SEROEPIDEMIOLOGY AND CHARACTERISATION OF NEUTRALISATION ESCAPE MUTANTS OF ENTEROVIRUS A71

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ABSTRACT

Enterovirus A71 (EV-A71) is an important emerging pathogen causing large epidemics of hand, foot and mouth disease (HFMD) in children. In Malaysia, since its first epidemic in 1997, recurrent cyclical epidemics have occurred every 2-3 years. Currently, no study of the seroepidemiology of EV-A71 infection has been done in Malaysia. The objectives of the current study were to determine the seroepidemiology of EV-A71 infection in children up to 12 years of age, and general urban and rural populations in Malaysia; to identify risk factors for seropositivity to EV-A71 in the rural population; and to generate EV-A71 neutralisation escape mutants to identify neutralisation epitopes. Neutralisation assay was performed to measure the neutralising antibody titres in 2141 serum samples from children, 460 samples from an urban Kuala Lumpur population, and 298 samples from rural Orang Asli populations. A significant association between subjects' age and seropositivity to EV-A71 was found among the children. Overall, EV-A71 seroprevalence rate and geometric mean titre (GMT) were significantly higher in the 7-12 years age group compared to the 1-6 years age group, and also in epidemic years (1997, 2000, 2003, 2006, 2008/2009, 2012) compared to non-epidemic years. The HFMD incidence rate was highest in children <2 years old. In the population study involving serum samples from healthy individuals aged 1-85 and 1-90 years old for urban and rural populations, respectively, EV-A71 seropositivity was strongly associated with increasing age in both populations. Potential risk factors associated with EV-A71 seropositivity were determined for the rural Orang Asli populations. Orang Asli children ≤12 years had significantly higher EV-A71 seropositivity rates than urban Kuala Lumpur children, and also higher rates in the age groups of 1-3, 4-6 and 7-12 years. Multivariate analysis confirmed that age ≤12 years and using untreated water were independently associated with EV-A71 seropositivity in the Orang Asli population. Lastly, as neutralising antibodies confer protection against

EV-A71 infection, more studies are needed to discover more neutralisation epitopes. EV-A71 neutralisation escape mutants were generated *in vitro* by exposing the virus to antibody pressure using a mouse monoclonal antibody (MAB979) for four passages. The amino acid changes were determined by sequencing. Two mutations were detected in the capsid protein of EV-A71 neutralisation escape mutants; threonine to isoleucine, located at the amino acid position 141 on VP2; and aspartic acid to asparagine, located at amino acid position 14 on VP1. Neutralising activity of the EV-A71 escape mutants was tested against EV-A71 monoclonal antibody MAB979, anti-EV-A71 positive mouse sera and human sera. The characterisation of the neutralisation escape mutants suggests that the neutralisation epitopes in humans and animals could be different. In summary, this first EV-A71 seroepidemiology study in Malaysia revealed that young age, using untreated water, and living within rural populations are risk factors associated with EV-A71 infection. The discovered neutralisation epitopes may contribute to development of vaccines or monoclonal antibody therapy.

ABSTRAK

Enterovirus A71 (EV-A71) adalah patogen penting yang menyebabkan wabak besar penyakit tangan, kaki dan mulut (HFMD) di kalangan kanak-kanak. Di Malaysia, sejak wabak yang pertama pada tahun 1997, wabak kitaran berulang berlaku setiap 2-3 tahun. Setakat ini, tiada kajian mengenai seroepidemiologi jangkitan EV-A71 dijalankan di Malaysia. Objektif kajian semasa adalah untuk menentukan seroepidemiologi jangkitan EV-A71 di kalangan kanak-kanak sehingga 12 tahun, dan populasi umum bandar dan kawasan pedalaman di Malaysia; faktor risiko untuk seropositiviti terhadap EV-A71 di kalangan penduduk pedalaman; dan menjana mutan kalis peneutralan bagi EV-A71 untuk mengenalpasti epitop peneutralan. Asai peneutralan dilakukan untuk mengukur titer antibodi peneutralan dalam 2141 sampel serum daripada kanak-kanak, 460 sampel dari penduduk Bandar Kuala Lumpur, dan 298 sampel dari penduduk Orang Asli di pedalaman. Satu kaitan yang signifikan antara umur subjek dan seropositiviti terhadap EV-A71 telah ditemui di kalangan kanak-kanak. Secara keseluruhan, kadar seroprevalens dan GMT terhadap jangkitan EV-A71 adalah lebih tinggi di kalangan kanak-kanak berumur 7-12 tahun berbanding 1-6 tahun, dan juga dalam tahun-tahun wabak (1997, 2000, 2003, 2006, 2008/2009, 2012) berbanding tahun-tahun bukan wabak. Kadar insiden HFMD adalah yang tertinggi di kalangan kanak-kanak berumur kurang daripada 2 tahun. Dalam kajian penduduk yang melibatkan sampel serum daripada individu yang sihat berumur 1-85 dan 1-90 tahun, masing-masing bagi penduduk bandar dan kawasan pedalaman, seropositiviti terhadap EV-A71 berkait rapat dengan peningkatan umur dalam kedua-dua populasi. Faktor-faktor risiko yang berkaitan dengan seropositiviti terhadap EV-A71 ditentukan untuk golongan penduduk Orang Asli. Kanak-kanak Orang Asli ≤12 tahun mempunyai kadar seropositiviti terhadap EV-A71 yang lebih tinggi berbanding kanak-kanak di Kuala Lumpur, juga pada kadar yang lebih tinggi dalam kumpulan umur 1-3, 4-6 dan 7-12 tahun. Analisis

multivariat mengesahkan umur ≤12 tahun dan menggunakan air yang tidak dirawat berkaitan secara tidak bersandar dengan seropositiviti terhadap EV-A71 dalam populasi Orang Asli. Akhir sekali, oleh kerana antibodi peneutralan memberikan perlindungan terhadap jangkitan EV-A71, lebih banyak kajian perlu dilakukan untuk mengetahui dengan lebih lanjut tentang epitop peneutralan. Mutan kalis peneutralan ini telah dihasilkan secara *in vitro* dengan mendedahkan virus tersebut kepada tekanan antibodi menggunakan antibodi monoklonal tikus (MAB979) untuk empat pemindahan. Perubahan asid amino ditentukan oleh penjujukan. Dua mutasi telah dikenalpasti pada dua protein capsid mutan kalis peneutralan bagi EV-A71; threonine kepada isoleucine, yang terletak di posisi asid amino 141 pada VP2; dan asid aspartik kepada asparagine, di posisi asid amino 14 pada VP1. Aktiviti peneutralan bagi mutan kalis peneutralan EV-A71 telah diuji dengan antibodi monoklonal EV-A71 MAB979, serum tikus dan serum manusia yang positif terhadap anti-EV-A71. Pencirian mutan kalis peneutralan tersebut mencadangkan bahawa epitop peneutralan dalam manusia dan haiwan mungkin berbeza. Secara ringkasnya, kajian seroepidemiologi terhadap EV-A71 yang pertama di Malaysia ini menunjukkan bahawa usia muda, menggunakan air yang tidak dirawat, dan hidup di kawasan pedalaman adalah faktor-faktor risiko yang berkaitan dengan jangkitan EV-A71. Epitop peneutralan yang ditemui boleh menyumbang kepada pembangunan vaksin atau terapi antibody monoklonal.

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°C	Degree Celsius
µg	Microgram
µl	Microliter
µM	Micromolar
x g	Gravitational acceleration
3Dpol	RNA-dependent RNA polymerase 3D
Anx2	Annexin II
ATCC	American Type Culture Collection
BE	Brain encephalitis
bp	Base pair
CCL5	Chemokine (C-C motif) ligand 5
cDNA	Complementary deoxyribonucleic acid
CI	Confidence intervals
CMC	Carboxylmethylcellulose
CNS	Central nervous system
CO_2	Carbon dioxide
CPE	Cytopathic effects
CSF	Cerebrospinal fluid
CV	Coxsackievirus
DMEM	Dulbecco's minimum Eagle's medium
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
DTT	Dithiothreitol
eIF4G	Eukaryotic initiation factor 4G
ER	Endoplasmic reticulum
EV-A71	Enterovirus A71
FBS	Foetal bovine serum
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte macrophage colony-stimulating factor
GMT	Geometric mean titres
HFMD	Hand, foot and mouth disease
IFN-α	Alpha interferon
IFN-β	Beta interferon
IFN-γ	Gamma interferon
Ig	Immunoglobulin
IL	Interleukin
IL-1Ra	IL-1 receptor antagonist
IP-10	Interferon gamma-induced protein 10
IRES	Internal ribosomal entry site
IVIg	Intravenous immunoglobulin
kb	Kilobase
KL	Kuala Lumpur
LB	Luria-Bertani
mAb	Monoclonal antibody
MCP-1	Monocyte chemoattractant protein-1
mg	Milligram
MHC	Major histocompatibility complex
MIP-1β	Macrophage inflammatory protein 1β
ml	Milliliter

mM	Milimolar
mRNA	Messenger ribonucleic acid
MYR	Malaysian ringgit
ng	Nanogram
nm	Nanometer
nt	Nucleotides
OR	Odds ratios
PABP	Poly(A)-binding protein
PCR	Polymerase chain reaction
PE	Pulmonary oedema
pfu	Plaque forming units
pg	Picogram
poly(I:C)	Polyriboinosinic:polyribocytidilic acid
PSGL-1	P-selectin glycoprotein ligand-1
R_0	Basic reproduction ratio
RD	Rhabdomyosarcoma
RE	Restriction enzyme
RNA	Ribonucleic acid
SCARB2	Scavenger receptor B2
SFM	Serum-free medium
TAE	Tris-acetate-EDTA buffer
$TCID_{50}$	Tissue culture infectious dose
TLR	Toll-like receptor
TNF	Tumour necrosis factor
USD	US dollar
UTR	Untranslated region
UV	Ultraviolet
VLP	Virus-like particles
VP	Viral protein
VPg	Viral protein genome-linked
w/v	Weight per volume

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Enterovirus A71 (EV-A71) is one of the major causative agents of hand, foot and mouth disease (HFMD) other than coxsackievirus A16 (CV-A16). EV-A71 was first isolated in California, USA in 1969. After 1970, EV-A71 had been isolated in epidemics around the world. Since 1997, large epidemics had been documented mainly in Asia regions including Taiwan (Ho *et al.*, 1999; Huang *et al.*, 2009; Wu *et al.*, 2013), mainland China (Ji *et al.*, 2012; Li *et al.*, 2013) and Singapore (Ang *et al.*, 2009; Ang *et al.*, 2015). A 3-year cyclical pattern of EV-A71-associated HFMD was documented in Japan (Iwai *et al.*, 2009), Singapore (Ang *et al.*, 2015) and recently in Cambodia (Horwood *et al.*, 2016). In 1997, the first EV-A71-associated epidemic was documented in Malaysia. The recurrence of an epidemic occurred in late 2000, 2003, 2005, early 2006, 2008/2009 and 2012 (Chan *et al.*, 2011; Chan *et al.*, 2012; Chua *et al.*, 2007; Chua & Kasri, 2011; Herrero *et al.*, 2003). A clear 3-year cyclical pattern of EV-A71-associated HFMD was shown in Sarawak, with epidemics occurring in the years 1997, 2000, 2003 and 2006 (Chan *et al.*, 2011; Sham *et al.*, 2014).

HFMD is usually characterised by low grade fever, lymphadenopathy, vesicles on the buccal mucosa and tongue, and vesicular or maculopapular rash on hands, feet and buttocks. Other than HFMD or herpangina, EV-A71 can also be associated with severe disease with cardiopulmonary complication such as cardiorespiratory failure, neurogenic pulmonary oedema or haemorrhage and myocarditis (Chan *et al.*, 2000; Ooi *et al.*, 2010; Yip *et al.*, 2013); and neurological complications such as aseptic meningitis, acute flaccid paralysis, encephalomyelitis and brainstem encephalitis (Chan *et al.*, 2000; Ong & Wong, 2015; Yip *et al.*, 2013). Severe disease occurs in children <2

years old with decreased risk as the age increases (Sabanathan *et al.*, 2014). Some risk factors associated with mild and severe HFMD/herpangina were defined previously. Most of the studies were conducted in main city and urban areas. The most important risk factor is young age, mainly <4 years old (Chang *et al.*, 2002; Fang *et al.*, 2014; Huang *et al.*, 2014; Li *et al.*, 2014). Other than that, attending kindergarten or nursery and living in rural areas were also identified risks (Chen *et al.*, 2014; Suzuki *et al.*, 2010; Zeng *et al.*, 2013). The key factor to reduce the transmission of EV-A71 is improved hygiene especially hand washing. Besides that, the risk of getting severe HFMD could be reduced by cleaning faucets after hand washing (Huang *et al.*, 2014) and being correctly diagnosed on the first visit to the hospital (Fang *et al.*, 2014).

To date, only molecular epidemiology of EV-A71 has been documented in Malaysia. Seroprevalence data on EV-A71 infection, however, has not been documented. This study is the first study to determine the seroepidemiology of EV-A71 infection in children and urban and rural general populations in Malaysia.

Neutralising antibodies are important as protective immunity in EV-A71 infection. The development of these antibodies may explain the 3-year cyclical pattern in EV-A71-associated HFMD (Gantt *et al.*, 2013). Infants <6 months old are usually protected by the maternal antibodies; however, these antibodies wane after 6 months of life (Luo *et al.*, 2009; Mao *et al.*, 2010; Tran *et al.*, 2011; Wang *et al.*, 2012). At this age, infants become vulnerable and susceptible to EV-A71 infection. The incidence rates of severe HFMD and fatality cases are thus high in this age group (Chang *et al.*, 2002). Neutralising antibodies bind to a region on surfaces of a virus called neutralisation epitopes and lead to the neutralisation of the virus. Many studies had identified these epitopes, mainly in animals (Chang *et al.*, 2010; Foo *et al.*, 2007; Jiang *et al.*, 2015; Kiener *et al.*, 2014; Liu *et al.*, 2011; Xu *et al.*, 2014; Zhang *et al.*, 2016) and one study described the B-cell epitopes in human (Gao *et al.*, 2012). The major neutralising

epitope was found on the capsid protein VP1, spanning the amino acids at position 208-222 (Foo *et al.*, 2007), but many recent studies found other neutralising epitopes on VP2, VP3 and VP4 capsids (Jiang *et al.*, 2015; Kiener *et al.*, 2014; Liu *et al.*, 2011; Xu *et al.*, 2014; Zhang *et al.*, 2016). This study further determined other neutralising epitopes by generating EV-A71 neutralisation escape mutants. Infectious clones carrying the escape mutations were constructed through site-directed mutagenesis. Mouse and human sera were used to determine the neutralising activity of these sera against these constructed virus escape mutants.

1.2 Objectives of the study

Currently, no study of seroepidemiology of EV-A71 infection has been done in Malaysia, either among children, or Malaysia urban and rural populations, unlike in other affected countries. Living in rural areas has been shown to be a risk factor for EV-A71, but risk factors associated with the infection in rural areas are not clearly defined. Neutralisation epitopes of EV-A71 are important in conferring protection against virus infection. Previous studies have used synthetic peptides to identify important epitopes. More studies are needed to discover more neutralisation epitopes. Thus, the main objectives of the study are to determine the seroprevalence and epidemiologic pattern of EV-A71 infection in Malaysia and to discover more neutralisation epitopes of EV-A71. The specific aims of the current study are:

1. To determine the seroepidemiology of EV-A71 infection among children up to 12 years in Kuala Lumpur, Malaysia.
2. To compare the seroepidemiology of EV-A71 infection among rural Orang Asli and urban Kuala Lumpur populations in West Malaysia.
3. To determine the risk factors associated with EV-A71 seropositivity in rural Orang Asli populations.

4. To discover more neutralisation epitopes by generating EV-A71 neutralisation escape mutants.

CHAPTER 2

LITERATURE REVIEW

2.1 The virology of EV-A71

2.1.1 Classification

Enteroviruses belong to the *Picornaviridae* family, which is divided into 26 genera: *Aphthovirus*, *Aquamavirus*, *Avihepatovirus*, *Avisivirus*, *Cardiovirus*, *Cosavirus*, *Dicipivirus*, *Enterovirus*, *Erbovirus*, *Gallivirus*, *Hepatovirus*, *Hunnivirus*, *Kobuvirus*, *Megrivirus*, *Mischivirus*, *Mosavirus*, *Oscivirus*, *Parechovirus*, *Pasivirus*, *Passerivirus*, *Rosavirus*, *Salivirus*, *Sapelovirus*, *Senecavirus*, *Teschovirus* and *Tremovirus* (Adams *et al.*, 2015).

Initially, the enteroviruses were classified into four subgroups based on their pathogenicity in humans and experimental animals and their cytopathic effects in tissue cultures: polioviruses (three serotypes), coxsackie group A (types 1-22, 24), coxsackie group B (types 1-6), and echoviruses (types 1-7, 9, 11-27, 29-34) (Ho, 2000; Solomon *et al.*, 2010; Yi *et al.*, 2011). After 1974, due to the limitations of this system, the enteroviruses were designated numerically beginning with enterovirus type 68. The original classifications have been replaced by a new typing method based on molecular and biological properties of the viruses (Nasri *et al.*, 2007). Thus, based on the phylogenetic clustering and the specific genes shared by the genome, the enteroviruses have been separated into four species (A-D), containing more than 100 types (Bessaud *et al.*, 2012; Wei *et al.*, 2011). The three serotypes of poliovirus were classified into the human enterovirus C species because they are genetically closely related (Brown *et al.*, 2003).

To date, *Enterovirus* species is divided into enterovirus A-H, enterovirus J and rhinovirus A-C (Table 2.1). EV-A71 is classified as a member of enterovirus species A (Brown & Pallansch, 1995).

2.1.2 Genome organisation and gene functions

EV-A71 is about 30 nm in size, non-enveloped and consists of icosahedral particles with single-stranded RNA of positive polarity (reviewed in Chan *et al.*, 2011). The RNA genome is approximately 7.4 kb in length, which encodes a long polyprotein with a single open reading frame and is flanked by untranslated regions (UTR) at the 5' and 3' ends (Kung *et al.*, 2014; Li *et al.*, 2011). The polyprotein can be cleaved into P1, P2 and P3 regions. The P1 region encodes the capsid protein which is comprised of four structural proteins VP1, VP2, VP3 and VP4. The P2 and P3 regions encode the nonstructural proteins including 2A-C and 3A-D, which are responsible for virus replication (reviewed in Chan *et al.*, 2011; Li *et al.*, 2011; Zhang *et al.*, 2010) (Figure 2.1).

2.1.2.1 Untranslated regions and their functions

The 5' UTR of the enteroviruses contain an internal ribosomal entry site (IRES) instead of the cap structure, unlike most of the mRNA in eukaryotic cells (Wang *et al.*, 2015). Type I IRES, which is approximately 500 nt, is mainly found in poliovirus, rhinovirus, coxsackievirus and EV-A71 (Huang & Shih, 2014; Lin *et al.*, 2009). In EV-A71, the 5' UTR consists of seven domains of conserved regions (Zell & Stelzner, 1997) (Figure 2.1). Domain I forms a cloverleaf structure whereas domains II-VI form the type I IRES, which is important for the cap-independent translation of the viral polyprotein (Balvay *et al.*, 2009). The enterovirus 3' UTR was initially thought to

Table 2.1: Current classification of *Enterovirus*

Species	Serotypes	Members
Enterovirus A	25	Coxsackievirus A2-8, A10, A12, A14, A16; enterovirus A71, A76, A89-92, A114, A119-121; simian enterovirus SV19, SV43, SV46; baboon enterovirus A13 (BA13)
Enterovirus B	63	Coxsackievirus A9, B1-B6; echovirus 1-7, 9, 11-21, 24-27, 29-33; enterovirus B69, B73-75, B77-88, B97-98, B100, B101, B106-107, B110-113; simian enterovirus SA5; swine vesicular disease virus SVDV-1, SVDV-2 (porcine variant of CV-B5)
Enterovirus C	23	Poliovirus 1-3; coxsackievirus A1, A11, A13, A17, A19-22, A24; enterovirus C95-96, C99, C102, C104-105, C109, C113, C116-118
Enterovirus D	5	Enterovirus D68, D70, D94, D111, D120
Enterovirus E	4	Enterovirus E1-E4
Enterovirus F	6	Enterovirus F1-F6
Enterovirus G	16	Enterovirus G1-G16
Enterovirus H	1	Enterovirus H1
Enterovirus J	6	Simian enterovirus SV6; enteroviruses J103, J108, J112, J115, J121
Rhinovirus A	80	Rhinovirus A1-2, A7-13, A15-16, A18-25, A28-34, A36, A38-41, A43, A45-47, A49-51, A53-68, A71, A73-78, A80-82, A85, A88-90, A94, A96, A100-109
Rhinovirus B	32	Rhinovirus B3-6, B14, B17, B26-27, B35, B37, B42, B48, B52, B69-70, B72, B79, B83-84, B86, B91-93, B97, B99-106
Rhinovirus C	55	Rhinovirus C1-55

Information in this table is adapted from information available on the Picornavirus Study Group (www.picornaviridae.com; www.picornastudygroup.com)

consist of two common stem-loop domains X and Y (Zoll *et al.*, 2009). However, stem-loop domain Z was identified in the 3' UTR of coxsackievirus B3 and all enterovirus B, and in enterovirus A, domain Z was proposed as a novel evolutionary marker for recombination (Kok & Au, 2013; Merkle *et al.*, 2002). The functional role of the enteroviral 3' UTR is unclear but it most probably plays a role in virus replication and translation (Kok *et al.*, 2011; Zoll *et al.*, 2009). In a previous study, it was also shown that tertiary structure is critical for the ability of the virus to replicate (Mirmomeni *et al.*, 1997).

2.1.2.2 Structural proteins

The P1 region of picornaviruses consists of four capsid proteins VP1-VP4 (Lin *et al.*, 2009). Five distinct particles have been identified in human enteroviruses: putative procapsid, provirion, mature infectious virus, the altered "A-particle" and empty capsid. VP0, VP1 and VP3 form the protomers; five protomers assemble into a pentameric assembly subunit, and twelve of these subunits further assemble into a naturally occurring empty capsid or procapsid, then into the provirion, a short-lived intermediate, through two proposed pathways. In the first pathway, after the twelve pentamers form the procapsid, the genome is inserted to form the provirion. In the second pathway, the twelve pentamers assemble around the viral genome and form the provirion. The procapsid serves as a reservoir of capsid components in infected cells and become the off-pathway assembly byproducts. After the formation of the provirion, VP0 is cleaved into VP2, which map to the capsid exterior, and VP4 which is internalised. The cleavage results in the formation of native virus, then to the A-particle upon binding to a cellular receptor. The transition to the A-particle is accompanied by expulsion of VP4 and externalisation of the VP1 N-terminal. An unknown secondary trigger causes some

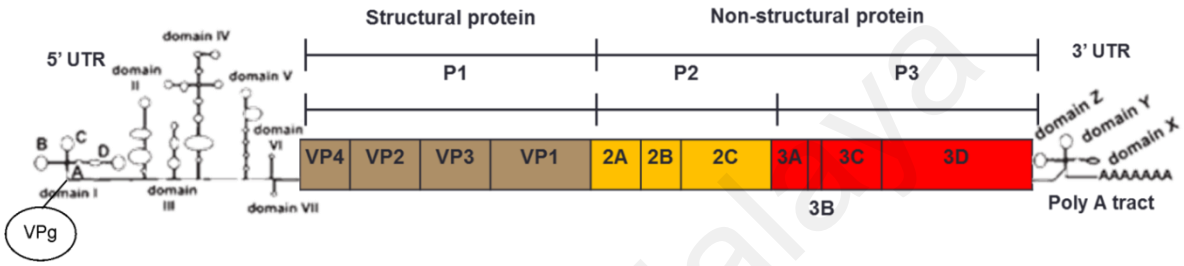


Figure 2.1: Schematic illustration of EV-A71 genome organisation. The figure was adapted with modifications from Chan *et al.* (2011). (VPg = viral protein genome-linked; UTR = untranslated region)

genome to be released, leaving behind an empty capsid (summarised by Shingler *et al.*, 2013).

Because VP1, VP2 and VP3 are located on the virion surface, these proteins are responsible for cellular receptor binding and are antigenic determinants (Liu *et al.*, 2014). The loop regions are the most variable regions and important neutralising immunogenic sites. The surface loops of VP1 are located around icosahedral fivefold axes. A difference between the loop of EV-A71 VP1 and that of other picornaviruses is the smaller loop size and shallower canyon (Plevka *et al.*, 2012). The most prominent and variable regions of VP2 and VP3 are called "puff" and "knob", respectively. These regions are involved in binding to non-immunoglobulin-like receptors in some picornaviruses and may have the same function in EV-A71 (Plevka *et al.*, 2012).

2.1.2.3 Non-structural proteins

The P2 and P3 regions represent the non-structural protein regions of the picornaviruses. The P2 region consists of 2A, 2B and 2C, while P3 consists of 3A, 3B, 3C and 3D. These proteins participate in virus replication.

The EV-A71 2A protein participates in the cleavage of the P1 region from the P2 and P3 regions (Huang *et al.*, 2011). This 2A protein has a protease activity, which cleaves the eukaryotic initiation factor 4G (eIF4G), which in turn, shuts off IRES-mediated translation initiation and host protein synthesis (Huang *et al.*, 2011; Shih *et al.*, 2011). The 2A protein also cleaves the poly(A)-binding protein (PABP) which inhibits cellular translation (Lin *et al.*, 2009; Shih *et al.*, 2011).

The enterovirus 2B protein is predominantly localised at the Golgi complex and contains two hydrophobic regions HR1 and HR2, which are amphipatic α-helix domain (de Jong *et al.*, 2003). The expression of 2B protein can alter cellular calcium homeostasis and modulates apoptosis (Campanella *et al.*, 2004; de Jong *et al.*, 2008; van

Kuppeveld *et al.*, 2005). This mechanism results in inhibition of protein trafficking through the Golgi complex (de Jong *et al.*, 2008).

The 2C protein plays a role in the assembly of mature virions by affecting the association of capsid precursors either with other capsid precursors or with RNA to facilitate viral RNA encapsidation (Vance *et al.*, 1997). In some picornaviruses such as poliovirus and coxsackievirus B3, 3A protein inhibits the ER-to-Golgi traffic (Choe *et al.*, 2005; Doedens & Kirkegaard, 1995). In poliovirus-infected cells, the 3A protein reduces the secretion levels of antiviral cytokines interleukin-6 (IL-6), IL-8 and beta interferon (IFN-β) (Dodd *et al.*, 2001). The poliovirus 3A protein also inhibits major histocompatibility complex (MHC) I-dependent antigen presentation (Deitz *et al.*, 2000) and suppresses tumour necrosis factor (TNF) mediated apoptosis by eliminating the TNF receptors from the cell membrane (Neznanov *et al.*, 2001).

In poliovirus, 3B protein, or viral protein genome-linked (VPg), is a 22-amino acid peptide that is covalently linked to its 5' end and is polyadenylated at its 3' end (Pathak *et al.*, 2008). VPg is the soluble product of the 3AB cleavage and serves as the protein primer for RNA replication. A "hot spot" spanning the 100-104 amino acid residues in the 3B (VPg) region binds to 3D polymerase and mediates the binding of 3AB protein to the polymerase (Strauss & Wuttke, 2007).

The EV-A71 3C protein is essential for viral replication (Lei *et al.*, 2013) and has protease activity that is responsible for the cleavage of the large virus polyprotein precursor into the functional, structural and replication proteins (Wang *et al.*, 2011). The EV-A71 3C protein can bind to multiple components of the innate immune system that lead to the suppression of type-I IFN.

RNA-dependent RNA polymerase 3D or $3D^{pol}$ of poliovirus catalyses the elongation of the RNA chain in a primer- and template-dependent manner (Burns *et al.*, 1989). This polymerase initiates viral transcription and replication by uridylating VPg (Jiang *et*

al., 2011). However, $3D^{pol}$ is error-prone which increases mutation rates resulting in quasispecies. This is important for the survival of the RNA virus population due to increased viral fitness and virulence under selective pressure (Pfeiffer & Kirkegaard, 2005).

2.2 Pathogenesis and clinical manifestations

2.2.1 Pathogenesis of EV-A71

Pathogenesis covers the mechanisms by which infection leads to disease. Pathogenic mechanisms include portal of entry, viral spread and host factors. Interactions between the virus and host factors determine the virulence of the viral disease.

EV-A71 is mainly transmitted via the faecal-oral route, and also through direct contact with infected saliva, vesicle fluid or respiratory droplets, or contaminated surfaces (Chan *et al.*, 2011). Upon entry, EV-A71 is presumed to initially replicate in the gastrointestinal system, and later spread to the respiratory system through the blood (Wang *et al.*, 2004). The virus later disseminates to the reticuloendothelial system (liver, spleen, bone marrow and lymph nodes), heart, lung, pancreas, skin, mucous membranes and CNS possibly through retrograde axonal pathway (Chen *et al.*, 2007; Wong *et al.*, 2010). Shedding of EV-A71 can occur in the throat until 2 weeks after an acute infection and EV-A71 can be isolated for up to 11 weeks later from stool (Chung *et al.*, 2001).

EV-A71 receptors play an important role in early stages of the viral infection, species and tissue tropism as well as virus pathogenesis. Several EV-A71 receptors have been identified including scavenger receptor B2 (SCARB2), P-selectin glycoprotein ligand-1 (PSGL-1), sialylated glycan, heparan sulphate and annexin II (Anx2) (reviewed by Yamayoshi *et al.*, 2014). SCARB2 is the most important receptor for EV-A71 and is most abundantly found in the lysosomal membrane, participating in membrane transport

and reorganisation of the endosomal/lysosomal compartment (Yamayoshi & Koike, 2011). This receptor is expressed in a wide variety of tissue including neurons in the CNS, lung pneumocytes, hepatocytes, renal tubular epithelium, splenic germinal centers and intestinal epithelium (Yamayoshi *et al.*, 2012; Yamayoshi *et al.*, 2014). The PSGL-1 receptor can be found restrictively in myeloid, lymphoid, dendritic lineages and platelets (Laszik *et al.*, 1996), but not in the neurons of the CNS and the tonsillar crypt epithelium (He *et al.*, 2013). Human PSGL-1, which is expressed on leukocytes, has a major role in early inflammation and has been proven as a functional receptor for EV-A71 (Nishimura *et al.*, 2009). PSGL-1 is involved in viral attachment and internalisation but not viral uncoating (Yamayoshi *et al.*, 2012). Sialylated glycans are abundantly expressed in gastrointestinal and respiratory epithelial cells (Yang *et al.*, 2009). Sialylated glycans are important for EV-A71 attachment to rhabdomyosarcoma (RD), SK-N-SH cells and DLD-1 intestinal cells. Incubation with other sialylated glycans significantly diminished EV-A71 attachment to RD and SK-N-SH cells and reduced EV-A71 replication in the DLD-1 intestinal cells (Su *et al.*, 2012; Yang *et al.*, 2009). Heparan sulphate is expressed in all cell types and plays important roles in a variety of biological events such as cell proliferation, cancer development and viral infection (Dulaney *et al.*, 2015; Tan *et al.*, 2012). This receptor has been shown to be an important attachment receptor in different cell lines for EV-A71 (Tan *et al.*, 2012) and other enteroviruses (Goodfellow *et al.*, 2001; Zautner *et al.*, 2006; Zautner *et al.*, 2003). Anx2 is abundantly expressed on endothelial cells, monocyte/macrophages, early myeloid cells and some tumour cells (Hajjar & Acharya, 2000). Anx2 binds to the VP1 capsid of the EV-A71 and enhances its infectivity (Yang *et al.*, 2011); however, viral entry and uncoating of EV-A71 via this receptor has not been reported (Yamayoshi *et al.*, 2014).

2.2.2 Clinical manifestations of EV-A71

EV-A71 is often associated with HFMD and herpangina. HFMD is a common childhood disease which usually occurs in children younger than 5 years old. It is commonly characterised by low grade fever, lymphadenopathy, vesicles on the buccal mucosa and tongue, and a vesicular or maculopapular rash on hands, feet and buttocks. The incubation period of this disease is usually 3 to 7 days. Besides EV-A71, CV-A16 and other enteroviruses including CV-A4, A5, A10, B2 and B5 are also associated with HFMD (Chan *et al.*, 2011). In herpangina, the lesions occur in the oral cavity including the fauces, uvula and soft palate. Most cases of HFMD and herpangina are mild and self-limiting and hospitalisation is not needed.

Unlike CV-A16 that usually causes mild infections, EV-A71 can be associated with more severe cardiopulmonary and neurological complications, mainly in children <2 years old, with decreased risk when age increases (Ho, 2000; Lu *et al.*, 2002; Sabanathan *et al.*, 2014). In many countries, EV-A71 has caused several cardiopulmonary complications such as cardiorespiratory failure, neurogenic pulmonary oedema or haemorrhage and myocarditis (Chan *et al.*, 2000; Ooi *et al.*, 2010; Yip *et al.*, 2013), and neurological complications such as aseptic meningitis, acute flaccid paralysis, encephalomyelitis and brainstem encephalitis (Chan *et al.*, 2000; Ong & Wong, 2015; Yip *et al.*, 2013). The rate of severe EV-A71 cases ranges from 0.1% to 1.1%, and the case-fatality rate ranges from 0.01% to 0.03% (Gao *et al.*, 2014; Sabanathan *et al.*, 2014; Xing *et al.*, 2014; Zhang *et al.*, 2011).

2.3 Immune responses and protection

Upon infection with EV-A71, host immune responses are activated to restrict virus dissemination and protect from virus invasion. Innate immunity often acts as the first defence mechanism (reviewed by Pathinayake *et al.*, 2015), followed by specific

cellular immunity which eradicates the virus. In EV-A71 infection, the formation of neutralising antibodies is important in protecting individuals from subsequent infection (reviewed in Huang *et al.*, 2012).

2.3.1 Immune responses in animal models and cell culture

Type-I IFNs provide first line defence against EV-A71 infection. Type-I IFNs include IFN-α, which is secreted by white blood cells, and IFN-β, which is produced by fibroblasts and several epithelial cells (Huang *et al.*, 2012). The administration of type-I IFNs was shown to protect against CV-A16 and EV-A71 infections in mice (Sasaki *et al.*, 1986). The introduction of polyriboinosinic:polyribocytidilic acid [poly(I:C)], a potent IFN inducer, improved survival rates and protected mice from EV-A71 infection (Liu *et al.*, 2005). IFN-α4, IFN-α6, IFN-α14 and IFN-α16 showed potent antiviral activity *in vitro* before, upon or after EV-A71 infection (Yi *et al.*, 2011). In human intestinal epithelial cells, EV-A71 infection induced toll-like receptor 7 (TLR7) and TLR8, which may be essential for IFN-β production. This finding might explain the mild gastrointestinal symptoms of EV-A71 infection (Chi *et al.*, 2013). EV-A71 replication together with massive proinflammatory cytokines production increases EV-A71 pathogenicity. The IL-6 level plays an important role in the immunopathogenesis of EV-A71 infection in mice. Upon infection with EV-A71, sustained high levels of IL-6 lead to more severe tissue damage and death in untreated mice, whereas mice treated with anti-IL-6 showed reduced tissue damage. These findings suggest potential treatment for severe EV-A71 infection (Khong *et al.*, 2011). In another study, mice that were systemically administered IL-6, IL-13 and IFN-γ developed mild pulmonary oedema (PE) and severe emphysema (Huang *et al.*, 2011). In EV-A71-susceptible human intestinal epithelial cells HT-29, upregulation of IL-6, chemokine (C-C motif) ligand 5 (CCL5) and interferon gamma-induced protein 10 (IP-10) may play a major

role in pathogenicity, but robust IFN-β production might provide sufficient local antiviral induction, thus resulting in mild gastrointestinal symptoms (Chi *et al.*, 2013). EV-A71 infection was shown to increase IP-10 expression, resulting in increasing serum and brain IFN-γ, and protecting the mice by reducing viral burden in multiple tissues (Shen *et al.*, 2013).

2.3.2 Immune responses in humans

Different cytokine expressions are observed in blood and CSF of EV-A71-induced HFMD patients with different severity. Many studies showed that brain encephalitis (BE) and PE are associated with significantly higher levels of IL-6, TNF-α and IL-1β (Gantt *et al.*, 2013; Griffiths *et al.*, 2012; Wang *et al.*, 2012; Ye *et al.*, 2015; Zhang *et al.*, 2013). Several cytokines were also shown to be elevated in serum and CSF in severe or critical patients compared to mild patients and normal controls, including IL-2, IL-8, IL-10, IL-13, IL-23, IL-33, IL-1 receptor antagonist (IL-1Ra), granulocyte colony-stimulating factor (G-CSF), IFN-γ, granulocyte macrophage colony-stimulating factor (GM-CSF), macrophage inflammatory protein 1β (MIP-1β), monocyte chemoattractant protein-1 (MCP-1), and IP-10 (Griffiths *et al.*, 2012; Wang *et al.*, 2012; Wang *et al.*, 2014; Ye *et al.*, 2015). IL-1β was found to impair heart function, but IL-1Ra and G-CSF, its natural antagonist, are promising as treatments for acute cardiac dysfunction and are currently being assessed (Griffiths *et al.*, 2012).

2.3.3 Neutralising antibodies and neutralisation

The formation of neutralising antibodies against EV-A71 infection may control EV-A71 infection and progress of the disease. Population levels of neutralising antibodies may explain the cyclical pattern of the HFMD epidemics caused by EV-A71 (Gantt *et al.*, 2013). The time intervals between outbreaks might be related to maternal immunity.

Infants who were born between 6 months to 1 year after an epidemic are still protected by the mother's immunity, which is the major immune barrier in protecting neonates from infections until a point of time where the antibody starts to wane (Mao *et al.*, 2010; Wang *et al.*, 2012). Higher age-specific incidence rates are observed in children aged 0 to 4 years old, and the rates decline as age increases (Ang *et al.*, 2009; Chen *et al.*, 2007). In this age group, the seroprevalence rate of EV-A71 neutralising antibodies was the lowest, especially in children aged >6 months to 2 years old (Akhmadishina *et al.*, 2014; Castro *et al.*, 2005; Ji *et al.*, 2012; Linsuwanon *et al.*, 2014; Tran *et al.*, 2011). Many studies show that 50-55% of neonates have detectable maternal EV-A71 neutralising antibodies, which declined to undetectable levels in 98-99% of infants by the age of 6 months old (Luo *et al.*, 2009; Mao *et al.*, 2010; Tran *et al.*, 2011; Wang *et al.*, 2012). The seroprevalence rate of EV-A71 neutralising antibodies was shown to increase with age (Akhmadishina *et al.*, 2014; Ang *et al.*, 2015; Ji *et al.*, 2012; Li *et al.*, 2013; Linsuwanon *et al.* 2014; Rabenau *et al.*, 2010; Tran *et al.*, 2011; Yang *et al.*, 2011). This supports the idea that adults have increased humoral immunity and are protected by long-lasting immunity (Rabenau *et al.*, 2010; Yang *et al.*, 2011).

Neutralising antibodies bind to neutralisation epitopes on the viral surface, leading to neutralisation of the virus. Monoclonal antibody (mAb) recognises more specific pathogen epitopes of its targeting pathogens. A summary of reported EV-A71 neutralising antibodies is shown in Table 2.2. In the context of EV-A71, it is ideal for a mAb to have a broad neutralising activity that recognises highly conserved region in all EV-A71 subgenotypes (Kiener *et al.*, 2014; Kok, 2015; Lim *et al.*, 2012; summarised by Ng *et al.*, 2015).

A previous study identified SP70 (amino acids 208-222 on VP1) as a neutralising epitope, and anti-SP70 neutralising antibodies were able to confer 80% *in vivo* protection against a lethal dose of EV-A71 (Foo *et al.*, 2007). Previous studies in China

Table 2.2: Summary of reported EV-A71 neutralising antibodies

Neutralising antibody	EV-A71 genotype	Epitope sequence	Amino acid position	Reference
mAb N1 mAb N3 mAb N4 mAb N6	Strain EV71/59, subgenotype B4	FGEHKQEKDLEYGAC	211-224, VP1	Chang *et al.*, 2011
mAb 4E8	Strain Hn2 (GQ994992), subgenotype C4	SSKSEYSLVI RIYMRMKHVR	240-250, VP1 250-260, VP1	Chang *et al.*, 2010
mAb 2G8	Strain AH/08/06 (HQ611148), subgenotype C4	YPTFGEHKQEKDLEYC	208-222, VP1	Deng *et al.*, 2015
anti-SP70 antibodies	Immunised with synthetic peptide SP70 derived from strain 5865/SIN/000009 (AF316321), subgenotype B4	YPTFGEHKQEKDLEYC	208-222, VP1	Foo *et al.*, 2007
mAb H3B10 mAb K8G2	Not known	KQEK	215-218, VP1	He *et al.*, 2012
mAb 7C7	EV-A71 subgenotype B5	EDSHP	142-146, VP2	Kiener *et al.*, 2012
mAb 10D3	Strain 5865/SIN/000009 (AF316321), subgenotype B4	P, A, E (P59L, A62D & E67D abolish neutralising activity)	59, 62, 67, VP3 "knob"	Kiener *et al.*, 2014
mAb D5 mAb H7 mAb C4	Immunised with EV-A71 VLPs (coexpression of P1 and 3CD proteins) derived from EV71 strain G082	VP1 protein, but not VP0 protein	Not known	Ku *et al.*, 2012
mAb MA28-7	Strain 1095, subgenotype C2	Glycine (VP1-145)	145, VP1	Lee *et al.*, 2013
mAb clone 22A12	Immunised with synthetic peptide SP70 derived from strain 5865/SIN/000009 (AF316321), subgenotype B4	YPTFGEHKQEKDLEYC	208-222, VP1	Li et al., 2009
mAb 4	Strain NUH0083/SIN/08 (FJ461781), subgenotype B5	IGDSVSRA	12-19, VP1	Lim *et al.*, 2013
mAb 51	Strain NUH0083/SIN/08 (FJ461781), subgenotype B5	KQEKD	215-219, VP1	Lim *et al.*, 2012
BB1A5	Strain52-3 (FJ600325), subgenotype C4	TEDSHPPYKQTQPGA (T141A, E142A, S144A & H145A abolish neutralising activity)	141-155, VP2	Xu *et al.*, 2014
anti-VP4N20 antibodies	Immunised with HBc-N149-VP4N20 VLPs derived from strain BJ08, subgenotype C4	GSQVSTQRSGSSHENSN SATE	1-20, VP4	Zhao *et al.*, 2013

mAb: monoclonal antibody

produced mAbs targeting the SP70 epitope which demonstrated strong neutralising activities (Deng *et al.*, 2015; Li *et al.*, 2009). Deng *et al.*, (2015) showed that mAb 2G8 generated from EV-A71 strain AH/08/06-immunised mice conferred full protection against lethal doses of EV-A71 in suckling mice. The mAb, which belonged to the IgM subclass, neutralised EV-A71 at the attachment stage. Mutational analysis showed that a K218A mutant partially escaped neutralisation, while an L220A mutant completely abolished binding and neutralisation. *In vitro* neutralisation assays demonstrated that these two substitutions are critical for antibody neutralisation in natural infection (Deng *et al.*, 2015). In the other study, mAb clone 22A12 generated by immunising mice with SP70 was shown to have strong neutralising activity against EV-A71 infection in an *in vitro* neutralisation assay (Li *et al.*, 2009). A study in Singapore also generated a mAb that specifically targets the amino acids 215-219 (KQEKD) spanning the SP70 epitope. MAb 51 was able to confer 100% protection in mice when passively administered prior to lethal challenge. In addition, mAb 51 also protected the mice against pathologic changes such as neuropil vacuolation and neuronal loss in the spinal cord (Lim *et al.*, 2012).

Apart from VP1, other studies have identified more neutralisation epitopes located on the other capsid proteins, VP2 and VP3. Kiener *et al.* (2012) generated a mAb 7C7 derived from mice immunised with the EV-A71-B5 strain. This mAb was mapped to amino acids 142-146 (EDSHP) on the VP2 EF loop (Kiener *et al.*, 2012). This epitope lies within the VP2-28 synthetic peptide, which was previously reported to have cross-neutralising activity (Liu *et al.*, 2011). Mutational analysis showed that aspartic acid/asparagine mutation of the EV-A71 BrCr strain (genotype A) at position 143 on VP2 did not interfere with antibody recognition; however, a serine to threonine mutation at position 144 resulted in abolished antigenicity of the VP2 protein in the subgenotype C4 Fuyang-08 strain (Kiener *et al.*, 2012). Another recent study in

Singapore demonstrated the first conformational epitopes on the VP3 capsid protein which are conserved in different EV-A71 genotypes. MAb 10D3 was generated by immunising the mice with an EV-A71-B4 strain. Eight escape mutants from different EV-A71 genotypes were isolated and all mutants were found to harbour mutations at three amino acid positions, either at positions 59, 62 or 67 in the VP3 "knob" region. Reverse genetically-engineered viruses were generated by introducing the mutations, either alone or in tandem. It was found that the viruses with P59L, A62D, A62P and E67D mutations abolished binding by mAb 10D3 and were able to evade neutralisation by the mAb (Kiener *et al.*, 2014).

Besides investigating neutralisation activities, mAbs and synthetic peptides are also used to identify the locations of EV-A71 neutralising or B-cell epitopes, specific either to humans or animals. These epitopes are important for future effective vaccine production and mAb treatment against EV-A71. Previously recognised epitopes are summarised in Table 2.3.

2.4 Epidemiology of EV-A71

2.4.1 Epidemics of EV-A71 infection/HFMD

2.4.1.1 Epidemics worldwide

EV-A71 was first isolated in California, USA in 1969, from a child diagnosed with aseptic meningitis (Ho, 2000). Since then, EV-A71 was isolated in epidemics in USA (1972), Australia (1972), Sweden (1973), Bulgaria (1975), Hungary (1978), Japan (1973 and 1978), and France (1979) (Ho, 2000; Hagiwara *et al.*, 1979). After the 1990s, many countries, especially in the Asia-Pacific region, have been affected by EV-A71 outbreaks: Taiwan (1998, 2002, 2004-2005, 2008-2009) (Ho *et al.*, 1999; Huang *et al.*, 2009; Wu *et al.*, 2013), mainland China (2008, 2010) (Ji *et al.*, 2012; Li *et al.*, 2013), Singapore (2000, 2002, 2005, 2009, 2012) (Ang *et al.*, 2009; Ang *et al.*, 2015), Vietnam

Table 2.3: Recognised neutralising/B-cell epitopes of EV-A71

Species	Neutralising/B-cell epitope	Amino acid position	Reference
Mouse	PESRESLAWQTATNPC	163-177 on VP1	Foo *et al.*, 2007
	YPTFGEHKQEKDLEYC	208-222 on VP1	Foo *et al.*, 2007
	HYRAHARDGVFDYYT	176-190 on VP3	Jiang *et al.*, 2015
	AGGTGTEDSHPPYKQ	136-150 on VP2	Liu *et al.*, 2011
	GSQVSTQRSGSHENSNSATE	1-20 on VP4	Zhang *et al.*, 2016
Rabbit*	DTGKVPALQAAE	40-51 on VP1	Gao *et al.*, 2012
	GEIDLPLEGTTN	91-102 on VP1	
	APKPDSRESPAW	160-171 on VP1	
	FGEHKQEKDLEY	211-222 on VP1	
Human*	DTGKVPALQAAE	40-51 on VP1	Gao *et al.*, 2012
	KVPALQAAEIGA	43-54 on VP1	
	LTIGNSTITTQE	16-27 on VP2	
	NRFYTLDTKLWE	61-72 on VP2	
	HQGALLVAVLPE	118-129 on VP2	
	YKQTQPGADGFE	148-159 on VP2	
	FHPTPCIHIPGE	28-39 on VP3	
	IHIPGEVRNLLE	34-45 on VP3	
	LLELCQVETILE	43-54 on VP3	
	RFPVSAQAGKGE	70-81 on VP3	
	NFTMKLCKDASD	223-234 on VP3	

*Recognised epitopes are B-cell epitopes

(2005, 2011-2012) (Khanh *et al.*, 2012; Tu *et al.*, 2007), Japan (1997, 2000) (Fujimoto *et al.*, 2002; Shimizu *et al.*, 1999), Cambodia (2012) (Horwood *et al.*, 2016), Thailand (2012) (Linsuwanon *et al.*, 2014; Puenpa *et al.*, 2014), Brunei (2006) (AbuBakar *et al.*, 2009), Korea (2008-2009) (Baek *et al.*, 2011), Hong Kong (1999, 2008) (Ma *et al.*, 2010), Austria (2002/2003) (Ortner *et al.*, 2009), and Australia (1999, 2000) (McMinn *et al.*, 2001). A 3-year cyclical pattern of EV-A71-associated HFMD was documented in Japan (Iwai *et al.*, 2009), Singapore (Ang *et al.*, 2015) and recently in Cambodia (Horwood *et al.*, 2016).

2.4.1.2 Epidemics in Malaysia

The first epidemic of HFMD in Malaysia occured in April 1997 in the state of Sarawak, and spread to peninsular Malaysia later in June. The HFMD was caused by highly neurovirulent EV-A71, which affected >2600 children and caused 48 deaths. In Sibu, Sarawak, subgenus B adenovirus was also isolated in all enterovirus-positive fatal cases (Cardosa *et al.*, 1999). Another epidemic occurred in late 2000, with 8 fatalities in Peninsular Malaysia due to CNS complications. A smaller epidemic occurred in 2003, beginning in Sarawak and later spreading to peninsular Malaysia. In 2005, EV-A71 recurred with 2 recorded deaths (Chua *et al.*, 2007). The epidemic spread to Sarawak in early 2006. More epidemics associated with EV-A71 occurred in 2008/2009 and 2012 (Chan *et al.*, 2011; Chan *et al.*, 2012; Chua *et al.*, 2007; Chua & Kasri, 2011; Herrero *et al.*, 2003). In Sarawak, which has a well-established surveillance system for HFMD, a clear 3-year cyclical pattern of EV-A71-associated HFMD was shown, with epidemics occurring in the years 1997, 2000, 2003 and 2006 (Chan *et al.*, 2011; Sham *et al.*, 2014).

2.4.2 Molecular epidemiology of EV-A71

EV-A71 genotypes are classified based on the nucleotide sequence identity of VP1, which has 80-85% identity (Chan *et al.*, 2010). Genotype A is represented by a single strain, prototype BrCr, which was first isolated in USA in 1969. There were no reports of epidemics caused by the BrCr strain until 2008, when the strain was isolated in a small epidemic in Anhui province, China. However, the source of the virus is unclear (Yu *et al.*, 2010). Genotype B has 5 lineages, B1-B5. B1 and B2 subgenotypes circulated between 1970 and 1990, and became predominant in mid-1980s. Subgenotypes B3 and B4 co-circulated with genotype C, and has emerged to caused epidemics in Southeast Asia since 1997. B5 strains were isolated in Japan (2003), Taiwan (2003, 2007-2008), Sarawak, Malaysia (2003) and Brunei (2006). A retrospective analysis identified the strain isolated in the Netherlands between 1963 and 1967 as subgenotype B0 (Podin *et al.*, 2006; van der Sanden *et al.*, 2009). Genotype C also has 5 lineages. Subgenotype C1, which replaced genotype B as the dominant strain, circulated in Europe and America at low levels in mid-1980s, except in Sydney where it has caused major community epidemic in 1986. Subgenotype C2 emerged in 1995 and caused large epidemics in Taiwan (1998), Australia (1999) and Japan (1997-1999, 2001-2002). C3 strains were isolated in Japan (1994) and China (1997), and emerged in Korea (2000) as the predominant strain in the EV-A71 epidemics there. Subgenotype C4 has been isolated in China since 1998 and has caused large epidemics there in recent years. This subgenotype was replaced by subgenotypes B5 and C5, which has been reported in Southern Vietnam and Taiwan (Brown *et al.*, 1999; McMinn, 2012; Solomon *et al.*, 2010; Wong *et al.*, 2010; Yi *et al.*, 2011; Yip *et al.*, 2013). Lastly, genotype D, which is genetically distinct from other EV-A71 strains, was isolated in India in 2002. This genotype was isolated from a child with acute flaccid paralysis and is represented by a single strain (Bessaud *et al.*, 2014; Deshpande *et al.*, 2003).

In Malaysia, only subgenotypes B3, B4, B5, C1 and C2 have been found to circulate despite the presence of many other different genotypes worldwide. During the first epidemic in 1997, four subgenotypes B3, B4, C1 and C2 were found to be co-circulating, in peninsular Malaysia. In 2000, only C1 and B4 subgenotypes were circulating in both peninsular Malaysia and Sarawak. Subgenotype C1, together with B5, caused a smaller epidemic in 2003. In 2005, subgenotype B5 strains were recognised to cause an epidemic, however, this B5 was found to be distinct from the B5 isolated in 2003. The B5 subgenotype was also the cause of an epidemic in Sarawak in early 2006. After 2007, subgenotype B5 strains have remained as the dominant circulating strains in Malaysia (Chan *et al.*, 2011; Chan *et al.*, 2012; Chua & Kasri, 2011; Yusof *et al.*, 2014).

The cyclical patterns of large epidemics are usually associated with genotype replacement; however, no associations have been found between genotypes and risks of severe neurological disease (Chan *et al.*, 2011; Chan *et al.*, 2012; Huang *et al.*, 2009; Tee *et al.*, 2010).

2.4.3 Seroepidemiology of EV-A71 infection

Seroepidemiological studies on EV-A71 infection are performed to determine the epidemiological patterns of infection and to monitor the seroprevalence rates of EV-A71 neutralising antibodies in the population. Seroepidemiological studies have been carried out previously in many Asian countries, including China (Ji *et al.*, 2012; Li *et al.*, 2013), Taiwan (Chang *et al.*, 2002; Lu *et al.*, 2002), Singapore (Ang *et al.*, 2011; Ang *et al.*, 2015; Ooi *et al.*, 2002), Cambodia (Horwood *et al.*, 2016), Vietnam (Tran *et al.*, 2011), Thailand (Linsuwanon *et al.*, 2014) and Japan (Hagiwara *et al.*, 1979; Mizuta *et al.*, 2009). Many of these studies found that after maternal antibody declines, EV-A71

seropositive rates increase with age, but do not differ significantly with gender. Worldwide seroepidemiological studies of EV-A71 are summarised in Table 2.4.

Many of these seroepidemiological studies found that after the maternal antibody declined, seropositive rates of EV-A71 antibodies increased with age, but did not differ significantly with gender. In a study in China, the seropositive rate of EV-A71 antibody increased between 22.5% and 87.5% in 1-4 years old group. The rate stabilised at about 80% in the age group of 5-15 years of age (Ji *et al.*, 2012). Similar seropositive rate was also shown in Thailand, Taiwan and Vietnam (Linsuwanon *et al.*, 2014; Lu *et al.*, 2002; Tran *et al.*, 2011). Relatively high seroprevalence rate was observed recently in Cambodia, where the EV-A71 seroprevalence among children was 88.8% although more stringent analyses were done with higher cut-off values (Horwood *et al.*, 2016). In Singapore, lower seropositive rates were observed. Overall seroprevalence rate was 26.9%, where the rate increased from 14.3% in children aged 1-6 years old to 27.8% in children aged 7-12 years old. The seropositive rate reached 38.8% in adolescents aged 13-17 years old (Ang *et al.*, 2011). In recent years, CV-A6 and CV-A16 infections were very prevalent in Singapore children and adolescents, where EV-A71 seroprevalence rate was only half of the CV-A6 and CV-A16 seroprevalence rates (26.9% vs. 62.7% and 60.6%, respectively) (Ang *et al.*, 2015). Lower seropositive rate of EV-A71 antibody in children <5 years old indicates that this age group is more susceptible to the infection. Before the occurrence of an epidemic, the seroprevalence or seropositive rate of EV-A71 antibody is usually low (Zhu *et al.*, 2010). This situation may account for the large scale epidemics of EV-A71 due to the accumulation of immunologically naïve younger population (Linsuwanon *et al.*, 2014). Besides that, this low rate was found to be associated with increased rates of mortality and severe cases mainly in children younger than 3 years of age during the EV-A71 epidemics (Chang *et al.*, 2002).

Table 2.4: Seroprevalence rates of EV-A71 infection in different age groups worldwide

Study population	Country	Seroprevalence of EV-A71		Reference
		Age group (years)	**Percentage (%)**	
831 individuals aged 1-5 years	Russia	1-2 3-5	5-20 19-83	Akhmadishina *et al.*, 2014
1200 individuals aged 1-17 years	Singapore	1-6 7-12 13-17	14.3 27.8 38.8	Ang *et al.*, 2011
700 individuals aged 1-17 years	Singapore	1-6 7-12 13-17	15.4 26.2 37.1	Ang *et al.*, 2015
539 people (before the 1998 epidemic)	Taiwan	< 0.6 0.5-0.9 1-1.9 2-2.9 3-5.9 6-11.9 12-19	36 4 4 22 36 63 66	Chang *et al.*, 2002
436 individuals aged 10 months to 75 years	Germany	0-3 3-6 6-10 10-15 >20	27.3 45.6 56.4 67.2 75	Diedrich *et al.*, 2009
238 patients aged <15 years with fever and exanthema	Brazil	0-3 12-15	14.8 69.2	Gomes *et al.*, 2002
1707 individuals aged 2-15 years	Cambodia 2006-2011 2000-2005	 2-15 2-7	 94.8 64.3	Horwood *et al.*, 2016
800 individuals aged 1 month to 15 years	Jiangsu, China	<6 months 7-11 months 1-4 5-15	6.7 5.0-10.0 22.5-87.5 >80.0	Ji *et al.*, 2012
3-40 serum samples of children aged 1 to ≤9 years from 2007-2009	Guangdong, China 2007 2008 2009 	 1 2 3-9 1 2 3-9 1 2 3-9	 0 <25 40 28.6 20 48.7 11.1 <10 36.8	Li *et al.*, 2013

Table 2.4, continued

Study population	Country	Seroprevalence of EV-A71		Reference
		Age group (years)	Percentage (%)	
1182 patients with HFMD/herpangina symptoms in 2008-2013 aged 0 to >18 years	Thailand	<6 months	6.7	Linsuwanon *et al.*, 2014
		>6 months-2	42.5	
		>2-6	48.3	
		>6-12	88.2	
		>12-18	80	
		>18	77.3	
442 individuals aged 0-15 years	Sao Paulo, Brazil	0-5	11	Luchs *et al.*, 2010
		6-15	14.6	
		>15	12.5	
459 pregnant women and their neonates	Taiwan	Pregnant mothers	63	Luo *et al.*, 2009
		<6 months	51	
83 residents in 2004	Yamagata, Japan	<6	24.1	Mizuta *et al.*, 2009
		6-14	44	
		>14	51.7	
258 individuals aged 1-20 years	Ningbo, China	1	13.5	Ni *et al.*, 2012
		2	22.2	
		3	37.8	
		4	62.2	
		5	67.6	
		10	86.5	
		20	83.8	
856 children <12 years old	Singapore	1-23 months	0.8	Ooi *et al.*, 2002
		2-5	12	
		>5	50	
696 individuals aged 1 to >60 years	Germany	1-4	12	Rabenau *et al.*, 2010
		5-9	49	
275 infants aged 1 year, 260 infants aged 18 months, 259 infants aged 2 years, 120 children aged 5-15 years	Ho Chi Minh, Vietnam	1	8.3	Tran *et al.*, 2011
		18 months	13.4	
		2	23.6	
		5-15	84	
472 individuals aged ≤15 years before 2008 outbreak; 83 individuals aged ≤15 years in 2010	Lu'an, China			Yu *et al.*, 2011
	Before 2008 outbreak (2006-2007)	<1	40	
		1	5.1	
		2-4	28.3	
		5-7	45.6	
		8-11	65.4	
		12-15	74.6	
	After 2008 outbreak (2010)	<1	43.7	
		1	5.6	
		2-4	66.6	
		5-7	71.4	
		8-11	72.7	
		12-15	77.7	

Table 2.4, continued

Study population	Country	Seroprevalence of EV-A71		Reference
		Age group (years)	**Percentage (%)**	
614 individuals aged <5 years	Shanghai, China	<0.5	28.6	Zeng *et al.*, 2012
		0.5-0.9	16.7	
		1-1.9	13.4	
		2-2.9	13.9	
		3-3.9	24.1	
		4-4.9	26.1	
555 pregnant mothers and 975 infants	Jiangsu, China	Prenatal women	85.3	Zhu *et al.*, 2012
		2 months	57.6	
		7 months	41.3	
		12 months	42.2	
		27-38 months	56.2	
900 individuals aged ≤5 years in 2005	China	≤5	32	Zhu *et al.*, 2010

2.4.4 Seroprevalence of EV-A71 infection in Malaysia

Despite numerous epidemics of EV-A71 in Malaysia, the seroepidemiologic patterns of EV-A71 infection among children and the general population, as well as the factors that contribute to the cyclical pattern of EV-A71 epidemics, are not well-studied. Many studies of EV-A71 seroepidemiology and EV-A71-associated risk factors described previously have been conducted in urban settings. It would be interesting to know the seroepidemiology and risk factors that contribute to higher seropositivity of EV-A71 infection among rural residents compared to the urban residents.

In Malaysia, urban areas are defined as gazetted areas and their adjoining built-up areas with a combined population of more than 10,000 persons, of which >60% of those aged ≥10 years are engaged in non-agricultural activities and have modern toilet facilities (Jaafar, 2004; Masron *et al.*, 2012). By 2010, 70% of Malaysia's population were in urban areas (Masron *et al.*, 2012). Some rural and remote areas which do not fulfil these criteria are populated by indigenous communities, or Orang Asli (Ngui *et al.*, 2011). In West Malaysia, the Orang Asli comprise 18 ethnic subgroups classified under three main groups, the Negrito, Senoi and Proto-Malay (Mohd Noor, 2012). Despite socioeconomic assistance provided by the Malaysian government, indigenous communities remain impoverished and marginalised, with substantially reduced life expectancy and higher infant mortality rates (Bedford, 2013). They live in rural areas with low levels of education, poor environmental sanitation, and lack of access to clean water (Lim *et al.*, 2009; Ngui *et al.*, 2011), contributing to a high prevalence of faecal-orally transmitted parasitic infections (Ngui *et al.*, 2011,Al-Delaimy *et al.*, 2014; Al-Mekhlafi *et al.*, 2013; Al-Mekhlafi *et al.*, 2008). However, the prevalence of faecal-orally transmitted viral infections such as EV-A71 in the indigenous community remains unknown.

2.5 Risk factors of HFMD/herpangina and severe EV-A71 infection

Several risk factors are associated with EV-A71-associated HFMD/herpangina and severe EV-A71 infection. The main risk factor associated with HFMD/herpangina and severe EV-A71 infection is young age between 0.5 to 4 years old (Chang *et al.*, 2002; Fang *et al.*, 2014; Huang *et al.*, 2014; Li *et al.*, 2014). Contact with HFMD/herpangina is also an associated risk factor for acquiring HFMD/herpangina (Chang *et al.*, 2002). In Taiwan, risk factors that increase seropositivity to EV-A71 infection include older age, attendance at a nursery or kindergarten, contact with HFMD/herpangina cases in 1998, families with greater number of children, and living in rural area (Chang *et al.*, 2002).

Because of a higher incidence of severe EV-A71 infection in young children, many studies have investigated risk factors for severe HFMD other than young age. In a cohort study in Taiwan, developmental retardation, which is a risk factor for cardiopulmonary failure, and delayed medical intervention, increased the risk of severe EV-A71-associated HFMD (Huang *et al.*, 2014). In China, it was found that factors associated with increased risk of developing severe HFMD were EV-A71 infection, high body temperature (>39°C) at presentation, living in a rural area, visiting a village clinic, low birth weight, and those who were under care of grandparents or caregivers with <6 years education (Chen *et al.*, 2014). A meta-analysis ruled out that fever >3 days, body temperature ≥37.5°C, lethargy, hyperglycemia, vomiting, infection by EV-A71, increased neutrophil count and home care were the risk factors, but not living in a rural area (Fang *et al.*, 2014). Another study in China also found that high respiratory rate, high blood glucose, increased percentage of lymphocytes, and high level of alanine aminotransferase increased the risk of severe HFMD (Li *et al.*, 2014). A study in Sarawak, Malaysia found that young age, fever, vomiting, mouth ulcers, breathlessness, poor urine output and cold limbs were risk factors for EV-A71-associated CNS disease (Ooi *et al.*, 2007). This study also found that the CNS disease was associated with

certain EV-A71 genotypes (Ooi *et al.*, 2007), although this has not been consistently shown in other studies.

In a hospital-based study, major social factors associated with severe HFMD were attending kindergarten and having rural-to-urban migrant worker parents (Zeng *et al.*, 2013). In Singapore, a case-control study found that hypoperfusion, seizure, altered mentation, meningeal irritation, tachycardia, tachypnoea, raised absolute neutrophil count and positive diagnosis of EV-A71 were risk factors for severe HFMD (Chew *et al.*, 2015). However, in Japan, the researchers found that attending child care centres was significantly associated with severe HFMD, but not age (Suzuki *et al.*, 2010).

Improved hygiene especially hand washing is a key factor in reducing transmission of the EV-A71. It was found that cleaning faucets after hand washing (Huang *et al.*, 2014) could reduce the risk of contracting severe HFMD.

2.6 Treatment and vaccine development against EV-A71

The incidence of severe EV-A71 infection in younger children has become a major concern in affected countries. The only treatment for severe EV-A71 infection so far is intravenous immunoglobulin (IVIg). IVIg is an intravenous injection of polyvalent IgG antibodies that are purified from pooled blood from thousands of donors. IVIg administration leads to changes of pro- and anti-inflammatory cytokine levels in severe EV-A71 patients, and contains neutralising antibodies which may provide necessary protection to the patients (Cao *et al.*, 2010; Jolles *et al.*, 2005; Ng *et al.*, 2015; Wang *et al.*, 2008). The administration of IVIg however raised some concerns. Firstly, different preparation methods lead to different compositions of IVIg products with different biological responses and different degrees of effectiveness. This leads to a lack of consistency in reported efficacy. Because the IgG antibodies are purified from thousands of blood donors, there are concerns of potential transmission of blood-borne

pathogens. Stringent blood screening is thus necessary and this increases the production cost and selling price. Lastly, IVIg contains the non-neutralising IgG3 isotype which was found to contribute to antibody-dependent enhancement events (Ameratunga *et al.*, 2004; Cao *et al.*, 2013; Han *et al.*, 2011; Ng *et al.*, 2015). Because of these concerns, mAb therapy could be an alternative to IVIg prophylaxis. MAbs are highly specific, bind to highly conserved regions present in all subgenotypes of EV-A71 which do not cross-react with non-EV-A71 viruses such as CV-A16 (Table 2.2; Chang *et al.*, 2010; Deng *et al.*, 2015; Kiener *et al.*, 2012; Kiener *et al.*, 2014; Li *et al.*, 2009; Lim *et al.*, 2012).

Much research is ongoing to develop an effective vaccine against EV-A71. Several types of vaccines have been reported, including inactivated vaccines, live-attenuated vaccines, subunit vaccines, synthetic peptides, virus-like particles (VLPs), baculovirus surface display and DNA vaccines (reviewed by Kok, 2015 and Ng *et al.*, 2015). To date in China, three inactivated vaccines have completed phase III clinical trials (Liang & Wang, 2014). These three vaccines were of C4 subgenotypes and were proven to provide protection against EV-A71-associated diseases when the C4 subgenotype was prevalent during the trial durations (Li *et al.*, 2014; Ng *et al.*, 2015; Zhu *et al.*, 2013; Zhu *et al.*, 2014). The inactivated vaccines are based on a particular EV-A71 strain, hence, cross-immunity and cross-neutralisation between different genogroups may be limited (Kiener *et al.*, 2013; Meng *et al.*, 2011; Ng *et al.*, 2015). However, in contrast to this, several studies have shown evidence of cross-neutralising antibody responses to EV-A71 infection in human and animals. These studies are summarised in Table 2.5.

Other issues are the induction of antibody-dependent enhancement events and suitable route of administration. Oral immunisation is the most suitable route as EV-A71 is transmitted via the faecal-oral route, and it could induce both mucosal and systemic immune responses (Ogra *et al.*, 2001; Wang *et al.*, 2010). As an alternative,

baculovirus-expressed vaccines such as baculovirus-expressed VLPs and baculovirus surface display have several advantages over inactivated vaccines. Unlike inactivated vaccines which may contain virus particles, the baculovirus-expressed vaccines contain no genomic material of EV-A71 and the baculovirus itself is harmless due to inability to replicate in mammalian cells (Kost *et al.*, 2005; Xu *et al.*, 2011). Thus, only biocontainment level 1 is required during manufacturing (Ng *et al.*, 2015). Furthermore, the use of formaldehyde in inactivated vaccines may disrupt the native conformation of the viral structural proteins (Wang *et al.*, 2012; Wilton *et al.*, 2014). This would reduce efficacy of the vaccine in eliciting immune responses, especially neutralising antibodies. As no inactivation is required for baculovirus-expressed vaccines, this issue would not cause similar problems with these vaccines (Ng *et al.*, 2015).

Table 2.5: Summary of studies investigating the cross-reactivity of neutralising antibody responses to enterovirus A71 infection in humans and animals

Cross-reactivity in human sera:

EV-A71 strains/genotypes	Source of patient sera	Findings	Reference
B1, B5 C2, C4A, C4B	Sera from 60 healthy adult vaccinees (aged 20-60 years) were collected 0, 21 and 42 days post-vaccination.	Strong cross-neutralising antibody responses in >85% of volunteers without pre-existing NtAbs against subgenotypes B1, B5 and C4A were observed. Weaker cross-neutralising antibody responses were found against C4B and C2 viruses.	Chou *et al.*, 2013
B4, B5 C2,C4a, C4b	34/37 paediatric patients aged 0-18 years with enteroviral infections; 3 healthy children as controls.	Infection with genotype B (B5) induced a strong genotype B EV-A71-specific but weaker genogroup C (C4b)-specific antibody-secreting cell (ASC) response in children. Neutralisation titres against genotype B viruses significantly correlated with genotype B EV-A71–specific ASC responses but not with genotype C EV-A71-specific ASC responses.	Huang *et al.*, 2015
A B1, B2, B3, B4, B5 C1, C2, C3, C4, C5	Sera from children < 5 years, infected with genotypes: C2 – 10 samples (1998) B4 – 5 samples (2000) C4 – 2 samples (2005) B5 – 5 samples (2008) C4 – 3 samples (2010)	Overall, all EV-A71-infected children had detectable NtAb titres against 11 EV-A71 genotypes. Homologous NtAb titres were not always higher than heterologous NtAb titres. Children infected with genotypes C2, C4, B4 and B5 had lower GMTs (≥4-fold difference) against genotype A than other genotypes but no clear antigenic variations between genotype B and C were observed.	Huang *et al.*, 2013
A B1, B4, B5 C2, C4, C5	5 groups of sera were collected: Anti-C2 (3 samples) Anti-B4 (4 samples) Anti-C4 (4 samples) Anti-C5 (1 sample) Anti-B5 (2 samples)	Human antiserum showed various NtAb titres against different viruses, indicating possible antigenic variation among viruses. The antigenic map showed that genotype B1 and B4 viruses clustered together while genotype C2 from 1998 was found in another antigenic cluster distinct from genotype B viruses. Genotype C4 viruses were more closely related antigenically to genotype C2. The reemergent genotype B5 viruses in 2003 and 2008 were antigenically different from the other genotype B and genotype C viruses.	Huang *et al.*, 2009

Table 2.5, continued

EV-A71 strains/genotypes	Source of patient sera	Findings	Reference
A B3, B4, B5 C1, C2, C3, C4, C5	160 sera (40 individuals in each age group; 20 in vaccine group and 20 in placebo group) inoculated with inactivated EV71 vaccine (FY-23K-B strain of subgenotype C4) were collected at 0, 56 and 360 days post-vaccination for cross-neutralisation assay. Individuals were aged 6-11 months, 12-23 months, 24-35 months and 36-71 months.	Cross-neutralising reactivity against major EV-A71 strains was observed in the vaccine groups. All sera exhibited similar low neutralising titres to the genotype C1 strain although these neutralising titres were higher than the positive control titre of 1:8.	Liu *et al.*, 2015
A B5 C4a	20 infected with B5 subgenotype and 10 infected with C4a subgenotype (≤3 years, 4-6 years and ≥7 years).	Children infected with B5 viruses had significantly lower neutralising antibody titres against genotype A virus and genotype C4a viruses than B5.	Luo *et al.*, 2012
B4, B5 C2, C4, C5	Sera from 119 infants and children (aged 6 months to 11 years) in two clinical trials of EV-A71 subgenotype C4 vaccine.	After two-dose vaccination, 49/53 participants in the initially seronegative group and 52/53 participants in the initially seropositive group showed <4-fold differences in NtAb titres against five EV-A71 strains, whereas corresponding values among sera from paediatric patients recovering from EV-A71-induced HFMD and subclinically infected participants were 8/8 and 41/43, respectively. The GMT of participants against five subgenotypes of EV-A71 increased significantly after vaccinations, irrespective of the baseline NtAb titre.	Mao *et al.*, 2013
A B2, B4, B5 C1, C2, C4	Sera from 83 residents in Yamagata aged 1->60 years were enrolled.	Residents previously infected with EV-A71 had NtAb against the different subgenotypes. The ranges of the NtAb titres against different subgenotype strains in the Yamagata residents differed by up to almost 4-fold.	Mizuta *et al.*, 2009

Table 2.5, continued

Cross reactivity in animal sera (mice, rabbits, monkeys):

EV-A71 strains/genotypes	Source of patient sera	Findings	Reference
A B1, B4 C2, C4	Sera from monkeys inoculated with EV-A71, followed by lethal challenge with the parental virulent strain EV-A71 (BrCr-TR).	The immunised monkey sera showed a broad spectrum of neutralising activity against different genotypes of EV-A71, including genotypes A, B1, B4, C2, and C4. The sera showed the highest homotypic neutralisation activity (genotype A) and the lowest neutralisation activity against genotype C2. The order of decreasing neutralisation activity of sera was: A>B1>C4>B4>C2.	Arita *et al.*, 2007
C4	Sera were collected from mice challenged with EV-A71.	An inactivated EV-A71 vaccine candidate offered complete protection from death induced by various circulating EV-A71 viruses to neonatal mice that were born to immunised female mice. The sera of the immunised dams and their pups showed high neutralisation titres against multiple circulating EV-A71 viruses.	Chang *et al.*, 2015
A B, B2, B3, B4, B5 C1, C2, C3, C4, C5 (18 isolates)	40 mAbs were used to type the antigenic profiles of 18 isolates from different EV71 genotypes.	The antigenic profiles characterised by the mAb panel did not correlate to their genotypes by phylogenetic classification. Ten isolates of genotype C4a were classified into four distinct antigenic types, but the panel mAbs still neutralised all virus genotypes.	Chen *et al.*, 2013
A B, B2, B3, B4, B5 C1, C2, C3, C4, C5 C2-like	Sera from rabbits immunised with purified viruses collected a week after final boost.	The reference EV-A71 viruses induced high homotypic neutralisation titres (1:256 to 1:4096). Genotype A virus consistently had >8-fold difference between homotypic and heterotypic NtAb titres but no clear pattern could be identified for genogroup B and C viruses. Genotype B2 and B5 viruses were highly immunogenic and induced high homotypic and heterotypic NtAb titres against all genogroup B and C viruses except the C2-like virus isolated in 2008.	Chia *et al.*, 2014

Table 2.5, continued

EV-A71 strains/genotypes	Source of patient sera	Findings	Reference
B4, B5 C4	Sera from mice and rabbit immunised with different EV71 immunogens (synthetic peptides, individual recombinant viral proteins, VLP, formalin-inactivated virions).	In mice, only VP1 elicited antibody responses with 1:128 neutralisation titre. The formalin-inactivated EV-A71 elicited antibodies that cross-neutralised (1:640) different EV-A71 genotypes in mice. In rabbit, sera cross-neutralised strongly against different genotypes of EV-A71 (1:6400).	Chou *et al.*, 2012
B3, B4, B5 C2, C4	Rhesus macaques were immunised with EV-A71-B5 VLP vaccine at day 0 and 21. The macaques were challenged with EV-A71- B3 on day 42. Blood samples were collected and processed at different intervals post-challenge.	Sera from vaccinated animals neutralised EV-A71- B3 *in vitro*. The profile of NtAb titres was similar for all four subgenotypes (B4, B5, C2 and C4). All vaccinated animals produced NtAbs against EV-A71 from all subgenotypes tested. EV-A71-B5 VLPs elicited antibodies that could cross-neutralise multiple EV-A71 subgenotypes. EV-A71-B3 infection also induced NtAbs against other EV-A71 subgenotypes.	Lim *et al.*, 2015
B3, B4, C1, C2, C3, C4, C5	Sera from mice immunised with formaldehyde-inactivated whole-virus vaccines derived from EV-A71 clinical isolates and mouse-adapted virus (MAV) were collected at day 14.	Antisera generated by immunisation with the MAV vaccine not only neutralised genotype B3 strains (≥1:512), but also neutralised strains of genotypes B4 (≥1:512) and C1 to C5 (1:256, 1:64, 1:256, 1:32 and 1:64, respectively).	Ong *et al.*, 2010
A B0, B1, B1*, B2 C2	Sera from rabbits hyperimmunised with EV-A71 isolates of subgenotypes B2 and C1.	Subgenotype B2-specific rabbit antiserum showed cross-neutralisation of B0, B1 and B2, but very low neutralisation of subgenogroup C1 or C2 viruses, probably explaining the global shift to genotype C in 1987 following a B2 epidemic. Anti-C1 rabbit serum neutralised both genotype B and C viruses.	van der Sanden *et al.*, 2010

NtAb: neutralising antibody; mAb: monoclonal antibody; GMT: geometric mean titre

CHAPTER 3

MATERIALS AND METHODS

3.1 Cells and viruses

3.1.1 Cell culture

Human rhabdomyosarcoma cell line (RD, ATCC # CRL-2061) was used in all experiments in this study. RD cells were propagated and maintained in 1X Dulbecco's minimum Eagle's medium (DMEM) supplemented with 10% heat-inactivated FBS (Hyclone, USA), 1% L-glutamine (200 mM) (Hyclone, USA), 1% non-essential amino acids (100X) (Hyclone, USA) and 1% penicillin/streptomycin (10,000 units/ml penicillin, 10,000 µg/ml streptomycin) (Hyclone, USA). The cells were incubated at 37°C in 5% CO_2 until 80-90% confluency was reached.

For cell passaging, the cell growth medium was removed from the flask. The cells were then rinsed with 0.12% trypsin. Two ml of the same trypsin was added into the flask, which was incubated for 5 minutes at 37°C to allow cell detachment. After complete detachment of the cells, an equal volume of the growth medium was added to the trypsinised cells. The cell suspension was split into a new 75 cm^2 tissue culture flask (TPP, Switzerland) with a ratio of 1:4. Ten ml of new cell growth medium was added and the cells were incubated at 37°C in 5% CO_2.

3.1.2 Virus strains and propagation

The virus strains used in this study were EV-A71 strain UH1/PM/97 (GenBank accession number AM396587) and EV-A71 strain 41 (GenBank accession number AF316321). Both strains are of subgenotype B4. Clinical isolates of EV-A71 subgenotype B5 from 2006 (GenBank accession number JN316092) and EV-A71 subgenotype C1 from 1997 (GenBank accession number JN316071) were also used.

Both strains were obtained from the Diagnostic Virology Laboratory, University of Malaya Medical Center in Kuala Lumpur, Malaysia.

To propagate the viruses, 75 cm^2 tissue culture flasks (TPP, Germany) of 70-80% confluent RD cells were infected with 100 µl of virus supernatant. The cells were incubated at room temperature for 1 hour and the virus inoculum was replaced with fresh DMEM supplemented with 2% heat-inactivated FBS. The infected cells were incubated at 37°C in 5% CO_2 until cytopathic effects (CPE) were observed at 3-5 days. When 70-80% of CPE was observed, the flasks were freeze-thawed once, then the culture supernatants were clarified by centrifugation at 14,000 x g for 10 minutes at 4°C. The supernatants were kept in -80°C until use.

3.1.3 Virus titration assay

Suspensions of 100 µl of RD cells were seeded in 96-well plates (BD Falcon, USA) at a density of 1.5 x 10^5 cells/well in DMEM supplemented with 10 % heat-inactivated FBS and incubated overnight at 37°C in 5% CO_2. The next day, 10-fold serial dilutions (from neat to 10^{-9}) of virus were prepared in serum-free DMEM (SFM) as diluent. Prior to infection, the growth medium (DMEM supplemented with 10% heat-inactivated FBS) was removed from each well, and aliquots of 0.1 mL from the appropriate dilutions were inoculated onto RD cells. The plates were incubated at room temperature for 1 hour with gentle rocking. The virus inocula were removed after 1 hour of adsorption. DMEM supplemented with 2 % heat-inactivated FBS were then added to the cells, which were then incubated at 37 °C in 5% CO_2. Plates were observed for CPE daily and results were recorded at day 5 post-incubation to determine the tissue culture infectious dose ($TCID_{50}$). $TCID_{50}$ is defined as the amount of virus that produces CPE in 50% of the cells. The $TCID_{50}$ values were calculated according to the formula by Reed and Muench (1938) and expressed as $TCID_{50}$/mL.

3.1.4 Virus plaque assay

Six-well plates with 5.0 x 10^5 RD cells/well or 24-well plates with 1.5 x 10^5 RD cells/well were prepared and incubated overnight at 37°C in 5% CO_2. Prior to virus infection, the growth medium (DMEM supplemented with 10% FBS) was removed, and an appropriate amount of SFM was added to the plates. An aliquot of 100 µl virus were inoculated into each well, and plates were incubated at room temperature for an hour with gentle rocking to allow virus attachment to the cells. After 1 hour of incubation, the virus inocula were removed and immediately replaced with 500 µl to 2 ml of plaque medium (DMEM supplemented with 2% FBS and 0.9% or 1.2% w/v carboxylmethylcellulose (CMC)). After 48 to 72 hours of incubation, the plaque medium was removed. The cells were fixed with 3.7% formaldehyde and stained with 0.5% crystal violet. The plaques were viewed against a white background. Plaque forming units per milliliter (pfu/ml) were calculated with the following formula:

$$\text{pfu/ml} = \frac{\text{Number of plaques x dilution factor}}{\text{Volume of inoculum (ml)}}$$

3.2 Seroprevalence studies of EV-A71

3.2.1 Serum samples

3.2.1.1 Children's sera

Serum samples were randomly picked from archived residual sera collected for routine virology and bacteriology tests in the Diagnostic Virology Laboratory, University of Malaya Medical Center, Kuala Lumpur. As this study aimed to measure background seroprevalence of EV-A71 infection, samples from patients suspected with HFMD were excluded to prevent bias as these samples are more likely be seropositive; this exclusion criterion has been previously used by several similar studies (Ang *et al.*,

2011; Li *et al.*, 2013; Ooi *et al.*, 2002; Rabenau *et al.*, 2010). A total of 1,769 sera from children aged between 1 to 12 years old, collected between 1995 and 2012, were tested for EV-A71 neutralising antibodies. Between 52 and 200 samples were collected for each year, except for 2009, when only 30 samples from children were available. Samples were divided into 1-6 years (pre-school) and 7-12 years (primary school) age groups for most analyses. A further 372 serum samples from children <1 year were analysed separately, as these may contain maternal antibodies. The study was approved by the hospital's medical ethics committee (reference number 872.7) and the Medical Research and Ethics Committee of the Ministry of Health, Malaysia (reference number NMRR-12-1038-13816). Our institution does not require informed consent for retrospective studies of archived and anonymised samples.

An additional 39 and 32 sera from children <3 years collected in 2013 were used to verify the concordance of neutralisation titres between the UH1/PM/97 strain, a clinical virus isolate from subgenotype B5 cultured in 2006, and an isolate from subgenotype C1 cultured in 1997.

3.2.1.2 Urban (Kuala Lumpur) and rural (Orang Asli) population sera

The urban samples were randomly selected from residual sera archived in the Diagnostic Virology Laboratory, University of Malaya Medical Centre. A total of 460 urban serum samples collected between 2010 and 2012 from patients aged 1 to 85 years were used. Samples from patients with suspected HFMD were excluded. A total of 298 rural serum samples were used, which had been previously collected from Orang Asli aged 1 to 90 years, between 2010 and 2012, to study prevalence of intestinal parasites. The sampled rural populations were from 14 Orang Asli villages in West Malaysia, located in the states of Selangor, Pahang, Perak, Malacca, and Negeri Sembilan (Al-Mekhlafi *et al.*, 2013; Al-Mekhlafi *et al.*, 2008; Lim *et al.*, 2009; Ngui *et al.*, 2011)

(Figure 3.1). None of the sampled individuals had active HFMD. Samples were divided into six age groups for analysis: 1-3 years, 4-6 years, 7-12 years, 13-17 years, 18-49 years and ≥50 years. The study was approved by the hospital's medical ethics committee (reference numbers 872.7 and 709.2).

Demographic data of each Orang Asli sample were provided by Dr. Romano Ngui from the Department of Parasitology, Faculty of Medicine, University Malaya and were only available for 248 samples. Data was chosen for the analysis of risk factors for EV-A71 seropositivity based on biological plausibility and previous publications.

3.2.2 Neutralisation assay

The selected serum samples were removed from -20°C storage and heat-inactivated at 56°C for 30 minutes. The neutralising titre of each serum was determined by a microneutralisation assay as described previously (Ang *et al.*, 2011), with modifications. Two-fold serial dilution of each serum sample was performed from 1:8 to 1:32. An aliquot of 90 µl of each dilution was mixed with 90 µl of 1000 $TCID_{50}$ of EV-A71 strain UH1/PM/97. The serum-virus mixture was then incubated at 37°C for 2 hours in 5% CO_2. Each serum dilution was transferred into a 96-well plate in triplicate. A suspension of 100 µl containing 1 x 10^4 RD cells was then added. Pooled positive sera of known titre were included in each assay as positive controls, using previously described criteria for reproducibility (Ang *et al.*, 2011). Reproducibility of positive titres was considered acceptable if there was a difference of less than one dilution with the same titre obtained on most assays (Ang *et al.*, 2011). Wells containing diluted serum, virus alone, and uninfected RD cells were also included as controls. The plates were incubated at 37°C in 5% CO_2 and examined for CPE after 5 days. The neutralising antibody titre was defined as the highest dilution that prevents the development of CPE

Figure 3.1: Locations of the rural Orang Asli villages where the samples were collected. The red solid circles indicate the villages or the areas where the villages are situated.

in 50% of the inoculated cells. A sample was considered positive if the neutralising titre was ≥1:8 (Ji *et al.*, 2012; Luo *et al.*, 2009).

3.2.3 National HFMD data

Overall national incidence rates of notified HFMD from 2006 to 2012 were available from the Ministry of Health, Malaysia. However, as the statutory notification of HFMD came into enforcement only in October 2006, cases prior to this were underreported. The monthly numbers of HFMD cases for each of the 13 states and 2 federal territories were available only between 2008 and 2014. The case definition for reporting HFMD is a child with mouth/tongue ulcers and/or maculopapular rash/vesicles on the palm and soles, with or without a history of fever. This is syndromic data without laboratory confirmation of the viral agent. As diagnostic virology facilities are not widely accessible, there is a scanty data on causative viral agents. EV-A71 epidemic years, with limited laboratory confirmation, were obtained from published reports (Chua *et al.*, 2007; Chua and Kasri, 2011; Chan *et al.*, 2011; Tee *et al.*, 2009), and defined as 1997, 2000, 2003, 2006, 2008/2009 and 2012.

3.2.4 Phylogenetic analysis

EV-A71 VP1 gene sequences of Malaysian isolates were retrieved from GenBank (Appendix I) and aligned with Geneious R6 (Biomatters Ltd, New Zealand). The best substitution model was determined using jModelTest v0.1.1 (Posada, 2008) as the general time reversible model with rate variation among sites (GTR+G). Phylogenetic trees were constructed using the Bayesian Markov Chain Monte Carlo method in BEAST 1.7.4 (Drummond and Rambaut, 2007), run for 30 million iterations with a 10% burn-in. All runs reached convergence with estimated sample sizes of >200. The clock model was uncorrelated lognormal relaxed and the tree prior was coalescent GMRF

Bayesian Skyride, allowing the generation of a plot of relative genetic diversity. The maximum clade credibility tree was viewed using FigTree 1.4 (Rambaut, 2012).

3.2.5 Statistical analysis

3.2.5.1 Analysis of the seroprevalence among children up to 12 years of age

Geometric mean titres (GMT) were calculated by log-transforming the positive neutralisation titres, using a value of 64 for titres >1:32. A two-sided type I error of 0.05 was used for statistical significance. Statistical analyses were performed using SPSS software version 22 (IBM SPSS Software, USA) and Stata version 12 (Stata Corp, College Station, Texas, USA), and graphs were drawn using GraphPad Prism 5 (GraphPad Software, USA).

3.2.5.2 Analysis of the seroprevalence between the urban Kuala Lumpur and rural Orang Asli populations

Fisher's exact test was used to compare differences in total and age group-specific seropositive rates between urban KL and rural Orang Asli populations. Independent-samples t-test was also used to compare the difference in mean ages between the urban and Orang Asli samples. Univariate logistic regression analysis was used to correlate age and EV-A71 seropositivity.

Regression analysis was also used to determine risk factors for EV-A71 seropositivity in the rural subjects for whom data was available. Independent variables were chosen based on previous literature and biological plausibility consistent with known transmission routes. In the initial univariate analysis, independent variables included age, gender, and factors relating to socioeconomic status, hygiene and living conditions. Household income is reported in Malaysian ringgit (MYR). All variables determined from the univariate analysis with *P*-values ≤0.25 were included in the

multivariate logistic regression analysis using the forward elimination model. Independent risk factors with *P*-values of <0.05 were considered statistically significant. Odds ratios (OR) were reported with 95% confidence intervals (CI). To assess the final model, the Hosmer and Lemeshow goodness-of-fit test was performed, and the area under the receiver operating characteristic curve was calculated. Statistical analyses were performed using SPSS software version 22 (IBM SPSS Software, USA), and graphs were drawn using GraphPad Prism 5 (GraphPad Software, USA).

3.3 Selection, purification and adaptation of resistant EV-A71

3.3.1 Bacterial culture

Luria-Bertani (LB) agar and broth were used to culture the bacteria. LB broth and agar were prepared according to Miller (1972), then autoclaved for 20 minutes at 121°C. The LB agar was supplemented with 50 µg/ml kanamycin then poured into petri dishes and solidified. The LB broth was supplemented with kanamycin prior to bacteria inoculation.

3.3.2 Generation and selection of EV-A71 escape mutants *in vitro*

EV-A71 neutralisation escape mutants were generated *in vitro* according to the methodology by Delang *et al.,* (2014) with modification. Different titres of EV-A71 strain UH1/PM/97 (ranging from 10 pfu to 1000 pfu) were incubated for 2 hours at 37°C with six two-fold dilutions (ranging from 1:16 to 1:512) of the commercial EV-A71 monoclonal antibody MAB979 (Merck Millipore, USA). After incubation, the antibody-virus mixtures were inoculated into the overnight-incubated RD cells in duplicate, and incubated again at 37°C for 1 hour. The antibody-virus mixtures were then replaced with DMEM supplemented with 2% heat-inactivated FBS and the plate was incubated at 37°C in 5% CO_2. The plate was observed every day for CPE

formation. After 3 days of incubation, the virus in the duplicate wells which was completely neutralised by the highest dilution of MAB979 antibody was selected and harvested.

Three 96-well plates containing confluent RD cells were further infected with the optimal virus titre (100 pfu/ml) and MAB979 antibody dilution (1:32). After 3 days of incubation, supernatant from 17 wells that showed complete virus-induced CPE were harvested. These virus supernatants may contain mutants that were able to escape neutralisation by MAB979 antibody. One mutant isolate from each plate was chosen and titrated in the presence of the same MAB979 antibody dilution. Six 10-fold dilutions of the neutralisation escape mutants were performed and incubated with 1:32 MAB979 antibody dilution for 2 hours at 37°C. The antibody-mutant mixtures were added to confluent RD cells in a 96-well plate and incubated for 1 hour at 37°C. After incubation, the inocula were replaced with DMEM supplemented with 2% heat-inactivated FBS and the plate was incubated at 37°C in 5% CO_2. After 3 days of incubation, escape mutants in the wells that showed complete CPE at the lowest virus input were harvested.

The selected escape mutants were further passaged in a 24-well plate, and then incubated with an equal volume of the optimal MAB979 antibody dilution for 2 hours at 37°C. The antibody-mutant mixtures were then added to the confluent RD cells in the plate and further incubated for 1 hour at 37°C. The inocula were replaced with DMEM supplemented with 2% heat-inactivated FBS containing the MAB979 antibody at 1:32 dilution. The virus supernatant was collected after 3 days of incubation, when complete CPE were observed in all wells.

3.3.3 Confirmation of the ability of the selected EV-A71 mutants to escape neutralisation

Neutralisation assay was performed to confirm that the mutant was capable of escaping neutralisation by MAB979 antibody. Two-fold dilutions of MAB979 antibody (ranging from 1:16 to 1:512) were mixed with equal volumes of 100 pfu escape mutant virus and incubated for 2 hours at 37°C. The antibody-mutant mixtures were then added to confluent RD cells in a 96-well plate and incubated for 1 hour at 37°C. After the incubation, the inocula were replaced with the DMEM supplemented with 2% heat-inactivated FBS and incubated for 3 days. At the same time, a similar set of assays was done using the EV-A71 strain UH1/PM/97 wild type virus as a comparison.

3.3.4 DNA sequencing of the selected EV-A71 neutralisation escape mutants

The P1 region of the EV-A71 escape mutants were sequenced to identify the mutations associated with the ability to replicate in the presence of the MAB979 antibody. The wild-type EV-A71 strain UH1 was also sequenced for comparison. Primers for PCR and sequencing were synthesised (Integrated DNA Technologies, USA), and are listed in Table 3.1.

Both wild-type and mutant EV-A71 viral RNA was extracted using the QIAamp viral RNA mini kit (QIAGEN, Germany) according to the manufacturer's manual. Briefly, 140 µl of viral supernatant containing the viral RNA was added to a 1.5 ml tube containing 560 µl AVL buffer mixed with the recommended amount of carrier RNA. The mixture was incubated at room temperature for 10 minutes, followed by addition of 560 µl of absolute ethanol. The mixture containing RNA was applied to the column and washed once with 500 µl of buffers AW1 and AW2. The RNA was eluted with 60 µl of RNA storage solution and kept at -80°C. The RNA was reversed transcribed into cDNA using SuperScript III reverse trancriptase (Invitrogen, USA) according to the

Table 3.1: Primers for PCR amplification and DNA sequencing analysis of the EV-A71 neutralisation escape mutants.

Primer	Sequence (5'→ 3')	T_a (°C)	Nucleotide position*
EV71-P1-F	CCATCCGGTGTGCAATAGAG	58	637-656
EV71-P1-R	CGAGTCCCGACACTATGTTA	58	3471-3490
EV71-VP2-F	TTAACAACGTACCCACCAAT	58	1879-1898
EV71-VP3-1F	GCAACTCCGGTTATCCCTAT	58	1635-1654
EV71-VP3-2F	AAACACACAGGTGAGCAGTC	58	2531-2550
EV71-VP4-F	CGGCAAACATCATAGTTGGT	58	1036-1055
EV71-F1	CCTCGAGTATGGAGCGTGTC	58	3098-3117
EV71-F2	CGGGTCTTTCATGGCCACAG	58	2081-2101
EV71-F3	CAAGTTCCATCAAGGAGCGC	58	1298-1311
VP3-FGAP1	GACGGCGTCTCAGCACCCAT	64	1767-1786
VP3-RGAP1	TGAGCATTTTACCTGTGGCCATGAA	64	2088-2112
VP3-VP1-FGAP2	TGGTCAGGGTCACTGGAGGTCAC	64	2046-2068
VP3-VP1-RGAP2	TGGGAGCACCAGGGGGAACA	64	2906-2925
VP1-FGAP3	TGTTCCCCCTGGTGCTCCCAA	64	2906-2926
VP1-RGAP3	CCCCAGACTGCTGGCCGAAC	64	3338-3357

*The nucleotide numbering refers to the EV-A71 UH1 strain (GenBank accession number AM396587)

manufacturer's manual. Briefly, the reaction mix was prepared with 0.5 µl of 10 µM EV71-P1-R, 2 µl of 10 mM dNTP mix, 2 µl of 10 pg-5 µg EV-A71 RNA and 8.5 µl of sterile, nuclease-free water, with a total volume of 13 µl. The mixture was heated at 65°C for 5 minutes and incubated on ice for at least 3 minutes. The contents were collected by brief centrifugation. Then, the following components were added and mixed by pipetting gently: 4 µl of 5X first-strand buffer; 1 µl of 0.1M DTT; 1 µl of RNaseOUT Recombinant RNase Inhibitor (40U/µl); and 1 µ of SuperScript III RT (200 units/µl). The mixture was further incubated at 55°C for 60 minutes and the reaction was inactivated at 70°C for 15 minutes. The cDNA was kept at -20°C until further experiments.

Amplification of both wild-type and mutant EV-A71 cDNA was performed using Q5 High-Fidelity DNA Polymerase (NEB, USA). The PCR reactions were prepared according to the manufacturer's manual. Briefly, the PCR mix was prepared with 5 µl of 5X Q5 reaction buffer, 0.5 µl of 10 mM dNTP mix, 1.25 µl of each primer combination (EV71-P1-F/EV71-P1-R, or VP3-FGAP1/VP3-RGAP1, or VP3-VP1-FGAP2/VP3-VP1-RGAP2, or VP1-FGAP3/VP1-RGAP3), 0.25 µl of Q5 high-fidelity DNA polymerase (2U/µl) and 1 µl of the EV-A71 cDNA template. Nuclease-free water was added to the reaction up to 25 µl. The PCR was performed with initial denaturation at 98°C for 30 seconds, 30 cycles of denaturation at 98°C for 10 seconds, annealing (at temperatures according to primer sets as shown in Table 3.1) for 20 seconds, and extension at 72°C for 2 minutes 30 seconds. The cycle ended with final extension at 72°C for 3 minutes. The PCR products were screened by horizontal gel electrophoresis with agarose gel (Vivantis, USA) prepared in 1X TAE buffer and pre-stained with GelRed nucleic acid stain (Biotium, USA). The DNA was mixed with 6X loading dye (Promega, USA) and loaded into the wells. VC 100 bp plus DNA ladder (Vivantis, USA) or GeneRuler 1 kb DNA ladder (Thermo Scientific, USA) was used to determine

the product sizes. Electrophoresis was carried out at 100V for 20 minutes and the DNA bands were visualised under UV illumination.

The PCR products were purified using Expin mini spin purification kit (GeneAll, Korea) according to the manufacturer's manual. DNA bands with correct sizes were carefully excised from the agarose gel using a clean scalpel blade. Buffer GB was added to the excised gel slices (300 µl per 100 mg of gel) and incubated at 50°C for 10 minutes. The DNA-containing mixtures were then applied to the spin columns and washed with 700 µl NW buffer. The DNA was eluted from the column with 40 µl of EB buffer and stored at -20°C until further experiments.

The purified PCR products were sent for sequencing with the sequencing primer sets (Table 3.1). The sequence results of the EV-A71 escape mutants were compared with the sequence of the EV-A71 wild-type virus and sequences from GenBank to identify the amino acid changes.

3.3.5 Construction of EV-A71 escape mutant infectious clones by site-directed mutagenesis

Infection clones that carried the mutations identified by DNA sequencing (Section 3.3.4) were constructed by site-directed mutagenesis. This was performed to confirm the neutralising capability of the EV-A71 neutralisation escape mutants. Clones were constructed to carry single identified mutations, or dual mutations. The pCMV-EV-A71 recombinant plasmid (Appendix II) based on the EV-A71 strain 41 (genotype B4), which was previously constructed using the Gibson-assembly method (unpublished data), was used as the template. Primer sets were designed in a back-to-back orientation by incorporating the desired nucleotide change at the 5' end of the forward primer, including at least 20-25 complementary nucleotides on the 3' end of the mutation. The reverse primer was designed so that the 5' ends of the two primers anneal back-to-back

(Table 3.2). For the clone that carried dual mutations, the plasmid that carried the mutation on the VP2 protein served as the backbone for the construction of the clone, amplified by the pD579N primer set.

Exponential amplification of the pCMV-EV-A71 recombinant plasmids was performed using Q5 High-Fidelity DNA Polymerase (NEB, USA). Large preparation (50 µl) of the PCR reactions was prepared according to the manufacturer's manual. Briefly, the PCR mix was prepared with 10 µl of 5X Q5 reaction buffer, 1 µl of 10 mM dNTP mix, 2.5 µl of pT210I-F and pT210I-R (primer set I), as well as pD579N-F and pD579N-R (primer set II), 0.5 µl of Q5 high-fidelity DNA polymerase (2U/µl) and 1 µl of the pCMV-EV-A71 recombinant plasmid template. Nuclease-free water was added to make the reaction up to 50 µl. For the clone with two mutations, 1 ng of the VP2-mutant plasmid backbone was used. The PCR was performed with initial denaturation at 98°C for 30 seconds; 2 cycles of denaturation at 98°C for 10 seconds, annealing at 72°C and 64°C for primer sets I and II, respectively, for 20 seconds; extension at 72°C for 5 minutes 30 seconds; followed by 20 cycles of denaturation at 98°C for 10 seconds; annealing at 72°C and 65°C for primer sets I and II, respectively, for 20 seconds; and extension at 72°C for 5 minutes 30 seconds. The PCR products were screened by horizontal gel electrophoresis as described above.

PCR products were purified using DNA clean and Concentrator-5 (Zymo Research, Germany) according to the manufacturer's manual. Briefly, in a 1.5 ml tube, 5 volumes of DNA binding buffer were added to one volume of DNA sample and mixed briefly by vortexing. The mixture was then transferred to a provided Zymo-Spin column in a collection tube and centrifuged for 30 seconds. The column was then washed twice with DNA wash buffer and the DNA was eluted with 10 µl nuclease-free water. The purified DNA was treated with T4 DNA ligase-T4 polynucleotide kinase-*Dpn*I (NEB, USA) to

Table 3.2: Primers involved in site-directed mutagenesis[a].

Primer	Sequence (5'→ 3')	Ta (°C)	Nucleotide position
pT210I-F	TTGAGGACAGCCACCCTCCTTAC	72	1964-1986
pT210I-R	TTCCTGTGCCGCCTGCCAC	72	1945-1963
pD579N-F	AATAGTGTGAGTAGGGCACTTAC	65	3070-3092
pD579N-R	TCCTATAGAGCTCTCTATCACATCTG	65	3044-3069

[a]Underlined nucleotides indicate substitution mutations

*The nucleotide numbering refers to EV-A71 strain 41 (GenBank accession number AF316321)

remove the non-mutated PCR template by *Dpn*I, and to circularise the linear double-stranded PCR product. Briefly, the treatment was prepared by mixing 1 µl of each enzyme in a PCR tube, then 1 µl of the enzyme mixture was taken out and mixed with 1 µl T4 ligase buffer and 8 µl purified DNA in a new tube. The mixture was incubated for 2 hours at room temperature prior to transformation.

3.3.6 Transformation of the plasmid carrying EV-A71 escape mutant infectious clones

The treated DNA was transformed into *E.coli* XL10-GOLD ultracompetent cells (Agilent Technologies, USA). The competent cells were first thawed on ice. An aliquot of 1 µl of β-mercaptoethanol was added into 20 µl of the XL10-GOLD competent cells and incubated for 5 minutes on ice. Then, 10 µl of the recombinant plasmids were added to the competent cells and incubated on ice for 30 minutes. The competent cells-plasmid mixture was subjected to heat-shock at 42°C for 45 seconds and immediately placed on ice for at least 2 minutes. An aliquot of 100 µl of super optimal broth with catabolite repression medium was added to the transformed XL10-GOLD competent cells and the suspension was shaken at 220 rpm for 1 hour at 37°C. After that, the suspension was spread onto a LB/kanamycin agar plate. The plate was incubated overnight at 37°C and stored at 4°C.

3.3.7 Plasmid extraction and sequencing

Transformed competent cells containing the insert of interest should grow on the agar plate. All colonies were picked and streaked on a new LB/kanamycin agar plate and incubated for 6 to 7 hours. Each colony was then inoculated in 5 ml LB/kanamycin broth and incubated overnight at 37°C with constant shaking.

The plasmids were purified using GeneAll Hybrid-Q kit (GeneAll, Korea) according to the manufacturer's manual. Briefly, 5 ml of bacteria culture was centrifuged and the supernatant was discarded. The pelleted bacterial cells were resuspended thoroughly with 250 µl buffer S1 containing RNase solution. Then, 250 µl of buffer S2 was added, the tube was inverted a few times, and the cell suspension was incubated for 5 minutes until it became clear and viscous. Next, 350 µl of buffer G3 was added to neutralise the cleared lysate and the lysate was centrifuged for 10 minutes. The supernatant was transferred carefully into a spin column by decanting or pipetting. The DNA binding column was washed with 500 µl buffer AW, followed by 700 µl buffer PW. Finally, the plasmid DNA was eluted with 50 µl of pre-heated (70°C) nuclease-free water. The plasmid DNA concentration was quantitated using a microplate reader (BioTek Instruments, USA).

The recombinant plasmids with the inserts of interest were confirmed by restriction enzyme (RE) digestion analysis. The RE digestion mix was prepared by mixing 5 µl of CutSmart Buffer (NEB, USA), 1 µl *BamHI* (NEB, USA), 1 µl *EcoRI* (NEB, USA), 1 µl plasmid DNA and nuclease-free water to a total volume of 45 µl. The reaction was incubated at 37°C for 2 hours. The digested product was viewed on agarose gel. The confirmed plasmids were sent for sequencing with two forward primers that targeted VP1 (F4, primer sequence: 5'-AGTATCTGGTATCAAACAAACTAC-3') and VP2 (F2, primer sequence: 5'-CATCCGGTGTGCAATAGAGC-3') capsid proteins. The sequence was analysed to ensure that the position of the substitution mutation was correct.

3.3.8 Plasmid purification

The colony with the correct substitution mutation was inoculated in 20 ml LB/kanamycin broth and incubated overnight at 37°C with constant shaking. The plasmids were purified using PureLink HiPure Plasmid DNA Purification Kit (Invitrogen, USA) according to the manufacturer's manual. Briefly, 2 ml of equilibration buffer was applied to the HiPure Mini Column and the solution was allowed to drain by gravity. The overnight bacterial culture was pelleted and all the supernatant was discarded. The pellet was resuspended with 0.4 ml lysis buffer and mixed by inverting the tubes a few times. After 5 minutes of incubation, 0.4 ml precipitation buffer was added and the tube was inverted a few times. The lysate was centrifuged and the supernatant was loaded into the equilibrated column by pipetting. The column was washed twice with 2.5 ml wash buffer and 0.9 ml elution buffer was added to the column. After all the elution buffer was collected in a 1.5 ml tube, 0.63 ml isopropanol was added to the eluate to precipitate the plasmid DNA. The mixture was mixed well and centrifuged for 30 minutes at 4°C. One ml of 70% ethanol was added to the plasmid DNA pellet and the tube was centrifuged again. The supernatant was discarded and the pellet was air-dried for about 10 minutes. Lastly, the plasmid DNA was resuspended in 50 µl nuclease-free water and stored at -20°C.

3.3.9 Transfection of the EV-A71 escape mutant infectious clones

RD cells were seeded into a 6-well plate at 5 x 10^5 cells/well and incubated overnight until the cells reached 70-90% confluent at transfection. The transfection mixture was prepared according to the Lipofectamine LTX DNA Transfection Reagent protocol (Invitrogen, USA). In a 1.5 ml tube, 12 µl Lipofectamine LTX Reagent was mixed with 150 µl Opti-MEM medium (Gibco, USA). In a separate 1.5 ml tube, 150 µl Opti-MEM medium was mixed together with 2.5 µg of purified plasmid DNA and 3 µl of PLUS

Reagent. Then, the diluted DNA was mixed with the diluted Lipofectamine LTX Reagent in a 1:1 ratio and incubated for 5 minutes at room temperature. The DNA-lipid complex was then added to the cells drop-by-drop. At 4 hours after transfection, the transfection medium was removed and fresh DMEM supplemented with 2% heat-inactivated FBS was added. The plate was incubated for 72 hours at 37°C.

After 72 hours of incubation, the plate was freeze-thawed once. The infectious DNA-transfected RD cells were harvested and cell debris was removed by centrifugation at 10,000 x g for 10 minutes at 4°C. The mutant virus-containing supernatant was kept at -80°C. To check virus viability, RD cells seeded in a 96-well plate were infected with the supernatant and incubated for 1 hour at room temperature with constant rocking. The inocula were replaced with DMEM supplemented with 2% heat-inactivated FBS and the plate was incubated at 37°C for 3-5 days or until CPE could be observed. The stock of the mutant virus was prepared by re-infected RD cells on a 6-well plate with the transfected virus supernatant. Plaque assay was done to quantitate mutant virus stock titres and also to see the plaque characteristics of the mutant viruses compared to the wild-type (refer to Section 3.1.4).

3.3.10 Neutralisation activity of the EV-A71 escape mutant infectious clones

3.3.10.1 Serum samples

Serum samples from 4 EV-A71-positive children paients aged <12 years old were used to study the neutralisation activity of the mutant viruses (sample numbers 17169, 17171, 17188 and 17204). All samples were collected in 2000 and had been previously found to be positive for anti-EV-A71 IgG (by Western blotting) and IgM (by IgM-capture ELISA and IgM-colloidal gold immunochromatographic assay, Beijing Wantai Biological Pharmacy Enterprise Co., Ltd., China), as well as neutralisation titre of >1:8.

Anti-EV-A71-positive mouse serum and pooled sera from healthy adults with neutralisation titre of >1:8 and from the laboratory were also used for the assay.

3.3.10.2 Neutralisation assay

Neutralisation assay was performed to confirm the ability of the infectious clones carrying the mutations to escape neutralisation by the MAB979 antibody, and, as comparison, the mouse antiserum. The serum samples from children and adults were used in the assay to determine the ability of the mutants to escape neutralisation by EV-A71 neutralising antibodies present in human sera.

Neutralisation assay was performed as described in Section 3.3.3, with both MAB979 antibody and the sera serially two-fold diluted from 1:8 to 1:512. A similar experiment was performed using the EV-A71 strain 41 wild-type virus as a comparison.

3.3.10.3 Construction of EV-A71 pentamer structure

Pentamer structure of EV-A71 was constructed to visualise the location of the VP2-T141I and VP1-D14N mutations, and to identify whether these mutations are internal or external. The EV-A71 (PDB: 3VBS) pentamer structure was constructed using UCSF chimera software.

CHAPTER 4

RESULTS

4.1 Epidemics of HFMD in Malaysia

4.1.1 Nationwide HFMD cases

Malaysia consists of Peninsular Malaysia, where most of the country's 16 states and federal territories are located, and East Malaysia, which consists of Sabah, Sarawak and Labuan. The monthly notified HFMD cases in each state and federal territory were available from 2008-2014 (Figure 4.1). The total annual HFMD cases in 2008-2014 were 15,564, 17,154, 13,394, 7,002, 34,519, 23,331 and 31,322 respectively. In 5 of the 7 years, HFMD cases increased around March and peaked around May-June. Sarawak had the highest number of HFMD cases nationwide, while Selangor reported the highest number of cases among the states in Peninsular Malaysia.

Only national overall HFMD incidence rates were available from 2006. Details of causative viruses are generally not available, as most HFMD cases are clinically diagnosed and diagnostic virology facilities are not widely accessible. However, published reports of laboratory-confirmed EV-A71 epidemic years (Chua & Kasri, 2011; Podin et al., 2007; Yusof et al., 2014) were in accordance with cyclical reported HFMD activity from the available surveillance data, showing that EV-A71 epidemics occurred in Malaysia every 2-3 years, in 1997, 2000, 2003, 2006, 2008/9, and 2012.

4.1.2 Age-specific incidence of HFMD in Kuala Lumpur and Malaysia

Age-specific incidence data was available only from 2011 to 2014 for the total HFMD cases nationwide and for the cases in Kuala Lumpur (Figure 4.2). The incidence rate of HFMD was the highest in those <2 years in both Kuala Lumpur (8.3, 43.7, 19.0 and 31.7 per 1000 population in 2011, 2012, 2013 and 2014, respectively) and Malaysia

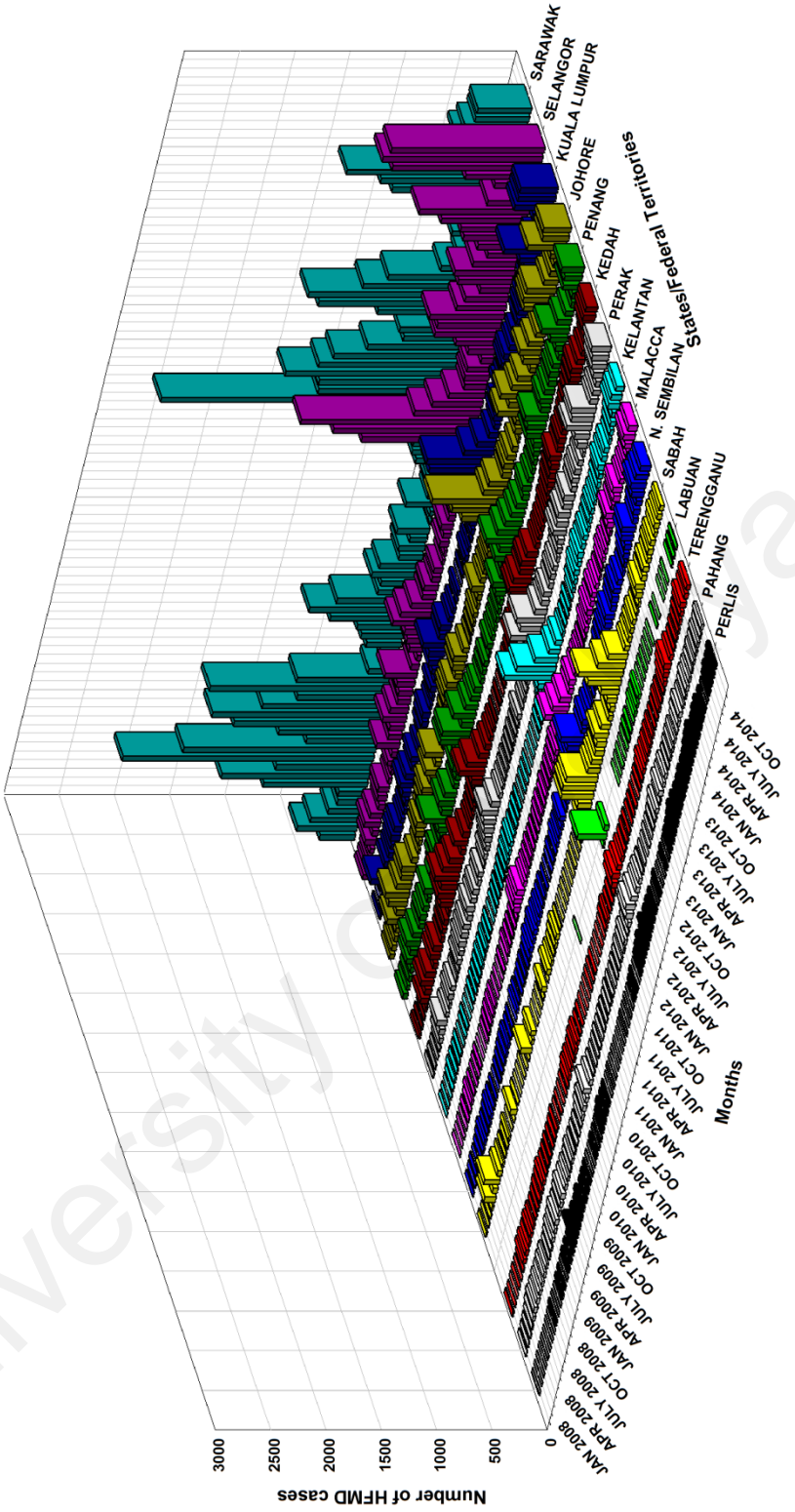


Figure 4.1: Monthly distribution of the number of notified HFMD cases in every state of Malaysia from 2008-2014. Data were obtained from the Ministry of Health, Malaysia.

(4.8, 22.9, 17.9 and 20.6 per 1000 population in 2011, 2012, 2013 and 2014, respectively). The rates decreased with increasing age, with the 7-12 years age group having the lowest incidence rates; in Kuala Lumpur, rates were 0.07, 1.1, 0.4 and 1.2 per 1000 population in 2011, 2012, 2013 and 2014 respectively, and overall in Malaysia, rates were 0.2, 0.9, 0.4 and 0.7 per 1000 population in 2011, 2012, 2013 and 2014, respectively.

4.2 Seroprevalence studies of EV-A71

4.2.1 Interpretation of neutralisation assay results

As explained in Section 3.2.2., the presence or absence of CPE in RD cells serves as an indicator in determining and interpreting the neutralising antibody titres. The neutralising antibody titre was defined as the highest dilution that prevents the development of CPE in 50% of the inoculated cells. Figure 4.3 shows the differences in the characteristics of infected cells, non-infected cells, and cells inoculated with seropositive or seronegative sera. In Figure 4.3C, the RD cells were alive and looked similar to the mock-infected RD cells (Figure 4.3A). The seropositive sera, which contain EV-A71 neutralising antibodies, fully inactivated or neutralised the virus during the 2-hour incubation period, thus the viruses were unable to infect the cells and cause CPE. The seronegative sera did not contain any neutralising antibodies, thus the viruses were not neutralised and were able to infect the cells, causing CPE seen in Figure 4.3D. The cells inoculated with seronegative sera looked similar to virus-infected cells (Figure 4.3B).

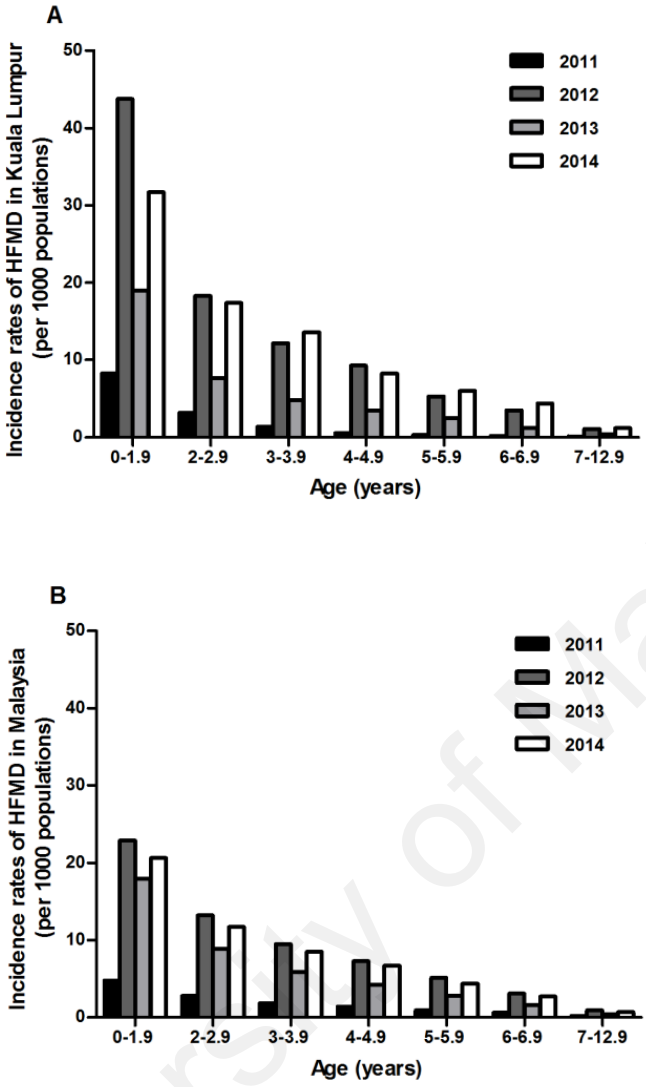


Figure 4.2: Age-specific incidence rates of HFMD cases in Kuala Lumpur and Malaysia. Incidence rates (per 1000 population) of HFMD cases in (A) Kuala Lumpur and (B) Malaysia were only available from the Ministry of Health, Malaysia for 2011 to 2014. Only combined data for cases aged <2 years was available.

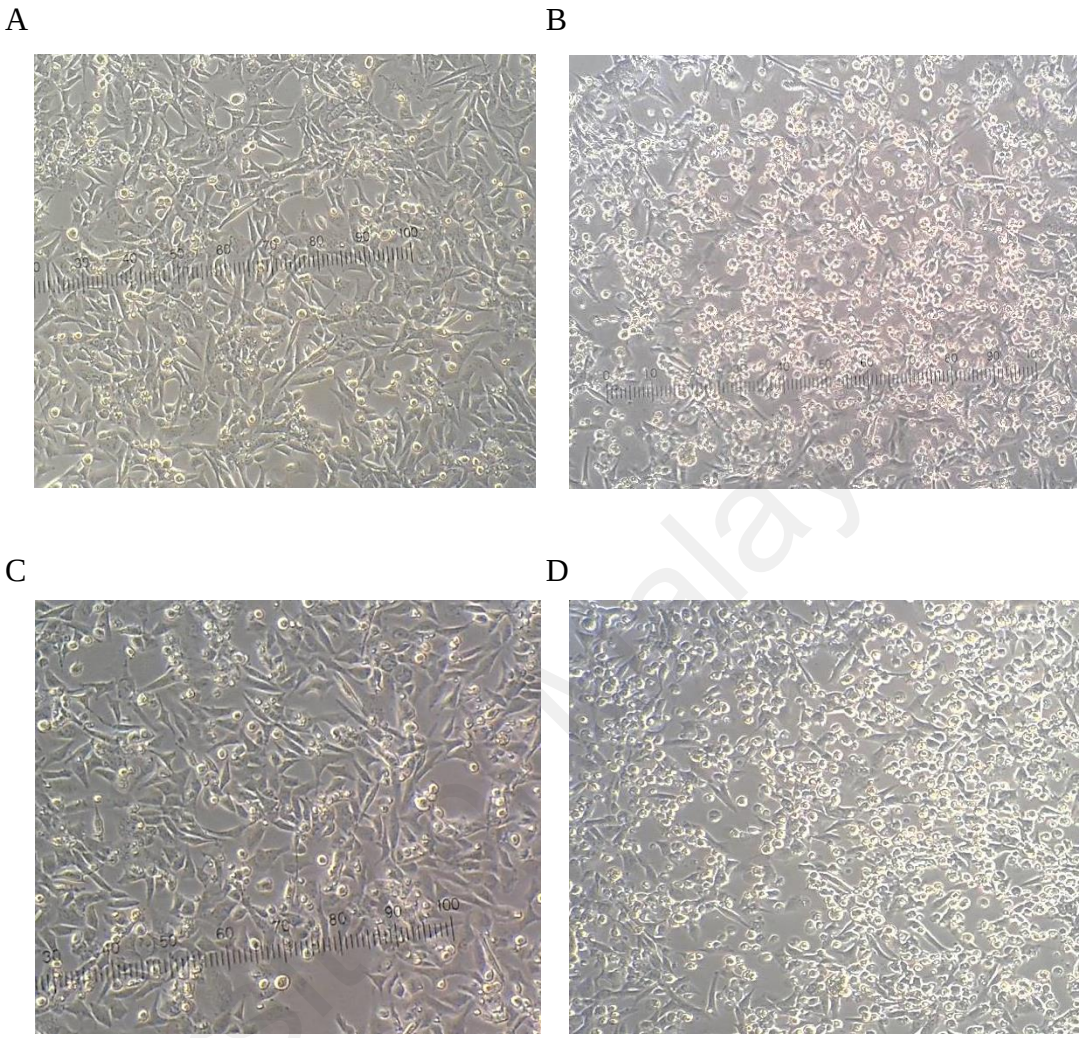


Figure 4.3: Characteristics of RD cells inoculated with (A) serum-free media only (mock-infected cells); (B) EV-A71 virus only (virus control); (C) seropositive sera; and (D) seronegative sera.

4.2.2 Verification of concordance of neutralisation titres between EV-A71 strains

A neutralisation assay was performed on additional serum samples from children <3 years old, comprising 39 sera from patients infected with EV-A71 B5 virus and 32 sera from patients with EV-A71 C1 virus. This was done to verify concordance of the neutralising antibody titres between these viruses with that of the subgenotype B4 virus which was used in the neutralisation assay in this study. These subgenotypes were reported to be circulating in Malaysia between 1997 and 2012 (Chan *et al.*, 2012).

High concordance in seropositive/seronegative status was obtained with the sera using either UH1 or B5 virus (97%, 38/39 sera) and UH1 or C1 virus (81%, 26/32 sera). Hence, these results support the use of the B4 virus alone for all the neutralisation assays.

4.2.3 Seroprevalence of EV-A71 in children up to 12 years in Kuala Lumpur, Malaysia

In Malaysia, the seroprevalence of EV-A71 infection is not well-documented. To date, much of the information regarding the epidemics of HFMD/EV-A71 infection were provided by syndromic surveillance and molecular data. Based on these data, the HFMD/EV-A71 epidemics in Malaysia occurred in a 3-year cyclical pattern, which was clearly shown in epidemics in Sarawak (Chan *et al.*, 2011; Sham *et al.*, 2014). One of the objectives of the present study was to determine the pattern of EV-A71 seroprevalence and age-dependent seroprevalence in children in Kuala Lumpur. The seroprevalence rates and GMT in epidemic and non-epidemic years were also compared to see if decreases in the rate and GMT among susceptible groups in between epidemics contributed to the cyclical pattern. Also, this study investigated how the emergence of different EV-A71 subgenotypes may have contributed to the cyclical pattern of epidemics.

4.2.3.1 EV-A71 seroprevalence and GMT decrease during non-epidemic years

EV-A71 seroprevalence was higher in the primary school 7-12 years age group (71.6%, 95% CI 68.2-74.7%) compared to the preschool 1-6 years age group (52.8%, 95% CI 49.8-55.9%; $P<0.001$) overall, and in 16 out of the 18 years analysed (with significant differences in 3 years; Table 4.1). The overall seroprevalence and GMT were significantly higher in epidemic years (seroprevalence 67.4%, 95% CI 63.8-70.9%; GMT 23.6, 95% CI 21.8-25.5) compared to non-epidemic years (seroprevalence 56.6%, 95% CI 53.6-59.5%; GMT 17.8, 95% CI 16.7-19.0; $P<0.001$) (Figure 4.4). As shown in Figure 4.4A, during epidemic years, the seroprevalence of children aged 1-2 years (52.5%, 95% CI 44.8-60.0%), 3-5 years (66.1%, 95% CI 58.7-72.8%), and 6-9 years (75.4%, 95% CI 69.1-80.8%) were significantly higher compared to non-epidemic years (1-2 years old: 39.6%, 95% CI 33.9-45.5%; 3-5 years old: 51.6%, 95% CI 45.8-57.4%; and 6-9 years: 64.4%, 95% CI 59.1-69). GMT also rose significantly during epidemic years (3-5 years old: 23.3, 95% CI 19.8-27.3; 6-9 years: 26.7, 95% CI 23.4-30.4; and 10-12 years old: 28.0, 95% CI 23.5-33.4) compared to non-epidemic years (3-5 years old: 18.1, 95% CI 15.7-20.9; 6-9 years: 18.1, 95% CI 16.2-20.2; and 10-12 years old: 20.4, 95% CI 18.0-23.1) (Figure 4.4B).

4.2.3.2 Age-dependent seroprevalence of EV-A71 in children

The increase and decrease of the EV-A71 seroprevalence and GMT during epidemic and non-epidemic years, respectively, are consistent with the observed general trend of EV-A71 seroprevalence spiking during reported EV-A71 epidemic years, and seroprevalence falling between epidemics (Figure 4.5A). These results showed that

Table 4.1: Seroprevalence rates of EV-A71 neutralising antibody in children, 1995-2012

Year	Age group (%)			
	1-6 years	**7-12 years**	**Total**	***P* value[a]**
1995	54.0 (27/50)	75.5 (40/53)	65.0 (67/103)	0.025
1996	47.8 (22/46)	68.4 (52/76)	60.7 (74/122)	0.035
1997	51.5 (34/66)	78.9 (56/71)	66.2 (90/137)	0.001*
1998	55.2 (16/29)	61.1 (22/36)	58.5 (38/65)	0.801
1999	55.0 (33/60)	65.0 (13/20)	57.5 (46/80)	0.602
2000	79.1 (53/67)	71.4 (35/49)	75.9 (88/116)	0.384
2001	42.3 (22/52)	64.3 (27/42)	52.1 (49/94)	0.040
2002	64.8 (35/54)	79.3 (23/29)	69.9 (58/83)	0.214
2003	57.1 (40/70)	72.7 (32/44)	63.2 (72/114)	0.113
2004	53.8 (64/119)	74.1 (60/81)	62.0 (124/200)	0.005
2005	28.7 (25/87)	58.0 (40/69)	41.7 (65/156)	<0.001*
2006	36.1 (22/61)	85.0 (17/20)	48.2 (39/81)	<0.001*
2007	45.0 (27/60)	74.2 (23/31)	54.9(50/91)	0.014
2008	65.3 (49/75)	78.9 (41/52)	70.9 (90/127)	0.115
2009	72.2 (13/18)	91.7 (11/12)	80.0 (24/30)	0.358
2010	42.9 (18/42)	70.6 (12/17)	50.9 (30/59)	0.084
2011	44.2 (19/43)	56.3 (9/16)	53.8 (28/59)	0.559
2012	77.1 (27/35)	76.5 (13/17)	76.9 (40/52)	1.000
TOTAL	52.8 (546/1034)	71.6 (526/735)	60.6 (1072/1769)	<0.001

[a]Fisher's exact test was used to test the associations between age group and seroprevalence.

*P<0.05 after Bonferroni correction of type 1 family wise error accounting for multiple comparisons across individual years.

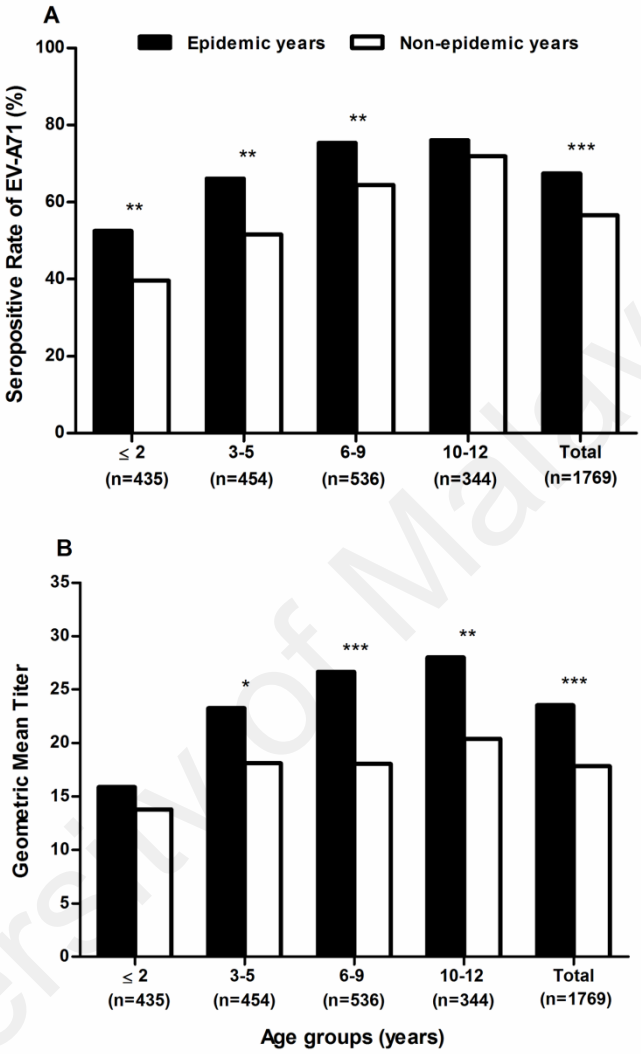


Figure 4.4: Age-specific seroprevalence and geometric mean titres of EV-A71 infection. (A) Seroprevalence rates and (B) geometric mean titres of EV-A71 infection by age, in epidemic and non-epidemic years (P<0.05*; P<0.01**; P<0.001***).

younger children aged 1-6 years old had lower seroprevalence in non-epidemic years, indicating greater susceptibility, which may explain the higher HFMD incidence in this age group (Figure 4.2). The higher seropositive rates and GMT levels seen during epidemic years are likely to reflect recent infection. HFMD incidence in older children aged 7-12 years is considerably lower; thus, the higher GMT levels seen during epidemics are more likely to represent re-exposure to EV-A71 or milder infection resulting in under-reporting. Taken together, both the incidence rates and the seroprevalence data suggested that HFMD caused by EV-A71 affects susceptible children aged 1-12 years, and most frequently affects younger children aged 1-6 years.

4.2.3.3 Phylogenetic analysis of the emergence of different EV-A71 subgenotypes in Malaysia

Many subgenotypes were co-circulating during EV-A71 epidemics. Subgenotypes B3, B4, C1 and C2 were present during the 1997 epidemic, but only subgenotypes B4 and C1 continued to circulate till 2001 and 2003, respectively (Figure 4.5C). After 2003, subgenotype B5 became the sole genotype circulating in Malaysia. A Bayesian Skyride plot was used to estimate the evolutionary dynamics of EV-A71 in Malaysia over time (Figure 4.5B). Sharp, transient rises of genetic diversity were observed in the reported epidemic years 1997, 2000, 2003, 2006, 2008/2009, and 2012. The decline in the effective population seen after the 1997 epidemic may coincide with purifying selection against subgenotypes B3 and C2. The decline in the effective population observed after the 2000 and 2003 epidemics may indicate purifying selection against subgenotypes B4 and C1, respectively. After 2006, when only subgenotype B5 was circulating, interepidemic viral diversity showed overall decline punctuated by spikes during the epidemic years of 2008/2009 and 2012.

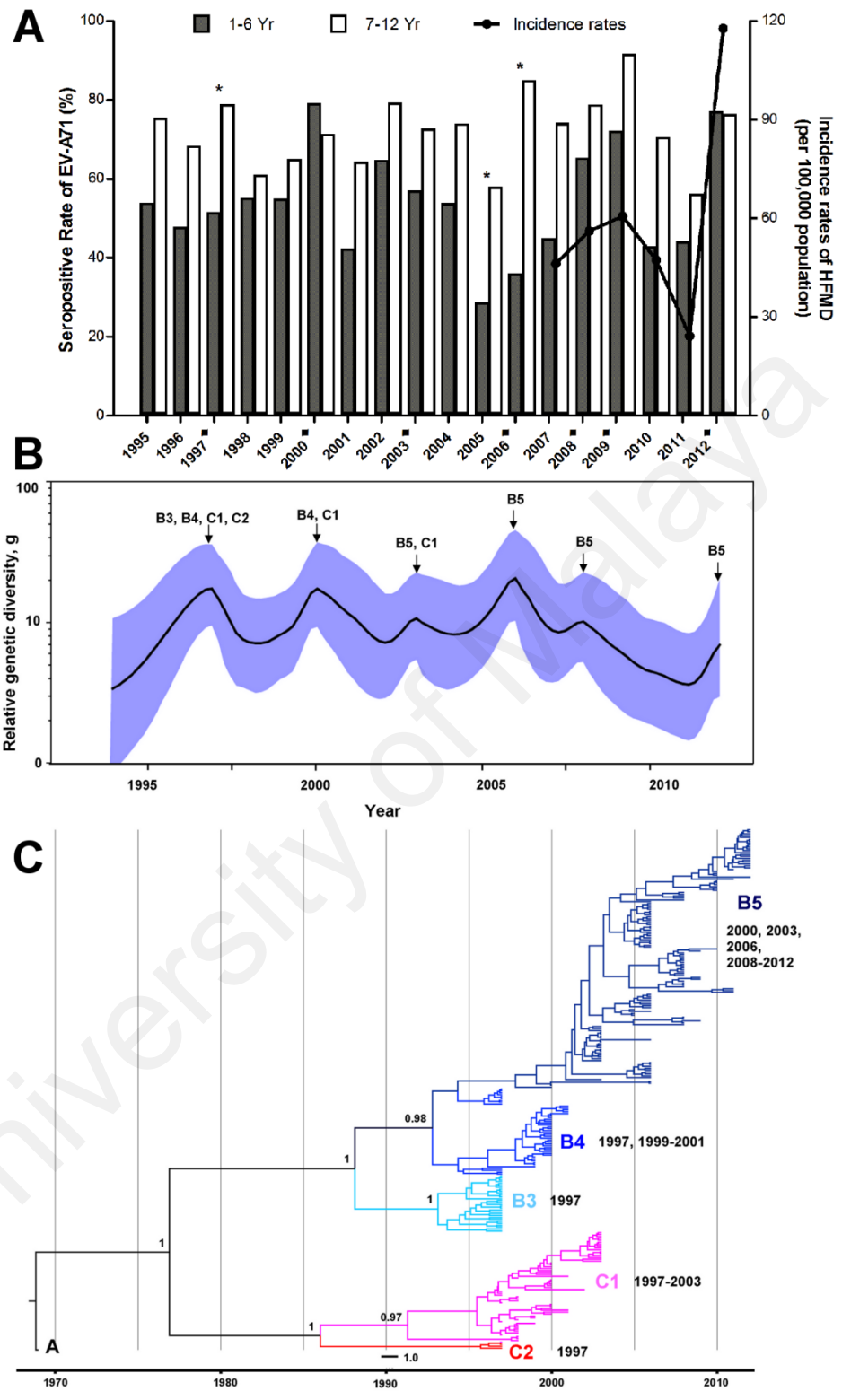


Figure 4.5: Age-dependent seroprevalence rates of EV-A71 infection in Kuala Lumpur, Malaysia from 1995-2012. (A) Seroprevalence rates of EV-A71 infection in 1-6 years (pre-school) and 7-12 years (primary school) age groups are shown. The asterisks indicate significant differences (P<0.05) in seroprevalence between the two age groups after Bonferroni correction for multiple comparisons across individual years. The black squares indicate reported EV-A71 epidemic years. The black line indicates the overall incidence rates of HFMD as reported by the Ministry of Health, Malaysia from 2007 (the statutory notification of HFMD was enforceable only from October 2006). (B) Bayesian Skyride plot estimating the genetic diversity of EV-A71 over time. (C) Maximum clade credibility tree based on VP1 sequences showing the EV-A71 subgenotypes present in Malaysia since the first epidemic in 1997. Posterior probability values are shown at the key nodes. EV-A71 BrCr from genotype A was used as the outgroup.

4.2.4 Seroprevalence and potential risk factors of EV-A71 infection among Orang Asli population in West Malaysia

Living in rural areas was reported as a risk factor for EV-A71 infection in previous studies (Chang *et al.*, 2002; Chen *et al.*, 2015; Zeng *et al.*, 2013). Many of these studies also investigated the EV-A71 risk factors in urban settings. To date, there have been no studies of the risk factors for EV-A71 infection in rural areas. In this study, the seroprevalence of EV-A71 infection between urban and rural populations were compared. The risk factors that contributed to EV-A71 seropositivity among the rural population were also determined.

4.2.4.1 Sociodemographic characteristics of urban KL and Orang Asli subjects

For the urban KL population, only age and gender data were available as the serum samples were selected from archived residual sera. For the 460 urban KL subjects, the mean age was 27.8 ± 22.5 years; 170 (37.0%) were ≤12 years, and 199 (43.3%) were female. Multivariate analysis was performed with age and gender as the predictors of seropositivity to EV-A71 in the urban KL population. The result confirmed that age ≤12 years (adjusted OR 2.88, 95% CI 1.90-4.37, P<0.001) was a predictor for EV-A71 seropositivity, but not gender (adjusted OR 1.15, 95% CI 0.76-1.76, P=0.507). For the 298 Orang Asli subjects, the mean age was 19.0 ± 17.4 years; 177 (59.4%) were ≤12 years, and 163 (54.7%) were female. As there were significant differences in age and gender between the urban and Orang Asli populations sampled (mean age, P<0.001; age ≤12 years, P<0.001; and gender, P=0.0023), further comparisons were only made between the same age groups of the two populations.

Full sociodemographic data was available for 248/298 (83.2%) Orang Asli subjects (Table 4.2), who were from the Proto-Malay ethnic group (Temuan subgroup, 74.8%) and Senoi group (Semai, 18.1%; Semoq Beri, 3.7%; and Jah Hut subgroups, 3.4%).

Findings for key socioeconomic indicators include 151/248 (60.9%) living in households with income <MYR 500/month (USD 123; in Malaysia, 0.5% of households are within this income class (Economic Planning Unit, Prime Minister's Department, Malaysia)), 131/248 (52.8%) with untreated water supply, and 190/248 (76.6%) who do not use a water-flush toilet for defaecation (Table 4.2).

4.2.4.2 Comparison of age-specific seropositivity rates of EV-A71 infection between urban KL and Orang Asli populations

Overall, a strong association between EV-A71 seropositivity and increasing age was seen in both urban KL (OR 1.02, 95% CI 1.01-1.03; *P*=0.001) and Orang Asli (OR 1.03, 95% CI 1.01-1.05; *P*<0.001) populations (Figure 4.6). For the urban KL population, the EV-A71 seropositivity rates increased gradually from 47.1% (95% CI 35.9-58.7%) in the youngest age group (1-3 years) to 84.2% (95% CI 78.0-88.9%) in the 18-49 years group, before declining significantly at the age of 50 years and older to 72.0% (95% CI 62.2-80.2%; *P*=0.025). Seropositive rates for the rural Orang Asli population showed a different trend, with very high rates in the youngest age groups, 1-3 years (81.8%, 95% CI 51.2-96.0), 4-6 years (97.1%, 95% CI 83.8-99.9%) and 7-12 years (96.2%, 95% CI 91.2-98.6). The seropositivity rates decreased, but not significantly, to 70-75% as age increased. The seropositivity rates of children in the 1-3, 4-6, and 7-12 years age groups were significantly higher in the Orang Asli population than in the urban KL population, while seropositivity of those aged >12 years were similar in both populations.

Overall, Orang Asli children ≤12 years had significantly higher seropositive rates than urban children (95.5% vs. 57.6%, *P*<0.001).

Table 4.2: Risk factors associated with EV-A71 seropositivity in Orang Asli populations (n = 248).

Variables	n	EV-A71 seropositive		Crude OR (95% CI)	*P* value	Adjusted OR (95% CI)	*P* value
		n	%				
Age (years)							
≤12	158	151	95.6	6.57 (2.67-16.17)	<0.001	8.07 (3.15-20.67)	<0.001*
≥13	90	69	76.7	1		1	
Gender							
Male	108	96	88.9	1.03 (0.47-2.29)	0.938		
Female	140	124	88.6	1			
Ethnic groups (subgroups)							
Proto-Malay (Temuan)	173	155	89.6	1.33 (0.58-3.03)	0.504		
Senoi (Semai, Semoq Beri, Jah Hut)	75	65	86.7	1			
States where villages are located							
Malacca[#]	4	4	100	-		-	
Negeri Sembilan	31	29	93.5	2.18 (0.47-10.07)	0.320	0.30 (0.04-2.11)	0.228
Pahang	44	42	95.5	3.15 (0.69-14.39)	0.139	2.60 (0.50-13.50)	0.256
Perak	54	45	83.3	0.75 (0.31-1.84)	0.530	1.10 (0.36-3.32)	0.868
Selangor	115	100	87.0	1		1	

Table 4.2, continued.

Variables	n	EV-A71 seropositive		Crude OR (95% CI)	*P* value	Adjusted OR (95% CI)	*P* value
		n	%				
Occupational status							
Child/student	167	159	95.2	7.05 (2.62-18.96)	<0.001	1.851 (0.18-18.70)	0.602
Not working (adult)	39	30	76.9	1.18 (0.43-3.26)	0.746	1.05 (0.35-3.14)	0.935
Working	42	31	73.8	1		1	
Attends school (≤17 years)							
No	39	37	94.9	1.04 (0.21-5.25)	0.958		
Yes	131	124	94.7	1			
Household income							
<MYR 500/month	151	133	88.1	0.85 (0.37-1.93)	0.696		
>MYR 500/month	97	87	89.7	1			
Water supply							
Untreated (river, well, rain water)	131	125	95.4	4.83 (1.88-12.37)	0.001	6.16 (2.29-16.56)	<0.001*
Treated pipe water	117	95	81.2	1		1	
Place of defecation							
Others (toilet without water-flush, bush, rivers)	190	173	91.1	2.38 (1.05-5.43)	0.039	1.23 (0.48-3.16)	0.673
Toilet with water-flush	58	47	81.0	1		1	

Table 4.2, continued.

Variables	n	EV-A71 seropositive		Crude OR (95% CI)	*P* value	Adjusted OR (95% CI)	*P* value
		n	%				
Wash hands before eating							
No	105	90	85.7	0.60 (0.27-1.32)	0.205	0.79 (0.33-1.88)	0.595
Yes	143	130	90.9	1		1	
Wear shoes							
No	161	144	89.4	1.02 (0.38-2.72)	0.974		
Sometimes	31	26	83.9	0.62 (0.17-2.24)	0.469		
Yes	56	50	89.3	1			

*Variables confirmed as independent risk factors for EV-A71 seropositivity by multivariate analysis.
[#]Not included in the univariate and multivariate analyses because all samples were seropositive

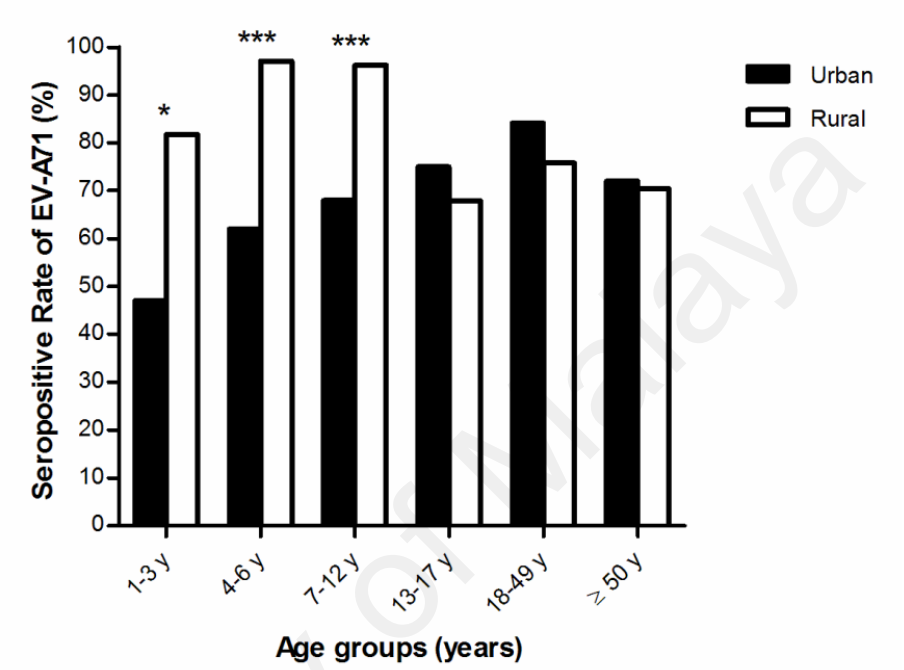


Figure 4.6: Comparison of EV-A71 seropositivity rates between urban KL and Orang Asli rural populations. The asterisks indicate significant differences in seropositive rates between the two populations by Fisher's exact test (P<0.05*; P<0.001***).

4.2.4.3 Risk factors for EV-A71 infection in Orang Asli populations

To determine sociodemographic, hygiene and lifestyle risk factors for EV-A71 seropositivity among the rural Orang Asli, univariate analysis was first performed (Table 4.2). The analysis identified six risk factors with *P* values <0.25: age ≤12 years, the states in which the Orang Asli villages are located, occupation of child/student, using untreated water supply (such as rivers or wells), defecating in places other than a water-flush toilet (such as non-flush toilets or in the open), and not washing hands before eating. Multivariate analysis confirmed two independent risk factors for EV-A71 seropositivity: age ≤12 years (adjusted OR 8.1, 95% CI 3.2-20.7, *P*<0.001) and using untreated water (adjusted OR 6.2, 95% CI 2.3-16.6, *P*<0.001). The final model had satisfactory fit and discrimination (goodness-of-fit, *P*=0.54; area under the curve = 0.79, 95% CI 0.70-0.89, *P*<0.001).

4.3. Characterisation of EV-A71 neutralisation escape mutants

Previous studies have identified neutralisation epitopes, not only in VP1 but also in other capsid proteins, in both humans and animals (Chang *et al.*, 2010; Foo *et al.*, 2007; Gao *et al.*, 2012; Jiang *et al.*, 2015; Kiener *et al.*, 2014; Liu *et al.*, 2011; Xu *et al.*, 2014; Zhang *et al.*, 2016). The present study aimed to identify more EV-A71 neutralisation epitopes by generating EV-A71 neutralisation escape mutants. A neutralisation escape mutant is a virus that has the ability to escape antibody-mediated neutralisation. The mutants usually carry mutations at a region which is critical for antigen-antibody interaction, known as a neutralisation epitope (Mateu, 1995). The monoclonal antibody MAB979 which was used in generating the EV-A71 neutralisation escape mutants in this study is a mouse monoclonal antibody which is anti-EV-A71-specific. It belongs to the IgG_1 subclass. In a previous study, MAB979 was shown to bind to a region of VP2

(amino acid sequence 136-150) which is highly conserved among EV-A71 genotypes (Liu *et al.*, 2011).

In this objective, an EV-A71 neutralisation escape mutant was isolated after four passages in the presence of MAB979, and the mutations associated with loss of neutralisation activity were identified. Infectious clones that carried the identified mutations were constructed to confirm the loss of neutralisation.

4.3.1 Confirmation of EV-A71 neutralisation escape mutants

To generate EV-A71 neutralisation escape mutants, the virus was incubated in the presence of the monoclonal antibody MAB979, harvested and further passaged. A single escape mutant virus was obtained, and sequencing showed two amino acid substitutions: threonine (T) to isoleucine (I), located at the amino acid position 141 on VP2 protein (EV-A71 VP2-T141I); and aspartic acid (D) to asparagine (N), at amino acid position 14 on the N-terminal end of VP1 protein (EV-A71 VP1-D14N). The amino acid substitutions are illustrated in Figure 4.7.

4.3.2 Construction of EV-A71 escape mutant infectious clones by site-directed mutagenesis

To further confirm the ability of the escape mutant to evade neutralisation activity, three EV-A71 mutant infectious clones were constructed. The plasmid backbone, pCMV-EV-A71 was originally constructed based on the EV-A71 strain 41, a genotype B4 virus, using the Gibson-assembly method (unpublished data). This method has been widely used for viruses, including flaviviruses, porcine reproductive and respiratory syndrome virus and plant viruses (Aubry *et al.*, 2014; Bordat *et al.*, 2015; Kulkarni *et al.*, 2014; Suhardiman *et al.*, 2015). The mutations were incorporated by exponential amplification, where the primers were designed in a back-to-back orientation as

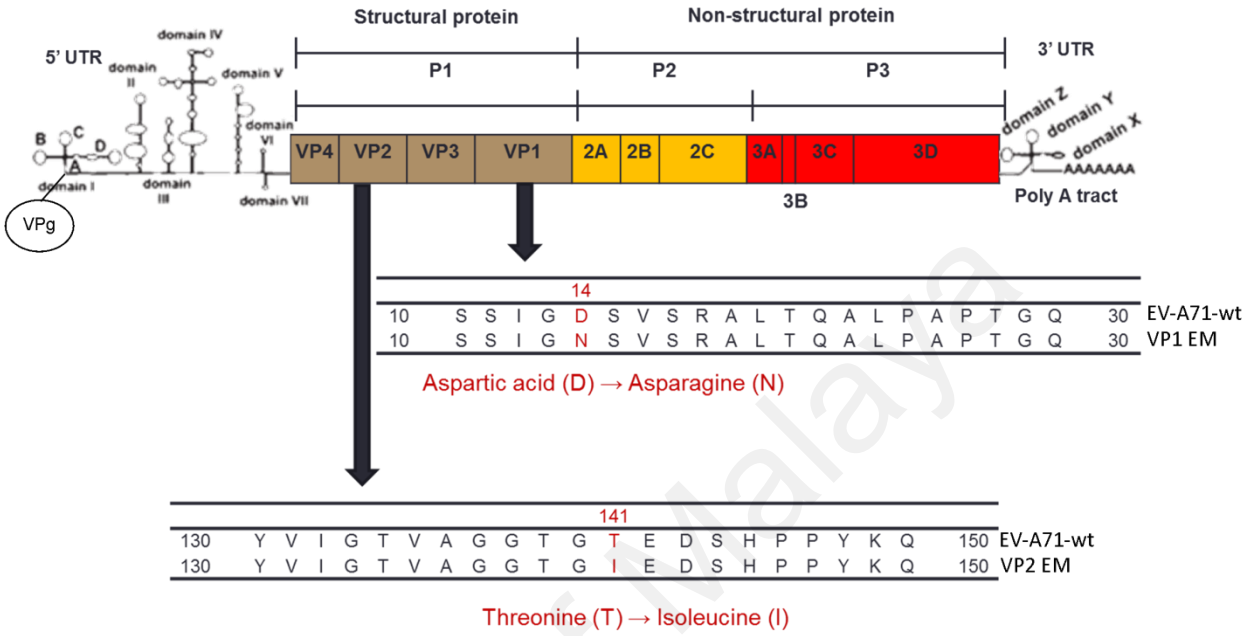


Figure 4.7: Amino acid substitutions in the neutralisation escape mutant identified by DNA sequencing. On the VP2 protein, the amino acid at position 141 changed from threonine (T) in the wild type EV-A71 UH1 strain to isoleucine (I) in the EV-A71 escape mutant (EV-A71 VP2-T141I). At position 14 on the VP1 protein, aspartic acid (D) in the wild type virus changed to asparagine (N) in the escape mutant (EV-A71 VP1-D14N). (wt = wild type; EM = escape mutant; VPg = viral protein genome-linked; UTR = untranslated region)

described in Section 3.3.5. Two plasmids carried one mutation each, either VP2-T141I or VP1-D14N, and another one carried both mutations (termed double mutation). Figure 4.8A shows the electrophoresis gel of the PCR products of all three clones at size 10.6 kbp.

The amplified plasmid products were transformed, purified and sequenced to ensure that the mutations were incorporated correctly. Before sending for DNA sequencing, the plasmids were subjected to RE digestion (*BamHI* and *EcoRI*) to ensure that the genes were inserted at the correct orientation. Figure 4.8B shows the electrophoresis gel of the RE digested plasmids.

4.3.3 Transfection and plaque morphology of EV-A71 escape mutant viruses

The pCMV-EV-A71 plasmid carrying the mutations was transfected into RD cells to allow replication of the infectious clones. The supernatant containing the infectious particles of the EV-A71 escape mutant was collected and quantitated using plaque assay. Plaque assay is commonly used to measure virus titre or the concentration of virus in a sample. Besides quantitating the escape mutant virus, the plaque assay was performed to compare the plaque size of the EV-A71 escape mutants with the wild-type EV-A71 (Figure 4.9). The EV-A71 double mutation and VP2-T141I escape mutant viruses showed smaller plaque size compared to the wild-type EV-A71, while there were no differences in the plaque morphology between the EV-A71 VP1-D14N escape mutant and the wild-type virus. Smaller plaques may indicate slower replication efficiency of the EV-A71 double mutation and VP2-T141I escape mutant viruses.

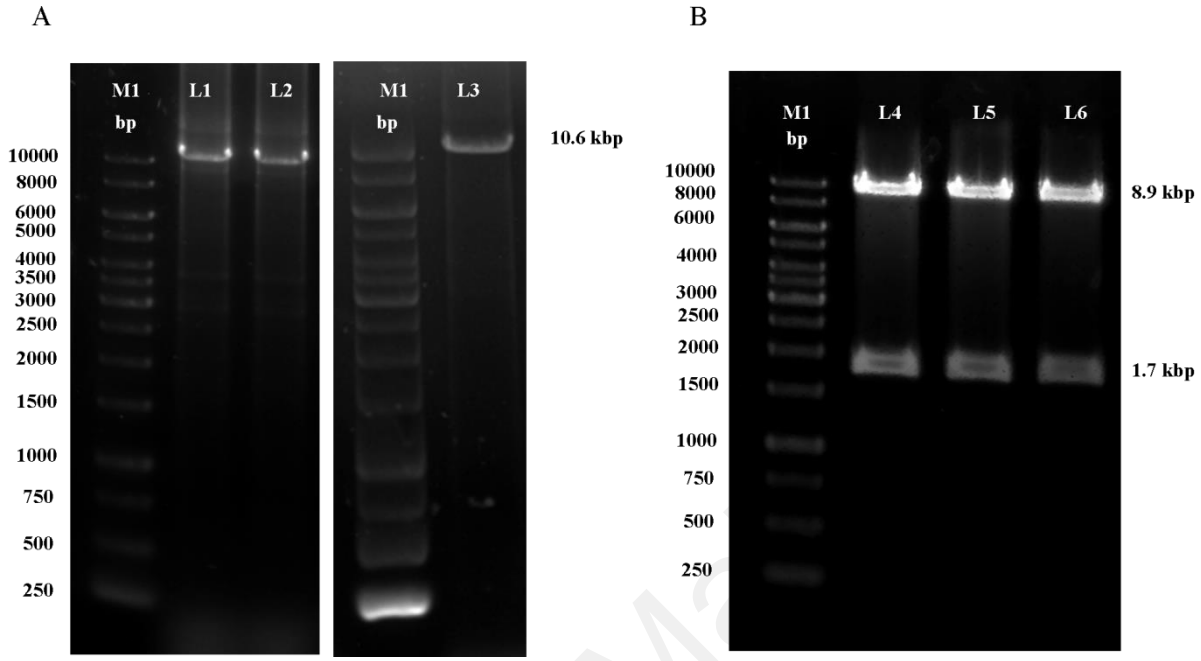
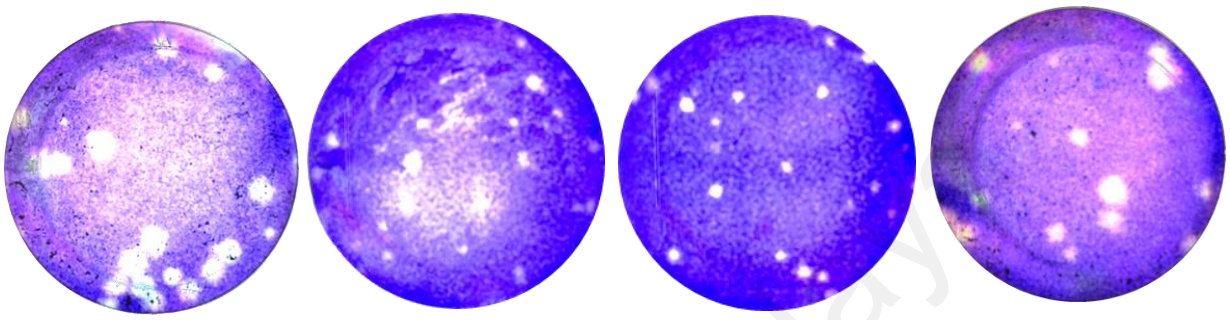


Figure 4.8: Agarose gel electrophoresis of products of site-directed mutagenesis by exponential amplification. (A) PCR products, pCMV-EV-A71 plasmid with the incorporation of the desired mutations. (B) *BamHI* and *EcoRI* digested pCMV-EV-A71 plasmids. Lane M1 contains 1 kb DNA ladder, with the sizes of the ladder and PCR products indicated in base pairs (bp) and kilobase pairs (kbp); lane L1 contains the pCMV-EV-A71 plasmid carrying the VP2-T141I mutation; lane L2 contains the pCMV-EV-A71 plasmid carrying the VP1-D14N mutation; lane L3 contains pCMV-EV-A71 plasmid carrying both mutations; lanes L4 to L6 contain the digested products of plasmids pCMV-EV-A71 carrying VP2-T141I, VP1-D14N and both mutations, respectively.



EV-A71 strain 41 (wild-type)

EV-A71double mutant VP2-T141I/ VP1-D14N

EV-A71 VP2-T141I

EV-A71 VP1-D14N

Figure 4.9: Plaque formation sizes of the EV-A71 strain 41 wild-type, EV-A71 double mutant VP2-T141I/VP1-D14N, EV-A71 VP2-T141I and EV-A71 VP1-D14N, at 72 hours post-infection.

4.3.4 Neutralisation of the EV-A71 neutralisation escape mutant viruses

Neutralisation assay was performed again to see the ability of the escape mutant viruses to evade the neutralising activity of MAB979, a panel of anti-EV-A71-positive sera from children, pooled adult sera and anti-EV-A71-positive mouse serum (Table 4.3). Some previous studies had shown that the mouse and human antibody epitopes are different, and present experiment could show whether the amino acid changes at these two positions are important neutralising epitopes of EV-A71 in mice and humans.

All sera and the MAB979 showed high neutralisation titres against the wild-type virus. The EV-A71 VP2-T141I/VP1-D14N mutant and EV-A71 VP2-T141I viruses escaped neutralisation by the MAB979 completely. The EV-A71 VP1-D14N was neutralised by MAB979 at a dilution of 1:64, which was just two-fold lower than that of the wild-type virus, indicating a non-significant effect. These results confirm that the mutation of the amino acid at position 141 on the VP2 capsid is important for neutralisation by MAB979, as it is falls within the previously reported neutralisation epitope site recognised by MAB979 (Liu *et al.*, 2011).

The neutralisation titres of the mouse serum decreased by 4-fold against both EV-A71 VP2-T141I/VP1-D14N mutant and the VP2-T141I escape mutant, and two-fold against the EV-A71 VP1-D14N, compared to wild-type. The fact that the escape mutants did not completely escape the neutralisation by the mouse serum suggests that the mutations do not occur on the major neutralising epitopes of EV-A71, but the amino acid changes do affect the neutralisation activity.

The escape mutants were incubated with human sera from patients with HFMD to see the effect of the mutations on the neutralisation activity of the sera. A pool of healthy adult sera with anti-EV-A71-positive neutralising antibodies and four individual sera from children that had detectable anti-EV-A71 IgG and IgM were used. There was no decrease in the neutralisation titres for pooled adult sera against both EV-A71 VP2-

T141I/VP1-D14N and VP2-T141I escape mutants when compared with that of the wild-type, and just a two-fold decrease in the titre against the EV-A71 VP1-D14N escape mutant. For the children's sera, the neutralisation titres for the mutant viruses were the same or decreased by two-fold compared with the wild-type in all but three instances, against the EV-A71 VP1-D14N escape mutant, the samples 17169 and 17171 showed 8- and 16-fold decrease, respectively; and another two sera (17188 and 17204) showed 4-fold decrease. Overall, the results suggest that the mutation on the VP2 capsid has little or no effect on the neutralisation activity of human sera, but the mutation on the VP1 capsid may have an important role.

A pentamer structure of the capsid proteins of EV-A71 was constructed (Figure 4.10). As previously reported, amino acid 141 on the EV-A71 VP2 is located within the EF loop (Liu *et al.*, 2011) which is well-exposed on the EV-A71 surface (Figure 4.10A; Xu *et al.,* 2014). The VP1-D14N mutation is located on the N-terminus of the VP1, which is buried within EV-A71 (Figure 4.10B; Lim *et al.*, 2013).

Table 4.3: Neutralisation titres of monoclonal antibody MAB979 and anti-EV-A71-positive human and mouse sera against EV-A71 wild-type virus and three neutralisation escape mutant viruses

EV-A71	MAB979	Mouse sera	Anti-EV-A71-positive children sera				Adult sera (pooled)
			17169	17171	17188	17204	
Strain 41 (wild-type)	128	128	128	64	64	128	128
VP2-T141I / VP1-D14N mutant	<8	32	64	64	32	64	128
VP2-T141I	<8	32	64	64	32	64	128
VP1-D14N	64	64	16	8	32	32	64

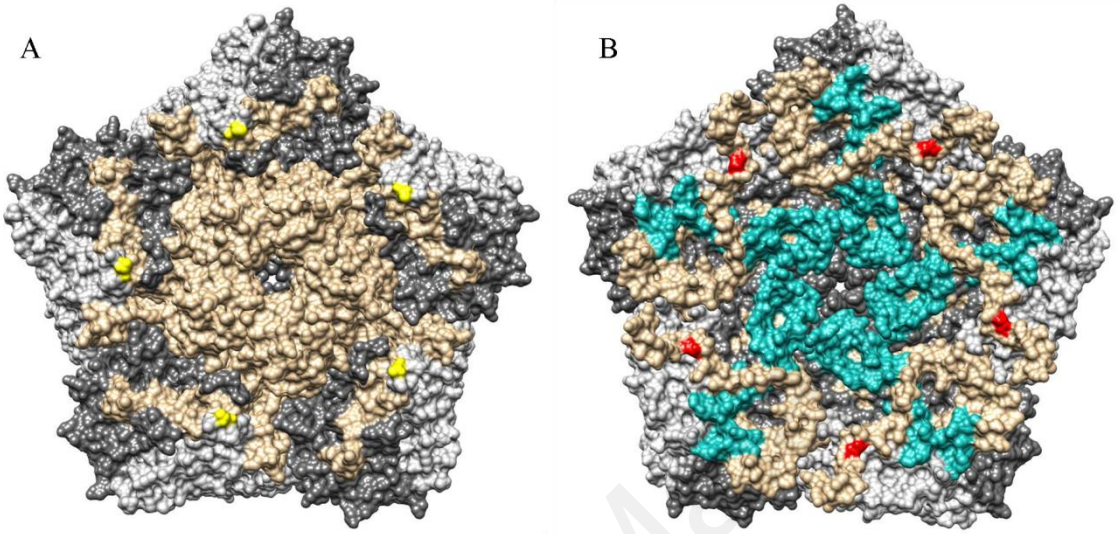


Figure 4.10: Pentamer structure of EV-A71 capsid proteins. (A) External view of the pentamer structure showing the VP2-T141I mutation (yellow), VP1 (brown), VP2 (dark grey) and VP3 (light grey) capsid proteins. (B) Internal view of the pentamer structure showing the VP1-D14N mutation (red) and the VP4 (cyan) capsid protein.

CHAPTER 5

DISCUSSION

5.1 Epidemics of HFMD in Malaysia

In Malaysia, HFMD became a statutorily notifiable disease only from October 2006, although national surveillance data does not include the causative viral agents. A notable exception is Sarawak, the worst affected state in Malaysia, which established sentinel and laboratory-based surveillance of HFMD in 1998. This clearly showed recurrent EV-A71 epidemics coinciding with large spikes in HFMD rates occurring at 3 year intervals (Podin *et al.*, 2007; Solomon *et al.*, 2010). In this study, it was found that national HFMD rates, which were not virus-specific, accorded with reported EV-A71 epidemics. Besides Malaysia, the 3-year cyclical pattern of the EV-A71 epidemics has also been noted in other countries such as Japan (Iwai *et al.*, 2009), Singapore (Ang *et al.*, 2015), and Cambodia (Horwood *et al.*, 2016).

The highest age-specific incidence of HFMD is seen in children <2 years old with a decreased rate in the older age groups (Figure 4.2). Previous studies in Taiwan and Singapore also reported higher incidence rates of HFMD in younger children, particularly in children younger than 4 years of age (Ang *et al.*, 2009; Chen *et al.*, 2007). Another study in Taiwan showed that the age-specific incidence rates of EV-A71 increased gradually after 6 months of age, from 1.71 per 100 person-years at 0-6 months to 4.97 per 100 person-years at 36 months of age (Lee *et al.*, 2012).

5.2 Seroprevalence studies of EV-A71

Seroprevalence studies of EV-A71 are a useful part of surveillance of EV-A71. To study EV-A71 seroprevalence, EV-A71 neutralising antibodies in serum samples were measured by neutralisation assay which is widely used in studies of EV-A71

seroprevalence (Ang *et al.*, 2011; Ji *et al.*, 2012; Luo *et al.*, 2009; Mizuta *et al.*, 2009; Rabenau *et al.*, 2010; Tran *et al.*, 2011). The serum samples were incubated with an optimised EV-A71 virus titre. *In vitro* culture in cells such as Vero (Horwood *et al.*, 2016; Tran *et al.*, 2011) or RD (Akhmadishina *et al.*, 2014; Ang *et al.*, 2011; Chang *et al.*, 2002; Diedrich *et al.*, 2009; Ji *et al.*, 2012; Luo *et al.*, 2009; Mao *et al.*, 2010; Ooi *et al.*, 2002) is often used. In this study, RD cells were used. Different studies used different strains of EV-A71, usually the current predominant strain in the country. In China, EV-A71 subgenotype C4 was used in seroprevalence studies (Ji *et al.*, 2012; Li *et al.*, 2013; Mao *et al.*, 2010). In Singapore (Ang *et al.*, 2011; Ang *et al.*, 2015) and Thailand (Linsuwanon *et al.*, 2014), EV-A71 subgenotype B5 was used. In studies in Brazil (Luchs *et al.*, 2010) and Germany (Diedrich *et al.*, 2009), the prototype EV-A71 strain BrCr was used. In the present study, a clinical EV-A71 strain UH1 of subgenotype B4 was used. This subgenotype previously circulated between 1997 and 2000 in Malaysia (Herrero *et al.*, 2003). Although this strain is not the current predominant strain in Malaysia, the use of this virus strain was supported by high concordance with neutralisation titres against EV-A71-B5 virus and EV-A71-C1 virus, which have both circulated in Malaysia recently, as explained in Section 4.2.2. The high concordance in seropositive/seronegative status which was obtained with the sera using either UH1 or B5 virus and UH1 or C1 virus was further supported by previous studies, as summarised in Table 2.5.

5.2.1 Seroprevalence of EV-A71 in children up to 12 years in Kuala Lumpur, Malaysia

This study reported the first EV-A71 seroprevalence in Kuala Lumpur, Malaysia, and is currently the only EV-A71 seroprevalence study done in Malaysia. In this study, seropositive children were identified from as early as 1995 and 1996, suggesting that

EV-A71 was already circulating before the first documented epidemic in 1997. It was shown that the overall EV-A71 seroprevalence and GMT in children were significantly higher during the epidemic years compared to non-epidemic years (67.4% vs. 56.6%), but it also showed that younger children were seropositive in non-epidemic years. The presence of seropositive young children in interepidemic years shows that ongoing transmission occurs between epidemics. This is supported by laboratory reports of EV-A71 isolated in low numbers during interepidemic years (Apandi *et al.*, 2011; Chan *et al.*, 2012; Huang *et al.*, 2012; Podin *et al.*, 2007). The high incidence rate of HFMD in the youngest age group is consistent with the significant differences in age-specific EV-A71 seroprevalence seen between non-epidemic and epidemic years in those <2 years old, particularly in the <6 month (from 47.7% to 64.0%, p=0.016) and 6 months to 1 year age groups (from 35.9% to 64.3%, p=0.0016).

The well-recognised cyclical pattern of EV-A71 epidemics seen in some countries has been attributed to the time taken for accumulation of enough susceptible children in the population. In Tokyo, the overall EV-A71 seroprevalence dropped to its lowest point in 6 years during the months just preceding an epidemic in 1973, including an absence of antibodies in children <4 years old (Hagiwara *et al.*, 1979). In Guangdong, China, seroprevalence gradually dropped from 2007 to 2009, before a large epidemic in 2010 (Li *et al.*, 2013). In Taiwan, there was evidence of fewer EV71 seroconversions in 1994-1997, before the 1998 epidemic (Lu *et al.*, 2002). A recent seroprevalence study in Cambodia showed that the EV-A71 had widely circulated and was undetected in the country at least a decade before the large epidemic in 2012, and that seroprevalence spikes every 2–3 years likely represented a cyclical pattern of epidemics (Horwood *et al.*, 2016). The present study charts seroprevalence in Kuala Lumpur over a long period of time, covering 18 years and 6 epidemics, and it showed that changes in population immunity in children appear to be the major driving force of the observed cyclical

epidemics. Specifically, the present study demonstrated that falls in seroprevalence were clearly associated with the occurrence of the subsequent epidemic. Seroprevalence in both 1-6 and 7-12 years age groups increased in epidemic years, suggesting that both groups are involved in disease burden and transmission. The higher HFMD rates seen in children aged 1-6 years is most likely due to their greater susceptibility (as shown by their lower seroprevalence rates in non-epidemic years), but it may also be due to under-reporting in older children, who often have milder disease (Chang *et al.*, 2004; Solomon *et al.*, 2010).

Estimation of the basic reproduction ratio (R_0), or the number of secondary cases arising from an infectious case, has been widely used to study the dynamics of transmission of infectious diseases such as SARS and influenza (Heffernan *et al.*, 2005). The R_0 of EV-A71 has been estimated as 5.48, which is considered as moderately infectious (Ma *et al.*, 2011). The EV-A71 R_0 was higher than the estimated CV-A16 R_0 of 2.5, suggesting that EV-A71 is more transmissible. For such a transmissible virus, the epidemic size is mainly dependent on the size of the susceptible population (Woolhouse & Gowtage-Sequeria, 2005). Following a viral epidemic, most of the population at risk would become immune. It may then take 2-3 years for the susceptible population to be replenished by newborns, and to be large enough for the R_0 to increase to >1, hence leading to a cyclical pattern of EV-A71 epidemics every 2-3 years. A similar study should be conducted to determine the R_0 to further understand EV-A71 transmission dynamics in Malaysia.

The present study also showed that Malaysian epidemics are characterised by peaks of increased genetic diversity, often with genotype changes. While the increased diversity may simply reflect a larger number of infections, it cannot be excluded that new variants with antigenic changes may escape population immunity and contribute to cyclical epidemics. Although found in less than a quarter of Malaysian EV-A71, the

positive selection pressure sites found at positions 98 and 145 of the VP1 protein have been previously reported (Tee *et al.*, 2010). These mutations appeared at the terminal branches with changes from E98K, E145Q and E145G. Amino acid position 98 is part of the BC loop and position 155 is part of the DE loop, both of which are immunogenic loops of VP1 (Chan *et al.*, 2012). A recent study measured cross-reactive neutralising antibody titres against viruses with mutations at residues 98, 145 and 164 (Huang *et al.*, 2015). Up to 4-fold neutralisation reduction was seen in sera from children, adults and rabbits tested against an EV-A71 VP1-98K/145Q/164E mutant, and all neutralisation titres were ≥ 1:16. However, viruses with all three mutations concomitantly have not yet been seen in nature. The significance of the antigenic variation will require more detailed longitudinal serological studies. If immune escape is not needed or plays only a minor role to produce the cyclical pattern of EV-A71 epidemics, a significant accumulation of susceptible children between epidemics will be enough to support large-scale transmission and another epidemic.

Overall, in other published studies, EV-A71-infected children have detectable neutralising antibody titres against all the EV-A71 genotypes (Huang *et al.*, 2013), and cross-protective immunity between genotypes is generally considered to be high (Chen *et al.*, 2013; Chia *et al.*, 2014). Previous studies in humans, monkeys, rabbits and mice showed that neutralisation antibody levels against different genotypes may vary, but overall human anti-serum generally does cross-neutralise strains of different genotypes (Table 2.5). Lower neutralisation titres may not reflect antigenic shift sufficient to lead to immune escape. To date, no cases of recurrent EV-A71 infection have been reported, suggesting the presence of life-long protective immunity against EV-A71. While enteroviruses clearly undergo antigenic evolution, complete immunological escape in EV-A71 seems to be rare, thus EV-A71 is generally considered to be a single serotype

antigenically. Any possible clinical significance and contribution of reduced cross-protective immunity towards new epidemics will require further confirmation.

5.2.2 Seroprevalence and potential risk factors of EV-A71 infection among Orang Asli populations in West Malaysia

As many previous studies of risk factors for EV-A71 were in urban settings (Ang *et al.*, 2009; Zeng *et al.*, 2013), the objective of this study was to determine the risk factors for seropositivity among rural Orang Asli communities, particularly hygiene and lifestyle factors which may facilitate the main route of faecal-oral transmission of the virus. This is the first serological survey of EV-A71 in rural Malaysian children conducted to date. The seropositive rates of Orang Asli children aged ≤12 years overall and within each childhood age group of 1-3, 4-6, and 7-12 years were considerably higher than that of urban KL children. The pattern of increasing seropositivity also differed; while the seropositivity of Orang Asli was already high at 81.8% by 1-3 years, seropositivity in urban KL children rose gradually from 47.1% at 1-3 years to 75% at 13-17 years. This suggests that the majority of Orang Asli children are infected at a much younger age, while urban KL children are infected not only in pre-school, but also in primary and secondary school; a similar trend of acquisition was seen in urban Singaporean children (Ang *et al.*, 2011). Young age, mainly younger than 4 years old, has been reported as one of the risk factors for EV-A71 infection (Ang *et al.*, 2015; Chang *et al.*, 2002; Li *et al.*, 2013). In this study, as most individuals in both urban and Orang Asli populations have been exposed to EV-A71 by 13 years, seropositivity rates of the two populations become similar from this point. In comparison to childhood seropositivity rates, rates in adolescents/adults began to level off or drop. This has also been described in studies in Taiwan (Wang *et al.*, 2012), Germany (Rabenau *et al.*, 2010), Vietnam (Tran *et al.*, 2011) and Thailand (Linsuwanon *et al.*, 2014). The most

likely explanation is the waning of measurable antibodies due to less exposure to EV-A71 in adults. It is not known if natural immunity is life-long. However, as EV-A71 infections are rare in adults (Ang *et al.*, 2011; Chang *et al.*, 2002), it is likely that they are protected by long-lasting, specific immunity even if detectable antibodies wane (Rabenau *et al.*, 2010; Wang *et al.*, 2012). A similar phenomenon is seen in individuals with hepatitis B vaccine-induced antibodies (Leuridan & Van Damme, 2011).

The higher seropositive rate in the young rural Orang Asli is most likely due to their poor living conditions and lifestyles compared to urban residents. The Orang Asli population in Malaysia live in poverty, with lower levels of education, poor healthcare and sanitation (Ahmed *et al.*, 2012). These are associated with high infection rates of parasitic diseases which are transmitted faecal-orally, such as intestinal helminthiasis, giardiasis and cryptosporidiosis (Ahmed *et al.*, 2012; Al-Mekhlafi *et al.*, 2013; Al-Mekhlafi *et al.*, 2011). Two independent risk factors for EV-A71 seropositivity were identified in this study. Age ≤12 years is a recognised risk for this childhood disease, as hygiene practices in children are usually poor. In a previous study in this population, younger children were also more likely than older children to have intestinal polyparasitism, likely due to poor personal hygiene (Al-Delaimy *et al.*, 2014). Using untreated water from rivers and wells is also an independent risk factor for EV-A71 seropositivity, as well as other faecal-orally acquired pathogens such as intestinal parasites (Ahmed *et al.*, 2011; Al-Delaimy *et al.*, 2014; Nasr *et al.*, 2013; Ngui *et al.*, 2011). Orang Asli communities are usually located close to rivers, which are essential for their daily activities, including washing, bathing, playing and swimming (Ahmed *et al.*, 2011). In these rural communities, children in particular were noted to prefer defecating in rivers rather than toilets, and this would result in higher risk of using contaminated water (Ahmed *et al.*, 2011; Al-Delaimy *et al.*, 2014; Nasr *et al.*, 2013; Ngui *et al.*, 2011). The higher seropositivity rates in Orang Asli children compared to

adults may also be due to differences in exposure to untreated water, for example children may play and swim more in rivers. Living in rural areas was also found to be a risk factor for EV-A71 and HFMD infection in Taiwan (Chang *et al.*, 2002) and China (Fang *et al.*, 2013; Mao *et al.*, 2010), and this was suggested to be due to similar contributory factors such as poorer public health conditions and lower socioeconomic status. Furthermore, severe EV-A71 infection in China has also been associated with rural residence (Fang *et al.*, 2013; Mao *et al.*, 2010) and, for children hospitalised in urban settings, having rural-to-urban migrant worker parents (Zeng *et al.*, 2013). This may be due to lower parental awareness of the need for medical attention, or poorer access to medical services.

Important strategies to prevent faecal-oral parasitic infections in Orang Asli have been suggested, which would also prevent enteroviral infections, including the provision of proper sanitation facilities and safe water supplies, and health education regarding good personal hygiene and good sanitary practices (Ahmed *et al.*, 2011; Nasr *et al.*, 2013). Orang Asli communities appear to have low levels of health education, with only 16% aware of the preventive measures against helminth infections (Nasr *et al.*, 2013). Good hand hygiene has been shown to significantly reduce EV-A71 transmission (Huang *et al.*, 2014; Xie *et al.*, 2015). This would be an important preventive strategy, as 42.3% of Orang Asli do not wash their hands prior to eating (this study), and in a separate study, 37.7% reported not washing their hands after defaecation (Nasr *et al.*, 2013). However, the preventive impact of hand-washing would be reduced if unclean water is used. Thus, teaching preventive measures for HFMD in health education campaigns should be accompanied by improvements in infrastructure. Parents should also be educated to recognise signs and symptoms of a very ill child, so they will seek healthcare at an early stage.

5.2.3 The need for EV-A71 vaccination for children in Malaysia

Promising EV-A71 vaccines have been recently reported, notably an inactivated vaccine which showed good efficacy, immunogenicity and safety in a phase 3 trial (Liang & Wang, 2014; Yee & Poh, 2016; Zhu *et al.*, 2013). The formulation of an effective vaccine programme depends on understanding of the epidemiology of the disease, which will vary between populations within a country. If this vaccine was introduced into routine immunisation programs, children would have to be vaccinated at least by the age of 6 months, and possibly earlier, before maternal antibody starts to wane, and at a time when the risk of severe disease is highest (Crawford & Graham, 2013; Li *et al.*, 2015; Zeng *et al.*, 2012; Zhu *et al.*, 2013). As most children in Malaysia and other Asian countries (Ji *et al.*, 2012; Ooi *et al.*, 2002) are seropositive by 5 years, an effective vaccine could prevent EV-A71 HFMD, as well as the severe neurological complications that mainly affect the very young (Lu *et al.*, 2002). This study provides data which will aid planning of future EV-A71 vaccine programs in Malaysia, and have identified Orang Asli children as a rural population at particularly high risk of infection compared to the urban KL children. Targeting rural children as a priority for vaccination may also impact urban transmission, in view of the increasing global trends of migration from rural to urban areas (Zeng *et al.*, 2013), which is also occurring in Malaysia (Masron *et al.*, 2012). Rural children may also need to be vaccinated at an earlier age than urban children. A similar strategy is used for measles in Sabah, Malaysia, where a higher incidence of measles has led to a policy of earlier initial vaccination at 6 months, compared to vaccination at 9-12 months in other states (Peng & Kassim, 1997; The Government of Malaysia's Official Portal, 2016). As HFMD and EV-A71 are undoubtedly under-recognised in rural areas, more epidemiological studies are needed in rural infants and children, to determine an appropriate age for vaccination.

5.3 Characterisation of EV-A71 neutralisation escape mutants

Several studies have been done to identify major neutralisation epitopes of EV-A71 by using synthetic peptides (Foo *et al.*, 2007; Liu *et al.*, 2011), generation of monoclonal antibodies (Chang *et al.*, 2011; Kiener *et al.*, 2012; Lim *et al.*, 2013) and generation of neutralisation escape mutants (Deng *et al.*, 2015; Kiener *et al.*, 2014; Xu *et al.*, 2014). A major neutralisation epitope of EV-A71 was first recognised by using synthetic peptides, mapped to the VP1 capsid protein at amino acid positions 208-222 and was named SP70 (Foo *et al.*, 2007). Later on, other studies located neutralisation epitopes on other capsid proteins VP2, VP3 and VP4. A neutralisation epitope was identified on VP2, which is mapped to amino acid positions 136-150 on the EF loop (Liu *et al.*, 2011). Another recent study located a conformational neutralisation epitope on the VP3 protein at amino acid positions 176-190 (Jiang *et al.*, 2015). An epitope lying on the N-terminus of the VP4 protein (amino acids 1-20) was also found to elicit cross-protective neutralising antibodies against different EV-A71 genotypes (Zhao *et al.*, 2013). The present study generated EV-A71 neutralisation escape mutants to identify neutralisation epitopes. This escape mutant was generated using the commercial anti-EV-A71 mAb MAB979 which was previously mapped to the EV-A71 VP2 protein (Liu *et al.*, 2011). In this study, two mutations were identified, first, threonine (T) to isoleucine (I) at position 141 of the VP2 EF loop, and secondly, aspartic acid (D) to asparagine (N) at position 14 of the VP1 N-terminus. Interestingly, the latter mutation has not been previously reported.

Many escape mutant studies focused on the effects of mutation of the neutralisation epitopes on neutralisation activity. A study by Deng *et al.* (2015) found that individual substitution of K218A within SP70 allowed partial escape from neutralisation by mAb 2G8, whereas L220A completely escaped neutralising activity and binding by the mAb. They also found that these two mutations are critical for neutralisation in human natural

infection (Deng *et al.*, 2015). A Western blot were done to see the effect of serine to threonine mutation at amino acid position 144, which was present in the EV-A71-C4 virus that recently emerged in China. The mutated virus could not be detected by the polyclonal sera from mice injected with either wild-type EV-A71-B5 or EV-A71-C4-Fuyang RG (a reverse genetic EV-A71-C4 strain). This finding suggested that this mutation abolished the antigenicity of the VP2 protein (Kiener *et al.*, 2012). The mutational analysis of three amino acid positions of the VP3 protein, P59L, A62D/P and E67D, abolished the binding and neutralising activity by specific mouse mAb 10D3, suggesting that these three amino acids are essential for neutralisation (Kiener *et al.*, 2014).

The escape mutants in the present study were also characterised. Three infectious clones were constructed by incorporating the VP2-T141I, VP1-D14N and VP2-T141I/VP1-D14N mutations to see the effects of these mutations on neutralisation by MAB979, anti-EV-A71-positive mouse serum, children patient's sera and adult sera. The viruses EV-A71 VP2-T141I and EV-A71 VP2-T141I/VP1-D14N escaped neutralisation by the mAb completely, whereas EV-A71 VP1-D14N showed 2-fold decrease compared to the wild-type virus. Since the target site of MAB979 was previously mapped to the VP2 neutralisation epitope corresponding to amino acids 136-150, the neutralisation escape of the two escape viruses carrying the VP2 mutation was as expected. However, the VP1 mutation did not significantly affect the neutralising activity, although a 2-fold decrease was seen.

In the neutralisation assay with anti-EV-A71-positive mouse serum, both EV-A71 VP2-T141I and EV-A71 VP2-T141I/VP1-D14N mutants showed a 4-fold decrease in neutralisation but only a 2-fold decrease in neutralisation against the EV-A71 VP1-D14N mutant by the mouse serum compared to the wild-type. These results showed that the mutation at the VP2-141 amino acid did not strongly abolish the neutralising activity

of the anti-EV-A71 mouse serum. This is highly likely due to the dominant mouse neutralisation epitope on the VP1 protein (amino acids 208-222), which enables the serum to neutralise the virus. However, the 4-fold decrease in the neutralisation titre compared to the EV-A71 wild-type virus showed that the VP2 mutation plays a critical role in neutralisation, as the amino acid T141 lies within the neutralisation epitope on the VP2 EF loop. The VP1-D14N mutation, which was discovered in this study, has a minor effect on the neutralising activity of the mouse serum. This mutation lies on the N-terminus of the VP1, which was previously recogised as immunoeactive but not neutralising epitopes. Anti-rabbit serum raised following injection of the N-terminal region (1-100) of VP1 protein elicited strong immune responses against the EV-A71 VP1 protein (Sivasamugham et al., 2006). Previously, a Western blot analysis showed that amino acids 11-21 contain EV-A71-specific antigenic sites (Zhang *et al.*, 2014). The truncated VP1 N-terminus protein containing amino acids 6-43 can be recognised anti-EV-A71 rabbit antisera (Zhang *et al.*, 2014). A study in Singapore generated a novel mouse mAb targeting amino acids 12-19 (IGDSVSRA) on the N-terminal region of the VP1. Although this mAb was found to be immunoreactive in Western blot and immunofluorescent assay, the mAb however did not possess neutralising activity (Lim *et al.*, 2013), which suggests that this epitope is a not a neutralisation epitope. In addition to this, another study documented a mAb targeting the amino acid residues 3-8 (RVADVI) on the N-terminal region of the VP1, which was also non-neutralising (Man-Li *et al.*, 2012). Thus, this may explain the minor effect of the VP1-D14N mutation on the neutralising activity by the mouse serum.

Besides the MAB979 and the mouse serum, the escape mutants were also tested against human sera in the neutralisation assay. Unlike with MAB979 and the mouse serum, there were no differences in the neutralisation titres of healthy adult pooled sera against both EV-A71 VP2-T141I and EV-A71 VP2-T141I/VP1-D14N mutants, while

only a 2-fold decrease in titre was seen against the EV-A71 VP1-D14N mutant compared to the wild-type. In children, one serum showed a 4-fold-decrease while the rest showed 2-fold decreases against the EV-A71 VP2-T141I and EV-A71 VP2-T141I/VP1-D14N mutants compared with the wild-type. As for the EV-A71 VP1-D14N mutant, two sera showed 8- and 16-fold decreases, and another two sera showed 4-fold decreases in neutralisation titres compared with the wild-type. There were no obvious differences in the neutralisation titres for human sera against all the escape mutants except in two children sera. There have not been many previous studies of neutralisation epitopes specific to humans. One study measured the reactivity of 205 human cord sera against the N- and C-terminus of the VP1 protein, and found that sera with high neutralising antibody titres were significantly more reactive with the N-terminal half of the VP1 compared to low-titre or negative sera, which suggested that this region is likely to have important neutralising antibody determinants important to humans (Tan & Cardosa, 2007). This could explain the decrease in titres observed with some children sera in the present study. Another study in China mapped 10 human anti-EV-A71 IgM epitopes and one human anti-EV-A71 IgG epitope on the viral capsid proteins using a panel of synthetic peptides (Table 2.3; Gao *et al.*, 2012). On the VP1 protein, both human anti-EV-A71 IgM and anti-EV-A71 IgG epitopes were mapped within the N-terminus to amino acid residues 40-51 (DTGKVPALQAAE) and 43-54 (KVPALQAAEIGA), respectively (Gao *et al.*, 2012). On the VP2 protein, only anti-EV-A71 IgM epitopes were recognised (Table 2.3; Gao *et al,* 2012). Only one amino acid residue lies within the VP2 neutralisation epitope, vp2-50 (148-159: YKQTQPGADGFE) (Gao *et al.*, 2012). This study showed that the epitopes may be different in humans and animals. This could explain why the mutations in the present study did not have major effects on the neutralising activity by human sera. However,

the number of sera used in the present study was not enough to confirm the importance of the mutations in humans.

The pentamer structure of EV-A71 showed that the location of the VP2-T141I mutation is exposed on the surface of the VP2 protein. This is in accordance with the fact that the VP2 mutation is located on the neutralisation epitope of the VP2. However, the VP1-D14N is located on the internal part of the EV-A71. Studies of the EV-A71 crystal structure showed that the N-terminal region of the VP1 is transiently exposed in the process of transition from provirion to the A-particle upon binding to a cellular receptor (Lim *et al.*, 2013; Shingler *et al.*, 2013). A recent study of the EV-A71 crystal structure showed that at least 18 amino acid residues from the VP1 N-terminus were transiently exposed during the uncoating process (Lyu *et al.*, 2015). In a study by Lim *et al.* (2013), the binding of mAb4 to its target epitope at the VP1 N-terminus occurred during the alterations of the native virus upon binding to the host cellular receptor (Lim *et al.*, 2013). This finding suggested that the mutation on the VP1 N-terminus in the present study might occur during the similar stage of infection. Further studies are needed to investigate the antibody binding to the N-terminal region of the VP1 during the EV-A71 infection.

Extensive studies on neutralisation with human sera are needed, not just with anti-EV-A71-positive children sera, but also with sera from all stages of EV-A71 infection. Further study of the neutralising antibody determinants and antibody binding within the N-terminal region of VP1, and neutralising epitope mapping in humans are also needed to develop a more effective vaccine and mAb therapy against EV-A71.

5.4 Limitation of the study

The main limitation of this study is the use of a convenience sample of residual diagnostic sera from a single hospital. However, it would be difficult to otherwise obtain such an extensive collection of serum samples from healthy children over many years. When compared to a random cluster survey, convenience sampling has also been shown to give similar estimates of seroprevalence to 5 vaccine-preventable viral diseases (Kelly *et al.*, 2002).

CHAPTER 6

CONCLUSION

In this study, the first seroepidemiological survey of EV-A71 in Malaysia was performed in children and adults in urban (Kuala Lumpur) and rural settings. Falls in seroprevalence rates in children 1-12 years old leading to higher levels of population susceptibility are the likely major reasons for the cyclical pattern of EV-A71 epidemics seen every 2-3 years in Malaysia over 18 years. Similar to other countries, the highest age-specific incidence of HFMD/EV-A71 infection in Malaysia occurred in children <2 years old. In the comparative seroepidemiological study among urban and rural populations, rural Orang Asli children had significantly higher seropositivity rates to EV-A71 than urban KL children. Untreated water supplies, poor hygiene practices and lack of adequate sanitary infrastructure are likely to play roles in the spread of the virus among this community, and these require further study. Increasing awareness of EV-A71 infection should be included alongside other parasitic infections as an important disease prevention strategy. Similar seroprevalence and epidemiological studies are needed in a wider range of urban and rural populations nationwide to fully define the risk factors for EV-A71 infection and inform future vaccination planning.

Using the monoclonal antibody MAB979, a neutralisation escape mutant was generated, which carried two mutations; one is located on the VP2 EF loop and another is located on the N-terminus of the VP1 capsid protein. The VP2 mutation only has minor effects on the neutralising activity by human sera as the VP2 neutralisation epitope was reported as a mouse-specific neutralisation epitope. On the other hand, the mutation in the VP1 N-terminus did not significantly affect neutralisation by the mouse serum, but decreased the neutralisation titre in some children's sera. This suggests that neutralisation epitopes in mice might be different from that of humans. More detailed

studies are needed to further discover the neutralisation epitopes in humans, within all the capsid proteins of EV-A71, to aid development of an effective vaccine and mAb therapy.

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APPENDICES

Appendix I: Malaysian EV-A71 VP1 sequences from 1997-2012

Accession number	Name	Year of isolation	Subgenotype
AF190565	1109_MAA_1997	1997	C1
AF190567	1112_MAA_1997	1997	C1
AY207612	03784_MAA_1997	1997	C1
JN316071	PM_14283_1997	1997	C1
AF190568	1113_MAA_1997	1997	C1
AY207638	0283_MAA_1997	1997	C1
AY207615	03750_MAA_1997	1997	C2
AM396584	Sha52_1997	1997	C2
AM396585	Sha71_1997	1997	C2
AY207611	03907_MAA_1997	1997	C2
AY207649	0091_MAA_1997	1997	B4
AF190560	1103_MAA_1997	1997	B4
AF190559	1102_MAA_1997	1997	B4
JN316108	PM_13091_1997	1997	B4
AY207646	0414_MAA_1997	1997	B4
AM396587	UH1_1997	1997	B4
AY207647	0128_MAA_1997	1997	B4
AY207640	0175_MAA_1997	1997	B4
AF190566	1110_MAA_1997	1997	B4
AY207637	0343_MAA_1997	1997	B4
AY207613	03300_MAA_1997	1997	B4
AY207643	0898_MAA_1997	1997	B4
AJ586873	Sha89_1997	1997	B4
JN316109	PM_13899_1997	1997	B3
AF190561	1105_MAA_1997	1997	B3
AY207644	0897_MAA_1997	1997	B3
JN316110	PM_13473_1997	1997	B3
AF190564	1108_MAA_1997	1997	B3
AY207616	0473_MAA_1997	1997	B3
AY207642	0899_MAA_1997	1997	B3
AY207648	0903_MAA_1997	1997	B3
AY207641	0036_MAA_1997	1997	B3
AY207645	0884_MAA_1997	1997	B3
AF190563	1107_MAA_1997	1997	B3
AF190562	1106_MAA_1997	1997	B3
AY207639	0245_MAA_1997	1997	B3
JN316111	PM_14716_1997	1997	B3
AY207636	04716_MAA_1997	1997	B3
AF190570	2334_MAA_1997	1997	B3
AF190569	2294_MAA_1997	1997	B3
AY207614	0870_MAA_1997	1997	B3
AF376072	MY104_9_SAR_1997	1997	B3

AB469182	SK_EV006_1997	1997	B3
DQ341367	MY821_3_SAR_1997	1997	B3
AF376076	MY755_3_SAR_1997	1997	B3
AM396588	Sha63_1997	1997	B3
AF190571	7202_MAA_1997	1997	B3
AF376078	MY860_3_SAR_1997	1997	B3
DQ341368	MY104_9_SAR_1997	1997	B3
AF376074	MY21_2_SAR_1997	1997	B3
AF376073	MY16_1_SAR_1997	1997	B3
AM396586	Sha66_1997	1997	B3
AF376075	MY6_2_SAR_1997	1997	B3
AF190576	1118_MAA_1998	1998	C1
JN316067	PM_17557_1998	1998	C1
AY207631	0557_MAA_1998	1998	C1
AY207630	0808_MAA_1998	1998	C1
JN316068	PM_17838_1998	1998	C1
AF190574	1116_MAA_1998	1998	C1
AF190573	1115_MAA_1998	1998	C1
AF190575	1117_MAA_1998	1998	C1
AF190572	1114_MAA_1998	1998	C1
JN316069	PM_17808_1998	1998	C1
AF376080	S10862_SAR_1998	1998	C1
AF376079	S10822_SAR_1998	1998	C1
AF376081	S11051_SAR_1998	1998	C1
AY207629	0838_MAA_1999	1999	C1
JN316070	PM_10749_1999	1999	C1
AY207653	0749_MAA_1999	1999	C1
JN316105	PM_12627_1999	1999	B4
JN316104	PM_12615_1999	1999	B4
JN316106	PM_12919_1999	1999	B4
AY207650	0919_MAA_1999	1999	B4
AY207651	0627_MAA_1999	1999	B4
AY207652	0615_MAA_1999	1999	B4
AY207626	0389_MAA_2000	2000	C1
JN316076	PM_17113_2000	2000	C1
AY207618	0807_MAA_2000	2000	C1
AY207625	0113_MAA_2000	2000	C1
JN316077	PM_17204_2000	2000	C1
JN316073	PM_15948_2000	2000	C1
AY207622	0948_MAA_2000	2000	C1
AY207620	0915_MAA_2000	2000	C1
AY207619	0836_MAA_2000	2000	C1
AF376087	S40221_SAR_2000	2000	C1
AY207621	0937_MAA_2000	2000	C1
AY207632	0832_MAA_2000	2000	C1
JN316079	PM_15774_2000	2000	C1
AY207634	0774_MAA_2000	2000	C1
JN316080	PM_17181_2000	2000	C1

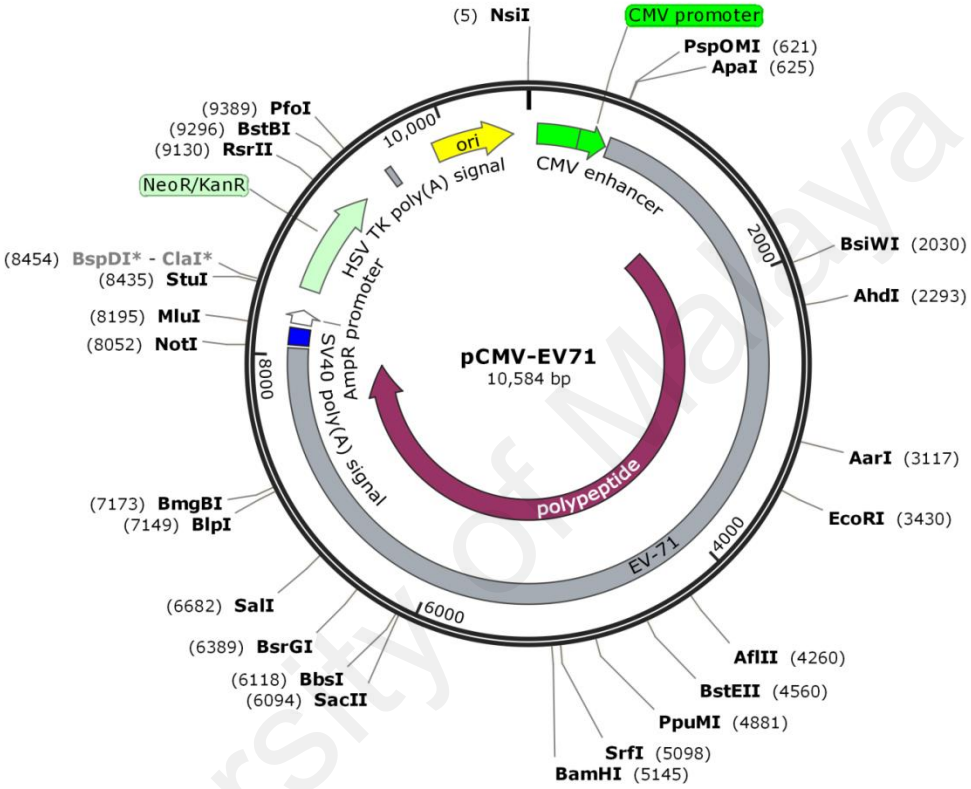
AY207635	05716_MAA_2000	2000	C1
JN316095	PM_17177_2000	2000	B5
AY207633	0815_MAA_2000	2000	B5
JN316097	PM_17467_2000	2000	B4
AY207628	0467_MAA_2000	2000	B4
AY207624	0066_MAA_2000	2000	B4
JN316099	PM_17164_2000	2000	B4
AY207617	0778_MAA_2000	2000	B4
JN316100	PM_17431_2000	2000	B4
AY207627	0431_MAA_2000	2000	B4
AY207623	0042_MAA_2000	2000	B4
AF376084	S21082_SAR_2000	2000	B4
AF376067	CN04104_SAR_2000	2000	B4
AF376069	SB0635_SAR_2000	2000	B4
JN316107	PM_16042_2000	2000	B4
AF376083	S12502_SAR_2000	2000	B4
AF376066	SB2864_SAR_2000	2000	B4
AF376082	S12172_SAR_2000	2000	B4
AF376085	S2861_SAR_2000	2000	B4
AF376071	CN9502_SAR_2000	2000	B4
AF376086	S40201_SAR_2000	2000	B4
AF376065	SB1647_SAR_2000	2000	B4
AF376068	CN062334_SAR_2000	2000	B4
AF376064	SB1191_SAR_2000	2000	B4
AF376070	CN0942_SAR_2000	2000	B4
JN316072	PM_19552_2001	2001	C1
JN316081	PM_19229_2001	2001	C1
DQ341360	J115_MAL_2001	2001	C1
JN316098	PM_20822_2001	2001	B4
JN316101	PM_20680_2001	2001	B4
JN316102	PM_20045_2001	2001	B4
JN316103	PM_20756_2001	2001	B4
DQ341365	PP37_MAL_2001	2001	B4
AY189154	S18191_SAR_2002	2002	C1
AY258316	CN30552_SAR_2003	2003	C1
AY258300	SB9522_SAR_2003	2003	C1
AY258296	SB9582_SAR_2003	2003	C1
AY258317	CN30014_SAR_2003	2003	C1
AY258298	SB9564_SAR_2003	2003	C1
AY258297	SB9579_SAR_2003	2003	C1
AY258302	SB9465_SAR_2003	2003	C1
AY258299	SB9533_SAR_2003	2003	C1
AY258301	SB9508_SAR_2003	2003	C1
AY258315	S19691_SAR_2003	2003	C1
AY258312	S19761_SAR_2003	2003	C1
AY258295	SB9604_SAR_2003	2003	C1
AY258314	S19731_SAR_2003	2003	C1
AY258294	SB9869_SAR_2003	2003	C1

JN316074	PM_25405_2003	2003	C1
JN316075	PM_24886_2003	2003	C1
DQ341363	S19841_SAR_2003	2003	B5
AY258308	S23141_SAR_2003	2003	B5
AY258309	S19871_SAR_2003	2003	B5
AY258307	S110031_SAR_2003	2003	B5
AY905550	SB10712_SAR_2003	2003	B5
AY258313	S19741_SAR_2003	2003	B5
AY258306	S110101_SAR_2003	2003	B5
AY258303	S110261_SAR_2003	2003	B5
AY258304	S110241_SAR_2003	2003	B5
AY258305	S110121_SAR_2003	2003	B5
AY905546	SB12282_SAR_2003	2003	B5
AY905545	SB12869_SAR_2003	2003	B5
AY905549	SB11977_SAR_2003	2003	B5
AY905547	SB12278_SAR_2003	2003	B5
AY258311	S19791_SAR_2003	2003	B5
AY258310	S19841_SAR_2003	2003	B5
DQ341362	SB12736_SAR_2003	2003	B5
AY905548	SB12007_SAR_2003	2003	B5
JN316083	PM_26165_2003	2003	B5
JN316084	PM_33034_2005	2005	B5
JN316085	PM_32286_2005	2005	B5
JN316086	PM_32308_2005	2005	B5
JN316082	PM_34589_2006	2006	B5
FM201324	EV71_MY1764589_2006	2006	B5
HQ676263	MY46_Sw_A_2006	2006	B5
HQ676262	MY45_Sw_A_2006	2006	B5
HQ676235	MY17_Sw_A_2006	2006	B5
HQ676254	MY37_Sw_A_2006	2006	B5
HQ676267	MY98_Sw_A_2006	2006	B5
FM201322	EV71_MY1764283_2006	2006	B5
FM201321	EV71_MY1764281_2006	2006	B5
HQ676252	MY34_Sw_A_2006	2006	B5
FM201327	EV71_MY1765058_2006	2006	B5
FM201326	EV71_MY1760517_2006	2006	B5
JN316090	PM_1687413_2006	2006	B5
JN316091	PM_1657636_2006	2006	B5
JN316093	PM_35017_2006	2006	B5
JN316092	PM_1657640_2006	2006	B5
HQ676255	MY38_Sw_A_2006	2006	B5
HQ676245	MY27_Sw_A_2006	2006	B5
HQ676260	MY43_Sw_A_2006	2006	B5
HQ676240	MY22_Sw_A_2006	2006	B5
HQ676259	MY42_Sw_A_2006	2006	B5
HQ676247	MY29_Sw_A_2006	2006	B5
HQ676241	MY23_Sw_A_2006	2006	B5
HQ676261_	MY44_Sw_A_2006	2006	B5

HQ676246	MY28_Sw_A_2006	2006	B5
JN316094	PM_1673313_2006	2006	B5
HQ676258	MY41_Sw_A_2006	2006	B5
HQ676248	MY30_Sw_A_2006	2006	B5
HQ676242	MY24_Sw_A_2006	2006	B5
HQ676257	MY40_Sw_A_2006	2006	B5
HQ676256	MY39_Sw_A_2006	2006	B5
HQ676244	MY26_Sw_A_2006	2006	B5
HQ676239	MY21_Sw_A_2006	2006	B5
HQ676249	MY31_Sw_A_2006	2006	B5
HQ676236	MY18_Sw_A_2006	2006	B5
HQ676234	MY16_Sw_A_2006	2006	B5
HQ676250	MY32_Sw_A_2006	2006	B5
HQ676243	MY25_Sw_A_2006	2006	B5
HQ676253	MY35_Sw_A_2006	2006	B5
HQ676266	MY97_Sw_A_2006	2006	B5
HQ676251	MY33_Sw_A_2006	2006	B5
HQ676237	MY19_Sw_A_2006	2006	B5
FM201325	EV71_MY_2006	2006	B5
JN316088	PM_34242_2006	2006	B5
HQ676238	MY20_Sw_A_2006	2006	B5
FM201323	EV71_MY1764454_2006	2006	B5
HM358812	EV0408_Penang_2008	2008	B5
HM358831	EV0338_Sabah_2008	2008	B5
HM358810	EV0336_Sabah_2008	2008	B5
HM358823	EV0911_Kedah_2008	2008	B5
HM358818	EV0764_Johor_2008	2008	B5
HM358816	EV0577_Pahang_2008	2008	B5
HM358822	EV0891_Johor_2008	2008	B5
HM358813	EV0466_Johor_2008	2008	B5
HM358815	EV0562_Penang_2008	2008	B5
HM358828	EV1035_Pahang_2008	2008	B5
HM358824	EV0943_Johor_2008	2008	B5
HM358830	EV1094_Johor_2008	2008	B5
HM358819	EV0811_Penang_2008	2008	B5
HM358825	EV0972_Johor_2008	2008	B5
HQ676264	MY47_Sw_A_2008	2008	B5
HM358827	EV1025_Penang_2008	2008	B5
JN316096	PM_2219140_2008	2008	B5
HM358817	EV0758_Sabah_2008	2008	B5
HM358820	EV0879_Bintulu_2008	2008	B5
HM358811	EV0372_Sabah_2008	2008	B5
HM358829	EV1078_Johor_2008	2008	B5
HM358821	EV0884_Johor_2008	2008	B5
HM358809	EV1075_Pahang_2008	2008	B5
HM358826	EV1019_Penang_2008	2008	B5
HM358814	EV0482_Sabah_2008	2008	B5
HQ676265	MY48_Sw_A_2008	2008	B5
HM358833	EV0076_KLumpur_2009	2009	B5
HM358832	EV0031_Johor_2009	2009	B5

HM358835	EV1945_Kuching_2009	2009	B5
HM358834	EV1705_Johor_2009	2009	B5
KC894881	EV1389-KLumpur_2010	2010	B5
KC894880	EV1312-Johor_2010	2010	B5
KC894879	EV1301-Melaka_2010	2010	B5
KC894878	EV1299-Melaka_2010	2010	B5
KC894877	EV1297-Melaka_2010	2010	B5
KC894876	EV1233-Kedah_2010	2010	B5
KC894872	EV0691-Terengganu_2010	2010	B5
KC894875	EV0994-Terengganu_2010	2010	B5
KC894873	EV0733-PPinang_2010	2010	B5
KC894874	EV0744-Johor_2010	2010	B5
KC894866	EV1056-Terengganu_2011	2011	B5
KC894869	EV0984-Sarawak_2011	2011	B5
KC894867	EV1268-Pahang_2011	2011	B5
KC894868	EV0978-Sarawak_2011	2011	B5
KC894865	EV1004-Terengganu_2011	2011	B5
KC894903	EV0997-Pahang_2012	2012	B5
KC894902	EV1325-Johor_2012	2012	B5
KC894899	EV1002-Johor_2012	2012	B5
KC894894	EV0891-Johor_2012	2012	B5
KC894883	EV0616-Johor_2012	2012	B5
KC894882	EV0615-Johor_2012	2012	B5
KC894900	EV1003-Johor_2012	2012	B5
KC894887	EV0673-Johor_2012	2012	B5
KC894884	EV0655-Kedah_2012	2012	B5
KC894889	EV0769-Johor_2012	2012	B5
KC894886	EV0665-Kelantan_2012	2012	B5
KC894888	EV0710-Johor_2012	2012	B5
KC894885	EV0659-Pahang_2012	2012	B5
KC894901	EV1170-Selangor_2012	2012	B5
KC894898	EV0961-Johor_2012	2012	B5
KC894890	EV0775-Johor_2012	2012	B5
KC894895	EV0894-Kedah_2012	2012	B5
KC894896	EV0896-Johor_2012	2012	B5
KC894897	EV0953-Johor_2012	2012	B5
KC894891	EV0779-Johor_2012	2012	B5
KC894893	EV0834-Johor_2012	2012	B5
KC894892	EV0791-Johor_2012	2012	B5
U22521	BrCr_1969	1969	A

Appendix II: Schematic illustration of the recombinant plasmid pCMV-EV-A71 and the restriction endonuclease restriction sites. This figure was created with SnapGene® Viewer version 2.8.1 (SnapGene®, USA)



PUBLICATIONS

Proceedings:

Seroprevalence of Enterovirus A71 infection in children up to 12 years of age in Kuala Lumpur, Malaysia at 19th Biological Sciences Graduate Congress, National University of Singapore, Singapore, 12-14th December 2014.

Seroprevalence of hand, foot and mouth disease caused by enterovirus-A71 among children in Malaysia

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Enterovirus A71 (EV-A71) is an important emerging pathogen, and in 2012, United States Centers for Disease Control and Prevention has listed it as one of global infectious disease threat. Since the first outbreak in Malaysia in 1997, hand, foot and mouth disease (HFMD) occurs every 2-3 years. There is no published data on HFMD/EV-A71 infection prior to this first epidemic. Thus, this study will determine the pattern and seroprevalence of EV-A71 infection during HFMD outbreaks in Malaysia. We randomly selected serum samples of children aged between 1 and 12 years old from year 1995 to 2012. The neutralizing antibody titers against EV-A71 were measured and the seropositive rates were determined and correlated with the incidence of HFMD nationwide. The seropositivity increased significantly with age (OR 1.15, 95% CI 1.12-1.19; $p<0.001$). The seropositive rate of EV-A71 infection is significantly lower in the 1-6 years old children, with 52.9% of the children being positive compared to 71.6% in the 7-12 years old. EV-A71 seropositivity was consistently higher in 7-12 years old group in 16 out of 18 years analyzed. The cyclical pattern is likely due to large accumulation of susceptible population in both younger and older children in between outbreaks enabling sustain transmission. In summary, children 1-12 years old, especially in the 1-6 years old contribute to the cyclical patterns of HFMD outbreaks observed in the last 18 years.

Research Articles:

NikNadia N, Sam IC, Khaidir N, Ngui R, Lim YA, Goh XT, Choy SH, Chan YF. Risk Factors for Enterovirus A71 Seropositivity in Rural Indigenous Populations in West Malaysia. PLoS One. 2016; 11(2): e0148767.

NikNadia N, I-Ching Sam, Sanjay Rampal, WMZ Wan Nor Amalina, Ghazali Nur Atifah, Khebir Verasahib, Chia Ching Ong, MohdAidinniza MohdAdib, Yoke Fun Chan. Cyclical patterns of hand, foot and mouth disease caused by enterovirus A71 in Malaysia. PLoS Negl Trop Dis. 2016; 10(3): e0004562.