. **77**:217-223.

Van Regenmortel M.H. (1989). The concept and operational definition of protein epitopes. *Philos. Trans. R. Soc. Lond. B. Biol. Sci*. **323**:451-466.

Van Regenmortel M.H. (1992). Molecular dissection of protein antigens. Structure of antigens. Vol. 1. CRC Press. Boca Raton. Florida. USA. 1-28.

Van Regenmortel M.H., Briand J.P., Muller S., Plaue S. (1988). Synthetic polypeptides as antigens. Wol. 19. Elsevier. Amsterdam. 1-227.

Van Wezel A.L., Van der Marel P., Hazendonk T.G., Boer Bak V., Henneke M.A. (1983). Antigenicity and immunogenicity of poliovirus capsid proteins. *Dev. Biol. Stand*. **55**:209-215.

Verstrepen W.A., Kuhn S., Kockx M.M., Van De Vyvere M.E., Mertens A.H. (2001). Rapid detection of enterovirus RNA in cerebrospinal fluid specimens with a novel single-tube real-time reverse transcription-PCR assay. *J. Clin. Microbiol*. **39**:4093-4096.

Vordermeier H.M., Whelan A.O, Cockle P.J., Farrant L., Palmer N., Hewinson R.G. (2001). Use of synthetic peptides derived from the antigens ESAT-6 and CFP-10 for differential diagnosis of bovine tuberculosis in cattle. *Clin. Diagn. Lab. Immunol.* **8**:571-578.

Vordermeier H.M., Whelan A.O., Hewinson R.G. (2003). Recognition of mycobacterial epitopes by T cells across mammalian species and use of a program that predicts human HLA-DR binding peptides to predict bovine epitopes. *Infect. Immun*. **71**:1980-1987.

Wallis C., Melnick J.L. (1967). Virus aggregation as the cause of the non-neutralizable persistent fraction. *J. Virol*. **1**:478-488.

Wang S.M., Lei H.Y., Huang M.C., Su L.Y., Lin H.C., Yu C.K., Wang J.L., Liu C.C. (2006). Modulation of cytokine production by intravenous immunoglobulin in patients with enterovirus 71-associated brainstem encephalitis. *J. Clin. Virol*. **37**:47-52.

Wang S.M., Lei H.Y., Huang M.C., Wu J.M., Chen C.T., Wang J.N., Wang J.R., Liu C.C. (2005). Therapeutic efficacy of milrinone in the management of enterovirus 71-induced pulmonary edema. *Pediatr. Pulmonol.* **39**:219-223.

Wang S.M., Lei H.Y., Su L.Y., Wu J.M., Yu C.K., Wang J.R., Liu C.C. (2007). Cerebrospinal fluid cytokines in enterovirus 71 brain stem encephalitis and echovirus meningitis infections of varying severity. *Clin. Microbiol. Infect.* **13**:677-682.

Wang S.Y., Lin T.L., Chen H.Y., Lin T.S. (2004). Early and rapid detection of Enterovirus 71 infection by an IgM-capture ELISA. *J. Virol. Methods.* **119**:37-43.

Wang S.M., Liu C.C., Tseng H.W., Wang J.R., Huang C.C., Chen Y.J., Yang Y.J., Lin S.J., Yeh T.F. (1999). Clinical spectrum of enterovirus 71 infection in children in southern Taiwan, with an emphasis on neurological complications. *Clin. Infect. Dis.* **29**:184-190.

Wang K.G., Sun L.Z., Jubelt B.,Waltenbaugh C. (1989). Cell-mediated immune responses to poliovirus. I. Conditions for induction, characterization of effector cells, and cross-reactivity between serotypes for delayed hypersensitivity and T-cell proliferative responses. *Cell. Immunol*. **119**:252-262.

Wang J.R., Tsai H.P., Chen P.F., Lai Y.J., Yan J.J., Kiang D., Lin K.H., Liu C.C., Su I.J. (2000). An outbreak of enterovirus 71 infection in Taiwan, 1998. II. Laboratory diagnosis and genetic analysis. *J. Clin. Virol*. **17**:91-99.

Wang J.R., Tuan Y.C., Tsai H.P., Yan J.J., Liu C.C., Su I.J. (2002). Change of major genotype of enterovirus 71 in outbreaks of Hand-Foot-and-Mouth Disease in Taiwan between 1998 and 2000. *J. Clin. Microbiol*. **40**:10-15.

Watkins-Riedel T., Woegerbauer M., Hollemann D., Hufnagl P. (2002). Rapid diagnosis of enterovirus infections by real-time PCR on the LightCycler using the TaqMan format. *Diagn. Microbiol. Infect. Dis*. **42**:99-105.

Whitton J.L., Cornell C.T., Feuer R. (2005). Host and virus determinants of picornavirus pathogenesis and tropism. *Nat. Rev. Microbiol*. **3**:765-776.

Wittwer C.T., Herrmann M.G., Gundry C.N., Elenitoba-Johnson K.S. (2001). Real-time multiplex PCR assays. *Methods*. **25**:430-442.

Wong K.T., Lum L.C., Lam S.K. (2000). Enterovirus 71 infection and neurologic complications. *N. Engl. J. Med*. **342**:356-358.

Wu C.N., Lin Y.C., Fann C., Liao N.S., Shih S.R., Ho M.S. (2001). Protection against enterovirus 71 infection in newborn mice by passive immunization with subunit VP1 vaccines and inactivated virus. *Vaccine.* **20**:895-904.

Wu B.W., Pan T.L., Leu Y.L., Chang Y.K., Tai P.J., Lin K.H., Horng J.T. (2007). Antiviral effects of Salvia miltiorrhiza (Danshen) against enterovirus 71. *Am. J. Chin. Med*. **35**:153-168.

Wu T.C., Wang Y.F., Lee Y.P., Wang J.R., Liu C.C., Wang S.M., Lei H.Y., Su I.J., Yu C.K. (2007). Immunity to avirulent enterovirus 71 and coxsackie A16 virus protects against enterovirus 71 infection in mice. *J. Virol*. **81**:10310-10315.

Yan J.J., Su I.J., Chen P.F., Liu C.C., Yu C.K., Wang J.R. (2001). Complete genome analysis of enterovirus 71 isolated from an outbreak in Taiwan and rapid identification of enterovirus 71 and coxsackievirus A16 by RT-PCR. *J. Med. Virol*. **65**:331-339.

Yu C.K., Chen C.C., Chen C.L., Wang J.R., Liu C.C., Yan J.J., Su I.J. (2000). Neutralizing antibody provided protection against enterovirus type 71 lethal challenge in neonatal mice. *J. Biomed. Sci.* **7**:523-528.

Zoll G.J., Melchers W.J., Kopecka H., Jambroes G., Van der Poel H.J., Galama J.M. (1992). General primer-mediated polymerase chain reaction for detection of enteroviruses: application for diagnostic routine and persistent infections. *J. Clin. Microbiol*. **30**:160-165.

APPENDIX I

Media for Bacterial Culture

LB (Luria-Bertani) Medium, per liter (Miller, 1972)

NaCl	10 g
Trytone	10 g
Yeast extract	5 g

For agar preparation, 15 g of Oxoid Agar Technical No. 3 was added before autoclaving at 121^{o}C at 15 Ib/inch^{2} (p.s.i.) for 20 min.

APPENDIX II

Materials for Tissue Culture

10x Minimum Essential Medium (MEM) Powder (Gibco BRL)

The powdered medium is supplemented with Earle's salt, L-glutamate, non-essential amino acids, without sodium bicarbonate. The MEM medium was prepared by dissolving the MEM powder in 1 L of sterile distilled water and further sterilized by membrane filtration.

MEM Growth Media

1x MEM	90.5 ml
1x Penicillin/Streptomycin	1.0 ml
Fetal Calf Serum (FCS)	5.0 ml
1.0 M HEPES Buffer	2.0 ml
7% Sodium Bicarbonate	1.5 ml

The media was stored at 4^{o}C until used.

MEM Reviving Media

1x MEM	85.5 ml
1x Penicillin/Streptomycin	1.0 ml
Fetal Calf Serum (FCS)	10.0 ml
1.0 M HEPES Buffer	2.0 ml
7% Sodium Bicarbonate	1.5 ml

The media was stored at 4^{o}C until used.

MEM Freezing Media

1x MEM	89.5 ml
1x Penicillin/Streptomycin	1.0 ml
Fetal Calf Serum (FCS)	5.0 ml
1.0 M HEPES Buffer	2.0 ml
7% Sodium Bicarbonate	1.5 ml
Dimethyl sulphoxide (DMSO)	1.0 ml

The media was prepared fresh before carrying out the procedure for cell freezing. DMSO was protected from light and stored at room temperature.

TN Buffer, per litre

Tris-HCL	50 mM
NaCl	50 mM

The pH was adjusted to 7.8 and the volume was prepared up to 1 L with sterile distilled water before further sterilization by membrane filtration.

30% Sucrose Gradient Cushion, per litre

A. Solution I

Tris-Acetate	20 mM
NaCl	1 M

The volume was prepared up to 600 ml with sterile distilled water.

B. Solution II

Sucrose	300 g

The volume was prepared up to 400 ml with sterile distilled water.

For a final 1 L preparation, both solutions I and II were added together before further sterilization by membrane filtration.

APPENDIX III

$TCID_{50}$ Assay

Determination of infective dose ($TCID_{50}$)

Dilution of virus stock	No. of wells showing CPE / No. of wells inoculated	Cumulative no. of wells infected	Cumulative no. of wells not infected	Calculated infectivity ratio	Calculated infectivity percentage (%)
10^{-3}	4/4	9	0	9/9	100
10^{-4}	3/4	5	1	5/6	83
10^{-5}	2/4	2	3	2/5	40
10^{-6}	0/4	0	7	0/7	0

$TCID_{50}$ was calculated using the Reed using and Muench formula.

1. The cumulative number of infected wells was obtained by adding all the wells which showed CPE at every dilution.
   (e.g. at dilution 10-5, 2 wells showed CPE. At dilution 10^{-4}, 3 wells showed CPE. Hence, the total number of wells which were showed CPE up to dilution factor 10^{-4} was 2 + 3 = 5)

2. The cumulative number of wells which were not infected with the virus was obtained by subtracting the number of wells which showed CPE from the number of wells inoculated.
   (e.g. at dilution 10^{-4}, 1 well did not show CPE. At dilution 10^{-5}, 2 wells did not show CPE. Hence, the total number of wells which did not show CPE up to the dilution factor 10^{-5} was 1 + 2 = 3)

3. The percentage of infectivity was calculated by dividing the total sum of the wells which showed CPE and those did not show CPE by the cumulative number of wells which showed CPE.
   (e.g. at dilution 10^{-4}, 5/5+1 = 0.83 = 83%)

4. The proportionate distance between the two dilutions, 10^{-4} and 10^{-5} , where 50% lies:

$$\frac{\text{Infectivity above 50\% - 50\%}}{\text{Infectivity above 50 \% - Infectivity below 50\%}} = \frac{83 - 50}{83 - 40} = 0.7$$

5. $TCID_{50}$ end point calculation: The negative logarithm of the dilution factor which was the closet to 50% was -4. Hence, the $TCID_{50}$ end point = -4.0 + (0.7) = -4.7. Therefore, the log of $TCID_{50}$ end point or titer = $10^{-4.7}$.

6. Interpretation of $TCID_{50}$ titer: A virus suspension with a $TCID_{50}$ of $10^{-4.7}$ per 0.1 ml represents one $TCID_{50}$: at such dilution, 50% of the cultures inoculated with the virus will be infected. A dilution of $10^{-2.7}$ will contain 10^2 $TCID_{50}$ in a volume of 0.1 ml.

APPENDIX IV

Materials for SDS-PAGE

Polyacrylamide Gels

	5% Separating Gel (10 ml total)	12% Stacking Gel (10 ml total)
30% acrylamide / 0.8% N,N`-methylene bisacrylamide	4,000 µl	1,700 µl
1.5 M Tris-HCl, pH 8.8	2,500 µl	-
1.0 M Tris-HCl, pH 6.8	-	1,250 µl
10% SDS	100 µl	100 µl
10% Ammonium persulphate	100 µl	100 µl
TEMED	5 µl	10 µl
dH_20	3,295 µl	6,840 µl

6x SDS Gel Loading Dye

Tris-HCl	0.35 M
SDS	10.28 %
Glycerol	36.0 %
Dithiothreitol (DTT)	0.6 M
Bromophenol Blue	0.012 %

The pH was adjusted to 6.8 and stored at -20^{o}C until used.

SDS Running Buffer

Tris-BASE	25 mM
Glycine	192 mM
SDS	0.1 %

The pH was adjusted to 8.3 and the volume was prepared up to 10 L with distilled water.

SDS Staining Solution

Ethanol	40 %
Acetic acid	10 %
Coomassie Brilliant Blue	0.1 %
Distilled water	50 %

The mixture was stored at 4^{o}C until used.

SDS Destaining Solution

Ethanol	40 %
Acetic acid	10 %
Distilled water	50 %

The mixture was stored at 4^{o}C until used.

APPENDIX V

Materials for Western Blot

Western Transfer Buffer

Tris-HCL	3.03 g
Glycine	1.44 g
Methanol	200 ml

The volume was prepared up to 1 L with distilled water and stored at 4^{o}C until used.

Western Blocking Buffer

Tris-HCL	20 mM
NaCl	150 mM
Tween 20	0.05 %
Bovine Serum Albumin (BSA)	1 %

Western Washing Buffer

Tris-HCL	20 mM
NaCl	150 mM
Tween 20	0.05 %

Articles

1. Foo D.G., MacAry P.A., Alonso S., Poh C.L. (2008). Identification of human $CD4^+$ T-cell epitopes on the VP1 capsid protein of Enterovirus 71. *Viral Immunol*. **21**.

2. Foo D.G., Ang R.X., Alonso S., Chow V.T., Quak S.H., Poh C.L. (2007). Identification of immunodominant VP1 linear epitope of Enterovirus 71 using synthetic peptides for detecting human anti-EV71 IgG antibodies in Western blot. *Clin. Microbiol. Infect.* **14**:286-288.

3. Foo D.G., Alonso S., Chow V.T., Poh C.L. (2007). Passive protection against lethal enterovirus 71 infection in newborn mice by neutralizing antibodies elicited by a synthetic peptide. *Microbes Infect.* **9**:1299-1306.

4. Foo D.G., Alonso S., Phoon M.C., Ramachandran N.P., Chow V.T., Poh C.L. (2007). Identification of neutralizing linear epitopes from the VP1 capsid protein of Enterovirus 71 using synthetic peptides. *Virus Res.* **125**:61-68.

5. Ho S.Y., Chua S.Q., Foo D.G., Locht C., Chow V.T., Poh C.L., Alonso S. (2007). The highly attenuated *Bordetella pertussis* BPZE1 strain as a potential live vehicle for the delivery of heterologous vaccine candidates. *Infect. Immun*. **76**:111-119.

Conference papers

1. Foo D.G., Poh C.L., Chow V.T., Alonso S. (2008). Synthetic Peptide-based Vaccine as A Potential Therapeutic Agent against Enterovirus 71. In: *1st International Singapore Symposium of Immunology, Singapore.*

2. Foo D.G., Poh C.L., Chow V.T., Alonso S. (2007). Synthetic Peptide-based Vaccine as A Potential Therapeutic Agent against Enterovirus 71. In: *Vaccine Congress, Amsterdam, The Netherlands.*

3. Foo D.G., Alonso S., Chow V.T., Poh C.L. (2007). Passive protection against lethal Enterovirus 71 infection in newborn mice by neutralizing antibodies elicited by a synthetic peptide. In: *NHG Annual Scientific Congress 2007, Singapore.*

4. Foo D.G., Alonso S., Phoon M.C., Rama N.P., Chow V.T., Poh C.L. (2006). Identification of Neutralizing Linear Epitopes from the VP1 Capsid Protein of Enterovirus 71 using Synthetic Peptides. In: *NHG Annual Scientific Congress 2006, Singapore.*
   (Best Poster Presentation Award under 'Basic Sciences' category)

6. Foo D.G., Alonso S., Phoon M.C., Rama N.P., Chow V.T., Poh C.L. (2006). Identification of Neutralizing Linear Epitopes from the VP1 Capsid Protein of Enterovirus 71 using Synthetic Peptides. In: *6th Louis Pasteur Conference on Infectious Diseases, Paris France.*

7. Ho S.Y., Foo D.G., Poh C.L., Chow V.T., Alonso S. (2006). Development of Vaccines against Enterovirus 71 using Bordetella Pertussis for Antigen Delivery. In: *6th Louis Pasteur Conference on Infectious Diseases, Paris France.*

Available online at www.sciencedirect.com
ScienceDirect
Virus Research 125 (2007) 61–68
Virus Research
www.elsevier.com/locate/virusres

Identification of neutralizing linear epitopes from the VP1 capsid protein of Enterovirus 71 using synthetic peptides

Damian Guang Wei Foo, Sylvie Alonso, Meng Chee Phoon, N.P. Ramachandran, Vincent Tak Kwong Chow, Chit Laa Poh *

Department of Microbiology, Yong Loo Lin School of Medicine, National University of Singapore, MD4A, 5 Science Drive 2, Singapore 117597, Singapore

Received 26 July 2006; received in revised form 1 December 2006; accepted 11 December 2006
Available online 12 January 2007

Abstract

Enterovirus 71 (EV71) is the main causative agent of Hand, foot and mouth disease (HFMD) and has been associated with severe neurological diseases resulting in high mortalities. Currently, there is no vaccine available and treatment is limited to palliative care. In this study, antisera were raised in mice against 95 overlapping synthetic peptides spanning the VP1 capsid protein of EV71. Two peptides, SP55 and SP70, containing amino acid 163–177 and 208–222 of VP1, respectively, are capable of eliciting neutralizing antibodies against EV71 in the *in vitro* microneutralization assay. SP70 was identified to be particularly potent in eliciting a neutralizing antibody titer comparable to that obtained with a whole virion-immune serum. Immunization of mice with either SP55 or SP70 triggered an EV71-specific IgG response as high as that obtained with the whole virion as immunogen. The IgG sub-typing revealed that the neutralizing antibodies elicited by both synthetic peptides are likely belonging to the IgG1 sub-type. Alignment with databases showed that the amino acid residues of SP70 are highly conserved amongst the VP1 sequences of EV71 strains from various subgenogroups. Altogether, these data indicate that SP70 represents a promising candidate for an effective synthetic peptide-based vaccine against EV71.

Keywords: Enterovirus 71; VP1 capsid protein; Synthetic peptides; Neutralizing antibodies

1. Introduction

Enterovirus 71 (EV71) was first reported in 1974 (Schmidt et al., 1974) and in recent years, it has caused large outbreaks with significant mortality in the Asia Pacific region. EV71 has been associated with an array of clinical diseases, ranging from mild exanthemas such as herpangina to aseptic meningitis, encephalitis and pulmonary edema. EV71 is also one of the main etiological agents of Hand, foot, and mouth disease (HFMD) (Hagiwara et al., 1978). In 1998, a HFMD outbreak caused by EV71 affected more than 100,000 young children in Taiwan and resulted in 78 deaths (Ho et al., 1999; Liu et al., 2000). During the period from September to October 2000, an outbreak of HFMD occurred in Singapore, affecting mostly children below 6 years of age and resulted in four fatal cases. The most recent epidemic occurring in Sarawak in February 2006 involved 8117 HFMD cases and caused nine deaths.

Currently, the only means of preventing and controlling the spread of EV71 during outbreaks is by public health surveillance and quarantine. A number of promising antiviral agents with activity against enteroviruses are still undergoing clinical trials. Among them, the 'WIN' group of compounds such as pleconaril is the most promising of these agents. Their antiviral activity is thought to be mediated by stabilization of the viral capsid and hence preventing the virus from uncoating upon receptor binding (Pevear et al., 1999). However, one major obstacle to the successful use of any antiviral agents in EV71-associated encephalitis is that most children may have already suffered irreversible brain damage due to the high viral load before treatment. Although the antiviral agent may have high level of activity against EV71, it is unlikely that much clinical improvement can be expected after treatment. Consequently, major research efforts are needed to emphasize on prevention of EV71 infection instead of therapy and this can be carried out via the development of effective vaccines.

* Corresponding author. Tel.: +65 6516 3674; fax: +65 6776 6872.
E-mail address: micpohcl@nus.edu.sg (C.L. Poh).

0168-1702/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.virusres.2006.12.005

Synthetic peptides are considered promising candidates for new-generation vaccines as large quantities of peptides can be chemically synthesized readily and safely. Other benefits of using peptide vaccines for immunization are the ability to immunize with a minimal structure, consisting of a well-defined immunogenic and protective epitope in order to stimulate an effective and specific protective immune response while avoiding potential undesirable effects. A peptide vaccine containing a neutralizing epitope can serve as an ideal vaccine as it is capable of inducing specific antibodies that can inhibit the interaction between the viral capsid protein and its cellular receptor.

EV71 is a small, non-enveloped, positive single-stranded RNA virus with four capsid proteins VP1, VP2, VP3 and VP4 where VP1 is composed of 297 amino acids and has been shown to be immunogenic. Immunization using a recombinant VP1 protein of EV71 was shown to confer protection against lethal EV71 infection in newborn mice, indicating that VP1 contains important antigenic sites that contribute to the neutralization of the virus (Wu et al., 2001).

In this study, characterization of the linear neutralization epitopes on the VP1 capsid protein of the Enterovirus 71 strain 41 (5865/SIN/00009) (belonging to genogroup B4 and isolated from a fatal case) was undertaken by the analysis of mice antisera raised against 95 diphtheria toxoid-conjugated synthetic peptides spanning the entire VP1 region. We tested the neutralizing abilities of the antisera elicited by the conjugated synthetic peptides and reported here two of them, designated SP55 and SP70, which successfully elicited high titers of neutralizing antibody against EV71.

2. Materials and methods

2.1. Design and synthesis of synthetic peptides

A set of 95 overlapping synthetic peptides spanning the entire sequence of the VP1 capsid protein of Enterovirus 71 strain 41 (Genbank accession no. AF316321) were synthesized at Mimotopes Pty Ltd. (Clayton Victoria, Australia). Each peptide contains 15 amino acid residues with 12 residues overlapping with the adjacent peptides. A cysteine residue was added at the C-terminal end for conjugation. Each peptide was chemically linked to the carrier protein, Diphtheria toxoid (DKP), through a linker, Maleimidocaproyl-*N*-hydroxysuccinimide.

2.2. Virus growth and purification

Enterovirus 71 strain 41 (5865/SIN/00009) as the homologous strain and representative EV71 strains from subgenogroups B2, B5, C2 and C4 as heterologous strains were propagated in rhabdomyosarcoma (RD) cells using minimum essential medium (Gibco, USA) supplemented with 5% fetal calf serum, 1% sodium pyruvate and 1.5% sodium bicarbonate. The infected cells were harvested and completely lysed by three cycles of freeze–thaw. The virus from the tissue culture was purified by precipitation with 7% polyethylene glycol 8000 and then centrifuged on a 30% sucrose cushion at 25,000 × *g* for 4 h. The virus titer was determined as $TCID_{50}$ on RD cells based on a typical cytopathic effect (CPE) produced by viral infection. Before being administered to mice, the homologous virus strain was inactivated by heating at 56 °C for 30 min. The amount of virion protein was quantified by the Bradford method (Bio-Rad Laboratories, USA).

2.3. Mice immunization

Groups of 5 adult (6 weeks old) female Balb/c mice were intraperitoneally immunized in a 50% emulsion of Freund's complete adjuvant with either 50 μg of conjugated synthetic peptides or heat-inactivated EV71 strain 41 (10 μg total protein). Two booster doses in 50% emulsion with Freund's incomplete adjuvant were given at 3 weeks intervals. Immune sera were collected 7 days after the last immunization.

2.4. Antibody detection

The levels of antibody to each synthetic peptide were measured by enzyme-linked immunosorbent assay (ELISA). The 96-well microtiter plates were coated overnight at 4 °C with 50 μl of 0.1 M carbonate buffer (pH 9.6) containing 10 μg/ml of unconjugated synthetic peptides or purified virions (EV71 strain 41). After blocking with 2% bovine serum albumin (BSA), plates were incubated with 50 μl of anti-serum at the indicated dilutions at 37 °C for 1 h and then washed three times with phosphate buffered saline (PBS) containing 0.1% Tween 20. Detection was performed using secondary horseradish peroxidase-conjugated goat anti-mouse IgG (H + L) antibody (Bio-Rad Laboratories, USA) at 37 °C for 1 h, followed by three washes. The reaction was revealed by the addition of the *o*-phenylenediamine dihydrochloride substrate (Sigma Aldrich, USA) and the absorbance at 450 nm was measured by an ELISA plate reader (Tecan Sunrise, USA). Each assay was performed three times independently.

The profiles of specific IgG sub-types in the mice anti-sera were determined by a commercially available mouse sub-type isotyping kit (Zymed Laboratories Inc., USA) according to the manufacturer's instructions using 10 μg/ml purified virions (EV71 strain 41) to coat the 96-well microtiter plates. The assay was performed three times independently.

2.5. Western blot

The total proteins of EV71 strain 41 were used to study the immunoreactivity of the mice hyperimmune sera. Briefly, viral proteins were transferred onto nitrocellulose membranes, blocked in 1% BSA-PBS and then incubated with the hyperimmune sera. A secondary alkaline phosphatase-conjugated goat anti-mouse IgG (H + L) antibody was used for detection using the WesternBreeze® chromogenic immunodetection system (Invitrogen, USA).

2.6. Neutralizing antibody assay

The presence of neutralizing antibodies was assayed in an *in vitro* microneutralization assay with RD cells. Mouse serum

samples were incubated at 56 °C for 30 min to inactivate the complement. Briefly, 25 μl of two-fold serial serum dilutions were mixed with equal volumes of 10^3 $TCID_{50}$ of virus in a 96-well microtiter plate and RD cell suspensions (final concentration 5×10^4 cells) were added 2 h later. After incubation for 48 h at 37 °C, the neutralizing antibody titer was read as the highest dilution of serum that inhibited virus growth. The assay was performed three times independently.

2.7. Computational analysis

The protein sequence of VP1 was analyzed for hydrophobic and hydrophilic regions using the Kyte and Doolittle method. The amino acid sequences of the synthetic peptides were used as query sequences in a protein–protein BLAST (blastp 2.2.14) search using the National Center for Biotechnology Information database. Alignment of the amino acid sequences was also undertaken by using the Clustal method of DNASTAR MegAlign.

3. Results

3.1. Identification of neutralizing synthetic peptide antisera

A set of 95 diphtheria toxoid-conjugated synthetic peptides consisting of 15 amino acids in length spanning the entire VP1 capsid protein of EV71 strain 41 were individually injected into groups of two mice. The antisera raised against each peptide were analyzed in an *in vitro* microneutralization assay. Two distinct antisera, obtained from mice immunized with synthetic peptides SP55 (amino acid residues 162–177) and SP70 (amino acid residues 208–222), showed significant neutralizing activi-

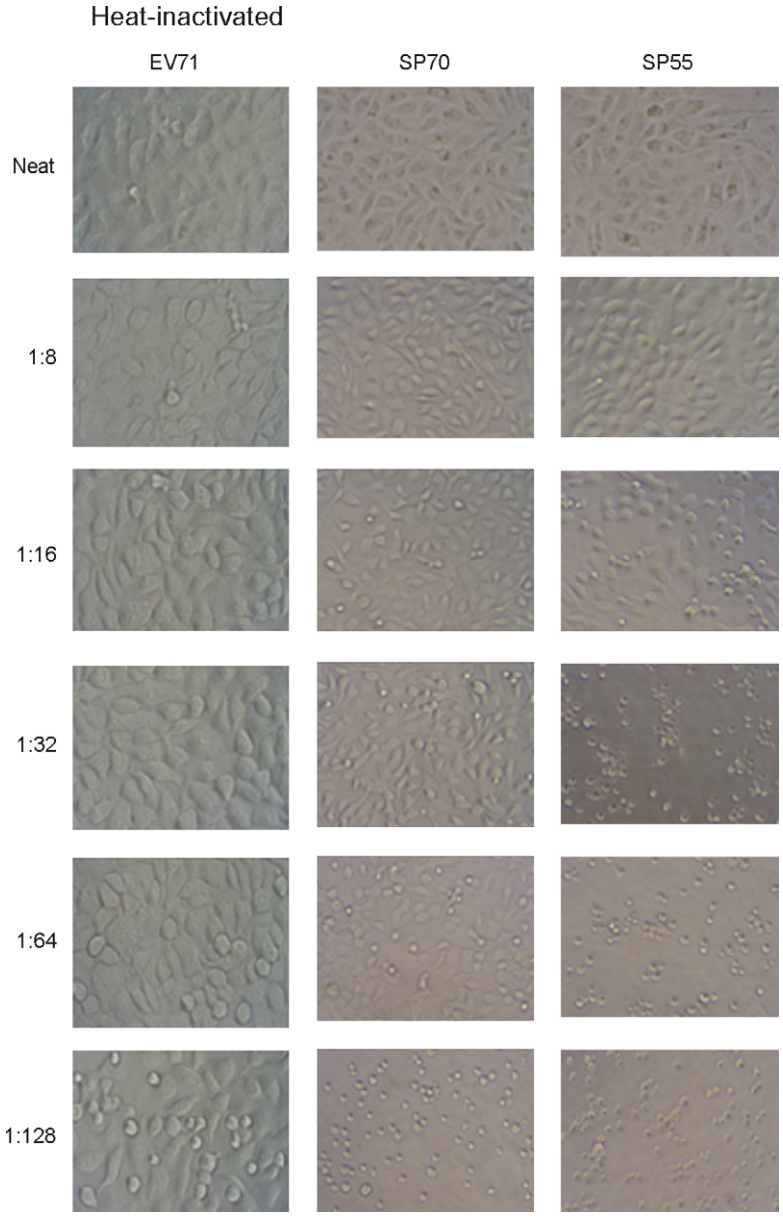


Fig. 1. Neutralization assay using antisera from mice immunized with heat-inactivated EV71, SP70 or SP55. Two-fold serial dilutions of each antiserum were incubated together with the virus and RD cells, and survival of cells was observed 48 h post-infection.

ties against infectivity of the homologous EV71 strain (data not shown).

To confirm the results obtained from this screening, groups of five mice were immunized with either the conjugated synthetic peptides SP55, SP70, SP12 or the heat-inactivated EV71, where SP12 (amino acid residues 34–48) and heat-inactivated EV71 represented negative and positive control groups, respectively.

The neutralizing activities of the antisera obtained from the SP55-, SP70-, SP12- or heat-inactivated EV71-immunized mice were analyzed in an *in vitro* microneutralization assay, using RD cells infected with 10^3 $TCID_{50}$ of EV71 strain 41. Complete protection of RD cells from CPE was observed with dilutions ranging from neat to 1:32 of the antiserum obtained from the SP70-immunized mice. The antiserum from the SP55-immunized mice also showed a neutralizing activity with complete protection from CPE at dilutions from neat to 1:8 whereas the heat-inactivated EV71-antiserum gave a complete protection from CPE at dilutions up to 1:64 (Fig. 1). Antisera from the naive and SP12-immunized mice did not confer any protection to RD cells from CPE (data not shown). Hence, these data demonstrated that the immune sera obtained from the SP70- and, to a lesser extent, the SP55-immunized mice contained neutralizing antibodies which suggest that neutralizing linear epitopes were present in both synthetic peptides SP70 and SP55.

3.2. Immunoreactivity of SP12, SP55 and SP70 antisera

The immunoreactivity of the antisera obtained from mice immunized with synthetic peptides SP12, SP55 or SP70 was assayed against viral total proteins by Western blot. For each antiserum tested, a single band was revealed at an apparent molecular weight (MW) of 32 kDa (Fig. 2). This band very likely corresponds to the VP1 monomer whose apparent MW is 32.7 kDa, indicating that the mice antisera raised against SP12, SP55 and SP70 contained specific VP1 antibodies that do not cross-react with other viral proteins.

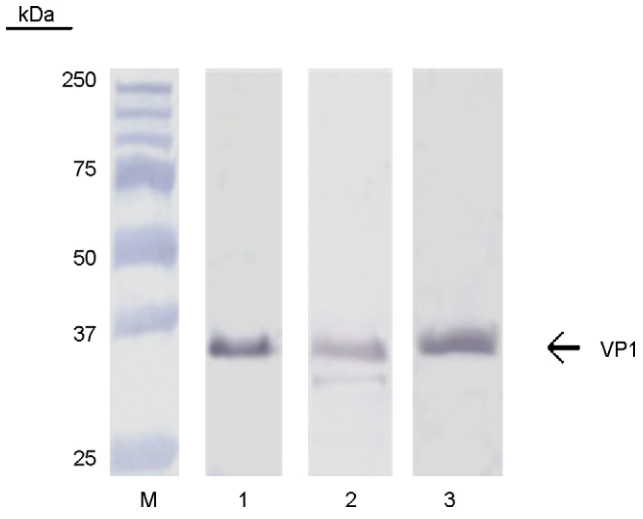


Fig. 2. Western blot analysis using the synthetic peptide-antisera against EV71 total proteins. The amount of viral proteins loaded in each lane was 10 μg. The lanes are as follows: lane M, molecular weight marker; lane 1, antiserum against synthetic peptide SP12; lane 2, antiserum against synthetic peptide SP55; lane 3, antiserum against synthetic peptide SP70. Arrow indicates the presence of VP1 in the respective lanes.

The immuno-specificity of all three antisera towards the 95 synthetic peptides was also assayed by ELISA where the synthetic peptides were individually coated onto the 96-well microtiter plates. A strong positive signal was obtained when antisera were incubated with the corresponding peptide used for immunization (homologous peptide). Furthermore, a positive signal was also obtained when antisera were incubated with the peptides whose amino acid sequences overlap the sequence of the homologous peptide (overlapping peptides). Interestingly, both the anti-SP12 and anti-SP55 antisera showed reactivity against the synthetic peptides SP19, SP20, SP21 and SP40, suggesting that SP12 and SP55 share common epitopes with SP19, SP20, SP21 and SP40 (Table 1).

3.3. Analysis of IgG responses elicited by synthetic peptides

To further characterize the antibody response raised upon immunization with the synthetic peptides SP12, SP55 or SP70, the total IgG response against the whole virion was measured by ELISA. The antibody levels elicited by the synthetic peptides were found to be as high as those obtained upon immunization with the heat-inactivated EV71 strain 41 (Table 2A). In addition, examination of the IgG sub-types revealed that IgG1 antibodies are predominantly produced in antisera elicited by the three respective synthetic peptides, and at levels comparable to the IgG1 level measured in the antiserum obtained from EV71-immunized mice. In contrast, the levels of IgG2a, IgG2b and IgG3 measured in the antisera from mice immunized with synthetic peptides were significantly lower than those obtained in the antiserum from EV71-immunized mice (Table 2B). These data indicate that the conjugated synthetic peptides SP12, SP55 and SP70 elicited mainly a Th2 immune response whereas the heat-inactivated EV71 triggered a mixed Th1/Th2 response.

3.4. In silico analysis of SP55 and SP70 amino acid sequences

The Kyte and Doolittle hydrophobicity profile of the VP1 capsid protein indicated that both synthetic peptides SP55 and SP70 contained amino acid sequences which are located within the major hydrophilic regions of the protein (Fig. 3), therefore, in regions likely to be exposed on the surface of VP1.

A protein–protein BLAST (blastp 2.2.14) search demonstrated that the amino acid sequences of SP55 and SP70 were highly specific for EV71 strains and no significant homology with other enteroviruses such as coxsackieviruses and echoviruses was found (data not shown). Alignment using the Clustal method of MegAlign (DNASTAR program) also indicated that the amino acid sequence of SP70 was found to be totally conserved amongst 25 other EV71 strains from sub-genogroups A to C4 whereas SP55 showed 80% identity with the genogroup A strain, BrCr-CA-70, from US and

Table 1
Immunospecificity of SP12-, SP55- and SP70-immune sera

Peptide	Amino acid position	Peptide sequence[a]	Anti-serum specificity		
			Reactivity with homologous peptide[b]	Reactivity with overlapping peptides[b]	Reactivity with other peptides[b]
SP12	34–48	AC – VSSHRLDTGEVPALQC – DKP	+++	SP10, SP11, SP13, SP14, SP15	SP19, SP20, SP21, SP40
SP55	163–177	AC – PESRESLAWQTATNPC – DKP	+++	SP54, SP56	SP19, SP20, SP21, SP40
SP70	208–222	AC – YPTFGEHKQEKDLEYC – DKP	+++	SP68, SP69, SP71, SP72	–

[a] AC denotes an acetyl group attached to the amino (N)-terminal end and DKP denotes the carrier protein (Diphtheria toxoid) conjugated at the carboxy (C)-terminal end.
[b] Peptide specificities were based on ELISA readings at 450 nm. The data represents the average of three independent experiments.

Table 2
Total and IgG sub-type responses in SP12-, SP55- and SP70-immune sera

Immunogen	$OD_{450\,nm}$
(A) Total IgG response	
SP55	0.87 (0.02)
SP70	0.88 (0.04)
SP12	0.87 (0.03)
Heat-inactivated virus	0.98 (0.01)
Naïve	0.17 (0.02)

Immunogen	$OD_{450\,nm}$			
	IgG1	IgG2a	IgG2b	IgG3
(B) IgG sub-type response				
SP55	0.47 (0.04)	0.10 (0.02)	0.09 (0.02)	0.11 (0.04)
SP70	0.80 (0.02)	0.18 (0.01)	0.14 (0.03)	0.12 (0.01)
SP12	0.72 (0.02)	0.11 (0.01)	0.10 (0.01)	0.17 (0.03)
Heat-inactivated virus	0.83 (0.03)	0.73 (0.01)	0.78 (0.01)	0.80 (0.02)
Naïve	0.08 (0.02)	0.09 (0.02)	0.08 (0.01)	0.07 (0.01)

(A) ELISA was carried out using individual sera at a dilution of 1:50. The 96-well microtiter plates were coated with purified virions (10 μg/ml). $OD_{450\,nm}$ readings are expressed as the average of five antisera per group. Standard deviation of OD readings is in parenthesis.
(B) ELISA was carried out using pooled sera ($n = 5$) at a dilution of 1:50. Wells of the 96-well microtiter plates were coated with the corresponding unconjugated synthetic peptide (10 μg/ml). The data represents the average of three separate experiments. Standard deviation of OD readings at 450 nm is in parenthesis.

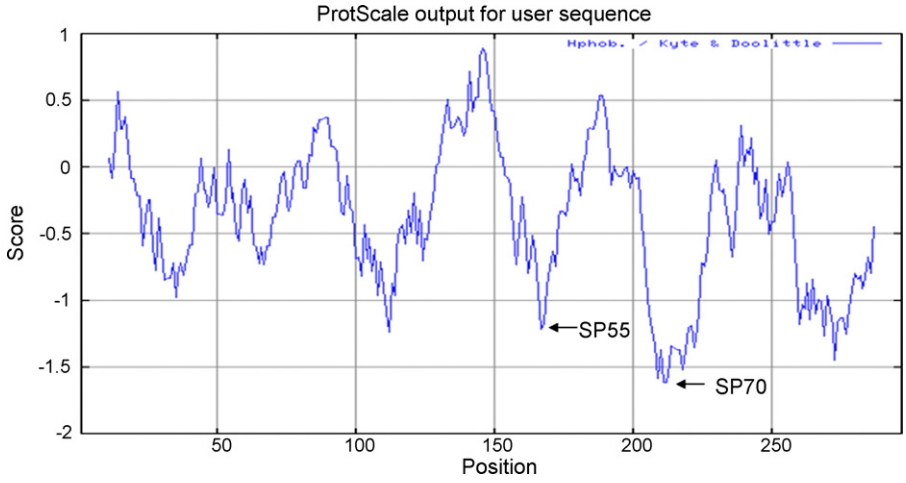


Fig. 3. Kyte and Doolittle hydrophobicity profiles of the VP1 capsid protein of EV71. The horizontal axis represents the amino acid position in VP1 and the vertical axis represents the hydropathy scores. Positive scores indicate hydrophobicity while negative scores indicate hydrophilicity. SP70 (amino acid residues 208–222) and SP55 (amino acid residues 163–177) are indicated on the plot.

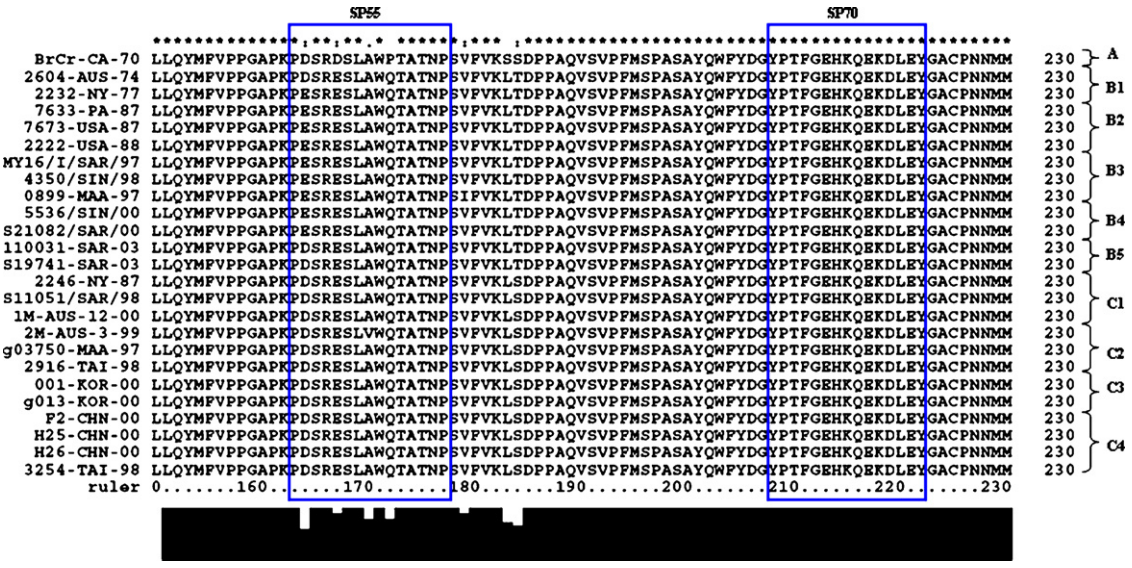


Fig. 4. Alignment of amino acid sequences represented by the synthetic peptides SP55 and SP70 against heterologous EV71 strains from different sub-genogroups based on the VP1 amino acid sequences.

85–100% homology with the other representative strains from sub-genogroups B1 to C4 (Fig. 4).

3.5. In vitro protection afforded by antisera from mice immunized with SP55, SP70 and heat-inactivated EV71 strain 41 against homologous and heterologous EV71 strains

To test that SP55- and SP70-antisera have neutralizing activities against heterologous strains of EV71, the mice antisera raised against each peptide were analyzed in an *in vitro* microneutralization assay, using RD cells infected with 10^3 $TCID_{50}$ of representative EV71 strains from other sub-genogroups such as B2, B5, C2 and C4. Antiserum from mice immunized with the heat-inactivated virion served as the positive control.

Complete protection of RD cells from CPE was observed with antiserum from mice immunized with the whole virion at a neutralizing titer of 1:64. The antiserum from the SP70-immunized mice showed neutralizing activities against homologous and heterologous EV71 strains with significant neutralizing antibody titer of 1:32 whereas the SP55-antiserum has a lower neutralizing antibody titer of 1:8 (Table 3). Hence, the data demonstrated the protective potential of SP70 and, to a lesser extend, SP55 against heterologous EV71 strains. This suggests that the neutralizing linear epitopes present in both SP70 and SP55 are highly conserved amongst representative EV71 strains from different sub-genogroups.

Table 3
Neutralizing antibody titers elicited by SP55, SP70 and heat-inactivated virion in mice against homologous and heterologous EV71 strains

Immunogen	Sub-genogroups of EV71				
	B2[a]	B4[b]	B5[a]	C2[a]	C4[a]
Whole virion[c]	1:32	1:64	1:64	1:64	1:64
SP55	1:8	1:8	1:8	1:8	1:8
SP70	1:16	1:32	1:16	1:32	1:32

[a] Heterologous EV71 strain.
[b] Homologous EV71 strains.
[c] Heat-inactivated EV71 strain 41 (5865/SIN/00009).

4. Discussion

Previous studies have shown that the VP1 capsid protein of EV71 constitutes a good sub-unit vaccine candidate by triggering the production of protective neutralizing antibodies in a murine model of infection (Wu et al., 2001; Chen et al., 2006; Chiu et al., 2006). However, the location of the protective neutralizing epitopes within the VP1 protein has not been established yet.

To approach this, we have used a Pepscan strategy in which 95 overlapping synthetic peptides were designed according to the primary sequence of the VP1 capsid protein. The diphtheria toxoid-conjugated synthetic peptides were injected into mice and the neutralizing activity of the antisera was determined. This strategy allows the identification of conformation-independent neutralizing linear epitope(s) that can thus be used in various vaccine designs. Two synthetic peptides, designated SP55 and SP70, were shown to elicit the production of neutralizing antibodies against EV71. The anti-SP70 antiserum was found to be almost as efficient as the heat-inactivated EV71 antiserum (highest dilutions preventing CPE at 1:32 and 1:64, respectively), in neutralizing the virus in an *in vitro* microneutralization assay. Moreover, the total IgG response specific to the whole virion which was measured in the antisera elicited by SP55 and SP70 was found to be as high as that measured in the EV71-immune serum. Previous studies have shown that whole virus particles offer the most effective protection against EV71 (Yu et al., 2000; Wu et al., 2001). Altogether, these data strongly suggest that synthetic peptides represent a promising approach for EV71 vaccine development.

An ideal synthetic peptide-based vaccine should contain both B-cell epitope(s) and T-cell epitope(s) that are able to induce a protective antibody response as well as a cytotoxic T-cell response important for killing infected host cells. The first generation of peptide vaccines which includes both B-cell and T-cell epitopes has been reported and shown to have a very good protective efficacy (Tam, 1996). In our study, the IgG sub-types analysis of the SP55- and SP70-antisera showed a strong IgG1 specific antibody response and a very low IgG2a, IgG2b and IgG3 antibody response which indicates a predominant Th2 immune response. These observations suggest that (i) both synthetic peptides SP55 and SP70 contain mainly B-cell epitopes and (ii) the neutralizing antibodies elicited by SP55 and SP70 belong to the IgG1 sub-type. The identification of T-cell epitope(s) within the VP1 capsid protein might be advantageous to further develop an effective synthetic peptide-based EV71 vaccine. This work is currently in progress.

In a recent study, the N-terminal moiety of the VP1 capsid protein was found to be the most immunogenic (Sivasamugham et al., 2006). However, both synthetic peptides SP55 and SP70 sequences are not located within the N-terminal moiety of VP1 but lie instead more towards the C-terminal part of the protein. The hydrophobicity profiles showed that SP55 and SP70 are located within the major hydrophilic regions of VP1 and hence they are expected to be exposed at the surface of the protein. Moreover, both synthetic peptides map in the region (amino acid residues 66–132) recently shown to be involved in the dimerization of VP1 capsid protein (Lal et al., 2006). The finding of neutralizing epitopes within this region further demonstrates their involvement in virus binding and entry into host cells. Our data also suggest that the N-terminal moiety of VP1 capsid protein may not be the best choice for sub-unit vaccine candidate.

Although the immunoreactivity of SP12-antiserum was shown to be as good as the SP55- and SP70-antisera, it does not contain any neutralizing antibodies. This suggests that the amino acid region of the VP1 capsid protein represented by synthetic peptide SP12 does not contain any functional domain involved in the virus binding and entry into host cells.

Several genogroups and sub-genogroups of EV71 have evolved since its first discovery in 1969, and frequent mutations of the viral genome constitute a major difficulty in vaccine development when using the whole viral particle as a potential vaccine candidate (McMinn, 2002). Many fatality cases due to EV71 were caused by strains from different sub-genogroups and studies have shown that the EV71 sub-genogroups are constantly evolving within the community (Brown et al., 1999; Mizuta et al., 2005; Wang et al., 2002) which emphasizes the importance of identifying the location of any conserved neutralizing epitope(s). Our blast search has shown that the amino acid sequence of synthetic peptide SP70 is totally conserved amongst 25 representative EV71 strains from different sub-genogroups and we have shown that the antisera raised against SP70 were able to neutralize heterologous EV71 strains, indicating cross-protection against EV71 infectivity.

A study has shown the changes in the survival rate of suckling mice upon *in vivo* EV71 challenge followed by administering intraperitoneally with mouse anti-EV71 serum containing different titers of neutralizing antibody (Yu et al., 2000). With reference to the multiplicity of infection (MOI), our study has a MOI value of 0.02 as compared to theirs with a lower MOI value of 0.0125. Based on their *in vivo* protection studies, they reported a 100% survival rate for suckling mice which received mouse anti-EV71 serum with a neutralizing antibody titer of 1:128 and a 60% survival rate for those which received mouse anti-EV71 serum with a lower neutralizing antibody titer of 1:64. Since their MOI value is approximately two-fold lower, their neutralizing antibody titers should decrease sequentially by two-fold when compared with our data. Therefore, for a 60% survival rate, the neutralizing antibody titer should decrease to 1:32 which corresponds to our neutralizing titer for antibody against synthetic peptide SP70. From extrapolation, this suggests that we should obtain a similar 60% survival rate for our suckling mice born to dams immunized with SP70 upon EV71 challenge. Another study has discussed on the discrepancy between levels of *in vitro* microneutralization activity and *in vivo* protection efficacy which suggests that *in vivo* functions of antibody, which cannot be measured and compared with the *in vitro* microneutralization assay, contribute significantly to protection that includes activation of complement cascades and mediating antibody-dependent cytotoxicity (Wu et al., 2001). Hence, demonstrating the *in vivo* protective efficacy of the synthetic peptide SP70 is necessary to further demonstrate its potential as a promising peptide vaccine candidate.

In conclusion, we have successfully identified two independent amino acid sequences within the VP1 capsid protein of EV71 strain 41 which contain neutralizing linear epitopes. These epitopes lie in the C-terminal moiety of the protein, in a region involved in VP1 dimerization. These sequences are highly conserved within the different sub-genogroups, making them promising and attractive candidates for synthetic peptide-based EV71 vaccines.

Acknowledgements

The authors wish to thank Miss Jasmine Chua Xin Jun for her experimental help throughout the course of this study. This research was supported by a Microbiology Vaccine Initiative grant number R182-008-067-731 awarded to C.L. Poh.

References

Brown, A.B., Oberste, M.S., Alexander Jr., J.P., Margery, L.K., Mark, A.P., 1999. Molecular epidemiology and evolution of Enterovirus 71 strains isolated from 1970 to 1998. J. Virol. 73, 9969–9975.

Chen, H.F., Chang, M.H., Chiang, B.L., Jeng, S.T., 2006. Oral immunization of mice using transgenic tomato fruit expressing VP1 protein from Enterovirus 71. Vaccine 24, 2944–2951.

Chiu, C.H., Chu, C., He, C.C., Lin, T.Y., 2006. Protection of neonatal mice from lethal Enterovirus 71 infection by maternal immunization with attenuated Salmonella enterica serovar Typhimurium expressing VP1 of Enterovirus 71. Microb. Infect. [Epub ahead of print].

Hagiwara, A., Tagaya, I., Yoneyama, T., 1978. Epidemic of hand, foot and mouth disease associated with Enterovirus 71 infection. Intervirology 9, 60–63.

Ho, M., Chen, E.R., Hsu, K.H., Twu, S.J., Chen, K.T., Tsai, S.F., et al., 1999. An epidemic of enterovirus infection in Taiwan. Taiwan Enterovirus Epidemic Working Group. N. Engl. J. Med. 341, 929–935.

Lal, S.K., Kumar, P., Yeo, W.M., Kar-Roy, A., Chow, V.T., 2006. The VP1 protein of human Enterovirus 71 self-associates via an interaction domain spanning amino acids 66–297. J. Med. Virol. 78, 582–590.

Liu, C.C., Tseng, H.W., Wang, S.M., Wang, J.R., Su, I.J., 2000. An outbreak of Enterovirus 71 infection in Taiwan, 1998; epidemiologic and clinical manifestations. J. Clin. Virol. 17, 23–30.

McMinn, P.C., 2002. An overview of the evolution of Enterovirus 71 and its clinical and public health significance. FEMS Microbiol. Rev. 26, 91–107.

Mizuta, K., Abiko, C., Murata, T., Matsuzaki, Y., Itagaki, T., Sanjoh, K., Sakamoto, M., Hongo, S., Murayama, S., Hayasaka, K., 2005. Frequent importation of Enterovirus 71 from surrounding countries into the local community of Yamagata, Japan, between 1998 and 2003. J. Clin. Microbiol. 43, 6171–6175.

Pevear, D.C., Tull, T.M., Seipel, M.E., Groarke, J.M., 1999. Activity of Pleconaril against enteroviruses. Antimicrob. Agents Chemother. 43, 2109–2115.

Schmidt, N.J., Lennette, E.H., Ho, H.H., 1974. An apparently new enterovirus isolated from patients with disease of the central nervous system. J. Infect. Dis. 129, 304–309.

Sivasamugham, L.A., Cardosa, M.J., Tan, W.S., Yusoff, K., 2006. Recombinant newcastle disease virus capsids displaying Enterovirus 71 VP1 fragment induce a strong immune response in rabbits. J. Med. Virol. 78, 1096–1104.

Tam, J.P., 1996. Recent advances in multiple antigen peptides. J. Immunol. Meth. 196, 17–32.

Wang, J.R., Tuan, Y.C., Tsai, H.P., Yan, J.J., Liu, C.C., Su, I.J., 2002. Change of major genotype of Enterovirus 71 in outbreaks of hand-foot-and-mouth disease in Taiwan between 1998 and 2000. J. Clin. Microbiol. 40, 10–15.

Wu, C.N., Lin, Y.C., Fann, C., Liao, N.S., Shih, S.R., Ho, M.S., 2001. Protection against Enterovirus 71 infection in newborn mice by passive immunization with subunit VP1 vaccines and inactivated virus. Vaccine 20, 895–904.

Yu, C.K., Chen, C.C., Chen, C.L., Wang, J.R., Liu, C.C., Yan, J.J., Su, I.J., 2000. Neutralizing antibody provided protection against enterovirus type 71 lethal challenge in neonatal mice. J. Biomed. Sci. 7, 523–528.

ELSEVIER

INSTITUT PASTEUR

Microbes and Infection 9 (2007) 1299–1306

www.elsevier.com/locate/micinf

Original article

Passive protection against lethal enterovirus 71 infection in newborn mice by neutralizing antibodies elicited by a synthetic peptide

Damian Guang Wei Foo, Sylvie Alonso, Vincent Tak Kwong Chow, Chit Laa Poh*

Department of Microbiology, Yong Loo Lin School of Medicine, National University of Singapore, MD4A, 5 Science Drive 2, Singapore 117597, Singapore

Received 27 March 2007; accepted 5 June 2007
Available online 10 June 2007

Abstract

Enterovirus 71 (EV71) infections could lead to high mortalities and neither vaccine nor therapeutic treatment is available. We investigated vaccination with a synthetic peptide SP70 representing a neutralizing linear VP1 epitope of EV71 strain 41 (subgenogroup B4) and passive transfer of anti-SP70 antibodies to protect suckling Balb/c mice against EV71 infectivity. When the mouse anti-SP70 antisera with a neutralizing antibody titer of 1:32 were passively administered to one-day-old suckling mice which had been challenged with a lethal dose of 1000 $TCID_{50}$ per mouse, the neutralizing anti-SP70 antibodies were able to confer 80% *in vivo* protection. In contrast, suckling mice which did not receive any anti-SP70 antisera did not survive the viral challenge at day 21 postinfection. Histological examination and real-time RT-PCR assays revealed viral infiltration in small intestines of EV71-infected mice. Interestingly, anti-SP70 antibodies play a major role in the inhibition of EV71 replication *in vivo* and significantly reduced the viral titer. In conclusion, EV71-neutralizing antibodies elicited by the synthetic peptide SP70 were able to confer good *in vivo* passive protection against homologous and heterologous EV71 strains in suckling Balb/c mice.

Keywords: Enterovirus 71; VP1 capsid protein; Synthetic peptide; Passive protection

1. Introduction

Enterovirus 71 (EV71) is a viral pathogen within the *Picornaviridae* family that causes clinical diseases in humans with manifestations such as herpangina, aseptic meningitis, encephalitis, pulmonary edema and hand, foot, and mouth disease (HFMD). EV71-infected children can develop severe neurological complications that lead to rapid clinical deteriorations and even death [1–5]. A significant increase in EV71 epidemics with high mortalities has been observed throughout the Asia-Pacific region since 1997. In 1998, an epidemic of EV71 infection affected more than 100,000 young children in Taiwan and approximately 400 children were hospitalized for pulmonary edema and neurogenic shock which resulted in 78 deaths [6–8]. In October 2000, an outbreak of HFMD caused by EV71 occurred in Singapore, affecting mostly children below 6 years of age and there were four fatalities. At present, there are no specific effective antiviral drugs available and only palliative care is provided. Preventive and control measures during EV71 outbreaks are limited to public health surveillance and isolation of infected children as there is no available vaccine against EV71.

EV71 contains a positive-stranded RNA which is enclosed by capsid proteins VP1, VP2, VP3, and VP4. These proteins are presumed to be similar to the picornaviral prototype capsid which is made up of the four structural proteins arranged in 60 repeating protomeric units of an icosahedron [9,10]. In addition to protecting the viral RNA from nuclease cleavage, the capsid recognizes the receptors on the surface of host cells [11–13] and displays antigenicity [14,15]. Newborn ICR mice born to dams which were immunized with the formalin-inactivated EV71 whole virion were protected against lethal viral challenge [16]. However, this strategy involves the use of whole viral particles which may implicate safety issues

* Corresponding author. Tel.: +65 6516 3674; fax: +65 6776 6872.
E-mail address: micpohcl@nus.edu.sg (C.L. Poh).

1286-4579/$ - see front matter © 2007 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.micinf.2007.06.002

during vaccine preparations. Another protection study has shown that the whole VP1 capsid protein is a potential vaccine candidate when it was able to confer protection against EV71 infection in suckling ICR mice, suggesting the presence of neutralizing epitope(s) within VP1 [17]. However, the neutralizing epitope(s) remained undefined. In our previous study, a synthetic peptide SP70 representing a neutralizing linear VP1 epitope of EV71 strain 41 (5865/SIN/00009) was successfully identified in eliciting high titers of neutralizing antibodies in Balb/c mice based on *in vitro* microneutralization assays [18]. However, passive protection of suckling Balb/c mice by anti-SP70 antibodies in an *in vivo* EV71 challenge study has not been reported. In this study, we demonstrated the *in vivo* protective efficacy of mouse antisera raised against the synthetic peptide SP70 which harbors a neutralizing linear epitope of the VP1 capsid protein of EV71 strain 41. We compared the efficacy with *in vivo* passive protection afforded by antisera raised against the heat-inactivated homologous EV71 strain and the synthetic peptide SP12 which represents an immunogenic but non-neutralizing linear VP1 epitope.

2. Materials and methods

2.1. Viruses

EV71 strain 41 (5865/SIN/00009) from the subgenogroup B4 was presented as the homologous strain in mice immunization and challenge studies. Representative EV71 strains (EV71/MS/7423/87) from the subgenogroup B2; 2933-Yamagata-03 from the subgenogroup B5; 1585-Yamagata-01 from the subgenogroup C2; and 75-Yamagata-03 from the subgenogroup C4 were presented as heterologous strains in challenge studies [1]. Viruses were grown in rhabdomyosarcoma (RD) cells at 37 °C in minimum essential medium (MEM) (Gibco, USA) supplemented with 5% fetal bovine serum, 1% sodium pyruvate and 1.5% sodium bicarbonate. Once the cells displayed cytopathic effect (CPE), they were harvested and completely lysed by three freeze–thaw cycles. Cellular debris was removed by centrifugation at 10,000 × g for 30 min. The virus was purified by precipitation with 7% polyethylene glycol 8000 (PEG 8000) and then subjected to centrifugation on a 30% sucrose cushion at 25,000 × g for 4 h. The 50% tissue culture infective dose ($TCID_{50}$) was determined in RD cells using the Reed and Muench formula [19]. Before being administered as an immunogen, the homologous EV71 strain was inactivated by heating at 56 °C for 30 min and the amount of virion protein was quantified by the Bradford assay (Bio-Rad Laboratories, USA). Viability of viruses after heat inactivation was carried out by introducing viral preparations into fresh medium containing RD cells to check for CPE.

2.2. Synthetic peptides

Diphtheria toxoid-conjugated synthetic peptides representing the amino acid sequence of SP12 or SP70 were synthesized by Mimotopes Pty Ltd (Clayton, Victoria, Australia) as described previously [18].

2.3. Mice

Inbred Balb/c mice were obtained from the Centre for Animal Resources of the National University of Singapore. All institutional guidelines for animal care and use were strictly followed throughout the experiments. Groups of mice ($n = 6$) at different age groups, ranging from one-day-old to two-week-old, were inoculated intraperitoneally with 100 µl of the homologous EV71 strain (1000 $TCID_{50}$). In another experiment, groups of mice ($n = 6$) at day 1 after birth were given different doses of the homologous EV71 strain (ranging from 1 $TCID_{50}$ to 1000 $TCID_{50}$ per mouse) via the intraperitoneal route. Mice ($n = 6$) in the control group were given 100 µl of phosphate buffered saline (PBS). Mice were monitored for the occurrence of mortality until three weeks postinfection. For immunization, groups of six-week-old female mice ($n = 5$) were intraperitoneally immunized with a 50% emulsion of Freund's complete adjuvant containing either 50 µg of conjugated synthetic peptides or the heat-inactivated EV71 strain 41 (10 µg total protein). Two booster doses in 50% emulsions with Freund's incomplete adjuvant were given at three weekly intervals. Sera were collected seven days after the final booster and checked for the presence of EV71-neutralizing antibodies using the *in vitro* microneutralization assay. Sera containing neutralizing antibodies were pooled and stored at −80 °C until use. For passive protection study, groups of mice ($n = 6$) at day 1 after birth were injected intraperitoneally with 100 µl of EV71 (1000 $TCID_{50}$ per mouse), followed by 100 µl of heat-treated (56 °C, 30 min) mice immune sera 24 h later. Suckling mice ($n = 6$) from control groups were either given naive sera or not given any antisera at all. Mice were monitored for body weight gain/loss and the occurrence of mortality until three weeks postinfection.

2.4. In vitro microneutralization assay

EV71-neutralizing antibodies were identified using the *in vitro* microneutralization assay as described previously [18]. The assay was carried out in triplicates.

2.5. Reverse transcription polymerase chain reaction (RT-PCR)

Organs from mice were aseptically harvested in MEM and were homogenized for total RNA extraction using the RNeasy extraction kit (Qiagen, USA) according to the manufacturers' instructions. The extracted RNA was then analyzed for the presence of EV71 using real-time Hybridization Probe RT-PCR for the detection of EV71 RNA as described previously [20]. Each assay was carried out in triplicates.

2.6. Immunohistochemical analysis

Cryosections with a thickness of 4 µm were made from frozen tissues and fixed on poly-L-lysine glass slides. Permeabilization of the fixed tissue sections was carried out by incubating with 0.2% Triton at room temperature for 10 min

followed by the addition of mouse anti-EV71 monoclonal antibody (Chemicon International, USA) and further incubation for 30 min. Slides were washed three times with PBS and bound antibodies were detected using secondary biotinylated anti-mouse IgG (Zymed Laboratories, USA). The reaction was visualized by the addition of aminoethyl carbazole substrate (Zymed Laboratories, USA) to give a red colored peroxidase stain and the slide was counterstained with haematoxylin (Zymed Laboratories, USA) to generate a blue background.

3. Results

3.1. Age of mice and viral dosage

EV71 infection was characterized by hairless skin lesions and paralysis of limbs in infected Balb/c mice. Both clinical manifestations persisted throughout the observation period (Fig. 1). Suckling Balb/c mice from different age groups were infected intraperitoneally with the homologous EV71 strain at a dose of 1000 $TCID_{50}$ per mouse. Mice which were infected at day 1 after birth did not survive by day 11 postinfection. With mice infected at day 4 after birth, death was delayed but all mice died by day 16 postinfection. Mice which were infected at day 7 or day 10 after birth had higher survival rates of 60% and 90%, respectively, by day 21 postinfection. All mice which were infected at day 14 after birth survived throughout the experimental period (data not shown). To determine the lethal viral dosage, groups of mice at day 1 after birth were infected intraperitoneally with different doses of viruses, ranging from 1 $TCID_{50}$ to 1000 $TCID_{50}$ per mouse. With an infective dose of 1000 $TCID_{50}$ virus, all mice died by day 11 postinfection whereas a 30% survival rate was observed for those which were infected with a reduced dosage (1 $TCID_{50}$ per mouse). Mice which were not infected did not show any sign of distress and all of them survived (Fig. 2A).

3.2. Passive protection against lethal homologous EV71 strain challenge in suckling mice

To assess the efficacy of passive protection by antisera raised against the homologous EV71 whole virion (anti-EV71) and the synthetic peptide SP70 (anti-SP70), suckling Balb/c mice born to naive dams were administered with the respective antisera one day after lethal challenge with the homologous EV71 strain (1000 $TCID_{50}$ per mouse). Groups of mice that received the anti-EV71 antisera with neutralizing antibody titers of 1:64 or 1:32 survived throughout the experimental period. The anti-SP70 antisera with a neutralizing antibody titer of 1:32 provided 80% protection whereas EV71-infected suckling mice which did not receive any antisera and those which received antisera either from SP12-immunized (anti-SP12) or naive mice did not survive by day 12 postinfection (Fig. 2B). The body weight of mice that were protected against EV71 infectivity rose steadily up to an average of approximately 12 g at day 21 postinfection. At day 9 postinfection, they weighed approximately 8 g on average when compared to unprotected mice that had an average body weight of approximately 3 g before they died from EV71 infection (Fig. 2C). When the neutralizing antibody titer of anti-EV71 antisera was reduced to 1:16, the survival rate of EV71-infected suckling mice dropped to 70% and subsequently to 50% when the neutralizing antibody titer was further reduced to 1:8 (Fig. 3A). Approximately 80% of suckling mice survived when they were given the anti-SP70 antisera with a neutralizing antibody titer of 1:32 and a 50% survival rate was observed with the antisera at reduced titer of 1:16. With a reduced neutralizing antibody titer of

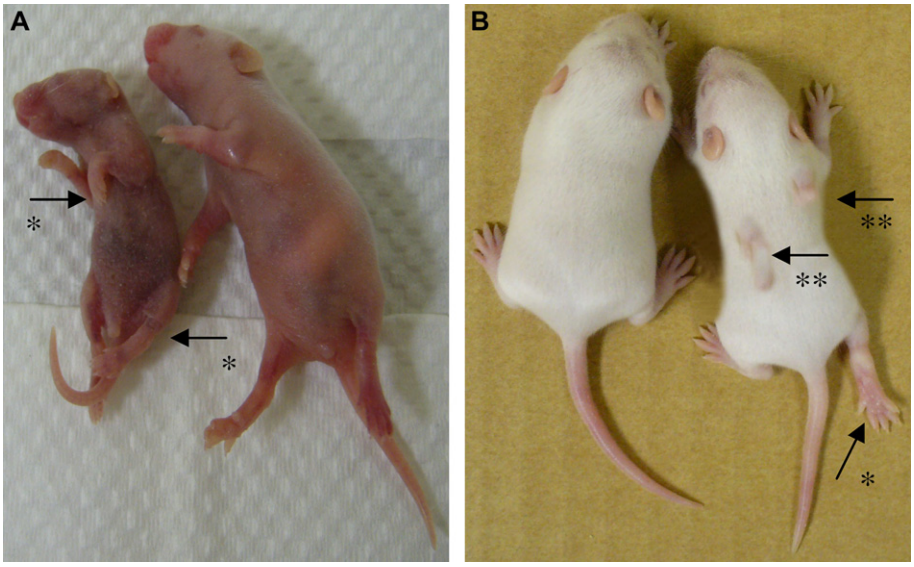


Fig. 1. Viral infection of suckling Balb/c mice with a lethal EV71 dose (1000 $TCID_{50}$ per mouse). (A) Mice at day 3 postinfection are shown. The mouse on the right side is a non-infected age-matched control. (B) Mice at day 9 postinfection are shown. The mouse on the left side is a non-infected age-matched control. Limb paralysis is represented by * and skin lesion is represented by **.

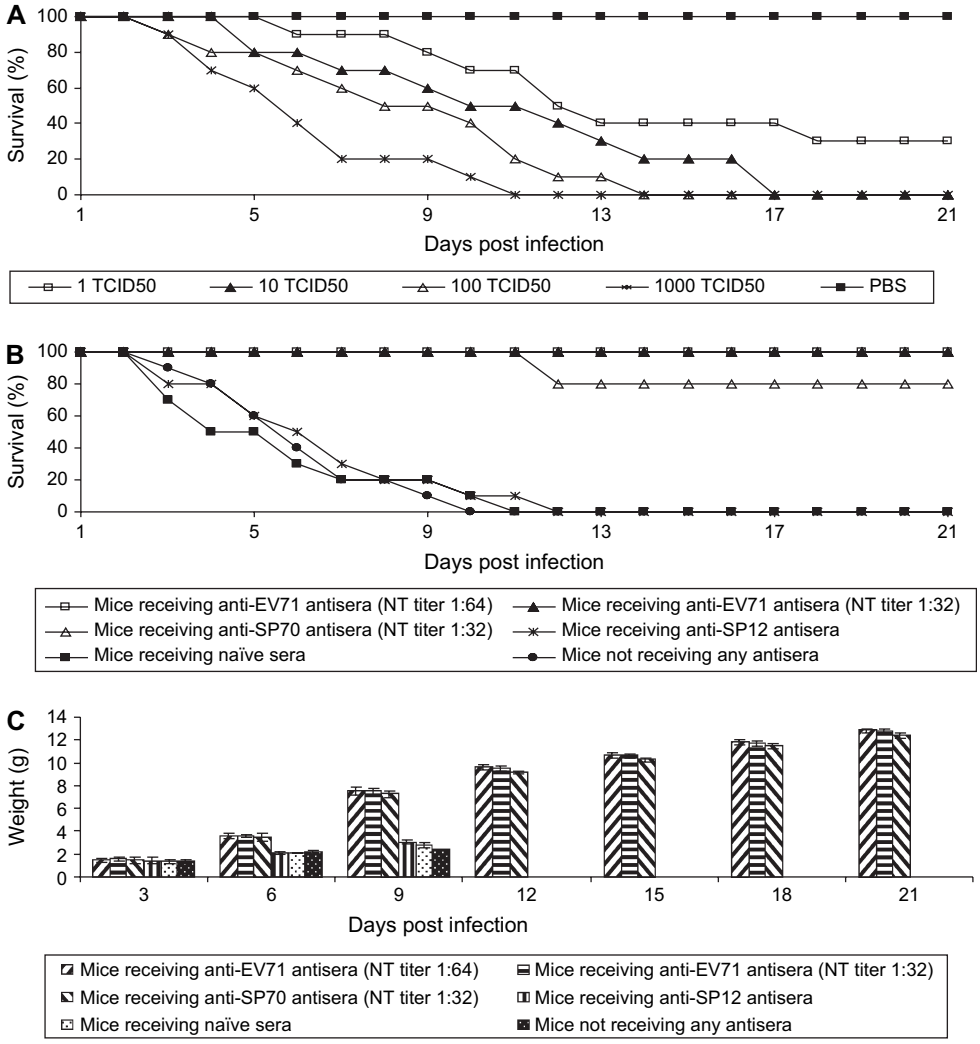


Fig. 2. *In vivo* infectivity of EV71. (A) Dose dependency of EV71-induced death. Groups of Balb/c mice ($n = 6$) at day 1 after birth were intraperitoneally infected with increasing doses of the homologous EV71 strain (ranging from 1 $TCID_{50}$ to 1000 $TCID_{50}$ per mouse). Mice in the control group were given 100 μl of PBS. Death was monitored daily until 21 days postinfection. (B) *In vivo* passive protection of suckling Balb/c mice after challenged with the homologous EV71 strain. Groups of mice ($n = 6$) at day 1 after birth were intraperitoneally infected with a lethal dose of 1000 $TCID_{50}$ per mouse. One day after infection, suckling mice were administered with antisera from EV71-, SP70- or SP12-immunized mice via the similar route. Mice in control groups were given naive sera or not administered with any antisera. Death was monitored daily until 21 days postinfection. (C) Monitoring of body weight gain/loss in suckling Balb/c mice after EV71 challenge. Body weights were recorded for all surviving mice every three days until 21 days postinfection.

1:8, the anti-SP70 antisera were still able to confer 20% protection (Fig. 3B).

3.3. Histological examination in EV71-infected suckling mice

Histological examinations revealed EV71 infiltration in small intestines of suckling Balb/c mice infected with the homologous EV71 strain. The presence of the virus was detected with the anti-EV71 monoclonal antibody. From micrographs of the cryosections, infected mice had intestinal structures which were damaged, thus indicating extensive viral replications. Mice which received the anti-EV71 or anti-SP70 antisera had intact intestinal structures and were protected against EV71 infectivity. However, mice which received the anti-SP12 antisera showed signs of tissue damage in their small intestines. The red colored peroxidase stain indicated EV71 infiltration of intestinal tissues (Fig. 4).

3.4. Detection of EV71 by real-time RT-PCR hybridization probe assay

Organs were harvested from Balb/c mice after challenge studies and RNA was extracted from homogenized tissues for the detection of EV71. The amplicons generated from the real-time RT-PCR assay were based on 204 base pairs PCR product of the VP1 region. Quantification analysis showed that the positive control has a Ct value of 13 which represents 5×10^8 EV71 copies and every 3.3 Ct value difference

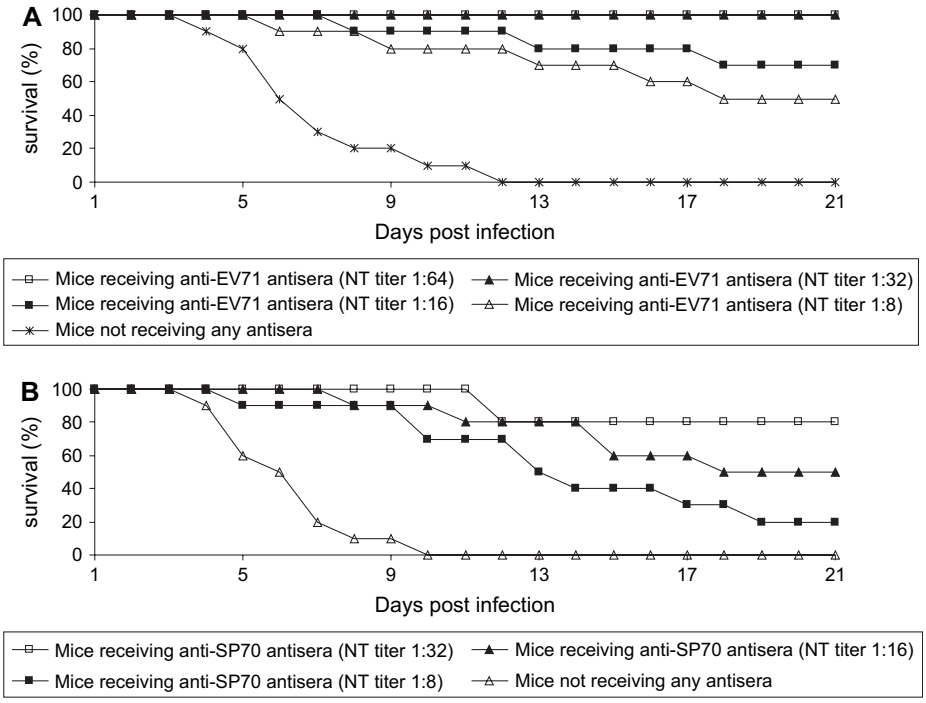


Fig. 3. *In vivo* passive protection study conferred by different EV71-neutralizing antibody titers. Groups of Balb/c mice ($n = 6$) at day 1 after birth were infected with the homologous EV71 strain (1000 $TCID_{50}$ per mouse) via the intraperitoneal route. One day after infection, suckling mice were intraperitoneally administered with serially diluted pooled antisera from (A) EV71-immunized mice or (B) SP70-immunized mice. Mice in control groups were not administered with any antisera. Death was monitored until 21 days postinfection.

represents a 10-fold difference in viral copies. Viral RNA samples from suckling mice which did not receive any antisera and those which received the anti-SP12 antisera or naive sera indicated a Ct value of 33. This represents the presence of 50 EV71 copies in the three respective samples. However, suckling mice which received either the anti-EV71 or anti-SP70 antisera had RNA transcripts with a higher Ct value of 37, representing approximately five EV71 copies (Fig. 5).

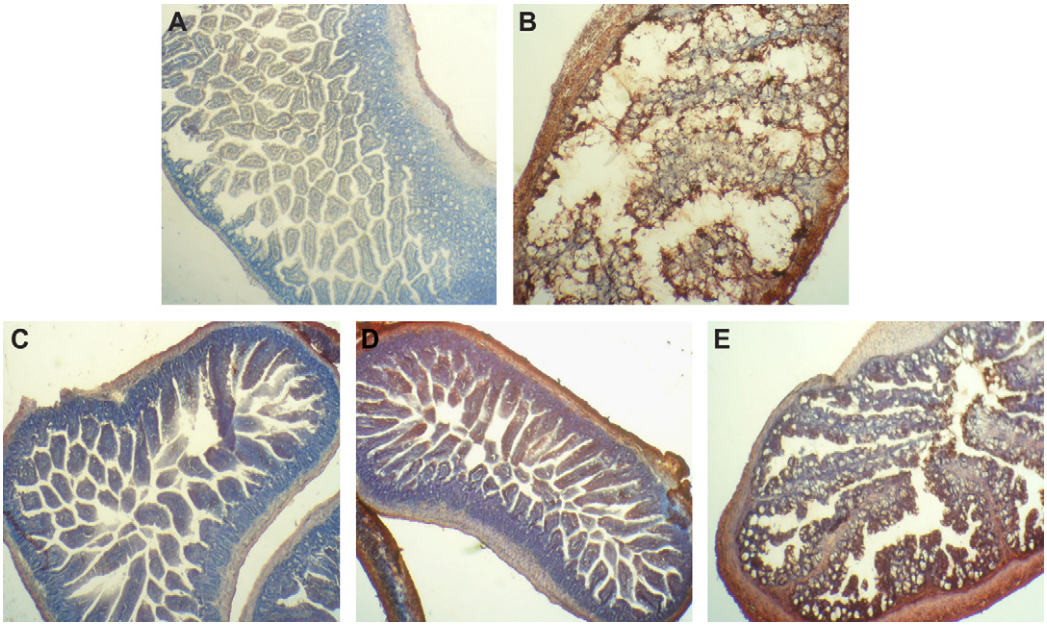


Fig. 4. Detection of EV71 infection in small intestines of suckling Balb/c mice after being challenged with the homologous EV71 strain (1000 $TCID_{50}$ per mouse). Intestinal structures from: (A) non-infected mice as the negative control; (B) EV71-infected mice as the positive control; (C) mice receiving the anti-EV71 antisera; (D) mice receiving the anti-SP70 antisera; and (E) mice receiving the anti-SP12 antisera. Cryosections of intestinal tissues were prepared and EV71 was detected with mouse anti-EV71 monoclonal antibody and biotinylated anti-mouse IgG before visualizing with aminoethyl carbazole substrate to give a red colored peroxidase stain as positive detection and haematoxylin to give a blue background.

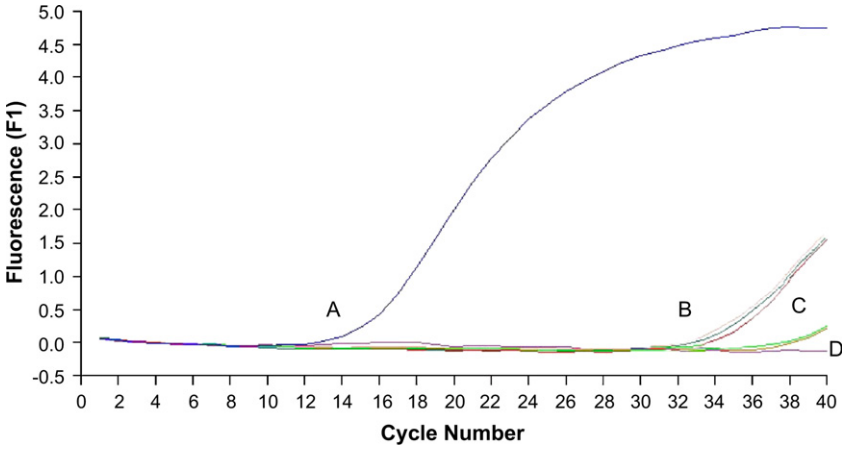


Fig. 5. Detection of EV71 by real-time RT-PCR hybridization probe assay in suckling Balb/c mice after challenge studies. (A) EV71 RNA transcript as the positive control with a Ct value of 13 representing 5×10^8 viral copies. (B) RNA transcripts from groups of suckling mice receiving pooled antisera from SP12-immunized mice ($n = 5$), pooled sera from non-immunized mice ($n = 5$) or no sera with a Ct value of 33 representing 50 viral copies. (C) RNA transcripts from groups of suckling mice receiving pooled antisera from EV71 whole virion- or SP70-immunized mice ($n = 5$) with a higher Ct value of 37 representing five viral copies. (D) The negative control includes all reagents without any RNA template.

3.5. Passive protection afforded by the anti-EV71 or anti-SP70 antisera against heterologous EV71 strains

Groups of suckling Balb/c mice born to naive dams were challenged with heterologous EV71 strains (1000 $TCID_{50}$ per mouse) and antisera raised against the homologous EV71 whole virion or synthetic peptide SP70 were administered at day 2 after infection. When EV71-infected suckling mice were administered with the anti-EV71 antisera at a neutralizing antibody titer of 1:32, all of them survived. Mice infected with EV71 strains from the subgenogroup B2 or B5 had 80% survival rates when they were administered with the anti-SP70 antisera with a neutralizing antibody titer of 1:32. Upon lethal challenge with EV71 strains from the subgenogroup C2 or C4, the anti-SP70 antisera protected 70% of infected suckling mice (Table 1).

Table 1
Survival rates of suckling mice from challenge with homologous or heterologous EV71 strains

EV71 subgenogroups	Mouse antisera[a]	Survival (%)
B2[c]	Anti-EV71	100
	Anti-SP70	80
B4[b]	Anti-EV71	100
	Anti-SP70	80
B5[c]	Anti-EV71	100
	Anti-SP70	80
C2[c]	Anti-EV71	100
	Anti-SP70	70
C4[c]	Anti-EV71	100
	Anti-SP70	70

[a] One day after EV71 challenge (1000 $TCID_{50}$ per mouse), suckling mice were administered with antisera from mice immunized with the heat-inactivated EV71 strain 41 (anti-EV71) or synthetic peptide SP70 (anti-SP70) and monitored until 21 days postinfection. Each group contained six mice.
[b] Homologous EV71 strain.
[c] Heterologous EV71 strains.

4. Discussion

Although immune responses such as elevated antibody level detected soon after immunization may demonstrate the immunogenicity of a vaccine, they do not necessarily provide a reliable guide to its efficacy in priming an *in vivo* protective response. Successful protection against viral infectivity in animal models has long been regarded as the best test for the efficacy of any vaccine. Benefits of vaccination could be derived either through active immunization or passive protection. Passive transfer of specific antibodies has been shown to reduce the severity of viral infections, including Japanese encephalitis infection [21], varicella infection [22] and coxsackievirus infection [23]. We have previously identified a neutralizing linear VP1 epitope, denoted as SP70 within the VP1 capsid protein of EV71 strain 41, which was able to elicit high titers of mouse EV71-neutralizing antibodies against the homologous EV71 strain and five heterologous EV71 strains from subgenogroups B2, B5, C2 and C4 [18]. In the present study, we have tested the *in vivo* functional potential of EV71-neutralizing antibodies elicited by the synthetic peptide SP70 in mice and have shown that the anti-SP70 antisera were able to confer *in vivo* passive protection of up to 80% survival rate in suckling Balb/c mice which have been challenged with a lethal dose of the homologous EV71 strain. Our previous study has shown that the amino acid sequence represented by SP70 was totally conserved amongst 25 EV71 strains from subgenogroups A, B1–B5 and C1–C4, which suggested possible cross-protection against infectivity of all EV71 strains [18]. Here, we have demonstrated that the anti-SP70 antisera with a neutralizing antibody titer of 1:32 were able to confer up to 80% *in vivo* passive protection in suckling Balb/c mice challenged with several heterologous EV71 strains. It is interesting to note that the level of protection was almost similar to that obtained against the homologous strain. This reflects the efficacy of *in vivo* protection conferred by passively transferred EV71-neutralizing antibodies elicited by SP70. We

have also shown that the neutralizing antibody titer plays a decisive role in the *in vivo* passive protection against EV71 infectivity. A significant reduction in the survival rate was correlated with suckling mice receiving antisera with progressively lower EV71-neutralizing antibody titers.

Natural viral infections commonly lead to the production of protective neutralizing antibodies recognizing both linear and conformational epitopes of viruses. A screening study based on ELISA has shown that human sera with high titers of neutralizing antibodies against EV71 strain MS/7423/87 reacted strongly with neutralizing conformational epitopes located at the N-terminal region of VP1 but exact locations of these epitopes were not well-defined [24]. The *in vivo* protective efficacy of these neutralizing antibodies has not been established as well. The neutralizing linear VP1 epitope (SP70) we have previously identified was located more towards the C-terminal part of the protein and mapped in the region involving VP1 dimerization [18]. In this study, we have demonstrated that the mouse antisera raised against this neutralizing linear VP1 epitope were able to confer good *in vivo* passive protection to suckling Balb/c mice against EV71 infectivity.

Antisera with a neutralizing antibody titer of 1:64 from mice immunized with formalin-inactivated EV71 strain Tainan/5079/98 were able to confer 65% passive protection to suckling ICR mice following challenge with the same viral strain [16]. Subsequently, another study reported that the passive administration of antisera raised against EV71 whole virions via the intraperitoneal route also offered 70% protection to newborn ICR mice against EV71 infectivity [17]. Although a number of immunogens with good *in vivo* passive protective potential have been identified, the majority of candidates that have been tested experimentally relied mainly on whole viral particles. In our study, the mouse antisera which were raised against EV71 whole virions did provide higher *in vivo* passive protection to suckling mice against lethal EV71 challenge when compared with the anti-SP70 antisera. This might be due to higher titers of neutralizing antibodies elicited by several neutralizing epitopes located on the virus other than that represented by the synthetic peptide SP70 alone. Unlike SP70, the immunogenic but non-neutralizing linear VP1 epitope represented by the synthetic peptide SP12 elicited antibodies which were unable to neutralize EV71 infectivity and hence infected suckling mice were not protected. The passively administered antisera raised against EV71 whole virions might also contain antibodies which could induce lysis of the virus-infected host cells through activation of complement cascades or by mediating antibody-dependent cellular cytoxicity (ADCC) activities. Conventional vaccine preparations using the whole virion as an inactivated vaccine might be ideal since it offered good protection against viral infection. However, the laborious and costly procedures involved in EV71 cultivation and purification as well as safety issues are of major concerns.

It is likely that different vaccine delivery systems can present the antigen differently. It is clear, however, that the delivery system must be considered carefully when assessing the immunogenicity and protective efficacy of any vaccine candidate. In a recent study, a vaccine strategy using live-attenuated *Salmonella enterica* serovar Typhimurium strains to express and deliver the VP1 protein of EV71 strain 2272 was shown to protect newborn ICR mice against infection caused by the same viral strain but immunization with live-attenuated vaccines has the problem of possible reversions to wild-type virulence [25]. The use of a synthetic peptide, however, has advantages of defined chemical composition, stability, safety and reduces the complexity of manufacturing. In this study, the synthetic peptide SP70 with its highly conserved amino acid sequence was able to elicit high titers of EV71-neutralizing antibodies that confer broad immunity against homologous and heterologous EV71 strains. Due to its small size, the synthetic peptide by itself may be poorly immunogenic. To increase the immunogenicity of a synthetic peptide, it could be linked to a larger carrier protein or by forming multiple antigenic peptides [26]. The use of either natural or artificial adjuvants has been found to enhance the immunogenicity of a synthetic peptide by promoting its uptake and activating dendritic cells (DCs) to initiate the immune response [27]. In the present study, the synthetic peptide SP70 was conjugated with a diphtheria toxoid which serves as a carrier protein and may help to prolong the half-life of SP70. The addition of a 50% emulsion of Freund's adjuvant to SP70 could have further enhanced its immunogenicity as well. The immunogenicity of a synthetic peptide could also be enhanced by designing a chimeric peptide construct incorporating both B-cell and T-cell epitopes. The neutralizing linear VP1 epitope represented by SP70 when linked to a T-helper cell epitope could be further explored as a chimeric peptide to raise antisera for *in vivo* protective efficacy evaluation.

In the absence of a vaccine for EV71, this study has demonstrated that the *in vivo* passive protection of suckling Balb/c mice by antisera raised against the synthetic peptide SP70 offers a potential therapeutic treatment for EV71 infections. The Fab region of the mouse anti-SP70 antibody which will recognize and bind to the neutralizing linear VP1 epitope can be selected as a potential candidate for humanized antibody production. Humanized monoclonal antibodies conferring good passive protection have been developed against the Venezuelan equine encephalomyelitis virus [28] and the respiratory syncytial virus [29]. A humanized monoclonal antibody which has specificity, avidity and neutralizing activity might be a viable treatment option against EV71 infection in humans.

Acknowledgements

Heterologous EV71 strains used in this study were gifts of Dr Katsumi Mizuta, Yamagata Prefectural Institute of Public Health, Yamagata, Japan. This investigation was supported by a Microbiology Vaccine Initiative Grant (R182-008-067-731) awarded to C.L. Poh.

References

[1] K. Mizuta, C. Abiko, T. Murata, Y. Matsuzaki, T. Itagaki, K. Sanjoh, M. Sakamoto, S. Hongo, S. Murayama, K. Hayasaka, Frequent

importation of enterovirus 71 from surrounding countries into the local community of Yamagata, Japan, between 1998 and 2003, J. Clin. Microbiol. 43 (2005) 6171–6175.
[2] K.T. Wong, L.C. Lum, S.K. Lam, Enterovirus 71 infection and neurologic complications, N. Engl. J. Med 342 (2000) 356–358.
[3] L.C. Lum, K.T. Wong, S.K. Lam, K.B. Chua, A.Y. Goh, W.L. Lim, B.B. Ong, G. Paul, S. AbuBakar, M. Lambert, Fatal enterovirus 71 encephalomyelitis, J. Pediatr. 133 (1998) 795–798.
[4] L.Y. Chang, C.Y. Lee, C.L. Kao, T.Y. Fang, C.Y. Lu, P.I. Lee, L.M. Huang, Hand, foot and mouth disease complicated with central nervous system involvement in Taiwan in 1980–1981, J. Formos. Med. Assoc. 106 (2007) 173–176.
[5] P.C. McMinn, An overview of the evolution of enterovirus 71 and its clinical and public health significance, FEMS. Microbiol. Rev. 26 (2002) 91–107.
[6] L.Y. Chang, T.Y. Lin, Y.C. Huang, Fulminant neurogenic pulmonary oedema with hand, foot and mouth disease, Lancet 352 (1998) 367–368.
[7] M. Ho, E.R. Chen, K.H. Hsu, S.J. Twu, K.T. Chen, S.F. Tsai, J.R. Wang, S.R. Shih, An epidemic of enterovirus 71 infection in Taiwan, N. Engl. J. Med. 341 (1999) 929–935.
[8] S.M. Wang, C.C. Liu, H.W. Tseng, J.R. Wang, C.C. Huang, Y.J. Chen, Y.J. Yang, S.J. Lin, T.F. Yeh, Clinical spectrum of enterovirus 71 infection in children in southern Taiwan, with an emphasis on neurological complications, Clin. Infect. Dis. 29 (1999) 184–190.
[9] M.G. Mateu, Antibody recognition of picornaviruses and escape from neutralization: a structural view, Virus Res. 38 (1995) 1–24.
[10] R.R. Ruekert, in: B.N. Fields, D.M. Knipe, P.M. Howley, et al. (Eds.), Picornaviridae: The Viruses and Their Replication, third ed., Fields Virology Lippincott-Raven Publishers, Philadelphia, 1996, pp. 609–640.
[11] C. Mendelsohn, E. Wimmer, V.R. Racaniello, Cellular receptors for poliovirus: molecular cloning, nucleotide sequence and expression of a new member of the immunoglobulin super family, Cell 56 (1989) 855–865.
[12] J.M. Bergelson, M.P. Sepley, B.M.C. Chan, M.E. Heinler, R.N. Finberg, Identification of the antigen VLA-2 as a receptor for echovirus 1, Science 255 (1992) 1718–1720.
[13] J.M. Greve, G. Davis, A.M. Meyer, C.P. Forte, S.C. Yost, C.W. Marlor, M.E. Kamarck, A. McClelland, The major human rhinovirus receptor is ICAM-1, Cell 56 (1989) 839–847.
[14] A.L. Van Wezel, P. Van der Marel, T.G. Hazendonk, V. Boer Bak, M.A. Henneke, Antigenicity and immunogenicity of poliovirus capsid proteins, Dev. Biol. Stand. 55 (1983) 209–215.
[15] D.M. Moore, K.M. Cowan, Effect of trypsin and chymotrypsin on the polypeptides of large and small plaque variants of foot-and-mouth disease virus: relationship to specific antigenicity and infectivity, J. Gen. Virol. 41 (1978) 549–562.
[16] C.K. Yu, C.C. Cheng, C.L. Cheng, J.R. Wang, C.C. Liu, J.J. Yan, I.J. Su, Neutralizing antibody provided protection against enterovirus type 71 lethal challenge in neonatal mice, J. Biomed. Sci. 7 (2000) 523–528.
[17] C.N. Wu, Y.C. Lin, C. Fann, N.S. Liao, S.R. Shih, M.S. Ho, Protection against enterovirus 71 infection in newborn mice by passive immunization with subunit VP1 vaccines and inactivated virus, Vaccine 20 (2001) 895–904.
[18] D.G.W. Foo, S. Alonso, M.C. Phoon, N.P. Ramachandran, V.T.K. Chow, C.L. Poh, Identification of neutralizing linear epitopes from the VP1 capsid protein of enterovirus 71 using synthetic peptides, Virus Res. 125 (2007) 61–68.
[19] L.J. Reed, H. Muench, A simple method of estimating fifty percent endpoints, Am. J. Hyg. 27 (1938) 4931–4937.
[20] E.L. Tan, V.T.K. Chow, G. Kumarasinghe, R.P.L. Tze, I.M. MacKay, T.P. Sloots, C.L. Poh, Specific detection of enterovirus 71 directly from clinical specimens using real-time RT-PCR hybridization probe assay, Mol. Cell. Probes 20 (2006) 135–140.
[21] J. Kimura-Kuroda, K. Yasui, Protection of mice against Japanese encephalitis virus by passive administration with monoclonal antibodies, J. Immunol. 141 (1988) 3606–3610.
[22] Y.C. Huang, T.Y. Lin, Y.J. Lin, R.I. Lien, Y.H. Chou, Prophylaxis of intravenous immunoglobulin and acyclovir in perinatal varicella, Eur. J. Pediatr. 160 (2001) 91–94.
[23] T.J. Geller, D. Condie, A case of protracted coxsackie virus meningoencephalitis in a marginally immunodeficient child treated successfully with intravenous immunoglobulin, J. Neurol. Sci. 129 (1995) 131–133.
[24] C.S. Tan, M.J. Cardosa, High-titred neutralizing antibodies to human enterovirus 71 preferentially bind to the N-terminal portion of the capsid protein VP1, Arch. Virol. (2007).
[25] C.H. Chiu, C. Chu, C.C. He, T.Y. Lin, Protection of neonatal mice from lethal enterovirus 71 infection by maternal immunization with attenuated *Salmonella enterica* serovar Typhimurium expressing VP1 of enterovirus 71, Microbes Infect. 8 (2006) 1–8.
[26] I. Haro, M.J. Gomara, Design of synthetic peptide constructs for the vaccine development against viral infections, Curr. Protein Pept. Sci. 5 (2004) 425–433.
[27] K.S. Rosenthal, D.H. Zimmerman, Vaccines: all things considered, Clin. Vaccine Immunol. 13 (2006) 821–829.
[28] A.R. Hunt, S. Frederickson, C. Hinkel, K.S. Bowdish, J.T. Roehrig, A humanized murine monoclonal antibody protects mice either before or after challenge with virulent Venezuelan equine encephalomyelitis virus, J. Gen. Virol. 87 (2006) 2467–2476.
[29] H. Fuller, C. Del Mar, Immunoglobulin treatment for respiratory syncytial virus infection, Cochrane Database Syst. Rev. 4 (2006) CD004883.

 10.1111/j.1469-0691.2007.01904.x

Identification of immunodominant VP1 linear epitope of enterovirus 71 (EV71) using synthetic peptides for detecting human anti-EV71 IgG antibodies in western blots

D. G. W. Foo[1], R. X. Ang[1], S. Alonso[1], V. T. K. Chow[1], S. H. Quak[2] and C. L. Poh[1,3]

[1]Department of Microbiology, Yong Loo Lin School of Medicine, National University of Singapore, [2]Department of Pediatrics, Children's Medical Institute, National University Hospital, Singapore and [3]Faculty of Life and Social Sciences, Swinburne University of Technology, Australia

ABSTRACT

A major IgG-specific immunodominant VP1 linear epitope of enterovirus 71 (EV71) strain 41 (5865/SIN/00009), defined by the core sequence LEGTTNPNG, was identified by Pepscan analysis. Oligonucleotides corresponding to the amino-acid sequence of synthetic peptide SP32 were cloned and over-expressed in *Escherichia coli* as a recombinant glutathione-*S*-transferase (GST)–SP32 fusion protein. In ELISAs, this protein did not react with human anti-EV71 IgG antibodies, but there was significant immunoreactivity according to western blot analysis. The amino-acid sequence of SP32 was highly specific for detecting EV71 strains in western blot analysis, and showed no immunoreactivity with monoclonal antibodies raised against other enteroviruses, e.g., CA9 and Echo 6.

Keywords Detection, enterovirus 71, immunodominant linear epitope, pepscan analysis, recombinant GST–SP32 fusion protein, synthetic peptide

Original Submission: 11 May 2007; **Revised Submission**: 23 July 2007; **Accepted**: 28 September 2007

Clin Microbiol Infect

Enterovirus 71 (EV71) is a single positive-stranded RNA virus [1], with infection often manifesting as hand, foot and mouth disease [2], characterised by ulcerating vesicles on the palate and vesicular lesions on the hands, feet and buttocks. The usual method for detecting EV71 in any clinical specimen involves viral isolation, followed by serotyping with serotype-specific antibodies and indirect immunofluorescence assays. An IgM-based ELISA for EV71 diagnosis has been reported to have high sensitivity and specificity. However, this approach is too laborious, as it involves the use of purified whole EV71 virions for detecting anti-EV71 antibodies [3–5]. Synthetic peptides corresponding to the VP1 protein of enteroviruses are broadly reactive in an IgG-based ELISA using sera from patients infected with enteroviruses, and this approach has the potential for serodiagnosis of a wide range of enterovirus serotypes [6]. To detect an enterovirus of unknown serotype, an assay with broad reactivity would be useful, but this would not help to identify a specific enterovirus.

In the present study, 95 overlapping synthetic peptides, designated SP1–SP95, that spanned the entire VP1 sequence of EV71 strain 41 (5865/SIN/00009) (GenBank accession no. AF316321) were synthesised by Mimotopes (Clayton, Australia). EV71 was propagated in RD cells using minimum essential medium (Gibco, Rockville, MD, USA), based on cytopathic effects produced as a result of virus infection. Serum samples were collected from children attending a paediatric outpatient clinic at the National University Hospital of Singapore, and these were screened for the presence of anti-EV71-neutralising antibodies using an in-vitro microneutralisation assay. A serum-neutralising titre of ≥1:8 was considered to be positive for EV71 exposure. Sera containing anti-EV71-neutralising antibodies (n = 40) were pooled and designated as EV71-positive sera. Sera without anti-EV71-neutralising antibodies (n = 40) were pooled and designated as EV71-negative sera. For assigning linear epitope(s) in ELISAs, the mean OD obtained from EV71-negative sera plus twice the standard deviation of the respective mean was used as the cut-off value.

Two IgG-specific immunoreactive VP1 linear regions were identified, designated as sites I and II. The first region, represented by synthetic peptides SP14 and SP15 (amino-acid residues 40–57), was not specific for detecting

Corresponding author and reprint requests: C. L. Poh, Faculty of Life and Social Sciences, Swinburne University of Technology, John Street, Hawthorn, Victoria 3122, Australia
E-mail: cpoh@groupwise.swin.edu.au

human anti-EV71 IgG, as both peptides also reacted with EV71-negative sera, perhaps because of the presence of non-specific IgG elicited previously against other enteroviruses that might harbour similar antigenic determinants. The second immunoreactive region was represented by synthetic peptides SP31, SP32 and SP33 (amino-acid residues 91–111), and these were highly specific for detecting human anti-EV71 IgG. This region, defined by the core sequence LEGTTNPNG, represents the major immunodominant VP1 linear epitope of EV71. When SP32 (DLPLEGTTNPNGYAN) was tested against EV71-positive sera, there was significant immunoreactivity towards SP32, which contained the core sequence.

The VP1 protein of the prototype EV71 BrCr strain (GenBank accession no. U00871), when produced as a recombinant His–VP1 fusion protein, was highly insoluble and therefore required denaturation and renaturation before it could be used as a capture antigen in western blots [7]. This insolubility could be attributed to several hydrophobic transmembrane regions within VP1. When a smaller peptide without these regions was used, the immunodominant VP1 linear epitope represented by SP32 was over-expressed as a soluble recombinant glutathione-*S*-transferase (GST)–SP32 fusion protein. Based on the Kyte and Doolittle hydrophobicity profile [8], this region (SP32) was shown to be mainly hydrophilic. Using a protein–protein BLAST search (blastp v.2.2.14; http://www.ncbi.nlm.nih.gov), the amino-acid sequence of SP32 was revealed to be highly homologous to EV71 strains, indicating its probable high sensitivity as a capture antigen for detecting human anti-EV71 IgG. Hence, a pair of positive- and negative-sense oligonucleotides was designed, based on the nucleotide sequence of SP32 (nucleotides 280–324 of the *VP1* gene). For annealing, both oligonucleotides were heated at 90°C for 3 min, and then incubated at 37°C for 15 min. Primers flanking the *VP1* gene (nucleotides 2442–3332 of the complete genome of EV71 strain 41) were also constructed: GST–VP1F, 5′-TGGATCCGGAGATAGAGTGGCAG (the *Bam*H1 restriction site is underlined) and GST–VP1R, 5′-GCCGAAGTCGACTCAAAGGG TAG (the *Sal*I restriction site is underlined). The vector pGEX-6p-1 (GE Healthcare Life Sciences, Little Chalfont, UK) was used to construct recombinant GST–SP32 and GST–VP1 fusion proteins, following the procedure recommended by the manufacturer.

The efficacy of the recombinant GST–SP32 fusion protein as a capture antigen for human anti-EV71 IgG was evaluated in IgG-based ELISA and western blot assays. No significant immunoreactivity was detected by ELISA, but because of its native state, the antigenic site represented by SP32 could have been masked by the larger GST protein moiety, thereby hindering antigen–antibody interactions. However, the fusion protein was immunoreactive against human anti-EV71 IgG in western blots, probably because of its denatured state, in which the steric hindrance caused by the GST protein would have been removed, thereby exposing the immunogenic region represented by SP32. Before being used as a capture antigen, EV71 was heat-inactivated at 56°C and the amount of viral protein was quantified using the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Both the recombinant GST–VP1 fusion protein and the whole EV71 virion were non-specific when used as capture antigens in western blots for detecting human anti-EV71 IgG. This was attributed to the presence of numerous common linear epitopes among enteroviruses [6,9–11]. It is believed that the cross-reactivity may be attributed to human anti-poliovirus IgG, present in most paediatric patients as a result of the national childhood immunisation programme.

In conclusion, the recombinant GST–SP32 fusion protein, harbouring the immunodominant VP1 linear epitope of EV71, demonstrated high specificity and sensitivity, and was a better capture antigen in western blots than the recombinant GST–VP1 fusion protein and the whole EV71 virion. This suggests that the recombinant GST–SP32 fusion protein could be further developed as a serological reagent for the detection of human anti-EV71 IgG, and could be used as a tool for identifying EV71 infection and as a confirmatory assay for recognising previous EV71 exposure.

ACKNOWLEDGEMENTS

The authors are grateful to J. X. J. Chua for her administrative work and experimental help with the ELISA screenings. This research was supported by Microbiology Vaccine Initiative (NUS) grant number R182-008-067-731 awarded to C. L. Poh. No information has been provided by the authors concerning the existence or absence of conflicting or dual interests.

REFERENCES

1. Palacios G, Oberste MS. Enterovirus as agents of emerging infectious diseases. *J NeuroVirol* 2005; **11**: 424–433.
2. Ho M, Chen ER, Hsu KH *et al*. An epidemic of enterovirus infection in Taiwan. *N Engl J Med* 1999; **341**: 929–935.
3. Tsao KC, Chang PY, Ning HC *et al*. Use of molecular assay in diagnosis of hand, foot and mouth disease caused by enterovirus 71 or coxsackievirus A 16. *J Virol Meth* 2002; **102**: 9–14.
4. Tano Y, Shimizu H, Shiomi M, Nakano T, Miyamura T. Rapid serological diagnosis of enterovirus 71 infection by IgM ELISA. *Jpn J Infect Dis* 2002; **55**: 133–135.
5. Wang SY, Lin TL, Chen HY, Lin TS. Early and rapid detection of enterovirus 71 infection by an IgM-capture ELISA. *J Virol Meth* 2004; **119**: 37–43.
6. Cello J, Samuelson A, Stalhandske P *et al*. Identification of group-common linear epitopes in structural and nonstructural proteins of enteroviruses by using synthetic peptides. *J Clin Microbiol* 1993; **31**: 911–916.
7. Shih SR, Li YS, Chiou CC *et al*. Expression of capsid protein VP1 for use as antigen for the diagnosis of enterovirus 71 infection. *J Med Virol* 2000; **61**: 228–234.
8. Foo DG, Alonso S, Phoon MC *et al*. Identification of neutralizing linear epitopes from the VP1 capsid protein of enterovirus 71 using synthetic peptides. *Virus Res* 2007; **125**: 61–68.
9. Samuelson A, Forsgren M, Johansson B, Wahren B, Sallberg M. Molecular basis for serological cross-reactivity between enteroviruses. *Clin Diagn Lab Immunol* 1994; **1**: 336–341.
10. Mertens TH, Pika U, Eggers HJ. Cross antigenicity among enteroviruses as revealed by immunoblot technique. *Virology* 1983; **129**: 431–442.
11. Samuelson A, Cello J, Skoog E *et al*. Enterovirus IgG ELISA using synthetic peptides as antigens. *Serodiagn Immunother Infect Dis* 1993; **5**: 93–96.

VIRAL IMMUNOLOGY
Volume 21, Number 2, 2008

Pp. 000–000
DOI: 10.1089/vim.2007.0089

Identification of Human CD4$^+$ T-Cell Epitopes on the VP1 Capsid Protein of Enterovirus 71

DAMIAN GUANG WEI FOO,[1] PAUL A. MACARY,[1] SYLVIE ALONSO,[1] and CHIT LAA POH[2]

ABSTRACT

The identification of human CD4$^+$ T-cell epitopes within a protein vaccine candidate is of great interest, as it provides a better understanding of the mechanisms involved in protective immunity and may therefore help in the design of effective vaccines and diagnostic tools. The entire amino acid sequence of the VP1 capsid protein from enterovirus 71 (EV71) strain 41 was submitted to analysis by the ProPred algorithm for the identification of potential promiscuous human CD4$^+$ T-cell epitopes. Three regions spanning amino acids 66–77, 145–159, and 247–261 of VP1 were predicted to bind more than 25 HLA-DR alleles. The corresponding synthetic peptides (SP1 to SP3) were then tested for their abilities to induce proliferation of CD4$^+$ T cells isolated from five human volunteers screened positive for previous EV71 exposure and one EV71-negative volunteer. Upon stimulation with either peptide, CD4$^+$ T-cell proliferative responses were observed for all EV71-positive volunteers, indicating the presence of EV71-specific memory CD4$^+$ T cells. The amplitude of the proliferative responses was peptide- and HLA-DR–dependent, and correlated well with the ProPred predicted binding efficiencies. Moreover, CD4$^+$ T cells from EV71-positive volunteers produced significant levels of IL-2 and IFN-γ upon stimulation, indicative of a T-cell differentiation into Th-1-type subset. Among the three peptides, SP2 induced the highest proliferative response and cytokine production. Moreover, SP2-induced proliferative response could be inhibited with anti–major histocompatibility complex (MHC) class II antibody, indicating that SP2 represents a MHC class II–restricted CD4$^+$ T-cell epitope. This study demonstrates that the ProPred algorithm can accurately predict the presence of human CD4$^+$ T-cell epitopes within the VP1 capsid protein of EV71, and therefore represents a useful tool for the design of subunit vaccines against EV71.

INTRODUCTION

Enterovirus 71 (EV71) is an emerging agent of infectious disease and belongs to the enterovirus A species (33). In 1969, EV71 was first isolated in California (37), and its association with hand, foot, and mouth disease (HFMD) was made in 1973 during the small epidemics in Sweden (5) and Japan (19). In 1975, the neurovirulence of EV71 was first reported in Bulgaria, where 44 people died of a polio-like disease (10). EV71 epidemics with neurological, cardiac, and/or pulmonary complications subsequently occurred worldwide, with a particular surge in the Asia-Pacific region during the last 10 years (36).

[1]Department of Microbiology and Immunology Programme, Centre for Life Sciences, National University of Singapore, Singapore.
[2]Faculty of Life and Social Sciences, Swinburne University of Technology, John Street, Hawthorn, Victoria, Australia.

The current strategy for preventing EV71 infection relies on public health surveillance and good hygiene practices. Presently, there is no specific drug against EV71 and treatments remain symptomatic only, providing relief from fever and pain. The "WIN" group of compounds is the most promising drug class so far, but it is rather ineffective in disease regression (2). Other anti-viral agents are currently being developed, including synthetic compounds like pyridazinyl oxime ethers (3) and the use of RNA interference (RNAi) technology (26). Natural products like allophycocyanin (38) and lactoferrin (25) have also been shown to exhibit anti-EV71 properties. Therapeutic antibodies against EV71 might be an interesting alternative to conventional drug treatments. We have recently described anti-EV71 neutralizing antibodies capable of effectively protecting suckling mice against homologous and heterologous EV71 challenges (14). However, a major concern with such therapeutic treatments is that they may be administered too late to the patient, after the virus has caused substantial and irreversible damage. Hence, the need for an effective vaccine against EV71 is urgent.

Natural viral infections usually lead to the production of protective neutralizing antibodies. Inactivated whole virions and subunit vaccines have been reported to effectively protect suckling mice against EV71 infection in passive experiments (14,46,47) or through maternally-transferred antibodies (9,46). In particular, the VP1 capsid protein has been shown to trigger the production of protective antibodies against EV71, highlighting VP1 as a promising vaccine candidate (8,9,14,46). In a recent study, the cord sera from 205 individuals were tested for the presence of anti-EV71 neutralizing antibodies, and the authors described that the N-terminal moiety of VP1 is likely to harbor EV71-neutralizing conformational epitopes (41). However, the mapping and the characterization of these regions have not been carried out.

Here we have identified three regions on the VP1 capsid protein of enterovirus 71 strain 41 (5865/SIN/00009) capable of inducing human EV71-specific $CD4^+$ T-cell proliferation. $CD4^+$ T cells play a crucial and pivotal role in adaptive immunity, for the development of an antibody-based immunity (11,12,40), as well as cytotoxic T-cell responses (17,22,32). Therefore the identification of $CD4^+$ T-cell epitopes is of great interest and might help to design more effective subunit vaccines.

MATERIALS AND METHODS

T-cell epitope prediction

The VP1 amino acid sequence of enterovirus 71 strain 41 (5865/SIN/00009) (Genbank accession no. AF316321) was loaded into the ProPred prediction software using the server (http://www.imtech.res.in/raghava/propred) to predict promiscuous epitopes. This server analyzes protein regions for their ability to bind to a total of 51 alleles belonging to 9 serologically defined HLA-DR molecules that are encoded by DRB1 and DRB5 genes. The quantitative matrices used in this prediction method include HLA-DR1 (2 alleles), HLA-DR3 (7 alleles), HLA-DR4 (9 alleles), HLA-DR7 (2 alleles), HLA-DR8 (6 alleles), HLA-DR11 (9 alleles), HLA-DR13 (11 alleles), HLA-DR15 (3 alleles), and HLA-DR51 (2 alleles). The server analyzed the amino acid sequence using each of the alleles independently and computed the binding strength of all the peptides. The prediction threshold was set at 3% and peptides predicted to bind at least 50% of the major histocompatibility complex (MHC) class II molecules were considered promiscuous for binding.

Virus propagation

Enterovirus 71 strain 41 (belonging to genogroup B4 and isolated from a fatal HFMD case in Singapore) was propagated in rhabdomyosarcoma (RD) cells as described previously (15). The cultured virus was purified with 7% polyethylene glycol 8000 followed by 30% sucrose gradient (15). The virus titer was determined as 50% tissue culture infective doses ($TCID_{50}$) in RD cells based on a typical cytopathic effect as a result of viral infection. Infected cells were seen to cluster, shrank and became pyknotic, and eventually degenerated and fell off of the surface (24) The virus was inactivated by heating at 56°C for 30 min, and the amount of viral protein was quantified by the Bradford assay (Bio-Rad Laboratories, California, USA) according to the manufacturer's instructions.

Peptide synthesis

Synthetic peptides (SP1 to SP4) were synthesized by Mimotopes Pty Ltd (Clayton, Victoria, Australia). Peptides were >90% in purity as assessed by high-performance liquid chromatography and were used without further purification. Approximately 1.7 mg of the peptide was dissolved in 340 μL dimethylsulfoxide (Sigma Chemical Co., St. Louis, USA) to obtain a 5 mM working concentration. For computational analysis, the amino acid sequences of the synthetic peptides were used as query sequences in a protein-protein BLAST (blastp 2.2.17) search using the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/BLAST).

HLA-DR typing

Blood samples from 20 volunteers of both genders, 25–31 y of age, were collected in tubes containing 4%

sodium citrate as an anticoagulant. Genomic and serological typing was performed by the WHO Immunology and Training Research Centre, Singapore, as a kind gift from the director, Professor Chan SH.

In vitro *microneutralization assay*

Human anti-EV71 neutralizing antibodies were identified using an *in vitro* microneutralization assay with RD cells as described previously (15). Human serum samples were incubated at 56°C for 30 min to heat-inactivate the complement before the assay. The assay was carried out in triplicate.

Isolation of peripheral blood mononuclear cells

Blood cells were subjected to separation by Ficoll-Hypaque gradient centrifugation at 400 g for 40 min. The buffy coats thus separated were diluted with phosphate-buffered saline (pH 7.2), supplemented with 2 mM EDTA (PBS/EDTA), and centrifuged at 300 g for 10 min. For removal of platelets, resuspended cells were centrifuged at 200 g for 15 min and the washing step was repeated once. After washing, peripheral blood mononuclear cells (PBMCs) were counted prior to magnetic labeling.

Culture and activation of monocyte-derived dendritic cells

PBMCs were resuspended in 80 μL of PBS/EDTA per 10^7 total cells, followed by incubation with CD14 MicroBeads (Miltenyi Biotec Inc., California, USA) for 15 min at 4°C. The suspension was then centrifuged at 300 g for 10 min and the pellet was resuspended at 10^8 cells in 500 μL of PBS/EDTA, followed by magnetic separation using LS columns (Miltenyi Biotec Inc., California, USA). Briefly, cell suspensions were applied to the top of the column and the unbound cells were passed through with 3×3 mL of PBS/EDTA supplemented with 0.5% bovine serum albumin (column buffer). CD14$^+$ cells were eluted with 5 mL column buffer. The cells were cultured for 6 d at 37°C in RPMI 1640 media supplemented with human interleukin 4 (IL-4) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (each at 20 ng/mL) to generate immature DCs (imDCs). To activate the imDCs, the cells were stimulated with peptides or EV71 whole virions (20 μg/mL) in the presence of LPS (0.5 μg/mL). When indicated, monoclonal anti-human MHC class II (HLA-DP, -DQ, and -DR) antibodies (Ancell Co., Minnesota, USA) were also added at 20 μg/mL.

T-cell proliferation assay

PBMCs were resuspended in 80 μL of PBS/EDTA per 10^7 total cells followed by incubation with CD4 MicroBeads (Miltenyi Biotec Inc.) for 15 min at 4°C. The isolation steps are similar to those for isolation of DCs as described above. CD4$^+$ T cells were resuspended in AIM-V® media (Invitrogen, Carlsbad, CA) supplemented with 10% autologous serum and were incubated in flat-bottomed 96-well plates for 7 d at 37°C in 5% CO_2 in air in the presence or absence of stimulated or non-stimulated DCs. The stimulator:responder cell ratio was 1:10. At 24 h before the end of incubation, the cells were pulsed with 0.5 μCi per well of [methyl-^{3}H] thymidine. After washing, the cells were harvested and counted on a liquid scintillation counter. Their radioactivity was expressed as the mean counts per min (cpm) of triplicate determinations. The stimulation index (SI) was calculated as the mean number of counts of cell-associated [^{3}H] thymidine per minute recovered from wells containing activated DCs divided by the mean number of counts of cell-associated [^{3}H] thymidine per minute recovered from wells containing imDCs.

Cytokine analysis

CD4$^+$ T-cell culture supernatants were collected at 96 h for cytokine measurement using the Bio-Plex™ suspension array system (Bio-Rad Laboratories) according to the manufacturer's instructions. The multiplex suspension bead array immunoassay was performed using the human 6-Plex kit (Bio-Rad Laboratories) to specifically evaluate cytokines of Th-1-type (IL-2 and IFN-γ) and Th-2-type (IL-4 and IL-10) subsets according to the manufacturer's protocol. The assay was carried out in triplicate.

RESULTS

Prediction of HLA-DR-restricted epitopes

Computer-generated algorithms that predict MHC class II binding peptides are based on the identification of HLA-DR allele-specific motifs in antigenic peptides and on the structural definition of HLA-peptide complexes. The primary amino acid sequence of the VP1 capsid protein of EV71 strain 41 was analyzed using the T-cell epitope prediction ProPred algorithm, which led to the identification of promiscuous regions potentially binding to HLA-DR molecules. Three regions (I, II, and III) were predicted to bind more than 50% of the 51 HLA-DR alleles available in the ProPred database, and were therefore considered as promiscuous CD4$^+$ T-cell epitopes by the program (Fig. 1). These regions map at amino acids 66–77, 145–159, and 247–261 of the VP1 protein (Table 1). The corresponding peptides, namely SP1, SP2, and SP3, respectively, were synthesized, as well as a fourth peptide SP4 (scrambled), representing a VP1 region with poor predictability for promiscuous

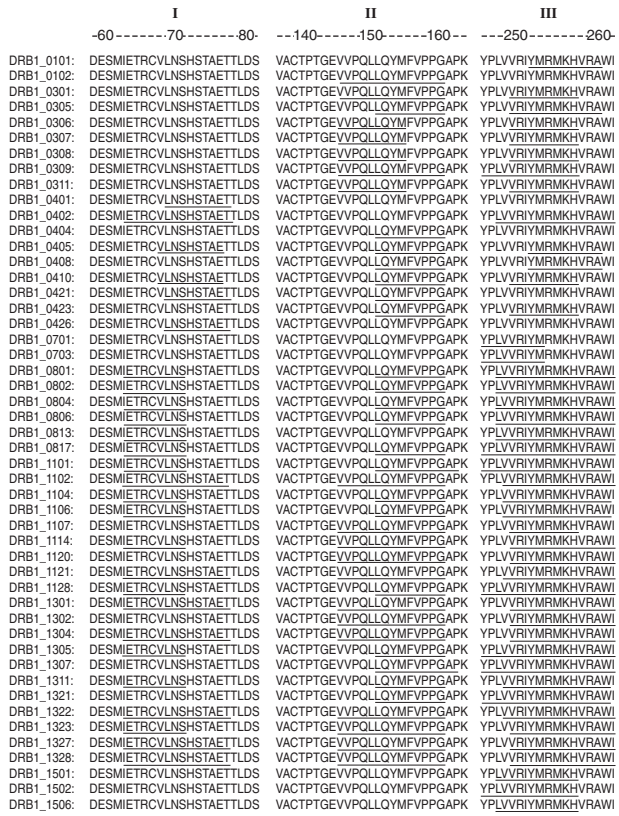


FIG. 1. An output of ProPred analysis of the VP1 amino acid sequence in HTML view II. The sequence was analyzed for binding to 51 HLA-DR alleles using the ProPred program at the default setting (threshold value of 3%). The peptide sequences predicted to bind more than 50% of the HLA-DR alleles available in the database are underlined.

binding (Table 1). A protein-protein BLAST (blastp 2.2.17) search indicated that the amino acid sequences of all four peptides were highly specific for EV71 strains and no significant homology with other enteroviruses was found (data not shown).

HLA-DR typing and detection of anti-EV71 antibodies in human volunteers

Of the 20 human volunteers recruited for this study, only six displayed HLA-DR alleles that were represented in the ProPred database. Among these six volunteers, five were sero-diagnosed positive for the presence of anti-EV71 neutralizing antibodies, with a neutralizing titer of 1:8 and above (Table 2). One volunteer was identified as EV71-negative (Table 2). All six volunteers were found to be negative for EV71 by cultural analysis, indicating that none of them were currently infected with EV71 (data not shown). In addition, Western blot analysis was performed using a recombinant GST-VP1 fusion protein as capture antigen to detect anti-VP1 IgG antibodies in the serum from each volunteer. As expected, a positive signal was observed when using serum samples from the five EV71-positive volunteers, whereas no signal was detected in the EV71-negative volunteer, demonstrating the presence of circulating anti-VP1 IgG antibodies in the EV71-positive volunteers, and confirming results of previous studies that described VP1 as a highly immunogenic antigen (8,9,14,15,46,47) (data not shown).

$CD4^+$ T-cell proliferative responses

$CD4^+$ T cells isolated from the EV71-positive and EV71-negative volunteers were stimulated with VP1-derived peptides (SP1–SP4) or with heat-inactivated EV71 whole virions using autologous monocyte-derived dendritic cells as professional antigen-presenting cells (APCs). The cellular proliferative responses were expressed as a stimulation index (SI), which corresponds to the ratio of the counts per minute (cpm) in the presence of antigen over the cpm without any antigenic stimulus. As expected, for the five EV71-positive volunteers, elevated $CD4^+$ T-cell proliferative responses were observed upon stimulation with EV71 whole virions (Fig. 2). However, stimulation with SP4 (scrambled peptide)

Table 1. Sequences and Locations Within VP1 of Predicted Promiscuous $CD4^+$T-Cell Epitopes

Peptide	*Predicted region*	*VP1 amino acid sequence*	*Position*
SP1	I	H-IETRCVLNSHSTAET-OH	66–77
SP2	II	H-EVVPQLLQYMFVPPG-OH	145–159
SP3	III	H-LVVRIYMRMKHVRAW-OH	247–261
SP4	NA	H-PTGQNTQVSSHRLDT-OH	27–41

[a]Peptides (SP1 to SP3) representing VP1 regions considered promiscuous for binding more than 25 different HLA-DR alleles. Peptide SP4 represents a scrambled peptide with poor predictability of promiscuous binding.

Abbreviation: NA, not applicable.

TABLE 2. EV71 EXPOSURE OF VOLUNTEERS AND PREDICTED PEPTIDE BINDING EFFICIENCIES

Volunteer	*HLA genotype*	*Serum anti-EV71 neutralizing antibody titer*[a]	*Anti-VP1 IgG antibody*[b]	*Predicted binding efficiency (%)*[c]			
				SP1	*SP2*	*SP3*	*SP4*
1	DRB1*0301	1:128	+	0	93	60	0
2	DRB1*0301	1:64	+	0	93	60	0
3	DRB1*0301	>1:256	+	0	93	60	0
4	DRB1*0405	>1:256	+	60	60	60	0
5	DRB1*1301	1:64	+	93	100	86	0
6	DRB1*0301	—	−	0	93	60	0

[a]Serum anti-EV71 neutralizing titer was determined using an *in vitro* microneutralization assay.

[b]Immunoblots using recombinant GST-VP1 fusion protein as capture antigen and 150 diluted serum sample from each volunteer as primary antibody. Plus signs represent the presence of a 58.7-kDa band corresponding to the GST-VP1 fusion protein, indicating the presence of specific anti-VP1 IgG antibody.

[c]Predicted binding efficiency of peptides based on a specific HLA-DR allele.

did not result in any significant proliferative response regardless of the HLA-DR allele tested. A weak but detectable CD4$^+$ T-cell proliferative response was observed for the EV71-negative volunteer upon stimulation with EV71 whole virions. CD4$^+$ T cells isolated from the EV71-positive volunteers proliferated significantly and with similar amplitudes upon stimulation with peptide SP2 (Fig. 2). However, the stimulation indices obtained upon stimulation with peptide SP1 or SP3 differ from one HLA-DR allele to another, and was either similar to or lower than the SI obtained upon SP2 stimulation. These data correlate with the ProPred analysis, which predicted for the five EV71-positive volunteers a higher binding efficiency for the VP1 region II (represented by SP2) as compared to regions I and III (represented by SP1 and SP3, respectively) (Table 2). Furthermore, stimulation with SP1 failed to induce significant CD4$^+$ T-cell proliferation for the EV71-positive volunteers with HLA genotype DRB1*0301 (volunteers 1–3), which again correlates with ProPred's predictions (Table 2). Similarly, the SI obtained upon SP3 stimulation correlated well with the ProPred analysis, which predicted that region III (corresponding to SP3 peptide) displays the highest binding efficiency for HLA genotype DRB1*1301. In addition, a background SI value was consistently obtained upon stimulation of CD4$^+$ T cells isolated from the EV71-negative volunteer with any of the stimulating antigens, indicating that the proliferative responses observed for volunteers 1–5 likely resulted from EV71-specific CD4$^+$ T cells (Fig. 2). Altogether, these data indicate that the ProPred program accurately predicted the binding efficiency for each peptide depending on the HLA-DR allele, and therefore represents a reliable approach to prediction and identification of potential human CD4$^+$ T-cell epitopes.

MHC class II-blocking experiment

To confirm that the observed CD4$^+$ T-cell proliferation was mediated by peptide presentation in association with MHC class II molecules, a MHC class II–blocking experiment was carried out using SP2 as the stimulating antigen. SP2 and anti-human MHC class II monoclonal antibodies were incubated with immature DCs (imDCs) from the five EV71-positive volunteers, and CD4$^+$ T-cell proliferative responses were measured as described above. The data showed that co-incubation with anti-

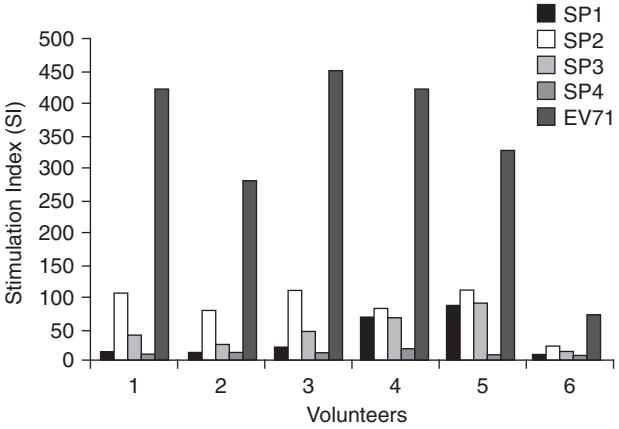


FIG. 2. Proliferation of CD4$^+$ T cells upon stimulation with peptides or EV71 whole virions. Heat-inactivated EV71 whole virions, peptides (SP1–SP4), or no antigen were first added to the monocyte-derived dendritic cells prepared from each volunteer before incubation with autologous CD4$^+$ T cells. [^{3}H] thymidine was added to the culture medium 24 h prior to counting. The stimulation index (SI) was calculated as the ratio of the mean cpm in the presence of activated DCs over the mean cpm in the presence of immature DCs (imDCs).

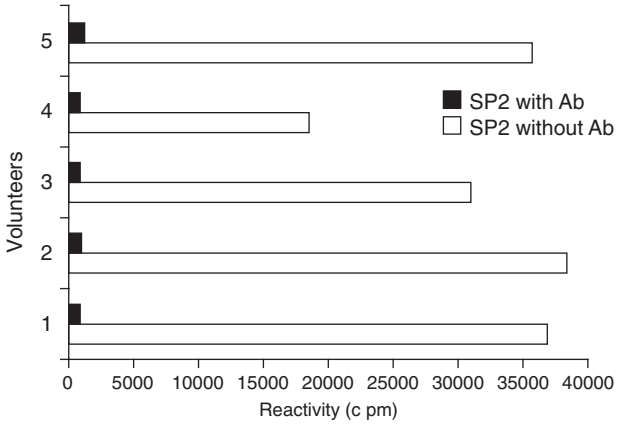


FIG. 3. Proliferation of SP2-stimulated $CD4^+$ T cells in the presence of anti-human MHC class II monoclonal antibodies. Peptide SP2 (20 μg/mL) was added to the monocyte-derived dendritic cells from each EV71-positive volunteer with or without MHC class II blocking antibodies (20 μg/mL). The mixtures were then incubated with autologous $CD4^+$ T cells and the stimulation index (SI) was calculated as previously described.

MHC class II antibodies resulted in a reduction of SP2-induced $CD4^+$ T-cell proliferation by more than 80% (Fig. 3). Therefore, this observation suggests that SP2 has to be presented by APCs in association with MHC class II molecules to effectively induce EV71-specific $CD4^+$ T-cell proliferation, thereby indicating that SP2 contains an MHC class II–restricted $CD4^+$ T-cell epitope.

Cytokine profile upon antigenic stimulation

To address whether the SP-induced stimulation of $CD4^+$ T-cells drives their differentiation into Th-1-type or Th-2-type subsets, the cytokine profile was determined. $CD4^+$ T cells from the six volunteers were co-incubated with autologous DCs primed with either peptides (SP1–SP4) or with heat-inactivated EV71 whole virions as described above, and the production of interleukins (IL)-2, IL-4, and IL-10, and interferon gamma (IFN-γ) was measured in the culture supernatants. Significant levels of IL-2 and IFN-γ were detected in the supernatant of the SP1-, SP2-, SP3-, and EV71-stimulated $CD4^+$ T cells from EV71-positive volunteers (Table 3). Similar to the proliferative responses, $CD4^+$ T cells from all EV71-positive volunteers secreted significantly ($p < 0.05$) higher levels of IL-2 and IFN-γ in response to SP2 as compared to SP1 and SP3. No significant differences in the production of IL-4 and IL-10 were observed upon stimulation with SP1–SP4 and in those with no antigenic stimulus (Table 3). Instead, significant but rather low levels of IL-4 were detected when using EV71 whole virions as stimulants for $CD4^+$ T cells from all EV71-positive, but also from EV71-negative, volunteers. Instead, a significant level of IL-10 was measured in EV71-stimulated $CD4^+$ T cells from EV71-positive volunteers only. Altogether, these data indicate that the levels of cytokine production correlate well with the $CD4^+$ T-cell proliferative responses, with SP2 triggering the highest cytokine production regardless of which HLA-DR allele is used. Significant production of IL-2 and IFN-γ by proliferating $CD4^+$ T cells also suggests their differentiation into a Th-1-type subset.

DISCUSSION

The host immune response developed upon any viral infection is primarily $CD4^+$ T-cell dependent, including the induction of a cytotoxic cellular response and the development of an efficient antibody response. Several studies of B-cell epitopes led to the identification of serotype- and group-common specific B-cell epitopes on the VP1 capsid protein of enteroviruses (6). The functional role of enterovirus-specific T cells, as well as the nature and specificity of their responses, have been less well-characterized so far. Although animal studies have been carried out to identify T-cell epitopes on four structural proteins (VP1–VP4) of enteroviruses (4,22,23,27,29,45), only a few studies based on the VP1 capsid protein have been carried out in humans (18,39).

Several approaches have been reported for the identification of T-cell epitopes. Overlapping synthetic peptides spanning the entire sequence of the protein candidate can be designed and assayed for their capability to activate $CD4^+$ T cells (1,35,43). However, such a systematic approach is costly, time-consuming, and tedious. Alternatively, computer programs can be employed to scan the protein of interest and predict regions likely to bind to MHC molecules. The discovery of MHC-binding motifs in proteins has led to the development of several algorithms based on the construction of a matrix of all possible amino acid side chain interactions for individual MHC-binding motifs (13,20). Such bioinformatics tools have been successfully employed to identify HLA-DR ligands derived from tumor antigens and endogenous proteins involved in autoimmune diseases (21,28). In addition, these programs have been reported to improve and accelerate the design of vaccines and diagnostic tests through the identification of promiscuous peptides in mycobacterial proteins (34,44).

In this study, the ProPred program was employed to predict HLA-DR binding ligands within the VP1 capsid protein of EV71 strain 41, since HLA-DR constitutes the dominant isotype of human MHC class II molecules (42). Activation of $CD4^+$ T cells is dependent upon the presentation of peptides by APCs in the context of MHC class II molecules. These peptides are generally approx-

TABLE 3. ANTIGEN-SPECIFIC CYTOKINE SECRETION BY STIMULATED CD4$^+$T CELLS

IL-2 (pg/mL)						
Volunteer	*SP1*	*SP2*	*SP3*	*SP4*	*EV71*	*No antigen*
1	4.8 (±1.9)	42.0 (±1.0)	13.9 (±3.1)	4.3 (±1.1)	102.5 (±2.2)	2.0 (±0.7)
2	6.2 (±2.9)	26.9 (±2.1)	20.3 (±1.5)	3.8 (±1.3)	107.7 (±2.8)	2.6 (±1.1)
3	3.3 (±1.3)	35.4 (±1.9)	19.8 (±1.6)	5.4 (±1.2)	106.2 (±3.0)	2.7 (±0.8)
4	28.1 (±4.6)	22.6 (±2.3)	23.0 (±1.8)	5.4 (±2.0)	108.2 (±1.7)	2.1 (±1.5)
5	38.3 (±1.7)	33.0 (±2.7)	34.2 (±2.4)	6.2 (±1.6)	106.4 (±4.5)	2.6 (±1.1)
6	4.1 (±1.7)	9.3 (±1.7)	4.8 (±1.1)	5.1 (±1.3)	79.4 (±2.7)	2.7 (±0.8)
IFN-γ (ng/mL)						
Volunteer	*SP1*	*SP2*	*SP3*	*SP4*	*EV71*	*No antigen*
1	12.9 (±1.8)	65.6 (±3.8)	40.3 (±3.8)	1.4 (±1.5)	684.5 (±3.4)	0.9 (±0.3)
2	13.7 (±1.7)	48.4 (±1.3)	31.5 (±3.3)	1.7 (±0.2)	236.1 (±2.3)	1.1 (±0.7)
3	17.4 (±1.2)	69.0 (±1.2)	40.8 (±2.2)	1.4 (±0.6)	957.6 (±2.1)	1.4 (±1.1)
4	34.4 (±2.1)	45.5 (±1.6)	32.0 (±1.3)	1.6 (±0.5)	643.8 (±3.6)	1.1 (±0.6)
5	46.4 (±2.9)	61.9 (±3.6)	48.2 (±1.2)	1.8 (±0.4)	384.3 (±2.8)	1.4 (±1.1)
6	0.8 (±0.5)	1.0 (±0.5)	0.8 (±0.3)	0.5 (±0.4)	38.3 (±1.2)	0.6 (±0.3)
IL-4 (pg/mL)						
Volunteer	*SP1*	*SP2*	*SP3*	*SP4*	*EV71*	*No antigen*
1	2.6 (±1.5)	4.3 (±4.5)	3.4 (±0.8)	2.5 (±1.0)	34.9 (±1.3)	2.6 (±1.2)
2	3.1 (±1.7)	3.4 (±2.7)	3.3 (±0.4)	2.2 (±1.9)	26.9 (±4.8)	2.1 (±1.3)
3	3.4 (±1.2)	3.9 (±3.3)	3.3 (±2.0)	2.6 (±1.4)	22.8 (±2.1)	2.6 (±1.3)
4	3.0 (±1.6)	3.1 (±0.6)	3.9 (±1.4)	3.2 (±1.6)	33.1 (±0.5)	2.7 (±1.6)
5	4.1 (±4.6)	3.9 (±3.3)	3.8 (±4.3)	3.2 (±1.5)	26.5 (±2.2)	2.2 (±0.9)
6	2.8 (±1.7)	2.6 (±1.6)	2.4 (±1.1)	2.5 (±0.8)	19.3 (±0.4)	2.2 (±0.8)
IL-10 (ng/mL)						
Volunteer	*SP1*	*SP2*	*SP3*	*SP4*	*EV71*	*No antigen*
1	1.2 (±0.1)	1.2 (±0.2)	1.2 (±0.2)	1.2 (±0.2)	31.5 (±0.2)	1.1 (±0.2)
2	1.2 (±0.2)	1.5 (±0.2)	1.2 (±0.2)	1.2 (±0.2)	22.9 (±0.3)	1.1 (±0.3)
3	2.1 (±0.2)	2.1 (±0.2)	2.3 (±0.3)	2.0 (±0.3)	23.9 (±0.8)	1.9 (±0.1)
4	1.6 (±0.2)	1.9 (±0.1)	1.8 (±0.1)	1.1 (±0.4)	24.4 (±0.2)	1.1 (±0.4)
5	2.0 (±0.3)	2.3 (±0.3)	2.6 (±0.2)	1.8 (±0.1)	11.5 (±0.3)	1.8 (±0.2)
6	1.3 (±0.4)	1.4 (±0.4)	1.3 (±0.3)	1.2 (±0.1)	3.1 (±0.1)	1.0 (±0.4)

Secretion of cytokines of Th-1-type (IL-2 and IFN-γ) and Th-2-type (IL-4 and IL-10) subsets by CD4$^+$ T cells upon stimulation by autologous monocyte-derived dendritic cells in response to peptides, EV71 whole virions (20 μg/mL), or no antigen. Values represent averages of data obtained from triplicate assays. Standard deviations are in parentheses.

imately 15 amino acids in length and are derived from internalized proteins that entered the endocytotic pathway. The MHC genes are the most polymorphic genes present in the genome, and the majority of the amino acid differences among the various alleles lie within the peptide-binding groove of the MHC class II molecules (31). Upon analysis, numerous regions within VP1 were predicted to bind one or more HLA-DR alleles, but only three regions (I, II, and III) were predicted to bind 50% or more HLA-DR alleles included in the ProPred database. These regions span amino acids 66–77, 145–159, and 247–261 of the VP1 protein, respectively. The corresponding peptides (SP1–SP3) were synthesized and shown to be able to induce the proliferation of CD4$^+$ T cells from five EV71-positive volunteers with different HLA-DR alleles, but not from an EV71-negative volunteer, indicating that the peptides have stimulated EV71-specific memory CD4$^+$ T cells. The stimulation indices obtained correlated well with the binding efficiencies predicted for each peptide and for each HLA-DR allele.

Among the three peptides tested, SP2 was identified to be the most capable of inducing significant $CD4^+$ T-cell proliferative responses among the five EV71-positive volunteers. Studies have shown that although several T-cell epitopes are present within the entire protein antigen, T cells tend to focus on only a few immunodominant epitopes, whereas discrete cryptic epitopes remain unseen by the host immune system (16,30). Therefore, SP2 most likely contains an immunodominant $CD4^+$ T-cell epitope. In addition, using anti-MHC class II antibody, we have demonstrated that SP2 is an MHC class II-restricted $CD4^+$ T-cell epitope.

Antigen-specific $CD4^+$ T cells producing IFN-γ have been shown to be essential for activation and maintenance of $CD8^+$ T-cell–mediated immune responses and for B-cell differentiation (17,32). A previous study on the identification of enterovirus cross-reactive T-cell epitopes suggested that IFN-γ release may be used as an indicator for specific T-cell activation (7). However, a poor correlation between the degree of antigen-specific T-cell proliferation and IFN-γ production has been previously reported, suggesting that proliferating and IFN-γ–producing T cells may belong to functionally different subsets (7). Instead, our results showed that the levels of IFN-γ secreted by peptide-stimulated $CD4^+$ T cells correlated well with their respective proliferative responses. In addition, the significant production of IL-2 and IFN-γ upon stimulation with SP1–SP3 clearly indicates a Th-1-subtype differentiation. Interestingly, a weak but significant proliferative response and cytokine production were observed by EV71-stimulated $CD4^+$ T cells from the EV71-negative volunteer. However, neither anti-EV71 neutralizing activity nor anti-VP1 antibodies were detected in the serum. It is likely that this volunteer might have been exposed to cross-reactive enteroviruses such as coxsackievirus 16 (CA16) or poliovirus as a result of the national childhood immunization program.

CONCLUSION

In conclusion, we have identified three potential human $CD4^+$ T-cell epitopes within the VP1 capsid protein of EV71. We have shown that the ProPred program has accurately predicted the presence of these epitopes and their binding efficiencies to three different HLA-DR alleles. Among these three epitopes, the one spanning amino acids 145–159 of VP1 appeared to be the best at inducing a high proliferation response of, and high cytokine levels by, $CD4^+$ T cells from the five EV71-positive human volunteers. However, to further demonstrate the promiscuous nature of this epitope, a larger number of human volunteers should be tested whose HLA-DR alleles are available in the ProPred database. In addition, to confirm that the antigenic stimulation induces proliferation of, and cytokine production by, EV71-specific $CD4^+$ T cells, more EV71 sero-negative volunteers should be tested. Because EV71 infection is endemic in Singapore, the proportion of sero-negative individuals is extremely low. This study is a first step toward the identification of promiscuous human $CD4^+$ T-cell epitopes within VP1, the major immunogenic and protective antigen against EV71. We believe that this approach will contribute to a better understanding of the immune correlates of protection against EV71 infection, and will help in developing more effective immuno-therapeutic and immuno-prophylactic strategies.

ACKNOWLEDGMENTS

This work was financed by a Microbiology Vaccine Initiative Grant (R182-008-067-731) from the National University of Singapore, awarded to C.L. Poh.

REFERENCES

1. Arend SM, Geluk A, van Meijgaarden KE, van Dissel JT, Theisen M, Andersen P, and Ottenhoff TH: Antigenic equivalence of human T-cell responses to *Mycobacterium* tuberculosis-specific RD1-encoded protein antigens ESAT-6 and culture filtrate protein 10 and to mixtures of synthetic peptides. Infect Immun 2000;68:3314–3321.
2. Barnard DL: Current status of anti-picornavirus therapies. Curr Pharm Des 2006;12:1379–1390.
3. Barnard DL, Hubbard VD, Smee DF, Sidwell RW, Watson KG, Tucker SP, and Reece PA: *In vitro* activity of expanded-spectrum pyridazinyl oxime ethers related to pirodavir: novel capsid-binding inhibitors with potent anti-picornavirus activity. Antimicrob Agents Chemother 2004; 48:1766–1772.
4. Beck MA, and Tracy SM: Murine cell-mediated immune response recognizes an enterovirus group-specific antigen(s). J Virol 1989;63:4148–4156.
5. Blomberg J, Lycke E, Ahlfors K, Johnsson T, Wolontis S, and Von Zeipel G: Letter: New enterovirus type associated with epidemic of aseptic meningitis and/or hand, foot, and mouth disease. Lancet 1974;13:112.
6. Cello J, Samuelson A, Stålhandske P, Svennerholm B, Jeansson S, and Forsgren M: Identification of group-common linear epitopes in structural and nonstructural proteins of enteroviruses by using synthetic peptides. J Clin Microbiol 1993;31:911–916.
7. Cello J, Strannegård, and Svennerholm B: A study of the cellular immune response to enteroviruses in humans: identification of cross-reactive T-cell epitopes on the structural proteins of enteroviruses. J Gen Virol 1996;77:2097–2108.

8. Chen HF, Chang MH, Chiang BL, and Jeng ST: Oral immunization of mice using transgenic tomato fruit expressing VP1 protein from enterovirus 71. Vaccine 2006;24: 2944–2951.

9. Chiu CH, Chu C, He CC, and Lin TY: Protection of neonatal mice from lethal enterovirus 71 infection by maternal immunization with attenuated *Salmonella enterica* serovar Typhimurium expressing VP1 of enterovirus 71. Microbes Infect 2006;8:1671–1678.

10. Chumakov M, Voroshilova M, Shindarov L, et al.: Enterovirus 71 isolated from cases of epidemic poliomyelitis-like disease in Bulgaria. Arch Virol 1979;60:329–340.

11. Collen T, Pullen L, and Doel TR: T cell-dependent induction of antibody against foot-and-mouth disease virus in a mouse model. J Gen Virol 1989;70:395–403.

12. Collen T, and Doel TR: Heterotypic recognition of foot-and-mouth disease virus by cattle lymphocytes. J Gen Virol 1990;71:309–315.

13. De Groot AS, Jesdale BM, Szu E, Schafer JR, Chicz RM, and Deocampo G: An interactive Web site providing major histocompatibility ligand predictions: application to HIV research. AIDS Res Hum Retroviruses 1997;13:529–531.

14. Foo DG, Alonso S, Chow VT, and Poh CL: Passive protection against lethal enterovirus 71 infection in newborn mice by neutralizing antibodies elicited by a synthetic peptide. Microbes Infect 2007;9:1299–1306.

15. Foo DG, Alonso S, Phoon MC, Ramachandran NP, Chow VT, and Poh CL: Identification of neutralizing linear epitopes from the VP1 capsid protein of enterovirus 71 using synthetic peptides. Virus Res 2007;125:61–68.

16. Gao Y, Wang C, and Splitter GA: Mapping T- and B-lymphocyte epitopes of bovine herpesvirus-1 glycoprotein B. J Gen Virol 1999;80:2699–2704.

17. Gao FG, Khammanivong V, Liu WJ, Leggatt GR, Frazer IH, and Fernando GJ: Antigen-specific CD4+ T-cell help is required to activate a memory CD8+ T cell to a fully functional tumor killer cell. Cancer Res 2002;62:6438–6441.

18. Graham S, Wang EC, Jenkins O, and Borysiewicz LK: Analysis of the human T-cell response to picornaviruses: identification of T-cell epitopes close to B-cell epitopes in poliovirus. J Virol 1993;67:1627–1637.

19. Hagiwara A, Tagaya I, and Yoneyama T: Epidemic of hand, foot and mouth disease associated with enterovirus 71 infection. Intervirology 1978;9:60–63.

20. Hammer J, Bono E, Gallazzi F, Belunis C, Nagy Z, and Sinigaglia F: Precise prediction of major histocompatibility complex class II-peptide interaction based on peptide side chain scanning. J Exp Med 1994;180:2353–2358.

21. Hammer J, Sturniolo T, and Sinigaglia F: HLA class II-peptide binding specificity and autoimmunity. Adv Immunol 1997;66:67–100.

22. Katrak K, Mahon BP, Minor PD, and Mills KH: Cellular and humoral immune responses to poliovirus in mice: a role for helper T cells in heterotypic immunity to poliovirus. J Gen Virol. 1991;72:1093–1098.

23. Kutubuddin M, Simons J, and Chow M: Identification of T-helper epitopes in the VP1 capsid protein of poliovirus. J Virol 1992;66:3042–3047.

24. Lennette EH, and Schmidt NJ: *Diagnostic Procedures for Viral and Rickettsial Infections*, 4th ed. Broadway. New York, 1969.

25. Lin TY, Chu C, and Chiu CH: Lactoferrin inhibits enterovirus 71 infection of human embryonal rhabdomyosarcoma cells *in vitro*. J Infect Dis 2002;186:1161–1164.

26. Lu WW, Hsu YY, Yang JY, and Kung SH: Selective inhibition of enterovirus 71 replication by short hairpin RNAs. Biochem Biophys Res Commun 2004;325:494–499.

27. Mahon BP, Katrak K, and Mills KH: Antigenic sequences of poliovirus recognized by T cells: serotype-specific epitopes on VP1 and VP3 and cross-reactive epitopes on VP4 defined by using CD4+ T-cell clones. J Virol 1992;66: 7012–7020.

28. Manici S, Sturniolo T, Imro MA, et al.: Melanoma cells present a MAGE-3 epitope to CD4+ cytotoxic T cells in association with histocompatibility leukocyte antigen DR11. J Exp Med 1999;189:871–876.

29. Minor PD: Antigenic structure of picornaviruses. Curr Top Microbiol Immunol 1990;161:121–154.

30. Novak EJ, Masewicz SA, Liu AW, Lernmark A, Kwok WW, and Nepom GT: Activated human epitope-specific T cells identified by class II tetramers reside within a CD4+ high, proliferating subset. Int Immunol 2001;13:799–806.

31. Oftung F, Lundin KEA, Geluk A, Shinnick TM, Meloen R, and Mustafa AS: Primary structure and MHC restriction of peptide defined T-cell epitopes from recombinantly expressed mycobacterial protein antigens. Med Principles Pract 1997;6:66–73.

32. Ossendorp F, Mengedé E, Camps M, Filius R, and Melief CJ: Specific T helper cell requirement for optimal induction of cytotoxic T lymphocytes against major histocompatibility complex class II negative tumors. J Exp Med 1998;187:693–702.

33. Palacios G, and Oberste MS: Enteroviruses as agents of emerging infectious diseases. J Neurovirol 2005;11:424–433.

34. Panigada M, Sturniolo T, Besozzi G, Boccieri MG, Sinigaglia F, Grassi GG, and Grassi F: Identification of a promiscuous T-cell epitope in *Mycobacterium tuberculosis* Mce proteins. Infect Immun 2002;70:79–85.

35. Ravn P, Demissie A, Eguale T, et al.: Human T cell responses to the ESAT-6 antigen from *Mycobacterium tuberculosis*. J Infect Dis 1999;179:637–645.

36. Sanders SA, Herrero LJ, McPhie K, Chow SS, Craig ME, Dwyer DE, Rawlinson W, and McMinn PC: Molecular epidemiology of enterovirus 71 over two decades in an Australian urban community. Arch Virol 2006;151:1003–1013.

37. Schmidt NJ, Lennette EH, and Ho HH: An apparently new enterovirus isolated from patients with disease of the central nervous system. J Infect Dis 1974;129:304–309.

38. Shih SR, Tsai KN, Li YS, Chueh CC, and Chan EC: Inhibition of enterovirus 71-induced apoptosis by allophycocyanin isolated from a blue-green alga *Spirulina platensis*. J Med Virol 2003;70:119–125.

39. Simons J, Kutubuddin M, and Chow M: Characterization of poliovirus-specific T lymphocytes in the peripheral blood of Sabin-vaccinated humans. J Virol 1993;67:1262–1268.

40. Snapper CM, and Mond JJ: Towards a comprehensive view of immunoglobulin class switching. Immunol Today 1993; 14:15–17.

41. Tan CS, and Cardosa MJ: High-titred neutralizing antibodies to human enterovirus 71 preferentially bind to the N-terminal portion of the capsid protein VP1. Arch Virol 2007;152:1069–1073.

42. Tsuji K, Aizawa M, and Sasazuki T: HLA 1991. *Proceedings of the Eleventh International Histocompatibility Workshop and Conference*. Oxford University Press, New York, 1992.

43. Vordermeier HM, Whelan AO, Cockle PJ, Farrant L, Palmer N, and Hewinson RG: Use of synthetic peptides derived from the antigens ESAT-6 and CFP-10 for differential diagnosis of bovine tuberculosis in cattle. Clin Diagn Lab Immunol 2001;8:571–578.

44. Vordermeier M, Whelan AO, and Hewinson RG: Recognition of mycobacterial epitopes by T cells across mammalian species and use of a program that predicts human HLA-DR binding peptides to predict bovine epitopes. Infect Immun 2003;71:1980–1987.

45. Wang KG, Sun LZ, Jubelt B, and Waltenbaugh C: Cell-mediated immune responses to poliovirus. I. Conditions for induction, characterization of effector cells, and cross-reactivity between serotypes for delayed hypersensitivity and T-cell proliferative responses. Cell Immunol 1989;119: 252–262.

46. Wu CN, Lin YC, Fann C, Liao NS, Shih SR, and Ho MS: Protection against lethal enterovirus 71 infection in newborn mice by passive immunization with subunit VP1 vaccines and inactivated virus. Vaccine 2001;20:895–904.

47. Yu CK, Chen CC, Chen CL, Wang JR, Liu CC, Yan JJ, and Su IJ: Neutralizing antibody provided protection against enterovirus type 71 lethal challenge in neonatal mice. J Biomed Sci 2000;7:523–528.

Address reprint requests to:
Dr. Sylvie Alonso
Centre for Life Sciences
28 Medical Drive, #03-05
Singapore 117456, Singapore

E-mail: micas@nus.edu.sg

Received October 4, 2007; accepted January 4, 2008

INFECTION AND IMMUNITY, Jan. 2008, p. 111–119 Vol. 76, No. 1
0019-9567/08/$08.00+0 doi:10.1128/IAI.00795-07

Highly Attenuated *Bordetella pertussis* Strain BPZE1 as a Potential Live Vehicle for Delivery of Heterologous Vaccine Candidates[▽]

Si Ying Ho,[1,2]† Shi Qian Chua,[1,2]† Damian G. W. Foo,[2] Camille Locht,[3,4] Vincent T. Chow,[2] Chit Laa Poh,[2] and Sylvie Alonso[1,2]*

Immunology Programme[1] and Department of Microbiology,[2] Yong Loo Lin School of Medicine, National University of Singapore, 28 Medical Drive, CeLS #03-06N, Singapore 117456, Singapore, and INSERM, U629,[3] and Institut Pasteur de Lille,[4] 1 rue du Prof. Calmette, F-59019 Lille, France

Received 11 June 2007/Returned for modification 4 October 2007/Accepted 10 October 2007

***Bordetella pertussis*, the causative agent of whooping cough, is a promising and attractive candidate for vaccine delivery via the nasal route, provided that suitable attenuation of this pathogen has been obtained. Recently, the highly attenuated *B. pertussis* BPZE1 strain has been described as a potential live pertussis vaccine for humans. We investigated here the use of BPZE1 as a live vehicle for heterologous vaccine candidates. Previous studies have reported the filamentous hemagglutinin (FHA), a major *B. pertussis* adhesin, as a carrier to express foreign antigens in *B. pertussis*. In this study, we also examined the BrkA autotransporter as a surface display system. Three copies of the neutralizing peptide SP70 from enterovirus 71 (EV71) were fused to FHA or in the passenger domain of BrkA, and each chimera was expressed in BPZE1. The FHA-$(SP70)_3$ and BrkA-$(SP70)_3$ chimeras were successfully secreted and exposed at the bacterial surface, respectively. Nasal administration of the live recombinant strains triggered a strong and sustained systemic anti-SP70 antibody response in mice, although the titers and neutralizing activities against EV71 were significantly higher in the sera of mice immunized with the BrkA-$(SP70)_3$-producing strain. These data indicate that the highly attenuated BPZE1 strain is a potential candidate for vaccine delivery via the nasal route with the BrkA autotransporter as an alternative to FHA for the presentation of the heterologous vaccine antigens.**

Live recombinant bacteria adapted to the respiratory tract appear to be attractive and promising vehicles for the presentation of vaccine antigens to the respiratory mucosa. *Bordetella pertussis*, the etiological agent of whooping cough, colonizes the human respiratory tract very efficiently and induces strong and protective local and systemic immune responses after a single nasal administration (39, 40, 49), with induction of immunity even at distant mucosal sites, such as the urogenital tract (41).

Consequently, *B. pertussis* has been successfully used as a live bacterial vector for the presentation of foreign antigens to the respiratory mucosa in mouse models (33, 38). However, suitable attenuation is mandatory in order to use *B. pertussis* as a live recombinant vector of vaccination. Recently, the highly attenuated *B. pertussis* BPZE1 strain was described (40). Mielcarek et al. reported markedly reduced lung inflammation in mice nasally infected with BPZE1, while the ability to colonize and induce protective immunity against pertussis infection was maintained. Furthermore, BPZE1 was found to induce protection in infant mice that was superior to the protection provided by the current acellular pertussis vaccines. These features make the *B. pertussis* BPZE1 strain an attractive live pertussis vaccine candidate and also a potential vehicle for vaccine delivery via the nasal route. The expression of heterologous antigens in BPZE1 and the ability of this strain to induce specific immune responses upon nasal administration of live recombinant bacteria have not been described previously.

Several heterologous antigens have been produced in recombinant *B. pertussis*, including the *Schistosoma mansoni* 28-kDa glutathione *S*-transferase (42), fragment C of tetanus toxin (50), transferrin-binding protein B (TbpB) from *Neisseiria meningitidis* (15), and HtrA from *Haemophilus influenzae* (3). These antigens have been fused to the filamentous hemagglutinin (FHA), a major adhesin of *B. pertussis* (34). FHA is a 220-kDa monomeric protein that is both surface exposed and secreted into the extracellular milieu (16, 29). It is highly immunogenic (2, 8, 52) and displays adjuvant properties (47), prompting its use as a carrier to present heterologous antigens to the respiratory mucosa. However, efficient secretion of FHA chimeras across the outer membrane requires a totally unfolded conformation of the passenger (24, 50), which limits the use of FHA as a carrier.

Autotransporters have been successfully used in *Salmonella* and *Escherichia coli* to present heterologous antigens at the bacterial surface (31, 32, 62), and they are able to translocate folded protein domains across the outer membrane (58). Autotransporters are large, secreted, often virulence-associated proteins of gram-negative bacteria (25). They display a characteristic domain structure that includes (i) a signal peptide at the N terminus; (i) a passenger domain, which encodes the functional part of the protein, and (iii) a C-terminal translocation unit, which is conserved in the autotransporter family. The latter domain consists of a beta barrel that is embedded in the outer membrane and through which the passenger domain is translocated to the cell surface (26). Most autotransporters

* Corresponding author. Mailing address: Immunology Programme and Department of Microbiology, Yong Loo Lin School of Medicine, National University of Singapore, 28 Medical Drive, CeLS #03-06N, Singapore 117456, Singapore. Phone: (65) 6516-3541. Fax: (65) 6778-2684. E-mail: micas@nus.edu.sg.
† S.Y.H. and S.Q.C. contributed equally to this work.
▽ Published ahead of print on 22 October 2007.

TABLE 1. *B. pertussis* strains used in this study

Strain	Relevant feature(s)	Reference
BPZE1	Attenuated Sm^r BPSM derivative lacking the *dnt* gene and producing inactive pertussis toxin and reduced tracheal cytotoxin	40
BPSQ5	BPZE1 derivative producing BrkA-$(SP70)_3$	This study
BPSY13.1	BPZE1 derivative producing FHA-$(SP70)_3$	This study
BPSY1	*brkA* knockout BPZE1 derivative	This study

are proteolytically processed, releasing an α-domain which comprises most of the passenger domain.

The *B. pertussis* BrkA autotransporter confers serum resistance by inhibiting the classical pathway of complement activation (6, 20) and plays a role in *B. pertussis* adhesion to and invasion of the host cells (19, 20). It is expressed as a 103-kDa precursor and is processed during secretion, which yields a 73-kDa N-terminal passenger domain and a 30-kDa C-terminal translocation unit (53). Following translocation, the cleaved passenger domain remains tightly associated with the bacterial surface (44). A truncated version of BrkA with a large deletion within its passenger domain has been reported and shown to be efficiently translocated across the outer membrane (45). We therefore hypothesized that this domain may be permissive for replacement at least in part by heterologous antigens.

Here, we report the expression of the neutralizing SP70 peptide from enterovirus 71 (EV71) in the highly attenuated *B. pertussis* BPZE1 strain using FHA or BrkA as a carrier. EV71 is a major causative agent of hand, foot, and mouth disease and has a propensity to cause severe neurological complications leading to significant morbidity and mortality in infants and children (36, 46). Since 1997, several outbreaks of EV71 infection have been reported in East and Southeast Asia, including Singapore and Japan, and its epidemic activity has been on the rise in the Asia-Pacific region (10, 12, 27). Several reports have indicated that the EV71 VP1 capsid protein is protective in animal models (13, 14, 55, 59) and is highly immunogenic in humans (57). We have recently shown that the SP70 peptide, spanning amino acids 208 to 222 of VP1, contains a neutralizing (23) and protective (22) B-cell epitope and is highly conserved among the EV71 subgenogroups.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. BPSY13.1, BPSY1, and BPSQ5 were derived from *B. pertussis* BPZE1, a streptomycin-resistant Tohama I derivative producing inactivated pertussis toxin, no dermonecrotic toxin, and background levels of tracheal cytotoxin (40). All *B. pertussis* strains were grown at 37°C for 72 h on Bordet-Gengou (BG) agar (Difco, Detroit, MI) supplemented with 1% glycerol, 10% defibrinated sheep blood, and 100 μg/ml streptomycin (Sigma Chemical Co., St Louis, MO). Liquid cultures were grown as described previously (37) in Stainer-Scholte medium containing 1 g/liter heptakis(2,6-di-*o*-methyl)-β-cyclodextrin (Sigma).

All DNA manipulations were carried out with chemically competent *Escherichia coli* One-Shot TOP10 (Invitrogen). The bacteria were grown at 37°C overnight on Luria-Bertani agar or in Luria-Bertani broth with vigorous shaking. When appropriate, 100 μg/ml ampicillin, 50 μg/ml ampicillin, or 10 μg/ml gentamicin was added to select for antibiotic-resistant strains.

Virus growth and purification. EV71 strain 5865/SIN/00009 (GenBank accession no. AF316321) was propagated in rhabdomyosarcoma (RD) cells using minimum essential medium (Gibco, United States) supplemented with 5% fetal calf serum, 1% sodium pyruvate, and 1.5% sodium bicarbonate. The virus particles were purified as described previously (23). Briefly, infected cells were lysed by subjecting them to freeze-thaw cycles. The virus particles were precipitated in 7% polyethylene glycol 8000 by centrifugation on a 30% sucrose cushion at 25,000 × *g* for 4 h. The virus titer was expressed as the 50% tissue culture infective dose with RD cells based on typical cytopathic effects (CPE) produced by viral infection. Before injection into mice, the virus suspension was heat inactivated at 56°C for 30 min. The amount of virion protein was quantified by the Bradford method (Bio-Rad Laboratories, United States).

Oligonucleotides, peptides, and antibodies. To circumvent any problems in protein translation due to poor codon usage (28), the original *sp70* DNA sequence was optimized to *B. pertussis* codon usage preference. To generate the FHA-$(SP70)_3$ construct, the upper and lower DNA strands of optimized *sp70* (5′-GATCGGCTACCCGACCTTCGGCGAGCACAAGCAGGAGAAGGACCTGGAGTACGA-3′ and 5′-GATCTCGTACTCCAGGTCCTTCTCCTGCTTGTGCTCGCCGAAGGTCGGGTAGCC-3′) were chemically synthesized and annealed, generating cohesive BglII-compatible ends. To generate the BrkA-$(SP70)_3$ construct, the upper and lower DNA strands of optimized *sp70* (5′-GATCTGTACCCGACCTTCGGCGAGCACAAGCAGGAGAAGGACCTGGAGTACTG-3′ and 5′-GATCCAGTACTCCAGGTCCTTCTCCTGCTTGTGCTCGCCGAAGGTCGGGTACA-3′) were chemically synthesized and annealed, generating cohesive BamHI-compatible ends.

Unconjugated SP70 peptide (22) was chemically synthesized at Mimotopes Pty. Ltd. (Clayton Victoria, Australia).

Rabbit anti-BrkA polyclonal antibodies were a kind gift from Rachel Fernandez (University of British Columbia, Canada).

Construction of recombinant *B. pertussis* strains. To construct the recombinant *B. pertussis* BPSY13.1 strain producing the FHA-$(SP70)_3$ chimera, a 1,620-bp HindIII PCR fragment was amplified from the BPZE1 chromosomal DNA using primers 5′-TTAAGCTTGCGAACGCGCTGCTGTGGG-3′ and 5′-TTAAGCTTCGCATCGGCGCTGCCCAGC-3′ (HindIII sites are underlined) and cloned into HindIII-opened plasmid pBR322 (7), yielding pBRSY0. The PCR fragment corresponded to nucleotides (nt) 5221 to 6840 of the *fhaB* open reading frame (ORF) and contained its unique BglII site. Three copies of the *sp70* DNA sequence were inserted in tandem and sequentially into the unique BglII site of pBRSY0. Insertion of one copy of *sp70* DNA into BglII-digested pBRSY0 restored a BglII site only at the 3′ end of the *sp70* DNA fragment, allowing insertion of a second *sp70* copy and then a third *sp70* copy, finally yielding pBRSY3. The 1,755-bp HindIII fragment from pBRSY3 was then cloned into HindIII-opened suicide plasmid pJQmp200rpsL18 (48), yielding pJQSY3. BPZE1 was electroporated with pJQSY3, allowing the *fha*-$(sp70)_3$ construct to integrate into the chromosomal DNA by allelic exchange (56) at the *fhaB* locus.

To construct the recombinant *B. pertussis* BPSQ5 strain, which express the BrkA-$(SP70)_3$ chimera, a 1-kb SalI-BamHI PCR fragment and a 945-bp BamHI-HindIII PCR fragment were cloned into pUC19 (60) using the corresponding restriction sites, yielding pUCSY2. Both PCR fragments were amplified from BPZE1 chromosomal DNA. The 1-kb SalI-BamHI PCR fragment was amplified using primers 5′-TTGTCGACGTAGTATCCCTTGGCCGCGC-3′ and 5′-TTGGATCCTGCGCATGCGGCGCGCC-3′ (SalI and BamHI sites are underlined) and encompassed the 5′ end of the *brkA* ORF (nt 1 to 151), its promoter region, and the first 529 nt of the adjacent *brkB* ORF, which is transcribed in the opposite direction. A 945-bp BamHI-HindIII PCR fragment was obtained using primers 5′-TTGGATCCACGCCGGCCAGGACGGCAA-3′ and 5′-TTAAGCTTCACGACCCAGGTTCCGCCC-3′ (BamHI and HindIII sites are underlined) and corresponded to nt 789 to 1735 of the *brkA* ORF. Three copies of the *sp70* gene fragment were then inserted in tandem and sequentially into BamHI-digested pUCSY2. Insertion of one copy of *sp70* DNA into BamHI-digested pUCSY2 restored a BamHI site only at the 3′ end of the *sp70* DNA fragment, which allowed insertion of a second copy and a third copy of *sp70*, finally yielding pUCSQ2. The 2,125-bp HindIII fragment from pUCSQ2 was finally cloned into HindIII-opened pJQmp200rpsL18, yielding pJQSQ1. BPZE1 was electroporated with pJQSQ1, which allowed the *brkA*-$(sp70)_3$ construct to integrate into the chromosomal DNA by allelic exchange at the *brkA* locus.

To construct the *brkA* knockout strain BPSY1, the 1,963-bp HindIII fragment from pUCSY2, which contained both the 1-kb SalI-BamHI and 945-bp BamHI-HindIII PCR fragments described above, was inserted into HindIII-opened pJQmp200rpsL18, yielding pJQSY1. BPZE1 was then electroporated with

pJQSY1, which allowed allelic exchange at the *brkA* chromosomal locus and resulted in deletion of nt 151 to 789 in the *brkA* ORF. BPSY1 therefore produced a truncated BrkA protein consisting of 103 amino acids.

Whole-cell extract preparation and supernatant concentration. Ten milliliters of mid- to late-exponential-phase bacteria in SSAB medium was centrifuged at 7,000 rpm for 15 min at room temperature. The supernatant was concentrated 10-fold using a 30-kDa-cutoff Ultra-4 centrifugal filter device (Amicon) according to the manufacturer's protocol. The bacterial pellet was resuspended in 500 μl of ultrapure water. An equal volume of 2× loading buffer was added before the preparation was heated at 95°C for 10 min. The chromosomal DNA was sheared by passing the suspension 10 times through a 27-gauge needle; this was followed by heating at 95°C for 15 min before 30 μl was loaded onto a sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel for Western blot analysis.

Immunodetection of FHA-$(SP70)_3$ and BrkA-$(SP70)_3$ chimeras. Concentrated (10×) culture supernatants or whole-cell extracts of the *B. pertussis* strains were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 8 or 12% polyacrylamide gels. The proteins were electrotransferred onto nitrocellulose membranes and incubated with mouse anti-SP70 polyclonal antibodies (23) diluted 1:100, mouse anti-FHA monoclonal antibodies diluted 1:250 (49), or rabbit anti-BrkA polyclonal antibodies diluted 1:30,000 (45) in Tris-buffered saline containing 0.1% Tween 20 and 2% bovine serum albumin. Alkaline phosphatase-conjugated goat anti-mouse or anti-rabbit immunoglobulin G (IgG) secondary antibodies (Sigma), both diluted 1:3,000, were used for chromogenic detection of the proteins after addition of the alkaline phosphatase substrate (nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate reagents; Sigma).

FACS. *B. pertussis* strains grown on BG agar were washed three times with sterile phosphate-buffered saline (PBS) supplemented with 5% glycerol. Fluorescence-activated cell sorting (FACS) was then conducted with the intact *B. pertussis* cells using a Coulter Epics machine (Beckman Coulter, Palo Alto, CA). Intact bacteria were incubated with rabbit anti-BrkA polyclonal antibodies (45) diluted 1:200 and then with Cy2-conjugated goat anti-rabbit IgG (Jackson Laboratories, West Grove, PA) diluted 1:100. Samples were analyzed with laser excitation at 488 nm, and the data were acquired using the EXPO version 2.0 software (Applied Cytometry Systems, Sheffield, United Kingdom) and analyzed with the WinMDI-2.8 software. The assay was performed two times independently.

Immunofluorescence. Intact *B. pertussis* cells were prepared as described above for FACS and spotted onto glass slides pretreated with 100 μl of 0.1% poly-L-lysine. The samples were examined with blue light excitation (488 nm) using an epifluorescence microscope (BX40; Olympus, Japan) at a magnification of ×1,000.

i.n. infection. The mice were kept under specific-pathogen-free conditions in individual ventilated cages, and all the experiments were carried out using the guidelines of the National University of Singapore animal study board. For colonization studies, 9-week-old outbred CD1 mice (Biopolis Research Center, Singapore) were each infected intranasally (i.n.) with 5×10^6 CFU of the different *B. pertussis* strains in 20 μl as described previously (1). At the indicated time points, four mice per group were sacrificed, and their lungs were aseptically removed and homogenized in PBS. Serial dilutions from individual lung homogenates were plated onto BG agar, and the total numbers of CFU per lung were determined after 4 to 5 days of incubation at 37°C. For immunization studies, groups of six 5-week-old BALB/c mice (Biopolis Research Center, Singapore) were infected i.n. twice at a 4-week interval with 5×10^6 CFU of the different *B. pertussis* strains in 20 μl. An additional group of six mice was inoculated intraperitoneally (i.p.) twice at a 4-week interval with 10 μg of heat-inactivated EV71 in a 50% emulsion of complete and incomplete Freund's adjuvant. At the indicated time points, the mice were bled at the retroorbital sinus.

Antibody detection. The levels of antibodies to SP70 and *B. pertussis* were measured by an enzyme-linked immunosorbent assay (ELISA). The 96-well microtiter plates (COSTAR; Corning) were coated overnight at 4°C with 50 μl of 0.1 M carbonate buffer (pH 9.6) containing 10 μg/ml of unconjugated SP70 peptide or total *B. pertussis* BPZE1 cell lysate. After blocking with 2% bovine serum albumin in PBS containing 0.1% Tween 20, 50 μl of serum diluted 1:50 (for anti-SP70 detection) or 1:800 (for anti-*B. pertussis* detection) was added to the wells. The plates were incubated at 37°C for 1 h, rinsed in PBS-0.1% Tween 20, and incubated at 37°C for 1 h with 50 μl of horseradish peroxidase-conjugated goat anti-mouse IgG(H+L) secondary antibodies (Sigma) at a 1:3,000 dilution. To detect the various IgG subtypes, horseradish peroxidase-conjugated goat anti-mouse IgG1, IgG2a, IgG2b, and IgG3 secondary antibodies (Jackson Laboratories) were used at a 1:5,000 dilution. The reaction was then developed using *o*-phenylenediamine dihydrochloride substrate (Sigma) at room temperature for 30 min in the dark and stopped by addition of 1 M sulfuric acid. The absorbance at 490 nm was determined with an ELISA plate reader (Tecan Sunrise, United States).

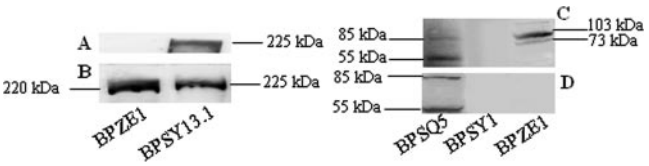


FIG. 1. Detection of FHA-$(SP70)_3$ and BrkA-$(SP70)_3$ chimeras by immunoblotting. Tenfold-concentrated culture supernatants from BPZE1 and BPSY13.1 cultures (A and B) or whole-cell extracts from BPZE1, BPSQ5, and BPSY1 cultures (C and D) were assayed by immunoblotting using anti-SP70 polyclonal antibodies (B and D), anti-FHA monoclonal antibodies (A), and anti-BrkA polyclonal antibodies (C). Fifty microliters of supernatant or 10 μl of cell extract was loaded.

EV71 neutralization assay. The presence of neutralizing antibodies against EV71 was determined by an in vitro microneutralization assay using RD cells, as described previously (23). Mouse serum samples were first incubated at 56°C for 30 min to inactivate complement activity. Briefly, 25 μl of twofold serial dilutions of heat-treated serum was coincubated with equal volumes containing 10^3 50% tissue culture infective doses of virus in a 96-well microtiter plate. Two hours later, 5×10^4 RD cells were added to each well and incubated at 37°C for 48 h. The cells were examined for CPE, and the neutralizing antibody titer was defined as the highest dilution of serum that inhibited virus growth by 100%, thereby preventing CPE. The assay was performed three times independently.

Statistical analysis. The results were analyzed using the unpaired Student *t* test. Differences were considered significant if the *P* value was <0.05.

RESULTS

Production of FHA-$(SP70)_3$ and BrkA-$(SP70)_3$ chimeras by *B. pertussis*. Up to 85% of the passenger domain of BrkA can be deleted without affecting the efficacy of translocation of the protein across the outer membrane (45). However, the passenger domain of BrkA has recently been found to possess adjuvant properties and immunogenic activities (9), which may be important when BrkA is used as a carrier for the display of vaccine candidates. We therefore decided to truncate the BrkA protein from amino acid A52 to H263, corresponding to a deletion of 32% of the passenger domain. Three copies of the 15-amino-acid SP70 neutralizing peptide from EV71 were then fused in tandem in the truncated passenger domain of BrkA. Three copies of SP70 were also inserted in tandem into full-length FHA. The chimeric proteins were designated BrkA-$(SP70)_3$ and FHA-$(SP70)_3$, respectively. The corresponding DNA constructs were introduced by allelic exchange into the *brkA* and *fhaB* chromosomal loci, respectively, of attenuated *B. pertussis* BPZE1, resulting in strains BPSQ5 and BPSY13.1, respectively.

The production of the FHA-$(SP70)_3$ and BrkA-$(SP70)_3$ chimeras by the recombinant strains was analyzed by immunoblotting using anti-SP70 and anti-FHA or anti-BrkA antibodies. A 225-kDa band corresponding to the predicted size of FHA-$(SP70)_3$ was detected in the culture supernatant of BPSY13.1 using anti-FHA and anti-SP70 antibodies (Fig. 1A and B, respectively). Two bands at 103 and 73 kDa, corresponding to full-length wild-type BrkA and its passenger domain, respectively, were detected in the whole-cell extract of BPZE1 using anti-BrkA antibodies (Fig. 1C). Similarly, two bands at 85 and 55 kDa, corresponding to the predicted sizes of full-length BrkA-$(SP70)_3$ and its passenger domain, respectively, were detected in the whole-cell extract of BPSQ5 using

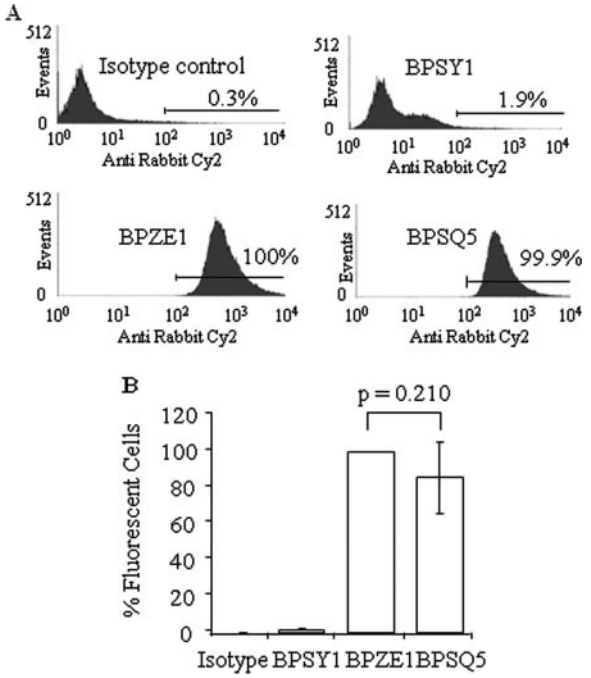


FIG. 2. Detection of the BrkA-$(SP70)_3$ chimera by FACS. Anti-BrkA polyclonal antibodies were coincubated with intact BPSY1, BPZE1, and BPSQ5 cells as indicated. The isotype control was BPZE1 bacteria stained with Cy2-conjugated secondary antibody. (A) Graphs representative of two independent experiments. (B) Average values for the two independent experiments. The results are expressed as means ± standard deviations.

anti-BrkA and anti-SP70 antibodies (Fig. 1C and D, respectively). No bands were detected in the *brkA* knockout BPSY1 strain using either antibody (Fig. 1C and D). FHA and BrkA were not detected by anti-SP70 antibodies (Fig. 1B and D, respectively).

These data demonstrate that FHA-$(SP70)_3$ and BrkA-$(SP70)_3$ were successfully produced by BPSY13.1 and BPSQ5, respectively. Moreover, they indicate that FHA-$(SP70)_3$ was efficiently secreted by BPSY13.1 into the extracellular milieu. In contrast and as expected, BrkA-$(SP70)_3$ was not secreted by BPSQ5 at an appreciable level (data not shown).

Cell surface exposure of the BrkA-$(SP70)_3$ chimera. To assess whether BrkA-$(SP70)_3$ was exposed at the bacterial surface of BPSQ5, FACS was performed with intact (nonpermeabilized) BPSQ5 cells using anti-BrkA antibodies. The parental BPZE1 and *brkA* knockout BPSY1 strains were used as positive and negative controls, respectively. As shown in Fig. 2, 100% of the parental BPZE1 cells exhibited surface exposure of BrkA. Similarly, the majority of BPSQ5 cells were found to be positive, and the difference from the parental strain was not statistically significant ($P = 0.21$) (Fig. 2B). As expected, the BPSY1 strain did not display any significant fluorescence.

The surface exposure of BrkA-$(SP70)_3$ was further confirmed by immunofluorescence analysis using anti-BrkA antibodies on intact BPSQ5, BPZE1, and BPSY1 cells. As shown in Fig. 3, BPZE1 and BPSQ5 cells displayed strong and comparable fluorescence signals (Fig. 3G and H, respectively), while no significant fluorescence emission was detected with the BPSY1 strain (Fig. 3F).

Due to a high background value, the anti-SP70 immune serum could not be used as the primary antibody in FACS and immunofluorescence studies to confirm the data obtained with the anti-BrkA immune serum (data not shown).

These results demonstrate that BrkA-$(SP70)_3$ is exposed at the bacterial surface of BPSQ5 at levels comparable to the levels of the wild-type BrkA protein in BPZE1.

Lung colonization by *B. pertussis* BPSY13.1 and BPSQ5. FHA and BrkA have been shown to play a role in the colonization efficiency of *B. pertussis* (1, 18). To study whether the recombinant BPSQ5 and BPSY13.1 strains retained the capacity to colonize the murine respiratory tract, mice were infected i.n. with either strain, and the colonization profiles were compared to those of the parental BPZE1 and *brkA* knockout BPSY1 strains.

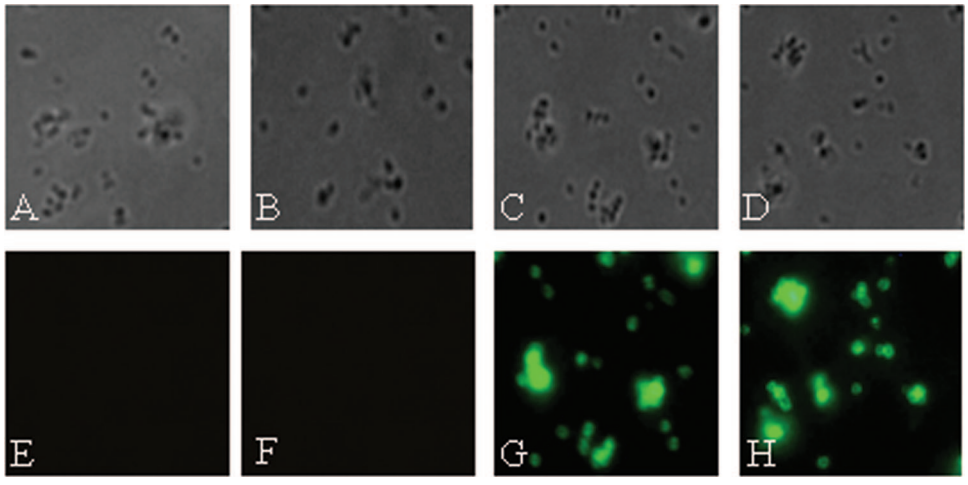


FIG. 3. Detection of the BrkA-$(SP70)_3$ chimera by immunofluorescence microscopy. Anti-BrkA polyclonal antibodies were coincubated with intact BPSY1 (B and F), BPZE1 (C and G), or BPSQ5 (D and H) cells. The isotype control (A and E) was BPZE1 bacteria stained with Cy2-conjugated secondary antibody. Panels A to D show corresponding phase-contrast images.

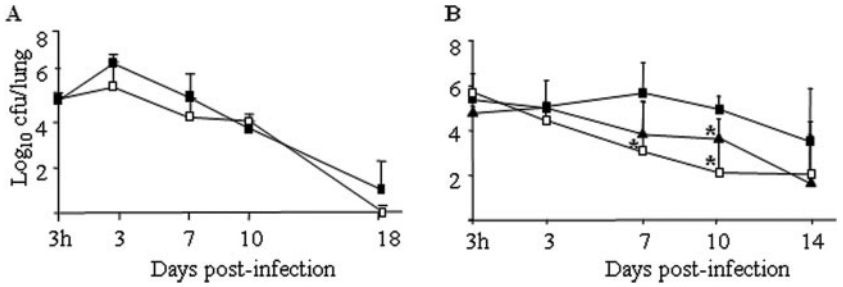


FIG. 4. Lung colonization by the recombinant *B. pertussis* strains. (A) CD1 mice were infected i.n. with 5×10^6 CFU of *B. pertussis* BPZE1 (solid squares in panels A and B), BPSY13.1 (open squares in panel A), BPSY1 (solid triangles in panel B), or BPSQ5 (open squares in panel B). The lungs of infected mice were harvested at the indicated time points, and appropriate dilutions of the lung homogenates were plated to determine the total number of CFU per lung. Four mice per group and per time point were assessed individually. Asterisk, $P < 0.05$ compared to BPZE1.

BPSY13.1 colonized the lungs as efficiently as the parental BPZE1 strain; a peak of multiplication was observed, followed by progressive clearance of the bacteria from the lungs (Fig. 4A), indicating that the insertion of three copies of SP70 into full-length FHA did not impair the adhesion function of the protein. However, similar to the colonization efficiency of the *brkA* knockout strain BPSY1, the colonization efficiency of BPSQ5 was found to be slightly but significantly ($P < 0.05$) reduced 7 and 10 days postinfection compared to that of BPZE1 (Fig. 4B). This observation suggests that the BrkA-$(SP70)_3$ chimera did not retain the full adhesion function of the wild-type BrkA protein and/or that other functions of BrkA, such as resistance to serum killing (20) and to antimicrobial peptides (21), were impaired in the BrkA-$(SP70)_3$ chimera, which might account for the reduced colonization ability observed with BPSQ5.

Systemic anti-SP70 and anti-*B. pertussis* antibody responses in mice. To examine the abilities of the two recombinant *B. pertussis* strains to trigger a systemic anti-SP70 antibody response upon nasal administration, groups of six BALB/c mice were infected i.n. twice at a 4-week interval with BPSY13.1, BPSQ5, or BPZE1. As a reference for anti-SP70 antibody production, an additional group of mice was inoculated i.p. with heat-inactivated EV71 using the same immunization schedule. The systemic anti-SP70 and anti-*B. pertussis* IgG responses were measured by ELISA 2 weeks after the boost.

Both BPSY13.1- and BPSQ5-infected mice developed a strong systemic anti-*B. pertussis* antibody response comparable to the response observed in the BPZE1-infected mice (Fig. 5A). As expected, no anti-*B. pertussis* antibody response was seen in the EV71-inoculated animals. However, the EV71-inoculated mice all showed high anti-SP70 antibody responses, while the naïve and BPZE1-infected mice displayed only background absorbance (Fig. 5B). Two of six BPSY13.1-infected mice (mice M5 and M6) produced significant anti-SP70 IgG antibody levels. In contrast, five of six BPSQ5-immunized mice produced significant anti-SP70 antibody levels, and the titers were significantly higher than the titers obtained for the BPSY13.1-immunized group ($P < 0.05$). However, the antibody titers measured for both groups of mice were found to be significantly lower than the titers measured for the EV71-inoculated group.

An anti-SP70 IgG subtype analysis was carried out for the immune sera from all the mouse groups and showed that there was production of significant levels of IgG2a/IgG2b antibodies in the BPSQ5- and BPSY13.1-immunized mice, indicative of a Th1-oriented immune response (Table 2).

The anti-*B. pertussis* and anti-SP70 antibody responses were monitored over a period of 8 weeks after the boost in the BPSY13.1- and BPSQ5-immunized groups and were found to be as high as the titers measured 2 weeks after the boost (data not shown), demonstrating that the antibody responses trig-

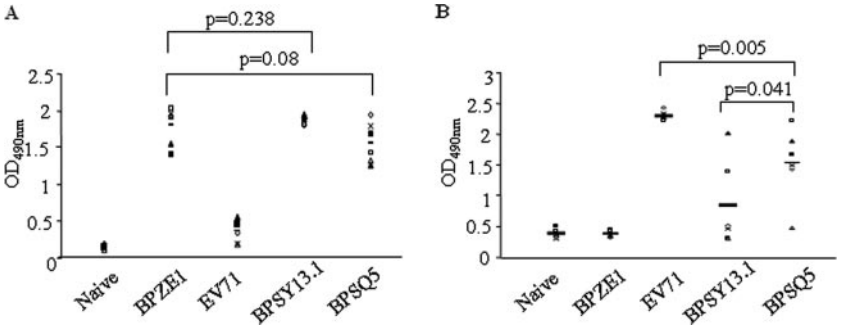


FIG. 5. Detection of specific antibody responses. Groups of six mice were infected i.n. twice at a 4-week interval with 5×10^6 CFU of BPZE1, BPSY13.1, or BPSQ5. The EV71 group was inoculated i.p. with 10 μg of inactivated virus using the same immunization schedule. The mice were bled 2 weeks after the boost, and the anti-*B. pertussis* (A) and anti-SP70 (B) IgG(H+L) titers were determined by ELISA with the individual sera diluted 1/800 and 1/50, respectively, using *B. pertussis* whole-cell lysate and SP70 peptide as coating antigens, respectively. ◇, mouse M1; ■, mouse M2; △, mouse M3; ×, mouse M4; ▲, mouse M5; □, mouse M6. The average is indicated by a horizontal line. OD_{490nm}, optical density at 490 nm.

TABLE 2. Isotype profiles of the immune sera

Immunogen	Mouse serum	Titer[a]			
		IgG1	IgG2a	IgG2b	IgG3
Naïve	Pooled	0.12	0.08	0.11	0.11
BPZE1	Pooled	0.10	0.10	0.12	0.11
EV71	Pooled	2.36	2.23	2.36	2.33
BPSQ5	M1	0.18	0.80	1.62	0.11
	M2	0.11	1.57	2.27	0.11
	M3	0.10	0.13	0.22	0.10
	M4	0.09	1.52	1.21	0.09
	M5	0.10	0.65	2.54	0.12
	M6	1.42	2.17	2.34	0.13
BPSY13.1	M1	0.22	0.25	0.78	0.07
	M2	0.08	0.08	0.08	0.09
	M3	0.09	0.07	0.07	0.07
	M4	0.27	0.71	0.08	0.08
	M5	2.16	1.42	1.33	0.17
	M6	0.39	2.00	2.03	0.19

[a] The anti-SP70 IgG1, IgG2a, IgG2b, and IgG3 titers were determined by ELISA using the sera diluted 1/50 and using SP70 peptide as the coating antigen. Sera from individual mice belonging to the naïve, BPZE1, and EV71 groups were pooled, while serum from each BPSY13.1- and BPSQ5-immunized mouse was tested individually.

TABLE 3. Neutralizing activities of the immune sera[a]

Immunogen	Mouse serum	Serum dilution[b]
Naïve	Pooled	NP
BPZE1	Pooled	NP
EV71	Pooled	1/128
BPSQ5	M1	1/32
	M2	1/2
	M3	NP
	M4	1/16
	M5	1/32
	M6	1/32
BPSY13.1	M1	NP
	M2	NP
	M3	NP
	M4	NP
	M5	1/16
	M6	NP

[a] Twofold serial dilutions of the sera from each group were incubated with EV71 before infection of RD cells, and the CPE were observed 48 h later. Sera from individual mice belonging to the naïve, BPZE1, and EV71 groups were pooled, while serum from each BPSY13.1- and BPSQ5-immunized mouse was tested individually.

[b] Highest dilution with which total protection was observed. NP, no protection.

gered by nasal administration of the recombinant strains was sustained.

Neutralizing activity of the immune sera against EV71. To evaluate the functional activities of the sera from the BPSY13.1- and BPSQ5-immunized mice, an in vitro EV71 neutralization assay was used. Serially diluted sera were coincubated with EV71 before infection of RD cells. CPE were determined 48 h later. The sera from the naïve, BPZE1-infected, and EV71-inoculated mice were pooled within groups, while the sera from the BPSY13.1- and BPSQ5-infected mice were analyzed individually.

In contrast to uninfected cells, which had a flattened and spindle-like shape, infected cells appeared to be rounded and swollen with microbodies, as described elsewhere (23; data not shown). As expected, the sera from the naïve and BPZE1-infected mice failed to protect RD cells from viral infection (Table 3). In contrast, the pooled serum from the EV71-inoculated mice provided complete protection to the cells up to a serum dilution of 1:128. For the six BPSY13.1-infected mice, only the serum from mouse M5, corresponding to the highest anti-SP70 IgG titer, displayed significant neutralizing activity against the virus. This serum conferred complete protection to the cells up to a dilution of 1:16. The sera from the five BPSQ5-infected mice, which were found to produce significant anti-SP70 antibodies, showed the ability to neutralize the virus, and complete protection was obtained with serum dilutions ranging from 1:2 to 1:32. Surprisingly, the highest neutralization titer did not correspond to the highest anti-SP70 antibody titer. For example, the sera from mice M1 and M5, which contained significantly different anti-SP70 antibody levels (Fig. 5B), were found to be equally able to neutralize EV71 in vitro.

These results show that nasal administration of BPSQ5 and, to a lesser extent, nasal administration of BPSY13.1 are able to trigger the production of systemic antibodies capable of neutralizing EV71 infection in vitro.

DISCUSSION

Despite high vaccination coverage, *B. pertussis* remains endemic in many areas, and reports of an increasing incidence of infection worldwide have been accumulating for the past 20 years (5, 11, 17). The resurgence of pertussis is believed to be due to waning vaccine-induced immunity in adults and to antigenic shift and adaptation of the circulating *B. pertussis* strains to the current acellular pertussis vaccines (43). Natural infection with *B. pertussis* has long been known to induce strong and long-lasting immunity that wanes later than vaccine-induced immunity (4). Furthermore, natural infection with *B. pertussis* induces measurable antigen-specific Th1 immune responses even in very young children (as young as 1 month of age) (35). However, the neonatal immune system is too immature for effective development of protective immunity upon administration of acellular vaccines (54). These observations suggest that live vaccines that can be administered by the nasal route in order to mimic as closely as possible natural infection may be attractive alternatives to the currently available subunit vaccines. Such a strategy would allow early immunization, possibly at birth, thereby reducing the incidence of pertussis in the very young and most vulnerable age groups. The highly attenuated *B. pertussis* BPZE1 strain has been described recently as a promising live pertussis vaccine (40). In this study we investigated the use of BPZE1 as a live vehicle to deliver a heterologous peptide vaccine candidate via the nasal route.

The heterologous antigens which have been produced in *B. pertussis* so far were fused to either full-length or truncated FHA (3, 15, 42, 50). To be efficiently secreted, an FHA chi-

mera must be in an unfolded conformation, precluding the fusion of any foreign antigens with cysteine residues susceptible to formation of disulfide bonds (24, 50). To further develop *B. pertussis* as a vehicle for vaccine delivery, we explored the use of the autotransporter BrkA as a surface display system. The efficient translocation across the outer membrane of folded protein domains containing disulfide bonds has been demonstrated for autotransporters in other bacterial species (58). We report here that insertion in tandem of three copies of the 15-amino-acid SP70 peptide within the full-length FHA and within the truncated passenger domain of BrkA does not impair the secretion and surface exposure, respectively, of the chimeras.

The colonization efficiency of BPSQ5, which produces the BrkA-$(SP70)_3$ chimera, was found to be slightly reduced compared to that of BPZE1, and the profile was comparable to that of a *B. pertussis brkA* knockout mutant. A previous study using a *B. pertussis brkA* knockout mutant showed that BrkA is involved in colonization efficacy in mice (18). Our findings further suggest that the A52-H263 region of the passenger domain of BrkA likely plays a role in this process. A role of BrkA in direct adherence to and invasion of the host cells has also been described (19, 20). However, whether the A52-H263 region of the BrkA passenger domain is part of the adhesion domain of the protein is not known. Other functions of BrkA, such as resistance to serum killing (20) and to antimicrobial peptides (21), might be impaired in the BrkA-$(SP70)_3$ chimera, which might account for the reduced colonization ability observed with BPSQ5.

Both BPSQ5- and BPSY13.1-immunized mice developed a strong anti-*B. pertussis* antibody response, and the titers were comparable to those obtained for mice immunized with parental strain BPZE1, demonstrating that fusion of three copies of SP70 to either FHA or BrkA did not alter the immunogenicity of the bacterial vector. The systemic anti-SP70 IgG response measured in the BPSQ5-immunized mice was found to be significantly higher than the response measured in the BPSY13.1-immunized mice. Several factors might account for the differential ability to trigger an anti-SP70 antibody response using BrkA or FHA as the carrier. These include the yield of each chimera produced by the recombinant strains and the size ratio between the carrier and the three copies of SP70 peptide, as well as the intrinsic immunogenicity of the carrier. Indeed, FHA is known to be highly immunogenic (2, 8, 52). However, this feature, if too prominent, may be a handicap as it may skew the immune response towards the carrier at the expense of the passenger antigen. Moreover, the subcellular localization of the chimera might also play a role in the presentation of the heterologous antigen. A recent study showed that *B. pertussis* BrkA, as well as autotransporters from other rod-shaped gram-negative bacteria, is localized at the bacterial pole, which may have profound implications for the nature and efficiency of the pathogen-host interactions (30). Thus, concentration of the BrkA-$(SP70)_3$ molecules at one pole of the bacteria might allow more efficient presentation to and processing by the host's antigen-presenting cells.

The lack of a suitable animal model to examine vaccine efficacy is a major obstacle to the development of EV71 vaccines. Mice are susceptible to EV71 infection in the first 4 days of life and then become completely resistant by 6 days of age (61, 51). EV71 infection has been found to be asymptomatic in all strains of adult mice tested, including BALB/c, C3H, ICR, CD28 knockout, and tumor necrosis factor alpha receptor knockout mice (59). Therefore, the efficacy of an EV71 vaccine candidate cannot be evaluated with actively immunized mice but can be addressed only using passive immunization, in which newborn (1-day-old) mice are challenged with EV71 and subsequently inoculated with the immune serum from actively immunized adult mice, as previously described (22, 61). We recently reported that anti-SP70 antisera with a neutralizing titer of 1:32 were able to confer up to 80% in vivo passive protection in suckling mice and that the survival rate correlated well with the neutralizing titers measured in vitro (22). Here, we found that the sera from the BPSQ5-immunized mice displayed significant neutralizing activities against EV71 in vitro and had titers of up to 1:32, demonstrating the presence of anti-SP70 neutralizing antibodies in the antisera. Moreover, the anti-SP70 serum antibody isotypes induced in the BSQ5-immunized mice were predominantly IgG2a and IgG2b, indicative of a Th1-oriented immune response. In contrast, parenteral administration of conjugated SP70 peptide induced a Th2 immune response with production of high levels of anti-SP70 IgG1 antibodies (23). This apparent discrepancy, very likely due to the vehicle used to deliver the SP70 peptide (live recombinant bacteria versus conjugated SP70 emulsified in Freund's adjuvant), suggests therefore that the neutralizing activity of the anti-SP70 antibodies is not restricted to one particular IgG isotype.

In conclusion, we describe here the feasibility of using the highly attenuated *B. pertussis* BPZE1 strain as a bacterial vector to deliver heterologous vaccine candidates, thereby allowing simultaneous protection against pertussis and the target disease. Since whooping cough and hand, foot, and mouth disease are two childhood diseases, combined immunization soon after birth would be highly desirable. Furthermore, we show that the BrkA autotransporter is a promising display system for foreign antigens in *B. pertussis*. This study opens up new avenues for the development of safe live attenuated *B. pertussis* as a vehicle for vaccine delivery via the nasal route.

ACKNOWLEDGMENT

This work was supported by National University of Singapore ARF grant R-182-000-077-112.

REFERENCES

1. **Alonso, S., K. Pethe, N. Mielcarek, D. Raze, and C. Locht.** 2001. Role of ADP-ribosyltransferase activity of pertussis toxin in toxin-adhesin redundancy with filamentous hemagglutinin during *Bordetella pertussis* infection. Infect. Immun. **69:**6038–6043.
2. **Alonso, S., N. Reveneau, K. Pethe, and C. Locht.** 2002. Eighty-kilodalton N-terminal moiety of *Bordetella pertussis* filamentous hemagglutinin: adherence, immunogenicity, and protective role. Infect. Immun. **70:**4142–4147.
3. **Alonso, S., E. Willery, G. Renauld-Mongenie, and C. Locht.** 2005. Production of nontypeable *Haemophilus influenzae* HtrA by recombinant *Bordetella pertussis* with the use of filamentous hemagglutinin as a carrier. Infect. Immun. **73:**4295–4301.
4. **Amsbaugh, D. F., Z. M. Li, and R. D. Shahin.** 1993. Long-lived respiratory immune response to filamentous hemagglutinin following *Bordetella pertussis* infection. Infect. Immun. **61:**1447–1452.
5. **Andrews, R., A. Herceq, and C. Roberts.** 1997. Pertussis notifications in Australia, 1991 to 1997. Commun. Dis. Intell. **21:**145–148.
6. **Barnes, M. G., and A. A. Weiss.** 2001. BrkA protein of *Bordetella pertussis* inhibits the classical pathway of complement after C1 deposition. Infect. Immun. **69:**3067–3072.
7. **Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heynecker,**

and H. W. Boyer. 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2:**95–113.
8. **Cahill, E. S., D. T. O'Hagan, L. Illum, A. Barnard, K. H. G. Mills, and K. Redhead.** 1995. Immune responses and protection against *Bordetella pertussis* infection after intranasal immunization of mice with filamentous hemagglutinin in solution or incorporated in biodegradable microparticles. Vaccine **13:**455–462.
9. **Cainelli Gebara, V., L. Risoleo, A. Lopes, V. Ferreira, W. Quintilio, F. Lepine, W. Silva, and I. Raw.** 2007. Adjuvant and immunogenic activities of the 73kDa N-terminal alpha-domain of BrkA autotransporter and Cpn60/60kDa chaperonin of *Bordetella pertussis*. Vaccine **25:**621–629.
10. **Cardosa, M. J., D. Perera, B. A. Brown, D. Cheon, H. M. Chan, K. P. Chan, H. Cho, and P. McMinn.** 2003. Molecular epidemiology of human enterovirus 71 strains and recent outbreaks in the Asia-Pacific region: comparative analysis of the VP1 and VP4 genes. Emerg. Infect. Dis. **9:**461–468.
11. **Celentano, L. P., M. Massari, D. Paramatti, S. Salmaso, and A. E. Tozzi.** 2005. Resurgence of pertussis in Europe. Pediatr. Infect. Dis. J. **24:**761–765.
12. **Chan, K. P., K. T. Goh, C. Y. Chong, E. S. Teo, G. Lau, and A. E. Ling.** 2003. Epidemic hand, foot and mouth disease caused by human enterovirus 71, Singapore. Emerg. Infect. Dis. **9:**78–85.
13. **Chen, H. F., M. H. Chang, B. L. Chiang, and S. T. Jeng.** 2006. Oral immunization of mice using transgenic tomato fruit expressing VP1 protein from enterovirus 71. Vaccine **24:**2944–2951.
14. **Chiu, C. H., C. Chu, C. C. He, and T. Y. Lin.** 2006. Protection of neonatal mice from lethal enterovirus 71 infection by maternal immunization with attenuated *Salmonella enterica* serovar Typhimurium expressing VP1 of enterovirus 71. Microbes Infect. **8:**1671–1678.
15. **Coppens, I., S. Alonso, R. Antoine, F. Jacob-Dubuisson, G. Renauld-Mongenie, E. Jacobs, and C. Locht.** 2001. Production of *Neisseria meningitidis* transferrin-binding protein B by recombinant *Bordetella pertussis*. Infect. Immun. **69:**5440–5446.
16. **Coutte, L., S. Alonso, N. Reveneau, E. Willery, B. Quatannens, C. Locht, and F. Jacob-Dubuisson.** 2003. Role of adhesin release for mucosal colonization by a bacterial pathogen. J. Exp. Med. **197:**735–742.
17. **De Serres, G., and B. Duval.** 2005. Pertussis vaccination beyond childhood. Lancet **365:**1015–1026.
18. **Elder, K. D., and E. T. Harvill.** 2004. Strain-dependent role of BrkA during *Bordetella pertussis* infection of the murine respiratory tract. Infect. Immun. **72:**5919–5924.
19. **Ewanowich, C. A., A. R. Melton, A. A. Weiss, R. K. Sherburne, and M. S. Peppler.** 1989. Invasion of HeLa 229 cells by virulent *Bordetella pertussis*. Infect. Immun. **57:**2698–2704.
20. **Fernandez, R. C., and A. A. Weiss.** 1994. Cloning and sequencing of a *Bordetella pertussis* serum resistance locus. Infect. Immun. **62:**4727–4738.
21. **Fernandez, R. C., and A. A. Weiss.** 1996. Susceptibilities of *Bordetella pertussis* strains to antimicrobial peptides. Antimicrob. Agents Chemother. **40:**1041–1043.
22. **Foo, G. W. D., S. Alonso, T. K. V. Chow, and C. L. Poh.** 2007. Passive protection against lethal enterovirus 71 infection in newborn mice by neutralizing antibodies elicited by a synthetic peptide. Microbes Infect. **9:**1299–1306.
23. **Foo, G. W. D., S. Alonso, M. C. Phoon, N. P. Ramachandran, T. K. V. Chow, and C. L. Poh.** 2007. Identification of neutralizing linear epitopes from the VP1 capsid protein of enterovirus 71 using synthetic peptides. Virus Res. **125:**61–68.
24. **Guedin, S., E. Willery, C. Locht, and F. Jacob-Dubuisson.** 1998. Evidence that a globular conformation is not compatible with FhaC-mediated secretion of the Bordetella pertussis filamentous haemagglutinin. Mol. Microbiol. **29:**763–774.
25. **Henderson, I. R., F. Navarro-Garcia, M. Desvaux, R. C. Fernandez, and D. Ala'Aldeen.** 2004. Type V protein secretion pathway: the autotransporter story. Microbiol. Mol. Biol. Rev. **68:**692–744.
26. **Henderson, I. R., F. Navarro-Garcia, and J. P. Nataro.** 1998. The great escape: structure and function of the autotransporter proteins. Trends Microbiol. **6:**370–378.
27. **Ho, M., E. R. Chen, K. H. Hsu, S. J. Twu, K. T. Chen, S. F. Tsai, J. R. Wang, and S. R. Shih.** 1999. An epidemic of enterovirus 71 infection in Taiwan. Taiwan Enterovirus Epidemic Working Group. N. Engl. J. Med. **341:**929–935.
28. **Ikemura, T.** 1985. Codon usage and tRNA content in unicellular and multicellular organisms. Mol. Biol. Evol. **1:**13–34.
29. **Jacob-Dubuisson, F., C. Locht, and R. Antoine.** 2001. Two-partner secretion in Gram-negative bacteria: a thrifty, specific pathway for large virulence proteins. Mol. Microbiol. **40:**306–313.
30. **Jain, S., P. van Ulsen, I. Benz, M. A. Schmidt, R. C. Fernandez, J. Tommassen, and M. B. Goldberg.** 2006. Polar localization of the autotransporter family of large bacterial virulence proteins. Infect. Immun. **188:**4841–4850.
31. **Kramer, U., K. Rizos, H. Apfel, I. B. Autenrieth, and C. T. Lattermann.** 2003. Autodisplay: development of an efficacious system for surface display of antigenic determinants in *Salmonella* vaccine strains. Infect. Immun. **71:**1944–1952.
32. **Lattemann, C. T., J. Maurer, E. Gerland, and T. F. Meyer.** 2000. Autodisplay: functional display of active β-lactamase on the surface of *Escherichia coli* by the AIDA-I autotransporter. J. Bacteriol. **182:**3726–3733.
33. **Locht, C.** 2000. Live bacterial vectors for intranasal delivery of protective antigens. Pharm. Sci. Technol. Today **3:**121–128.
34. **Locht, C., P. Bertin, F. D. Menozzi, and G. Renauld.** 1993. The filamentous hemagglutinin, a multifaceted adhesion produced by *Bordetella* spp. Mol. Microbiol. **9:**653–660.
35. **Mascart, F., V. Verscheure, A. Malfroot, M. Hainaut, D. Pierard, S. Temerman, A. Peltier, A.-S. Debrie, J. Levy, G. Del Giudice, and C. Locht.** 2003. *Bordetella pertussis* infection in 2-months-old infants promotes type 1 T cell responses. J. Immunol. **170:**1504–1509.
36. **McMinn, P. C.** 2002. An overview of the evolution of enterovirus 71 and its clinical and public health significance. FEMS Microbiol. Rev. **26:**91–107.
37. **Menozzi, F. D., C. Gantiez, and C. Locht.** 1991. Identification and purification of transferrin- and lactoferrin-binding proteins of *Bordetella pertussis* and *Bordetella bronchiseptica*. Infect. Immun. **59:**3982–3988.
38. **Mielcarek, N., S. Alonso, and C. Locht.** 2001. Nasal vaccination using live bacterial vectors. Adv. Drug Deliv. Rev. **51:**55–70.
39. **Mielcarek, N., J. Cornette, A. Schacht, R. Pierce, C. Locht, A. Capron, and G. Riveau.** 1997. Intranasal priming with recombinant *Bordetella pertussis* for the induction of a systemic immune response against a heterologous antigen. Infect. Immun. **65:**544–550.
40. **Mielcarek, N., A-S. Debrie, D. Raze, J. Bertout, C. Rouanet, A. B. Younes, C. Creusy, J. Engle, W. E. Goldman, and C. Locht.** 2006. Live attenuated *B. pertussis* as a single-dose nasal vaccine against whooping cough. PLoS Pathogens **2:**0001–0009.
41. **Mielcarek, N., I. Nordstrom, F. Menozzi, C. Locht, and J. Holmgren.** 2000. Genital antibody responses in mice after intranasal infection with an attenuated candidate vector strain of *Bordetella pertussis*. Infect. Immun. **68:**491–495.
42. **Mielcarek, N., G. Riveau, F. Remoue, R. Antoine, A. Capron, and C. Locht.** 1998. Homologous and heterologous protection after single intranasal administration of live attenuated recombinant *Bordetella pertussis*. Nat. Biotechnol. **16:**454–457.
43. **Mooi, F. R., I. H. van Loo, and A. J. King.** 2001. Adaptation of *Bordetella pertussis* to vaccination: a cause for its reemergence? Emerg. Infect. Dis. **7:**526–528.
44. **Oliver, D. C., G. Huang, and R. C. Fernandez.** 2003. Identification of secretion determinants of the *Bordetella pertussis* BrkA autotransporter. J. Bacteriol. **185:**489–495.
45. **Oliver, D. C., G. Huang, E. Nodel, S. Pleasance, and R. C. Fernandez.** 2003. A conserved region within the *Bordetella pertussis* autotransporter BrkA is necessary for folding of its passenger domain. Mol. Microbiol. **47:**1367–1383.
46. **Palacios, G., and M. S. Oberste.** 2005. Enterovirus as agent of emerging infectious diseases. J. Neurovirol. **11:**424–433.
47. **Poulain-Godefroy, O., F. D. Menozzi, S. Alonso, C., Vendeville, A. Capron, C. Locht, and G. Riveau.** 2003. Adjuvant activity of free *Bordetella pertussis* filamentous hemagglutinin delivered by mucosal routes. Scand. J. Immunol. **58:**503–510.
48. **Quandt, J., and M. F. Hynes.** 1993. Versatile suicide vectors which allow direct selection for replacement in Gram-negative bacteria. Gene **127:**15–21.
49. **Renauld-Mongenie, G., N. Mielcarek, J. Cornette, A.-M. Schacht, A. Capron, G. Riveau, and C. Locht.** 1996. Induction of mucosal immune responses against a heterologous antigen fused to filamentous hemagglutinin after intranasal immunization with recombinant *Bordetella pertussis*. Proc. Natl. Acad. Sci. USA **93:**7944–7949.
50. **Reveneau, N., S. Alonso, F. Jacob-Dubuisson, A. Mercenier, and C. Locht.** 2001. Tetanus toxin fragment C-specific priming by intranasal infection with recombinant *Bordetella pertussis*. Vaccine **20:**926–933.
51. **Roberts, G. B. S., and J. F. Boyd.** 1987. The histopathology of enterovirus infections in new-born mice. J. Infect. **15:**45–56.
52. **Shahin, R., D. F. Amsbaugh, and M. F. Leef.** 1992. Mucosal immunization with filamentous hemagglutinin protects against *Bordetella pertussis* respiratory infection. Infect. Immun. **60:**1482–1488.
53. **Shannon, J. L., and R. C. Fernandez.** 1999. The C-terminal domain of the *Bordetella pertussis* autotransporter BrkA forms a pore in lipid bilayer membranes. J. Bacteriol. **181:**5838–5842.
54. **Siegrist, C. A.** 2001. Neonatal and early life vaccinology. Vaccine **19:**3331–3346.
55. **Sivasamugham, L. A., M. J. Cardosa, W. S. Tan, and K. Yusoff.** 2006. Recombinant Newcastle disease virus capsids displaying enterovirus 71 VP1 fragment induce a strong immune response in rabbits. J. Med. Virol. **78:**1096–1104.
56. **Stibitz, S.** 1994. Use of conditionally counterselectable suicide vectors for allelic exchange. Methods Enzymol. **235:**458–465.
57. **Tan, C. S., and M. J. Cardosa.** 2007. High-titer neutralizing antibodies to human enterovirus 71 preferentially bind to the N-terminal portion of the capsid protein VP1. Arch. Virol. **157:**1069–1073.
58. **Veiga, E., V. De Lorenzo, and L. A. Fernandez.** 2004. Structural tolerance of bacterial autotransporters for folded passenger protein domains. Mol. Microbiol. **52:**1069–1080.
59. **Wu, C. N., Y. C. Lin, C. Fann, N. S. Liao, S. R. Shih, and M. S. Ho.** 2001.

Protection against lethal enterovirus 71 infection in newborn mice by passive immunization with subunit VP1 vaccines and inactivated virus. Vaccine **20:** 895–904.

60. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.
61. **Yu, C. K., C. C. Chen, C. L. Chen, J. R. Wang, C. C. Liu, J. J. Yan, and I. J. Su.** 2000. Neutralizing antibody provided protection against enterovirus type 71 lethal challenge in neonatal mice. J. Biomed. Sci. **7:**523–528.
62. **Zhu, C., F. Ruiz-Perez, Z. Yang, Y. Mao, V. L. Hackethal, K. M. Greco, W. Choy, K. Davis, J. R. Butterton, and E. C. Boedeker.** 2006. Delivery of heterologous protein antigens via hemolysin or autotransporter systems by an attenuated *ler* mutant of rabbit enteropathogenic *Escherichia coli*. Vaccine **24:**3821–3831.

Editor: A. Camilli