Clinical characteristics, virology and host genetic markers of Hand, Foot and Mouth Disease in Southern Vietnam

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Doctor of Philosophy
2018

Kellogg College
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This thesis is a special gift I would like to give to my beloved Dad, who is fighting bravely against pneumonia and colon cancer in the hospital, right now.



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A thesis submitted for the degree of
Doctor of Philosophy

January 2018

ABSTRACT

Hand, foot and mouth disease (HFMD) has been associated with large outbreaks among young children, including severe illness and fatality, in the Asia-Pacific Region since 1997. Severe illness is most often associated with enterovirus A71 (EV-A71). Vietnam experienced a large sustained outbreak of almost 200.000 hospitalised cases and over 200 deaths in 2011-12, the large majority occurring in southern Vietnam.

Retrospective data from 2009 to 2015 showed that Vietnam experienced 3 phases of HFMD outbreak time: before the outbreak (2009-2010), during the outbreak (2011-2012) and after the outbreak (2013-2015). Clinical features were slightly different between phases, partly reflected the effect of national intervention strategies.

Between July 2013 and July 2015 1547 children were enrolled. Four serotypes of EV-A71, Coxsackie virus (CV) A6, A10 and A16 were responsible for 1005/1327 (75.7%) of diagnosed cases. We found an unexpected dominance of EV-A71 among both in- and outpatients, and a strong association with severe illness. CV-A6 and A10 emerged in Vietnam during our study period and replaced CV-A16. CV-A10 was associated with different clinical and laboratory characteristics.

The relative genetic diversity of subgenogroup B5 increased sharply in 2012, leading to subgenogroup switch from C4 to B5 and then remained stable from 2013

to 2015. Subgenogroup B5 dominantly circulated in Vietnam during the study time and might import to Vietnam from two independent sources.

Three potential single nucleotide polymorphism (SNPs), rs10235866 represents gene NPTX2/TMEM130, rs817854 and rs10739235 represent gene RAD23B (RAD23 homolog B), were detected to be associated with severe disease. Results from my study, covering demography, clinical features, virology and host genetic factors, provides a full view of HFMD in Southern Vietnam and therefore may have great impact for disease management and control.

ACKNOWLEDGEMENTS

This project would not been completed without the support from many people. First of all, I would like to express my deepest gratitude to my three supervisors: Dr H Rogier Van Doorn, Dr Catherine Louise Thwaites and Dr Le Van Tan. You have guided me through extremely new and interesting tasks during my PhD student time. You gave me valuable advices not only in scientific fields but also in emotional control in my tough time. Thank you for being so patient with me.

I am also thankful to four special people who influenced my decision (to be a PhD). They are Professor Jeremy Farar and Professor Tran Tinh Hien, who showed me that science is interesting and that I can do many joyful things with science; Dr Ha Manh Tuan and Dr Ho Lu Viet – my former chiefs in Children's Hospital 2, who supported and encouraged me from the beginning of my PhD until now.

I would like to say thank you to my colleagues at the Oxford Clinical Research Unit, particularly Emerging Infection team for your tremendous help and support whenever I need. You showed me what the real team work is and what really matters to me is that we have built genuine friendship after all. Also I greatly appreciate Dr Khor Chiea Chuen, Dr Li Zheng and staff from Genome Institute of Singapore (GIS) for supporting me with host genetic part, the study staff in Children's Hospital 1, Children's Hospital 2 and Hospital for Tropical Diseases for their great work and all patients/parents who participated in my study. Without them, this project could not be completed.

Last but not least, I would like to give thanks, with all my heart, to my Dad, Mom and my whole family, for loving me, being proud of me and supporting me unconditionally in every single step of my life.

DECLARATION

The majority of work presented in this thesis is my own and was conducted under the supervision of Dr Hidrink Rogier Van Doorn, Dr Le Van Tan and Dr Catherine Louise Thwaites at the Oxford Clinical Research Unit (OUCRU) in Ho Chi Minh City, Vietnam. EV-A71 sequencing was carried out in collaboration with my colleagues from Emerging Infection team at OUCRU. Host genetic part was conducted in the lab of Dr Khor Chiea Chuen at Genome Institute of Singapore (GIS), with the support of Dr Li Zheng. Results from this thesis have not been submitted for any degree or other qualification elsewhere.

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ABBREVIATIONS

HFMD	Hand, Foot and Mouth Disease
EV/EVs	Enterovirus/Enteroviruses
EV-A71	Enterovirus A71
CV	Coxsackievirus
CNS	Central Nervous System
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
VP	Viral Protein
NCR	None coding region
ORF	Opening
CSF	Cerebral spinal fluid
PCR	Polymerase chain reaction
RT-PCR	Reverse Transcription Polymerase Chain
ANS	Autonomic Nervous System
MoH	Ministry of Health
WHO	World Health Organisation
IVIg	Intravenous Immunoglobulin
IL	Interleukin

IFN	Interferon
CI	Confident Interval
OR	Odds ratio
HRV	Heart Rate Variability
SCARB2	Scavenger Receptor Class B Member 2
PSGL-1	P-selectin glycoprotein ligand-1
TNF	Tumor necrosis factor
IgM/ IgA/ IgG	Immunoglobulin M/A/G
GWAS	Genome Wide Association Study
SNP	single nucleotide polymorphism
HLA	human leukocyte antigen
OUCRU	Oxford Clinical Research Unit
IQR	Interquantile
CRP	C-reactive Protein
WBC	White Blood Cell
CH1	Children's Hospital 1
CH2	Children's Hospital 2
HTD	Hospital of Tropical Diseases
ICD10	International Classification of Diseases 10th

ID	Infection Department
OPC	Out-Patient Clinic
PICU	Peadiatric Intensive Care Unit
ED	Emergency Department
GIS	Genome Institute of Singapore
QC	Quality Control
Q-Q	Quantile – Quantile
HWE	Hardy–Weinberg equilibrium
PCA	Principle Component Analysis
HPD	Highest Posterior Density

1. BACKGROUND

1.1. HAND, FOOT AND MOUTH DISEASE: A GENERAL OVERVIEW

Hand, Foot and Mouth Disease (HFMD) is a common viral infection, usually seen in children, especially those who are under five years old. *Enterovirus* species A is the causative agent of HFMD. Serotypes Enterovirus A71 (EV-A71), coxsackievirus A6 (CV-A6), CV-A10 and CV-A16 are the most frequently detected pathogens. The typical presentation of the disease includes rash or vesicles on hands, feet, buttocks and painful mouth ulcers, with or without fever. Most of the cases are mild to moderate in severity and patients often recover completely within a week. However, some patients may progress to more severe disease with neurological complications such as acute meningitis, encephalitis, paralysis and cardiopulmonary distress.

Large outbreaks of severe disease emerged in the Asia-Pacific region, initially in 1997 with an outbreak in Sarawak, Malaysia and the disease has remained as one of the most threatening health problems in the region since then. There is no specific antiviral treatment to date. An inactivated EV-A71 vaccine has recently been licensed in China but its use remains limited within some localities in China. New antiviral agents and vaccines, especially bivalent/multivalent vaccines, are under development.

1.2. EPIDEMIOLOGY

1.2.1. History of HFMD

Symptoms of HFMD, including vesicles in hand, feet, buttocks and painful mouth ulcers, were first reported by J. H. Seddon in 1957 in a newsletter sent to Research Committee of the New Zealand Council [1]. Almost at the same time, in Toronto, Canada, Robinson et al. described 60 cases with fever, oral and facial lesions, skin rash and other respiratory and intestinal symptoms. Cell culture of throat swabs and stool samples from Toronto's cases yielded viruses belonging to *Enterovirus* A (then called Coxsackie A viruses). However, a clear association between virus and disease was not confirmed yet [2].

The disease was first named "hand, foot and mouth disease" by Alsop et al. in an outbreak in Birmingham, UK in 1959 with 83 cases among which CV-A16 was isolated from the vesicle fluid of one patient [3]. After that, by the 1970s, minor outbreaks were reported in the United States of America, England, New Zealand and Japan with mild and completely recovered cases, confirming the association between CV-A16, CV-A10 and the disease [4–6].

EV-A71 was first isolated in 1969 from a 9 month old child with encephalitis during an outbreak in California [7]. In 1972 a report in New York mentioned ten cases with central nervous system (CNS) infection (aseptic meningitis, encephalitis). Some of these also had the characteristic HFMD rash and all cases were EV-A71 positive by culture [8]. Almost at the same time, in summer of 1972 -1973, 39 patients in Melbourne, Australia were admitted to hospital with CNS infection symptoms. Five

of them also presented with rash. Of the five, two cases had HFMD, other cases had either “fine maculopapular, diffuse erythematous” or “impetigo”. EV-A71 was isolated from all 39 cases [9]. Since then, several outbreaks of EV-A71 associated HFMD have occurred in Sweden 1973 [10], Japan 1973 [11] and 1978 [12], Australia 1986 [13]. Following the outbreak in Sarawak, Malaysia in 1997, HFMD has emerged and remained one of the most important public health problems in the Asia-Pacific region [14].

1.2.2. Notable outbreaks of HFMD in Asia Pacific region

Numerous HFMD outbreaks occurred around the world before 1997. Japan experienced two major epidemics in 1973 and 1978. The first one (1973) included more than 3200 cases, among which EV-A71 was isolated from 66 cases and CNS disorders were also reported in this outbreak [5,11,15]. The second one (1978) was a large-scale epidemic throughout the country with 36301 cases. CNS involvement was also reported but less frequently than in 1973 [16].

From April to August 1997, a severe epidemic of HFMD occurred in Sarawak, Malaysia with 2628 cases reported. 889 children were hospitalized, 39 among them had aseptic meningitis or acute flaccid paralysis. 29 children died due to progressive cardiac failure and pulmonary edema. All fatal cases were young children (median age of 1.5 years), had a history of fever, progressed to cardiopulmonary distress and died quickly after admission. EV-A71 was identified as the causative agent of this outbreak [14]. This outbreak marked the beginning of HFMD spread through the

Asia-Pacific region and the disease has continued to cause severe and continuing outbreaks until now.

Taiwan in 1998, reported 129,106 cases from a national surveillance system among which 78 were fatal. EV-A71 was the dominant pathogen detected in 469 cases, followed by CV-A16 [17]. In Fuyang, China, from 1 March to 9 May 2008, 6,049 HFMD cases were reported from local healthcare facility of which 353 (6%) were severe and 22 (0.4%) were fatal. EV-A71 and CV-A16 were the predominant pathogens and a recombinant EV-A71 / CV-A16 virus was also found in this outbreak [18]. Since the Fuyang outbreak HFMD and EV-A71 have remained endemic in China, with over 1 million of new cases reported each year and an estimated HFMD incidence of 1.2 per 1,000 person–years [19].

Year	Clinical presentation	Age	Outcome / Incidence	EV-A71 diagnosis
Australia				
Feb-Sep 1999, Perth	14 cases with neurological complications	12/14 cases (86%)<4 years old	4 developed long-term neurological sequelae [20]	EV-A71
2000-2001 Sydney	200 HFMD cases admitted 9 with CNS complications 5 pulmonary edema	NA	3 cases with long-term neurological sequelae [21]	EV-A71 in all pulmonary edema cases
Brunei Darussalam				
2006	1681 cases	Mostly <5 years	3 fatal cases	EV-A71 in 34 cases [22]
China				
2007	1149 HFMD cases 11 severe	84.4% <5 years old	3 fatal cases	52.4% (55/105) (+) EV-A71 C4 [23]
2008 Jan - May	61459 cases	NA	36 fatal cases	54.5% of 582 tested cases (+) EV-A71 [24]
2008 Fuyang city	6049 cases 353 severe [18]	< 5 years	22 fatal cases	NA

2009	1155525 cases 13810 severe	75% <3years	353 fatal cases	41% of confirmed cases (+) EV-A71 [19]
2010	1774669 cases	NA	905 fatal cases	94.12% of deaths (+) EV-A71 [19]
2012	1619706 [19]	NA	509 fatal cases	NA
2013	1832141 [25]		256 fatal cases	all death cases (+) EV-A71
Taiwan				
1998	129106 reported cases 405 severe	mostly <5years	37 fatal cases	44/59 severe survive cases (+) EV-A71 34/37 death cases (+) EV-A71 [17]
2000	291 severe	NA	41 fatal cases	Fatal cases (+) EV-A71 [26]
2001	389 severe	NA	55 fatal cases [26]	Fatal cases (+) EV-A71
1998-2005	1548 severe	75% <2years	245	EV-A71 (+) rate ranging from 11-100% [26,27]
2008	19530 cases 714 Hospital admissions HFMD/herpangina [28]	NA	1 fatal cases	18.1% EV-A71

Japan				
1998	36.5 cases/sentinel site/year 2400 sites [29]	< 5 years old	NA	NA
1999-2005	42.7 cases/sentinel site/year 3000 sites [29]	NA	NA	NA
2000	205365 cases	90% <5 years [30]		EV-A71 were causative agents
2003	172659			
Malaysia				
1997	2628 HFMD cases 889 cases admitted 39 severe	Age median of fatal cases 1.5 years	29 fatal cases	6 fatal cases (+) EV-A71 [31]
1997	4625 admitted cases	NA	11 [32]	EV-A71 in all fatal cases
1998-2005	2950 cases/ 4290 specimens [33]	NA	NA	EV-A71 predominant
Mongolia				
2008	3210 HFMD	NA	NA	102/245 cases were (+) EV-A71 [34]

South Korea				
2008-2009	719 suspected cases 2008: 200 cases 2009: 519 cases	NA	NA	447 (62%) (+) EV 18 cases (+) CV-A10 in 2008 91 cases [35]
Singapore				
2000	3790 cases	NA	3 fatal cases	73% of 104 diagnosed cases (+) EV-A71 [36]
2002	15282 reported cases	NA	1640.5 per 100000 population [36]	
2001	5187 cases	1 fatal case < 4 years 2 fatal cases< 11months [36]	3 fatal cases	
2007	20003 reported cases [37]	NA	NA	NA
Thailand				
July 2012	39000 cases	3 fatal cases, of which 1 cases 16 years old	Incidence rate 62.7/100000 person-years	EV-A71 B5 detected in all fatal cases [38]

Cambodia				
April-June 2012	74 cases	50 fatal cases Median ages: 2 years old	NA	EV-A71 C4 was detected in most of fatal cases [39,40]
Vietnam				
2005 [41]	764 diagnosed cases 51 severe	<5 years	1 fatal case	441 cases (+) EV 173 cases (+) EV-A71 214 cases (+) CV-A16
2006-2007	305 severe cases	NA	None	39 cases (+) EV-A71 [42]
2007	5719 cases	Mostly < 5 years	23 fatal cases	NA
2008	10958 cases	NA	25 fatal cases [42]	NA
2009	10632 cases	NA	23 fatal cases [42]	NA
2011-2012	116000 cases	Mostly < 5 years	200 fatal cases	EV-A71 C4 [43]

Table 1.1. Notable outbreaks of HFMD since 1997 in the Asia Pacific region

1.2.3. Vietnam situation

The first case of EV-A71 associated disease in Vietnam was reported in 2003 by the Pasteur Institute, Ho Chi Minh City in an outbreak of encephalitis associated with HFMD in southern Vietnam. In 2005, 764 HFMD cases were admitted to Children's Hospital 1, one of the two main children's hospitals in Ho Chi Minh City, with a diagnosis of HFMD. Of those, 96.2% were under five years old. Enteroviruses were detected by virus isolation followed by sequencing of 411 viruses (53.8%), 173 of which were EV-A71 (43.1%). 51 cases (29.5%) had experienced neurological complications and 3 of them died (39). Surveillance from the same hospital in 2007, 2008, 2009 reported the number of new and fatal cases as 5719 and 23, 10958 and 25, and 10638 and 23, respectively [43].

In 2011-2012, Vietnam experienced the largest and most severe HFMD outbreak with 174,677 new cases and 200 deaths reported. Most of the 2011 cases were from southern Vietnam. The disease spread to northern Vietnam in 2012 [43] and has remained a major public health issue until now. EV-A71 was the main cause of severe disease, accounting for 132/174 (76%) of the tested throat swabs taken from patients with severe HFMD during the 2011 outbreak. Virologically, EV-A71 subgenogroup C4 was dominant, while subgenogroup C5 was (still) sporadically detected. However, in 2013 subgenogroup C4 was replaced by B5 [44].

1.2.4. Incidence and mortality rate

Many countries in the Asia–Pacific region now have established surveillance systems for HFMD such as China, Hong Kong, Singapore, Taiwan, Malaysia and Vietnam. The incidence of EV-A71 associated HFMD has increased dramatically since the Sarawak outbreak in 1997 although it varies according to country and year [45]. The largest epidemiological study by Xing et al, collecting data from over 7 million HFMD cases across China from 2008-2012, showed an incidence of 1.2 per 1000 person – years from 2010-2012 and the highest was among children from 12-23 months old (38.2 per 1000 person-years in 2012) [19]. A Taiwanese study from 2008-2009 also showed differences in incidence rate when classifying patients into age-groups, increasing from 17.1 per 1000 person-years at 0–6 months of age to 40.9, 57.4, and 49.7 per 1000 person-years at 7–12, 13–24, and 25–36 months of age, respectively [46]. In Hong Kong, data from all reported cases in 2008 gave an annual incidence of 1.4 per 100,000 general population and children under 5 years old had the highest incidence of 27.9/100,000 [47]. A study in Singapore reported an increase in annual incidence rate per 100,000 population from 125.5 in 2001 to 435.9 in 2007. Similar to Hong Kong, the highest incidence was also in children less than 4 years old [37].

A systematic review of 14 studies in Singapore, southern Vietnam, South Korea, Hong Kong and China from 1998 - 2013 reported a pooled case-fatality rate of 1.7%. This rate was highest in mainland China (1.8%) followed by other regions (Vietnam and Singapore) (1.5%) and Hong Kong (1.1%). The overall pooled case-fatality rate also increased from 1.5% in 1998 – 2007 to 1.8% in 2008 – 2012 [45]. Data from

Taiwan in 1998 showed a trend of case-fatality rate in different age groups. The case-fatality rates were 89.4, 51.9 and 6.6 per 100,000 in infants 7-12 months, children 1-3 years old and over 3 years, respectively [48]. In Vietnam, Khanh et al reported a case-fatality rate in EV-A71-associated HFMD of 0.5% in a hospital – based study in Ho Chi Minh City in 2011, which was lower than the rate from a previous study in the same hospital from 2005 [41]. However, in these two studies, case-fatality rates were calculated based on number of cases admitted to one large tertiary-care pediatric hospital only and thus do not represent a population level case-fatality rate.

1.3. VIROLOGY

HFMD is caused by different serotypes of *Enterovirus* A, a species within the genus *Enterovirus* of the family *Picornaviridae*. While CV-A16, (first isolated in 1959) and EV-A71 are traditionally regarded as common causes of HFMD, in recent years CV-A6 has emerged and caused outbreaks of HFMD worldwide. Other serotypes associated with HFMD are CV-A2-8, 10, 12, 14, and EV-A76 and 89-92 (which belong to the species *Enterovirus* A) and Coxsackie B1-B5 (which belongs to the species *Enterovirus* B) [49–51].

1.3.1. Enteroviruses

Enteroviruses (EV) are one of the most common viruses causing human disease worldwide. Most infections are asymptomatic and self-limiting. However, some serotypes of virus are associated with a wide range of clinical manifestations, from

mild febrile illness to severe and potentially forms, which can be fatal, such as meningitis, encephalitis, myelitis, myocarditis, acute flaccid paralysis and neonatal sepsis – like disease [52]. *Enterovirus* B is the most common pathogen associated with aseptic meningitis worldwide, while *Enterovirus* A serotypes such as EV-A71 and Coxsackieviruses are the most common species isolated from patients with HFMD. In recent years, *Enterovirus* D68 has emerged and caused large outbreaks of respiratory illness with an epidemiological link with acute flaccid paralysis in the United States and other countries [53,54].

Because of the high genetic diversity and high rate of mutation and recombination, enteroviruses can evolve to form new serotypes/variants, which may subsequently emerge [55–57]. Currently, there are 13 identified species of enteroviruses of which 7 species are known to infect humans. They are enteroviruses A-D and rhinoviruses A-C. Currently, there are more than 100 serotypes of enterovirus A-D that have been identified (Figure 1.1).

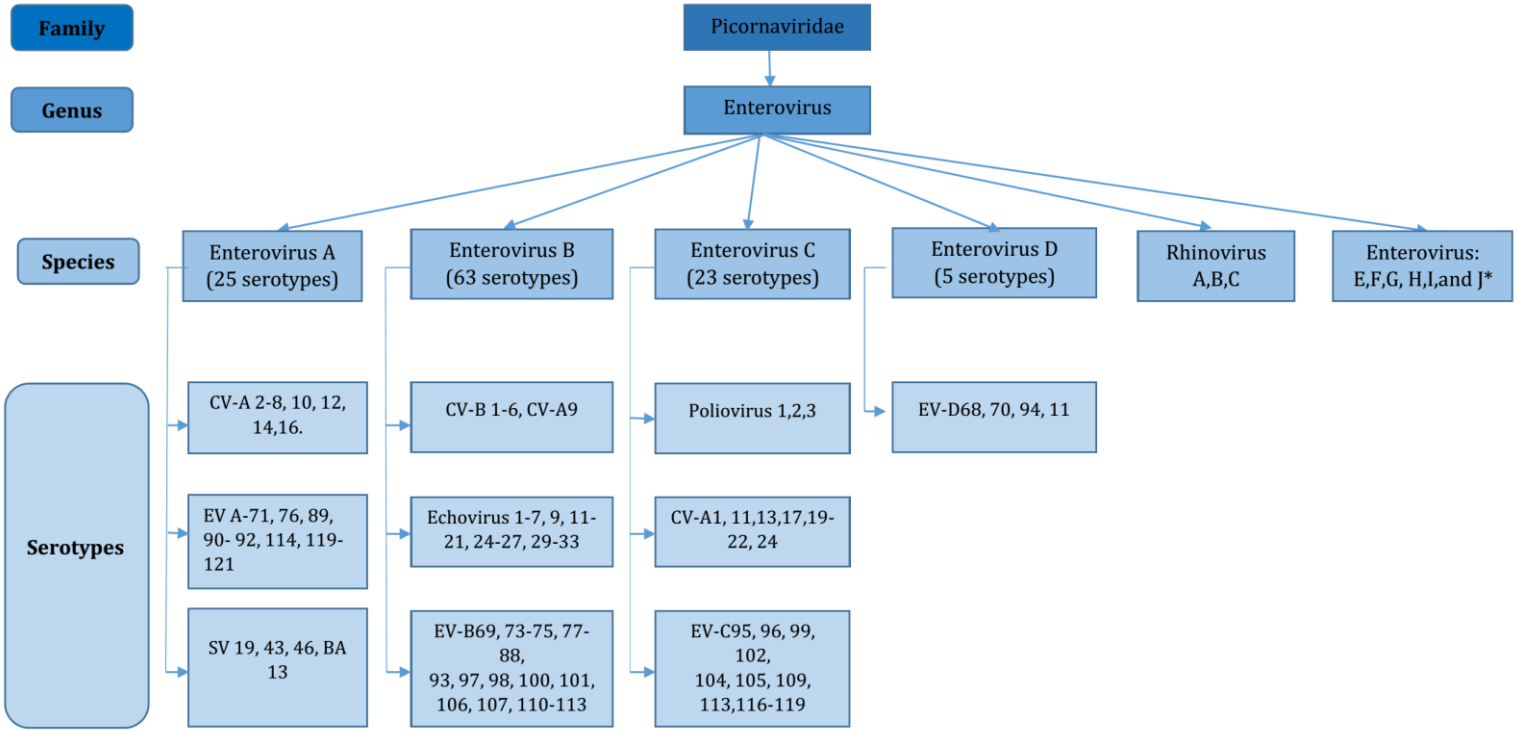


Figure 1.1. Figure Enterovirus classification

(derived from http://www.picornaviridae.com/Enterovirus)

*: Enterovirus species E, F (former name *bovine Enterovirus*), Enterovirus species G (former name *porcine Enterovirus*), H, J (former name *simian Enterovirus A) and I*, are commonly seen in animals

EV=Enterovirus, CV= Coxsackievirus; SV= Simian Enterovirus; BA= baboon Enterovirus

The main transmission route of enteroviruses A-D is feco – oral but other modes of transmission like respiratory droplets, mucosa of the eyes and recently, swimming in contaminated water have also been reported [58–60]. After ingestion, viruses multiply primarily in the gastrointestinal tract but they can also multiply in other tissues such as respiratory mucosa, tonsils, nerves and muscles before spreading through the blood stream to target organs.

1.3.1.1. Virion structure

Enteroviruses, like other genera of the family *Picornaviridae*, are small (30 nm in diameter) with a non-enveloped protein capsid and a single strand of positive – sense RNA. The non-enveloped structure without the lipid layer provides enteroviruses A-D the ability to resist organic solvents and non-ionic detergents and remain stable within acid pH. Enteroviruses A-D can only be inactivated by UV light, chloride and formaldehyde. These chemical and physical characteristics permit viruses to survive for days to weeks outside the hosts.

The enterovirus A-D virion is an icosahedral capsid comprising of 60 protomeric units. Each protomer is formed by four capsid proteins: VP1, VP2, VP3 and VP4 of which, proteins VP1, VP2 and VP3 are exposed on the surface of the virion and VP4 beneath them and, therefore, VP4 can not be detected by the host antibodies. VP2 and VP3 are located along the threefold axis and VP1 along the fivefold axis of symmetry. Five protomeric units assemble to form a pentamer and twelve pentamers, in turn, assemble to compose a viral mature virion. The apex of each pentamer (fivefold axis)

forms a “star-shape” promontory with a deep cleft surrounded (also called a “canyon”). The canyon is the viral receptor binding site. Most known neutralization sites are clustered on VP1 [61].

1.3.1.2. Viral genome

The RNA of enteroviruses is about 7.4kb in length. A virally encoded polypeptide VPg (3B) is at the 5’ end of the genome, followed by a noncoding region (NCR). The 5’ NCR are regions with a high level of nucleotide identity among enteroviruses and therefore, these regions have been used to design probes and primers to detect enterovirus RNA generically. In contrast, VP1 is the most variable region of the genome, and is therefore used for serotype identification.

The longest part of viral genome is a single open reading frame (ORF). This ORF is subdivided into three regions, designated P1 to P3. The P1 region encodes the four capsid proteins: VP4, VP2, VP3 and VP1. VP1 is the main site for neutralization as it contains type-specific epitopes correlating with the serotype [62,63] although other neutralizing epitopes have also been found on VP2 and VP3 [64,65]. VP4 is not exposed to the viral surface and it has a role in viral binding to human cells, leading to the uncoating of virus. The P2 and P3 regions encode non-structural proteins: 2A, 2B and 2C (P2) and 3A, 3B, 3C and 3D (P3). These proteins are involved in the replication of viral RNA, together with their intermediates, and are therefore essential for the viral life cycle. The single ORF is followed by a short 3’ NCR and a terminal polyadenylated tail (Figure 1.2) [66].

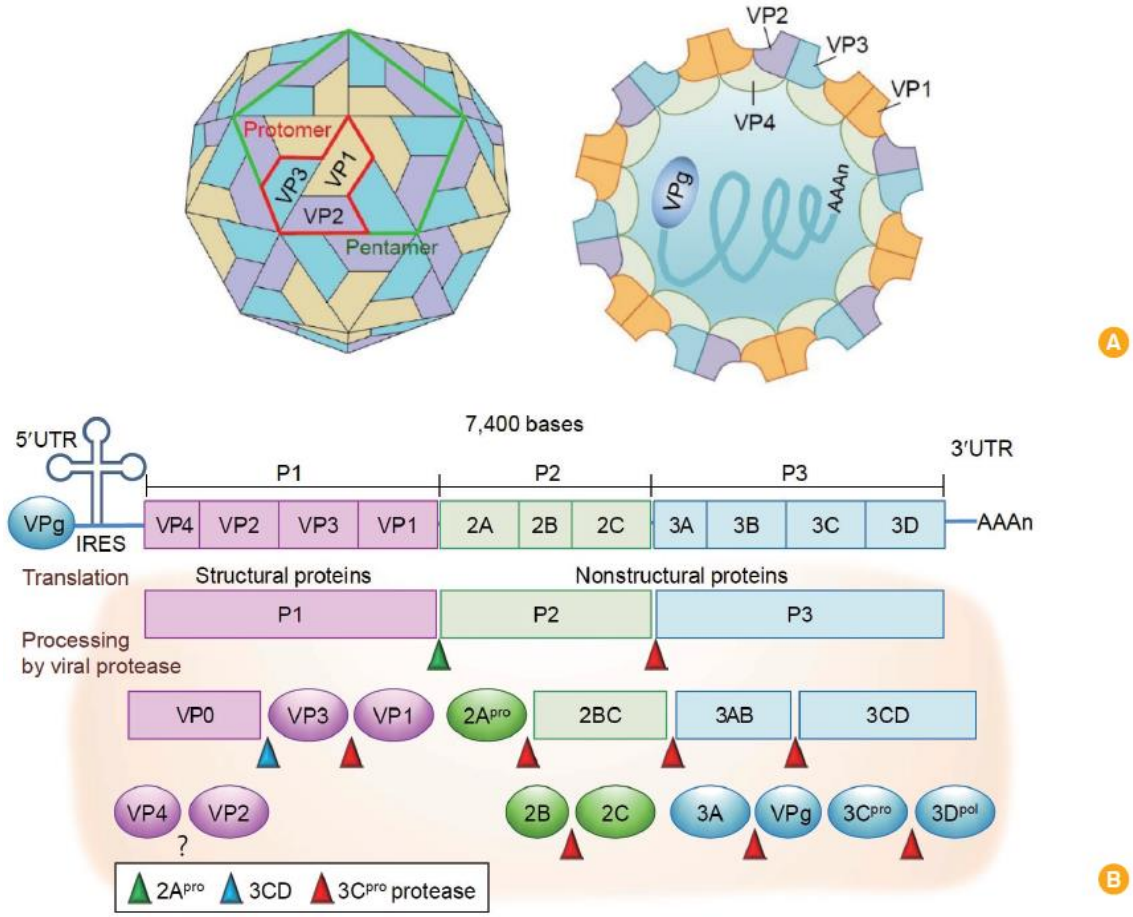


Figure 1.2. Virion structure and genome organisation of Enteroviruses

Figure obtained from reference [66]

1.3.1.3. Viral entry and replication

1.3.1.3.1. Enterovirus receptors

Enteroviruses can cause variety of diseases due to the ability to bind to different receptors such as poliovirus receptor (CD155) for poliovirus [67], intercellular adhesion molecule 1 (ICAM1) for most of rhinoviruses [68], complement decay-accelerating factor (DAF or CD55) for group B Coxsackieviruses [69], scavenger receptor class B membrane 2 (SCARB2) and sialomucin-like leukocyte membrane protein (PSGL1) for EV-A71 [70] (more details in section 1.6.1.1).

1.3.1.3.2. Viral entry and uncoating

Enterovirus entry process includes three particle types: 160S, 135S (also called the A-particle) and 80S particles [71]. The uncoating process starts with the changing of the 160S particle (native and infectious form of the virion) to form the 135S particle. Those changing includes capsid expansion, externalization of the capsid protein VP1 N terminus and release of myristoyl–VP4. The 135S particle then releases RNA genome, through iron-permeable channels, yielding the 80S particle (empty particle) [71,72].

1.3.1.3.3. Translation and replication

Translation is mediated by an internal ribosome entry site (IRES) [73]. Replication occurs on virus-induced, tubule vesicular replication organelle (ROs). The formation of ROs required viral protein 2BC and 3A and some host proteins such as acyl-CoA-bindingdomain-containing protein

(ACBD3), phosphatidyl inositol 4-kinase-β (PI4KB), and oxysterol-binding protein (OSBP) [74,75]. However, the exact mechanism of ROs formation is still unclear. The essential enzyme of the replication process is the viral RNA-dependent RNA polymerase $3D^{pol}$ [72]. Replication is initiated at the 5' and 3' UTRs and requires a primer, the viral peptide 3B (also known as VPg) coupled to two uredines. $3D^{pol}$, together with $3CD^{pro}$ and 3AB bind to structural RNA elements. Genome replication starts with synthesis of a negative-strand (−) RNA that serves as a template for synthesis of new (+) RNA molecules [72].

1.3.1.3.4. Virion assembly and release

New synthesis (+) RNA molecules can either enter a new round of replication or be packaged into progeny virions. The proteolytic processing starts with the presence of $3CD^{pro}$ protein, yielding capsid proteins VP0, VP1 and VP3. VP0 then cleaves into VP2 and VP4. Capsid proteins then self-organize into protomers and pentamers and assemble into provirions [72]. Mature virions exit the host cell via non-lytic release of extracellular vesicles or via cell lysis, completing the life circle of enterovirus [72].

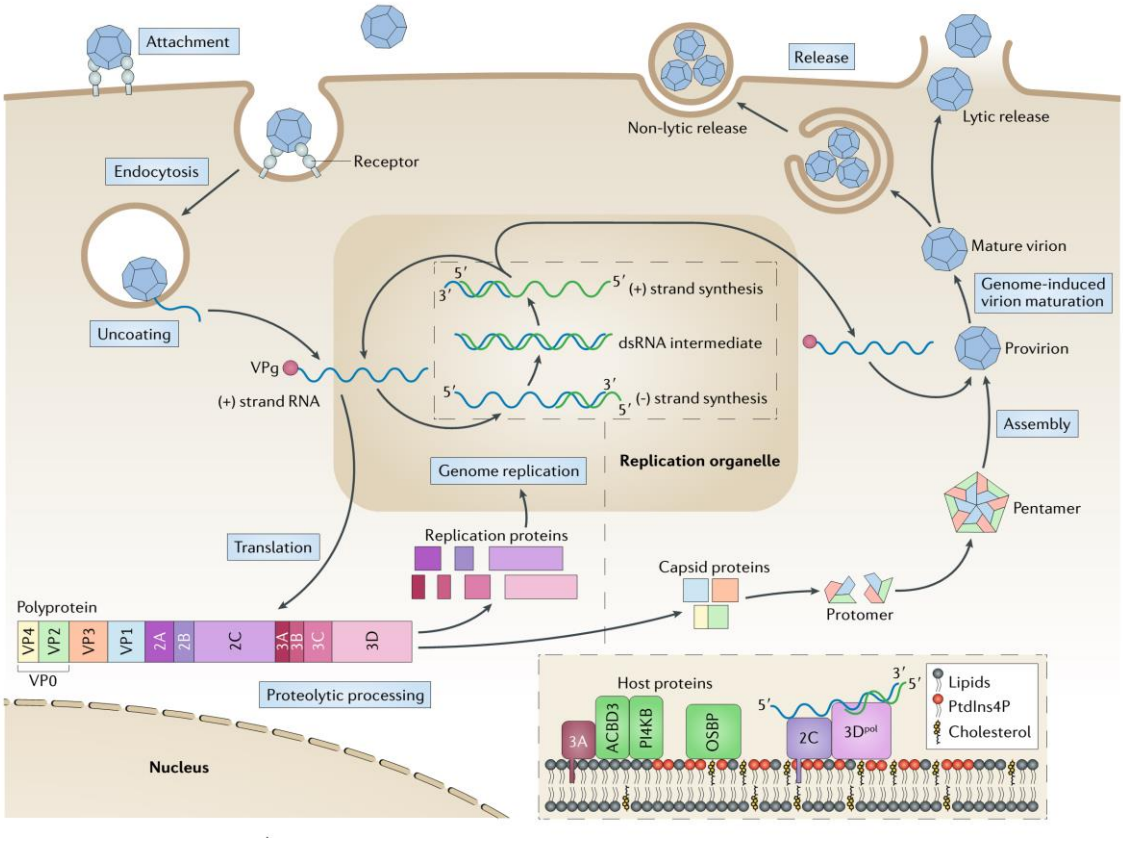


Figure 1.3. Enterovirus life circle

Figure obtained from reference [72]

After binding to receptors and endocytic uptake in the cell, the virion delivers its positive-strand (+) RNA genome. The viral genome is linked to the viral protein VPg (3B), served as a primer for replication.

Genome is translated into replication proteins (2A–2C and 3A–3D) and capsid proteins (VP0, VP1 and VP3). Replication occurs on membranous replication organelles. Mature virions exit the host cell via non-lytic release of extracellular vesicles or via cell lysis. Capsid proteins self-arranged to form protomers, pentamers and assembly to mature virions before release.

1.3.1.4. Enterovirus detection

1.3.1.4.1. Specimen types for virology tests

Many types of clinical samples can be used for laboratory diagnosis of enterovirus infection, including swabs from skin vesicles (when available, albeit difficult to collect, especially in young children), throat swabs, rectal swabs, stool, blood and cerebrospinal fluid (CSF) samples. Among them, the throat swab has the highest yield, following by vesicle, ulcer and rectal swabs [76]. EV-A71 virus can persist in the gastro-intestinal tract for up to several weeks and can be detected in rectal swabs and stool samples during this period.

Because enteroviruses can cause asymptomatic infection, viral detection in the upper respiratory or gastro-intestinal tract may not reflect disease causation [42,76]. Viruses detected from vesicle swabs, however, reflect an on-going infection. However, not all patients with HFMD have the typical vesicles that can be used for routine diagnosis. Blood and CSF samples are less sensitive for virus detection by PCR and/or cell culture. Ooi et al., in a study in 2007, recommended a combination of a throat swab plus a vesicle swab or a rectal swab (in patients without vesicles) to maximize detection rate [76].

1.3.1.4.2. Virus isolation and antibody-based diagnostic methods

In the pre-molecular era virus isolation was regarded as the most reliable method for viral detection, including enteroviruses. Enteroviruses were sub-classified originally by their ability to grow in various cell lines and to cause diseases in animals [77] .

However, virus isolation requires a high level of expertise, is time – consuming and relatively insensitive. Besides, some enterovirus (EV) serotypes, particularly in the EV-A group, do not grow well in cultured cell lines [61].

Antibody-based assays are another way to diagnose acute or past EV infection. This method is however not specific because of the cross-reactivity and reactivity from past infection.

1.3.1.4.3. Nucleic acid amplification based diagnostic methods

The most advanced and significantly developed techniques to detect EVs are nucleic acid amplification based methods, including reverse transcription polymerase chain reaction (RT-PCR). Depending on the purpose of the test, primers can be designed to target different parts of the virus genome. The first sets of primers and probes for universal detection of EVs were reported from the 1990s [78,79], followed by many others in the next few years [80–83]. Overall, results proved that RT-PCR is a sensitive, specific, rapid and useful method to detect EVs [84,85]. All sets of universal detection of EVs are targeted at the highly conserved 5'-NCR regions of the EV genome. For species–specific detection, primers are targeted at the VP1 coding region. By using PCR primers/probes targeted at genetically variable regions as VP1, several serotype specific PCR (EV-A71 and CV-As) have been successfully developed.

In addition to the traditional RT-PCR, real-time RT-PCR has also been developed to detect EVs. By using DNA-binding fluorophores or fluorophore – labelled oligonucleotide probes during the amplification process, it can shorten the amplification time and a (semi) quantitative result is obtained. Additionally, real time

RT-PCR method does not require a post amplification-analysis step (e.g. agarose gel-based electrophoresis) and thus reduces the risk of contamination.

1.3.1.4.4. Enterovirus serotype determination

In addition to serotype specific PCRs employing primers/probes targeted at genetically variable regions of enterovirus genomes as mentioned above, enterovirus serotype determination can be achieved by employing generic primers to amplify a fragment of variable enterovirus genome (VP1, VP2 and VP4). Subsequently, generated amplicons are sequenced, and the obtained sequences are compared with a known sequence databases to identify the specific EV serotype [63,86]. As the VP1 sequence contains specific clusters correlated with serotypes, most of the available techniques focus on this coding region, although other region as VP2 and VP4 have also been used in some studies. This method has great advantage in detecting new EV serotypes which were previously classified as non-typeable EVs by the traditional antibody-based approach [87]. It also reduces the turnaround time significantly from weeks to days and therefore can be used in clinical practice.

1.3.1.5. Circulation of HFMD causing by enteroviruses.

Over 20 EV serotypes have been reported to cause HFMD worldwide. Since the first detection from HFMD patients in Canada in 1957, CV-A16 has been reported to be the major pathogen in many outbreaks around the world. The largest reported CV-A16 HFMD in Europe was in England and Wales in 1994, with 952 cases. In the Asia-Pacific region, CV-A16 was also the main etiological agent in Japan (1981-2007) [88,89],

Vietnam 2005 [41], Singapore (2001-2007)[37], Thailand (2008-2011) [90] and China [91].

In recent years, reports from many countries in the region showed the emergence of other serotypes besides CV-A16 and EV-A71. In 2008, a CV-A6 outbreak occurred in Finland and the virus was reported as an "unusual causative agent of HFMD" and predicted it could emerge to be the dominant cause of HFMD in the coming years [92]. Indeed, from 2009-2011, a study in Linyi, China showed a change in etiological pattern of HFMD with a significant drop of EV-A71 (to almost 11%) and a rapid increase in other serotypes, including CV-A6 (6%) and CV-A12 (10%). Similarly, Taiwan also reported an increase in the detection rate of CV-A6 in the same period of time, from 15% (110/712) in 2007 to 22% (153/687) in 2009 [93]. Similarly, CV-A6 replaced CV-A16 and EV-71 to become the predominant pathogen in some parts of China (accounting for 47.8% of HFMD cases in Shenzhen 2013) [94] and Thailand (33.5% of HFMD in 2012) [95]. Unlike CV-A16 and other enteroviruses which mostly cause mild disease in children, the newly emerging CV-A6 has occasionally been reported to cause severe HFMD in children [96] and adults [97,98]. Other serotypes including CV-A10 and CV-A12 were also among the top five common HFMD pathogens in different studies in China [99] and Thailand [95].

Since the Sarawak outbreak in 1997, EV-A71 started to cause new series of HFMD outbreaks in the Asia-Pacific region [100]. Cyclical epidemics of 2-3 years have been observed in many areas where EV-A71 associated HFMD is endemic, including Japan, Malaysia and Singapore [33,101,102] (Figure 1.4). While the underlying mechanism of this phenomenon remains unknown, it is hypothesized that herd immunity among

older children and the presence of a large enough birth cohort of unprotected children play a role [103].

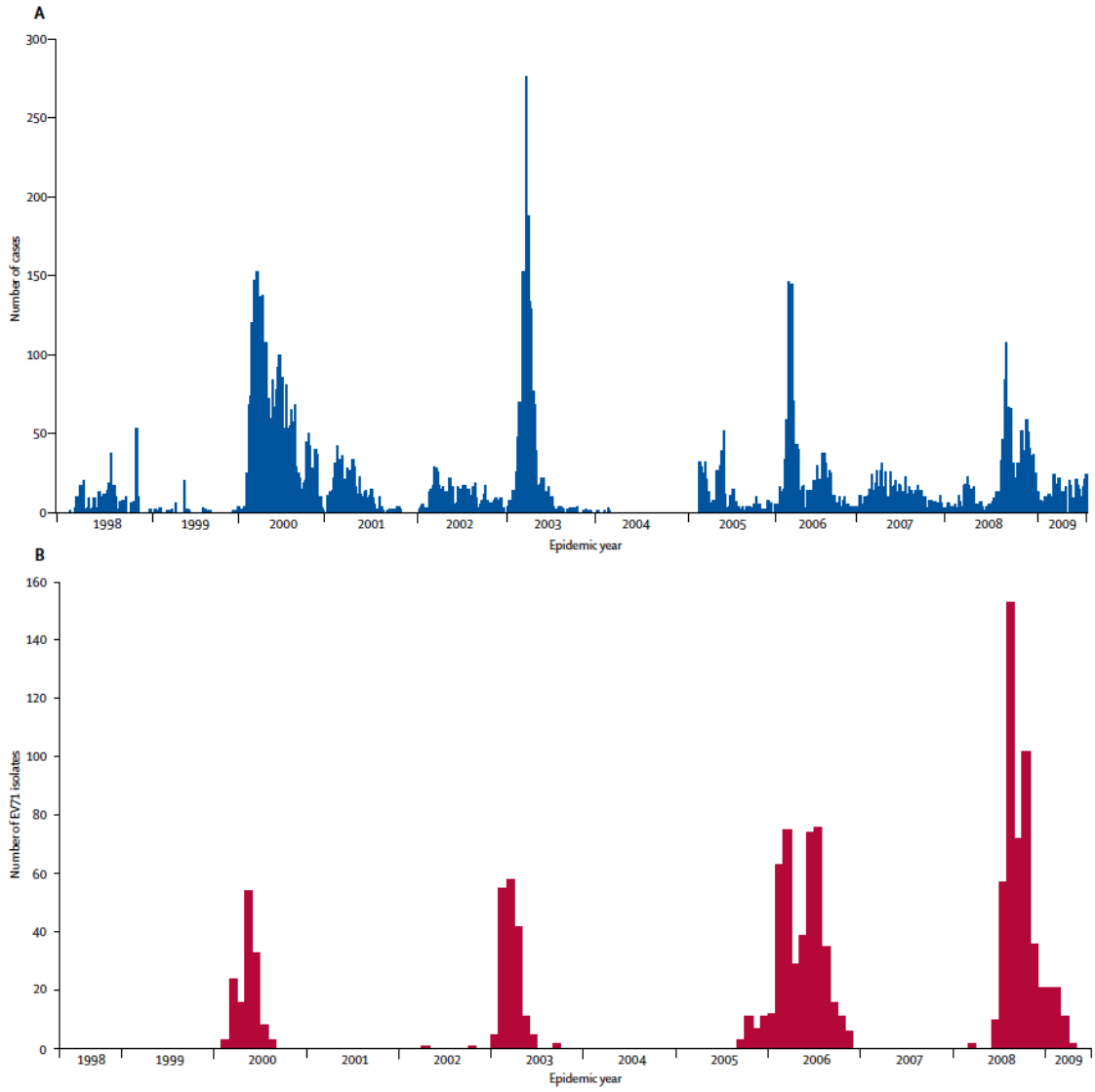


Figure 1.4. Distribution of (A) HFMD cases and (B) EV-A71 isolates in sentinel clinics in Sarawak, Malaysia from March 1998 to mid-2009, with 2-3 year cyclical pattern.

Figure obtained from reference [100].

1.3.2. Enterovirus A71 (EV-A71)

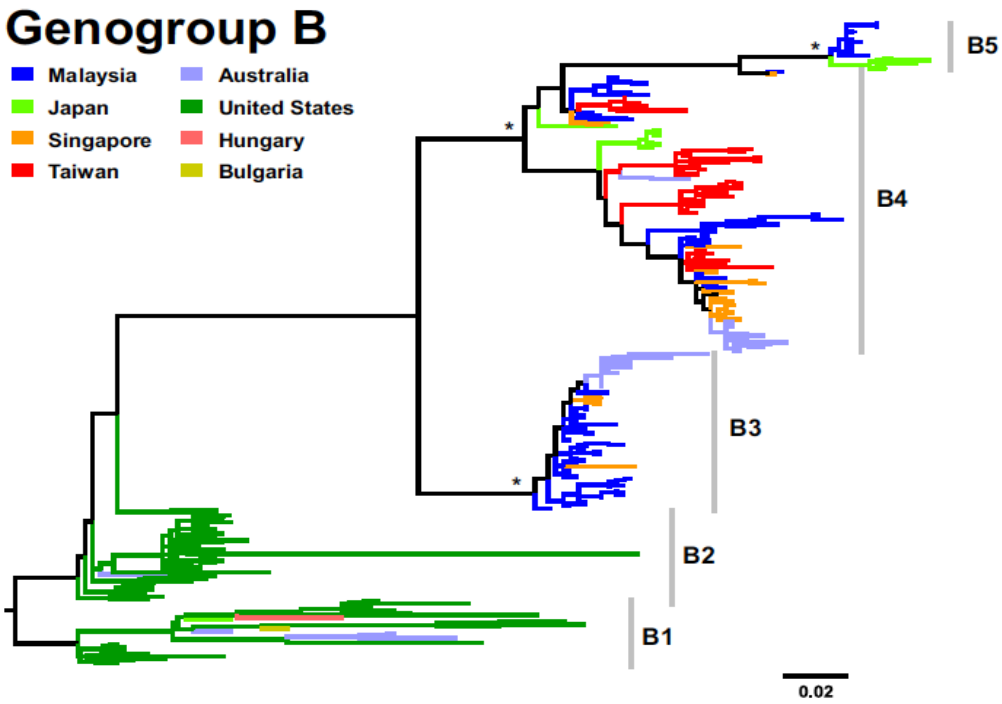
EV-A71 is most closely related to CV-A16, from which it was estimated to evolve in 1941 [104]. It is classified into three genogroups: A, B and C, and then further divided into 11 subgenogroups, depending on sequence variation of VP1 [104]. More recently, novel genogroups (D, E, F, and G) have been described in India (D and G) and parts of Africa (genogroups E and F) [105–107]. Genogroup A consists of the prototype strain (BrCr) only, while genogroup B and C are classified into five subgenogroups B1 to B5 and C1 to C5, respectively [66] (Figure 1.5).

1.3.2.1. Phylogenetic relationship of EV-A71 subgenogroups.

VP1 sequence based phylogenetic analysis showed that subgenogroups B1 to B5, C1,C2 and C4 are distributed globally, with viral sequences found in many countries around the world, while subgenogroup C3 and C5 were only detected in South Korea and Vietnam, respectively [104,108]. Within genogroup B, subgenogroups B1 and B2, which caused HFMD in the early 70s, have been replaced by B3, B4 and B5 in recent years [109–111] These subgenogroups, together with viruses of genogroup C, continue to circulate and cause disease in the Asia-Pacific region.

Subgenogroup C1 was first identified in Australia and the United States in 1980s and still circulates globally until now. The 1990s was the time of genogroup C expansion when large numbers of novel subgenogroups such as C2, C4, C5 were detected, causing outbreaks associated with severe disease and fatality in Taiwan [17,112], China [18,24], Singapore [37,113,114] and Vietnam [41,43].

A



B

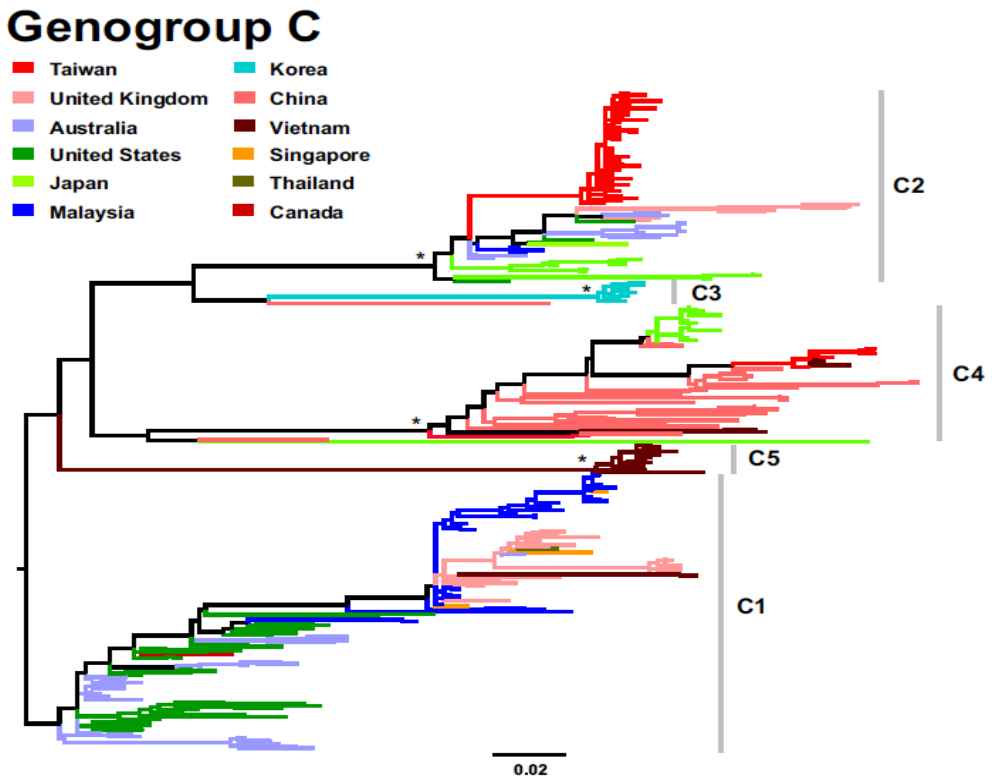


Figure 1.5. Phylogenies of EV-A71 VP1 sequence genes of (A) genogroup B and (B) genogroup C from 1970 to 2008.

Figures obtained from reference [104].

Results from analysis of 200 whole genome sequences of EV-A71 in Vietnam from 2011 to 2013 showed that EV-A71 isolates belonged mostly to two subgenogroups: C4 (154 sequences) and B5 (38 sequences). The rest (8 viruses) were subgenogroup C5. Subgenogroup C4 was likely to have originated from China and Hong Kong, as they were closely related to those from these two countries. Subgenogroup B5 seemed to be imported into Vietnam from Malaysia or Taiwan, and has replaced C4 in 2013 [44]. In parallel there is long-term persistence of subgenogroup C5 in Vietnam since 2003 with a low level of endemicity [108].

EV-A71 isolates of the same subgenogroup from different places also showed complex relatedness [104]. Clusters of sequences from the same subgenogroup, in one location could be interspersed with clusters from other locations. This result suggested that an outbreak in different countries, although by the same subgenogroup, could be a result of different viral lineages [32,108]. When observing the distribution of subgenogroups by time and places from rooted trees, sequences collected earlier were close to the root while sequences collected later located further from the root. Tee et al. suggested that there was a temporal subgenogroup replacement at global level.

Changing in dominant subgenogroups has been mentioned in reports from Taiwan and Vietnam [33,41,115]. Japan and Taiwan have experienced the same situation, when different predominant subgenogroups caused large outbreaks at different times [116,117]. Shifting in dominant subgenogroups can potentially lead to large outbreaks, especially with newly identified strains, for which most of the people in the affected community were likely lacking immunity [100]. From 2011-2012,

Vietnam experienced an explosive outbreak of HFMD caused by subgenogroup C4 that was most likely imported from other countries in the region.

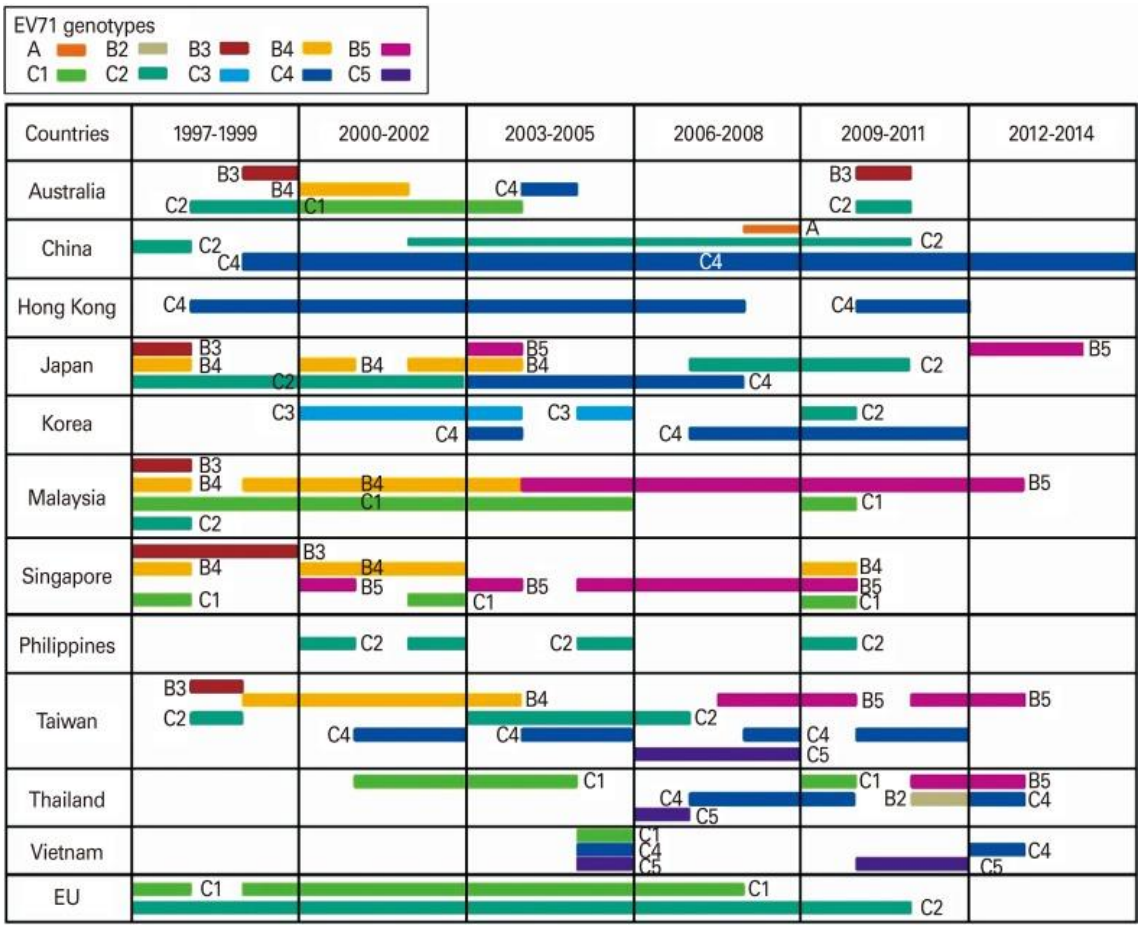


Figure 1.6. EV-A71 subgenogroups identified from 1997 to 2014 of the Asia Pacific regions. EU data includes data from Austria, Germany, UK and The Netherlands.

Figure obtained from reference [66].

1.3.2.2. EV-A71 phylodynamics

Before the 1990s, genogroup B showed a slight change in relative genetic diversity. This change could reflect sporadic occurrence of EV-A71 associated HFMD outbreaks in some parts of the world. The late 1990s witnessed a sharp increase of genogroup B relative genetic diversity, with the identification of subgenogroup B3 in 1999 [104] and B4 in1998-2000 outbreaks in Taiwan [118]. Interestingly, data from global surveillance of EV-A71 laboratory confirmed cases strongly corresponded to these dynamics of the skyline plot, suggesting that an increase of relative genetic diversity may lead to EV-A71 epidemics [104,119]. Similarly, the skyline plot of genogroup C shows a significant rise of relative genetic diversity in the mid-1980s with the introduction of subgenogoup C1. The viral diversity of genogroup C viruses then remained stable until it increased again at the end of 1990s, coinciding with major HFMD outbreaks in Taiwan caused by genogroup C2 (Figure 1.7).

In Vietnam, sequence analysis revealed a dramatic increase in terms of relative genetic diversity of C4 from the end of 2011, reaching a peak at December 2011, then remaining at this high level of diversity within the next 4 months before gradually declining in 2012. In contrast, B5 viral diversity increased remarkably from the second half of 2012. B5 then replaced C4 at the end of 2012 and its genetic diversity continued to increase in 2013 (Figure 1.8) [44].

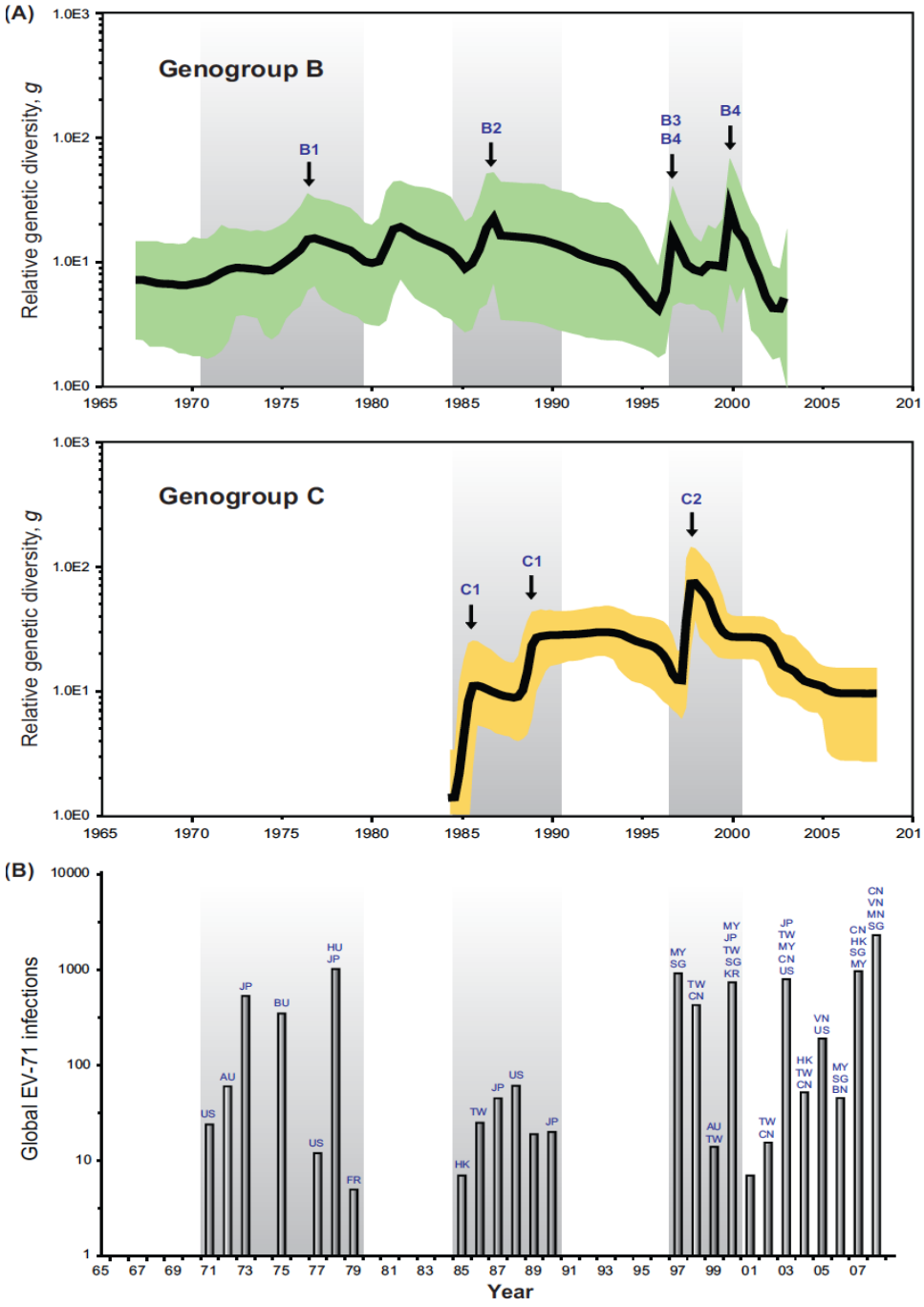


Figure 1.7. (A) Relative genetic diversity of genogroup B and (B) Relative genetic diversity of genogroup C from 1965 to 2010; (C) Global laboratory confirmed EV-A71 infection in the same period of time.

Obtained from reference [104].

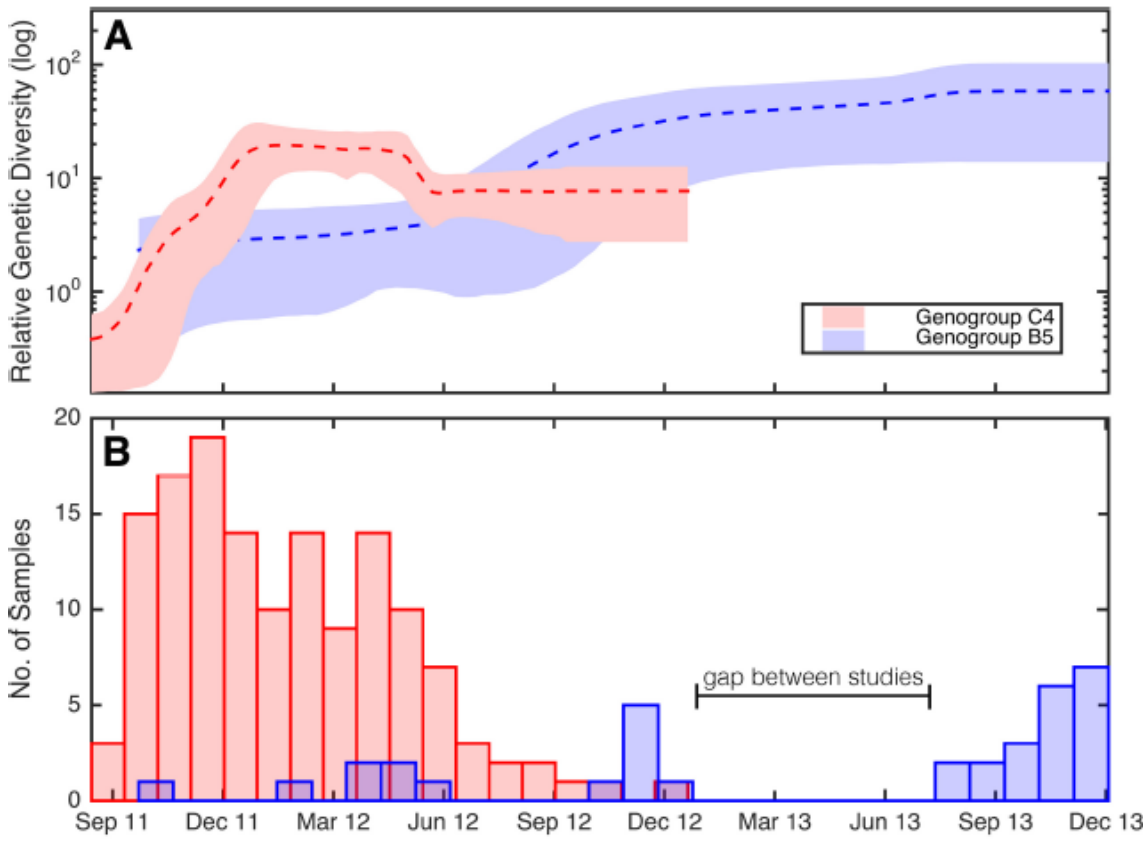


Figure 1.8. (A) Skyline plot showing relative genetic diversity of two main lineages circulated in Vietnam: C4 (red) and B5 (blue); (B) Sampling prevalence of C4 and B5 during the study period.

Obtained from reference [44].

1.4. CLINICAL MANIFESTATIONS

1.4.1. Clinical diagnosis

HFMD is diagnosed mostly by clinical judgment, based on typical skin lesions, with or without mouth ulcers. Mouth ulcers are multiple and painful lesions in the posterior oral cavity such as uvula, tonsils and palate although ulcers can also be seen in other parts of the mouth like buccal mucosa, tongue and lips. Patients may or may not have fever. Typically, the skin lesions are macular and vesicular. The usual location of rash is on the palms of the hands and the soles of the feet but they can also be found on buttocks, knees and elbows. Studies in China and Singapore reported that a macular rash and the lack of mouth ulcers are risk factors of severe HFMD [120,121].

1.4.2. Clinical course of HFMD according to World Health Organization guidelines

World Health Organization guidelines classify HFMD patients into four groups according to severity. The first and mildest group includes patients with just skin lesions and/or mouth ulcers with or without fever, and no signs of neurological complications. The second group includes patients with signs and symptoms of neurological involvement such as myoclonus, tremor, ataxia and cranial nerve palsies. Patients with autonomic nervous system (ANS) dysregulation are classified in the third group. ANS dysregulation symptoms includes cold sweating, mottled skin, tachycardia, tachypnea, hypertension and hyperglycemia. The fourth and most severe group includes patients with cardiopulmonary failure. Patients in this group may experience hypotension, shock and pulmonary edema. Not all patients with

neurological involvement will process to more severe stages. According to Khanh et al, in a study of 3129 children in 2011 , progression rates varied, from 4% to 22% depending on types neurological symptoms presented [43] (Figure 1.9).

1.4.3. Vietnam Ministry of Health guidelines

The Vietnamese Ministry of Health (MoH) in 2011 also issued guidelines for HFMD diagnosis and treatment with a grading system that was different from WHO guidelines. Here, HFMD is divided into four grades. Grade 1, grade 3 and grade 4, similar to the WHO classification but there is a difference in the grade 2 classification, which includes features of central nervous system (CNS) involvement, such as myoclonus, fever >39 ^{0}C or ataxia. Grade 2 is then divided into subgroups according to myoclonus reporting: 2A is myoclonus reported by parents/care-givers only and grade 2B is myoclonus observed by a physician. Grade 2B includes two subgroups: 2B1 is myoclonus observed by medical staff or history of myoclonus and lethargy or pulse higher than 130 bpm; 2B2 is ataxia, nystagmus, limb weakness, cranial nerve palsies, persistent high fever or pulse higher than 150 bpm [122]. The different grades are important as they will have different treatment implications. Grade 2B2 patients are given IVIg immediately while grade 2B1 patients are first monitored closely for 6 hours. And if patients do not improve, IVIg will be given (Figure 1.10).

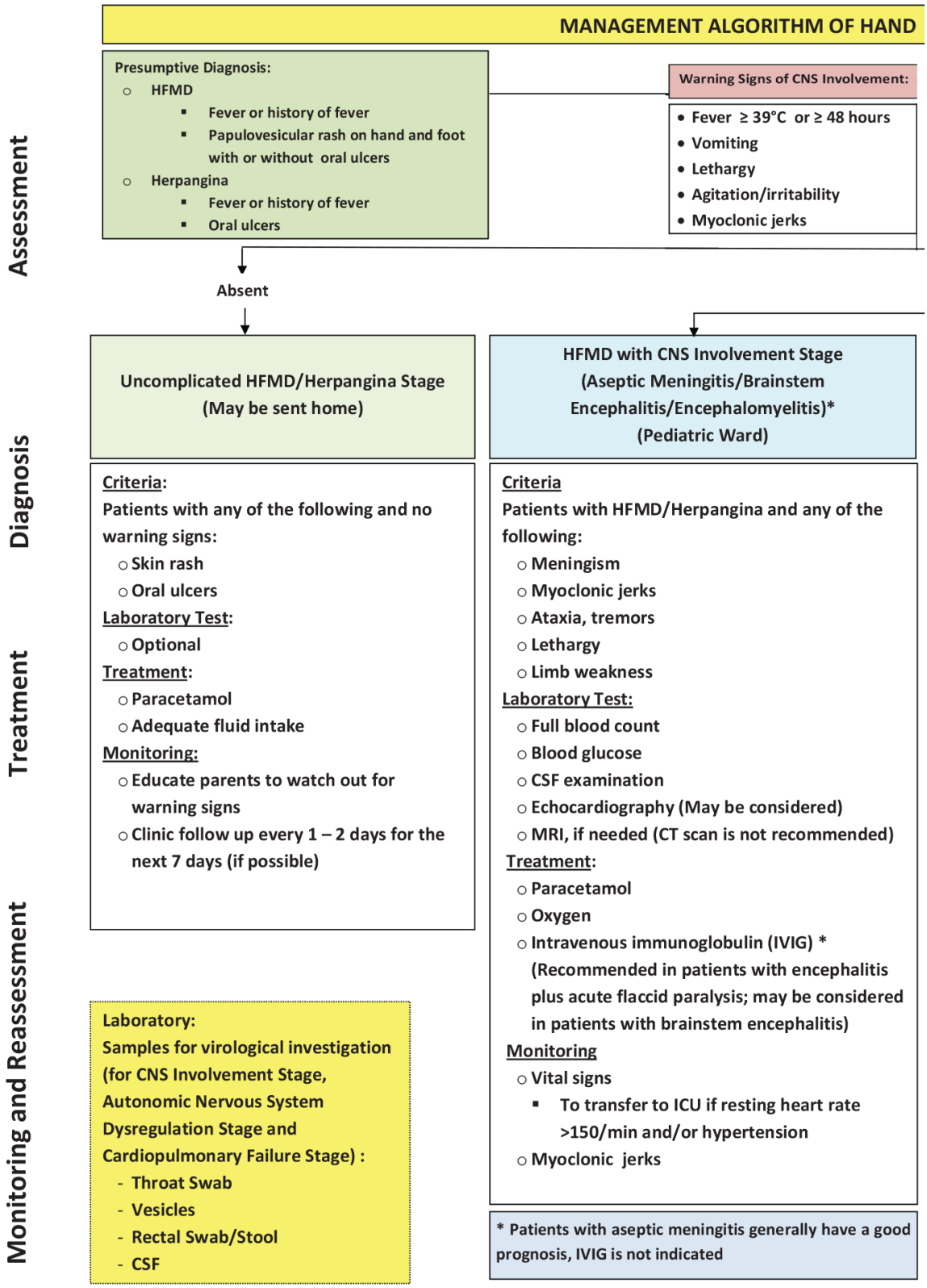
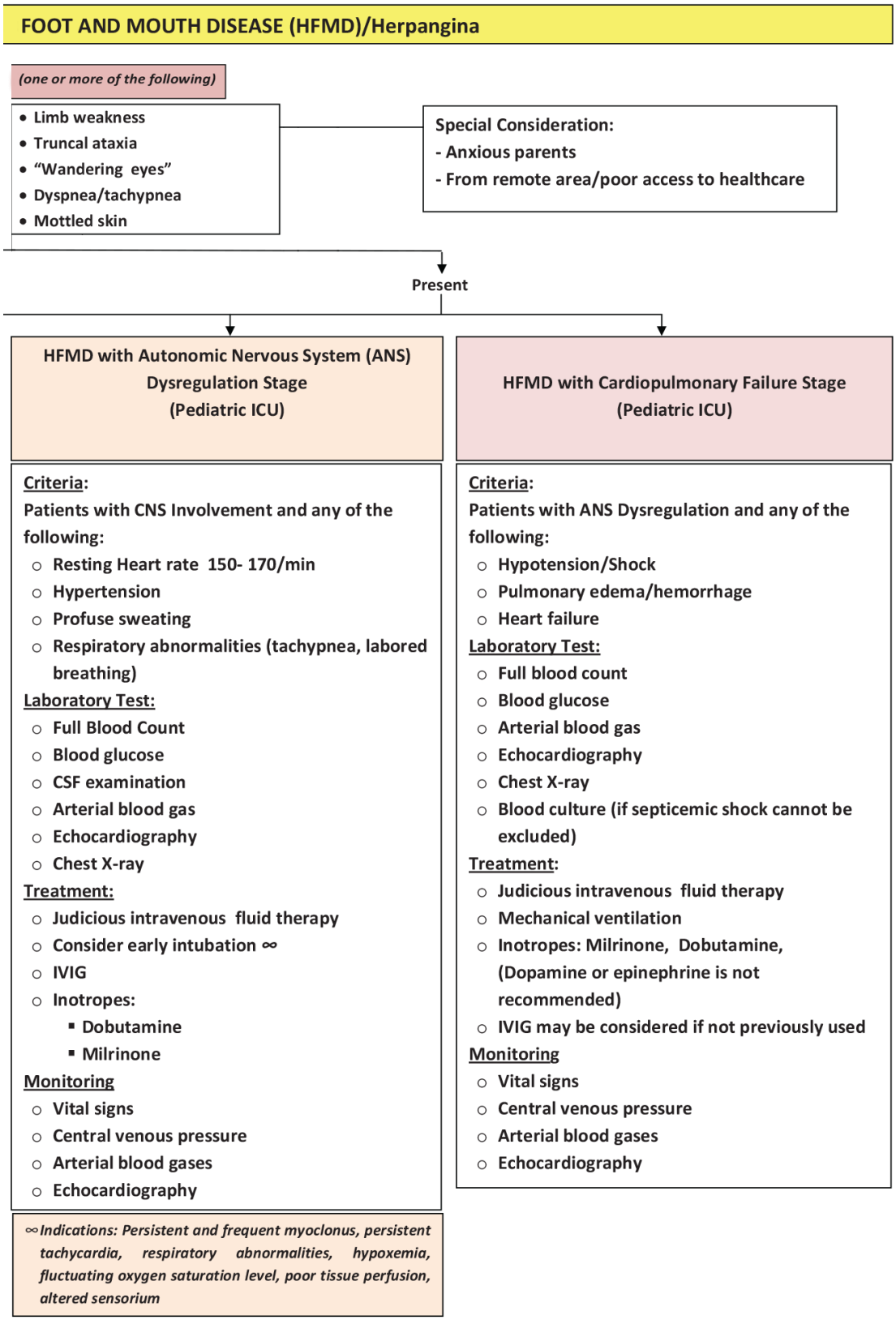


Figure 1.9. WHO guideline for diagnosis, assessment and management of HFMD.

Obtained from reference [40]


FOOT AND MOUTH DISEASE (HFMD)/Herpangina
(one or more of the following)
• Limb weakness
• Truncal ataxia
• "Wandering eyes"
• Dyspnea/tachypnea
• Mottled skin
Special Consideration:
- Anxious parents
- From remote area/poor access to healthcare
Present
HFMD with Autonomic Nervous System (ANS) Dysregulation Stage (Pediatric ICU)
HFMD with Cardiopulmonary Failure Stage (Pediatric ICU)
Criteria:
Patients with CNS Involvement and any of the following:
○ Resting Heart rate 150- 170/min
○ Hypertension
○ Profuse sweating
○ Respiratory abnormalities (tachypnea, labored breathing)
Laboratory Test:
○ Full Blood Count
○ Blood glucose
○ CSF examination
○ Arterial blood gas
○ Echocardiography
○ Chest X-ray
Treatment:
○ Judicious intravenous fluid therapy
○ Consider early intubation ∞
○ IVIG
○ Inotropes:
▪ Dobutamine
▪ Milrinone
Monitoring
○ Vital signs
○ Central venous pressure
○ Arterial blood gases
○ Echocardiography
∞ Indications: Persistent and frequent myoclonus, persistent tachycardia, respiratory abnormalities, hypoxemia, fluctuating oxygen saturation level, poor tissue perfusion, altered sensorium
Criteria:
Patients with ANS Dysregulation and any of the following:
○ Hypotension/Shock
○ Pulmonary edema/hemorrhage
○ Heart failure
Laboratory Test:
○ Full blood count
○ Blood glucose
○ Arterial blood gas
○ Echocardiography
○ Chest X-ray
○ Blood culture (if septicemic shock cannot be excluded)
Treatment:
○ Judicious intravenous fluid therapy
○ Mechanical ventilation
○ Inotropes: Milrinone, Dobutamine, (Dopamine or epinephrine is not recommended)
○ IVIG may be considered if not previously used
Monitoring
○ Vital signs
○ Central venous pressure
○ Arterial blood gases
○ Echocardiography

Classification	Signs/symptoms	Suggested Management
Grade 1	Oral ulcers and/or vesicular rash on the hands, feet, and/or the buttocks	OPD care, with advice sheet for family Careful observation for warning signs
Grade 2a	Grade 1 AND Myoclonic jerks observed by the family (not witnessed by medical staff) Legarthy, agitation/irritability Fever ≥ 39°C for ≥ 48 hours. Vomiting	Hospitalization Oral phenobarbitone Vital Signs Monitoring: every 4-6 hours following standard protocols
Grade 2b - Group 1	Grade 1 AND Myoclonic jerks witnessed by medical staff or by the family (≥ 2 jerks/30 minutes or 1 jerk and stupor) Resting pulse rate > 130/min but <150/min (adjusted for fever*)	Admit to HDU/PCIU IV Phenobarbitone Antipyretics Vital Signs Monitoring: every 1-3 hours for minimum 6 hours until stable
Grade 2b - Group2	Grade 1 AND witnessed myoclonic jerks accompanied by one of the following findings: Continuous limb tremor, limb weakness or paralysis, or drowsiness (provided no hypoglycemia) Resting pulse rate > 150 /min (adjusted for fever*) Fever ≥ 39.5 ^{0}C (rectal) and unresponsive to antipyretics > 4 hours	Give oxygen IV Phenobarbitone Antipyretics Start IVIG – 2g/kg in two divided doses Check: FBC, CRP, blood sugar, and consider lumbar puncture Vital Signs Monitoring: every 1-3 hours for > 6hrs
Grade 3	Serious complications in CNS or cardiopulmonary systems: Pulse >170 /min* Profuse sweating Stage 1 hypertension (SBP > 95th centile for age) Respiratory abnormalities: tachypnea, labored breathing Muscle hypertonia Coma (Glasgow coma score < 10)	Oxygenation Consider need for ventilation IV Phenobarbitone and IVIG Milrinone if stage 2 hypertension (SBP > 99th centile for age + 5 mm Hg) Dobutamine if HR> 170 bpm Consider additional fever control measures Invasive blood pressure monitoring Check: FBC, CRP, blood sugar, and consider lumbar Puncture Vital signs monitoring: every 30-60 mins for ≥ 6 hrs.

Grade 4	Severe complications: Acute pulmonary edema Cardiac collapse	SpO2 < 92% with cannula oxygen 6 litres/min) Respiratory arrest or gasping respiration Intubation and ventilation IV Phenobarbitone Dobutamine; Fluid challenge Antipyretics, Access CVC plus invasive BP monitoring Vital signs monitoring: every 15-30 mins for ≥ 6 hrs.

Figure 1.10. Vietnamese MoH HFMD Classification and Management Guidelines (March 2012)

Obtained from reference [122]

1.4.4. Management

Most HFMD cases are benign and self-limiting. Patients usually recover completely after a week without any specific treatment. The skin lesions heal without scarring. Dehydration due to poor intake because of the painful mouth lesions is the most common complaint in HFMD patients. Coinfection with bacteria is rare although coinfection with other enteroviruses has been reported frequently in endemic areas. About 10-30% HFMD cases admitted to hospital in Asia progressed to a more severe stage with neurological complications such as aseptic meningitis, encephalitis or acute flaccid paralysis [17,41,123]. Myoclonic jerks, tremor, ataxia, nerve palsies were observed in cases with neurological complications. The most severe type of HFMD involves cardiopulmonary distress which has high mortality and morbidity [124]. Fatal cases usually present with fever, less skin lesions and progress rapidly to severe stages, starting with poor peripheral perfusion, tachycardia, elevated blood

pressure and tachypnea. Myocardial dysfunction and pulmonary edema occur at the very last stage of the disease [123,125].

There is no specific antiviral treatment for HFMD to date. Most available treatment approaches are just supportive. As the disease progresses rapidly, the most crucial thing to reduce mortality and morbidity of HFMD is to train physicians how to assess patients and recognize warning signs as early as possible so that patients can be admitted and monitored closely.

Patients with uncomplicated HFMD can be treated at home with appropriate counselling to parents/care givers, on how to take care of the patients and how to recognize warning signs to come back to see the doctor immediately.

Patients with warning signs such as myoclonus, fever for more than 2 days, temperature higher than 39^0C, tremor, persistent vomiting (from grade 2) need to be admitted to hospital for closer monitoring and treatment. Depending on patients' condition, different treatment approaches are recommended by the guidelines such as antipyretics, anticonvulsants, sedation, intubation or inotropes [42].

Some treatment approaches are recommended in many countries for severe HFMD cases, although there is a lack of supportive evidence. These include intravenous immunoglobulin (IVIg), milrinone, magnesium sulphate and hemodialysis.

The use of IVIg was widely accepted in many countries during large HFMD outbreaks such as China, Malaysia, Taiwan and Korea [17,31,126–128] based on several theories. The first theory is that IVIg may help to neutralize enteroviruses, with

evidence from other small studies where IVIg was used as an additional therapy in treatment of neonates with enteroviral sepsis and X-linked agammaglobulinemia patients with chronic enteroviral infections of the central nervous system [129,130]. Viremia is however acute and transient in patients with most enteroviral infections, including HFMD. The second theory is the immunomodulatory ability of IVIg which may reduce the levels of "harmful" cytokines and chemokines such as interleukin 6 (IL-6), IL-10, IL-13, IL-8, interferon (IFN)-gamma-induced protein (IP)-10, monocyte chemoattractant protein (MCP)-1 and monokine induced by IFN-gamma (MIG). These substances are present with high levels in severe HFMD cases [131,132]. Wang et al., in a study in 2006, measured plasma cytokine concentrations before and within 12-24 hours after IVIg administration. Results showed the reduction of IFN-gamma, IL-6, IL-8, IL-10, and IL-13 levels in severe HFMD patients who received IVIg and suggested that IVIg may be considered in patients with EV-A71 brainstem encephalitis [133]. Studies in Malaysia and Taiwan demonstrated that early use of IVIg may stop disease progression and provide better outcomes [123,134]. However, as IVIg is expensive and also has side effects, the use of IVIG needs to be supported by well-designed randomized clinical trials.

For patients from grade 3 onward, the Vietnamese MoH guidelines recommend the use of milrinone, a phosphodiesterase 3 inhibitor which can decrease sympathetic tachycardia, reduce levels of IL-13 and improved outcome of patients in a Taiwanese study [135]. A randomized control trial in Children's Hospital 1, Ho Chi Minh City, including 41 patients with pulmonary edema and/or neurogenic shock (grade 3) showed that milrinone could reduce mortality rate in the first week (18.2% (4/22) in

the milrinone compared with 57.9% (11/19) in the control group) [136]. Pure inotropes like dopamine or epinephrine are associated with worse outcome, while milrinone have both inotropic and vasodilator properties [137,138].

Magnesium sulphate is another potential treatment approach for severe HFMD cases with ANS dysregulation, with positive results when used empirically in severe EV-A71 HFMD cases in Vietnam. A clinical trial has been completed to prove the clinical effects of this treatment and results are awaited [139] .

For critically ill patients, a last resort approach applied in some countries, including Vietnam is hemodialysis, which had positive effects on clinical outcomes and cytokine level reduction [140]. However, more data need to be collected to prove the real effect of this method.

1.4.5. Antiviral agents

There is no licensed antiviral agent for EV-A71 treatment available until now although numerous have been tested since the 1990s, targeting different stages of the viral life circle [141]. Most of these were only tested in vitro, using various cell types. Vapendavir, a novel enteroviral protease inhibitor (PI) SG85 and capsid binder, showed potential ability to inhibit the replication of 21 subgenogroups (including genogroups A, B and C) of EV-A71 in vitro and currently under clinical trial of rhinovirus treatment but not yet underwent in vivo test against EV-A71 [142]. Only bovine lactoferrin, pleconaril, shRNA, siRNA, rupintrivir, ribavirin and 17-AAG have been tested *in vivo* [143–148], but none have shown benefit in clinical trials.

1.4.6. Vaccines

Similar to other viral infections, without specific antiviral agents, the most effective way to control HFMD is vaccination. Five inactivated EV-A71 vaccines have been developed and tested since 2010 [149] in Taiwan, mainland China and Singapore. Taiwan tested the first EV-A71 (B4 strain) vaccine on human subjects. A single dose of a 5 or 10 μg antigen could induce a 4-fold or greater increase in neutralization antibody titers in healthy adult volunteers [150] and produced cross-neutralizing antibodies at different levels against B1, B5 and C4 subgenogroups [151]. Singapore also reported positive results from a phase I trial of an EV-A71 B2 vaccine. In 2013, China introduced three EV-A71 C4 vaccines developed independently by different companies. All these were evaluated in phase 3 trials and were reported to be safe and effective with protection rates against HFMD of >90% and against other EV-A71 associated diseases (including severe disease) of >80%). Cross-neutralizing activity against B4, B5, C2, C5 was also stronger than with the B2-based vaccine [149]. However, like the vaccines in Taiwan and Singapore, the C4-bases Chinese vaccines could not protect against HFMD caused by CV-A16 [66,149,151]. Two among three Chinese vaccines have been licensed for commercial use in China in 2015 and 2016 [66]. However, due to the lack of ability of those vaccines to protect against other enteroviruses, associated with severe HFMD there remains a need to develop bivalent or multivalent vaccines to control (severe) HFMD.

1.5. RISK FACTORS FOR SEVERITY

1.5.1. Risk factors for progression/severity

Although the majority of HFMD patients recover completely without any specific treatment, a small proportion of patients progress to more severe stages of the disease with neurological involvement and/or further cardiopulmonary distress [152]. Children with severe disease usually present with subtle early warning signs/symptoms before deteriorating rapidly to more severe disease [42]. Many signs/symptoms have been proposed as risk factors for severity in different studies. Ooi et al. described a duration of fever ≥ 3 days (P = <0.0001, Odds ratio (OR): 6.52, 95% confidence interval (CI): 2.83 – 14.99), high fever (temperature ≥ 38.5 °C) (P <0.0192, OR: 2.27, 95%CI: 1.14 – 4.51) and lethargy (P <0.001, OR: 3.18, 95%CI: 1.60 – 6.335) as three independent risk factors of central nervous system (CNS) involvement [123]. These factors were confirmed by He and Chang in studies in Taiwan [103,120,153]. Myoclonal jerks, recurrent vomiting and limb weakness were also considered predictors for neurological complications [103,152,154]. The type and position of skin lesions and mouth ulcers were reported in some studies to be associated with severe HFMD [121]. The presence of a maculopapular rash and lack of mouth ulcers appeared to be risk factor for severity in some studies in mainland China and Taiwan [121,155,156]. However, a meta-analysis from 2014, using data from 19 HFMD studies, showed no association between skin/mouth lesions and disease progression. Other factors which increased the risk of progression included young age, raised white blood cell count and EV-A71 infection [153].

However, most patients with CNS involvement do not progress further to develop cardiopulmonary distress. Only children with ANS symptoms such as mottled skin, sweating, tachycardia, tachypnea, hypertension and hyperglycemia were more likely to progress to fulminant cardiac distress and pulmonary edema [157]. Two other clinical / laboratory markers which may be reliable parameters in predicting severe cases were heart rate variability (HRV) and troponin I levels. According to those studies, severe patients had extremely low HRV compared with non-severe patients [158] and a raised troponin I result was associated with an increased risk of progression. However, those two parameters were costly and in many cases, could not be used routinely in the resource limited clinical settings where HFMD is hyperendemic [42].

1.5.2. Risk factors for mortality

Some studies have suggested that inappropriate treatment could increase the risk of mortality [120]. The regionally common types of treatment of infectious diseases including antibiotics, glucocorticoids, and antipyretics were evaluated to see if these could affect outcome. Antibiotics and different antipyretics showed no effect on patient's outcome while glucocorticoids, usually prescribed to reduce the inflammatory response, was reported to increase the risk for fatality of HFMD patients significantly [25,155,159]. Studies from China have also suggested first admission to a low level commune hospital is a risk factor for mortality [25]. However, because of the variety of health care system settings and capacity in different countries, more data from other countries are needed to confirm this finding.

1.6. HOST GENETIC STUDIES

1.6.1. Host factors

1.6.1.1. Virus receptors

Viruses use receptors to attach to host cells to uncoat the virion and release RNA into the host cell cytoplasm. Some EV-A71 receptors have been identified, including human scavenger receptor B2 (SCARB2), human P-selectin glycoprotein ligand 1 (PSGL-1), dendritic cell specific intercellular adhesion molecule-3 grabbing nonintegrin (DC-SIGN), annexin A2 (Anx2), heparan sulfate (HS), and sialylated glycans [160–165], of which SCARB2 and PSGL-1 are specific EV-A71 receptors. SCARB2, also known as lysosomal integral membrane protein II, LGP85, or CD36b like-2, is ubiquitously expressed in many human tissues and cell types, mostly located in the lysosomal membrane. SCARB2 participates in membrane transportation, reorganization of the endosomal/lysosomal compartment and mediation of β-glucocerebrosidase delivery from the endoplasmic reticulum to lysosomes [70]. SCARB2 can also shuttle between the lysosomal compartments and plasma membrane, where it is an EV-A71 receptor. Yamayoshi et al. showed that SCARB2 on the cell surface can bind to EV-A71, forming the SCARB2 - EV-A71 complex. This complex help EV-A71 to internalize [160,166].

PSGL-1 is a sialomucin-like leukocyte membrane protein. It plays an important role in gathering and rolling leukocytes from blood vessels to affected sites during acute infection [161]. T-cells expressing PSGL-1 are susceptible to EV-A71 infection and after attachment, viral particles can be internalized by a caveolin-dependent pathway

[162]. PSGL-1 is expressed predominantly on leukocytes, and does not mediate entry into non-leukocyte cells. According to Nishimura et al., based on sequencing data, about 80% of EV-A71 isolates did not bind to PSGL-1 and thus EV-A71 can be classified into PSGL-1-binding (PB) and non-binding strains (non-PB) [167]. The receptor spectrum of EV-A71 may be associated with clinical outcome and brainstem invasion or cardiopulmonary edema though direct viral invasion.

1.6.1.2. Immune response

Few studies have been conducted to investigate the host immune response to EV-A71 infection. Inflammatory mediators were shown to play roles in the pathogenesis of EV-A71 associated severe HFMD, especially pulmonary edema [168]. High level of interleukin 6 (IL-6) and interferon gamma were found in CSF samples of patients with pulmonary edema in studies in Taiwan [169,170]. Taiwanese studies also reported raised levels of IL-6 (especially in the first 2 days) [170], tumor necrosis factor alpha (TNF-a) and IL-10, IL-13 in plasma of patients with pulmonary edema. Those cytokines were thought to increase the vascular permeability, and hence contribute to pulmonary edema [131,132]. Some chemokines, including monocyte chemoattractant protein (MCP-1), IL-8 and monokine induced by interferon gamma (MIG) were reported to be present at high levels in plasma of patients with brainstem encephalitis [132].

In terms of the cellular immune response, HFMD patients with pulmonary edema presented with low lymphocyte counts, particularly low CD4, CD8 and natural killer cells (NK) [171]. A reduction in interferon gamma (IFN-g), IL-1 beta, IL-6 in

stimulated peripheral mononuclear cell of patients with pulmonary edema was also reported by Chang et al. in the same study [171]. Although a clear mechanism of severe HFMD, particularly pulmonary edema is still under research, the development of pulmonary edema in severe EV-A71 associated HFMD is believed to be strongly related to dysregulation of the systemic and CNS inflammatory response [100].

In terms of humoral immunity, enterovirus infections generally result in immunoglobulin M (IgM), IgA and IgG responses that can be long lasting. As shown in vaccine trials and serological studies, cross neutralization between enterovirus serotypes, especially between EV-A71 and other EV-A serotypes, does not occur. Therefore, patients infected with one serotype may have a second episode of HFMD if subsequently infected with other serotypes. Consequently, the implementation a monovalent EV-A71 vaccine may be able to reduce the number of severe HFMD cases. However, multivalent vaccines would be required to fully control HFMD. This is highly challenging as over 20 enterovirus serotypes can cause HFMD.

1.6.2. Host genetics

The outcome of a particular infectious disease is a result of a complex interaction between the pathogens and the hosts [172]. The most crucial purpose of host genetic analysis is to identify genetic risk factors for a particular disease. There are many technologies and tools to investigate this host-pathogen interaction, including candidate gene studies, genetic linkage studies/genome wide linkage studies, genome-wide association study (GWAS) and Exome / whole genome sequencing.

1.6.2.1. Candidate gene study

Candidate gene studies were used as the main genetic approach before the genome-wide association studies era [173]. Here, selected genes are tested for an association with a particular trait of a disease. Candidate genes are chosen based on known biological characteristics of the disease or results from previous laboratory or animal studies. Therefore, this method is appropriate for diseases which mechanisms are well - understood. One example of this approach was the identification of PSEN, APP and PSEN2 mutations in Alzheimer's disease [174]. However, as there are up to 30.000 genes in the human genome and the complex mechanism of disease, in most cases, it is unlikely that one gene or a group of genes could contribute to a particular phenotype and the chance of a randomly selected gene could influence a trait is extremely low [175].

1.6.2.2. Genetic linkage study

Genetic linkage studies identify and map loci (regions of the genome) by observing related individuals [176]. Using panels of microsatellite markers spaced at intervals of approximately 10 cM across the genome, genetic linkage studies scan for marker alleles correlated with a phenotype within a pedigree [177]. This method can be applied in both major gene disorders and complex diseases. However, based on the concept that different alleles can be connected with the trait in different pedigrees [176], this kind of study only scan search for markers within families and therefore is not suitable to be applied for common diseases in a larger populations.

1.6.2.3. Genome-wide association study (GWAS)

The principle of genome-wide association studies (GWAS) is similar to genetic linkage, in which both types of studies aim to detect the association between one or more genetic polymorphisms and a trait. The difference between GWAS and genetic - linkage is that GWAS assumes that human populations share common ancestry so if an allele is associated with a trait, this association will happen in the whole population [178]. GWAS screens across the human genome to measure and analyze the DNA sequence variations, and tries to detect genetic risk factors (if any) of a common disease in the population [179]. The most common study design of GWAS is a case-control study, which compares the frequencies of polymorphisms between cases and controls. With the development of new BeadChips, which allow to scan for hundreds of thousands of single nucleotide polymorphisms (SNPs) in a short time, GWAS, particularly case-control studies have become a widely accepted approach for both rare and more common diseases [172,178,179]. However, this type of study also has limitations due to the multiple comparisons, leading to false positive results. Therefore, GWAS usually requires replication and validation phases to confirm the findings.

1.6.2.4. Whole genome sequencing and exome sequencing

Whole genome sequencing is one of the most advanced genetic technique to identify genetic variants that affect heritable phenotypes, including important disease-causing mutations. However, the human genome is large and complicated, and completion took more than 200 scientists over a decade in a project that cost almost

$3 billion to complete [180] (1990 – 2003). Today, advances in next generation sequencing technologies makes whole human genome sequencing more straightforward, quick and much cheaper ($US 1000 per genome). However, it remains relatively expensive and data management / bioinformatics may be challenging for host genetic associated studies.

Exome sequencing is a method to solve the cost- and time-consuming problems of whole-genome sequencing. The exome only accounts for about 1% of the human genome but carries about 85% of disease associated genetic variants. Since the first clinical success in diagnosing a causal variant of a rare form of inflammatory bowel disease in an infant [181], exome sequencing has been used more widely to diagnose novel diseases and find causative mutations for known disease phenotypes.

1.6.2.5. Genetic studies in HFMD

Very few studies have been conducted to study the host genetic factors associated with severe HFMD. A study in Taiwan in 2001 showed that a polymorphism of the cytotoxic T lymphocyte antigen–4 (CTLA-4) at position 49 of exon 1 had a higher frequency of the G/G genotype in patients with EV-A71 associated meningoencephalitis than in those without (18/31 vs. 14/47; P = 0.045) and non-HFMD controls (18/31 vs. 25/93l; P = 0.007) [182]. Chang et al., in a study in 2013, collecting data from 219 EV-A71 associated HFMD patients and 97 control cases in Taiwan, performed HLA-typing. The results showed that HLA-33 was significantly associated with EV-A71 susceptibility. HLA-33 is more often found in Asian than white-Caucasian populations. Chang and his team, therefore suggested it might be a

reason explaining why EV-A71 associated HFMD occurred more frequently in Asian countries. HLA-A2, in the same study, was also reported to be associated with cardiopulmonary failure [183].

In recent years, China has led the field with a series of studies about host factors of susceptibility and severe HFMD, focusing on the association of known polymorphisms in genes coding for cytokines and chemokines. A case-control study in China, including 186 EV-A71 HFMD patients and 233 controls, found three SNPs: the G allele (genotypes AG or GG) in the *CCL2–2518A/G,* the A allele (genotypes AA or AG) in the CXCL10−201A/G and the C allele (genotypes CC or CT) in the IL8+781C/T associated with severe HFMD in the Chinese population [184]. Another study targeted different polymorphisms such as interferon gamma (IFN-γ)+874 T/A, interleukin 10 (IL-10)−1082 G/A [185] and chemokine (C–C motif) ligand 2 (CCL2-2510A/G) [186].

In terms of GWAS, Zou et al., in a study in 2016, showed that the GG genotype of promoter variant rs2843710 was associated with susceptibility and severity of EV-A71 infection [187]. This variant is a polymorphisms of the type I IFN signaling pathway genes such as interferon-induced GTP-binding protein Mx (MX1) and oligoadenylate synthestase (OAS1), which were already described to be linked with susceptibility and severity of HFMD [188,189]. However, the P value in this study was still far from the usually accepted P value of gene-association studies, i.e. 10^{-8} [190] and hence this finding could still be an artifact of multiple comparisons.

Another group of Chinese scientists also reported that the OAS3 rs1859330 G/A genetic polymorphism may be associated with severe HFMD. It is the coding gene for the 2'5'-oligoadenylate synthetase (OAS), an interferon (IFN)-induced protein, that plays an important role in the antiviral action of IFN [191]. Similarly to Zou's study, this study also generated a P value which was not significant enough for a genome - wide association result.

Collectively, (EV-A71 associated) HFMD has emerged, has become endemic and has become one of the most important public health issues in Vietnam and the Asia-Pacific region over the last decades. There have been many studies in the region and in Vietnam, yet, several questions remain unanswered about the epidemiology, virology, host genetics and clinical management of this important infection. A better understanding of these components of this emerging infection is essential for the region and, with the pandemic potential of HFMD and EV-A71, at a global level. Therefore a comprehensive research project was conducted, aiming at filling knowledge gaps about epidemiology, virology, clinical, and host genetics of HFMD in Vietnam.

1.7. HYPOTHESIS OF THE STUDY

- There might be multiple HFMD pathogens co-circulating in Southern Vietnam between 2013 and 2015 and clinical phenotypes might vary according to pathogens.
- There might be a replacement of EVA71 subgenogroups during the study period.
- There might be specific host genetic markers associated with severe HFMD.

- The epidemiology and clinical phenotypes of HFMD between 2009 and 2015 might be different.

1.8. AIMS OF THE STUDY

- To prospectively identify the circulating pathogens in southern Vietnam and their related clinical manifestations and risk factors for severity between 2013 and 2015 in the entire illness severity spectrum.
- To retrospectively describe the clinical presentation, epidemiology and management of HFMD from 2009-2013 in Children's Hospital 2 and compare these between different years: before, during and after the 2011-12 outbreak.
- To describe the evolution of EV-A71 in southern Vietnam between 2013 and 2015.
- To identify host genetic markers associated with severe HFMD.

2. CLINICAL AND AETIOLOGICAL STUDY OF HAND, FOOT AND MOUTH DISEASE IN IN-AND OUTPATIENTS IN SOUTHERN VIETNAM, 2013-2015

2.1. INTRODUCTION

In Vietnam, EV-A71 was first detected in 2003. In 2005 the first outbreak of HFMD was reported, of which while CV-A16 and EV-A71 were the leading causes, EV-A71 was responsible for cases with neurological complications [41]. Since then increasing numbers of severe cases of HFMD have occurred. In 2008, HFMD became a notifiable disease and on average 10,000 cases were recorded annually between 2008 and 2010. In 2011–2012, there was a dramatic increase in hospitalized and fatal cases with a total of over 200,000 children admitted, and more than 200 deaths [43]. HFMD currently occurs throughout the country but the highest burden of disease is recorded in the southern provinces in terms of absolute numbers and proportion of severe and fatal cases [43].

Better understanding of the underlying etiology of HFMD is needed, but there are few data on the current situation in Vietnam. Most published previous work has focused on hospitalized patients with EV-A71-associated disease and the epidemiology and etiology of milder disease is poorly characterized. However, in recent years, clinical and national surveillance data from the region have shown the

emergence of other EV-A serotypes (including CV-A6, CV-A10) as the cause of hospitalized cases of HFMD including cases of severe disease [19,96].

This chapter presents a detailed characterization of HFMD in southern Vietnam, focusing on the etiologic agents and the associated epidemiological and clinical characteristics in southern provinces between July 2013 and July 2015. Hitherto, most studies have only focused on severe disease and inpatients; here my team and I enrolled >1500 patients covering a wider spectrum of severity, including outpatients.

2.2. MATERIALS AND METHODS

2.2.1. Study setting

A prospective observational study was conducted at outpatient clinics, clinical wards and Pediatric Intensive Care Units of three tertiary referral hospitals for the southern provinces of Vietnam: Children's Hospital 1, Children's Hospital 2 and the Hospital for Tropical Diseases, all located in Ho Chi Minh City.

2.2.2. Patient enrolment

All patients presenting to the three study hospitals between July 2013 and July 2015 with a clinical diagnosis of HFMD according to the guidelines of the Vietnamese Ministry of Health [122] were eligible to participate in the study.

2.2.3. HFMD case classification

Patients were classified as severe or non-severe using a grading system issued by the Vietnamese Ministry of Health [124]. These guidelines were based on guidelines from the World Health Organization (WHO) [42] but provide more detailed staging useful for diagnosis and treatment, and is more applicable to clinical practice in Vietnam. In this study severe HFMD was defined as grade 2B or higher (Figure 1.10).

2.2.4. Clinical data and samples

Demographic and clinical data were recorded for all patients on enrolment to the study and daily until discharge or day 7 for inpatients (whichever sooner). For outpatients similar data were collected by telephone on days 2, 4 and 6. Inpatients who were discharged before day 7 and all outpatients returned for a follow-up visit between days 7 and 14 after enrolment.

Rectal and throat swabs were collected at enrolment in addition to baseline hematology and biochemistry data obtained as part of routine care. An additional blood sample was collected at the follow-up visit.

2.2.5. Enterovirus detection and serotype determination

2.2.5.1. Viral RNA extraction

Viral RNA was extracted from clinical specimens (either throat or rectal swabs, Figure 2.1) using QIAamp viral RNA Mini kit (QIAgen GmbH, Hilden, Germany), following manufacturer's instructions. In short, 140 µl of clinical samples were first

mixed with 20 µl of equine arteritis virus (EAV) functioning as an internal control [192,193], the obtained mixture was then lysed using 560 µl of AVL buffer containing carrier RNA (provided with the kit) at room temperature for 10 minutes to inactivate RNases and to ensure isolation of intact viral RNA. Subsequently, 560 µl of absolute ethanol was added to the extraction tube and the mixture was then thoroughly mixed using vortex. Next, the sample was loaded onto the QIAamp Mini spin column (provided with the kit), followed by a centrifugation step to capture viral nucleic acid on the silica membrane of the spin column and to get rid of contaminating protein. In the next step, contaminants were further washed away using 500 µl of washing buffets AW1 and AW2, respectively (provided in the kit). Finally, viral RNA was recovered in 60 µl of buffet AVE (provided with the kit), and stored at -80 ^{0}C until used.

2.2.5.2. Multiplex real-time RT-PCR for detection EVs and EV-A71

2.2.5.2.1. Primers and probes

Primers and probes used for simultaneous detection of EVs and EV-A71 were derived from a previous method paper published by my research group at OUCRU [194], and are listed in Table 2.1.

2.2.5.2.2. Multiplex real-time RT-PCR procedure.

Multiplex real-time RT-PCR was carried out as described elsewhere [194] using the SuperScriptR III One-Step qRT-PCR System with PlatinumR Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA). Each PCR reaction has a total volume of 25 µl

consisting of 12.5 µl of 2X RT-PCR reaction mix (provided with the kit), 0.5 µl enzyme Mix (provided with the kit), primers and probes at appropriate concentrations (1 µl each, table 2.1) and 5 µl isolated RNA sample. The PCR reaction was then performed in a LightCycler 480 II platform (Roche Diagnostics GmbH, Mannheim, Germany) utilizing a thermal condition consisting of one cycle of 60 ^{0}C for 3 minutes, followed by 15 minutes at 53 ^{0}C and 2 minutes at 95 ^{0}C, and 45 cycles of 15 seconds at 95 ^{0}C, 1 minutes at 53 ^{0}C (including fluorescence acquisition) and 15 seconds at 72 ^{0}C.

Name	Sequence (5'→3')	Final concentration	Postition	Note
EAV-F primer	CATCTCTTGCTTTGCTCCTTAG	400 nM	1847–1868	Internal control [193]
EAV-R primer	AGCCGCACCTTCACATTG	400 nM	1980–1997	
EAV-probe	FAM-CGCTGTCAGAACAACATTATTGCCCAC-BHQ1	100 nM	1926–1964	
ENT-F	CCCTGAATGCGGCTAAT	400 nM	452 - 468	Enterovirus specific primers and probe [195]
ENT-R	ATTGTCACCATAAGCAGCC	400 nM	579 - 597	
ENTr-probe	Cy5-ACCCAAAGTAGTCGGTTCCG-BHQ3	200 nM	531 - 551	
EV-A71-634 F	GGAGAACACAARCARGAGAAAGA	400 nM	634 - 656	Enterovirus 71 specific primers and probe [43]
EV-A71-743R	ACYAAAGGGTACTTGGAYTTVGA	400 nM	721 - 743	
EV-A71-probe	Cyan500-TGATGGGCACDTTCTCRGTGCG-BHQ1	40 nM	686 - 707	

(FAM = Carboxyfluorescein; Cy5 = Cyanine 5; Cyan500 = Cyan 500 NHS ester; BHQ = black hole quencher; R = A and G; Y = T and C; V = A, C, and G; D = A, G, and T)

Table 2.1. Primer and probe sequences used in multiplex real time RT-PCR for Enterovirus and Enterovirus A71 detection.

Obtained from reference [194].

2.2.5.2.3. Multiplex real time RT-PCR result interpretation.

A sample was positive if negative controls were all negative and tested sample had a crossing point (Cp) value equal or less than 40. A sample was negative if negative controls were all negative, internal controls showed a Cp value in the range from 32 to 35, the positive control had a Cp value of about 30 and tested sample had Cp value >40 or no amplification signal. A sample was considered as inconclusive if any negative control was positive and/or the internal control showed a Cp value >35, which required the experiment to be repeated.

For each patient, a real-time RT-PCR on throat swab was performed first. If throat swab was positive, the patient was concluded to have enterovirus infection. If the real-time RT-PCR result of throat swab was negative, the rectal swab was tested. If rectal swab's result was positive, patient was enterovirus PCR positive. Otherwise, the patient was enterovirus PCR negative (Figure 2.1).

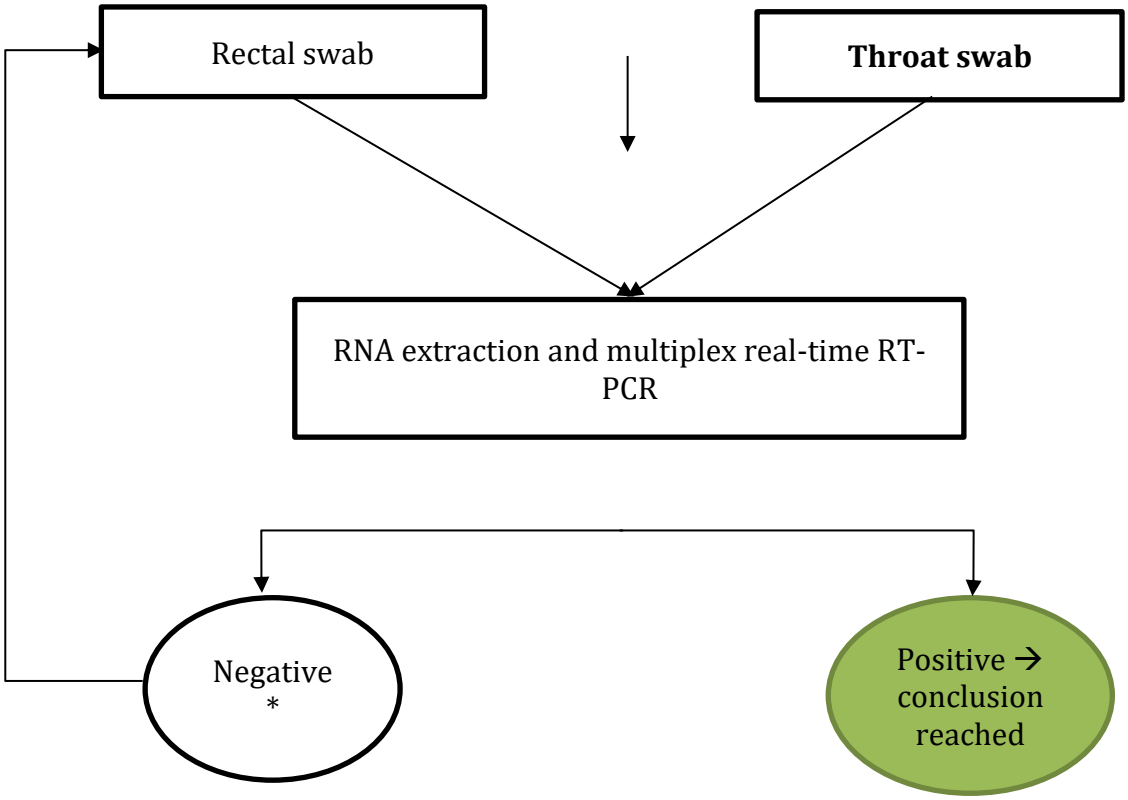


Figure 2.1. Flow chart showing the workflow for real time RT-PCR testing of clinical samples

*The analysis is ended when real time RT-PCR of rectal swab is completed.

Obtained from reference [194]

2.2.5.3. Enterovirus serotype determination

For enterovirus serotype determination, all samples that were EVs real time RT-PCR positive but EV-A71 real time RT-PCR negative by the multiplex real-time RT-PCR were subjected to an additional serotyping step using a VP1-based approach. The workflow included two rounds of PCR amplification prior to a sequencing step of the obtain PCR amplicons. The PCR reactions were performed using SuperSript OneStep RT-PCR Platinum (Invitrogen), and a previously published set of VP1 primers by Nix et al in 2006 [196] (Table 2.2).

2.2.5.3.1. First round PCR

First round PCR was carried out using 10 µl of 2X reaction Mix (included in the SuperSript kit), 0.5 µl of enzyme mix, 1 µl of primer mix (as showed in Table 2.2), 5 µl of RNA and molecular grade water (to achieve a final reaction volume of total 20 µl). The thermal cycling condition consisted of a reverse transcription step which included one cycle at 55 ^{0}C for 3 minutes, 22 ^{0}C for 10 minutes, and 42 ^{0}C for 30 minutes, and an amplification step at 95 ^{0}C for 1 minute followed by 40 cycles of 94 ^{0}C for 30 seconds, 42 ^{0}C for 30 seconds and 60 ^{0}C for 1 minute, and final extension at 72 ^{0}C for 7 minutes.

2.2.5.3.2. Second round PCR

The second round PCR reaction was performed in a final reaction volume of 25 µl consisting of 23 µl of Platinum Super mix (Invitrogen), 0.5 µl of each of the AN89 and AN88 primers (Table 2.2), and 1 µl of the first round RT-PCR product. The

thermal cycling condition for the 2nd PCR was as follow: a denaturation step at 95^{0}C for 1 minute followed by 40 cycles of 94 ^{0}C for 20 seconds, 55 ^{0}C for 30 seconds, and 72 ^{0}C for 20 seconds, and a final extension step at 72 ^{0}C for 7 minutes.

PCR products with an expected size of ~320bp were then analyzed on 2% agarose gels. Finally, the amplified products were purified using cold ethanol based precipitation approach and subjected to the sequencing step (see below).

Name	Sequence (5'→3')	Working concentration (uM)	Position	Note
SO224	GCIATGYTIGGIACICAYRT	A mixture of these primers was made to contain a concentration of 10ug/ml of each **(Primer mix)**	1977 - 1996	1st round RT-PCR
SO222	CICCIGGIGGIAYRWACAT		2969 - 2951	1st round RT-PCR
AN32	GTYTGCCA		3009 - 3002	1st round RT-PCR
AN33	GAYTGCCA		3009 - 3002	1st round RT-PCR
AN34	CCRTCRTA		3111 - 3104	1st round RT-PCR
AN35	RCTYTGCCA		3009 - 3002	1st round RT-PCR
AN89	CCAGCACTGACAGCAGYNGARAYNGG	10	2602 - 2627	2nd round PCR (forward)
AN88	TACTGGACCACCTGGNGGNAYRWACAT	10	2977 - 2951	2nd round PCR (reverse)
AN232	CCAGCACTGACAGCA	10	2602 - 2616	Sequencing
AN233	TACTGGACCACCTGG	10	2977 - 2963	Sequencing

Table 2.2. Primers and probes used for serotyping PCR and sequencing [196]

2.2.5.3.3. Sequencing

Purified PCR products were sequenced using AN232 and AN233 primers (Table 2.2) and the BigDye Terminator V3.1 Cycle Sequencing kit (Applied Biosystems, Carlsbad, CA, USA) in an ABI 3130XL DNA sequencer (Applied Biosystems). The sequencing reaction contained 4 µl of sequencing mix (Applied Biosystem), 2µl of 5X buffer (provided with the kit), 8ul of water, 1µl of primer (either AN232 or AN233), and 5 µl of purified DNA. The thermal cycling condition consisted of one cycle at 95 ^{0}C for 1 minute followed by 30 cycles at 94 ^{0}C for 20 seconds, 60 ^{0}C for 2 minutes. Products were purified using cold ethanol based procedure. In brief, 4 µl of STOP solution (containing 2 volume of home-brewed EDTA, 2 volume of home-brewed Sodium Acetate, and 1 volume of Glycogen (Roche Diagnostics GmbH, Mannheim, Germany)) was added to each sequencing reaction tube, followed by 60 µl of absolute ethanol previously stored at -20 ^{0}C. The mixture was thoroughly mixed and was then transferred to a 1.5ml Eppendorf tube for a subsequent precipitation step by centrifugation, and two washing steps with 30 µl of ethanol 70%. Finally, the pellets containing dye-terminated sequences were vacuum dried for 25 minutes, re-suspended in 25 µl of Hi-Di solution (Applied Biosystems), and sequenced in an ABI 3130XL DNA sequencer (Applied Biosystems).

The obtained VP1 sequencing reads were first assembled and then manually edited using Contig Express (a component of Vector NTI Suit 7 (Informax Inc., NY, USA). Finally, the sequences were analysed using an online EVs typing tool available at *http://www.rivm.nl/mpf/enterovirus/typingtool/* to determine specific enterovirus serotype [197].

2.2.6. Statistical analysis

Data were analysed with R 3.2.5 [198]. Continuous variables were expressed using median and interquartile range (IQR), categorical variables using frequency. Fisher's exact test with p-value by Monte Carlo simulation, t-test, Kruskal - Wallis and independent test were used to test categorical, continuous (normal distribution), continuous (skewed) and categorical variables with ordinal variables, respectively. Multiple comparisons were adjusted using Holm - Bonferroni correction. To determine factors associated with disease progression, all factors with p-value <0.05 were tested in a multivariate regression model.

2.2.7. Ethics

The study was approved by the Oxford Tropical Research Ethics Committee (OxTREC) and by the Institutional Review Boards of all enrolling hospitals. Parents or guardians of children all gave informed consent to their child participating in the study.

2.3. RESULTS

2.3.1. Patients and characteristics

Between July 2013 and July 2015 a total of 1547 patients were enrolled into the study. Baseline characteristics are given in Table 1. Patients were enrolled relatively early after onset of symptoms (median 1 day, IQR: 1 – 2). The median age of children was 21.2 months (IQR: 12.4 – 25.7; actual age range 6.8 – 51). 1423 (92%) patients had mild HFMD (grade 1 or 2A) on admission/ enrolment and 1355 (87.6%)

patients were classified with mild disease at discharge, with 119 patients progressing to a higher grade during hospitalization (Tables 2.3 and 2.4)

	Total (n=1547)	Outpatient (n=590)	Inpatient (n=957)	P value
Demographics				
Gender (male)	933 (61.3)	344 (58.3)	589 (61.5)	0.2
Age (months)	21.2 (12.4 – 25.7)	20.2 (13.2 – 30.3)	16.8 (12.1 – 24)	***
Living in Ho Chi Minh City	893 (57.7)	357 (60.5)	536 (56)	0.08
Illness day on admission (days)	1.4 (1 – 2)	1.2 (1 – 2)	1.5 (1 – 2)	***
Duration of hospital stay (days)	4.1 (2 – 5)	NA	4.1 (2 – 5)	NA
Clinical signs and symptoms				
Fever	1311 (73.2)	377 (63.9)	755 (78.9)	***
Rash	1310 (85.6)	541 (91.7)	769 (82.3)	***
Type of rash				*
Mostly vesicular	63 (4.9)	46 (8.5)	17 (2.2)	*
Mostly macular	601 (46.2)	215 (39.7)	386 (50.9)	
Both	636 (48.9)	281(51.8)	355 (46.8)	
Mouth lesion	1345 (88.2)	506 (85.8)	839 (89.7)	*
Lips	64 (5)	61 (10.3)	3 (0.4)	***
Tongue	512 (39.9)	188 (31.9)	324 (46.8)	***
Palate	959 (74.6)	434 (73.6)	525 (75.8)	0.5
Buccal	256 (20)	201(34.1)	55 (7.9)	***
Headache	22 (1.9)	19 (5.5)	3 (0.4)	***
Cough	347 (22.5)	207 (35.1)	140 (14.7)	***
Runny nose	326 (21.2)	214 (36.3)	112 (11.8)	***
Conjunctivitis	17 (1.1)	12 (2)	5 (0.5)	*
Vomiting	331 (21.4)	145 (24.6)	186 (19.5)	*
Diarrhoea	86 (5.6)	33 (5.6)	53 (5.6)	1
Drowsiness	35 (2.3)	9 (1.5)	26 (2.8)	0.1
Irritability	213 (13.8)	145 (24.6)	68 (7.2)	***
Myoclonus	261 16.9)	64 (10.8)	197 (20.6)	***
Sweating	13 (0.84)	6 (1)	7 (0.7)	0.5
Lethargy	12 (0.8)	2(0.3)	10 (1.1)	0.1

Ataxia	4 (0.4)	0	4 (0.4%)	1
Tremor	30 (3.1)	0	30 (3.2)	1
Nystagmus	3 (0.3)	0	3 (0.3)	1
Limb weakness	5 (0.5)	0	5 (0.5)	1
Temperature (°C)	37.5 (35.6 – 38.2)	37 (36.9 – 37.5)	38 (37.4 – 38.5)	***
Pulse (bpm)	120 (110 – 130)	110 (100 – 110)	124 (120 – 130)	***
Systolic blood pressure (mmHg)	90 (85 – 90)	90 (80 – 90)	90 (90 – 97)	***
Diastolic blood pressure (mmHg)	55 (50 – 60)	50 (50 – 60)	60 (55 – 60)	***
Body mass index	17.5 (15.6 – 18.6)	17.7 (16 – 18.7)	17.4 (15.5 – 18.5)	0.2
Blood results				
Cell count (10^9/mL)	12.5 (9.7 – 15.9)	11 (8.8 – 13.6)	13.6 (10.7 – 17.1)	***
Neutrophil (%)	48.7 (38.4 – 59.3)	46.2 (37.1 – 57.5)	50.3 (39.5 – 60.6)	***
Lymphocyte (%)	37.6 (27.8 – 47.7)	39.2 (29.3 – 48.2)	36.5 (26.9 – 46.5)	**
Platelet count (10^9/mL)	300 (248 – 359)	306 (256.5 – 354)	296 (241 – 364)	0.4
Blood glucose (mg/dL)	99 (83 – 114)	112 (112 – 126)	88 (76 – 101)	***
CRP (mg/L)	7.6 (2.4 – 22)	3 (1 – 7.5)	13 (6 – 28.1)	***

Table 2.3. Patient characteristics on admission/ enrolment and comparison between in and outpatients

*Note: data are presented as number (%); denominators may vary slightly; NA: none applicable; * p <0.05, ** p<0.01 and *** p<0.001*

Continious variables were expressed by median (IQR). Categorical variables were expressed by frequency (percentage).

	Highest Grade before discharge					
Grade at admission	Grade 1	Grade 2A	Grade 2B1	Grade 2B2	Grade 3	Grade 4
Grade 1	1	**21**	**1**	0	**3**	0
Grade 2A	*15*	724	**28**	**8**	**32**	0
Grade 2B1	0	*2*	62	**9**	**11**	**1**
Grade 2B2	0	*1*	*1*	7	**3**	0
Grade 3	0	*1*	0	0	24	**2**
Grade 4	0	0	0	0	0	0

Table 2.4. Patient grades on admission and discharge

Grey squares are patients with same grade recorded at admission and discharge (non-progressed). In bold in the open squares are patients who progressed from low grades to a higher grade (more severe) during hospital stay.

2.3.2. Enterovirus serotype detection and monthly distribution

Overall, samples from 1327 patients (85.8%) were positive for enterovirus by multiplex real time PCR. Serotyping and specific PCR analyses identified a total of 20 EV serotypes in HFMD patients in southern Vietnam during the study period. The four dominant serotypes were EV-A71, CV-A6, CV-A10 and CV-A16 with EV-A71 being the most common (Figure 2.2).

Of 138 patients in whom other EV serotypes were detected, CV-A4 was identified in 21.7%, followed by CV-A12 (19.6%), CV-A2 (15.2%), CV-A8 (7.2%), CV-A5 (5.1%), other *Enterovirus* A (1.4 %), and *Enterovirus* B (23.9 %) (Figure 2.3).

Temporally, the four common enterovirus serotypes (including EV-A71, CV-A6, CV-A10 and CV-A16) replaced each other during the entire study period. This phenomenon was seen in both inpatient and outpatient groups (Figures 2.4, 2.5 and 2.6).

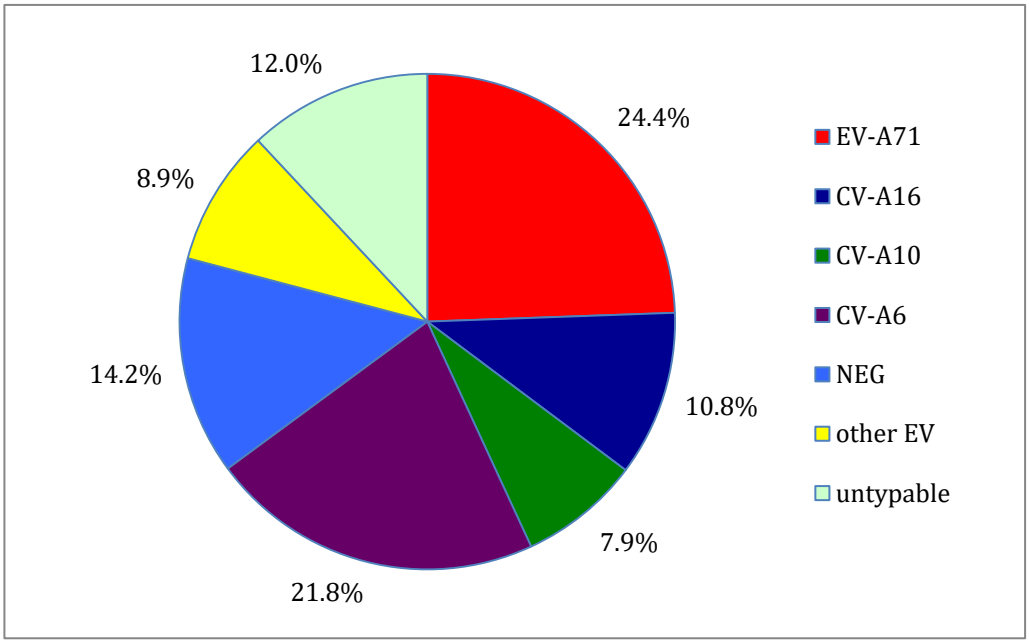


Figure 2.2. Pie chart showing the proportions of four dominant serotypes of EV-A detected among 1547 patients with clinical HFMD enrolled between July 2013 and July 2015

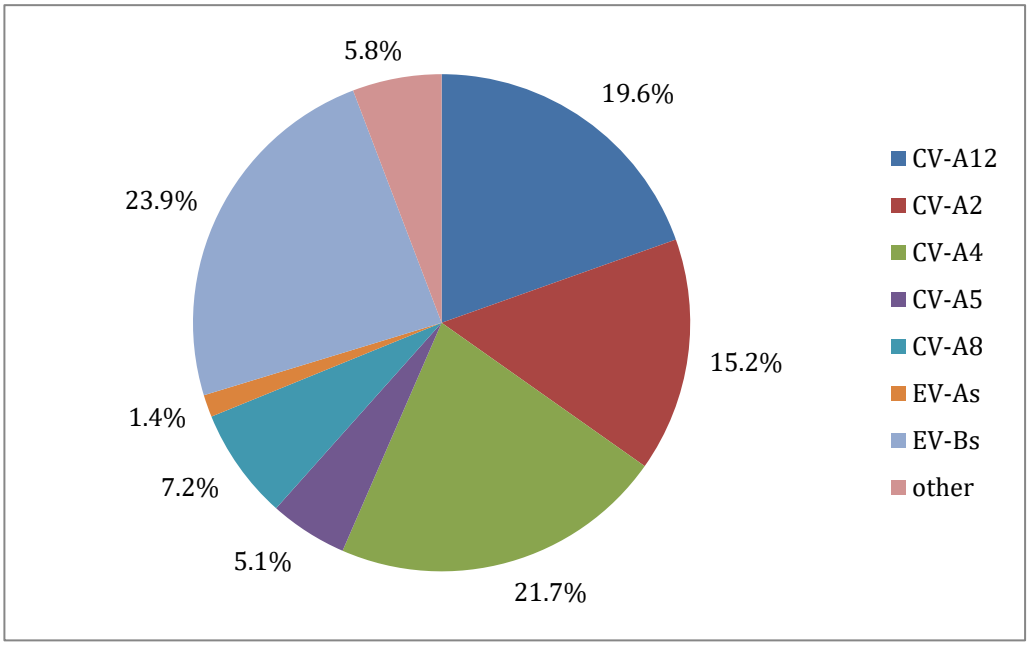


Figure 2.3. Pie chart showing the proportions of other serotypes of EV detected among 138 patients with clinical HFMD enrolled between July 2013 and July 2015

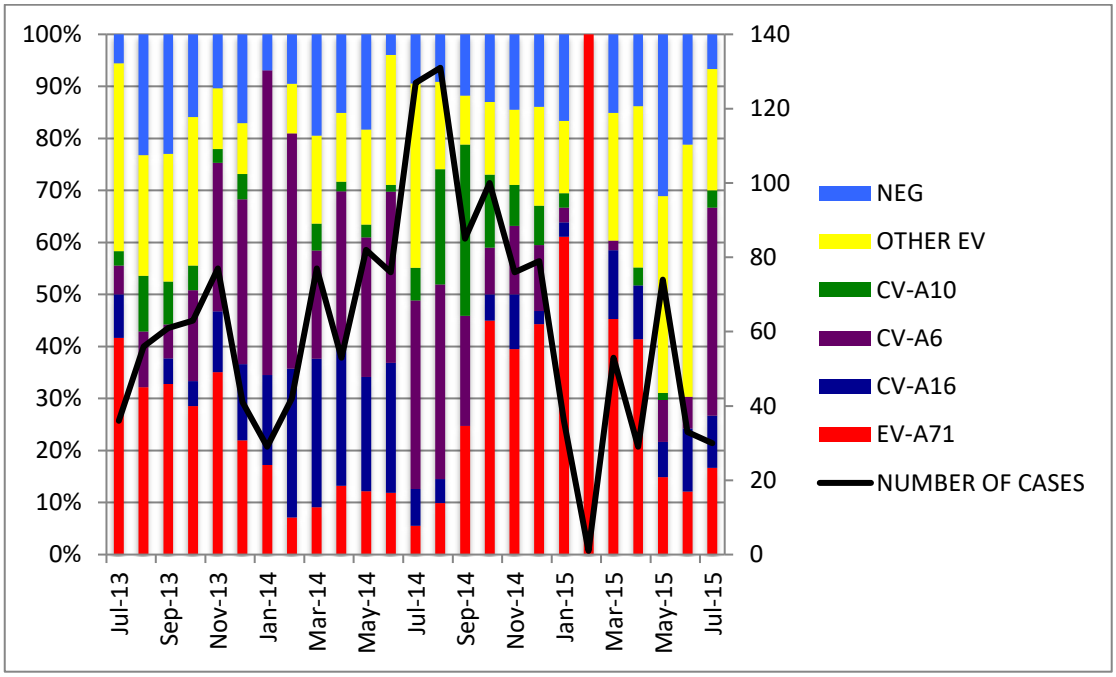


Figure 2.4. Monthly distribution of enterovirus serotypes - all 1547 patients.

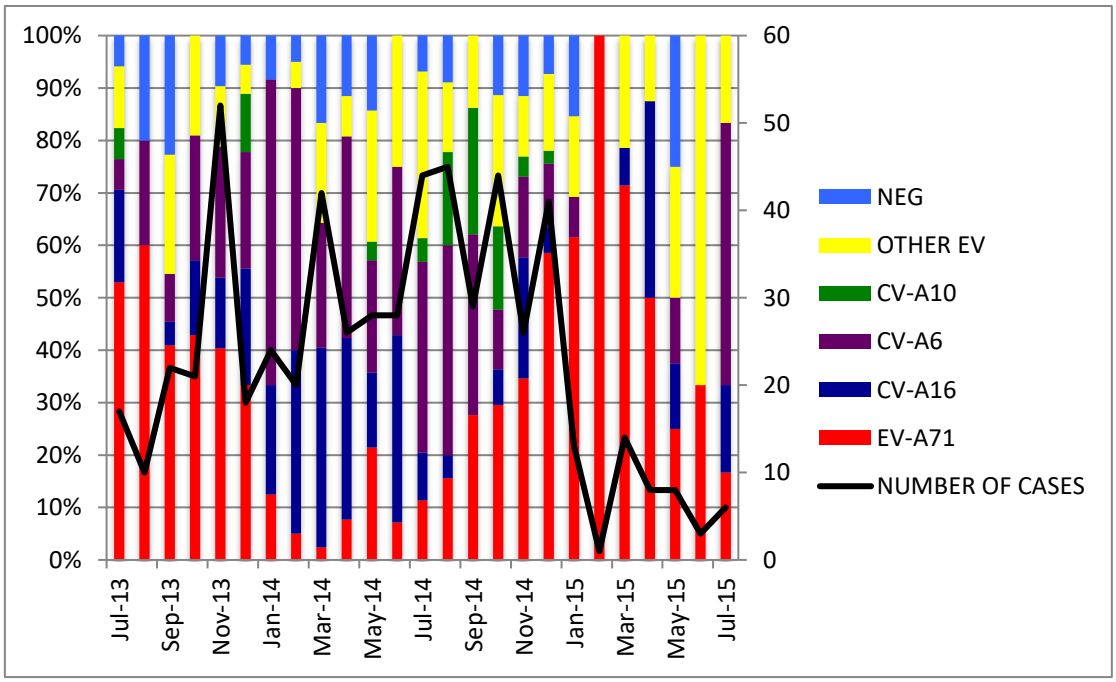


Figure 2.5. Monthly distribution of enterovirus serotypes - 590 outpatients

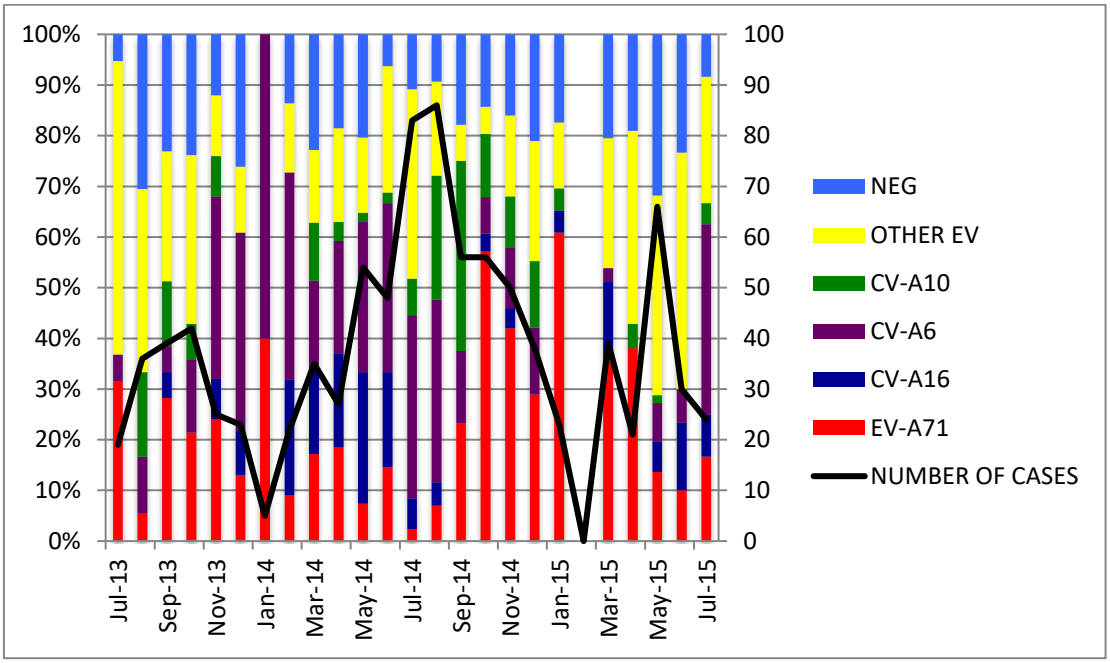


Figure 2.6. Monthly distribution of enterovirus serotypes - 947 inpatients

Figure 2.4, 2.5 and 2.6, Other EV includes typeable and untypeable samples

2.3.3. Demographics, clinical characteristics and laboratory data by pathogens

When stratifying the data by pathogens, there was considerable homogeneity in terms of demographics, clinical characteristics and laboratory data between patients infected with different enterovirus A serotypes. Variables where there were statistically differences between patient groups are shown in Table 2.5.

Patients with EV-A71-associated HFMD were significantly older than others (21.9 months, IQR: 14.2 – 32.1), while patients with CV-A10-associated HFMD were youngest (14.3 months, IQR: 10.8 –19.7) (Table 2.5).

EV-A71 was detected at a higher frequency among patients with severe disease (grade 2B or more) compared to the other viruses ($p<0.01$) (Figure 2.7). Likewise, other clinical presentations (including duration of hospital stay, myoclonal jerks, tremors, and irritability) reflecting clinically severe HFMD were more often observed among EV-A71 infected patients (Table 2.5). Compared with other groups, CV-A10 infected patients had fewer skin lesions but more often had mouth ulcers (78/122 (64%) and 117/122 (96%), respectively. CV-A10 infected patients also had higher values for both white blood cell counts and C-reactive protein (CRP) than the other groups (Table 2.5).

Characteristic	CV-A10 (n=122)	CV-A16 (n=167)	CV-A6 (n=337)	EV-A71 (n=378)	Negative (n=220)	Other EV (n=323)	p- value
Age (months)	**14.3 (10.8 – 19.7)**	19.1 (14.2-27)	16.5 (11.9 – 22.4)	**21.9 (14.2 – 32.1)**	16.9 (11.5-25.3)	18.6 (22.4 – 24.9)	<0.001
Length of hospital stay (days)	3 (2-5)	3(2-4)	3(2-5)	**4 (3-6)**	3 (2-5)	3 (2-5)	<0.001
Myoclonus	15 (12)	18 (11)	49 (15%)	**90 (24)**	42 (19)	48 (15)	0.002
Irritability	13 (11%)	18 (11%)	49 (15%)	**89 (24%)**	42 (19%)	48 (15)	<0.001
Tremor	0 (0)	0 (0)	1 (1)	**25 (12)**	2 (1%)	2 (1)	<0.001
Erythema	**78 (64)**	155 (93)	330 (98)	345 (95)	166 (78)	236 (74)	<0.001
Mouth Ulcers	**117 (96)**	156 (93)	294 (87)	311 (85)	179 (84)	288 (90)	<0.001
WBC (k/UL)	**14.65 (12.18 - 18.1)**	12.2 (9.2-14.5)	12.5 (9.9 – 16.6)	11.7 (9.5 – 14.7)	11.85 (8.93 – 16.25)	12.9 (10.07 – 16.22)	<0.001
Platelet (k/UL)	294 (250-364)	298 (251-342)	308 (249.7 - 359.2)	309 (261-376.75)	288 (232.5 - 357.15)	290 (236.5 - 349)	0.026
Glucose (mg/dl)	94.95 (82-111)	99.5 (81.1-112.2)	101 (85.36-117.5)	102 (86-118)	97 (79-108)	98 (79-113)	0.01
CRP (UI/L)	**17.35 (6-34.23)**	7 (1.4-20)	10 (4-26)	**5 (1.2-10)**	8 (2.46-23)	8 (3.5-25)	<0.001

Table 2.5. Clinical characteristics and lab results by pathogens

Note: data are presented as number (%) for categorical variables and median, interquartile range for continuous variables; denominators may vary slightly. In bold text are factors which are significantly different when comparing with other groups. P-values are adjusted for multiple comparison.

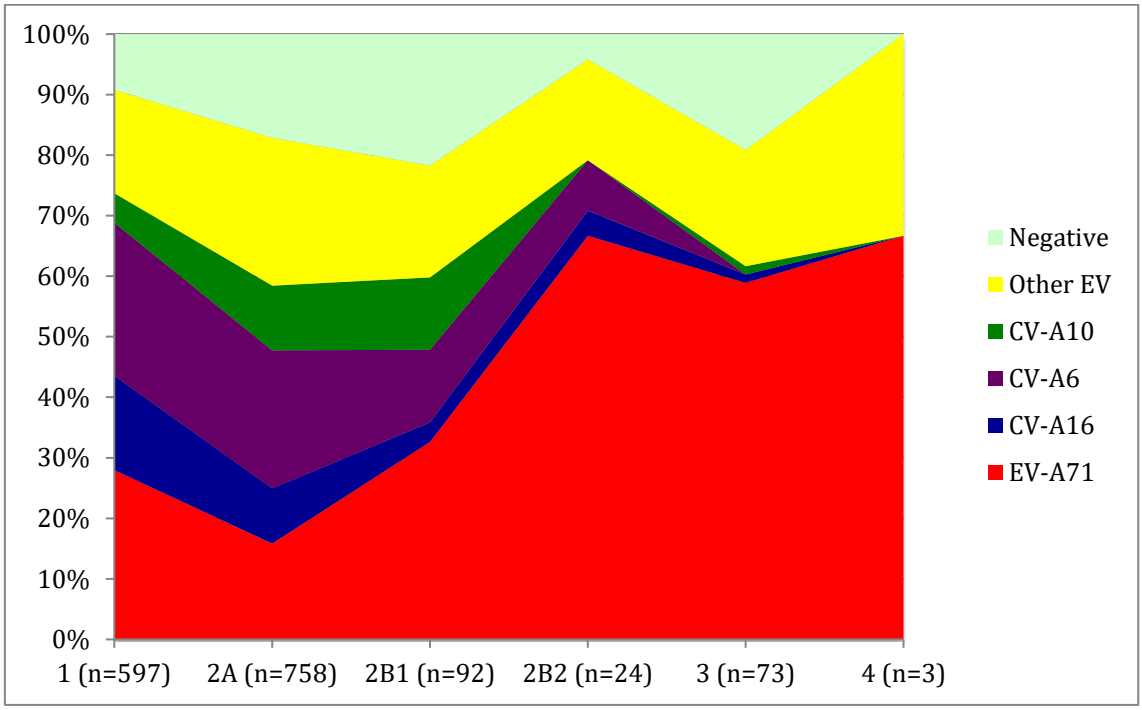


Figure 2.7. Distribution of pathogen detected by severity

Other EV included typeable and untypeable samples. Grade 4 included 3 patients, of which 2 positive with EV-A71 and 1 positive with CV-A4.

2.3.4. Comparison between in- and out patients

There were considerable differences in terms of demographics, clinical presentations and laboratory test results between inpatients and outpatients (Table 2.3), while the etiological patterns and monthly distribution of the detected enterovirus serotypes were similar between the two groups (Figures 2.5 and 2.6). Notably, outpatients were older, more likely to have a rash and had higher blood glucose levels than inpatients. Meanwhile, inpatients presented to the hospital at a later time of illness, had higher temperature, and higher blood cell count and CRP than outpatients (Table 2.3).

2.3.5. Factors associated with progression to severe disease

119 inpatient cases (8.8%) progressed to a more severe grade of HFMD, of whom 98 cases (6.3%) progressed from non-severe grades to severe grades. A further 21 cases progressed within the mild grade category; i.e. from grade 1 to grade 2A (Table 2.4). Patients progressing from mild to severe grades were less likely to have a rash and mouth lesions on admission compared to those who did not. Types of rash were also different between the two groups. Vesicular and mixed rash were seen more frequently in the non-progressing group while progressing patients more often presented with a macular rash (Table 2.6). Myoclonus, tremor and limb weakness at presentation were also less likely seen in non-progressing group. Patients who progressed to the most severe grades (grade 3 & 4) with cardiopulmonary complications and/or autonomic dysfunction had a higher incidence of drowsiness and lethargy at presentation. Patients in this group also presented with a higher

heart rate and systolic blood pressure, in addition to higher blood glucose and lower CRP than non-progressing patients or patients with milder disease (Table 2.6).

Multivariate analysis showed that admission features of vesicular rash, increased blood pressure, high heart rate and elevated blood glucose were associated with increased risk of progression to severe grades while higher CRP showed a trend towards protection from progression (Table 2.7). However, when analyzing only cases who progressed to very severe disease (grade 3 & 4 - with autonomic dysfunction and/or cardiopulmonary complications), the presence of skin rash was associated with protection from progression. When a rash was present, a macular type rash was significantly associated with increased risk of progression (Table 2.8).

	Non – progressed- non severe to severe (n= 859)	Progressed – non severe to severe (n=98)	P1values	Non-progressed to cardiopulmonary complication (n=907)	Progressed to autonomic dysfunction and/or cardiopulmonary complication (n=50)	P2 values
Demographics						
Gender (male)	517 (61.7)	72 (60.5)	0.8	558 (61.5)	31 (62)	1
Age (months)	16.6 (12.1 – 23.5)	18.7 (14.9 – 26.9)	0.2	16.7 (12.1 – 23.7)	18.7 (11.2 – 26.9)	0.7
Ho Chi Minh City origin	474 (55.2)	62 (63.3)	0.1	505 (55.7)	31 (62)	0.6
Illness day on admission (days)	1 (1 - 2)	2 (1 - 2)	0.002	1 (1 – 2)	2 (1 – 2)	0.05
Duration of hospital stay (days)	3 (2 -5)	6 (5 – 7)	<0.001	4 (2 – 5)	6 (5 – 7)	<0.001
Clinical signs and symptoms						
Rash	698 (83.4)	71 (73.2)	0.02	737 (83.3)	32 (65.3)	0.001
Type of rash			<0.001			<0.001
Mostly vesicular	13 (1.9)	4 (5.6)		15 (2.1)	2 (6.1)	
Mostly macular	339 (49.3)	47 (66.2)		360 (49.7)	26 (78.8)	
Both	335 (48.8)	20 (28.2)		350 (48.3)	5 (15.2)	
Mouth lesion	767 (91.5)	72 (74.2)	<0.001	808 (91.2)	31 (63.3)	<0.001
Lips	3 (0.5)	0	1	3 (0.4)	0	1

Tongue	305 (46.8)	19 (46.3)	1	314 (46.2)	10 (74.1)	0.06
Palate	499 (76.5)	26 (63.4)	0.06	518 (76.5)	7 (43.8)	0.005
Buccal	55 (8.4)	0	0.07	55 (8.1)	0	0.6
Headache	3 (0.4)	0	1	3 (0.4)	0	1
Cough	127 (14.9)	13 (13.5)	0.9	133 (14.8)	7 (13.5)	1
Runny nose	104 (12.2)	8 (8.2)	0.3	107 (11.9)	5 (10.2)	1
Conjunctivitis	4 (0.5)	1 (1)	0.4	4 (0.5)	1 (2)	0.2
Vomiting	167 (19.5)	19 (19.4)	1	175 (19.3)	11 (22)	0.6
Diarrhoea	50 (5.8)	3 (3.1)	0.3	5 1(5.6)	2 (4.1)	1
Drowsiness	20 (2.4)	6 (6.6)	0.03	21 (2.4)	5 (11.6)	0.005
Irritability	61 (7.2)	7 (7.2)	1	66 (7.3)	2 (4.1)	0.6
Myoclonus	167 (19.5)	30 (30.6)	0.01	185 (20.4)	12 (24)	0.6
Sweating	6 (0.7)	1 (1)	0.5	7 (0.8)	0	1
Lethargy	7 (0.8)	3 (3.1)	0.08	7 (0.8)	3 (6)	0.01
Ataxia	4 (0.5)	0	1	4 (0.5)	0	1
Tremor	22 (2.6)	8 (8.8)	0.005	29 (3.2)	1 (2.3)	1
Nystagmus	3 (0.4)	0	1	3 (0.3)	0	1
Limb weakness	2 (0.2)	3 (3.3)	0.008	4 (0.5)	1 (2.4)	0.2
Temperature (°C)	38 (37.3 – 38.5)	38 (37.5 – 38.7)	0.4	38 (37.4 – 38.5)	37.8 (37.5 – 38.2)	0.3
Pulse (bpm)	122 (120 – 130)	130 (122 – 142)	<0.001	124 (120 – 130)	130 (120 – 142)	0.001
Systolic blood pressure (mmHg)	90 (90 – 95)	98 (90 – 104.5)	<0.001	90 (90 – 95)	98 (90 – 105)	<0.001

Diastolic blood pressure (mmHg)	60 (55 – 60)	60 (55 – 60)	0.004	60 (55 – 60)	60 (54 – 60)	0.3
Body mass index	17 (15.5 – 18.6)	16 (15.5 – 18.2)	0.2	16.8 (15.5 – 18.6)	16.2 (14.9 – 17.9)	0.1
Blood results						
Cell count (10^9/mL)	13.6 (10.6 – 17.2)	13.1 (11.5 – 16.5)	1	13.6 (10.6 – 17.2)	13.2 (11.7 – 17.1)	0.7
Neutrophil (%)	50 (39.5 – 60.4)	45.6 (40.6 – 63.1)	0.054	50.2 (39.5 – 60.5)	54.5 (40.1 – 63.3)	0.3
Lymphocyte (%)	37 (27 – 46.6)	33 (25 – 45.8)	0.06	36.7 (27.1 – 46.4)	35.2 (23.9 – 48.2)	0.3
Platelet count (10^9/mL)	294 (237.5 – 361.5)	320 (277 – 412)	0.051	295 (240 – 364)	322 (299.8 – 383.5)	0.1
Blood glucose (mg/dL)	87 (75 – 100)	97 (84.3 – 108.5)	<0.001	88 (75 – 101)	94 (85 – 106.7)	0.008
CRP (mg/L)	14 (6 – 29)	6 (2.1 – 21)	<0.001	13.2 (6 – 28.2)	6 (3 – 22.5)	0.004

Table 2.6. Comparison between progressing and non-progressing cases

P1: P values for cases progressed from a non-severe grade to a severe grade

P2: P values for cases progressed from a grade without autonomic dysfunction / cardiopulmonary involvement to a grade with autonomic dysfunction / cardiopulmonary involvement.

Factors	Odd Ratios (95%CI)	P value
Types of rash (vesicular)	8.7 (1.4 – 43)	0.01
CRP	0.85 (0.72 – 1)	0.055
Blood glucose	2.9 (1.04 – 8.3)	0.04
Pulse	1.54 (1.2 - 2)	<0.001
SBP	2.3 (1.4 – 3.8)	0.001

Table 2.7. Multivariate analysis on factors associated with disease progression – from non-severe (grade 1-2A) to severe (all-severe cases, grade 2B or higher)

Factors	Odds Ratio (95%CI)	P value
Type of rash (macular)	3.2 (1.09 – 11.7)	0.04
Having skin lesions	0.03 (0.0007 – 1.15)	0.03
Pulse	1.4 (0.9 – 1.9)	0.08
Drowsiness	3.7 (0.8 – 14.7)	0.07

Table 2.8. Multivariate analysis on factors associated with disease progression – from non-severe (grade 1-2A) to severe with autonomic dysfunction and/or cardiopulmonary complications (grade 3-4)

Note for table 5 &6: In my model, lab values (blood glucose and CRP) were calculated by ln2. For pulse and SBP, changes were counted by every 10bpm and 10mmHg, respectively.

2.4. DISCUSSION

This is the first longitudinal study and in-depth analysis of etiology and associated clinical phenotypes of both in- and outpatients with HFMD admitted to three tertiary referral hospitals for southern provinces in Vietnam, where to date the majority of HFMD cases from Vietnam have been reported. Between July 2013 and July 2015, a total of 1547 HFMD patients were recruited. Between 2011 and 2012 Vietnam experienced the largest outbreak of HFMD in terms of both hospitalized cases and fatal cases, with over 200,000 cases and 200 deaths [43]. The number and proportion of severe cases recorded in the present study was less than anticipated based on the previous 2 years observations. Whilst the underlying mechanism remains unknown, our previous research has shown that genetically EV-A71, the main cause of severe HFMD, has undergone a switch from subgenogroup C4 to subgenogroup B5 at the end of 2012 [44,66], and since then B5 has continued to circulate in southern Vietnam (data not shown). Intriguingly, in Vietnam we do not see the cyclical 2-3 year pattern of HFMD and its pathogens as reported from Japan and Malaysia [89,199].

This study is one of the few to record the characteristics of mild cases of HFMD, finding a significant proportion168/590 (28.5%) of EV-A71 among outpatients. The proportion of EV-A71 infected patients in mild and moderately severe cases (grade 1 and 2A) was remarkable in this study, with a detection rate of around 20%. All these recovered completely without any complications. On the other hand, as EV-A71 was detected more frequently among severe patients, a higher rate of neurological symptoms among EV-A71 positive patients was also found.

This is the first study to report CV-A6-associated HFMD, previously not detected in Vietnam, including during the 2005 HFMD outbreak [41] and indeed this was the second most frequently detected pathogen after EV-A71. From October 2013 to October 2014, CV-A6 emerged, replacing CV-A16 and together with EV-A71 becoming the 2 most common pathogens in southern Vietnam, similar to reports from mainland China. Likewise other countries world-wide also described simultaneous emergence of CV-A6 [88,96,98,200–202].

The four main serotypes (EV-A71, CV-A6, CV-A16 and CV-A10) continuously varied in dominance and detection rates and replaced each other during the study period, while accounting for 1005/1327 (75.7%) of all EV positive HFMD cases. This challenges future intervention strategies including vaccine development and implementation, but in turn emphasizes much needed effort for continuous investigation of molecular epidemiology of HFMD pathogens in endemic countries.

Several differences in clinical features of cases were detected. Inpatients presented significantly less often with rash than outpatients. This may represent either a health seeking or diagnostic bias: either parents are triggered to go to hospital when patients have a rash (and mild HFMD with subtle rash is less likely to present), or doctors only diagnose HFMD with subtle rash as HFMD when (characteristic) more severe symptoms of neurological involvement develop. Clinical phenotypes also appeared to differ according to underlying viral etiology. Patients testing positive for CV-A10 had a typical clinical presentation, with low frequency of skin rash but high frequency of mouth ulcers. Furthermore, they had higher white blood cell counts and C-reactive protein levels, which may suggest more severe illness or

bacterial co-infection. The lower median age of CV-A6 and CV-A10 infected patients suggests the absence of background (maternal) immunity and recent introductions into Vietnam, which merits further analysis into the evolutionary process of the pathogens and the level of cross immunity between common HFMD causing enteroviruses.

Similar to previous reports from Taiwan and China, several clinical features on admission were documented in this chapter, which were indicative of progression to more severe stages of the disease. The 119 patients who progressed to more severe stages of disease presented with less rash and mouth lesions and if presenting with rash, showed a macular rash as the predominant type.

2.5. CONCLUSION

This study represents the most comprehensive descriptive HFMD study from Vietnam. The analysis of 1547 in- and outpatients revealed overall dominance of EV-A71, especially among more severe illness but also among outpatients, detailed serotype emergence and replacement, specific serotype-associated clinical features and clinical and laboratory data associated with progression to more severe illness. These findings are essential for planning public health measures and improving clinical management.

3. LONGITUDINAL DESCRIPTION OF CLINICAL PICTURE, MANAGEMENT AND OUTCOME OF HFMD FROM 2009 TO 2015

3.1. INTRODUCTION

The first outbreak of HFMD in Vietnam occurred in 2005 [41]. The numbers of cases gradually increased although remained confined to the southern part of the country until 2011 when a significant outbreak occurred [43] (See Chapter 1 section 1.3.2) and cases occurred throughout the country.

With the support of the World Health Organization (WHO), the Vietnamese Ministry of Health (MoH) responded to the outbreak of 2011 with a wide range of interventions. These included strengthening of the national surveillance system through sentinel surveillance system, and improving clinical management of cases [203]. According to the surveillance system guidelines, all hospitals had to report newly admitted cases (including fatal cases) to the Department of Health, using a standardized report form. Clinical case definition was based on the MoH guideline for diagnosis and management of HFMD. From 2012, there was a template hospital chart, designed especially for HFMD, that was used in all hospitals to ensure that all cases were recorded and reported However data on weekly reported cases were only available for the period after the 2011-2012 outbreak, and showed that in Vietnam HFMD circulated year - round with a small peak from March to May and a larger peak from September to December, while retrospective data showed that in

2011 the highest number of HFMD cases was recorded in September with one major peak between June and December [204].

The prospective observational study described in Chapter 2 covered the period from July 2013 until July 2015. Therefore to evaluate the treatment strategies and to better understand the epidemiology of HFMD over time, a comprehensive retrospective study was conducted, aiming at synthetizing epidemiology, clinical features (including severity) and treatment of HFMD over time (2009 – 2015) using retrospective data from one of the three centers of the prospective study.

The study aims were as follows:

- To retrospectively collect basic information from patients admitted to CH2 with a clinical diagnosis of HFMD from 2009 – 2013.
- To describe the clinical, epidemiologic, demographic characteristics and treatment of HFMD in Ho Chi Minh City between 2009 and 2013 and to identify to what extent these aspects of the epidemic may have changed over time.
- To identify clinical and/or laboratory factors that may predict disease outcome/progression.

3.2. MATERIALS AND METHODS

3.2.1. Study setting

A retrospective study was conducted using data from the hospital information system and clinical case records of HFMD patients admitted to Children's Hospital 2 (CH2), Ho Chi Minh City between January 2009 and June 2013. This hospital applied

a computerized database systems from 2008 and hence it was practical and affordable to extract list of patients. Moreover, Children's Hospital 2, Children's Hospital 1 and Hospital for Tropical Disease are at the same level and have similar roles in serving patients in Ho Chi Minh City therefore data from one hospital was fairly representative of all 3 ones. These data were combined with prospectively collected data from my HFMD research program in three referral hospitals: Children's Hospital 1, Children's Hospital 2 and Hospital of Tropical Diseases (Chapter 2).

3.2.2. Approach and data collection

Firstly, a complete list of HFMD patients admitted to CH2 from January 2009 to June 2013 was obtained from the hospital data system (cases with final diagnosis of B08.4 code - ICD10). A list of patients then was randomly selected using R 3.2.5 [198] , including 750 cases from each calendar year for the period between January 2009 and December 2012. For the period between January and June 2013, I selected 375 since the last period of the year were covered by the prospective study described in Chapter 2. From the cases selected, demographic and clinical data were then retrospectively collected from the hospital records using case record form (Supplementary Appendix 8.5).

3.2.3. Data analysis

Data were analysed using R 3.2.5. Continuous variables were expressed using median and interquartile range (IQR), categorical variables using frequency. Fisher's exact test with p-value by Monte-Carlo simulation, t-test and Kruskal -

Wallis were used to test categorical, continuous (normal distribution) and continuous (skewed) variables, respectively. Multiple comparisons were adjusted using Holm - Bonferroni correction. To determine factors associated with disease progression, all factors with p-value <0.05 were tested in a multivariate regression model.

3.3. RESULTS

3.3.1. Patients' characteristics

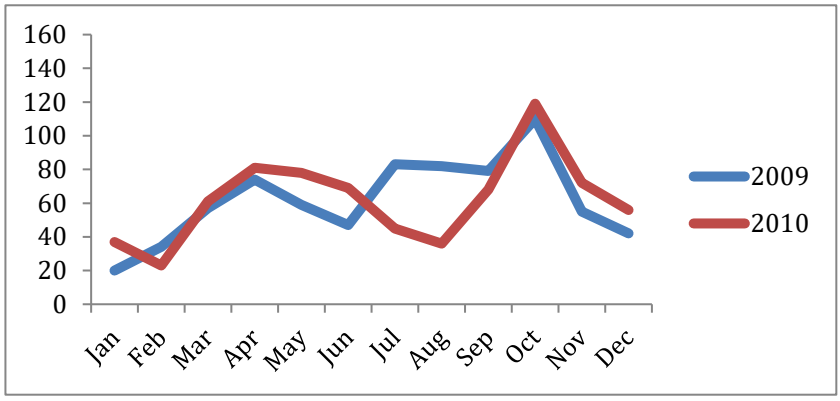
In total, 4313 patients (including 1484 (for the period 2009-2010: before the outbreak), 1494 (2011-2012: during the outbreak) and 1333 (2013- July 2015: after the outbreak) were included in the analysis. Patients' baseline characteristics are presented in Table 1. The median age was 18.4 months old (IQR: 12.3 – 25.5). There were more boys admitted than girls (2664/4313, 61.8%). Most patients (4074, 4313/94.5%) had mild (grade 1 and 2a) disease and the median time from onset of the illness to admission was 2 days (IQR: 2 – 3). Duration of hospitalization was 3 days (IQR 2 – 4).

3.3.2. Monthly distribution of HFMD cases over time

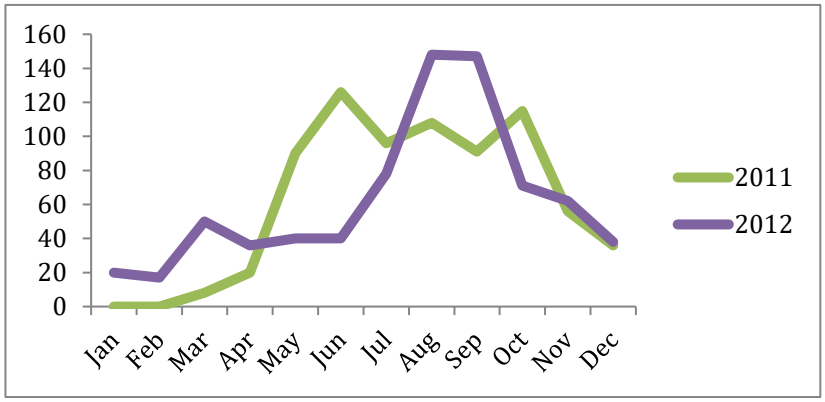
Before the outbreak (2009 – 2010), there were two endemic peaks each year: one small peak from March to May and a bigger one from September to November. However, this pattern was not observed during the outbreak, with the number of cases increasing dramatically from April to June 2011 and remaining high until

month year. After the outbreak, the number of cases fluctuated but with a single peak in the middle of the year (from April to July) (Figures 3A, 3B and 3C).

A.



B.



C.

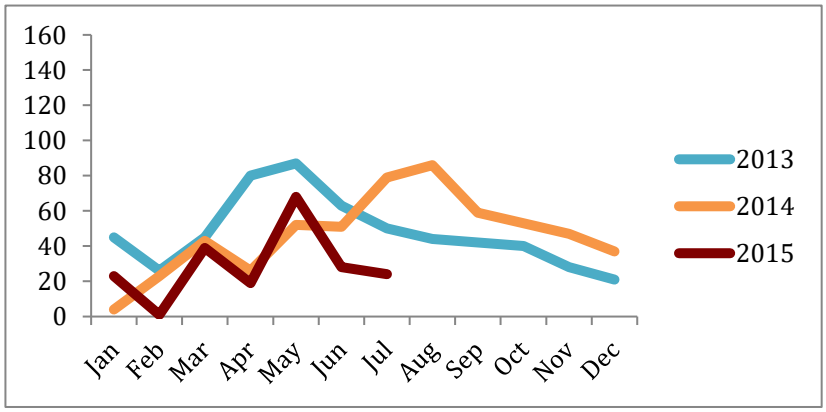


Figure 3.1. Monthly case distribution

A. Before the outbreak B. During the outbreak C. After the outbreak

3.3.3. Comparison of patients' characteristics before, during and after the outbreak

Patients' characteristics changed during the 3 phases in terms of demographics and clinical features, as shown in table 1. The phase 3 group was different from two other groups: patients were younger (16.5 months, IQR: 11.9 - 23.9) and admitted to hospital earlier (2 days, IQR: 1-2).

The referral patterns were different within hospitals between the 3 phases. Specifically, after admission to the hospital, 1299/1333 (97.6%) of patients after the outbreak were sent directly to infectious disease ward (ID), higher than before (1369/1483, 92%) and during the outbreak (1395/1494, 93.4%), while after the outbreak, less patients were transferred to ID ward from other wards/hospitals than before, with rates of transferred cases before, during and after the outbreak were 7.1% (105/1483), 5.9% (88/1494) and 1.7% (23/1333), respectively.

Grading at discharge, especially grade 1 and grade 2a, varied between groups. More than half of patients before the outbreak were diagnosed with HFMD grade 1 at discharge (884/1483, 59.5%). This rate dropped significantly to just about one-fourth during the outbreak (25.6%, 383/1494) and only 5.9% (79/1333) after the outbreak. In contrast, the proportion of patients with grade 2a increased from 39% (582/1483) before the outbreak to 68.9% (1030/1494) during the outbreak and almost doubled the rate after the outbreak (83.7%, 1116/1333).

In terms of clinical features, patients after the outbreak were less likely to have skin lesions but they often presented with mouth ulcers. Neurological symptoms such as

myoclonus, irritability, and vomiting were seen more often in patients admitted before and during the outbreak (Table 3.1).

3.3.4. Comparison between severe and non-severe cases

Among the 4313 patients included in the study, there were 239 severe cases (Grade 2b1 and above) (5.5%). There were considerable differences between the two groups in terms of demographics, clinical characteristics and lab results. Notably, severe patients were older, and had higher and a longer duration of fever. Myoclonus, tremor and vomiting were also recorded more frequently in severe patients. Severe patients had higher white blood cell and platelet counts and a higher level of blood glucose (Table 3.2). Multivariable analysis showed that high and prolonged fever, myoclonus, tremor and raised blood glucose were associated with an increased risk of severe disease (Table 3.3).

Characteristics	**Total (n= 4313)**	**Before outbreak (n=1483)**	**During outbreak (n=1494)**	**After outbreak (n=1333)**	**P value**
Gender (Male)	2664 (61.8)	901 (61)	931 (62)	832 (62)	0.7
Age (moths)	18.4 (12.3 - 25.5)	18.1 (12.7 - 25.2)	18.5(12.4 -27.3)	16.5 (11.9 - 23.9)	***
Time before admission (days)	2 (2-3)	2 (2-3)	2 (2-3)	2 (1-2)	***
Grade at discharge 1 2a 2b1 2b2 3 4	 1346 (31.2) 2728 (63.3) 139 (3.2) 47 (1) 51 (1.2) 2 (0.05)	 884 (59.5) 582 (39) 8 (0.5) 3 (0.2) 8 (0.5) 1 (0.1)	 383 (25.6) 1030 (68.9) 40 (2.7) 26 (1.7) 14 (0.9) 1 (0.07)	 79 (5.9) 1116 (83.7) 91 (6.8) 18 (1.4) 29 (2.2) 0	***
Admission ward ED ID Other wards Transferred from other hospital	 26 (0.6) 4063 (94.2) 90 (2) 126 (2.9)	 12 (0.8) 1369 (92) 41 (2.8) 64 (4.3)	 11 (0.7) 1395 (93.4) 39 (2.6) 49 (3.3)	 9 (0.7) 1299 (97.6) 10 (0.7) 13 (1)	***
Fever	3473 (81.2)	1112 (75)	1274 (86)	1087 (82)	***
Duration of fever	1 (1-2)	1 (1-2)	1 (1-2)	1 (1-2)	0.06
Erythema	3722 (87.8)	1409 (95)	1309 (89)	1004 (78)	***
Mouth ulcers	3260 (79.8)	1124 (79)	1001 (73)	1135 (88)	***
Myoclonus	2017 (47.2)	879 (60)	798 (54)	340 (26)	***
Irritability	652 (18.6)	251 (22)	305 (28)	96 (8)	***
Tremor	241 (5.8)	82 (6)	92 (6)	67 (5)	0.4
Vomit	1116 (27.6)	382 (30)	483 (33)	251 (19)	***
WBC (K/ul)	13.2 (10-15.9)	12.3 (9.9 - 15.5)	12 (9.5-15.4)	13.4 (10.5-17)	***
Platelets (K/ul)	319 (254-372)	311.5 (258-372.2)	319 (262-381)	294 (240-359)	***
Blood glucose	86.7 (73-98)	79 (67-92)	85(74.3-99)	87 (75-100)	***

Table 3.1. Characteristics of admitted patients before, during and after the outbreak.

*Note: data are presented as number (%); denominators may vary slightly; NA: none applicable; * p <0.05, ** p<0.01 and *** p<0.001.*

3.3.5. Comparison of severe cases in different periods of time

When stratifying the 239 severe patients for the three different periods of the study, the total number of severe cases (Grade 2b1 or higher) was highest in phase 3 (after the outbreak) (n=138/1333, 10.3%), followed by phase 2 (during the outbreak) (n=81/1494, 5.4%) and phase 1 (before the outbreak) (n=20/1483, 1.3%). The majority of patients enrolled after the outbreak had grade 2b1 (91/138, 66%), followed by grade 2b2 and 3 combined (21/138, 34%). For the period during the outbreak, half of the severe cases had grade 2b2 or higher (2b2: 26/81, 32%; 3: 14/81, 17% and 4: 1/81, 1%). In phase 2 (during the outbreak), severe patients were admitted to the hospital later than in phase 3 (after the outbreak). The proportion of patients that were transferred from other hospitals was higher in phase 1 and 2 than number of transferred patients of phase 3(Table 3.4).

Clinical presentations were significantly different between phase 3 (after the outbreak) and 1 and 2 (before and during the outbreak). Overall, severe patients had higher temperatures, longer duration of fever and were more likely to have neurological symptoms in phases 1-2 than in phase 3 (Table 3.4).

In terms of treatment, intravenous immunoglobulin (IVIg) was given more often in phases 1 and 2. Most of the severe patients (grade 2b1 and above) in phase 1 received IVIg (80%, 16/20 cases). This rate reduced to 71.6% (58/81) of similarly severe patients in phase 2 and just 40.5% (56/138) in phase 3. The proportion of patients requiring endotracheal intubation and mechanical ventilation was also different between 3 phases: 20% (4/20) in phase 1, 12.3% (10/81) in phase 2 and only 0.7% (1/138) in phase 3. The proportion of patients receiving milrinone,

inotropes and renal replacement therapy was not significantly different between the different phases of the study (Table 3.4).

Characteristics	Non-severe (n=4074)	Severe (n=239)	P value
Gender (Male)	2514 (62)	150 (63)	0.9
Age (months)	17.5 (12.2 - 25.2)	21.1 (15 - 29.4)	***
Time before admission (days)	2 (2-3)	2 (1-3)	*
Fever	3276 (81)	197 (83)	0.9
Body temperature (°C)	38.4 (37.8 - 39)	38.8 (37.8 - 39.5)	***
Duration of fever (days)	1 (1-2)	2 (1-3)	***
Erythema	3538 (88)	184 (84)	0.1
Mouth ulcer	3107 (80)	153 (75)	0.1
Myoclonus	1851 (46)	166 (69)	***
Irritability	605 (18)	27 (23)	0.07
Tremor	151 (3.8)	90 (38.5)	***
Vomit	1032 (27)	84 (36)	**
WBC (k/ul)	12.5 (10-15.9)	13.2 (10.6-16.6)	*
Platelets (k/ul)	308 (254-370)	339 (258-408.5)	**
Blood glucose (g/dl)	84 (72-97)	100 (86.5-118)	***

Table 3.2. Characteristics of severe and non-severe patients

*Note: data are presented as number (%); denominators may vary slightly; NA: none applicable; * p <0.05, ** p<0.01 and *** p<0.001*

Factors	Odd ratios (95% CI)	P value
Duration of fever	1.3 (1.12 - 1.53)	<0.001
Body temperature	1.5 (1.1 - 2.0)	0.008
Myoclonus	3.2 (1.9 - 5.5)	<0.001
Tremor	13.5 (8.9 - 20.8)	<0.001
Blood glucose	1.3 (1.2 - 1.5)	<0.001

Table 3.3. Multivariable analysis of severe cases

Note: For table 4, changes in blood glucose was counted by every 10mg/dl

Characteristics	**Before outbreak (n=20)**	**During outbreak (n=81)**	**After outbreak (n=138)**	**P value**
Gender	15 (75)	53 (65)	82 (59)	0.4
Age	21.6 (14.6 - 29)	25.3 (16-36.5)	19.5 (14.7-27)	0.02
Admission ward ED ID Other wards Transferred from other hospital	 1 (5) 16 (80) 1 (5) 2 (10)	 7 (9) 67 (83) 4 (5) 3 (4)	 1(0.7) 136 (98.5) 1 (0.7) 0	 ***
Grade at discharge 2b1 2b2 3 4	 8 (40) 3 (15) 8 (40) 1 (5)	 40 (49) 26 (32) 14 (17) 1 (1)	 91(66) 18 (13) 3 (2.1) 0	***
Time before admission (days)	2 (2-2.7)	2 (2-3)	2 (1-2)	***
Fever	19 (95)	77 (96)	101 (73)	***
Body temperature	39.5 (38.9-39.9)	39.5 (39-39.9)	38 (37-38.9)	***
Duration of fever	3 (2.5-4)	3(2-3)	2 (1-3)	***
Erythema	20 (100)	80 (99)	84 (72)	***
Mouth ulcers	14 (74)	53 (76)	86 (74)	1

Myoclonus	20 (100)	67 (83)	79 (57)	***
Irritability	3(21)	24 (44)	20(15)	***
Tremor	8 (40)	50 (62)	32 (24)	***
Vomit	10 (50)	38 (48)	36 (26)	**
Lab results				
WBC	14.7 (11.1-18.7)	13.2(11-15.9)	13.1 (9.9-17.2)	0.7
Platelets	341(281.5-443)	352 (286.5-399.5)	301.5 (229.8-413)	0.2
Blood glucose	101 (75-121)	106 (90 -125)	96.7 (86.5-111)	0.09
Treatment				
IVIg	16 (80)	58 (71.6)	56 (40.6)	***
Milrinone	1 (5)	6 (7.4)	9 (6.5)	0.2
Intubation	4 (20)	10 (12.3)	1 (0.7)	***
Inotropes	3 (15)	7 (8.6)	7 (5)	0.16
CRRT	0	1 (1.2)	0	0.4

Table 3.4. Comparison of all severe cases before, during and after outbreak.

*Note: data are presented as number (%); denominators may vary slightly; NA: none applicable; * p <0.05, ** p<0.01 and *** p<0.001*

3.4. DISCUSSION

This chapter reported a descriptive study about the epidemiology, clinical characteristics, patient management and outcomes of HFMD in Vietnam from 2009 to 2015, encompassing the period from 2 years before the explosive outbreak in 2011-2012 to 2 year and a half afterwards.

In terms of seasonality, the obtained data show that in southern Vietnam HFMD consistently did have a major epidemic peak occurring around August and November each year (corresponding to the beginning of new school year), and a smaller epidemic peak sporadically happening from March to May, corresponding to the period when there was a transition between dry and rainy seasons, especially before the 2011-2012 outbreak. This observed pattern is similar to data from China and Japan where scientists used seasonal models to explain and predict the distribution of HFMD [205–208]. High temperatures and humidity were considered advantageous for the disease to spread [206,208,209]. The observed evidence so far may explain why most of HFMD cases occur in the southern part of the country, where the climate is warm all year round. More in-depth modelling tied to more detailed incidence and climate data would allow better understanding of this.

The analyses from this study have shown that HFMD patients admitted to CH2 after the outbreak were younger than before and during the outbreak. This may be attributed to several factors. Firstly, the pathogens causing HFMD have altered during this time. In 2005 CV-A16 and EV-A71 were the main causes, while CV-A6 and CV-A10, particularly CV-A6, have emerged from 2013 onward to become

dominant coxsackieviruses causing HFMD in Vietnam (Chapter 2). Consequently, many young children would not have maternal immunity against these two viruses and hence be more susceptible to the infections. Secondly, as the epidemic progressed, it is likely that there was increasing awareness among both medical staff and parents that younger children are more at risk of severe disease. Therefore older children were likely to be managed at home.

During the outbreak (phase 2), the Vietnamese MoH's response focused on three main strategies: strengthening of the surveillance system, training of clinical staff and raising public awareness [203]. Data from our study show the likely impact of these strategies. After the outbreak, increased awareness amongst parents may have resulted in them seeking medical treatment and presenting to hospital earlier than the period during and after the 2011-2012 outbreak, reflected by the earlier stage of disease at admission than before.

In terms of medical staff's awareness of the diagnosis of HFMD and ability to manage the disease appropriately, our data indicated impact of policy. Before the outbreak, more than half of admitted patients had mild stages of disease not requiring hospitalization (grade 1), while this proportion dropped to 25.6% and 5.9%, in phases 2 and 3, respectively. This is especially important as unnecessary admission puts a great burden on hospitals and ward staff, both economically and in terms of staff workload. In terms of economic burden, a study in China in 2017 showed the mean total cost for mild outpatients were 201 Chinese Yuan (about 30 USD). The cost increased by almost five times to 1072 Chinese Yuan (160 USD) for mild inpatients [210]. With the policy of free medical care for all children under six years

old in Vietnam, unnecessary admission of mild cases posed a high burden for both health care system and the society as a whole. A comprehensive study of social-economic burden of HFMD in Vietnam is therefore needed to ascribe the burden of HFMD and to guide the development and implementation of intervention strategies in Vietnam, and is currently underway.

Increased burden on hospital staff may affect the quality of care for severe patients, especially in limited settings of Vietnam hospitals where nurse/doctor: patient ratios are already compromised compared to developed settings [211].

Misdiagnosis also appeared to be more common before the outbreak, with about 3% of patients who were first diagnosed with other diseases and admitted to other wards then being diagnosed with HFMD and transferred to infectious disease ward. After the outbreak, this rate decreased dramatically to 0.7%. This is likely due to increased awareness of medical staff about HFMD and increased familiarity with its presentation.

At a national level, there were a total of 29 fatal cases reported between 2013 and 2015 as compared to 207 fatal cases in 2011-2012 [43,212]. Patients in phase 3 (after the outbreak) admitted to hospital earlier than in phase 1 and 2 (before and during the outbreak). This prompt and timely admission and management may have reduced progression, leading to a better outcome. Notably, data synthesized from over 7 million HFMD cases in China showed that fatality mostly occurred within 2 – 4 days after illness onset [19], suggesting that early hospital admission may have played a role in improved outcome.

Similar to previous reports from Taiwan and China, we have documented several clinical features on admission, which were indicative of severe cases. Severe patients presented with higher temperature and longer fever [121,123,154]. Myoclonus, tremor and high blood glucose were also associated with severe cases. Although the retrospective nature of this study means that identification and evaluation of prognostic features is limited, the consistency of our findings with previous work and the prospective study (Chapter 2) should be noted.

Overall, this data provides important background information regarding HFMD characteristics in terms of demographic, clinical features and lab results which can be used as a basis for further epidemiological study. However the study was limited in terms of retrospective nature and included data from only one hospital. It may therefore not be represent the whole situation of Southern Vietnam and hence effort should be made to collect data from other hospitals to strengthen the results.

Collectively, the data pointed that early hospital admission, accurate clinical case classification and prompt management are key components for HFMD outbreak response.

3.5. CONCLUSION

This study systematically extracted data from seven consecutive years, analyzing the difference in patient demographics, clinical features and indicators of health-system responses with respect to HFMD before, during and after a major outbreak in Southern Vietnam. Results from this longitudinal study provide a picture of HFMD

features and the alteration of these features during time. These findings are also essential in planning future public health interventions and disease control strategy.

4. EVOLUTION OF ENTEROVIRUS A71 CAUSING HAND FOOT AND MOUTH DISEASE IN VIETNAM FROM 2013 TO 2015

4.1. INTRODUCTION

Enterovirus A71 (EV-A71)–associated HFMD is a major health problem in the Asia-Pacific region since the first noticeable outbreak occurring in Sarawak, Malaysia in 1997 (section 1.2.2).

Based on sequence analysis of the structural viral protein 1 (VP1), EV-A71 is classified into 3 genogroups (A, B and C). However, in recent years, newly described genogroups of EV-A71 have been reported from India (genogroups D and G) [105,213], Central Africa, and Madagascar (genogroups E and F) [106,107]. The discovery of these novel genogroups in new geographic regions further illustrates the pandemic potential of enteroviruses in general and EV-A71 in particular, and suggests that the number of circulating genogroups and/or the genetic diversity of EV-A71 is far greater than previously estimated [106].

Outbreaks of EV-A71 are usually associated with a switch of the predominant subgenogroup [100,104] and/or recombination events [55]. Likewise, sequence data from 2005 to 2011 from Vietnam showed a subgenogroup switch from C5 to C4 in 2011 [41,43], coinciding with an explosive outbreak that caused 174,677 hospitalizations and 200 deaths [43]. More recent data, from months of the study described in Chapter 2, shows that EV-A71 subgenogroup C4 was replaced by

subgenogroup B5 in 2013 in Vietnam, while C5 was sporadically detected. In contrast, C4 subgenogroup has been circulating in China as the dominant subgenogroup and has caused over one million cases a year since 2008 [214]. Of note, subgenogroup C4 was also responsible for the recent outbreak of severe HFMD in Cambodia in 2012 [39,215].

Together, these data emphasize that active molecular surveillance and understanding of the long-term evolution of EV-A71 is required, especially in countries where the virus is endemic, as such knowledge is essential to inform the development of appropriate intervention strategies, in particular the selection of appropriate and representative strains for vaccine development.

Because the evolutionary history of EV-A71 subgenogroups at a global scale has been extensively described elsewhere [104,108], the analysis in this chapter focused on Vietnamese sequences obtained from my project (chapter 2) and other Vietnamese sequences available in GenBank as per July 2017, mostly from southern provinces and particularly from Ho Chi Minh City: a metropole with a population of over 10 million.

4.2. MATERIALS AND METHODS

4.2.1. Clinical samples and RNA extraction

Viral sequences reported in this chapter were derived from samples collected from the above observational study (Chapter 2). EV-A71 real time RT-PCR positive samples with a Cp value <30 ware selected for direct whole-genome sequencing.

Viral RNA extraction was performed using the QIAamp viral RNA kit as described Chapter 2. Isolated RNA was then subjected to whole-genome amplification and sequencing on a MiSeq platform (illumina) using a workflow developed by my research group, shown in Figure 4.1.

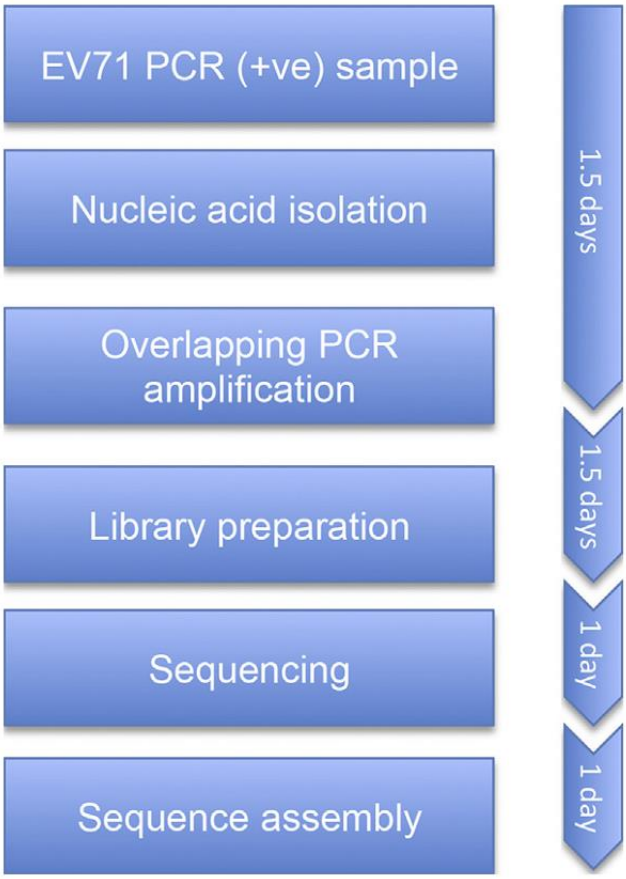


Figure 4.1. workflow for whole genome sequencing analysis of EV-A71 from chlldren with hand, foot and mouth disease from clinical samples

Figure obtained from reference [216].

4.2.2. RT-PCR

Whole genome sequences of EV-A71 were amplified from isolated RNA using 4 overlapping RT-PCRs. The PCR primers sequences are listed in Table 4.1.

Name	Sequence (5'→3')	Note	Nucleotide position
FL5F	TTAAAACAGCCTGTGGGTTGYACC	Amplicon 1	1 - 1128
FL7R	AACCTRTTCACBGAAACATC		
FL14F	AARGAC ATYTTCACTGAAATGGC	Amplicon 2	960 - 3180
FL9R	ACTAAAGGGTACTTGGACTTVGA		
FL15F	GGATTRGTWGGGGAGATAGACCT	Amplicon 3	2699 - 2721
FL16F	GGATTRGTWGGAGAGATAGACCT		2699 – 2721
FL17F	GGATTRGTWGGGGAGATAGATCT		2699 – 2721
FL18F	GGATTRGTWGGAGAGATAGATCT		2699 – 2721
FL19R	GTCACTTCAATRTCRCAGTCCAT		4827 – 4805
FL20R	GTCACTTCAATRTCRCAATCCAT		4827 - 4805
FL10F	CAAACACCGWATTGAACCTGT	Amplicon 4	4440 - 7430
FL3R2	GCTATTCTGGTTATAACAAATTTACC		

Table 4.1. Primer sequence used in RT-PCR

RT-PCR reactions were performed using the SuperScript III One-Step RT-PCR system with Platinum Taq DNA High Fidelity (Invitrogen, Carlsbad, CA, USA) in a Mastercycler (Eppendorf, Hamburg, Germany). Each RT-PCR reaction contained 12.5 µl of 2x reaction buffer (provided with the kit), 800 nM of each primer (Table 4.1), 5 µl of RNA and molecular grade water to make up a total volume of 25 µl. Thermal cycling conditions consisted of: RT- PCR1 (amplicon 1): 60 ^{0}C for 3 minutes, 53 ^{0}C for 30 minutes, 94 ^{0}C for 2 minutes; followed by 45 cycles of 94 ^{0}C for 15 seconds, 53 ^{0}C for 30 seconds, and 68 ^{0}C for 3.5 minutes and, finally, 5 minutes at 68 ^{0}C; RT-PCR2 (amplicon 2): 45 ^{0}C for 30 minutes, 94 ^{0}C for 2 minutes; followed by 45 cycles of 94 ^{0}C for 15 seconds, 53 ^{0}C for 30 seconds, and 68 ^{0}C for 3.5 minutes and, finally, 5 minutes at 68 ^{0}C; RT-PCRs 3 and 4 (amplicons 3 and 4): 60 ^{0}C for 3 minutes, 50 ^{0}C for 30 minutes, 94 ^{0}C for 2 minutes; followed by 45 cycles of 94 ^{0}C for 15 seconds, 50 ^{0}C for 30 seconds, and 68 ^{0}C for 3.5 minutes and, finally, 68 ^{0}C for 5 minutes.

4.2.3. DNA Quantitation and pooling

Individual PCR products were quantified by a fluorescence-based dsDNA quantification method using the Quant-iT *dsDNA Assay* Kit (Invitrogen) in a Qubit fluorometer (Invitrogen), following the manufacturer's instructions. In brief, 2µl of DNA, from clinical samples or standard DNA provided with the kit, was mixed with 198 µl Qubit solution (prepared from Qubit reagent and Qubit buffet – provided with the kit). The mixture was incubated at room temperature for 2 minutes and the DNA concentration in each sample was measured using a Qubit 2.0 fluorometer and pooled DNA samples with a concentration of 0.2 ng/µl were prepared.

4.2.4. Library preparation

Pooled DNA samples were subjected to a library preparation step using the Nextera XT DNA sample preparation kit (Illumina, San Diego, CA, USA), as detailed below.

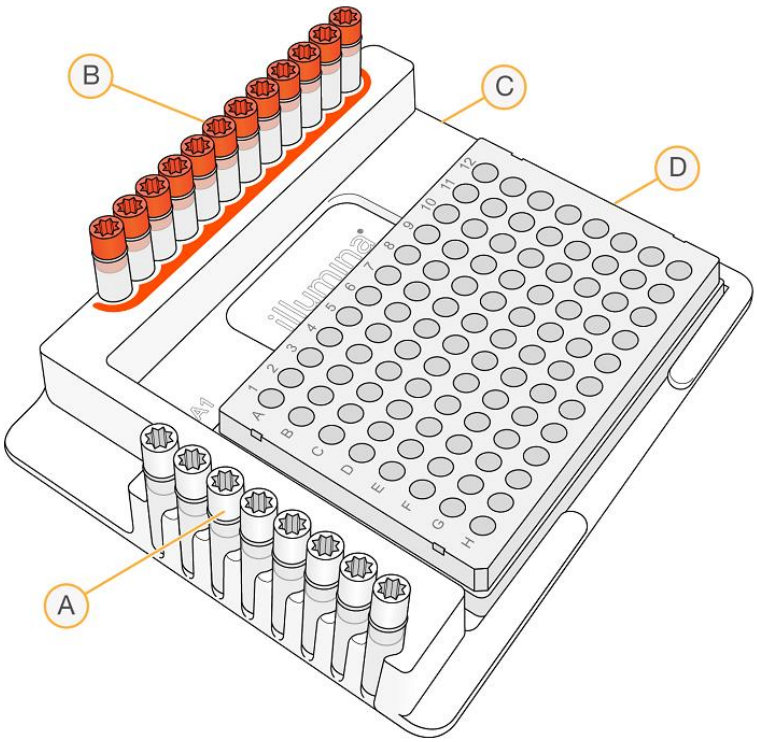
4.2.4.1. DNA tagmentation

This step uses Nextera transposome to randomly cut amplified PCR products into fragments for subsequent adapter ligation and PCR amplification to enrich for initial input. In brief, 10µl of tagment DNA buffet (provided with the kit), 5µl of DNA and 5µl of Amplicon Tagment Mix (provided with the kit) were added to each well of a 96-well PCR plate. The plate was then incubated at 55 ^{0}C for 5 minutes and cooled down to 10 ^{0}C in a thermal cycler. Next, 5 µl of neutralize tagment buffet was added and the plate was incubated at room temperature for 5 minutes.

4.2.4.2. DNA library amplification

Index 1 (i7) and index 2 (i5) adapters (provided with the kit) were arranged across a TruSeq Index Plate Fixture (as shown in Figure 4.2)

5 µl of each index adapter and 15 µl of Nextera PCR Master Mix (provided with the kit) were added to each well of the tagmented DNA prepared above. The mixture was then subjected to an amplification step using a thermal cycling condition consisting of 72 ^{0}C for 3 minutes, 95 ^{0}C for 30 seconds; 12 cycles of 95 ^{0}C for 10 seconds, 55^{0}C for 30 seconds, and 72^{0}C for 30 seconds, and, finally, 72 ^{0}C for 5 minutes and hold at 10 ^{0}C. The total volume of each reaction after this step is 50µl.



A Rows A–H: Index2 (i5) adapters (white caps)

B Columns 1–12: Index1 (i7) adapters (orange caps)

C TruSeq Index Plate Fixture

D Hard-Shell PCR plate

Figure 4.2. Setup of TruSeq Index Plate Fixture for 96 Libraries

Obtained from reference [217]

4.2.4.3. Library cleaning up

This step uses AMPure XP beads (Beckman Coulter, Inc., Fullerton, CA, USA) to purify the prepared DNA library and selectively capture DNA fragments of around 500bp in length. 30 µl of AMPure XP beads was added to each well preloaded with DNA prepared above. The plate was then incubated at room temperature for 5 minutes and placed on a magnetic stand for 2 minutes. Next, 200 µl of 80% ethanol was added to each well. The supernatant was removed, and the pellets were air dried for 15 minutes. Finally, 52.5 µl of suspension buffer (provided with the kit) was added and 50 µl of supernatant was transferred to a new plate after incubation.

4.2.4.4. Library normalization

This process normalizes the quantity of each library to ensure more equal library representation in the pooled library. 20 µl of cleaned-up sample was transferred to a 96-well PCR plate, followed by 45 µl of mix (containing library normalization additives 1 and 800 µl library normalization beads 1(both provided with the kit)). The plate was then shaken at 1,800 rpm for 30 minutes. The supernatant was discarded and 45 ul of library normalization ash 1 (provided with the kit) was added to each well. The plate was shaken again at 1,800 rpm for 5 minutes. The supernatant was discarded and 30 µl NaOH (0.1M) was added. Finally, after another shaking step, 20 µl of supernatant was transferred to a new plate, pre-loaded with 20 µl of library normalization storage buffer 1 (provided with the kit).

4.2.4.5. Library pooling, quality assessment and MiSeq sample loading.

Each sample was diluted at 1000-fold in molecular grade water and 5 µl each library was transferred to a new tube. The DNA quality was assessed using KAPA library quantification Kit (KAPA Biosystems, Wilmington, Massachusetts, USA). 4µl diluted sample was mixed well with 6µl KAPA mix (provided with the kit) and the PCR was run at the following conditions: 95 ^{0}C for 5 minutes, 34 cycles of 95 ^{0}C for 30 seconds and 60 ^{0}C for 45 seconds. DNA quantification was performed by reviewing the background-subtracted (normalized) amplification curves and the Cq scores (quantification cycle) for replicate data points (DNA Standards, libraries and controls), and exclusion of obvious outliers. Replicate data points should differ by ≤0.2 cycles.

After quantification, 24µl sample and 576µl hybridization buffer were loaded onto a thawed Miseq reagent cartridge.

4.2.5. MiSeq sequencing and sequence assembly

Sequencing of the prepared library was carried out using the Miseq reagent kit v2 (300 cycles, Illumina) on an Illumina Miseq platform. For each run, 96 samples were multiplexed and differentiated by double indexes using the Nextera XT Index Kit (Illumina).

The obtained sequences were processed to remove PCR primer sequences using CLC Genomic Workbench (QIAgen) and sequence assembly was performed using the Geneious 8.1.5 software package (Biomatters, San Francisco, CA, USA) utilizing a

reference-based mapping tool (i.e. the consensus sequence was obtained by mapping individual reads of each sample to a reference sequence) [216].

4.2.6. Quality control check of whole genome sequences prior to phylogenetic analysis

Using the Geneious software (Biomatters), genetic distances were first calculated among the included sequences for phylogenetic analysis to identify and remove duplicate sequences. Recombinant sequences (see below) were also removed. The remaining sequences were then assessed using TempEst 1.5 [218] in order to check whether there was sufficient signal in the data to proceed with subsequent analyses, especially those carried out in BEAST (see below). The TempEst output was a linear regression of root-to-tip genetic distances of input sequences. A correlation coefficient of $R2 > 0.7$ was applied to remove outliers, whose sampling dates were not in agreement with their genetic divergence and phylogenetic positions. Outliers could include but were not limited to recombinants, sequences of low quality due to sequencing/assembly errors or sequences with mislabeling of the sampling dates. These were therefore uninformative for subsequent phylogeographics analysis.

4.2.7. Phylogenetic analysis

All alignments were carried out using the multiple-sequence alignment tool available in Geneious 8.1.5 (Biomatters).

To assess the phylogenetic relationships between Vietnamese viruses from the present study and global sequences, a dataset consisting of 490 whole-genome

sequences (114 sequences obtained from this study and 376 representative sequences downloaded from GenBank as of July 2017, covering all subgenogroups from different countries worldwide) was used. Maximum – likelihood (ML) trees were re-constructed using a nucleotide substitution model (SYM) and Gamma distribution of among – site rate variation available in IQ-TREE, version 1.4.3 [219]. Support for individual nodes was assessed using a bootstrap of 1000 replicates. I then used FigTree software (http://tree.bio.ed.ac.uk/software/figtree/) to visualize the obtained ML tree.

The evolution rates and divergence time of EV-A71 subgenogroup B5 were estimated for both VP1 (n=101, including 73 from this study) and whole genome sequences (n=159, including 124 from this study). VP1 sequence data also included sequences from northern Vietnam. All analyses were carried out in BEAST, version 1.8.3 [220]. For all analyses, whole genome and VP1 data were analyzed using the General Time Reversible (GTR) + Gamma 4 nucleotide substitution model and the VP1 Tamura and Nei 1993 (TN93) + Gamma 4 nucleotide substitution model, respectively. They were best fitting models, as previously generated and tested with IQ-TREE. Relaxed Bayesian skyline analysis available in BEAST was first employed to identify the suitable Bayesian Markov Chain Monte Carlo framework that gave the best convergence (effective sample size – ESS of all parameters above 200 was accepted). Output files were then visualized using Tracer 1. Lengths of chain of 100 million and 200 million were used for VP1 and whole genome analysis, respectively.

Data were tested with two clock models (strict and relaxed) and two coalescent modes (constant size and Bayesian skyline). The model with a Bayes factor closest

to 0 was considered the best fitting model and was used to estimate the relative genetic diversity overtime and evolution rates. Maximum clade credibility (MCC) trees, generated in BEAST, were then visualized using FigTree.

4.2.8. Selection pressure

Analysis of selection pressure was done using three different methods (Estimate selection for each codon, Z test of selection and Fisher's Exact test of selection) available in MEGA5 [221].

4.3. RESULTS

4.3.1. EV-A71 sequences

In total, 135 complete genomes of EV-A71 were successfully sequenced. This comprises 14% of the current collection of whole-genome sequences globally (n=991) and 68% of whole genome sequences from Vietnam (n=200) deposited to GenBank.

4.3.2. Phylogeny of EV-A71 and circulation of EV-A71 subgenogroups in Vietnam

Phylogenetic analysis of 490 complete VP1 sequences representing all (sub)genogroups, including the newly discovered genogroups D, E, F and G revealed that 135 EV-A71 sequences generated as part of the present study belonged to subgenogroups B5 (n= 125) and C4 (n=10) (Figure 4.3). An overview of the monthly distribution of EV-A71 subgenogroup C4 and B5 sequenced in southern Vietnam

between 2013 and 2015 is presented in Figure 4.6. Viruses of subgenogroup C4 were only detected between September 2014 and March 2015.

Phylogenetic analysis revealed there were two viral lineages of subgenogroup B5 that were circulating and causing HFMD in Vietnam: one related closely to the Malaysian lineage and the other related to Taiwanese strain (Figure 4.4). As for C4 viruses sequenced as part of the present study, they clustered closely together with C4 sequences from Vietnam from between 2011 and 2012 (Figure 4.5). Together with Cambodian C4 sequences, they formed a distinct viral lineage, which suggests they originated from the same source, most likely China [108].

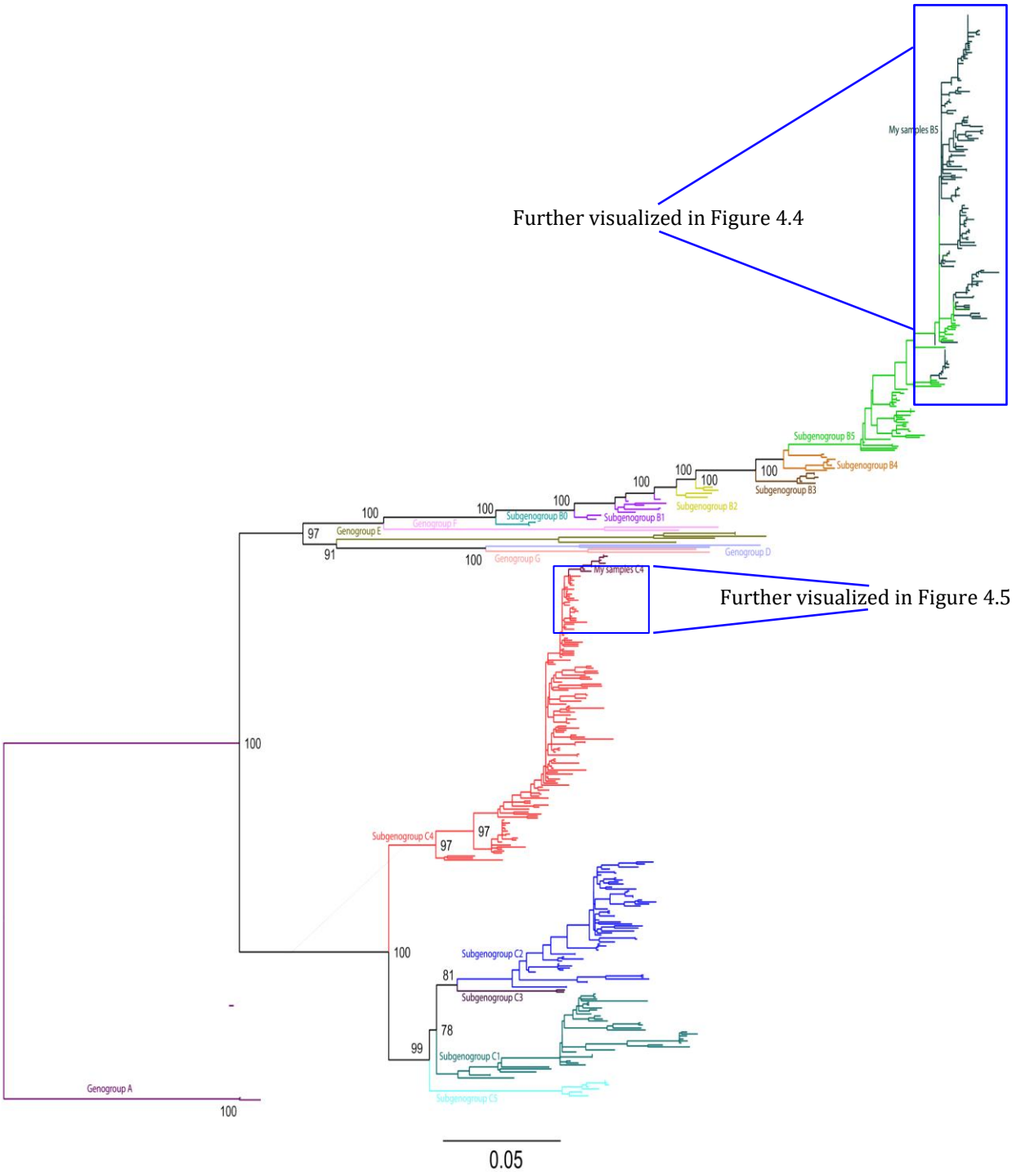


Figure 4.3. Maximum likelihood tree of 490 representative VP1 sequences of all (sub)genogroups of EV-A71 collected in this study (n=114) and from GenBank (n=376).

Branches were color – coded according to genogroups and subgenogroups. Numbers indicated bootstrap values.



Figure 4.4. A subtree (from the Maximum Likelihood Tree shown in Figure 4.3) showing two lineages of subgenogroup B5 circulating in Vietnam: one originated from Malaysian lineage and the other one originated from Taiwanese lineage.

The dark green clusters are sequences from this study and the bright green clusters are global sequences.



Figure 4.5. A subtree (from the maximum likelihood tree shown in Figure 4.3) showing the tight clustering of subgenogroup C4 in Vietnam.

Dark red indicates sequences obtained from this study and red indicates global sequences, including sequences from Vietnam previously published.

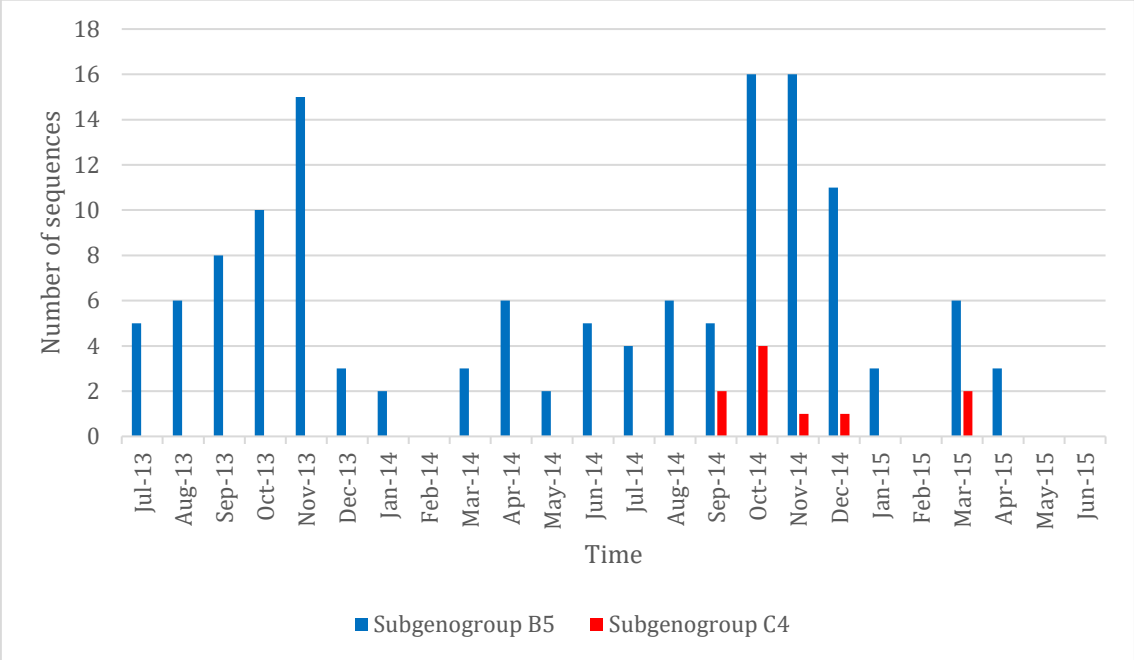


Figure 4.6. Temporal distribution of EV-A71 subgenogroup B5 and C4 2013 – 2015 from children with HFMD in three hospitals in Ho Chi Minh City.

4.3.3. Demographics of Vietnamese EV-A71 subgenogroup B5 in Vietnam

Bayesian skyline analysis was used to reveal temporal changes in the relative genetic diversity of EV-A71 subgenogroup B5 in Vietnam. Notably, the relative genetic diversity of subgenogroup B5 increased sharply in 2012, and then remained relatively stable at high level from 2013 to 2015 (Figure 4.7), coinciding with a complete switch from C4 to B5 in Vietnam in 2013 [44].

The most recent common ancestor of EV-A71subgenogroup B5 viruses circulating in Vietnam was estimated in 2005 – 2009: March 2006 (95 HPD – highest posterior density- June 2005 - January 2007) for whole genome sequence based analysis and May 2008 (95 HPD Dec 2006 – Sep 2009) for VP1 sequence based analysis.

The different result of most common ancestor between VP1 and whole genome in this study is due to the differences in reference sequences used. Most of published sequences from Vietnam are restricted to gthe VP1 gene with limited numbers of whole genome sequences available. The results from whole genome sequences should be more accurate because although VP1 is the most diverse region, changing at other non-structural regions was also reported [222].

4.3.4. Phylogeography of EV-A71 subgenogroup B5 in Vietnam

Understanding the spatial distribution of an emerging pathogen within an endemic country is essential for outbreak control. Phylogeographic analysis was employed to reveal the transmissibility of EV-A71 subgenogroup B5 between provinces within Vietnam using both whole genome and VP1 datasets. Detail analysis for all provinces

(13 provinces for whole genome dataset, and 16 provinces for VP1 dataset which included additional sequences from northern areas) from where the patients came were uninformative due to the small sample sizes of corresponding datasets. The results of phylogeographic analyses for main discrete geographic locations in Vietnam showed high fluidity within southern Vietnam (Figure 4.8). Inference for viral movement between south and north is prohibited by a small number of viruses used for analysis. However, the viruses from the north were observed to cluster tightly and form a distinct cluster on the MCC tree (Figure 4.9), suggesting a low level of viral movement between south and north in Vietnam.

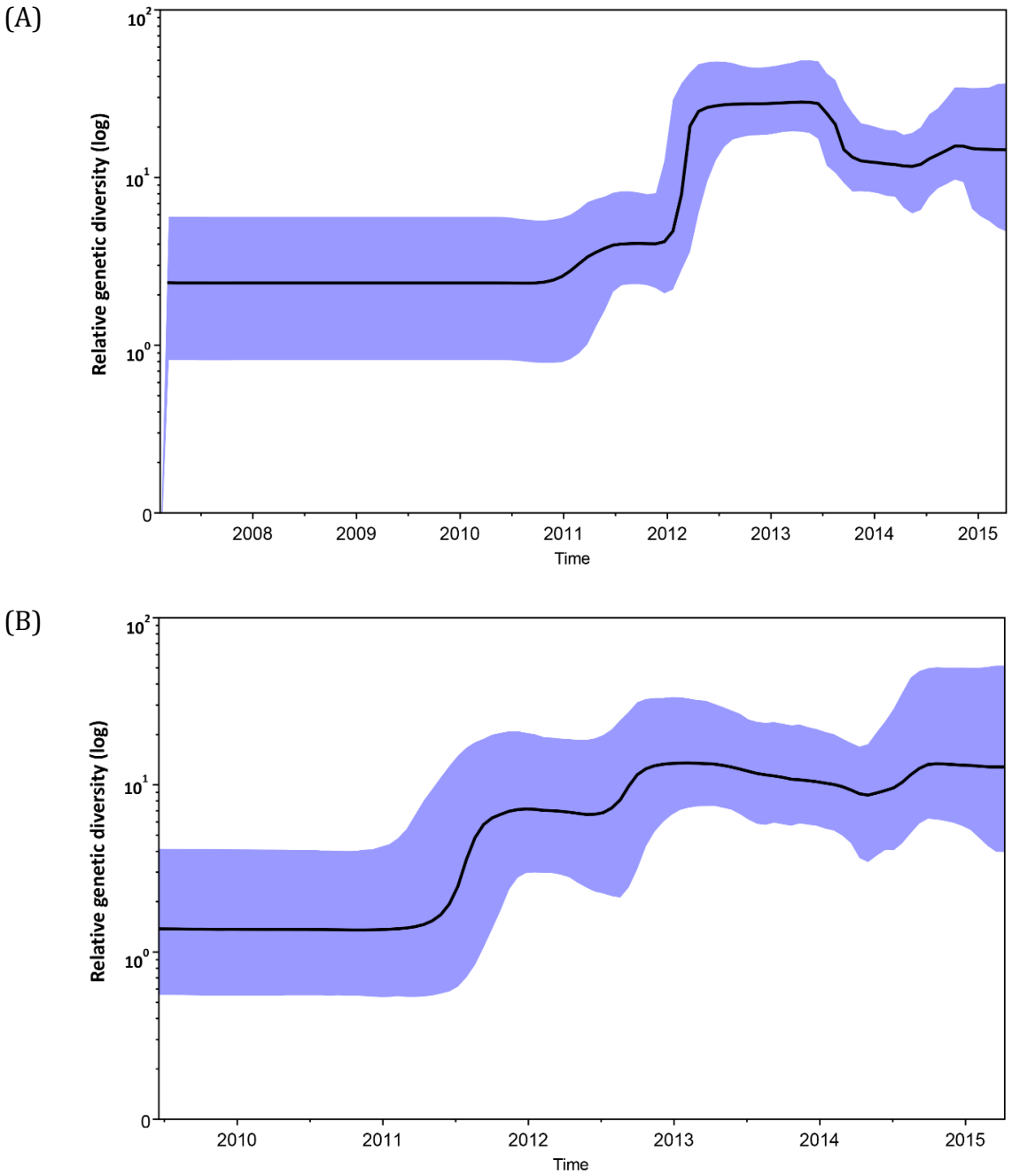


Figure 4.7. Bayesian skyline plots illustrating the relative genetic diversity of EV-A71 subgenogroup B5 in Vietnam over time from (A) whole-genome sequences and (B) VP1 sequences.

The black line indicates the mean while the blue area shows the upper and lower 95% HPD values

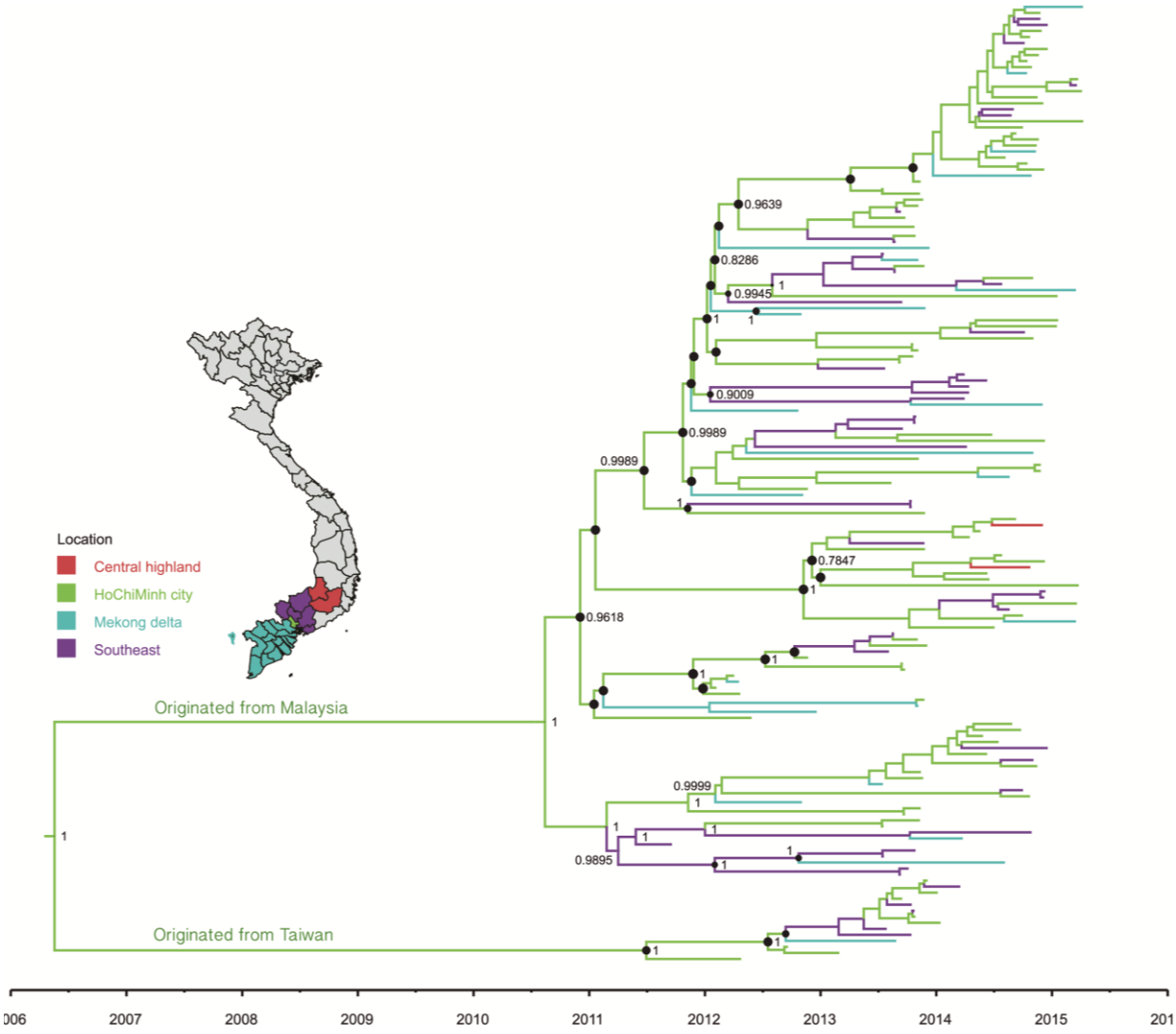


Figure 4.8. MCC tree of 159 EV-A71 subgenogroup B5 whole genome sequences. Branches were color-coded by location sampling.

Posterior probabilities ≥70% and state probabilities ≥70% (black circles) are indicated at all nodes. An accompanying map obtained from https://mapchart.net was added to panel A to illustrate 5 discrete states in Vietnam.

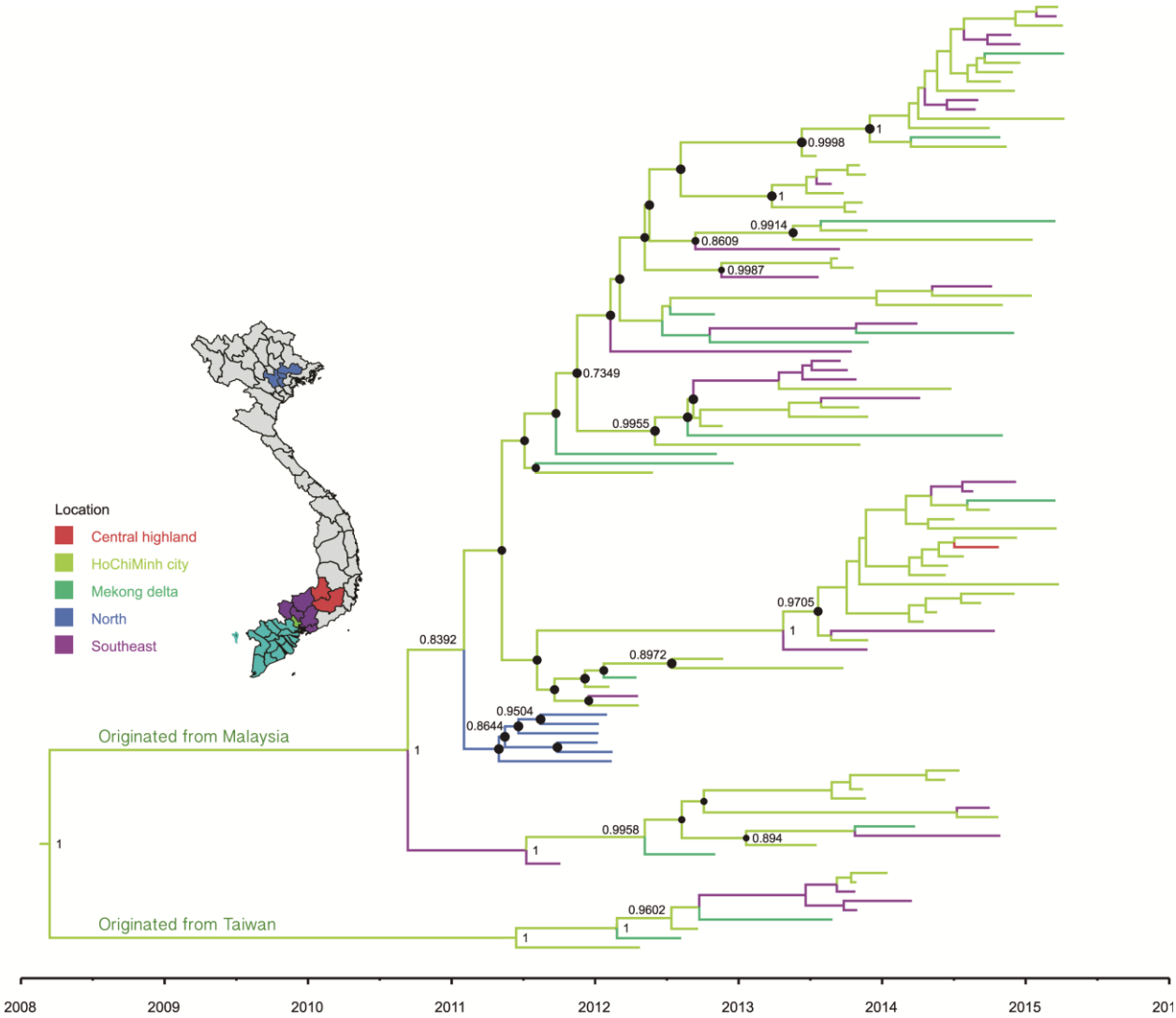


Figure 4.9. MCC tree of EV-A71 subgenogroup B5 VP1 sequences. Branches were color-coded by location sampling.

Posterior probabilities ≥70% and state probabilities ≥70% (black circles) are indicated at all nodes. An accompanying map obtained from https://mapchart.net was added to panel A to illustrate 5 discrete states in Vietnam.

4.3.5. Nucleotide substitution rate and selection pressure of subgenogroup B5

The rate of nucleotide substitution among whole-genome and VP1 sequences of EV-A71 subgenogroup B5 was estimated at 3.9 x 10^{-3} and 5.12 x 10^{-3} substitutions/site/year, respectively.

To explore the degree of selective pressure on VP1 and complete coding region of EV-A71 subgenogroup B5, the rate of nonsynonymous (dN) and synonymous substitution (dS) was estimated. Maximum likelihood based analysis revealed the estimates of mean dN/dS values were 0.0465 and 0.0428 for VP1 and complete coding region, respectively. This suggested that EV-A71 subgenogroup B5 evolution was driven by strong negative selection. Investigation for dN/dS ratios of individual codons did not reveal any sites that underwent positive selection pressure.

Due to the small number of EV-A71 subgenogroup C4 sequenced in the present study in-depth phylogenetic analysis and back-to-back comparison of associated demographics and clinical outcomes of these two subgenogroup was deemed uninformative.

4.4. DISCUSSION

The circulation and the evolutionary history of EV-A71 subgenogroups in southern Vietnam between 2013 and 2015 was described using samples collected as part of the clinical study described in Chapter 2. Unlike other countries in the region such

as China, subgenogoup B5 was predominant during this period of time, while viruses of subgenogroup C4 were only sporadically detected at the end of 2014.

Following a switch of predominant subgenogroup among clinically detected viruses from C4 to B5 in 2013 [44], the sporadic detection of C4 in late 2014 and the tight clustering of those C4 sequences with previously reported C4 sequences from Vietnam suggested that after causing the large outbreak in 2011-2012, this subgenogroup C4 continues to circulate at a low level of transmission in Vietnam and/or only caused (asymptomatic) infection that was not severe enough for the patients to come to the hospital.

Subgenogroup B5 viruses exclusively circulate in the Asia-Pacific region and have been responsible for large HFMD outbreaks in Malaysia, Brunei and Taiwan [22,223,224] with an estimated time of emergence of around 2001.2 (HPD 2000.6 – 2001.8) [104]. In terms of genetic diversity, B5 has established two separate lineages that have previously been reported in Malaysia and Taiwan, respectively [104,108,225], and recently these two lineages were imported into Vietnam with the Malaysian lineage being the dominant viruses up to 2013. Likewise, my analysis showed that Vietnamese B5 viruses also belonged to these two different lineages, and the Malaysian strains remains predominant in Vietnam until 2015.

As for the phylogeographic pattern, diverged lineages of subgenogroup B5 have been endemic in multiple Asian countries with occasional viral movement across the countries [108], while viral movement between localities within an endemic country is more prominent. The latter is in agreement with the observed

phylogeograpgic pattern of EV-A71 subgenogroup B5 in Vietnam obtained in the present study and a recent report from Vietnam [108].

Observational data has shown that in the past subgenogroup switches often accompany large EV-A71-associated HFMD outbreaks [100,104,224,226].

However, the work presented here shows that following the subgenogroup switch from C4 to B5 in 2013, there was a decrease of the numbers of reported cases and both the numbers and proportion of fatalities in Vietnam. The reasons underlying this form key research questions for future HFMD research and I have begun to address some in this thesis. Possibilities include a complex interplay between cross-immunity, pathogen evolution, herd immunity, and public health responses (Chapter 3). For pathogen evolution, result in this chapter was only partially in agreement with previous studies. Notably, results obtained to date (including from this study) have consistently shown that EV-A71 (including subgenogroup B5) evolution is under strong purifying selection and the rate of nucleotide substitution is around 4 – 5 × 10-3 substitutions/site/year. However, while my analysis of B5 sequences did not reveal any specific amino acid residue that underwent positive selection, a recent study using B5 viral sequences from Vietnam generated by VP1 sequencing of viral culture isolates and worldwide showed that amino acid residues 43 and 145 of the VP1 protein were under positive selection [108], which may have important implication for vaccine development. Similarly, a switch from E to K at residue 43 of VP1 protein among B5 viruses in 2013 reported in a recent study [108] was not observed in any of our B5 sequences (data not shown). Of note, all EV-A71 genomes obtained in this study were recovered directly from clinical samples

without in vitro culture while samples from Thao's study came from human rhabdomyosarcoma (RD) (ATCC CCL136) and African green monkey kidney (Vero) (ATCC CCL81) cell lines [108]. Whilst the extent to which EV-A71 may adapt to in vitro cell culture system has been a neglected area of research, it is without a doubt that our results more accurately reflect the genetic diversity of the pathogen in human populations.

This analysis revealed that Vietnamese B5 viruses had their most recent common ancestor in ~2008. Of note, we have previously reported that EV-A71 subgenogroup B5 was first sporadically detected in hospitalized HFMD patients in southern Vietnam in 2011 [44], and then became dominant subgenogroup causing HFMD in Vietnam in 2013. The results presented here are thus in accordance to previous findings from VP1 sequences, which suggested that emerging subgenogroups may cryptically circulate in endemic countries for 2-3 year prior to their appearance [104].

Over the last years, despite the decrease in terms of both the number of severe/fatal cases of HFMD, on average, around 80,000 HFMD cases have been reported in Vietnam. This suggested the high endemicity/transmissibility of HFMD causing pathogens including EVA71, which is in parallel with the observed skyline plots of subgenogroup B5. Indeed, skyline analyses revealed the relative genetic diversity of subgenogroup B5 increased sharply in 2012, and then remained stable from 2013 to 2015. It confirmed our previous results showing that the relative genetic diversity of B5 rapidly increased in 2012 and remained high in 2013 [44]. While the present study represented the first to reconstruct a long-term relative genetic diversity of

EV-A71 in Vietnam for a period over 5 years (2011 – 2015), the result is in accordance with previous findings for EV-A71 subgenogroups that have established high transmissibility in a locality (e.g. subgenogroup C4 in China) [108].

Whilst the findings in this chapter represent significant new knowledge in this area, this study has some limitations. Firstly, the results from this chapter are only based on surveillance of the major hospitals in Ho Chi Minh City, which admitted patients from all southern provinces. The circulating viruses in the north and central parts of Vietnam were obviously missed. Secondly, surveillance based on disease (HFMD) did not refect a full picture of virus circulation and different serotypes or subgenogroups may vary in the pathogenicity. To better reveal the evolutionary history of EV-A71 in Vietnam, it is imperative that a more comprehensive sampling approach is used. Thirdly, only EV-A71 positive samples with a Cp value of <30 were subjected to sequencing, which may have resulted in an underestimation of the diversity of EV-A71 and subgenogroup circulation. Of note, subgenogroup C5 was previously reported to continuously circulate in Vietnam between 2003 and 2013, but was not detected in the present study.

4.5. CONCLUSION

This chapter's results describe in detail the predominantly circulating EV-A71 subgenogroups in southern Vietnam and the evolution of subgenogroup B5 (the major subgenogroup in Vietnam from 2013 – 2015). Together with etiological results described in chapter 2, this contributes to a more complete picture of the

causative agents of HFMD in southern Vietnam, and thus may be helpful in vaccine development and public health intervention.

5. A GENOME-WIDE ASSOCIATION STUDY OF SEVERE HAND FOOT AND MOUTH DISEASE CASES

5.1. INTRODUCTION

Severe HFMD is a result of complex interactions between pathogens and host immune responses.Although EV-A71 was detected more frequently in severe HFMD cases, there is still no clear evidence that a particular subgenogroup can associated with disease severity [227,228]. Results from the Singapore genetic study, showing the association of HLA-33 with susceptibility of EV-A71 infection and HLA-A2 with the development of cardiopulmonary complication in HFMD patients [183], suggested that host genetics may play a role in the mechanism of severe HFMD.

The International HapMap project created a large database, recording patterns of genetic variation and linkage disequilibrium of different populations. With the introduction of advanced genotyping chips and advanced computer systems, allowing screening for hundreds of thousands of SNPs across the whole human genome, genome wide association study (GWAS) became affordable and feasible. Compared with other genetic approaches, GWAS appears to be the most suitable method for HFMD initial screening in terms of timing and cost-benefit (more details in section 1.6.1.3).

The host genetic approach has been applied to detect genetic factors associated with HFMD susceptibility and severity and already some candidate genes have been identified [187,188,191]. However, because non-significant P values at GWAS level

were achieved in these studies (P value should be less than 10^{-8}), more efforts should be undertaken to confirm and validate those findings and to expand the number of potential genetic associations.

To explore the potential contribution of host genetic to development of severe HFMD, herein I conducted a GWAS study of DNA samples from Vietnamese patients with HFMD of varying disease severity. I hypothesize that there are host genetic variations associated with severe HFMD.

5.2. MATERIALS AND METHODS

5.2.1. Samples for HFMD cases

Blood samples for genotyping were collected from patients enrolled in the prospective study described in Chapter 2. The study was conducted at Children's Hospital 1, Children's Hospital 2 and the Hospital for Tropical Diseases: the three largest referral hospitals for HFMD treatment in southern Vietnam. All study sites and the Oxford Tropical Research Ethics Committee (OxTREC) approved the study protocol and the application of host genetics analysis.

Patients were clinically diagnosed with HFMD and disease severity was classified according to MoH guidelines. Severe HFMD was defined as grade 2B or higher regardless of etiology, and non-severe HFMD cases included patients with grade 1 or 2A illness with positive EV-A71 results. Two ml of whole blood was collected at enrollment day from all patients and stored at -80 ^{0}C for further use.

5.2.2. Samples for control

Controls were otherwise healthy individuals, diagnosed with primary closed angle glaucoma from the Vietnam National Institute of Ophthalmology in Hanoi, Hue University Hospital in Da Nang, Viet Tiep general hospital in Hai Phong and Ho Chi Minh Ophthalmology hospital, Vietnam. These individuals have no reported history of serious infections, and have been previously described by Khor CC et al [229]. The controls were genotyped on the same genome-wide genotyping array as the HFMD cases using the Illumina OmniExpress 24 bead-array (Illumina). This bead-array has been applied in previous studies and is an effective and powerful technique to screen for genetic associations [229,230]. This practice of genotyping cases and controls using the same array minimizes experimental artifacts and biases which would otherwise occur [231].

We calculated the control population according to the number of severe enrolled cases. The inclusion of >1357 controls, matched to >129 severe HFMD cases, provides optimal scaling (>10 to 1 control to case ratio) to maximize statistical power to detect genetic associations [232]. At the time of selecting controls, 129 severe HFMD cases were available for genotyping. More controls and severe cases from the prospective study will be added for a more powerful statistic result in the future. However, in this thesis, I focused on the result from the first phase of GWAS screening.

5.2.3. Genotyping process

5.2.3.1. DNA extraction

DNA extraction of whole blood was performed at OUCRU before sending to the Genome Institute of Singapore (GIS). Samples were purified and extracted using the QIAamp DNA Mini Kit (QIAgen), following the manufacturer's instructions. 20 µl QIAGEN protease was first added to a 1.5ml micro centrifuge tube, followed by 200 µl of sample and 200 µl of buffer AL. The mixture was then incubated at 56 ^{0}C for 10 minutes. Next, 200 µl of ethanol 100% was added to the mixture. Then the mixture was applied to the QIAamp Mini spin column (placed in a 2ml collection tube) and the column was centrifuged at 6000 × g. DNA was then washed thoroughly with buffer AW1 and AW2. Finally, total DNA was recovered in 200 µl of buffer AE and stored at -80 ^{0}C until use.

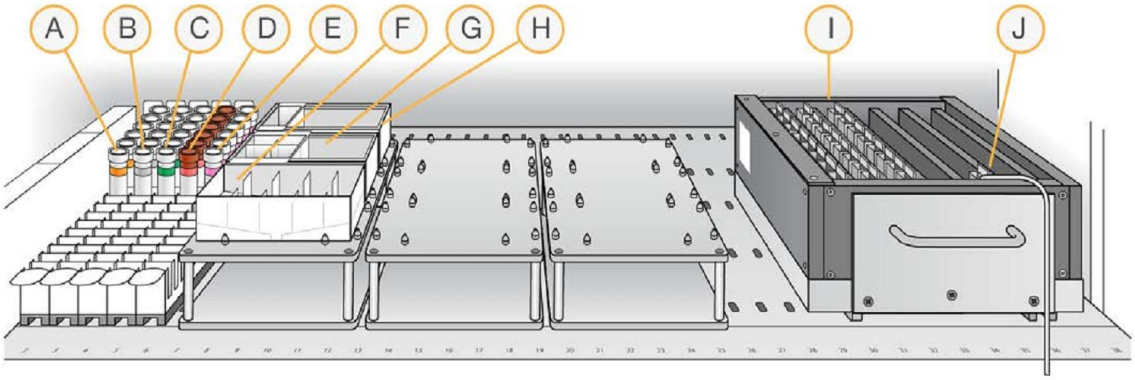
5.2.3.2. DNA quantitation and quality control

Extracted DNA samples were quantified, using the PicoGreen ®dsDNA quantitation kit (Thermo Fisher Scientific, Invitrogen, USA), following manufacturer's instructions. In short, DNA samples and PicoGreen were diluted 100 to 200 times in TE buffer. Diluted PicoGreen solution was mixed with the diluted DNA in 96 well plates in a volume ratio of 1:1. Each plate contained a duplicate set of standards ranging from 0 to 1000ng/ml. DNA measurement was performed using a GENios Tecan reader (Tecan, Zurich, Switzerland) at 485 nm excitation / 535 nm emission and DNA concentrations were calculated using Optima 2.0 R3 software.

5.2.3.3. Genotyping

DNA samples were quantified again after reception at GIS using the Picogreen dsDNA assay. SNP genotyping was done using the Illumina Human OmniExpress-24 BeadChip (Illumina, California, CA, USA), following the manufacturer's instructions. Briefly, cases and controls were randomly applied on plates and were genotyped with the Illumina Human OmniExpress-24 BeadChip (Illumina). DNA was first denatured by adding 20µl of multi - sample amplification 1 mix, and 4 µl NaOH to each well, neutralized with 34 µl multi-sample amplification 2 mix and amplified by adding 38 µl of multi – sample amplification master mix, then incubated overnight at 37 ^{0}C in the Illumina Hybridization Oven. This step ensured that each DNA sample was amplified sufficiently for genotyping. Amplified DNA then was enzymatically fragmented by adding 25 µl fragmentation solution to each well containing sample and placing the plate on the 37 ^{0}Cheat block for 1 hour, precipitated by adding 50 µl of precipitation solution, incubating at 37 ^{0}C for 5 minutes then adding 155 µl 100% 2-propanol to each well and resuspended with 23 µl of resuspention, hybridization and wash solution prior to the hybridizing step. Resuspended DNA samples were then loaded to BeadChips and these were put into a flow-through Hyb chamber (Illumina). The chambers were incubated at 48 ^{0}C for 16-24 hours, allowing the samples to hybridize to the BeadChip. After incubation, BeadChips were washed to remove the unhybridized DNA. The last step was to extend primers hybridized to DNA on the BeadChip with labeled nucleotides. The labeled extended primers then underwent a multi-layer staining process on the chamber rack. This step was done automatically with a Tecan eight – tip robot (Tecan, Zurich, Switzerland). Reagents

included resuspension, hybridization, and wash solution, XStain BeadChip solution 1, XStain BeadChip solution 2, Two-Color Extension Master Mix, XStain BeadChip solution 3, XStain BeadChip solution 4, Superior Two-Color Master Mix, Anti-Stain Two-Color Master Mix and Reagent used to prepare BeadChips for hybridization. The robot was set up following the manufacture's manual and run with option XStain Tasks | XStain LCG BeadChip (at the control computer). Stained BeadChips were read by IScan system and data was ready for analysis.



A LX1

B LX2

C EML

D SML

E ATM

F XC3 in Full Reservoir

G RA1 in Half Reservoir

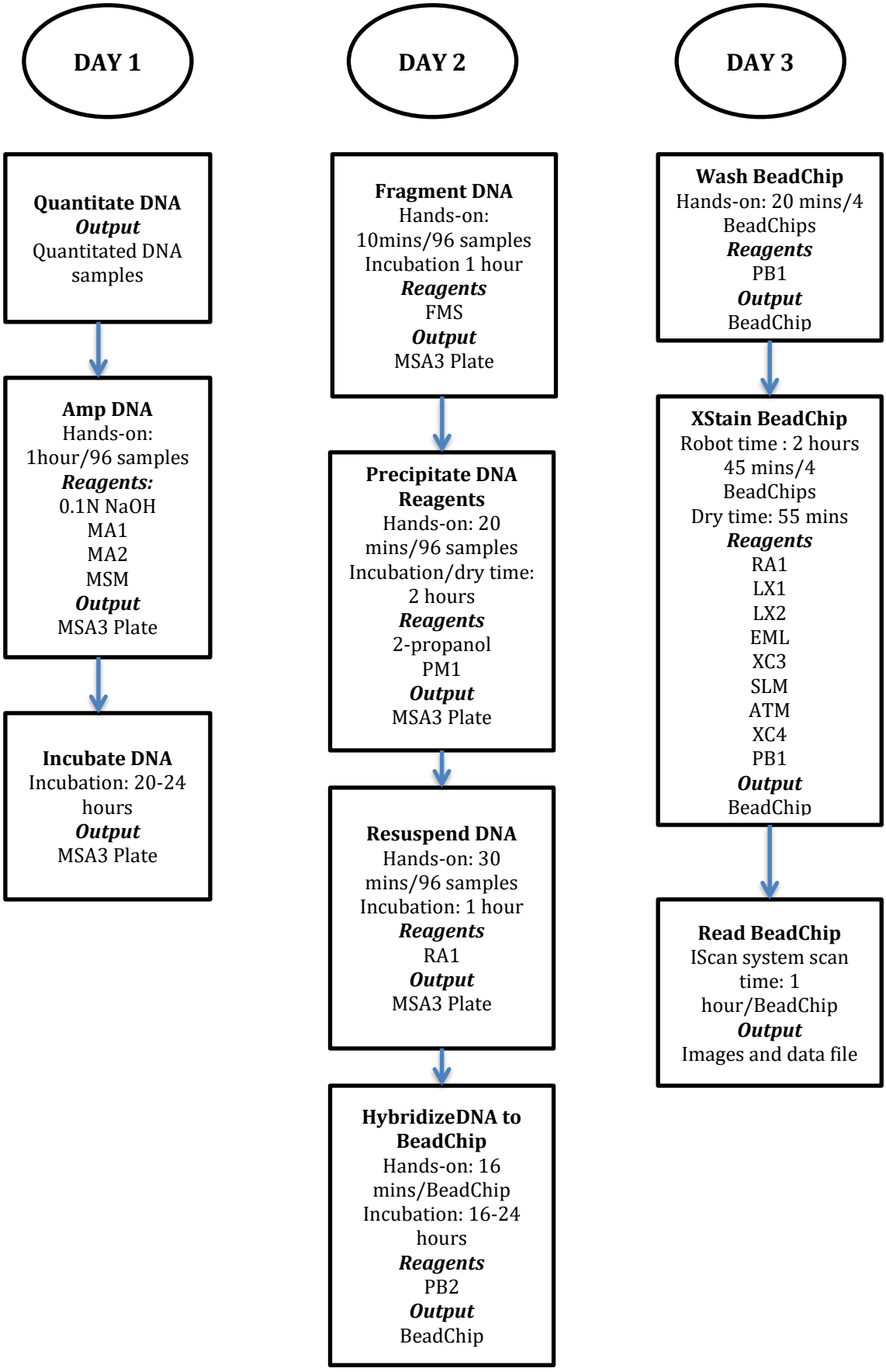
H 95% Formamide / 1 mM EDTA in Quarter Reservoir

I 24 BeadChips in Chamber Rack

J Temperature Probe

Figure 5.1. Setting Tecan robot for XStain step.

Obtained from reference [233]


DAY 1
Quantitate DNA
Output
Quantitated DNA samples
Amp DNA
Hands-on: 1hour/96 samples
Reagents:
0.1N NaOH
MA1
MA2
MSM
Output
MSA3 Plate
Incubate DNA
Incubation: 20-24 hours
Output
MSA3 Plate
DAY 2
Fragment DNA
Hands-on: 10mins/96 samples
Incubation 1 hour
Reagents
FMS
Output
MSA3 Plate
Precipitate DNA
Reagents
Hands-on: 20 mins/96 samples
Incubation/dry time: 2 hours
Reagents
2-propanol
PM1
Output
MSA3 Plate
Resuspend DNA
Hands-on: 30 mins/96 samples
Incubation: 1 hour
Reagents
RA1
Output
MSA3 Plate
HybridizeDNA to BeadChip
Hands-on: 16 mins/BeadChip
Incubation: 16-24 hours
Reagents
PB2
Output
BeadChip
DAY 3
Wash BeadChip
Hands-on: 20 mins/4 BeadChips
Reagents
PB1
Output
BeadChip
XStain BeadChip
Robot time : 2 hours 45 mins/4 BeadChips
Dry time: 55 mins
Reagents
RA1
LX1
LX2
EML
XC3
SLM
ATM
XC4
PB1
Output
BeadChip
Read BeadChip
IScan system scan time: 1 hour/BeadChip
Output
Images and data file

ATM—Anti-Stain Two-Color Master Mix, FMS—Fragmentation solution, MA1—Multi-Sample Amplification 1 Mix, MA2—Multi-Sample Amplification 2 Mix, MSM—Multi-Sample Amplification Master Mix, PB1—Reagent used to prepare BeadChips for hybridization, PB2—Humidifying buffer used during hybridization, PM1—Precipitation solution, RA1—Resuspension, hybridization, and wash solution, SML—Superior Two-Color Master Mix, EML—Two-Color Extension Master Mix, LX1—XStain BeadChip solution 1, LX2—XStain BeadChip solution 2, XC3—XStain BeadChip solution 3, XC4—XStain BeadChip solution 4.

Figure 5.2. Flowchart for genotyping process. XStain BeadChip step was done automatically by Tecan robot.

Figure obtained from reference [233].

5.2.4. Data processing and statistical analysis

Data was analyzed with PLINK version 1.7 [234]. Stringent quality control (QC) was applied to remove poorly performing Single Nucleotide Polymorphisms (SNPs) and samples before running association tests.

5.2.4.1. Quality control

5.2.4.1.1. Sample quality control

Marker genotyping efficiency (the proportion of samples with a genotype call for each marker) is an indicator of the quality of SNP assays. Samples with an overall genotyping call rate (genotyping efficiency) < 95% were removed from analysis. All remaining samples were then screened for potential biological relationship among the patients using the principle of variability in allele sharing, according to the degree of relationship (individuals at different level of relatedness have different level of DNA identity). We calculated the Identity-by-state (IBS) and compared all samples by pairs. IBS refers to alleles that are the same, irrespective of whether they are inherited from a recent ancestor [235]. For those pairs of individuals who showed evidence of relatedness, we removed the one with the lower genotyping call rate. This relatedness could be due to duplicated samples or biological relationship such as siblings. After relationship verification, we performed principle component analysis (PCA). This was done to account for spurious associations resulting from ancestral differences of individuals SNPs. We then generated PC plots with R statistic program.

5.2.4.1.2. SNPs quality control

We exclude X and Y chromosomes from the analysis for several reasons. Firstly, previous studies have not showed any strong association between gender and severe HFMD disease. Secondly, the X chromosome contains about 5% of human genes but they were commonly excluded from GWAS study because the lack of coverage on GWAS chips (including the BeadChip we used in this study), differences in the number of genes or variants on the X chromosome compared to the autosomes, differences in the minor-allele frequency of variants on the X chromosome and complicated statistic issues when dealing with sex chromosomes [236].

All SNPs which had > 5% missing genotypes were removed from the data. The remaining SNPs then underwent Hardy - Weinberg equilibrium (HWE) test. The Hardy–Weinberg principle states that allele and genotype frequencies in a population will remain constant from generation to generation in the absence of other evolutionary influences. Deviations from HWE can arise in the presence of a common deletion or polymorphism. If an excess of heterozygotes is observed, this may indicate the presence of over-dominant selection or the occurrence of outbreeding and usually due to some reasons: the locus is under selection or 'null alleles' may be present which are leading to a false observation of excess homozygotes. Null alleles are usually caused by a mutation in the primer binding site leading to an allele that will not amplify [190]. The HWE test was applied and SNPs with a HWE of P< 10-5 were cut. Lastly, SNPs with a minor allele frequency below 1% were excluded from the analysis.

5.2.4.2. Case control association analysis

5.2.4.2.1. Quantile - quantile plots (Q-Q plots)

A Q-Q plot is a graphic technique to detect if two data sets (case and control groups in this study) have the same distribution. It is constructed by ranking a set of values of a statistic from smallest to largest and plotting them against their expected values. When case and control groups have similar distribution, the observed line should be close to the expected line in a Q-Q plot. For case - control analysis, we conducted Q-Q plots with R.

5.2.4.2.2. Association test

In this GWAS study, association between severe HFMD and controls was tested using a standard case/control test (in PLINK software). P values of multiple tests were then adjusted with the Holm - Bonferroni correction. A list of SNPs exceeding $P < 10^{-4}$ was recorded. Genome-wide inflation factors were also calculated to ensure any findings were not biased by substantial population substructure or different genotyping call rates between cases and controls. Analysis of linkage disequilibrium and association results visualization were performed with Haploview [237]. The blanks/gaps within chromosomes are areas with SNPs of low genotyping call rate, usually seen in centromere regions.

5.3. RESULTS

5.3.1. Quality control results

Total 288 cases and 1357 controls were included in the GWAS. After exclusion of samples for per-sample call rates of less than 95%, relatedness and spurious association, 271 cases and 1262 controls were eligible for analysis (Figure 5.3). A summary of clinical and virological characteristics of the included patients is described in Table 5.1.

693045 SNPs were generated with the Illumina Human OmniExpress-24 BeadChip (Illumina, USA) and included at the beginning of the QC process. After QC exclusion, a total of 577578 SNPs were appropriate for further analysis (Figure 5.4).

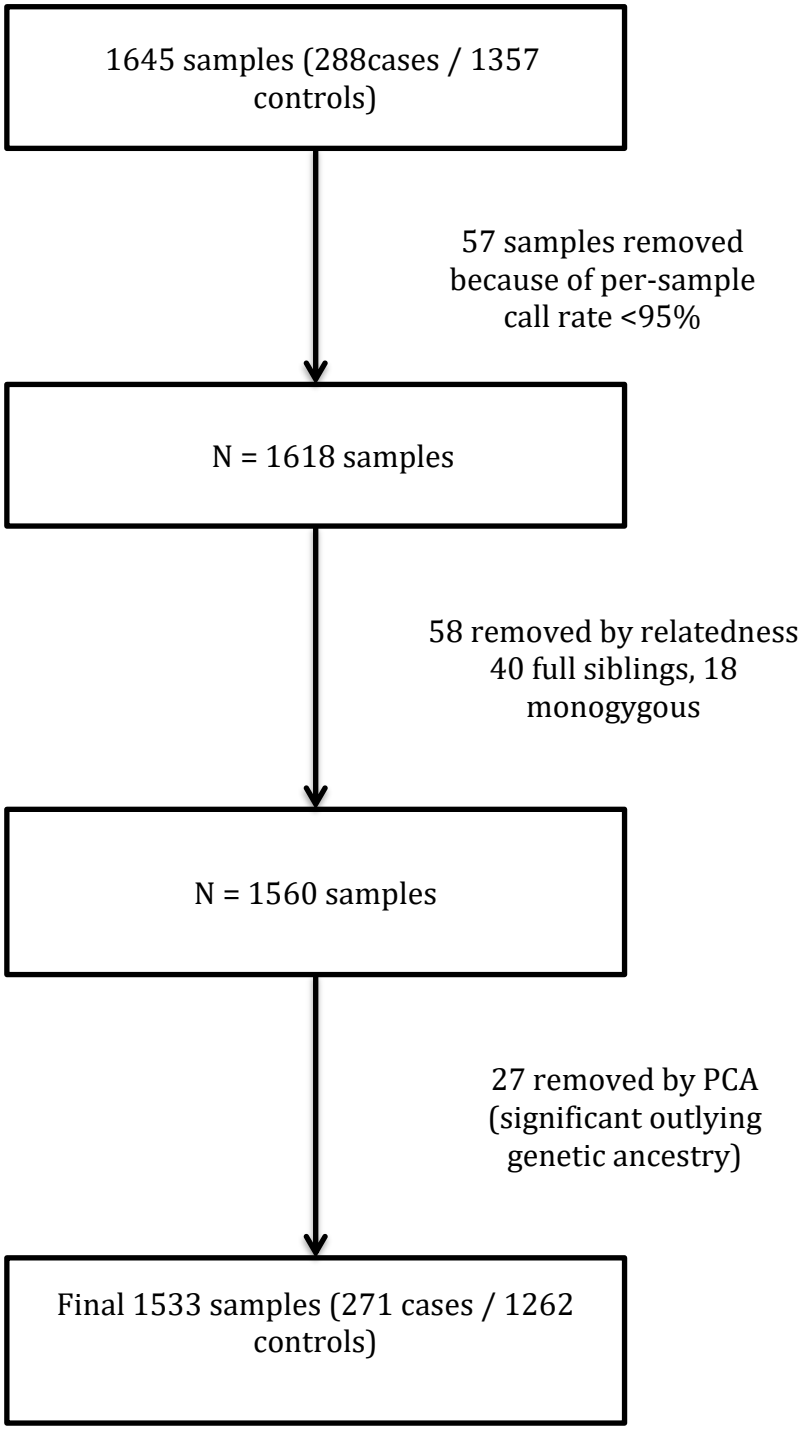


Figure 5.3. Data process for per-sample quality control.

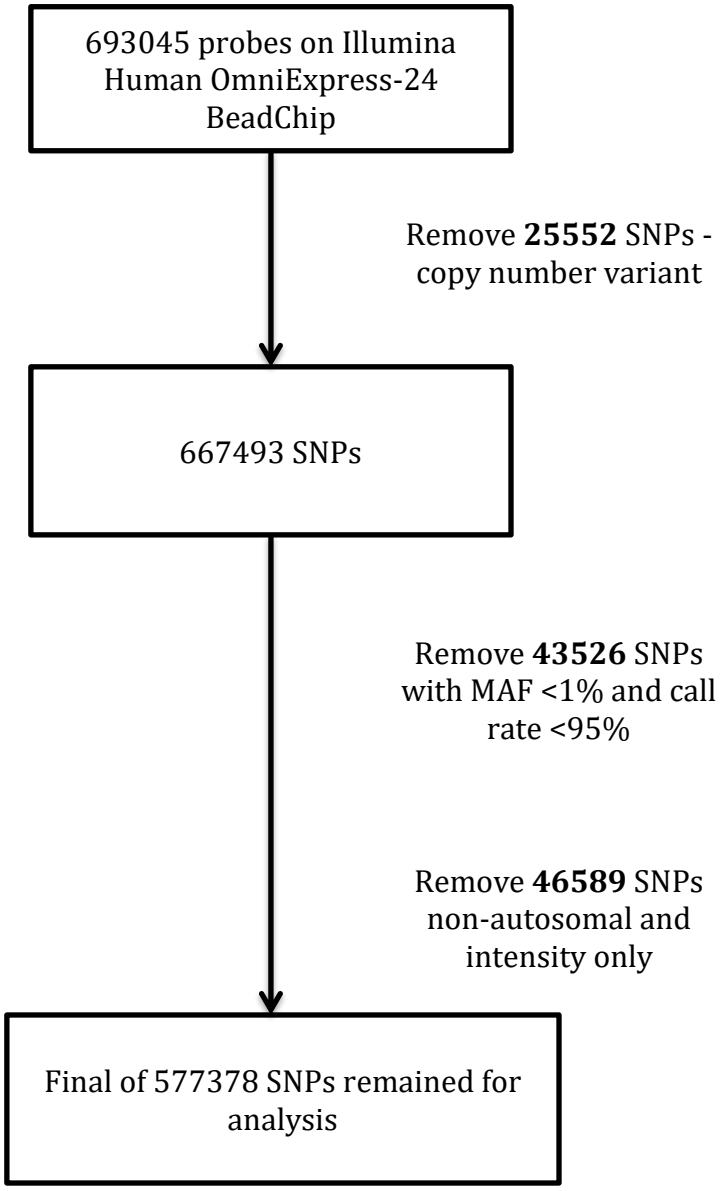
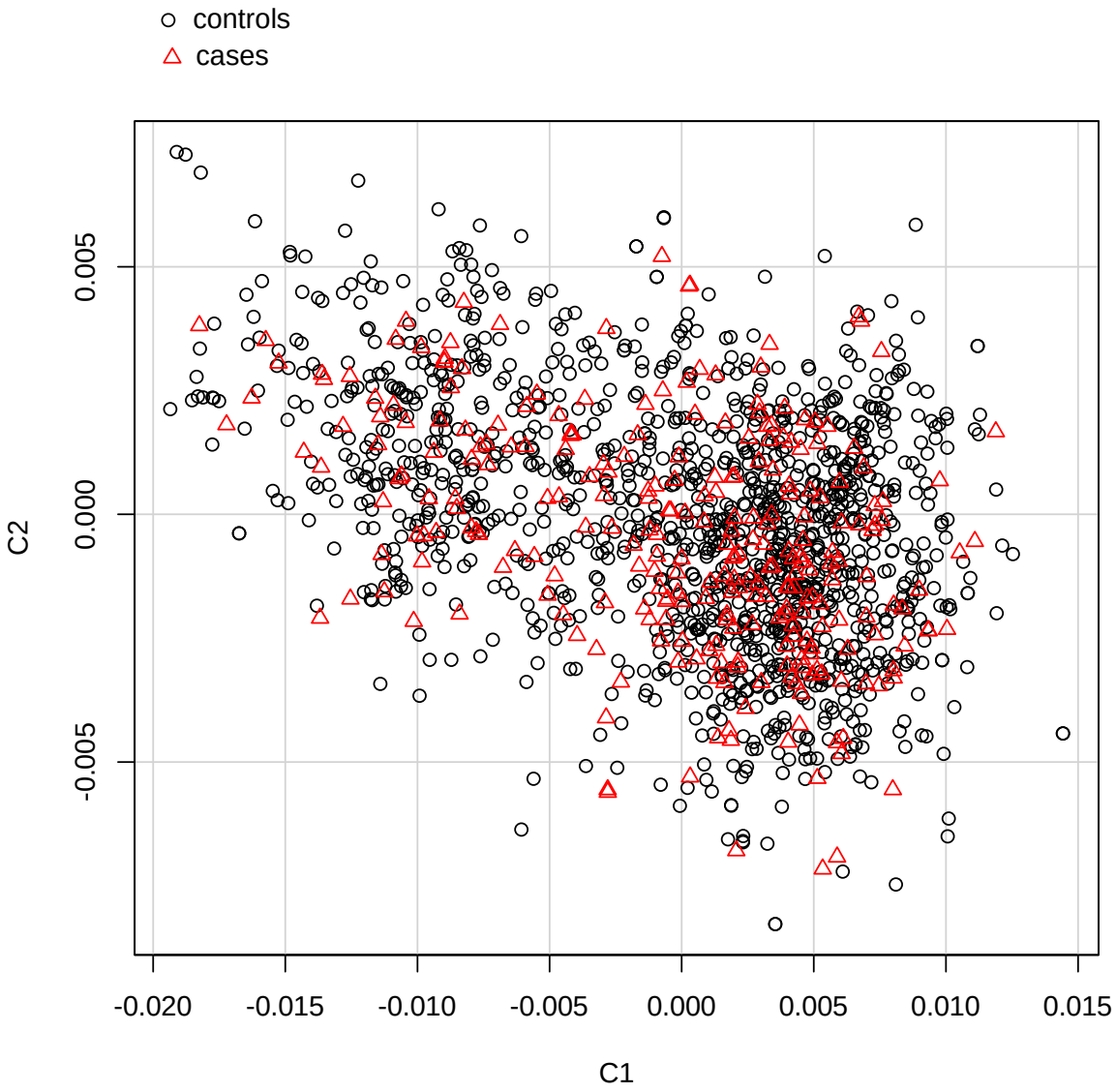


Figure 5.4. Data process for per-SNP quality control

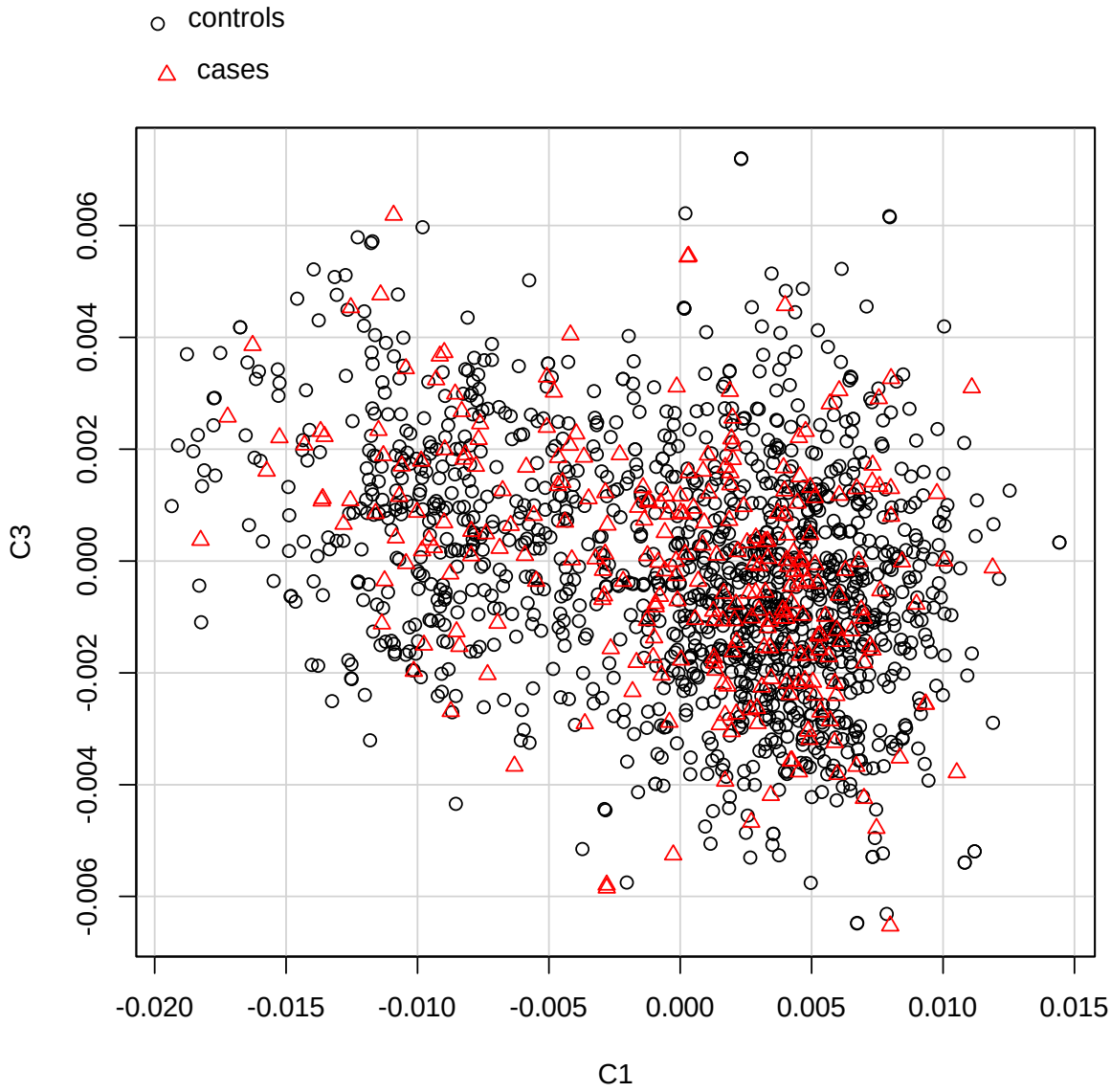
Characteristics	**N = 271**
Gender (Male)	158 (58.3)
Age (months - median - IQR)	19.3 (13.5 - 30.8)
Grade at discharge	
1 2a 2b1 2b2 3 4	84 (31) 2 (0.7) 78 (28.8) 29 (10.7) 73 (26.9) 5 (1.9)
PCR results	
EV-A71 CV-A6 CV-A10 Other EV Negative	185 (68.3) 12 (4.4) 11 (4) 36 (13.3) 27 (10)

Table 5.1. Clinical description of 271 HFMD cases.



C1: Principle component 1; C2: Principle component 2

Figure 5.5. Ancestry analysis using principle components of HFMD cases and controls (PC1 vs PC2) after removing outlying cases.



C1: Principle component 1, C2: Principle component 2

Figure 5.6. Ancestry analysis using principle components of HFMD cases and controls (PC1 vs PC3) after removing outlying cases.

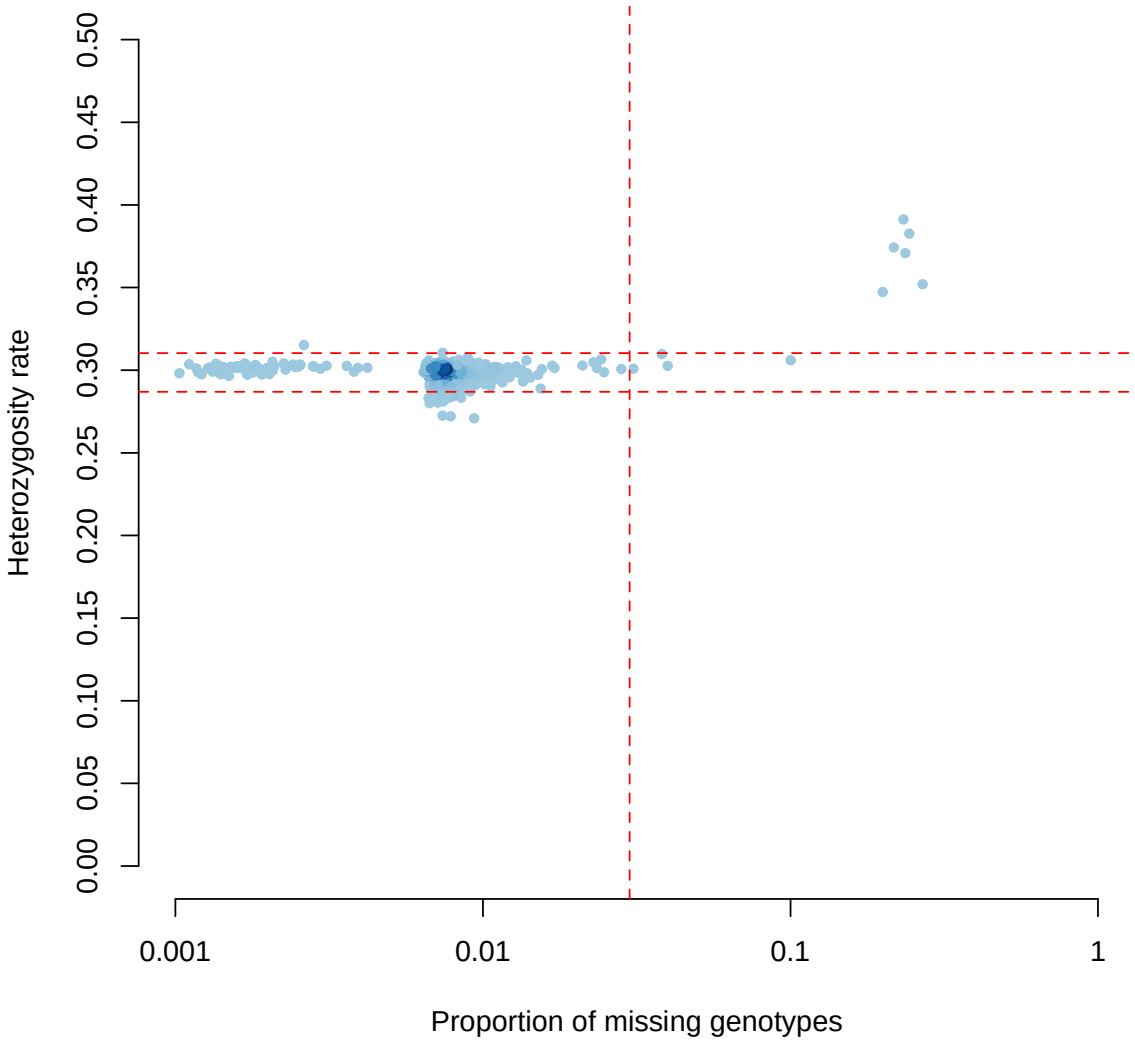


Figure 5.7. Heterozygosity rate and missing genotypes.

SNPs in the upper right of the graph (lots of genetic variability and high missing genotypes) will be removed. Shading indicates sample density and dashed lines denote QC thresholds.

5.3.2. Genetic screening results

After quality control, 86 non-severe cases and 185 severe cases were tested with 1262 controls. Association tests were performed between different groups of HFMD cases versus controls to screen for potential loci, which showed significant difference from others in severe groups. 4 groups were included in the association test for single SNP analysis: i) all HFMD cases vs controls, ii) all severe HFMD cases vs controls, iii) all non-severe HFMD cases vs controls and iv) all EV-A71 - positive severe HFMD cases vs controls.

5.3.2.1. Severe cases versus controls

185 severe cases and 1262 controls were included in the test. 137 SNPs with $P < 10^{-4}$ were potential candidates for genetic association (Table 5.2). Among these, there were 4 SNPs, including rs817854 on chromosome 9, rs10235866 on chromosome 7, rs1298238 on chromosome 1 and rs10739235 on chromosome 9 that were significantly different from other SNPs (P values = 3.63 x 10-7, 6.44 x 10-7 , 7.33 x 10-7 and 7.37 x 10-7). Those P values were also relatively close to the normal threshold of significant P value of genome-wide association at 10^{-8}. When combining with Odds ratios, rs10235866, rs10739235 and rs817854 were considered as promising candidates, with higher OR than two other candidates. In this study I used the significant threshold of 10^{-6} for severe cases instead of 10^{-8}. This threshold could distinguish the three mentioned SNPs, although it was not an expected significant value. This was also one of the limitations of this study (mentioned later in

discussion part). Figure 5.8 and 5.9 shows the frequencies of those SNPs across the chromosome.

5.3.2.2. Severe EV-A71-positive cases versus controls

When testing for association in severe HFMD cases associated with EV-A71 (n=99), rs10235866, rs10739235 and rs817854 also showed highly significant with P values above the 10^{-8} threshold (2.74 x 10-10 and 9.62 x 10-11, respectively, Figure 5.10 and 5.11)). Table 5.3 includes all SNPs with a P value < 10^{-4}.

5.3.2.3. All cases versus controls and non-severe cases versus controls screening

An association test for single SNPs was performed with 271 HFMD cases and 1262 controls. No significant SNPs across all chromosomes was observed although there were still 126 SNPs with a P value < 10^{-4} (Figure 5.14 and 5.15)

Similarly, the association test for non-severe cases generated a list of SNPs with P values < 10^{-4} (116 SNPs) but no P values reached the GWAS significance threshold (Figure 5.12 and 5.13).

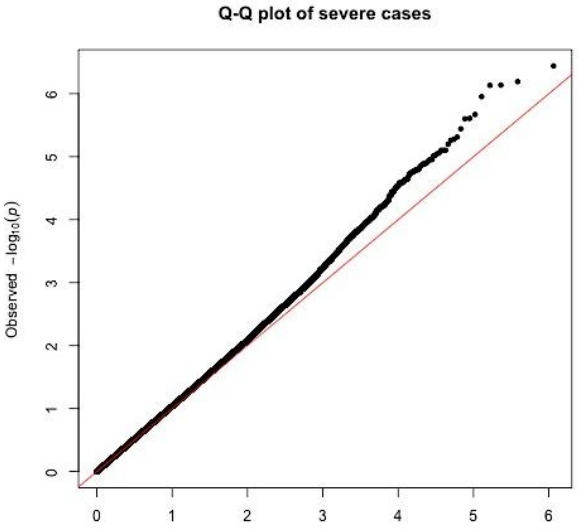
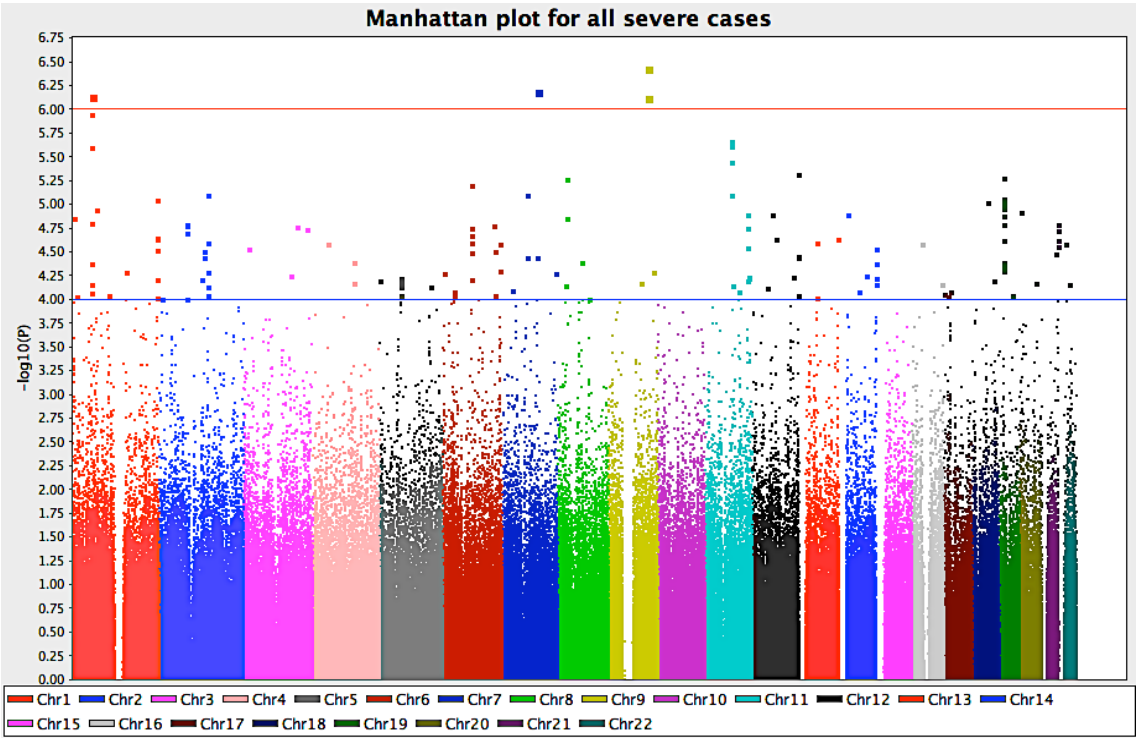


Figure 5.8. Q-Q plot of single SNPs analysis of severe HFMD cases versus controls with inflation factor = 1.05. The dot line and red line were closed to each other, indicating that two cohorts had similar distribution.



Blue line: threshold for potential SNPs (P< 10-4)

Red line: threshold for significant SNPs (P< 10^{-6})

Figure 5.9. Manhattan plot showing genotyped SNPs plotted according chromosomal location (X-axis: chromosome, Y-axis: - log $_{10}$ P generated from association test) of severe HFMD cases and controls.

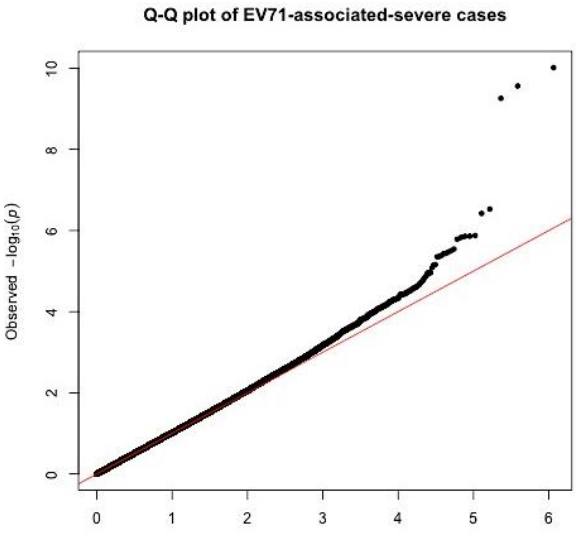
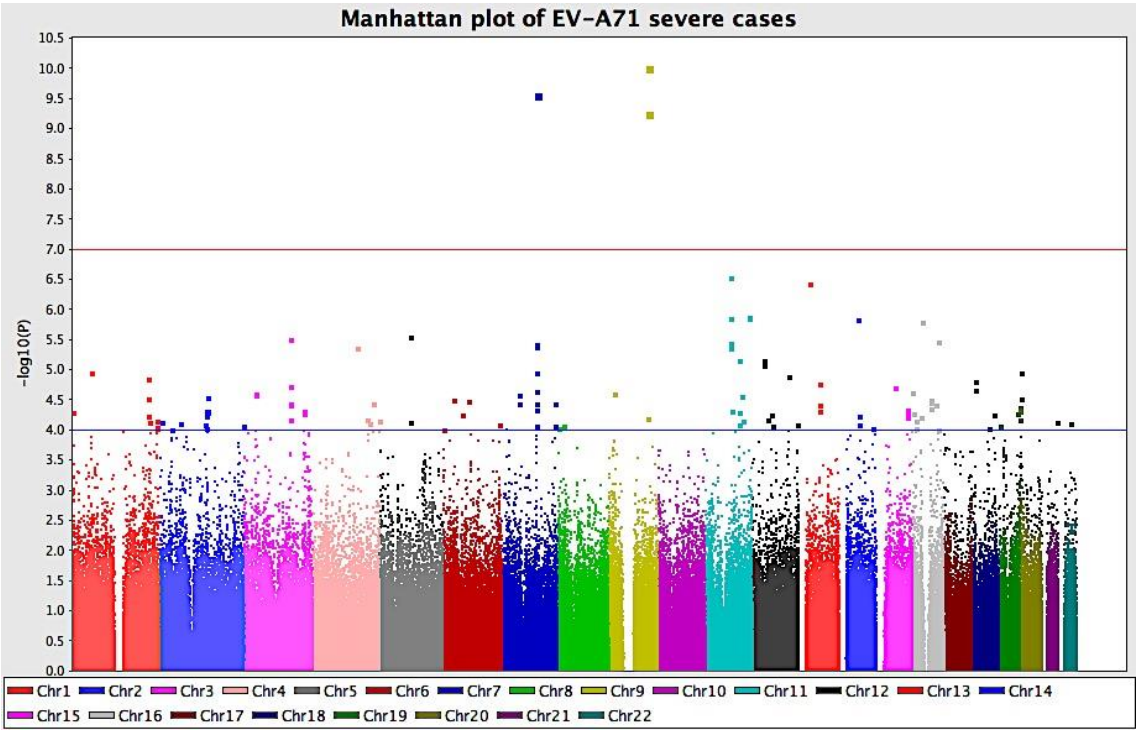


Figure 5.10. Q-Q plot of single SNPs analysis of severe HFMD EVA71-positive cases versus controls. The dot line and red line were closed to each other, indicating that two cohorts had similar distribution.



Blue line: threshold for potential SNPs

Red line: threshold for significant SNPs

Figure 5.11. Manhattan plot showing genotyped SNPs plotted according chromosomal location (X-axis: chromosome, Y-axis: - log 10 P generated from association test) of severe HFMD EVA71-positive cases and controls.

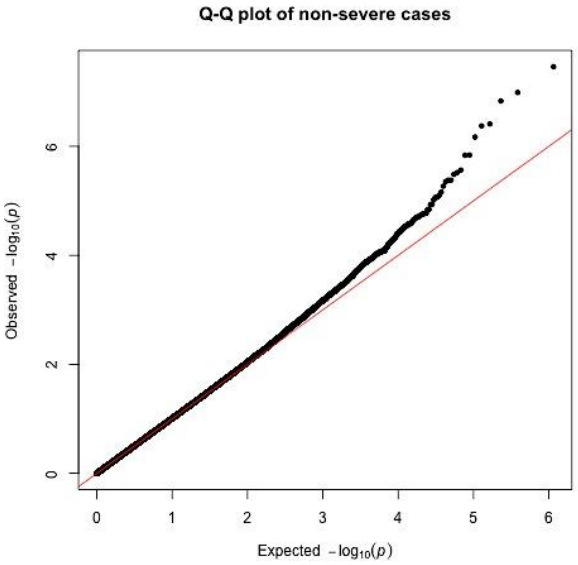
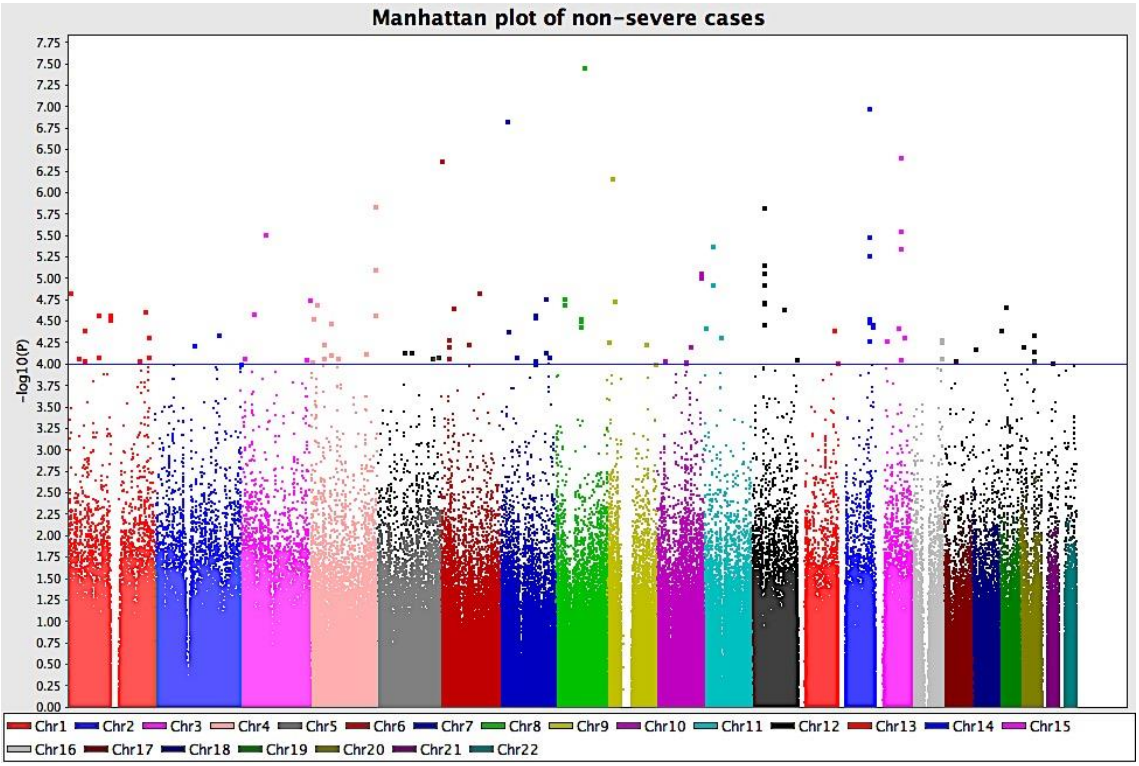


Figure 5.12. Q-Q plot of single SNPs analysis of non-severe HFMD cases versus controls. The dot line and red line were closed to each other, indicating that two cohorts had similar distribution.



Blue line: threshold for potential SNPs.

Figure 5.13. Manhattan plot showing genotyped SNPs plotted according chromosomal location (X-axis: chromosome, Y-axis: - log $_{10}$ P generated from association test) of non-severe HFMD cases and controls

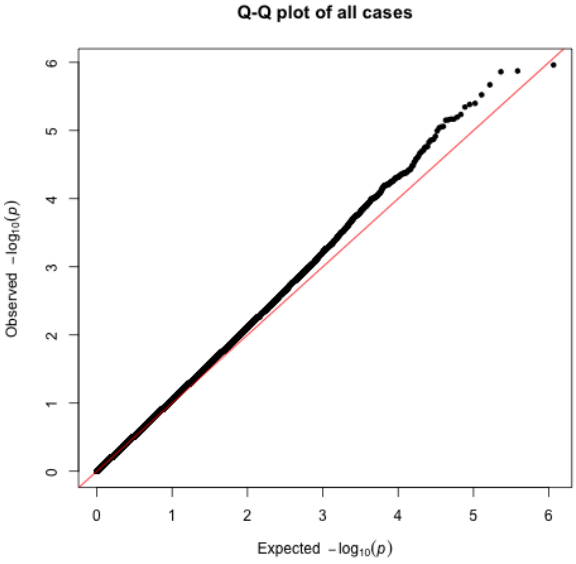
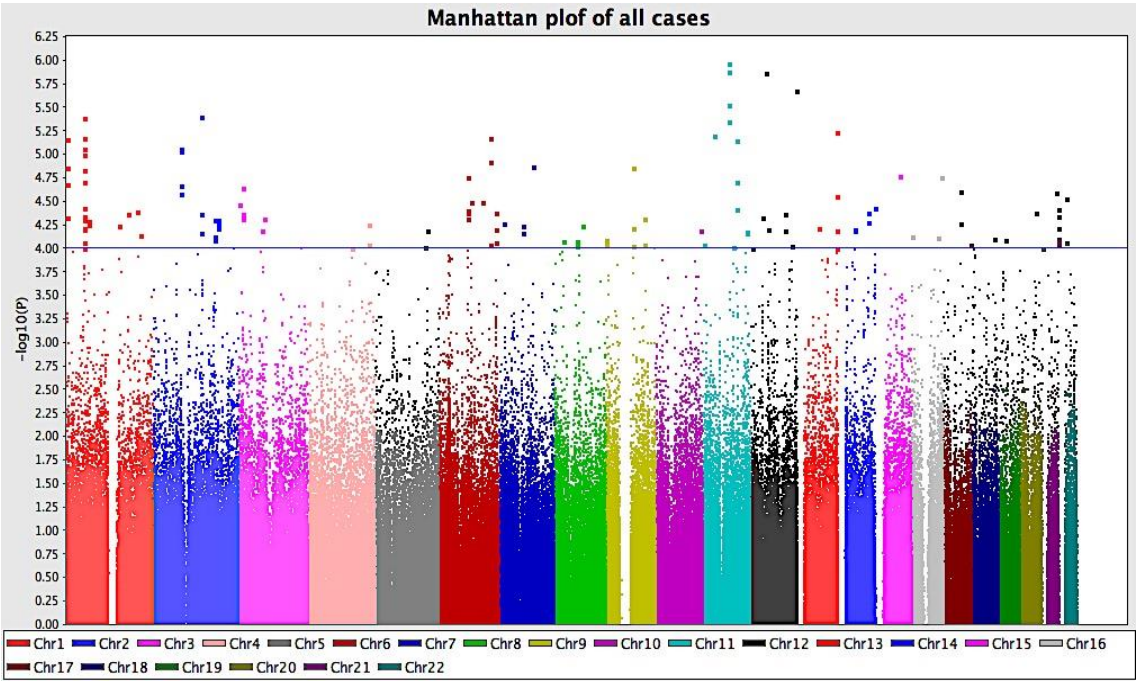


Figure 5.14. Q-Q plot of single SNPs analysis of all HFMD cases versus controls. The dot line and red line were closed to each other, indicating that two cohorts had similar distribution.



Blue line: threshold for potential SNPs

Figure 5.15. Manhattan plot showing genotyped SNPs plotted according chromosomal location (X-axis: chromosome, Y-axis: - log $_{10}$ P generated from association test) of all HFMD cases and controls.

CHR	SNP	POSITION	A1	F_A	F_U	A2	CHISQ	P	OR
9	rs817854	110113919	A	0.06216	0.01864	G	25.88	3.63E-07	1.49
7	rs10235866	98269772	A	0.2081	0.1157	G	24.78	6.44E-07	2.009
1	rs1298238	53633248	G	0.3973	0.2724	A	24.53	7.33E-07	1.761
9	rs10739235	110047794	G	0.06354	0.01983	A	24.52	7.37E-07	3.354
1	rs874349	53631902	A	0.2935	0.185	G	23.72	1.12E-06	1.83
11	rs12803970	73002434	A	0.03514	0.007534	G	22.46	2.14E-06	4.797
11	rs12790863	72979231	G	0.03784	0.008737	A	22.19	2.47E-06	4.462
1	rs874348	53631805	A	0.3743	0.2571	G	22.16	2.51E-06	1.728
11	rs7127940	72976988	G	0.03552	0.007949	A	21.46	3.62E-06	4.596
12	rs1877978	130985182	G	0.6054	0.4782	A	20.89	4.87E-06	1.674
19	rs2438589	12395022	A	0.3946	0.2791	G	20.72	5.31E-06	1.683
8	rs328095	26340103	G	0.273	0.1743	A	20.66	5.49E-06	1.778
6	rs2120535	82093262	G	0.5811	0.4556	A	20.38	6.35E-06	1.657
2	rs6758115	137069775	G	0.4324	0.3154	A	19.96	7.89E-06	1.654
7	rs2049635	70244289	A	0.1676	0.09231	G	19.96	7.90E-06	1.979
11	rs11235712	72994199	G	0.03784	0.009516	A	19.96	7.91E-06	4.093
19	rs4297803	12399733	A	0.3919	0.2791	G	19.78	8.70E-06	1.664
1	rs2802728	243518151	G	0.1484	0.07815	A	19.71	9.00E-06	2.055
18	rs616345	43554371	A	0.04121	0.01111	C	19.62	9.46E-06	3.825
19	rs10423583	12397800	A	0.3913	0.2789	G	19.57	9.68E-06	1.662
19	rs10407898	12426234	G	0.3361	0.2303	A	19.31	1.11E-05	1.691
19	rs9676962	12441244	A	0.3288	0.2244	G	19.31	1.11E-05	1.693
1	rs7414744	69323222	A	0.06757	0.02538	G	19.26	1.14E-05	2.783
20	rs8182997	1497623	A	0.3973	0.2857	G	19.15	1.21E-05	1.648
11	rs2887763	121568179	A	0.1892	0.1101	G	19.06	1.27E-05	1.885
14	rs1951594	25084632	C	0.5892	0.4679	A	19.01	1.30E-05	1.631
12	rs11575216	56755457	C	0.03804	0.009936	G	19.01	1.30E-05	3.941
19	rs2943690	12378371	A	0.3907	0.2797	C	18.98	1.32E-05	1.651

8	rs328096	26340833	G	0.2541	0.162	A	18.87	1.40E-05	1.762
1	rs10915578	4723917	G	0.05135	0.01664	A	18.84	1.42E-05	3.199
1	rs1320594	53610372	A	0.3919	0.2821	C	18.66	1.56E-05	1.64
19	rs10422077	12427288	A	0.327	0.2248	G	18.6	1.61E-05	1.676
2	rs13416390	77520451	G	0.1919	0.113	A	18.6	1.61E-05	1.864
21	rs9977482	46899119	A	0.2377	0.1492	G	18.58	1.63E-05	1.778
2	rs10180141	77515995	G	0.1919	0.1131	A	18.55	1.66E-05	1.862
6	rs17826169	146953914	C	0.3676	0.2607	A	18.51	1.69E-05	1.648
3	rs6440662	149979591	A	0.1595	0.08849	G	18.45	1.74E-05	1.954
6	rs996136	82087297	G	0.5757	0.4563	A	18.43	1.76E-05	1.616
11	rs10790451	121570520	A	0.2243	0.1391	G	18.4	1.79E-05	1.79
3	rs4854984	178982493	G	0.1114	0.05401	A	18.38	1.81E-05	2.196
21	rs2236472	46903412	A	0.2378	0.1502	G	18.33	1.86E-05	1.766
2	rs10179301	77501554	C	0.2	0.12	A	18.21	1.98E-05	1.833
6	rs2323252	82099191	C	0.573	0.4548	A	18.08	2.12E-05	1.608
1	rs4658566	243566904	A	0.1378	0.0733	G	17.95	2.27E-05	2.021
1	rs12239876	243546954	C	0.1459	0.07924	A	17.93	2.29E-05	1.986
13	rs9555784	111948823	G	0.3838	0.2768	A	17.93	2.29E-05	1.628
12	rs7134781	66943175	A	0.5054	0.3899	G	17.88	2.35E-05	1.599
19	rs1862507	12436574	G	0.327	0.2266	A	17.86	2.38E-05	1.658
21	rs2236470	46903021	G	0.2378	0.1512	A	17.83	2.42E-05	1.752
13	rs9526518	49601364	G	0.3703	0.4873	A	17.73	2.55E-05	0.6186
2	rs11896100	139177661	C	0.03243	0.007924	A	17.73	2.55E-05	4.197
6	rs6454218	82075973	A	0.2	0.3067	G	17.72	2.56E-05	0.5652
4	rs16853108	41330982	G	0.03243	0.00793	A	17.7	2.58E-05	4.193
6	rs2022999	162933253	G	0.3541	0.4707	A	17.7	2.59E-05	0.6164
22	rs3788317	19889825	A	0.273	0.1807	C	17.69	2.60E-05	1.703
16	rs938355	27828751	A	0.05946	0.02179	G	17.68	2.61E-05	2.838
21	rs9977268	46907287	A	0.2396	0.1503	G	17.55	2.80E-05	1.781
11	rs1074086	121575095	G	0.2459	0.1585	A	17.52	2.84E-05	1.732

3	rs425456	11920880	G	0.03611	0.009547	A	17.49	2.89E-05	3.887
14	rs11160756	104113482	G	0.3892	0.5056	A	17.48	2.90E-05	0.6231
1	rs4658568	243577313	G	0.1378	0.07409	A	17.38	3.07E-05	1.998
6	rs472577	147836218	A	0.5622	0.4465	G	17.37	3.08E-05	1.592
2	rs4662627	129762727	A	0.5108	0.3966	G	17.35	3.11E-05	1.588
6	rs962983	82064465	G	0.2011	0.3067	A	17.27	3.24E-05	0.5691
21	rs2835688	38651111	G	0.3115	0.2143	A	17.21	3.35E-05	1.658
12	rs12298735	130064448	A	0.0625	0.02389	G	17.1	3.55E-05	2.724
7	rs17530700	98273043	G	0.06486	0.02536	A	17.08	3.58E-05	2.666
7	rs1881010	70217631	A	0.1838	0.1094	G	17.07	3.60E-05	1.834
2	rs10928858	129774095	A	0.3595	0.2573	G	17.06	3.63E-05	1.62
2	rs10928860	129774475	C	0.3595	0.2573	A	17.06	3.63E-05	1.62
12	rs11060443	130060137	A	0.06216	0.02379	G	17.04	3.66E-05	2.72
4	rs13114676	117540588	C	0.5135	0.401	A	16.84	4.07E-05	1.577
8	rs7008059	68945041	G	0.08919	0.04084	A	16.83	4.09E-05	2.3
19	rs7256140	12409924	A	0.3568	0.2555	C	16.83	4.09E-05	1.616
14	rs7154572	104105414	A	0.5811	0.467	G	16.81	4.14E-05	1.583
1	rs11206109	53606080	A	0.3054	0.2105	G	16.77	4.21E-05	1.649
19	rs11880217	12418795	A	0.3568	0.256	G	16.68	4.43E-05	1.612
6	rs6925670	162936958	A	0.5676	0.4548	G	16.47	4.95E-05	1.573
19	rs6511787	12446244	A	0.5081	0.397	G	16.46	4.98E-05	1.569
1	rs821427	153275977	G	0.4297	0.3231	A	16.41	5.10E-05	1.578
2	rs6430628	137093578	G	0.2027	0.1256	A	16.38	5.17E-05	1.77
9	rs7029319	126888086	A	0.3892	0.2861	G	16.37	5.21E-05	1.59
7	rs6974964	154152834	A	0.1514	0.08565	G	16.35	5.27E-05	1.904
6	rs2236277	2959513	A	0.3967	0.5095	G	16.34	5.30E-05	0.6331
3	rs6767367	132653045	G	0.2378	0.1545	A	16.25	5.56E-05	1.708
14	rs12435874	78540214	C	0.1703	0.1003	A	16.22	5.63E-05	1.84
11	rs1975922	123547510	G	0.07838	0.03447	A	16.17	5.79E-05	2.382
12	rs645510	118106433	A	0.3505	0.2516	G	16.16	5.82E-05	1.606

14	rs11850979	104096558	G	0.3946	0.5063	A	16.12	5.94E-05	0.6355
14	rs2896489	104108743	G	0.3946	0.5063	A	16.12	5.94E-05	0.6355
11	rs1841876	123549724	A	0.07838	0.03452	G	16.11	5.99E-05	2.378
5	rs2545493	60336004	A	0.05978	0.02303	G	16.09	6.05E-05	2.697
6	rs1413720	82036764	A	0.2514	0.3574	G	16.08	6.08E-05	0.6037
1	rs10754803	243513047	G	0.3108	0.217	A	16.07	6.10E-05	1.627
6	rs1419027	82078440	A	0.2028	0.3056	G	16.07	6.10E-05	0.5781
2	rs4848628	121536569	C	0.1405	0.2331	A	16.06	6.14E-05	0.5378
5	rs7379059	1143298	G	0.4405	0.3344	A	16.02	6.27E-05	1.567
11	rs10892771	121568262	A	0.2405	0.1573	G	16.01	6.32E-05	1.697
18	rs7230656	60314838	A	0.07568	0.03291	G	15.99	6.37E-05	2.406
18	rs7235438	60314872	G	0.07568	0.03291	A	15.99	6.37E-05	2.406
5	rs2270822	60459040	A	0.05946	0.023	G	15.95	6.51E-05	2.686
4	rs4560484	117543972	A	0.5109	0.4012	C	15.9	6.68E-05	1.559
9	rs16906728	93397205	A	0.08649	0.04002	G	15.89	6.70E-05	2.271
20	rs6104014	43740693	A	0.08649	0.04002	G	15.89	6.70E-05	2.271
1	rs1288367	53605884	A	0.3459	0.456	G	15.83	6.93E-05	0.6311
22	rs16987316	29643294	G	0.2364	0.1541	A	15.83	6.95E-05	1.7
16	rs9938042	85020087	A	0.04595	0.01545	G	15.81	6.99E-05	3.069
14	rs2273175	104160141	G	0.3108	0.4195	A	15.81	6.99E-05	0.624
8	rs6586986	21326878	A	0.2351	0.1533	G	15.77	7.16E-05	1.698
11	rs3740769	76938121	G	0.4022	0.2994	A	15.76	7.18E-05	1.574
5	rs159371	60444276	G	0.06216	0.02474	A	15.74	7.26E-05	2.613
2	rs47150	137220743	G	0.4027	0.3002	A	15.74	7.26E-05	1.571
5	rs17107181	147064881	C	0.1033	0.05151	A	15.72	7.33E-05	2.121
12	rs11181627	43061751	A	0.1514	0.08677	C	15.65	7.61E-05	1.877
7	rs10951200	28592608	A	0.07568	0.03331	G	15.56	8.00E-05	2.376
14	rs1188147	56433472	A	0.05135	0.01862	G	15.51	8.20E-05	2.853
6	rs374205	32196873	G	0.06044	0.02377	A	15.5	8.23E-05	2.642
11	rs3905431	95714113	A	0.1162	0.06101	G	15.5	8.25E-05	2.024

17	rs2386144	19587603	C	0.2324	0.3348	A	15.49	8.28E-05	0.6017
11	rs7114828	95717607	A	0.1005	0.04992	G	15.48	8.36E-05	2.127
1	rs3737989	53600078	G	0.3135	0.221	A	15.45	8.49E-05	1.609
17	rs8081687	1094119	A	0.07297	0.03175	G	15.38	8.79E-05	2.401
6	rs536996	147839691	A	0.5811	0.4721	G	15.34	8.99E-05	1.551
5	rs976080	60217651	A	0.05946	0.02345	C	15.33	9.01E-05	2.633
1	rs1934709	102968036	G	0.0973	0.04794	A	15.32	9.06E-05	2.141
12	rs11609780	129892922	A	0.1757	0.1062	G	15.31	9.15E-05	1.794
2	rs12616701	137062927	A	0.4189	0.3166	G	15.3	9.17E-05	1.556
19	rs540818	35710685	G	0.07027	0.1447	A	15.3	9.18E-05	0.4467
5	rs290506	60298360	A	0.06284	0.02542	G	15.3	9.18E-05	2.571
6	rs2523544	31333562	A	0.106	0.05393	G	15.3	9.19E-05	2.08
1	rs634478	10888791	A	0.1811	0.1105	G	15.26	9.39E-05	1.779
17	rs6502201	12593743	C	0.1595	0.09363	A	15.25	9.43E-05	1.837
5	rs158933	60238590	A	0.06284	0.02546	G	15.24	9.46E-05	2.567
1	rs2039838	243582314	A	0.1378	0.07726	G	15.23	9.54E-05	1.909
13	rs6561490	49540727	A	0.3703	0.4786	C	15.21	9.61E-05	0.6406
2	rs290047	77529369	A	0.173	0.1043	G	15.18	9.77E-05	1.796
8	rs13261974	89220059	A	0.3784	0.4865	G	15.14	9.97E-05	0.6424
2	rs4669232	8123746	A	0.3757	0.2774	G	15.14	9.98E-05	1.568

Table 5.2. List of 138 SNPs with P value < 10^{-4} in association test of severe HFMD cases vs controls (all severe cases)

CHR	SNP	POSITION	A1	F_A	F_U	A2	CHISQ	P	OR
9	rs10739235	110047794	G	0.09677	0.01983	A	41.9	9.62E-11	5.297
7	rs10235866	98269772	A	0.2737	0.1157	G	39.85	2.74E-10	2.88
9	rs817854	110113919	A	0.08947	0.01864	G	38.48	5.54E-10	5.175
11	rs7127940	72976988	G	0.04787	0.007949	A	26.28	2.96E-07	6.275
13	rs7989117	28479260	A	0.2316	0.1086	G	25.8	3.79E-07	2.473
11	rs1975922	123547510	G	0.1053	0.03447	A	23.38	1.33E-06	3.295
11	rs1841876	123549724	A	0.1053	0.03452	G	23.31	1.38E-06	3.29
11	rs12790863	72979231	G	0.04737	0.008737	A	23.3	1.39E-06	5.641
14	rs1211769	55051375	G	0.05789	0.01268	A	23.17	1.48E-06	4.786
16	rs938355	27828751	A	0.07895	0.02179	G	22.97	1.64E-06	3.848
5	rs1423633	87092264	A	0.1211	0.04444	G	21.91	2.86E-06	2.961
3	rs6767367	132653045	G	0.2842	0.1545	A	21.72	3.15E-06	2.173
16	rs4788477	72754901	G	0.1421	0.05714	A	21.57	3.41E-06	2.733
11	rs12803970	73002434	A	0.04211	0.007534	G	21.42	3.68E-06	5.791
7	rs17530700	98273043	G	0.08421	0.02536	A	21.39	3.74E-06	3.534
7	rs17252863	97184423	A	0.1064	0.03685	G	21.19	4.17E-06	3.112
4	rs4371629	125013856	A	0.05263	0.0115	G	21.09	4.38E-06	4.776
11	rs11235712	72994199	G	0.04737	0.009516	A	21.06	4.45E-06	5.175
11	rs16922748	95704425	G	0.04211	0.007924	A	20.22	6.91E-06	5.503
12	rs2352616	31115662	A	0.2105	0.3728	G	20.16	7.11E-06	0.4486
12	rs4931393	31115346	A	0.2105	0.3715	C	19.87	8.28E-06	0.4511
7	rs879187	98273346	A	0.08421	0.02694	G	19.32	1.10E-05	3.321
1	rs874349	53631902	A	0.3158	0.185	G	19.31	1.11E-05	2.033
20	rs674110	3728026	G	0.3158	0.185	A	19.31	1.11E-05	2.033
12	rs4981021	104149999	G	0.5947	0.4319	A	18.98	1.32E-05	1.931
1	rs1414530	216798830	A	0.3211	0.4842	G	18.86	1.41E-05	0.5038
18	rs4798618	8216997	G	0.2526	0.138	A	18.62	1.59E-05	2.112

13	rs4477582	57712738	G	0.3263	0.1959	A	18.44	1.75E-05	1.989
3	rs6779839	132494523	G	0.05789	0.01506	A	18.29	1.90E-05	4.02
15	rs17525891	52014185	G	0.05376	0.01314	A	18.22	1.96E-05	4.268
18	rs12454728	8213200	G	0.2474	0.1355	A	18.02	2.19E-05	2.098
7	rs10262556	98317100	A	0.07895	0.02536	C	17.96	2.26E-05	3.295
16	rs9939865	337613	A	0.05851	0.01549	G	17.85	2.39E-05	3.95
3	rs13075567	34665118	G	0.5	0.3478	A	17.77	2.49E-05	1.875
9	rs930543	13749977	A	0.1737	0.0832	G	17.75	2.52E-05	2.316
7	rs3734931	47847751	A	0.06316	0.01783	G	17.66	2.64E-05	3.714
3	rs11129601	34662157	A	0.5158	0.3629	G	17.65	2.66E-05	1.87
11	rs12797633	104519419	A	0.2263	0.1208	C	17.59	2.75E-05	2.128
2	rs7577825	139144543	A	0.07895	0.02575	G	17.48	2.91E-05	3.243
1	rs7553463	216788184	A	0.3263	0.483	G	17.4	3.03E-05	0.5185
20	rs658495	3737495	G	0.2	0.1022	A	17.39	3.05E-05	2.196
16	rs4132615	52704510	G	0.4521	0.3057	A	17.35	3.12E-05	1.874
6	rs374205	32196873	G	0.07527	0.02377	A	17.3	3.20E-05	3.343
16	rs4784241	52715409	G	0.4474	0.3027	A	17.2	3.36E-05	1.865
6	rs768790	73388290	A	0.05914	0.01605	G	17.2	3.36E-05	3.853
16	rs4386133	52706844	A	0.4474	0.3029	G	17.14	3.47E-05	1.863
7	rs11974761	151660361	A	0.4842	0.3362	G	17.08	3.58E-05	1.853
3	rs6770608	132728006	A	0.3211	0.1957	G	17.06	3.62E-05	1.943
7	rs1534664	151660102	A	0.4842	0.3364	G	17.05	3.64E-05	1.852
7	rs17659820	47860510	A	0.06316	0.01824	G	17.03	3.67E-05	3.629
7	rs17440093	98294265	A	0.07895	0.02615	G	17.01	3.71E-05	3.192
4	rs406542	171510501	A	0.03684	0.007154	G	17.01	3.72E-05	5.308
3	rs16839757	132588834	A	0.05263	0.01347	G	16.99	3.76E-05	4.069
16	rs9926714	66154700	A	0.05263	0.01347	C	16.99	3.76E-05	4.069
13	rs559648	57703846	A	0.3105	0.1875	C	16.98	3.78E-05	1.952

20	rs17778888	1179237	A	0.1842	0.09231	G	16.77	4.23E-05	2.22
16	rs12932515	52712547	A	0.4474	0.3047	C	16.68	4.42E-05	1.847
15	rs7164531	88814356	A	0.1935	0.09865	G	16.58	4.67E-05	2.193
7	rs1994410	98316837	C	0.07895	0.02655	A	16.56	4.71E-05	3.143
13	rs9563465	57678083	A	0.3105	0.1887	G	16.55	4.74E-05	1.936
11	rs12421551	76812803	G	0.1947	0.1002	A	16.53	4.78E-05	2.171
2	rs6430462	134599227	A	0.2211	0.3676	G	16.52	4.82E-05	0.4883
3	rs2593858	172133487	G	0.5737	0.4223	A	16.48	4.91E-05	1.841
20	rs6078117	1180222	G	0.3	0.1804	A	16.48	4.91E-05	1.947
2	rs603666	137199326	A	0.04737	0.01149	G	16.48	4.93E-05	4.278
2	rs563709	137219133	G	0.04737	0.01151	A	16.43	5.04E-05	4.271
1	rs12033324	2307481	A	0.06667	0.01984	C	16.42	5.08E-05	3.529
11	rs3905431	95714113	A	0.1368	0.06101	G	16.41	5.11E-05	2.44
16	rs2235649	1837406	A	0.3936	0.2583	G	16.34	5.30E-05	1.864
19	rs11881006	51032396	A	0.08947	0.03249	G	16.33	5.33E-05	2.926
3	rs551228	172133060	C	0.5737	0.4231	A	16.32	5.35E-05	1.835
12	rs89962	52915172	A	0.06842	0.02139	C	16.23	5.61E-05	3.359
15	rs7164988	88814209	G	0.2053	0.1085	A	16.21	5.66E-05	2.122
18	rs17071470	61365168	A	0.2474	0.14	G	16.21	5.67E-05	2.02
6	rs10498805	55290936	G	0.06842	0.02141	A	16.21	5.68E-05	3.357
2	rs7569670	134600686	G	0.5579	0.4087	A	16.16	5.82E-05	1.826
1	rs1414529	216798806	G	0.2316	0.3774	A	16.16	5.84E-05	0.4971
14	rs17127681	55092680	A	0.04211	0.009509	G	16.11	5.97E-05	4.579
16	rs277895	25169839	G	0.2181	0.3629	A	16.06	6.13E-05	0.4897
15	rs11073770	88820515	C	0.2211	0.1205	A	16.04	6.21E-05	2.071
9	rs7029300	110152533	C	0.1632	0.0793	A	15.94	6.52E-05	2.264
20	rs7269259	1179082	A	0.2	0.1054	G	15.9	6.67E-05	2.122
4	rs6832907	153982885	G	0.06316	0.01903	A	15.9	6.67E-05	3.475

12	rs4489809	43262044	G	0.08065	0.0278	A	15.89	6.73E-05	3.068
3	rs7620792	132694401	A	0.2895	0.1739	G	15.84	6.88E-05	1.935
16	rs7195536	11442028	G	0.03684	0.007582	A	15.83	6.92E-05	5.007
1	rs10754803	243513047	G	0.3421	0.217	A	15.81	6.99E-05	1.876
16	rs9921338	11416839	A	0.04301	0.009905	G	15.78	7.10E-05	4.493
4	rs28628755	188915984	A	0.1316	0.05864	G	15.75	7.22E-05	2.432
11	rs10789545	106637578	A	0.5211	0.3757	G	15.75	7.22E-05	1.808
21	rs16998067	41733181	A	0.05789	0.01664	C	15.71	7.40E-05	3.632
21	rs2837580	41738635	G	0.05789	0.01664	A	15.71	7.40E-05	3.632
5	rs6885160	87099119	G	0.09574	0.03657	A	15.7	7.43E-05	2.79
1	rs17007616	220579058	A	0.1	0.03922	G	15.69	7.45E-05	2.722
2	rs12466138	5529462	G	0.1105	0.04556	A	15.67	7.56E-05	2.603
2	rs17399724	60052940	A	0.2842	0.1704	C	15.63	7.71E-05	1.934
4	rs2349306	161766489	A	0.07368	0.02462	C	15.59	7.87E-05	3.151
22	rs137507	33282971	A	0.1421	0.06577	G	15.59	7.88E-05	2.353
11	rs3016513	95714608	G	0.1421	0.06582	A	15.56	8.01E-05	2.351
12	rs11609780	129892922	A	0.2	0.1062	G	15.55	8.05E-05	2.104
14	rs11850008	56404641	G	0.1684	0.08373	A	15.52	8.14E-05	2.216
6	rs2064225	163794927	A	0.1474	0.06939	G	15.51	8.19E-05	2.318
2	rs4662627	129762727	A	0.5421	0.3966	G	15.48	8.33E-05	1.801
7	rs11978256	151660298	G	0.4789	0.338	A	15.47	8.37E-05	1.8
7	rs817765	98230657	G	0.4947	0.3525	A	15.47	8.39E-05	1.799
19	rs1036592	1920712	G	0.5585	0.4117	A	15.46	8.43E-05	1.808
12	rs11575216	56755457	C	0.04255	0.009936	G	15.44	8.53E-05	4.428
2	rs6715941	241993027	G	0.2263	0.126	A	15.42	8.60E-05	2.029
8	rs2736064	15710219	A	0.2684	0.1585	G	15.41	8.63E-05	1.948
1	rs10927011	243579112	G	0.3474	0.2231	A	15.34	9.00E-05	1.854
1	rs4658566	243566904	A	0.1526	0.0733	G	15.31	9.11E-05	2.277

2	rs13020966	134595521	A	0.2421	0.1384	G	15.28	9.29E-05	1.989
18	rs6650676	47861856	A	0.2158	0.1185	G	15.27	9.30E-05	2.048
16	rs6498181	11427193	A	0.04211	0.009905	G	15.25	9.40E-05	4.394
1	rs4658558	243505841	A	0.3316	0.2102	G	15.25	9.42E-05	1.864
8	rs1523255	3849209	G	0.2737	0.4179	A	15.22	9.56E-05	0.5248
14	rs8014274	97199831	A	0.07368	0.02496	G	15.22	9.58E-05	3.107
2	rs10490658	37136398	G	0.3579	0.5044	A	15.16	9.86E-05	0.5477
6	rs2236277	2959513	A	0.3632	0.5095	G	15.14	9.99E-05	0.549
16	rs989925	75141060	G	0.1368	0.06305	A	15.14	9.99E-05	2.356

Table 5.3. List of SNPs with P-value < 10-4 in association test of severe HFMD cases (EVA71 positive only) vs controls

5.4. DISCUSSION

During HFMD outbreaks, the number of patients requiring hospitalization can be exceptionally high. This poses a high burden on the health care systems of the affected countries. The identification of genetic makers associated with severe disease can help to better understand disease pathophysiology and guide the development of clinical management strategies, thereby reducing the burden of HFMD. However, to date there have been few studies addressing the association between host genetics and clinical phenotypes of HFMD, while the obtained results from such GWAS-based studies were far from accepted significance levels ($P < 10^{-8}$). For example, a recent study in China showed that rs2843710 of type I interferon receptor 1 (IFNAR1) was associated with HFMD susceptibility and severity in Chinese patients with a P value of 0.0001 and 0.028, respectively [187].

In this chapter, the Illumina Human OmniExpress-24 BeadChip with a coverage of >600K SNPs across the human genome was employed to screen total of 271 Vietnamese patients with HFMD (including 185 with severe HFMD), representing the most comprehensive GWAS study to date. The analysis has revealed two potential genetic variations, namely rs10235866 of gene NPTX2/TMEM130 and rs10739235 of gene RAD23B on chromosomes 7 and 9, respectively, which may be associated with severe HFMD and severe EV-A71 associated HFMD.

The Illumina Human OmniExpress-24 BeadChip used in the present study also included IFNAR1. However, the genetic variations of IFNAR1 described in the Chinese study was not detected in the present study (p-value 0.036, which was

much lower than 10^{-8}. This may be attributed to the difference in terms of patient populations or false positivity of the signal of the original study. An additional study with a larger sample size on the same population is thus needed to confirm and validate the findings among Chinese patients.

5.4.1. Roles of two detected candidate genes.

SNP rs10235866 is a single nucleotide variant of gene NPTX2/TMEM130. This gene, named neuronal pentraxin II, is 11 kb in length, contains four introns, and is located on chromosome 7q21.3-q22.1 [238,239]. It encodes neuronal pentraxins, a synaptic protein which stimulates synapse formation. This protein also plays a role in clustering of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors at established synapses, leading to non-apoptotic cell death of dopaminergic nerve cells [239]. In human, this kind of cells are found in substantia nigra pars compacta. The NPTX2 gene was reported to be upregulated in patients with Parkinson's disease [240], suggesting that NPTX2 may be involved in its pathogenesis. However, the full underlying mechanism is still unknown.

SNP rs10739235 and rs817854 are two single nucleotide variants both belong to gene RAD23B (RAD23 homolog B), coding for Nucleotide Excision Repair Protein. The RAD23 protein was shown to be involved in nucleotide excision repair (NER) in vitro [241]. It also interacts with the nucleotide excision activity of 3-methyladenine-DNA glycosylase (MPG), suggesting a role in DNA damage recognition in base excision repair [241,242]. Some studies have suggested that

RAD23B may be related to different kinds of cancers but no strong evidence was given [243,244].

These two genes did not show any association with infection or viral infection in previous studies. Most of the available reports just mentioned about their roles in non-communicable diseases (as mentioned above). Therefore, once the significance of those genes are confirmed, functional studies should be conducted to investigate the roles of these genes to viral infection.

5.4.2. Limitation of this study

The first GWAS screening revealed two potential SNPs associated with a severe HFMD phenotype. However, due to the reduction of severe HFMD in Vietnam in the study period, I could not recruit a sufficient number of severe cases for this first GWAS stage and therefore this result is not statistically powerful enough. The P values of the two detected SNPs in this screening phase were close to the accepted significance threshold of genome-wide association. The analysis is this an exploratory phase in itself. As the HFMD program is still recruiting patients, I expect to have access to samples from more severe cases to repeat the analysis and improve the statistical power for the present GWAS discovery stage.

Moreover, the principle of GWAS is to measure and analyze DNA sequence variation across the human genome to identify if a particular gene is associated with severity/susceptibility of a disease [179]. Thus the results in this chapter (if proven to be GWAS signal) only suggested which SNPs/loci should be used for further analysis wherein SNP functionality is studies.

5.4.3. Future directions

More cases (both HFMD cases and controls) should be collected and analyzed using the same technique and statistical analysis to improve statistical power. Once reaching the GWAS formal significance value, PCR primers, covering most significant SNPs will be designed and tested on another patient cohort in a replication stage to confirm candidate SNPs. Detected genes then will undergo different process in vitro and in vivo to understand the mechanism/ role of those genes to clinical phenotypes before a conclusion is reached.

5.5. CONCLUSION

Three potential candidate genetic variations were detected using GWAS, which may be associated with severe HFMD, specifically NPTX2 (represented by SNP rs10235866) and RAD23B (represented by SNP rs10739235 and rs817854). A GWAS replication stage is needed to confirm the association of these SNPs and severe HFMD, and is currently ongoing.

6. GENERAL DISCUSSION AND FUTURE DIRECTIONS

Since the Sarawak outbreak in 1997, (EV-A71-associated) HFMD has become endemic and a notifiable infectious disease in countries of the Asia-Pacific region with pandemic potential. Consequently, there has been a dramatic increase in terms of research activities undertaken to answer the key questions about this neglected infection prior to 1997.

A literature research on PubMed using the term 'Hand, Foot and Mouth Disease' resulted in over 2000 articles, of which only 212 were published before 1997. Undoubtedly, tremendous progress has been made on various aspects of this emerging infection over the last decades. Yet there remain significant gaps in our knowledge, especially about clinical management and intervention strategies. Herein, I will discuss the progress that has been made on HFMD research over the last years, focusing on the main areas of my DPhil research (including the clinical, epidemiological, etiological, pathogen evolution and host genetics aspects of the disease), whilst other aspects have been covered elsewhere in Chapter 1. The section asks some key questions for future research.

During 4 years of my DPhil project, I have set up an observational study in three large hospitals of Ho Chi Minh City, Vietnam, collecting clinical data and samples from 1547 HFMD patients. 20 EV serotypes were detected within these samples and 135 whole genome sequences were obtained. I also used GWAS to screen for genetic markers associated with severe HFMD and found two potential SNPs which can be

used for further genetic studies. A retrospective study was also conducted in Children's Hospital 2 with information from 4314 hospital records to describe the 7 year progress of HFMD in Southern Vietnam. Those work, putting together, has fulfilled the gaps of HFMD understanding from many angles.

6.1. CLINICAL ASPECTS

In terms of clinical features, although many HFMD studies have been conducted in Vietnam and the region, most of these have focused on hospitalized (severe) cases. This study was, therefore, the first to collect the clinical data and specimens from both out- and inpatients with HFMD in parallel. Notably, I also reported the associated clinical phenotypes for specific dominant pathogens (CV-A6, CV-A10, CV-A16 and EV-A71), which showed a slight difference between these common causative agents of HFMD. More specifically, CV-A10 infected patients were likely to have mouth ulcers than skin lesions. They also had higher white blood cell counts and C-reactive protein levels, which suggest CV-A10 infection may trigger more aggressive responses from the host immune system, and hence more severe illness.

In view that there is no specific treatment for HFMD and vaccines against EV-A71 have been developed for commercial use in China only, and that most of severe HFMD patients progress within 72 hours [19], the identification risk factors associated with severe clinical phenotypes to develop a reliable prognostic model for progression remains key to clinical management of severe HFMD. My analysis identified macular rash, high blood glucose, high pulse and elevated systolic blood pressure as risk factors associated with development of severe HFMD. However, due

to the low number of severe cases admitted during the study period of my DPhil research, building and validating a prognostic model for severe HFMD was unachievable. Future studies should therefore focus on these aspects, which would require a combined clinical and modelling effort.

6.2. EPIDEMIOLOGY AND SOCIAL ECONOMIC BURDEN

By combining data from 7 consecutive years (2009 – 2015), this study revealed for the first time annual peaks of HFMD in Vietnam, with one large peak from August to November (coinciding with the start of the school year after the summer break) and a smaller one from March to May (coinciding with the transition from dry to wet season in southern Vietnam). This pattern was similar to that in southern provinces of China [206,208]. Warm and humid weather was reported to be a favorable factor for the disease and may influence the monthly distribution of HFMD cases [206,208]. Of note, recent studies have also successfully predicted the incidence of HFMD based on weather variability and seasonal factors [205–208,245]. A comprehensive modeling study utilizing data collected across Vietnam is thus able to provide more insights into the epidemiology of HFMD in Vietnam at a nationwide level and for distinct geographic locations (e.g. south, central and north, highlands and river deltas).

Following the explosive outbreak of HFMD in 2011-2012, with support from WHO, the Vietnamese Ministry of Health developed and implemented a wide range of intervention strategies, including strengthening of the surveillance systems, staff training and raising public awareness about the disease [203]. Although, a direct

link was not established due to the retrospective nature of the present study, one of the interesting findings of my work when looking at the data from HFMD cases from 2009 to 2015 was the change in clinical management and public awareness about the disease. The improvement of medical staff training in diagnosis and treatment of the disease was reflected by a decreased rate of misdiagnosis/misclassification and unnecessary admissions after the outbreak. The reduction of unnecessary admissions could also help to alleviate the social-economic burden on the national health care system and society, especially parents of affected children. Likewise, awareness improvement among the general public and medical staff was indicated by the time since onset of illness that the children were first diagnosed/seen by a doctor. After the outbreak, better understanding about the disease allowed parents to seek for medical advice and treatment earlier. This earlier diagnosis, together with close and appropriate monitoring and management, may have contributed to a reduction in terms of the numbers of patients progressing to severe disease, leading to better outcome. Of note, similar findings were reported in a recent study from China [19].

Evaluating the impact of intervention strategies during an epidemic is challenging. However, collectively my research has found suggestions that accurate case classification, early hospital admission/diagnosis and appropriate clinical monitoring and management are key components to consider in response to emerging infection outbreaks.

6.3. AETIOLOGY

Enterovirus A is the causative agent of HFMD, and the serotype EV-A71 is associated with the more severe clinical phenotypes. However, the prevalence of other enterovirus A serotypes associated with HFMD in Vietnam and their associated clinical phenotypes remains poorly understood. Using a combination of multiplex real-time RT PCR and partial VP1 sequencing, I analyzed a total of 1547 HFMD patients (957 inpatients and 590 outpatients) in southern Vietnam between July 2013 and July 2015, and identified 20 EV serotypes co-circulating in southern Vietnam during the study period. The main pathogens included EV-A71, CV-A16, CV-A6 and CV-A10. These serotypes co-circulated in Vietnam and replaced each other during the study period. Remarkably, CV-A6 and CV-A10 were detected in Vietnam for the first time, and CV-A6 has emerged to become the most common of the non-EV-A71 EV-As detected in HFMD patients. Together, data from this study and those from countries in the region including China [91], suggest complex patterns of etiology and serotype replacement among HFMD patients, and emphasize the need for continued monitoring and surveillance of circulating pathogens and the challenge to develop bivalent/multivalent vaccines to control HFMD. To support this, it is also important to assess the inter-relatedness among HFMD causing pathogens as well as between other enteroviruses, particularly the level of cross-immunity in natural infection and post-vaccination.

One intriguing finding in this study was the high proportion of EV-A71 among non-severe cases (including outpatients) (20%). While this result was somehow unexpected, it was in accordance with a recent report in China [91]. Also, viruses of

the family *Picornaviridae* are ubiquitous and often cause asymptomatic infections and/or mild disease. Yet, the underlying mechanisms determining the disease phenotype remain unknown. There may be a complex interaction between the make-up of the host genetics background and immune system and the pathogen that shapes the clinical manifestations, meriting in-depth studies, including study of the host genetics of severe HFMD (Chapter 5).

6.4. EVOLUTION OF EV-A71

The main EV-A71 subgenogroup causing infections in patients enrolled in my cohort from 2013 to 2015 was B5, which had a relatively stable genetic diversity. Results from a previous study conducted by my colleagues and I illustrated A subgenogroup switch from C4 to B5 in 2012. My findings therefore further reveal B5 continued to be the predominant subgenogroup of EV-A71 in Vietnam after 2012 [44].

Phylogenetic analysis of the B5 viruses in this study revealed 2 distinct viral lineages (one originating from Malaysia and the other from Taiwan), co-circulating in southern Vietnam, suggesting two independent viral importation events from Malaysia and Taiwan. In terms of phylogeography, B5 sequences from northern Vietnam formed a distinct cluster, albeit a small sample size, while sequence from other parts of the South showed a high level of viral movement across provinces. Clearly, additional analysis with a larger sample size is needed to confirm these preliminary findings. The observed phylogeographic pattern of EV-A71 within Vietnam is in parallel with previous findings suggesting a low level of viral movement across distantly connected locations (i.e. southern and northern parts of

Vietnam), and may partially explain why the explosive outbreak of EV-A71 in 2011 was confined to the southern part of Vietnam.

Subgenogroup switches often accompany large/severe EV-A71 associated HFMD outbreak, and therefore may play a role in disease severity. The number of severe cases in my cohort was less than anticipated based on the observations from the previous 2 years when C4 was the dominant pathogen [41,43]. This reduction coincided with a subgenogroup switch from C4 to B5 in 2013, suggesting that the B5 subgenogroup in Vietnam may have undergone some changes/mutation affecting the virulence and pathogenicity of the virus. Alternatively, this might have been a consequence of the interplay between the pathogen, public health response and population immunity. In terms of pathogen evolution, Kataoka et al, using non-human primate models, reported a strong in vivo selection of 145E at residue 145 of VP1 variants, associated with the development of viremia and neuropathogenesis. By observing the presence of 145E in different subgenogroups, Kataoka's results suggest that a higher proportion of 145E in C4 may be due to the more severe phenotype [246]. However, this result should be treated with caution, as mutations can occur when adapting virus on cell cultures and primates. Furthermore, the majority of B5 viruses sequenced in this study carried the 145E residue which is not strongly associated with severe phenotypes.

One of the other remaining key questions for EV-A71-associated HFMD is why EV-A71 infection in Vietnam does not exhibit the 2-3 cyclical pattern as observed in other countries in the region as Malaysia, Taiwan and Japan, even though a similar

complex pattern of subgenogroup replacement and viral movement across provinces was documented.

Interestingly, a recent report from China, utilizing nationwide data from 13.7 million cases from 2008 to 2015, also showed that, similar to Vietnam, HFMD in China did not exhibit the 2-year periodicity for all serotypes. [91]. Collectively, the epidemic patterns of HFMD in the region are complex, with huge implications for vaccine implementation. I hypothesize that herd immunity, pathogen virulence, public health responses and other factors have a combined effect on the dynamics of HFMD in individual countries, including Vietnam. Heading to the future, these are key areas for research with a multidisciplinary approach.

6.5. HOST GENETIC FACTORS

Using GWAS, I reported for the first time a list of candidate SNPs. Three particularly interesting SNPs: rs10235866, rs10739235 and rs817854, have a P value relatively close to the significance threshold of GWAS and therefore may be associated with severe HFMD in Vietnam.

Likewise, in recent years, efforts from countries in the region (China) have also revealed some promising SNP candidates, albeit with P values at a level well below the GWS significance [187,189,191]. Clearly, more severe cases, which are currently being enrolled, are needed to test whether the association between these SNPs and severe HFMD are significant.

To further exclude the possibility of false positive GWAS signals, it is imperative that specific PCR primers, targeting the candidate SNPs will be used to validate my initial findings in another cohort with similar characteristics as the screening cohort. Lastly, the chosen SNPs will need to undergo functional studies both in vitro and in vivo to identify the biological contribution of such associated genes/SNPs to the pathogenesis of severe HFMD.

6.6. FURTHER DIRECTIONS

Over the last years, we have made extensive progress in understanding the epidemiology, etiology, virology and to some extent the immunology of HFMD, and in developing effective vaccines for EV-A71-associated HFMD. However, the outbreaks of (EV-A71-associated) HFMD in the Asia-Pacific region still present many challenges that need to be overcome for a better response to the event of emerging infections, thereby providing the best care we possibly can to the affected patients. Yet, to this end, we still do not know what are the best treatment approaches we should offer to the severe patients, owing to a failure to conduct appropriate clinical trials during the recent outbreak peaks and the fading interest from the funders once epidemics are over. Addressing the question of how to best conduct intervention studies during an outbreak, and maintaining interest from major funders on pathogens with pandemic potential are both highly challenging, but are crucial to improve patient outcomes.

For the future directions of HFMD research, clinical trials of current supportive management strategies and antivirals aiming at improving HFMD case management

is undoubtedly one of the main areas for future research. Other needed efforts to elucidate cross-immunity, socio-economic burden, host genetics associations and pathogen evolution.

The whole HFMD program is still ongoing in Vietnam and will enroll more patients until the end of 2018, expected to reach 3000 cases. We will continue to test for HFMD pathogens and monitor EV serotype replacement. A study of the social-economic burden of HFMD has been conducted by my group to assess the disease burden in more details among both inpatients and outpatients. Cross-reactive immune responses to different EV serotypes in sera from HFMD patients will also be tested using samples from this study. This work will deepenunderstanding of the host immune response, which is essential for vaccine development. I will continue the host genetics part of this project: another 100 severe HFMD cases were collected already and DNA samples are ready to be shipped to GIS for GWAS analysis. The whole program, once completed, will give a fuller picture of HFMD in Vietnam from all different aspects, including epidemiology, clinical features, etiology, social – economy and host genetic factors.

7. REFERENCES

1. Seddon JH. Research Newsletter,. Research Committee of the New Zealand Council, College of General Practitioners. 1961;2.

2. Robinson CR, Doane FW, Rhodes AJ. Report of an Outbreak of Febrile Illness with Pharyngeal Lesions and Exanthem: Toronto, Summer 1957--Isolation of Group A Coxsackie Virus. Can Med Assoc J. 1958 Oct 15;79(8):615–21.

3. Alsop J, Flewett TH, Foster JR. "Hand-Foot-and-Mouth Disease" in Birmingham in 1959. Br Med J. 1960 Dec 10;2(5214):1708–11.

4. Duff MF. Hand-foot-and-mouth syndrome in humans: coxackie A10 infections in New Zealand. Br Med J. 1968 Jun 15;2(5606):661–4.

5. Tagaya I, Moritsugu Y. Epidemic of hand, foot and mouth disease in Japan. Jpn. J. Med. Sci. Biol. 1973 Jun;26(3):143–7.

6. Adler JL, Mostow SR, Mellin H, Janney JH, Joseph JM. Epidemiologic investigation of hand, foot, and mouth disease. Infection caused by coxsackievirus A 16 in Baltimore, June through September 1968. Am. J. Dis. Child. 1970 Oct;120(4):309–14.

7. Schmidt NJ, Lennette EH, Ho HH. An apparently new enterovirus isolated from patients with disease of the central nervous system. Journal of infectious diseases. 1974;129(3):304–309.

8. Deibel R, Gross LL, Collins DN. Isolation of a new enterovirus (38506). Proc. Soc. Exp. Biol. Med. 1975 Jan;148(1):203–7.

9. Kennett ML, Birch CJ, Lewis FA, Yung AP, Locarnini SA, Gust ID. Enterovirus type 71 infection in Melbourne. Bulletin of the World Health Organization. 1974;51(6):609.

10. Blomberg J, Lycke E, Ahlfors K, Johnsson T, Wolontis S, von Zeipel G. Letter: New enterovirus type associated with epidemic of aseptic meningitis and-or hand, foot, and mouth disease. Lancet. 1974 Jul 13;2(7872):112.

11. Hagiwara A, Tagaya I, Yoneyama T. Epidemic of Hand, Foot and Mouth Disease Associated with Enterovirus 71 Infection. Intervirology. 1978;9(1):60–3.

12. Ishimaru Y, Nakano S, Yamaoka K, Takami S. Outbreaks of hand, foot, and mouth disease by enterovirus 71. High incidence of complication disorders

of central nervous system. Archives of disease in childhood. 1980;55(8):583–588.

13. Gilbert GL, Dickson KE, Waters MJ, Kennett ML, Land SA, Sneddon M. Outbreak of enterovirus 71 infection in Victoria, Australia, with a high incidence of neurologic involvement. Pediatr. Infect. Dis. J. 1988 Jul;7(7):484–8.

14. Chan LG, Parashar UD, Lye MS, Ong FGL, Zaki SR, Alexander JP, Ho KK, Han LL, Pallansch MA, Suleiman AB. Deaths of children during an outbreak of hand, foot, and mouth disease in Sarawak, Malaysia: clinical and pathological characteristics of the disease. Clinical infectious diseases. 2000;31(3):678–683.

15. Tagaya I, Tachibana K. Epidemic of hand, foot and mouth disease in Japan, 1972-1973: difference in epidemiologic and virologic features from the previous one. Japanese Journal of Medical Science and Biology. 1975;28(4):231–234.

16. Tagaya I, Takayama R, Hagiwara A. A large-scale epidemic of hand, foot and mouth disease associated with enterovirus 71 infection in Japan in 1978. Japanese Journal of Medical Science and Biology. 1981;34(3):191–196.

17. Ho M, Chen ER, Hsu KH, Twu SJ, Chen KT, Tsai SF, Wang JR, Shih SR. An epidemic of enterovirus 71 infection in Taiwan. Taiwan Enterovirus Epidemic Working Group. N. Engl. J. Med. 1999 Sep 23;341(13):929–35.

18. Zhang Y, Zhu Z, Yang W, Ren J, Tan X, Wang Y, Mao N, Xu S, Zhu S, Cui A, Zhang Y, Yan D, Li Q, Dong X, Zhang J, Zhao Y, Wan J, Feng Z, Sun J, Wang S, Li D, Xu W. An emerging recombinant human enterovirus 71 responsible for the 2008 outbreak of hand foot and mouth disease in Fuyang city of China. Virol. J. 2010 May 12;7:94.

19. Xing W, Liao Q, Viboud C, Zhang J, Sun J, Wu JT, Chang Z, Liu F, Fang VJ, Zheng Y, Cowling BJ, Varma JK, Farrar JJ, Leung GM, Yu H. Hand, foot, and mouth disease in China, 2008-12: an epidemiological study. Lancet Infect Dis. 2014 Apr;14(4):308–18.

20. McMinn P, Stratov I, Nagarajan L, Davis S. Neurological manifestations of enterovirus 71 infection in children during an outbreak of hand, foot, and mouth disease in Western Australia. Clinical infectious diseases. 2001;32(2):236–242.

21. Nolan MA, Craig ME, Lahra MM, Rawlinson WD, Prager PC, Williams GD, Bye AME, Andrews PI. Survival after pulmonary edema due to enterovirus 71 encephalitis. Neurology. 2003 May 27;60(10):1651–6.

22. AbuBakar S, Sam I-C, Yusof J, Lim MK, Misbah S, Hooi P-S. Enterovirus 71 Outbreak, Brunei. Emerg Infect Dis. 2009 Jan;15(1):79–82.

23. Zhang Y, Tan X-J, Wang H-Y, Yan D-M, Zhu S-L, Wang D-Y, Ji F, Wang X-J, Gao Y-J, Chen L. An outbreak of hand, foot, and mouth disease associated with subgenotype C4 of human enterovirus 71 in Shandong, China. Journal of Clinical Virology. 2009;44(4):262–267.

24. Yang F, Ren L, Xiong Z, Li J, Xiao Y, Zhao R, He Y, Bu G, Zhou S, Wang J, Qi J. Enterovirus 71 Outbreak in the People's Republic of China in 2008. Journal of Clinical Microbiology. 2009 Jul;47(7):2351.

25. Luo K-W, Gao L-D, Hu S-X, Zhang H, Deng Z-H, Huang W, Sun Q-L, Zhang F, Zhang S-Y, Chen Y. Hand, Foot, and Mouth Disease in Hunan Province, China, 2009-2014: Epidemiology and Death Risk Factors. PLoS ONE. 2016;11(11):e0167269.

26. Lin T-Y, Twu S-J, Ho M-S, Chang L-Y, Lee C-Y. Enterovirus 71 Outbreaks, Taiwan: Occurrence and Recognition. Emerg Infect Dis. 2003 Mar;9(3):291–3.

27. Chen K-T, Chang H-L, Wang S-T, Cheng Y-T, Yang J-Y. Epidemiologic Features of Hand-Foot-Mouth Disease and Herpangina Caused by Enterovirus 71 in Taiwan, 1998 2005. PEDIATRICS. 2007 Aug 1;120(2):e244–52.

28. Chen S-P, Huang Y-C, Li W-C, Chiu C-H, Huang C-G, Tsao K-C, Lin T-Y. Comparison of clinical features between coxsackievirus A2 and enterovirus 71 during the enterovirus outbreak in Taiwan, 2008: a children's hospital experience. J Microbiol Immunol Infect. 2010 Apr;43(2):99–104.

29. Taniguchi K, Hashimoto S, Kawado M, Murakami Y, Izumida M, Ohta A, Tada Y, Shigematsu M, Yasui Y, Nagai M. Overview of infectious disease surveillance system in Japan, 1999-2005. J Epidemiol. 2007 Dec;17 Suppl:S3-13.

30. Editorial Committee of Infectious Agents Surveillance Report, National Institute of Infectious Diseases. Annual report on findings of infectious agents in Japan, 2003. Jpn. J. Infect. Dis. 2004;57 Suppl:1–143.

31. Chan LG, Parashar UD, Lye MS, Ong FG, Zaki SR, Alexander JP, Ho KK, Han LL, Pallansch MA, Suleiman AB, Jegathesan M, Anderson LJ. Deaths of children during an outbreak of hand, foot, and mouth disease in sarawak, malaysia: clinical and pathological characteristics of the disease. For the Outbreak Study Group. Clin. Infect. Dis. 2000 Sep;31(3):678–83.

32. Shimizu H, Utama A, Yoshii K, Yoshida H, Yoneyama T, Sinniah M, Yusof MA, Okuno Y, Okabe N, Shih SR, Chen HY, Wang GR, Kao CL, Chang KS, Miyamura T, Hagiwara A. Enterovirus 71 from fatal and nonfatal cases of hand, foot and

mouth disease epidemics in Malaysia, Japan and Taiwan in 1997-1998. Jpn. J. Infect. Dis. 1999 Feb;52(1):12–5.

33. Podin Y, Gias EL, Ong F, Leong Y-W, Yee S-F, Yusof MA, Perera D, Teo B, Wee T-Y, Yao S-C, Yao S-K, Kiyu A, Arif MT, Cardosa MJ. Sentinel surveillance for human enterovirus 71 in Sarawak, Malaysia: lessons from the first 7 years. BMC Public Health. 2006 Jul 7;6:180.

34. Zhang Y, Nan L, Wu G, Tan X, Xu D, Gu S, Zhu S, Yan D, An H, Xu W. The epidemiologic and virological analysis of an outbreak of hand, foot, and mouth disease in Inner Mongolia in 2007. Bing Du Xue Bao. 2009 May;25(3):159–65.

35. Ryu W-S, Kang B, Hong J, Hwang S, Kim A, Kim J, Cheon D-S. Enterovirus 71 infection with central nervous system involvement, South Korea. Emerging Infect. Dis. 2010 Nov;16(11):1764–6.

36. Chan KP, Goh KT, Chong CY, Teo ES, Lau G, Ling AE. Epidemic hand, foot and mouth disease caused by human enterovirus 71, Singapore. Emerging Infect. Dis. 2003 Jan;9(1):78–85.

37. Ang LW, Koh BK, Chan KP, Chua LT, James L, Goh KT. Epidemiology and control of hand, foot and mouth disease in Singapore. Ann Acad Med Singapore. 2009;38:106–112.

38. Linsuwanon P, Puenpa J, Huang S-W, Wang Y-F, Mauleekoonphairoj J, Wang J-R, Poovorawan Y. Epidemiology and seroepidemiology of human enterovirus 71 among Thai populations. Journal of Biomedical Science. 2014 Feb 18;21(1):16.

39. Seiff A. Cambodia unravels cause of mystery illness. Lancet. 2012 Jul 21;380(9838):206.

40. Sabanathan S, Tan LV, Thwaites L, Wills B, Qui PT, Rogier van Doorn H. Enterovirus 71 related severe hand, foot and mouth disease outbreaks in South-East Asia: current situation and ongoing challenges. J Epidemiol Community Health. 2014 Jun;68(6):500–2.

41. Tu PV, Thao NTT, Perera D, Huu TK, Tien NTK, Thuong TC, How OM, Cardosa MJ, McMinn PC. Epidemiologic and virologic investigation of hand, foot, and mouth disease, southern Vietnam, 2005. Emerging Infect. Dis. 2007 Nov;13(11):1733–41.

42. World Health Organization. A Guide to Clinical Management and Public Health Response for Hand, Foot and Mouth Disease (HFMD). World Health Organization; 2011.

43. Khanh TH, Sabanathan S, Thanh TT, Thoa LPK, Thuong TC, Hang V thi T, Farrar J, Hien TT, Chau N van V, van Doorn HR. Enterovirus 71-associated Hand, Foot, and Mouth Disease, Southern Vietnam, 2011. Emerg Infect Dis. 2012 Dec;18(12):2002–5.

44. Geoghegan JL, Tan LV, Kühnert D, Halpin RA, Lin X, Simenauer A, Akopov A, Das SR, Stockwell TB, Shrivastava S, Ngoc NM, Uyen LTT, Tuyen NTK, Thanh TT, Hang VTT, Qui PT, Hung NT, Khanh TH, Thinh LQ, Nhan LNT, Van HMT, Viet DC, Tuan HM, Viet HL, Hien TT, Chau NVV, Thwaites G, Grenfell BT, Stadler T, Wentworth DE, Holmes EC, Van Doorn HR. Phylodynamics of Enterovirus A71-Associated Hand, Foot, and Mouth Disease in Viet Nam. J. Virol. 2015 Sep;89(17):8871–9.

45. Zhao YY, Jin H, Zhang XF, Wang B. Case-fatality of hand, foot and mouth disease associated with EV71: a systematic review and meta-analysis. Epidemiol. Infect. 2015 Oct;143(14):3094–102.

46. Lee M-S, Chiang P-S, Luo S-T, Huang M-L, Liou G-Y, Tsao K-C, Lin T-Y. Incidence rates of enterovirus 71 infections in young children during a nationwide epidemic in Taiwan, 2008-09. PLoS Negl Trop Dis. 2012;6(2):e1476.

47. Ma E, Chan KC, Cheng P, Wong C, Chuang SK. The enterovirus 71 epidemic in 2008--public health implications for Hong Kong. Int. J. Infect. Dis. 2010 Sep;14(9):e775-780.

48. Lu C-Y, Lee C-Y, Kao C-L, Shao W-Y, Lee P-I, Twu S-J, Yeh C-C, Lin S-C, Shih W-Y, Wu S-I, Huang L-M. Incidence and case-fatality rates resulting from the 1998 enterovirus 71 outbreak in Taiwan. J. Med. Virol. 2002 Jun;67(2):217–23.

49. Wang J, Teng Z, Cui X, Li C, Pan H, Zheng Y, Mao S, Yang Y, Wu L, Guo X, Zhang X, Zhu Y. Epidemiological and serological surveillance of hand-foot-and-mouth disease in Shanghai, China, 2012–2016. Emerging Microbes & Infections. 2018 Jan 24;7(1):8.

50. Yang F, Zhang T, Hu Y, Wang X, Du J, Li Y, Sun S, Sun X, Li Z, Jin Q. Survey of enterovirus infections from hand, foot and mouth disease outbreak in China, 2009. Virol. J. 2011 Nov 6;8:508.

51. Park SH, Choi SS, Oh SA, Kim CK, Cho SJ, Lee JH, Ryu SH, Pak SH, Jung SK, Lee JI, Park GY, Choi SM, Chae YZ, Kang BH, Cheon DS, Kim HS. Detection and characterization of enterovirus associated with herpangina and hand, foot, and mouth disease in Seoul, Korea. Clin. Lab. 2011;57(11–12):959–67.

52. Khetsuriani N, Lamonte-Fowlkes A, Oberst S, Pallansch MA, Centers for Disease Control and Prevention. Enterovirus surveillance--United States, 1970-2005. MMWR Surveill Summ. 2006 Sep 15;55(8):1–20.

53. Greninger AL, Naccache SN, Messacar K, Clayton A, Yu G, Somasekar S, Federman S, Stryke D, Anderson C, Yagi S, Messenger S, Wadford D, Xia D, Watt JP, Van Haren K, Dominguez SR, Glaser C, Aldrovandi G, Chiu CY. A novel outbreak enterovirus D68 strain associated with acute flaccid myelitis cases in the USA (2012-14): a retrospective cohort study. Lancet Infect Dis. 2015 Jun;15(6):671–82.

54. Messacar K, Schreiner TL, Maloney JA, Wallace A, Ludke J, Oberste MS, Nix WA, Robinson CC, Glodé MP, Abzug MJ, Dominguez SR. A cluster of acute flaccid paralysis and cranial nerve dysfunction temporally associated with an outbreak of enterovirus D68 in children in Colorado, USA. Lancet. 2015 Apr 25;385(9978):1662–71.

55. McWilliam Leitch EC, Cabrerizo M, Cardosa J, Harvala H, Ivanova OE, Koike S, Kroes ACM, Lukashev A, Perera D, Roivainen M, Susi P, Trallero G, Evans DJ, Simmonds P. The association of recombination events in the founding and emergence of subgenogroup evolutionary lineages of human enterovirus 71. J. Virol. 2012 Mar;86(5):2676–85.

56. Gaunt E, Harvala H, Österback R, Sreenu VB, Thomson E, Waris M, Simmonds P. Genetic characterization of human coxsackievirus A6 variants associated with atypical hand, foot and mouth disease: a potential role of recombination in emergence and pathogenicity. J. Gen. Virol. 2015 May;96(Pt 5):1067–79.

57. Puenpa J, Vongpunsawad S, Österback R, Waris M, Eriksson E, Albert J, Midgley S, Fischer TK, Eis-Hübinger AM, Cabrerizo M, Gaunt E, Simmonds P, Poovorawan Y. Molecular epidemiology and the evolution of human coxsackievirus A6. J. Gen. Virol. 2016 Dec;97(12):3225–31.

58. Jacques J, Moret H, Minette D, Lévêque N, Jovenin N, Deslée G, Lebargy F, Motte J, Andréoletti L. Epidemiological, molecular, and clinical features of enterovirus respiratory infections in French children between 1999 and 2005. J. Clin. Microbiol. 2008 Jan;46(1):206–13.

59. Leveque N, Laurent A. A Novel Mode of Transmission for Human Enterovirus Infection Is Swimming in Contaminated Seawater: Implications in Public Health and in Epidemiological Surveillance. Clin Infect Dis. 2008 Sep 1;47(5):624–6.

60. Nilsson EC, Jamshidi F, Johansson SMC, Oberste MS, Arnberg N. Sialic acid is a cellular receptor for coxsackievirus A24 variant, an emerging virus with pandemic potential. J. Virol. 2008 Mar;82(6):3061–8.

61. Boivin G, Mazzulli T, Petric M, Couillard M. Diagnosis of Viral Infections*. Clinical Virology, Third Edition. 2009 Jan 1;265–94.

62. International Committee on Taxonomy of Viruses, King AMQ, editors. Virus taxonomy: classification and nomenclature of viruses: ninth report of the

International Committee on Taxonomy of Viruses. London ; Waltham, MA: Academic Press; 2012.

63. Oberste MS, Maher K, Kilpatrick DR, Pallansch MA. Molecular evolution of the human enteroviruses: correlation of serotype with VP1 sequence and application to picornavirus classification. Journal of virology. 1999;73(3):1941–1948.

64. Mateu MG. Antibody recognition of picornaviruses and escape from neutralization: a structural view. Virus Res. 1995 Sep;38(1):1–24.

65. Minor PD, Ferguson M, Evans DM, Almond JW, Icenogle JP. Antigenic structure of polioviruses of serotypes 1, 2 and 3. J. Gen. Virol. 1986 Jul;67 (Pt 7):1283–91.

66. Yi E-J, Shin Y-J, Kim J-H, Kim T-G, Chang S-Y. Enterovirus 71 infection and vaccines. Clin Exp Vaccine Res. 2017 Jan;6(1):4–14.

67. Mendelsohn CL, Wimmer E, Racaniello VR. Cellular receptor for poliovirus: molecular cloning, nucleotide sequence, and expression of a new member of the immunoglobulin superfamily. Cell. 1989 Mar 10;56(5):855–65.

68. Greve JM, Davis G, Meyer AM, Forte CP, Yost SC, Marlor CW, Kamarck ME, McClelland A. The major human rhinovirus receptor is ICAM-1. Cell. 1989 Mar 10;56(5):839–47.

69. Shafren DR, Bates RC, Agrez MV, Herd RL, Burns GF, Barry RD. Coxsackieviruses B1, B3, and B5 use decay accelerating factor as a receptor for cell attachment. J. Virol. 1995 Jun;69(6):3873–7.

70. Yamayoshi S, Fujii K, Koike S. Receptors for enterovirus 71. Emerg Microbes Infect. 2014 Jul;3(7):e53.

71. Belnap DM, Filman DJ, Trus BL, Cheng N, Booy FP, Conway JF, Curry S, Hiremath CN, Tsang SK, Steven AC, Hogle JM. Molecular tectonic model of virus structural transitions: the putative cell entry states of poliovirus. J. Virol. 2000 Feb;74(3):1342–54.

72. Baggen J, Thibaut HJ, Strating JRPM, van Kuppeveld FJM. The life cycle of non-polio enteroviruses and how to target it. Nat. Rev. Microbiol. 2018 Jun;16(6):368–81.

73. Lozano G, Martínez-Salas E. Structural insights into viral IRES-dependent translation mechanisms. Curr Opin Virol. 2015 Jun;12:113–20.

74. Suhy DA, Giddings TH, Kirkegaard K. Remodeling the endoplasmic reticulum by poliovirus infection and by individual viral proteins: an autophagy-like origin for virus-induced vesicles. J. Virol. 2000 Oct;74(19):8953–65.

75. Strating JR, van Kuppeveld FJ. Viral rewiring of cellular lipid metabolism to create membranous replication compartments. Curr. Opin. Cell Biol. 2017;47:24–33.

76. Ooi MH, Solomon T, Podin Y, Mohan A, Akin W, Yusuf MA, del Sel S, Kontol KM, Lai BF, Clear D, Chieng CH, Blake E, Perera D, Wong SC, Cardosa J. Evaluation of Different Clinical Sample Types in Diagnosis of Human Enterovirus 71-Associated Hand-Foot-and-Mouth Disease. J Clin Microbiol. 2007 Jun;45(6):1858–66.

77. Cecil RLF, Goldman L, Schafer AI. Goldman's Cecil Medicine,Expert Consult Premium Edition -- Enhanced Online Features and Print, Single Volume,24: Goldman's Cecil Medicine. Elsevier Health Sciences; 2012.

78. Chapman NM, Tracy S, Gauntt CJ, Fortmueller U. Molecular detection and identification of enteroviruses using enzymatic amplification and nucleic acid hybridization. Journal of clinical microbiology. 1990;28(5):843–850.

79. Rotbart HA. Enzymatic RNA amplification of the enteroviruses. Journal of Clinical Microbiology. 1990;28(3):438–442.

80. Petitjean J, Freymuth F, Kopecka H, Brouard J, Boutard P, Lebon P. Detection of enteroviruses in cerebrospinal fluids: enzymatic amplification and hybridization with a biotinylated riboprobe. Mol. Cell. Probes. 1994 Feb;8(1):15–22.

81. Abebe A, Johansson B, Abens J, Strannegård O. Detection of enteroviruses in faeces by polymerase chain reaction. Scand. J. Infect. Dis. 1992;24(3):265–73.

82. Rotbart HA. Nucleic acid detection systems for enteroviruses. Clin. Microbiol. Rev. 1991 Apr;4(2):156–68.

83. Zoll GJ, Melchers WJ, Kopecka H, Jambroes G, van der Poel HJ, Galama JM. General primer-mediated polymerase chain reaction for detection of enteroviruses: application for diagnostic routine and persistent infections. J. Clin. Microbiol. 1992 Jan;30(1):160–5.

84. Ramers C, Billman G, Hartin M, Ho S, Sawyer MH. Impact of a diagnostic cerebrospinal fluid enterovirus polymerase chain reaction test on patient management. JAMA. 2000 May 24;283(20):2680–5.

85. Romero JR. Reverse-transcription polymerase chain reaction detection of the enteroviruses. Arch. Pathol. Lab. Med. 1999 Dec;123(12):1161–9.

86. Oberste MS, Maher K, Kilpatrick DR, Flemister MR, Brown BA, Pallansch MA. Typing of human enteroviruses by partial sequencing of VP1. Journal of clinical microbiology. 1999;37(5):1288–1293.

87. Oberste MS, Maher K, Flemister MR, Marchetti G, Kilpatrick DR, Pallansch MA. Comparison of classic and molecular approaches for the identification of untypeable enteroviruses. Journal of clinical microbiology. 2000;38(3):1170–1174.

88. Kobayashi M, Makino T, Hanaoka N, Shimizu H, Enomoto M, Okabe N, Kanou K, Konagaya M, Oishi K, Fujimoto T. Clinical manifestations of coxsackievirus A6 infection associated with a major outbreak of hand, foot, and mouth disease in Japan. Japanese journal of infectious diseases. 2013;66(3):260–261.

89. Iwai M, Masaki A, Hasegawa S, Obara M, Horimoto E, Nakamura K, Tanaka Y, Endo K, Tanaka K, Ueda J, Shiraki K, Kurata T, Takizawa T. Genetic changes of coxsackievirus A16 and enterovirus 71 isolated from hand, foot, and mouth disease patients in Toyama, Japan between 1981 and 2007. Jpn. J. Infect. Dis. 2009 Jul;62(4):254–9.

90. Puenpa J, Theamboonlers A, Korkong S, Linsuwanon P, Thongmee C, Chatproedprai S, Poovorawan Y. Molecular characterization and complete genome analysis of human enterovirus 71 and coxsackievirus A16 from children with hand, foot and mouth disease in Thailand during 2008-2011. Arch. Virol. 2011 Nov;156(11):2007–13.

91. Yang B, Liu F, Liao Q, Wu P, Chang Z, Huang J, Long L, Luo L, Li Y, Leung GM, Cowling BJ, Yu H. Epidemiology of hand, foot and mouth disease in China, 2008 to 2015 prior to the introduction of EV-A71 vaccine. Euro Surveill. 2017 Dec;22(50).

92. Osterback R, Vuorinen T, Linna M, Susi P, Hyypiä T, Waris M. Coxsackievirus A6 and hand, foot, and mouth disease, Finland. Emerging Infect. Dis. 2009 Sep;15(9):1485–8.

93. Lo S-H, Huang Y-C, Huang C-G, Tsao K-C, Li W-C, Hsieh Y-C, Chiu C-H, Lin T-Y. Clinical and epidemiologic features of Coxsackievirus A6 infection in children in northern Taiwan between 2004 and 2009. Journal of Microbiology, Immunology and Infection. 2011 Aug 1;44(4):252–7.

94. Li J-L, Yuan J, Yang F, Wu Z-Q, Hu Y-F, Xue Y, Zhou B-P, Jin Q. Epidemic characteristics of hand, foot, and mouth disease in southern China, 2013: Coxsackievirus A6 has emerged as the predominant causative agent. Journal of Infection. 2014 Sep;69(3):299–303.

95. Puenpa J, Mauleekoonphairoj J, Linsuwanon P, Suwannakarn K, Chieochansin T, Korkong S, Theamboonlers A, Poovorawan Y. Prevalence and characterization of enterovirus infections among pediatric patients with hand foot mouth disease, herpangina and influenza like illness in Thailand, 2012. PLoS ONE. 2014;9(6):e98888.

96. Yang F, Yuan J, Wang X, Li J, Du J, Su H, Zhou B, Jin Q. Severe hand, foot, and mouth disease and coxsackievirus A6-Shenzhen, China. Clin. Infect. Dis. 2014 Nov 15;59(10):1504–5.

97. Stewart CL, Chu EY, Introcaso CE, Schaffer A, James WD. Coxsackievirus A6-induced hand-foot-mouth disease. JAMA Dermatol. 2013 Dec;149(12):1419–21.

98. Ramirez-Fort MK, Downing C, Doan HQ, Benoist F, Oberste MS, Khan F, Tyring SK. Coxsackievirus A6 associated hand, foot and mouth disease in adults: Clinical presentation and review of the literature. Journal of Clinical Virology. 2014 Aug;60(4):381–6.

99. Liu X, Mao N, Yu W, Chai Q, Wang H, Wang W, Wang L, Wang Z, Xu W. Genetic characterization of emerging coxsackievirus A12 associated with hand, foot and mouth disease in Qingdao, China. Arch. Virol. 2014 Sep;159(9):2497–502.

100. Solomon T, Lewthwaite P, Perera D, Cardosa MJ, McMinn P, Ooi MH. Virology, epidemiology, pathogenesis, and control of enterovirus 71. Lancet Infect Dis. 2010 Nov;10(11):778–90.

101. Mizuta K, Abiko C, Murata T, Matsuzaki Y, Itagaki T, Sanjoh K, Sakamoto M, Hongo S, Murayama S, Hayasaka K. Frequent Importation of Enterovirus 71 from Surrounding Countries into the Local Community of Yamagata, Japan, between 1998 and 2003. J Clin Microbiol. 2005 Dec;43(12):6171–5.

102. Hosoya M, Kawasaki Y, Sato M, Honzumi K, Kato A, Hiroshima T, Ishiko H, Suzuki H. Genetic diversity of enterovirus 71 associated with hand, foot and mouth disease epidemics in Japan from 1983 to 2003. Pediatr. Infect. Dis. J. 2006 Aug;25(8):691–4.

103. Chang L-Y, King C-C, Hsu K-H, Ning H-C, Tsao K-C, Li C-C, Huang Y-C, Shih S-R, Chiou S-T, Chen P-Y, Chang H-J, Lin T-Y. Risk factors of enterovirus 71 infection and associated hand, foot, and mouth disease/herpangina in children during an epidemic in Taiwan. Pediatrics. 2002 Jun;109(6):e88.

104. Tee KK, Lam TT-Y, Chan YF, Bible JM, Kamarulzaman A, Tong CYW, Takebe Y, Pybus OG. Evolutionary genetics of human enterovirus 71: origin, population dynamics, natural selection, and seasonal periodicity of the VP1 gene. J. Virol. 2010 Apr;84(7):3339–50.

105. Saxena VK, Sane S, Nadkarni SS, Sharma DK, Deshpande JM. Genetic Diversity of Enterovirus A71, India. Emerging Infectious Diseases. 2015 Jan;21(1):123–6.

106. Bessaud M, Razafindratsimandresy R, Nougairède A, Joffret M-L, Deshpande JM, Dubot-Pérès A, Héraud J-M, de Lamballerie X, Delpeyroux F, Bailly J-L.

Molecular comparison and evolutionary analyses of VP1 nucleotide sequences of new African human enterovirus 71 isolates reveal a wide genetic diversity. PLoS ONE. 2014;9(3):e90624.

107. Bessaud M, Pillet S, Ibrahim W, Joffret M-L, Pozzetto B, Delpeyroux F, Gouandjika-Vasilache I. Molecular characterization of human enteroviruses in the Central African Republic: uncovering wide diversity and identification of a new human enterovirus A71 genogroup. J. Clin. Microbiol. 2012 May;50(5):1650–8.

108. Thao NTT, Donato C, Trang VTH, Kien NT, Trang PMMT, Khanh TQ, Nguyet DT, Sessions OM, Cuong HQ, Lan PT, Huong VTQ, van Doorn HR, Vijaykrishna D. Evolution and Spatiotemporal Dynamics of Enterovirus A71 Subgenogroups in Vietnam. J. Infect. Dis. 2017 Dec 12;216(11):1371–9.

109. Alexander JP, Baden L, Pallansch MA, Anderson LJ. Enterovirus 71 infections and neurologic disease--United States, 1977-1991. J. Infect. Dis. 1994 Apr;169(4):905–8.

110. Chumakov M, Voroshilova M, Shindarov L, Lavrova I, Gracheva L, Koroleva G, Vasilenko S, Brodvarova I, Nikolova M, Gyurova S, others. Enterovirus 71 isolated from cases of epidemic poliomyelitis-like disease in Bulgaria. Archives of virology. 1979;60(3–4):329–340.

111. Brown BA, Oberste MS, Alexander JP, Kennett ML, Pallansch MA. Molecular Epidemiology and Evolution of Enterovirus 71 Strains Isolated from 1970 to 1998. J Virol. 1999 Dec;73(12):9969–75.

112. Kung S-H, Wang S-F, Huang C-W, Hsu C-C, Liu H-F, Yang J-Y. Genetic and antigenic analyses of enterovirus 71 isolates in Taiwan during 1998-2005. Clin. Microbiol. Infect. 2007 Aug;13(8):782–7.

113. Shimizu H, Utama A, Onnimala N, Li C, Li-Bi Z, Yu-Jie M, Pongsuwanna Y, Miyamura T. Molecular epidemiology of enterovirus 71 infection in the Western Pacific Region. Pediatrics international. 2004;46(2):231–235.

114. McMinn P, Lindsay K, Perera D, Chan HM, Chan KP, Cardosa MJ. Phylogenetic Analysis of Enterovirus 71 Strains Isolated during Linked Epidemics in Malaysia, Singapore, and Western Australia. J Virol. 2001 Aug;75(16):7732–8.

115. Cardosa MJ, Perera D, Brown BA, Cheon D, Chan HM, Chan KP, Cho H, McMinn P. Molecular Epidemiology of Human Enterovirus 71 Strains and Recent Outbreaks in the Asia-Pacific Region: Comparative Analysis of the VP1 and VP4 Genes. Emerg Infect Dis. 2003 Apr;9(4):462–8.

116. Lin K-H, Hwang K-P, Ke G-M, Wang C-F, Ke L-Y, Hsu Y-T, Tung Y-C, Chu P-Y, Chen B-H, Chen H-L, Kao C-L, Wang J-R, Eng H-L, Wang S-Y, Hsu L-C, Chen H-

Y. Evolution of EV71 genogroup in Taiwan from 1998 to 2005: an emerging of subgenogroup C4 of EV71. J. Med. Virol. 2006 Feb;78(2):254–62.

117. Huang Y-P, Lin T-L, Kuo C-Y, Lin M-W, Yao C-Y, Liao H-W, Hsu L-C, Yang C-F, Yang J-Y, Chen P-J, Wu H-S. The circulation of subgenogroups B5 and C5 of enterovirus 71 in Taiwan from 2006 to 2007. Virus Res. 2008 Nov;137(2):206–12.

118. Wang J-R, Tuan Y-C, Tsai H-P, Yan J-J, Liu C-C, Su I-J. Change of major genotype of enterovirus 71 in outbreaks of hand-foot-and-mouth disease in Taiwan between 1998 and 2000. J. Clin. Microbiol. 2002 Jan;40(1):10–5.

119. Bible JM, Pantelidis P, Chan PKS, Tong CYW. Genetic evolution of enterovirus 71: epidemiological and pathological implications. Reviews in Medical Virology. 2007 Nov;17(6):371–9.

120. He Y, Yang J, Zeng G, Shen T, Fontaine RE, Zhang L, Shi G, Wang Y, Li Q, Long J. Risk Factors for Critical Disease and Death from Hand, Foot and Mouth Disease: The Pediatric Infectious Disease Journal. 2014 Sep;33(9):966–70.

121. Zhang D, Li R, Zhang W, Li G, Ma Z, Chen X, Du Z, Li Z, Guo P, Lin Z, Lu J, Hao Y. A Case-control Study on Risk Factors for Severe Hand, Foot and Mouth Disease. Sci Rep. 2017 Jan 13;7:40282.

122. Vietnam Ministry of Health. Guideline for Hand - Foot - Mouth Disease Management. 2012. Available from: http://bvdkquangnam.vn/ao-to-nckh/ao-to-nhan-vien-bv/137-cm-nang-chn-oan-va-x-tri-bnh-tay-chan-ming-tr-em.html

123. Ooi MH, Wong SC, Mohan A, Podin Y, Perera D, Clear D, del Sel S, Chieng CH, Tio PH, Cardosa MJ, Solomon T. Identification and validation of clinical predictors for the risk of neurological involvement in children with hand, foot, and mouth disease in Sarawak. BMC Infect. Dis. 2009;9:3.

124. Huang C-C, Liu C-C, Chang Y-C, Chen C-Y, Wang S-T, Yeh T-F. Neurologic complications in children with enterovirus 71 infection. New England Journal of Medicine. 1999;341(13):936–942.

125. Ooi MH, Wong SC, Lewthwaite P, Cardosa MJ, Solomon T. Clinical features, diagnosis, and management of enterovirus 71. The Lancet Neurology. 2010 Nov;9(11):1097–105.

126. Lin T, Chang L, Hsia S, Huang Y, Chiu C, Hsueh C, Shih S, Liu C, Wu M. The 1998 Enterovirus 71 Outbreak in Taiwan: Pathogenesis and Management. Clinical Infectious Diseases. 2002 May;34(S2):S52–7.

127. Choi CS, Choi YJ, Choi UY, Han JW, Jeong DC, Kim HH, Kim JH, Kang JH. Clinical manifestations of CNS infections caused by enterovirus type 71. Korean J Pediatr. 2011 Jan;54(1):11–6.

128. Wang J-N, Yao C-T, Yeh C-N, Huang C-C, Wang S-M, Liu C-C, Wu J-M. Critical management in patients with severe enterovirus 71 infection. Pediatrics International. 2006 Jun 1;48(3):250–6.

129. McKinney RE, Katz SL, Wilfert CM. Chronic enteroviral meningoencephalitis in agammaglobulinemic patients. Rev. Infect. Dis. 1987 Apr;9(2):334–56.

130. Johnston JM, Overall JC. Intravenous immunoglobulin in disseminated neonatal echovirus 11 infection. Pediatr. Infect. Dis. J. 1989 Apr;8(4):254–6.

131. Wang S-M, Lei H-Y, Huang K-J, Wu J-M, Wang J-R, Yu C-K, Su I-J, Liu C-C. Pathogenesis of Enterovirus 71 Brainstem Encephalitis in Pediatric Patients: Roles of Cytokines and Cellular Immune Activation in Patients with Pulmonary Edema. J Infect Dis. 2003 Aug 15;188(4):564–70.

132. Wang S-M, Lei H-Y, Yu C-K, Wang J-R, Su I-J, Liu C-C. Acute Chemokine Response in the Blood and Cerebrospinal Fluid of Children with Enterovirus 71-Associated Brainstem Encephalitis. J Infect Dis. 2008 Oct 1;198(7):1002–6.

133. Wang S-M, Lei H-Y, Huang M-C, Su L-Y, Lin H-C, Yu C-K, Wang J-L, Liu C-C. Modulation of cytokine production by intravenous immunoglobulin in patients with enterovirus 71-associated brainstem encephalitis. Journal of Clinical Virology. 2006 Sep;37(1):47–52.

134. Chang L-Y, Hsia S-H, Wu C-T, Huang Y-C, Lin K-L, Fang T-Y, Lin T-Y. Outcome of enterovirus 71 infections with or without stage-based management: 1998 to 2002. Pediatr. Infect. Dis. J. 2004 Apr;23(4):327–32.

135. Wang S-M, Lei H-Y, Huang M-C, Wu J-M, Chen C-T, Wang J-N, Wang J-R, Liu C-C. Therapeutic efficacy of milrinone in the management of enterovirus 71-induced pulmonary edema. Pediatr. Pulmonol. 2005 Mar 1;39(3):219–23.

136. Chi C-Y, Khanh TH, Thoa LPK, Tseng F-C, Wang S-M, Thinh LQ, Lin C-C, Wu H-C, Wang J-R, Hung NT, Thuong TC, Chang C-M, Su I-J, Liu C-C. Milrinone therapy for enterovirus 71-induced pulmonary edema and/or neurogenic shock in children: a randomized controlled trial. Crit. Care Med. 2013 Jul;41(7):1754–60.

137. Wang S-M, Liu C-C. Enterovirus 71: epidemiology, pathogenesis and management. Expert Review of Anti-infective Therapy. 2009 Aug 1;7(6):735–42.

138. Wang S-M, Ho T-S, Shen C-F, Liu C-C. Enterovirus 71, one virus and many stories. Pediatr Neonatol. 2008 Aug;49(4):113–5.

139. Qui PT, Khanh TH, Trieu HT, Giang PT, Bich NN, Thoa LPK, Nhan LNT, Sabanathan S, Van Doorn R, Toan ND, Merson L, Dung NTP, Khanh LP, Wolbers M, Hung NT, Chau NVV, Wills B. Intravenous magnesium sulfate for the management of severe hand, foot, and mouth disease with autonomic nervous system dysregulation in Vietnamese children: study protocol for a randomized controlled trial. Trials. 2016 Feb 19;17:98.

140. Cao L-J, Geng W-J, Xu M-X, Huo X-M, Wang X-D, Shi X-N. [Effect of continuous hemofiltration on inflammatory mediators and hemodynamics in children with severe hand, foot and mouth disease]. Zhongguo Dang Dai Er Ke Za Zhi. 2016 Mar;18(3):219–23.

141. Tan CW, Lai JKF, Sam I-C, Chan YF. Recent developments in antiviral agents against enterovirus 71 infection. J Biomed Sci. 2014 Feb 12;21(1):14.

142. Tijsma A, Franco D, Tucker S, Hilgenfeld R, Froeyen M, Leyssen P, Neyts J. The capsid binder Vapendavir and the novel protease inhibitor SG85 inhibit enterovirus 71 replication. Antimicrob. Agents Chemother. 2014 Nov;58(11):6990–2.

143. Weng T-Y, Chen L-C, Shyu H-W, Chen S-H, Wang J-R, Yu C-K, Lei H-Y, Yeh T-M. Lactoferrin inhibits enterovirus 71 infection by binding to VP1 protein and host cells. Antiviral Res. 2005 Jul;67(1):31–7.

144. Tan EL, Tan TMC, Tak Kwong Chow V, Poh CL. Inhibition of enterovirus 71 in virus-infected mice by RNA interference. Mol. Ther. 2007 Nov;15(11):1931–8.

145. Li Z-H, Li C-M, Ling P, Shen F-H, Chen S-H, Liu C-C, Yu C-K, Chen S-H. Ribavirin reduces mortality in enterovirus 71-infected mice by decreasing viral replication. J. Infect. Dis. 2008 Mar 15;197(6):854–7.

146. Zhang G, Zhou F, Gu B, Ding C, Feng D, Xie F, Wang J, Zhang C, Cao Q, Deng Y, Hu W, Yao K. In vitro and in vivo evaluation of ribavirin and pleconaril antiviral activity against enterovirus 71 infection. Arch. Virol. 2012 Apr;157(4):669–79.

147. Zhang X, Song Z, Qin B, Zhang X, Chen L, Hu Y, Yuan Z. Rupintrivir is a promising candidate for treating severe cases of enterovirus-71 infection: evaluation of antiviral efficacy in a murine infection model. Antiviral Res. 2013 Mar;97(3):264–9.

148. Tsou Y-L, Lin Y-W, Chang H-W, Lin H-Y, Shao H-Y, Yu S-L, Liu C-C, Chitra E, Sia C, Chow Y-H. Heat shock protein 90: role in enterovirus 71 entry and assembly and potential target for therapy. PLoS ONE. 2013;8(10):e77133.

149. Chong P, Liu C-C, Chow Y-H, Chou A-H, Klein M. Review of enterovirus 71 vaccines. Clin. Infect. Dis. 2015 Mar 1;60(5):797–803.

150. Cheng A, Fung C-P, Liu C-C, Lin Y-T, Tsai H-Y, Chang S-C, Chou A-H, Chang J-Y, Jiang R-H, Hsieh Y-C, Su I-J, Chong PC-S, Hsieh S-M. A Phase I, randomized, open-label study to evaluate the safety and immunogenicity of an enterovirus 71 vaccine. Vaccine. 2013 May 7;31(20):2471–6.

151. Chou A-H, Liu C-C, Chang J-Y, Jiang R, Hsieh Y-C, Tsao A, Wu C-L, Huang J-L, Fung C-P, Hsieh S-M, Wang Y-F, Wang J-R, Hu M-H, Chiang J-R, Su I-J, Chong PC-S. Formalin-inactivated EV71 vaccine candidate induced cross-neutralizing antibody against subgenotypes B1, B4, B5 and C4A in adult volunteers. PLoS ONE. 2013;8(11):e79783.

152. Chang L-Y, Lin T-Y, Hsu K-H, Huang Y-C, Lin K-L, Hsueh C, Shih S-R, Ning H-C, Hwang M-S, Wang H-S. Clinical features and risk factors of pulmonary oedema after enterovirus-71-related hand, foot, and mouth disease. The Lancet. 1999;354(9191):1682–1686.

153. Fang Y, Wang S, Zhang L, Guo Z, Huang Z, Tu C, Zhu B-P. Risk factors of severe hand, foot and mouth disease: A meta-analysis. Scandinavian Journal of Infectious Diseases. 2014 Jul;46(7):515–22.

154. Lu H-K, Lin T-Y, Hsia S-H, Chiu C-H, Huang Y-C, Tsao K-C, Chang L-Y. Prognostic implications of myoclonic jerk in children with enterovirus infection. J Microbiol Immunol Infect. 2004 Apr;37(2):82–7.

155. Chong CY, Chan KP, Shah VA, Ng WYM, Lau G, Teo TES, Lai SH, Ling AE. Hand, foot and mouth disease in Singapore: a comparison of fatal and non-fatal cases. Acta Paediatr. 2003 Oct;92(10):1163–9.

156. Chang LY, Lin TY, Huang YC, Tsao KC, Shih SR, Kuo ML, Ning HC, Chung PW, Kang CM. Comparison of enterovirus 71 and coxsackie-virus A16 clinical illnesses during the Taiwan enterovirus epidemic, 1998. Pediatr. Infect. Dis. J. 1999 Dec;18(12):1092–6.

157. Fu Y-C, Chi C-S, Lin N-N, Cheng C-C, Jan S-L, Hwang B, Hsu S-L, Gong C-L, Chen Y-T, Chiu Y-T. Comparison of heart failure in children with enterovirus 71 rhombencephalitis and cats with norepinephrine cardiotoxicity. Pediatr Cardiol. 2006 Oct;27(5):577–84.

158. Lin M-T, Wang J-K, Lu FL, Wu E-T, Yeh S-J, Lee W-L, Wu J-M, Wu M-H. Heart rate variability monitoring in the detection of central nervous system complications in children with enterovirus infection. Journal of Critical Care. 2006 Sep;21(3):280–6.

159. Ma H, He F, Wan J, Jin D, Zhu L, Liu X, Liu Q, Zhang G, Ding Z, Fontaine RE, Zhu B-P, Jian H, Zhang L, Xu W, Zeng G. Glucocorticoid and pyrazolone treatment

of acute fever is a risk factor for critical and life-threatening human enterovirus 71 infection during an outbreak in China, 2008. Pediatr. Infect. Dis. J. 2010 Jun;29(6):524–9.

160. Yamayoshi S, Yamashita Y, Li J, Hanagata N, Minowa T, Takemura T, Koike S. Scavenger receptor B2 is a cellular receptor for enterovirus 71. Nature Medicine. 2009 Jul;15(7):798–801.

161. Nishimura Y, Shimojima M, Tano Y, Miyamura T, Wakita T, Shimizu H. Human P-selectin glycoprotein ligand-1 is a functional receptor for enterovirus 71. Nature Medicine. 2009 Jul;15(7):794–7.

162. Tan CW, Poh CL, Sam I-C, Chan YF. Enterovirus 71 uses cell surface heparan sulfate glycosaminoglycan as an attachment receptor. J. Virol. 2013 Jan;87(1):611–20.

163. Yang B, Chuang H, Yang KD. Sialylated glycans as receptor and inhibitor of enterovirus 71 infection to DLD-1 intestinal cells. Virol. J. 2009 Sep 15;6:141.

164. Ren X-X, Ma L, Liu Q-W, Li C, Huang Z, Wu L, Xiong S-D, Wang J-H, Wang H-B. The molecule of DC-SIGN captures enterovirus 71 and confers dendritic cell-mediated viral trans-infection. Virol. J. 2014 Mar 12;11:47.

165. Yang S-L, Chou Y-T, Wu C-N, Ho M-S. Annexin II binds to capsid protein VP1 of enterovirus 71 and enhances viral infectivity. J. Virol. 2011 Nov;85(22):11809–20.

166. Yamayoshi S, Ohka S, Fujii K, Koike S. Functional Comparison of SCARB2 and PSGL1 as Receptors for Enterovirus 71. Journal of Virology. 2013 Mar 15;87(6):3335–47.

167. Nishimura Y, Lee H, Hafenstein S, Kataoka C, Wakita T, Bergelson JM, Shimizu H. Enterovirus 71 binding to PSGL-1 on leukocytes: VP1-145 acts as a molecular switch to control receptor interaction. PLoS Pathog. 2013;9(7):e1003511.

168. Huang P-N, Shih S-R. Update on enterovirus 71 infection. Current Opinion in Virology. 2014 Apr;5:98–104.

169. Wang S-M, Lei H-Y, Su L-Y, Wu J-M, Yu C-K, Wang J-R, Liu C-C. Cerebrospinal fluid cytokines in enterovirus 71 brain stem encephalitis and echovirus meningitis infections of varying severity. Clin. Microbiol. Infect. 2007 Jul;13(7):677–82.

170. Lin T-Y, Hsia S-H, Huang Y-C, Wu C-T, Chang L-Y. Proinflammatory cytokine reactions in enterovirus 71 infections of the central nervous system. Clin. Infect. Dis. 2003 Feb 1;36(3):269–74.

171. Chang L-Y, Hsiung CA, Lu C-Y, Lin T-Y, Huang F-Y, Lai Y-H, Chiang Y-P, Chiang B-L, Lee C-Y, Huang L-M. Status of cellular rather than humoral immunity is correlated with clinical outcome of enterovirus 71. Pediatr. Res. 2006 Oct;60(4):466–71.

172. Khor C-C, Hibberd ML. Revealing the molecular signatures of host-pathogen interactions. Genome biology. 2011;12(10):1.

173. Farrell MS, Werge T, Sklar P, Owen MJ, Ophoff RA, O'Donovan MC, Corvin A, Cichon S, Sullivan PF. Evaluating historical candidate genes for schizophrenia. Mol. Psychiatry. 2015 May;20(5):555–62.

174. Zou Z, Liu C, Che C, Huang H. Clinical genetics of Alzheimer's disease. Biomed Res Int. 2014;2014:291862.

175. Hattersley AT, McCarthy MI. What makes a good genetic association study? Lancet. 2005 Oct 8;366(9493):1315–23.

176. Dawn Teare M, Barrett JH. Genetic linkage studies. Lancet. 2005 Sep 17;366(9490):1036–44.

177. Evans DM, Cardon LR. Guidelines for genotyping in genomewide linkage studies: single-nucleotide-polymorphism maps versus microsatellite maps. Am. J. Hum. Genet. 2004 Oct;75(4):687–92.

178. Cordell HJ, Clayton DG. Genetic association studies. Lancet. 2005 Sep 24;366(9491):1121–31.

179. Bush WS, Moore JH. Chapter 11: Genome-Wide Association Studies. Lewitter F, Kann M, editors. PLoS Computational Biology. 2012 Dec 27;8(12):e1002822.

180. Warr A, Robert C, Hume D, Archibald A, Deeb N, Watson M. Exome Sequencing: Current and Future Perspectives. G3: Genes, Genomes, Genetics. 2015 Aug 1;5(8):1543–50.

181. Worthey EA, Mayer AN, Syverson GD, Helbling D, Bonacci BB, Decker B, Serpe JM, Dasu T, Tschannen MR, Veith RL, Basehore MJ, Broeckel U, Tomita-Mitchell A, Arca MJ, Casper JT, Margolis DA, Bick DP, Hessner MJ, Routes JM, Verbsky JW, Jacob HJ, Dimmock DP. Making a definitive diagnosis: successful clinical application of whole exome sequencing in a child with intractable inflammatory bowel disease. Genet. Med. 2011 Mar;13(3):255–62.

182. Yang KD, Yang MY, Li CC, Lin SF, Chong MC, Wang CL, Chen RF, Lin TY. Altered cellular but not humoral reactions in children with complicated enterovirus 71 infections in Taiwan. J. Infect. Dis. 2001 Mar 15;183(6):850–6.

183. Chang L-Y, Chang I-S, Chen W-J, Huang Y-C, Chen G-W, Shih S-R, Juang J-L, Shih H-M, Hsiung CA, Lin T-Y, Huang L-M. HLA-A33 Is Associated With Susceptibility to Enterovirus 71 Infection. PEDIATRICS. 2008 Dec 1;122(6):1271–6.

184. Li J, Chen Z, Lv T, Han Z. Genetic polymorphism of CCL2−2518, CXCL10−201, IL8+781 and susceptibility to severity of Enterovirus-71 infection in a Chinese population. Inflammation Research. 2014 Jul;63(7):549–56.

185. Yang J, Zhao N, Su N-L, Sun J-L, Lv T-G, Chen Z-B. Association of interleukin 10 and interferon gamma gene polymorphisms with enterovirus 71 encephalitis in patients with hand, foot and mouth disease. Scand. J. Infect. Dis. 2012 Jun;44(6):465–9.

186. Han Z, Li J, Chen Z. Genetic polymorphism of CCL2-2510 and susceptibility to enterovirus 71 encephalitis in a Chinese population. Arch. Virol. 2014 Sep;159(9):2503–7.

187. Zou R, Zhang G, Li S, Wang W, Yuan J, Li J, Wang Y, Lin Y, Deng Y, Zhou B, Gao GF, Liu Y. A functional polymorphism in IFNAR1 gene is associated with susceptibility and severity of HFMD with EV71 infection. Sci Rep. 2015 Dec 18;5:18541.

188. Zhang X, Xu H, Chen X, Li X, Wang X, Ding S, Zhang R, Liu L, He C, Zhuang L, Li H, Zhang P, Yang H, Li T, Liu W, Cao W. Association of functional polymorphisms in the MxA gene with susceptibility to enterovirus 71 infection. Hum Genet. 2014 Feb 1;133(2):187–97.

189. Cai Y, Chen Q, Zhou W, Chu C, Ji W, Ding Y, Xu J, Ji Z, You H, Wang J. Association analysis of polymorphisms in OAS1 with susceptibility and severity of hand, foot and mouth disease. International Journal of Immunogenetics. 2014 Oct;41(5):384–92.

190. Balding DJ. A tutorial on statistical methods for population association studies. Nat. Rev. Genet. 2006 Oct;7(10):781–91.

191. Tan Y, Yang T, Liu P, Chen L, Tian Q, Guo Y, He H, Liu Y, Chen Z. Association of the OAS3 rs1859330 G/A genetic polymorphism with severity of enterovirus-71 infection in Chinese Han children. Arch. Virol. 2017 Aug;162(8):2305–13.

192. Dung TTN, Phat VV, Nga TVT, My PVT, Duy PT, Campbell JI, Thuy CT, Hoang NVM, Van Minh P, Le Phuc H, Tuyet PTN, Vinh H, Kien DTH, Huy HLA, Vinh NT, Nga TTT, Hau NTT, Chinh NT, Thuong TC, Tuan HM, Simmons C, Farrar JJ, Baker S. The validation and utility of a quantitative one-step multiplex RT real-time PCR targeting rotavirus A and norovirus. J. Virol. Methods. 2013 Jan;187(1):138–43.

193. Hue KDT, Tuan TV, Thi HTN, Bich CTN, Anh HHL, Wills BA, Simmons CP. Validation of an internally controlled one-step real-time multiplex RT-PCR assay for the detection and quantitation of dengue virus RNA in plasma. J. Virol. Methods. 2011 Nov;177(2):168–73.

194. Thanh TT, Anh NT, Tham NT, Van HMT, Sabanathan S, Qui PT, Ngan TT, Van TTM, Nguyet LA, Ny NTH, Thanh LTM, Chai OK, Perera D, Viet DC, Khanh TH, Ha DQ, Tuan HM, Wong KT, Hung NT, Chau NVV, Thwaites G, van Doorn HR, Van Tan L. Validation and utilization of an internally controlled multiplex Real-time RT-PCR assay for simultaneous detection of enteroviruses and enterovirus A71 associated with hand foot and mouth disease. Virol. J. 2015;12:85.

195. Beld M, Minnaar R, Weel J, Sol C, Damen M, van der Avoort H, Wertheim-van Dillen P, van Breda A, Boom R. Highly sensitive assay for detection of enterovirus in clinical specimens by reverse transcription-PCR with an armored RNA internal control. J. Clin. Microbiol. 2004 Jul;42(7):3059–64.

196. Nix WA, Oberste MS, Pallansch MA. Sensitive, seminested PCR amplification of VP1 sequences for direct identification of all enterovirus serotypes from original clinical specimens. J. Clin. Microbiol. 2006 Aug;44(8):2698–704.

197. Kroneman A, Vennema H, Deforche K, v d Avoort H, Peñaranda S, Oberste MS, Vinjé J, Koopmans M. An automated genotyping tool for enteroviruses and noroviruses. J. Clin. Virol. 2011 Jun;51(2):121–5.

198. R Development Core Team. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2010.

199. NikNadia N, Sam I-C, Rampal S, WanNorAmalina W, NurAtifah G, Verasahib K, Ong CC, MohdAdib M, Chan YF. Cyclical Patterns of Hand, Foot and Mouth Disease Caused by Enterovirus A71 in Malaysia. PLoS Negl Trop Dis. 2016 Mar;10(3):e0004562.

200. Lu Q-B, Zhang X-A, Wo Y, Xu H-M, Li X-J, Wang X-J, Ding S-J, Chen X-D, He C, Liu L-J, Li H, Yang H, Li T-Y, Liu W, Cao W-C. Circulation of Coxsackievirus A10 and A6 in Hand-Foot-Mouth Disease in China, 2009–2011. Zhang C, editor. PLoS ONE. 2012 Dec 18;7(12):e52073.

201. Hayman R, Shepherd M, Tarring C, Best E. Outbreak of variant hand-foot-and-mouth disease caused by coxsackievirus A6 in Auckland, New Zealand: New Zealand coxsackievirus A6 outbreak. Journal of Paediatrics and Child Health. 2014 Oct;50(10):751–5.

202. Hongyan G, Chengjie M, Qiaozhi Y, Wenhao H, Juan L, Lin P, Yanli X, Hongshan W, Xingwang L. Hand, Foot and Mouth Disease Caused By Coxsackievirus A6, Beijing, 2013: The Pediatric Infectious Disease Journal. 2014 Dec;33(12):1302–3.

203. World Health Organization. Hand, foot and mouth disease factsheet [Internet]. WPRO. [cited 2017 Sep 20]. Available from: http://www.wpro.who.int/vietnam/topics/hand_foot_mouth/factsheet/en/

204. Nguyen NTB, Pham HV, Hoang CQ, Nguyen TM, Nguyen LT, Phan HC, Phan LT, Vu LN, Tran Minh NN. Epidemiological and clinical characteristics of children who died from hand, foot and mouth disease in Vietnam, 2011. BMC Infect. Dis. 2014 Jun 18;14:341.

205. Urashima M, Shindo N, Okabe N. Seasonal models of herpangina and hand-foot-mouth disease to simulate annual fluctuations in urban warming in Tokyo. Jpn. J. Infect. Dis. 2003 Apr;56(2):48–53.

206. Zhao D, Wang L, Cheng J, Xu J, Xu Z, Xie M, Yang H, Li K, Wen L, Wang X, Zhang H, Wang S, Su H. Impact of weather factors on hand, foot and mouth disease, and its role in short-term incidence trend forecast in Huainan City, Anhui Province. Int J Biometeorol. 2017 Mar;61(3):453–61.

207. Wang P, Zhao H, You F, Zhou H, Goggins WB. Seasonal modeling of hand, foot, and mouth disease as a function of meteorological variations in Chongqing, China. Int J Biometeorol. 2017 Feb 10;

208. Liu S, Chen J, Wang J, Wu Z, Wu W, Xu Z, Hu W, Xu F, Tong S, Shen H. Predicting the outbreak of hand, foot, and mouth disease in Nanjing, China: a time-series model based on weather variability. Int J Biometeorol. 2017 Oct 30;

209. Phung D, Nguyen HX, Nguyen HLT, Do CM, Tran QD, Chu C. Spatiotemporal variation of hand-foot-mouth disease in relation to socioecological factors: A multiple-province analysis in Vietnam. Sci. Total Environ. 2018 Jan 1;610–611:983–91.

210. Zheng Y, Jit M, Wu JT, Yang J, Leung K, Liao Q, Yu H. Economic costs and health-related quality of life for hand, foot and mouth disease (HFMD) patients in China. PLOS ONE. 2017 Sep 21;12(9):e0184266.

211. World Health Organization. Health Service Delivery Profile Vietnam 2012 [Internet]. Available from: http://www.wpro.who.int/health_services/service_delivery_profile_vietnam.pdf

212. World Health Organization. Hand, Foot, and Mouth Disease Situation Update [Internet]. Report No.: 47 8,. Available from: http://www.wpro.who.int/emerging_diseases/hfmd-biweekly_20151229.pdf?ua=1

213. Deshpande JM, Nadkarni SS, Francis PP. Enterovirus 71 isolated from a case of acute flaccid paralysis in India represents a new genotype. Current Science. 2003;1350–1353.

214. Tan X, Huang X, Zhu S, Chen H, Yu Q, Wang H, Huo X, Zhou J, Wu Y, Yan D, Zhang Y, Wang D, Cui A, An H, Xu W. The persistent circulation of enterovirus 71 in People's Republic of China: causing emerging nationwide epidemics since 2008. PLoS ONE. 2011;6(9):e25662.

215. Duong V, Mey C, Eloit M, Zhu H, Danet L, Huang Z, Zou G, Tarantola A, Cheval J, Perot P, Laurent D, Richner B, Ky S, Heng S, Touch S, Sovann L, van Doorn R, Tan Tran T, Farrar JJ, Wentworth DE, Das SR, Stockwell TB, Manuguerra J-C, Delpeyroux F, Guan Y, Altmeyer R, Buchy P. Molecular epidemiology of human enterovirus 71 at the origin of an epidemic of fatal hand, foot and mouth disease cases in Cambodia. Emerg Microbes Infect. 2016 Sep 21;5(9):e104.

216. Tan LV, Tuyen NTK, Thanh TT, Ngan TT, Van HMT, Sabanathan S, Van TTM, Thanh LTM, Nguyet LA, Geoghegan JL, Ong KC, Perera D, Hang VTT, Ny NTH, Anh NT, Ha DQ, Qui PT, Viet DC, Tuan HM, Wong KT, Holmes EC, Chau NVV, Thwaites G, van Doorn HR. A generic assay for whole-genome amplification and deep sequencing of enterovirus A71. J. Virol. Methods. 2015 Apr;215–216:30–6.

217. Illumina. Nextera XT DNA Library Prep Kit- Reference Guide [Internet]. 2017. Available from: https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/samplepreps_nextera/nextera-xt/nextera-xt-library-prep-reference-guide-15031942-02.pdf

218. Rambaut A, Lam TT, Max Carvalho L, Pybus OG. Exploring the temporal structure of heterochronous sequences using TempEst (formerly Path-O-Gen). Virus Evol. 2016 Jan;2(1):vew007.

219. Nguyen L-T, Schmidt HA, von Haeseler A, Minh BQ. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. Mol. Biol. Evol. 2015 Jan;32(1):268–74.

220. Drummond AJ, Suchard MA, Xie D, Rambaut A. Bayesian phylogenetics with BEAUti and the BEAST 1.7. Mol. Biol. Evol. 2012 Aug;29(8):1969–73.

221. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 2011 Oct;28(10):2731–9.

222. Wu W-H, Kuo T-C, Lin Y-T, Huang S-W, Liu H-F, Wang J, Chen Y-MA. Molecular epidemiology of enterovirus 71 infection in the central region of Taiwan from 2002 to 2012. PLoS ONE. 2013;8(12):e83711.

223. Huang S-W, Hsu Y-W, Smith DJ, Kiang D, Tsai H-P, Lin K-H, Wang S-M, Liu C-C, Su I-J, Wang J-R. Reemergence of enterovirus 71 in 2008 in taiwan: dynamics of genetic and antigenic evolution from 1998 to 2008. J. Clin. Microbiol. 2009 Nov;47(11):3653–62.

224. Chua KB, Kasri AR. Hand foot and mouth disease due to enterovirus 71 in Malaysia. Virologica Sinica. 2011 Aug;26(4):221–8.

225. Donato C, Hoi LT, Hoa NT, Hoa TM, Van Duyet L, Dieu Ngan TT, Van Kinh N, Vu Trung N, Vijaykrishna D. Genetic characterization of Enterovirus 71 strains circulating in Vietnam in 2012. Virology. 2016;495:1–9.

226. Huang S-W, Kiang D, Smith DJ, Wang J-R. Evolution of re-emergent virus and its impact on enterovirus 71 epidemics. Exp. Biol. Med. (Maywood). 2011 Aug;236(8):899–908.

227. Liu Y, Fu C, Wu S, Chen X, Shi Y, Zhou B, Zhang L, Zhang F, Wang Z, Zhang Y, Fan C, Han S, Yin J, Peng B, Liu W, He X. A novel finding for enterovirus virulence from the capsid protein VP1 of EV71 circulating in mainland China. Virus Genes. 2014 Apr;48(2):260–72.

228. Ooi MH, Wong SC, Podin Y, Akin W, del Sel S, Mohan A, Chieng CH, Perera D, Clear D, Wong D, Blake E, Cardosa J, Solomon T. Human enterovirus 71 disease in Sarawak, Malaysia: a prospective clinical, virological, and molecular epidemiological study. Clin. Infect. Dis. 2007 Mar 1;44(5):646–56.

229. Khor CC, Do T, Jia H, Nakano M, George R, Abu-Amero K, Duvesh R, Chen LJ, Li Z, Nongpiur ME, Perera SA, Qiao C, Wong H-T, Sakai H, Melo MB de, Lee M-C, Chan AS, Azhany Y, Dao TLH, Ikeda Y, Perez-Grossmann RA, Zarnowski T, Day AC, Jonas JB, Tam POS, Tran TA, Ayub H, Akhtar F, Micheal S, Chew PTK, Aljasim LA, Dada T, Luu TT, Awadalla MS, Kitnarong N, Wanichwecharungruang B, Aung YY, Mohamed-Noor J, Vijayan S, Sarangapani S, Husain R, Jap A, Baskaran M, Goh D, Su DH, Wang H, Yong VK, Yip LW, Trinh TB, Makornwattana M, Nguyen TT, Leuenberger EU, Park K-H, Wiyogo WA, Kumar RS, Tello C, Kurimoto Y, Thapa SS, Pathanapitoon K, Salmon JF, Sohn YH, Fea A, Ozaki M, Lai JSM, Tantisevi V, Khaing CC, Mizoguchi T, Nakano S, Kim C-Y, Tang G, Fan S, Wu R, Meng H, Nguyen TTG, Tran TD, Ueno M, Martinez JM, Ramli N, Aung YM, Reyes RD, Vernon SA, Fang SK, Xie Z, Chen XY, Foo JN, Sim KS, Wong TT, Quek DT, Venkatesh R, Kavitha S, Krishnadas SR, Soumittra N, Shantha B, Lim B-A, Ogle J, Vasconcellos JPC de, Costa VP, Abe RY, Souza BB de, Sng CC, Aquino MC, Kosior-Jarecka E, Fong GB, Tamanaja VC, Fujita R, Jiang Y, Waseem N, Low S, Pham HN, Al-Shahwan S, Craven ER, Khan MI, Dada R, Mohanty K, Faiq MA, Hewitt AW,

Burdon KP, Gan EH, Prutthipongsit A, Patthanathamrongkasem T, Catacutan MAT, Felarca IR, Liao CS, Rusmayani E, Istiantoro VW, Consolandi G, Pignata G, Lavia C, Rojanapongpun P, Mangkornkanokpong L, Chansangpetch S, Chan JCH, Choy BNK, Shum JWH, Than HM, Oo KT, Han AT, Yong VH, Ng X-Y, Goh SR, Chong YF, Hibberd ML, Seielstad M, Png E, Dunstan SJ, Chau NVV, Bei J, Zeng YX, Karkey A, Basnyat B, Pasutto F, Paoli D, Frezzotti P, Wang JJ, Mitchell P, Fingert JH, Allingham RR, Hauser MA, Lim ST, Chew SH, Ebstein RP, Sakuntabhai A, Park KH, Ahn J, Boland G, Snippe H, Stead R, Quino R, Zaw SN, Lukasik U, Shetty R, Zahari M, Bae HW, Oo NL, Kubota T, Manassakorn A, Ho WL, Dallorto L, Hwang YH, Kiire CA, Kuroda M, Djamal ZE, Peregrino JIM, Ghosh A, Jeoung JW, Hoan TS, Srisamran N, Sandragasu T, Set SH, Doan VH, Bhattacharya SS, Ho C-L, Tan DT, Sihota R, Loon S-C, Mori K, Kinoshita S, Hollander AI den, Qamar R, Wang Y-X, Teo YY, Tai E-S, Hartleben-Matkin C, Lozano-Giral D, Saw SM, Cheng C-Y, Zenteno JC, Pang CP, Bui HTT, Hee O, Craig JE, Edward DP, Yonahara M, Neto JM, Guevara-Fujita ML, Xu L, Ritch R, Liza-Sharmini AT, Wong TY, Al-Obeidan S, Do NH, Sundaresan P, Tham CC, Foster PJ, Vijaya L, Tashiro K, Vithana EN, Wang N, Aung T. Genome-wide association study identifies five new susceptibility loci for primary angle closure glaucoma. Nature Genetics. 2016 May;48(5):556.

230. Aung T, Ozaki M, Lee MC, Schlötzer-Schrehardt U, Thorleifsson G, Mizoguchi T, Igo RP, Haripriya A, Williams SE, Astakhov YS, Orr AC, Burdon KP, Nakano S, Mori K, Abu-Amero K, Hauser M, Li Z, Prakadeeswari G, Bailey JNC, Cherecheanu AP, Kang JH, Nelson S, Hayashi K, Manabe S-I, Kazama S, Zarnowski T, Inoue K, Irkec M, Coca-Prados M, Sugiyama K, Järvelä I, Schlottmann P, Lerner SF, Lamari H, Nilgün Y, Bikbov M, Park KH, Cha SC, Yamashiro K, Zenteno JC, Jonas JB, Kumar RS, Perera SA, Chan ASY, Kobakhidze N, George R, Vijaya L, Do T, Edward DP, de Juan Marcos L, Pakravan M, Moghimi S, Ideta R, Bach-Holm D, Kappelgaard P, Wirostko B, Thomas S, Gaston D, Bedard K, Greer WL, Yang Z, Chen X, Huang L, Sang J, Jia H, Jia L, Qiao C, Zhang H, Liu X, Zhao B, Wang Y-X, Xu L, Leruez S, Reynier P, Chichua G, Tabagari S, Uebe S, Zenkel M, Berner D, Mossböck G, Weisschuh N, Hoja U, Welge-Luessen U-C, Mardin C, Founti P, Chatzikyriakidou A, Pappas T, Anastasopoulos E, Lambropoulos A, Ghosh A, Shetty R, Porporato N, Saravanan V, Venkatesh R, Shivkumar C, Kalpana N, Sarangapani S, Kanavi MR, Beni AN, Yazdani S, Lashay A, Naderifar H, Khatibi N, Fea A, Lavia C, Dallorto L, Rolle T, Frezzotti P, Paoli D, Salvi E, Manunta P, Mori Y, Miyata K, Higashide T, Chihara E, Ishiko S, Yoshida A, Yanagi M, Kiuchi Y, Ohashi T, Sakurai T, Sugimoto T, Chuman H, Aihara M, Inatani M, Miyake M, Gotoh N, Matsuda F, Yoshimura N, Ikeda Y, Ueno M, Sotozono C, Jeoung JW, Sagong M, Park KH, Ahn J, Cruz-Aguilar M, Ezzouhairi SM, Rafei A, Chong YF, Ng XY, Goh SR, Chen Y, Yong VHK, Khan MI, Olawoye OO, Ashaye AO, Ugbede I, Onakoya A, Kizor-Akaraiwe N, Teekhasaenee C, Suwan Y, Supakontanasan W, Okeke S, Uche NJ, Asimadu I, Ayub H, Akhtar F, Kosior-Jarecka E, Lukasik U, Lischinsky I, Castro V, Grossmann RP, Megevand GS, Roy S, Dervan E, Silke E, Rao A, Sahay P, Fornero P, Cuello O, Sivori D, Zompa T, Mills RA, Souzeau E,

Mitchell P, Wang JJ, Hewitt AW, Coote M, Crowston JG, Astakhov SY, Akopov EL, Emelyanov A, Vysochinskaya V, Kazakbaeva G, Fayzrakhmanov R, Al-Obeidan SA, Owaidhah O, Aljasim LA, Chowbay B, Foo JN, Soh RQ, Sim KS, Xie Z, Cheong AWO, Mok SQ, Soo HM, Chen XY, Peh SQ, Heng KK, Husain R, Ho S-L, Hillmer AM, Cheng C-Y, Escudero-Domínguez FA, González-Sarmiento R, Martinon-Torres F, Salas A, Pathanapitoon K, Hansapinyo L, Wanichwecharugruang B, Kitnarong N, Sakuntabhai A, Nguyn HX, Nguyn GTT, Nguyn TV, Zenz W, Binder A, Klobassa DS, Hibberd ML, Davila S, Herms S, Nöthen MM, Moebus S, Rautenbach RM, Ziskind A, Carmichael TR, Ramsay M, Álvarez L, García M, González-Iglesias H, Rodríguez-Calvo PP, Fernández-Vega Cueto L, Oguz Ç, Tamcelik N, Atalay E, Batu B, Aktas D, Kasım B, Wilson MR, Coleman AL, Liu Y, Challa P, Herndon L, Kuchtey RW, Kuchtey J, Curtin K, Chaya CJ, Crandall A, Zangwill LM, Wong TY, Nakano M, Kinoshita S, den Hollander AI, Vesti E, Fingert JH, Lee RK, Sit AJ, Shingleton BJ, Wang N, Cusi D, Qamar R, Kraft P, Pericak-Vance MA, Raychaudhuri S, Heegaard S, Kivelä T, Reis A, Kruse FE, Weinreb RN, Pasquale LR, Haines JL, Thorsteinsdottir U, Jonasson F, Allingham RR, Milea D, Ritch R, Kubota T, Tashiro K, Vithana EN, Micheal S, Topouzis F, Craig JE, Dubina M, Sundaresan P, Stefansson K, Wiggs JL, Pasutto F, Khor CC. Genetic association study of exfoliation syndrome identifies a protective rare variant at LOXL1 and five new susceptibility loci. Nat. Genet. 2017;49(7):993–1004.

231. Kiryluk K, Li Y, Scolari F, Sanna-Cherchi S, Choi M, Verbitsky M, Fasel D, Lata S, Prakash S, Shapiro S, Fischman C, Snyder HJ, Appel G, Izzi C, Viola BF, Dallera N, Del Vecchio L, Barlassina C, Salvi E, Bertinetto FE, Amoroso A, Savoldi S, Rocchietti M, Amore A, Peruzzi L, Coppo R, Salvadori M, Ravani P, Magistroni R, Ghiggeri GM, Caridi G, Bodria M, Lugani F, Allegri L, Delsante M, Maiorana M, Magnano A, Frasca G, Boer E, Boscutti G, Ponticelli C, Mignani R, Marcantoni C, Di Landro D, Santoro D, Pani A, Polci R, Feriozzi S, Chicca S, Galliani M, Gigante M, Gesualdo L, Zamboli P, Battaglia GG, Garozzo M, Maixnerová D, Tesar V, Eitner F, Rauen T, Floege J, Kovacs T, Nagy J, Mucha K, Pączek L, Zaniew M, Mizerska-Wasiak M, Roszkowska-Blaim M, Pawlaczyk K, Gale D, Barratt J, Thibaudin L, Berthoux F, Canaud G, Boland A, Metzger M, Panzer U, Suzuki H, Goto S, Narita I, Caliskan Y, Xie J, Hou P, Chen N, Zhang H, Wyatt RJ, Novak J, Julian BA, Feehally J, Stengel B, Cusi D, Lifton RP, Gharavi AG. Discovery of new risk loci for IgA nephropathy implicates genes involved in immunity against intestinal pathogens. Nat. Genet. 2014 Nov;46(11):1187–96.

232. Thorleifsson G, Magnusson KP, Sulem P, Walters GB, Gudbjartsson DF, Stefansson H, Jonsson T, Jonasdottir A, Jonasdottir A, Stefansdottir G, Masson G, Hardarson GA, Petursson H, Arnarsson A, Motallebipour M, Wallerman O, Wadelius C, Gulcher JR, Thorsteinsdottir U, Kong A, Jonasson F, Stefansson K. Common sequence variants in the LOXL1 gene confer susceptibility to exfoliation glaucoma. Science. 2007 Sep 7;317(5843):1397–400.

233. Illumina. Illumina Infinium HTS Assay Protocol Guide [Internet]. 2017. Available from: https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/humanomniexpress-24/infinium_hts_assay_protocol_user_guide_15045738_a.pdf

234. Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MAR, Bender D, Maller J, Sklar P, de Bakker PIW, Daly MJ, Sham PC. PLINK: A Tool Set for Whole-Genome Association and Population-Based Linkage Analyses. The American Journal of Human Genetics. 2007 Sep;81(3):559–75.

235. Powell JE, Visscher PM, Goddard ME. Reconciling the analysis of IBD and IBS in complex trait studies. Nat. Rev. Genet. 2010 Nov;11(11):800–5.

236. Wise AL, Gyi L, Manolio TA. eXclusion: Toward Integrating the X Chromosome in Genome-wide Association Analyses. Am J Hum Genet. 2013 May 2;92(5):643–7.

237. Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics. 2005 Jan 15;21(2):263–5.

238. Scherer SW, Cheung J, MacDonald JR, Osborne LR, Nakabayashi K, Herbrick J-A, Carson AR, Parker-Katiraee L, Skaug J, Khaja R, Zhang J, Hudek AK, Li M, Haddad M, Duggan GE, Fernandez BA, Kanematsu E, Gentles S, Christopoulos CC, Choufani S, Kwasnicka D, Zheng XH, Lai Z, Nusskern D, Zhang Q, Gu Z, Lu F, Zeesman S, Nowaczyk MJ, Teshima I, Chitayat D, Shuman C, Weksberg R, Zackai EH, Grebe TA, Cox SR, Kirkpatrick SJ, Rahman N, Friedman JM, Heng HHQ, Pelicci PG, Lo-Coco F, Belloni E, Shaffer LG, Pober B, Morton CC, Gusella JF, Bruns GAP, Korf BR, Quade BJ, Ligon AH, Ferguson H, Higgins AW, Leach NT, Herrick SR, Lemyre E, Farra CG, Kim H-G, Summers AM, Gripp KW, Roberts W, Szatmari P, Winsor EJT, Grzeschik K-H, Teebi A, Minassian BA, Kere J, Armengol L, Pujana MA, Estivill X, Wilson MD, Koop BF, Tosi S, Moore GE, Boright AP, Zlotorynski E, Kerem B, Kroisel PM, Petek E, Oscier DG, Mould SJ, Döhner H, Döhner K, Rommens JM, Vincent JB, Venter JC, Li PW, Mural RJ, Adams MD, Tsui L-C. Human chromosome 7: DNA sequence and biology. Science. 2003 May 2;300(5620):767–72.

239. Hsu YC, Perin MS. Human neuronal pentraxin II (NPTX2): conservation, genomic structure, and chromosomal localization. Genomics. 1995 Jul 20;28(2):220–7.

240. Moran LB, Hickey L, Michael GJ, Derkacs M, Christian LM, Kalaitzakis ME, Pearce RKB, Graeber MB. Neuronal pentraxin II is highly upregulated in Parkinson's disease and a novel component of Lewy bodies. Acta Neuropathol. 2008 Apr;115(4):471–8.

241. Sugasawa K, Masutani C, Uchida A, Maekawa T, van der Spek PJ, Bootsma D, Hoeijmakers JH, Hanaoka F. HHR23B, a human Rad23 homolog, stimulates

XPC protein in nucleotide excision repair in vitro. Mol. Cell. Biol. 1996 Sep;16(9):4852–61.

242. van der Spek PJ, Visser CE, Hanaoka F, Smit B, Hagemeijer A, Bootsma D, Hoeijmakers JH. Cloning, comparative mapping, and RNA expression of the mouse homologues of the Saccharomyces cerevisiae nucleotide excision repair gene RAD23. Genomics. 1996 Jan 1;31(1):20–7.

243. Liu C-Y, Wu MC, Chen F, Ter-Minassian M, Asomaning K, Zhai R, Wang Z, Su L, Heist RS, Kulke MH, Lin X, Liu G, Christiani DC. A Large-scale genetic association study of esophageal adenocarcinoma risk. Carcinogenesis. 2010 Jul;31(7):1259–63.

244. Jorgensen TJ, Helzlsouer KJ, Clipp SC, Bolton JH, Crum RM, Visvanathan K. DNA repair gene variants associated with benign breast disease in high cancer risk women. Cancer Epidemiol. Biomarkers Prev. 2009 Jan;18(1):346–50.

245. Du Z, Xu L, Zhang W, Zhang D, Yu S, Hao Y. Predicting the hand, foot, and mouth disease incidence using search engine query data and climate variables: an ecological study in Guangdong, China. BMJ Open. 2017 Oct 6;7(10):e016263.

246. Kataoka C, Suzuki T, Kotani O, Iwata-Yoshikawa N, Nagata N, Ami Y, Wakita T, Nishimura Y, Shimizu H. The Role of VP1 Amino Acid Residue 145 of Enterovirus 71 in Viral Fitness and Pathogenesis in a Cynomolgus Monkey Model. PLoS Pathog. 2015 Jul;11(7):e1005033.

8. APPENDICES

8.1. INFORMED CONSENT FORM FOR HFMD OBSERVATIONAL STUDY

INFORMED CONSENT FORM

WHAT IS THIS PROGRAM ABOUT?

Your child is invited to participate in a program to improve the understanding of hand, foot and mouth disease: the virus causing it, how the body reacts to the virus and how reactions and viruses vary in Ho Chi Minh City. We will also look at the DNA/genetic code of children with the virus to find out why most children recover well from the disease, but some children get very sick.

Up to 5,400 children will participate in this program which is run by the Oxford University Clinical Research Unit, the Hospital for Tropical Diseases, Children's Hospital 1 and Children's Hospital 2, and is approved by the ethical committees of each institution.

DOES MY CHILD HAVE TO BE IN THIS PROGRAM?

Your child's participation in the program is your choice. If you decide you do not want your child to participate, your child will still receive standard medical care and will not lose any benefits they would normally have. During the time your child participates in this program, you are free to stop your child's participation at any time.

WHAT WILL HAPPEN IF MY CHILD IS IN THE PROGRAM?

If your child is a part of the program, s/he will have the following procedures:

TODAY: A doctor will examine your child and take a swab sample from your child's nose, throat and rectum. Taking blood when your child is not hospitalized is not part of routine care. If you agree, we will take 4ml of blood to check blood and chemistry levels (2ml), to test how your child's body responds to the infection and to look at how his/her genetic code affects their infection (2ml).

IF YOUR CHILD IS WELL ENOUGH TO GO HOME: As part of standard clinical care your doctor may ask you to bring your child back to the hospital to review his/her condition until your child is well again. For the program we will also ask you a few questions to see how your child is feeling in 2 days, 4 days and 6 days. If you're not coming to the hospital on those days we will phone you.

IF YOUR CHILD IS HOSPITALIZED: A doctor from the ward who is also in the program will visit your child each day they are in the hospital to examine them.

If your child has spinal fluid taken from his/her back as part of routine care, we will collect an additional 0.5mL to test for viruses that causes hand, foot and mouth disease.

FOR ALL PATIENTS: In addition, we will ask you to come back to the hospital one time in about 8-14 days, when your child will be examined by a doctor and will have 2 ml of blood taken as a follow-up sample. Then your child's part in the program is finished.

WILL MY CHILD BENEFIT FROM BEING IN THE PROGRAM?

Your child will not benefit directly from being in this program. You will receive reimbursement for the outpatient visit, and results (blood count, CRP, glucose) of some of the extra tests through your child's doctor. The results of most of the extra tests done for this program will not be available right away so they will not be reported back to your child's doctor. Your child's participation in the program may help patients in the future by giving important information about the virus and how to treat hand, foot and mouth disease.

HOW WILL THE COSTS BE COVERED?

At the first visit, you will be reimbursed 200,000 VND for the cost of a doctor visit and the study laboratory tests. All research laboratory tests will be paid for by the program. You will also be reimbursed for travel costs when you come back for the scheduled follow-up visit at day 8-14. This amount is 200,000 VND if you live in Ho

Chi Minh City, and more if you live farther. The study staff can tell you what the travel cost is for your district.

You are responsible to cover all of your child's hospital costs as per usual. In case your child has health problems resulting from the program, we will make sure the cost to treat this is covered by the program.

ARE THERE RISKS OF BEING IN THE PROGRAM?

Being in this program means that your child will have swabs taken and blood collected for tests that are not part of regular care. Taking swabs can be uncomfortable and can cause gagging (in the throat) or sneezing (in the nose). Needle pokes to take blood may cause brief pain in the arm or hand. Possible risks from having blood drawn include bruising, bleeding, and very rarely, infections where the blood was taken.

Host genetics blood test is not part of routine care. Some people may consider it an invasion of privacy to have information collected for research and to studies performed on their DNA/genetic code. Results will be made available to other researchers but your child will not be identified and no one will know whose genetic information it is. Your child's DNA will be stored in a freezer for future studies about how DNA can affect who gets a disease and how severe it becomes and may be sent out of the country to do these tests. You can choose if your child will have these tests or not by checking the box on the last page of this form.

HOW DO WE ADDRESS THE RISKS OF THE PROGRAM?

All of your child's information and samples will be coded by a number instead of using their name so that no one knows who they belong to. This will help to protect your child's privacy. All information about your child will be kept in a safe place where only project staff can access it. If you decide at any time to take your child out of the program, no new information will be collected. However, information collected on your child up until that point will be used.

HOW DO I GET MORE INFORMATION?

You are encouraged to ask any questions related to this program during the time of participation. If you have any questions about this program, its procedures, risks and benefits, or alternatives please call Dr. Hoang Minh Tu Van at 0902337583.You can also reach the study coordinator to change your appointment or ask questions at {*coordinator number*}.

If you have any questions about your child's rights as a subject in this program or about what will happen to the samples taken, you may want to talk to Dr Hoang Minh Tu Van, or if you want to speak to someone outside of the program you may contact the Ethics Committee at the {*Hospital Name*} at {*EC Phone Number*}.

INFORMED CONSENT FORM

(to be signed by the participant's parent or guardian)

- I have read the information given to me and freely agree for my child to be in this program. I have also had a chance to discuss it with the study staff and been given a copy to keep.
- I have been told about the risks and benefits. I got answers that I could understand to all my questions.
- I consent to study staff collecting information about my child's health and using this information for future medical research about hand foot and mouth disease or the response to infection.
- I understand that I may take my child out of the program at any time. If my child stops the program, it will **NOT** affect their future care. If I decide for my child to stop the program, I agree that the information collected about my child up to the point when my child stops may continue to be used.

(please check one)

☐ I AGREE or ☐ I DO NOT AGREE

that samples may be stored for tests to be done in the future and that these may be done outside of Vietnam.

(please check one)

☐ I AGREE or ☐ I DO NOT AGREE

that that genetic tests on my blood samples can be performed and that this may be done outside of Vietnam.

Participant Number [___] [___][___][___][___]

Patient's name: _____

Signature of Person Giving Consent:	Full Name:	Relationship to Patient:	Date of Signature:
_____	_____	_____	___/___/_____

I, the undersigned, have fully explained the relevant information of this program to the person named above and will provide her/him with a copy of this signed and dated informed consent form.

Investigator/Designee Signature	Full Name	Date of Signature
_____	_____	___/___/_____

If the person giving consent cannot read the form themselves, a witness must be present and sign here: I was present throughout the entire informed consent process with the participant. This form was read accurately to the volunteer, all questions from the volunteer were answered and the volunteer has agreed to take part in the research.

Witness Signature	Full Name	Date of Signature
_____	_____	___/___/_____

8.2. CASE REPORT FORM FOR OUTPATIENT – OBSERVATIONAL STUDY

Demographics	**DEMO**
Participant number 03EI-[__|__]-[__|__|__|__]	Initials [__|__|__|__|__|__]

General information
1. Date of enrolment: [__|__]/[__|__]/[__|__] (dd/mm/yy)
2. Patient's Full name:* [_____]
3. Ward of enrolment: ○OPC ○ ID Ward ○ Emergency ward ○PICU
4. Gender: ○male○female
5. Date of birth: [__|__]/[__|__]/[__|__|__|__] *dd mm yyyy*
6. Address: a. Home number/street:* [_____] b. Ward: [_____] c. District: [_____] d. City/Province [_____]
7. Contact details * a. Home phone: [ ] b. Mobile phone: [ ]

** Information are not entered into the database*

Completed by: _____ *Date completed:* _____

03EI Observational study of HFMD

Inclusion and Exclusion Criteria	**INEX**
Participant number 03EI-[__|__]-[__|__|__|__]	Initials [__|__|__|__|__|__]

INCLUSION CRITERIA		
1. Less than 72hrs of symptom history	○Yes	○No
2. Age 0-12 years of age	○Yes	○No
3. Clinical diagnosis of HFMD	○Yes	○No
4. Informed consent given by parent or legal guardian	○Yes	○No
5. Willing to return for follow-up	○Yes	○No
EXCLUSION CRITERIA		
1. Another clinical diagnosis more likely	○ Yes	○ No

Completed by: _____ *Date completed:* _____

History	**HIST**
Participant number 03EI-[__|__]-[__|__|__|__]	Initials [__|__|__|__|__|__]

History

1. Date of onset of illness [__|__]/[__|__]/[__|__] (dd/mm/yy)
2. Does the patient attend:

O Daycare facility O Kindergarten O School O Stay atHome

If the child attends one of the above, write the address:

a. Number/street: [_____]
b. Ward: [_____]
c. District: [_____]
d. City/Province: [_____]

3. How many adults normally live in your house (including parent/guardian)?
   .....**[__|__]**
4. How many children under the age of 15 (including other people's children) normally live in your house? **[__|__]**

During the past 2 weeks, was there

5. someone in household with HFMD: O Yes O No
6. someone at daycare/kindergarten/school with HFMD: O Yes O No
7. any other contact with someone with HFMD: O Yes O No

Completed by: _____ *Date completed:* _____

Symptoms and Signs	**EXAM**
Participant number 03EI-[__|__]-[__|__|__|__]	Initials [__|__|__|__|__|__]

Symptoms and Signs

Since onset of illness, did the child experience:

1. Headache : ○Yes ○ No ○ Not documented
2. Fever: ○Yes ○ No ○ Not documented
3. Cough: ○Yes ○ No ○ Not documented
4. Runny nose: ○Yes ○ No ○ Not documented
5. Vomiting: ○Yes ○ No ○ Not documented
6. Diarrhea: ○Yes ○ No ○ Not documented
7. Drowsiness: ○Yes ○ No ○ Not documented
8. Irritability: ○Yes ○ No ○ Not documented
9. Myoclonus: ○Yes ○ No ○ Not documented
10. Sweating ○ Yes ○ No ○ Not documented
11. Lethargy ○ Yes ○ No ○ Not documented
12. Temperature: [__|__].[__] °C
13. Pulse [__|__|__]/min
14. Blood pressure [__|__|__]/[__|__|__] mmHg
15. Respiratory rate [__|__]/min
16. Respiratory pattern ○ Normal ○Tachypnea ○ Irregular ○ wheeze ○stridor ○ Cheyne-stokes ○ Apnea
17. Conjunctivitis: ○Yes ○ No ○ Not documented
18. Weight: [__|__].[__] Kg
19. Height: [__|__|__] Cm
20. Head circumference: [__|__] Cm

Symptoms and Signs	**EXAM**
Participant number 03EI-[__|__]-[__|__|__|__]	Initials [__|__|__|__|__|__]

Document location of rash and how many spots/vesicles visible at ENROLMENT

Hands and Feet

○0 ○ 1-5 ○>5

Knees and Elbows

○0 ○ 1-5 ○>5

Buttocks

○0 ○ 1-5 ○>5

Descibe rash: ○ mostly vesicular ○ mostly macular ○ both

Mouth Lesions

Location of ulcers: ☐Tongue ☐Palate ☐Buccal ☐Lips

Completed by: _____ *Date completed:* _____

Follow up	**FU**
Participant number 03EI-[__|__]-[__|__|__|__]	Initials [__|__|__|__|__|__]

Sampling	
1. Throat swab in VTM:	○ Yes ○ No
2. Rectal swab in VTM :	○ Yes ○ No
3. EDTA bloodfor routine laboratory	○ Yes ○No
4. EDTA blood for serology/host genetics	○ Yes ○No

Results from routine laboratory tests:	
5. Date sample taken:	[__|__]/[__|__]/[__|__] (dd/mm/yy)
6. White blood cell count	[__|__],[__] * 10^9/ml
7. Neutrophil %	[__|__],[__] %
8. Lymphocyte %	[__|__],[__] %
9. Platelet count	[__|__|__],[ ] * 10^9/ml
10. Blood glucose	[__|__],[__]
11. CRP	[__|__],[__]

Completed by: _____ *Date completed:* _____

Follow up	**FU**
Participant number 03EI-[__|__]-[__|__|__|__]	Initials [__|__|__|__|__|__]

DAY 2 of ILLNESS ASSESSMENT:

Indicate one option: ○ A ○ B ○ C ○ D

A. The patient attends the out-patient clinic for assessment: please complete this form.
B. The patient doesn't attend the out-patient clinic and the information is gathered by phone: please complete this form.
C. The patient doesn't attend the out-patient clinic and cannot be contacted by phone. Leave this form and the summary sheets blank.
D. The patient is admitted to the hospital: if the hospital of admission is HTD, CH1 or CH2: contact the local study coordinator

Leave the rest of this form and "Day 6 of illness assessment" blank

---

Date of collecting information [__|__]/[__|__]/[__|__|__|__] (dd/mm/yyyy)

1. Has the patient improved since the visit during the acute illness? ○ Yes ○ No
2. Did the patient attend any other clinic or hospital since the last visit? ○ Yes ○ No

   a. If yes, give the name and reason?

   [_____]
   [_____]

   b. If yes, what did they diagnose there?
      ○ Hand, Foot and Mouth Disease,
      ○ Something else

Completed by: _____ *Date completed:* _____

Follow up	**FU**
Participant number 03EI-[__|__]-[__|__|__|__]	Initials [__|__|__|__|__|__]

DAY 4 of ILLNESS ASSESSMENT:

Indicate one option: ○ A ○ B ○ C ○ D

A. The patient attends the out-patient clinic for assessment: please complete this form.
B. The patient doesn't attend the out-patient clinic and the information is gathered by phone: please complete this form.
C. The patient doesn't attend the out-patient clinic and cannot be contacted by phone. Leave this form and the summary sheets blank.
D. The patient is admitted to the hospital: if the hospital of admission is HTD, CH1 or CH2: contact the local study coordinator

Leave the rest of this form and "Day 6 of illness assessment" blank

---

Date of collecting information [__|__]/[__|__]/[__|__|__|__] (dd/mm/yyyy)

3. Has the patient improved since the visit during the acute illness? ○ Yes ○ No
4. Did the patient attend any other clinic or hospital since the last visit? ○ Yes ○ No

a. If yes, give the name and reason?

[_____]
[_____]

b. If yes, what did they diagnose there?
○ Hand, Foot and Mouth Disease,
○ Something else

Completed by: _____ *Date completed:* _____

Follow up	**FU**
Participant number 03EI-[__|__]-[__|__|__|__]	Initials [__|__|__|__|__|__]

DAY 6 of ILLNESS ASSESSMENT:

Indicate one option: ○ A ○ B ○ C ○ D

A. The patient attends the out-patient clinic for assessment: please complete this form.
B. The patient doesn't attend the out-patient clinic and the information is gathered by phone: please complete this form.
C. The patient doesn't attend the out-patient clinic and cannot be contacted by phone. Leave this form and the summary sheets blank.
D. The patient is admitted to the hospital: if the hospital of admission is HTD, CH1 or CH2: contact the local study coordinator to enroll the patient in the inpatient arm of this study.

Leave the rest of this form blank

Date of collecting information [__|__]/[__|__]/[__|__|__|__] (dd/mm/yyyy)

1. Has the patient improved since the last visit or phone call? ○Yes ○No
2. Has the patient completely recovered now? ○Yes ○No

   a. If no, describe:

   [_____]
   [_____]

3. Did the patient attend any other clinic or hospital since the last visit or phone call? ○ Yes ○ No

   If yes, give the name and reason?

   [_____]
   [_____]

   a. If yes, what did they diagnose there?
      ○ Hand, Foot and Mouth Disease,
      ○ Something else

Completed by: _____ *Date completed:* _____

Follow-up at day 8-15, if discharged before day 8 (Day of enrolment is day 1).

1. Date of follow-up [_|_]/[_|_]/[_|_] (dd/mm/yy)
2. Outcome at follow-up: ○ Full recovery without complication ○Incomplete recovery ○Death ○Other, specify: _____
3. If Incomplete recovery ☐Seizures ☐Hypertonicity ☐Paralysis ☐Swallowing difficulties ☐Other, specify: [_____]

Sampling
4. EDTA taken ○Yes ○ No

Completed by: _____ *Date completed:* _____

8.3. CASE REPORT FORM FOR INPATIENT – OBSERVATIONAL STUDY

Inclusion and Exclusion Criteria	**INEX**
Participant number 03EI-[__|__]-[__|__|__|__]	Initials [__|__|__|__|__|__]

General information
1. Date of enrolment: [__|__]/[__|__]/[__|__] (dd/mm/yy)
2.
3. Patient's Full name:* [_____]
4. Hospital number:* [_____]
5. Ward of enrolment: ○ OPC ○ ID Ward ○ Emergency ward ○ PICU
6. Gender: ○ male ○ female
7. Date of birth: [__|__]/[__|__]/[__|__|__|__] *dd mm yyyy*
8. Address: a. Home number/street:* [_____] b. Ward: [_____] c. District: [_____] d. City/Province [_____]
9. Contact details * a. Home phone: [ ] b. Mobile phone: [ ]

** Information are not entered into the database*

Completed by: _____ *Date completed:* _____

Inclusion and Exclusion Criteria	**INEX**
Participant number 03EI-[__|__]-[__|__|__|__]	Initials [__|__|__|__|__|__]

B: INCLUSION CRITERIA		
1. Age 0-12 years of age	○ Yes	○ No
2. Clinical diagnosis of HFMD	○ Yes	○ No
3. Admitted to Hospital	○ Yes	○ No
4. Informed consent given by parent or legal guardian	○ Yes	○ No
5. Willing to return for follow-up	○ Yes	○ No
EXCLUSION CRITERIA		
1. Another clinical diagnosis more likely	○ Yes	○ No

Completed by: _____ *Date completed:* _____

History	**HIST**
Participant number 03EI-[__|__]-[__|__|__|__]	Initials [__|__|__|__|__|__]

History

1. Date of onset of illness [__|__]/[__|__]/[__|__] (dd/mm/yy)
2. Does the patient attend:

   ○ Daycare facility ○ Kindergarten ○ School ○ Stay at Home

   If the child attends one of the above, write the address:

   a. Number/street: [_____]
   b. Ward: [_____]
   c. District: [_____]
   d. City/Province: [_____]

3. How many adults normally live in your house (including parent/guardian)?
   .....**[__|__]**
4. How many children under the age of 15 (including other people's children) normally live in your house? **[__|__]**

During the past 2 weeks, was there

5. someone in household with HFMD: ○ Yes ○ No
6. someone at daycare/kindergarten/school with HFMD: ○ Yes ○ No
7. any other contact with someone with HFMD: ○ Yes ○ No

Completed by: _____ *Date completed:* _____

History	**HIST**
Participant number 03EI-[__|__]-[__|__|__|__]	Initials [__|__|__|__|__|__]

C: History

8. Date of admission to this hospital [__|__]/[__|__]/[__|__|__|__] (dd/mm/yyyy)
9. Transfer from another hospital ○ Yes ○ No
   If yes: Which hospital [_____]
   When was the patient admitted there: [__|__]/[__|__]/[__|__|__|__] (dd/mm/yyyy)
10. What grade of HFMD did the child have on the day s/he was first admitted to hospital

○ Grade 1 ○ Grade 2a ○ Grade 2b(1) ○ Grade 2b(2) ○ Grade 3 ○ Grade 4

11. Did the patient get any of the following, since onset of illness prior to enrolment?
    a. Cardiac arrest ○ Yes ○ No
    b. Mechanical Ventilation ○ Yes ○ No
    c. Hemofiltration ○ Yes ○ No
    d. Intravenous Immunoglobulin ○ Yes ○ No
    e. Milrinone ○ Yes ○ No
    f. IV Magnesium ○ Yes ○ No
    g. Phenobarbital ○ Yes ○ No
    h. Corticosteroids / Methylprednisolone ○ Yes ○ No
    i. Antibiotics ○ Yes ○ No

Completed by: _____ *Date completed:* _____

08RS – Neurodevelopmental outcomes in severe HFMD

Symptoms and Signs	**EXAM**
Participant number 08RS-[__|__]-[__|__|__]	Initials [__|__|__|__|__|__]

Symptoms and Signs

Since onset of illness, did the child experience:

1. Headache : ○ Yes ○ No ○ Not documented
2. Fever: ○ Yes ○ No ○ Not documented
3. Cough: ○ Yes ○ No ○ Not documented
4. Runny nose: ○ Yes ○ No ○ Not documented

5. Vomiting: ○ Yes ○ No ○ Not documented
6. Diarrhea: ○ Yes ○ No ○ Not documented
7. Drowsiness: ○ Yes ○ No ○ Not documented
8. Irritability: ○ Yes ○ No ○ Not documented
9. Myoclonus: ○ Yes ○ No ○ Not documented
10. Sweating ○ Yes ○ No ○ Not documented
11. Lethargy ○ Yes ○ No ○ Not documented

12. Temperature: [__|__].[__] °C
13. Pulse [__|__|__]/min
14. Blood pressure [__|__|__]/[__|__|__] mmHg
15. Respiratory rate [__|__]/min
16. Respiratory pattern ○ Normal ○Tachypnea ○ Irregular ○ wheeze ○stridor ○ Cheyne-stokes ○ Apnea
17. Conjunctivitis: ○ Yes ○ No ○ Not documented

18. Enrollment Grade HFMD:
    - ○ Grade 1
    - ○ Grade 2a
    - ○ Grade 2b(1)
    - ○ Grade 2b(2)
    - ○ Grade 3
    - ○ Grade 4
    - ○ Not documented

19. Weight: [__|__].[__] Kg
20. Height: [__|__|__] Cm
21. Head circumference: [__|__] Cm

08RS – Neurodevelopmental outcomes in severe HFMD

Symptoms and Signs	**EXAM**
Participant number 08RS-[__|__]-[__|__|__]	Initials [__|__|__|__|__|__]

Document location of rash and how many spots/vesicles visible at ENROLMENT

Hands and Feet
○ 0 ○ 1-5 ○ >5

Knees and Elbows
○ 0 ○ 1-5 ○ >5

Buttocks
○ 0 ○ 1-5 ○ >5

Descibe rash: ○ mostly vesicular ○ mostly macular ○ both

Mouth Lesions
Location of ulcers: ☐Tongue ☐Palate ☐Buccal ☐Lips

Completed by: _____ *Date completed:* _____

Neurological exam	**NEU**
Participant number 03EI-[__|__]-[__|__|__|__]	Initials [__|__|__|__|__|__]

ADMISSION NEUROLOGICAL EXAMINATION

1. Has the child had Phenobarbital within the last 12 hours? ○ Yes: oral ○ Yes: intravenous ○ No

Blantyr Coma Score

A state of unrousable coma is reached at a score of <3

ʃRub knuckles on patient's sternum ᶻFirm pressure on thumbnail bed with horizontal pencil

2. Best motor response	Localises painful stimulusʃ	2	○
	Withdraws limb from painᶻ	1	○
	Nonspecific or absent response	0	○
3. Verbal response	Appropriate cry	2	○
	Or inappropriate cry	1	○
	None	0	○
4. Eye movements	Directed (e.g follows mother's face)	1	○
	Not directed	0	○
5. Total		0-5	[__]

GLASGOW COMA SCORE

6. Admission GCS documented ○ Yes ○ No

   If Yes, state components of GCS:

   E: [__] / 4 V: [__] / 5 M: [__] / 6

7. Pupils react to light **Right** ○ Yes ○ No **Left** ○ Yes ○ No
8. Nystagmus ○ Yes ○ No ○ Not documented
9. Tremor ○ Yes ○ No ○ Not documented
10. Ataxia ○ Yes ○ No ○ Not documented
11. Limb weakness ○ Yes ○ No
    a. If Yes, specify: Upper limb ○ Right ○ Left
    b. Lower limb ○ Right ○ Left

Completed by: _____ *Date completed:* _____

Laboratory tests	**LAB**
Participant number 03EI-[__|__]-[__|__|__|__]	Initials [__|__|__|__|__|__]

Sampling	
1. Throat swab in VTM:	○ Yes ○ No
2. Rectal swab in VTM :	○ Yes ○ No
3. EDTA blood for routine laboratory	○ Yes ○ No
4. EDTA blood for serology/host genetics	○ Yes ○ No

Results from routine laboratory tests:	
5. Date sample taken:	[__|__]/[__|__]/[__|__] (dd/mm/yy)
6. White blood cell count	[__|__],[__] * 10^9/ml
7. Neutrophil %	[__|__],[__] %
8. Lymphocyte %	[__|__],[__] %
9. Platelet count	[__|__|__],[ ] * 10^9/ml
10. Blood glucose	[__|__],[__]
11. CRP	[__|__],[__]

Completed by: _____ *Date completed:* _____

DAILY EXAMINATION	**-----**
Participant number 03EI-[__|__]-[__|__|__|__]	Initials [__|__|__|__|__|__]

	Record highest value from the hospital chart recorded since the time of last measurement						
DAY	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**	**Day 8**
Date of assessment	___/___/___	___/___/___	___/___/___	___/___/___	___/___/___	___/___/___	___/___/___
Time of assessment							
Temperature (°C)	[__|__].[__]	[__|__].[__]	[__|__].[__]	[__|__].[__]	[__|__].[__]	[__|__].[__]	[__|__].[__]
Pulse (/min)	[__|__|__]	[__|__|__]	[__|__|__]	[__|__|__]	[__|__|__]	[__|__|__]	[__|__|__]
Blood pressure (mmHg)	_____/_____	_____/_____	_____/_____	_____/_____	_____/_____	_____/_____	_____/_____
Respiratory rate (/min)	[__|__]	[__|__]	[__|__]	[__|__]	[__|__]	[__|__]	[__|__]
Skin/mouth lesion	Unchanged [ ] Disappear [ ] Blurr [ ] New lesion [ ] Describe [ ]	Unchanged [ ] Disappear [ ] Blurr [ ] New lesion [ ] Describe [ ]	Unchanged [ ] Disappear [ ] Blurr [ ] New lesion [ ] Describe [ ]	Unchanged [ ] Disappear [ ] Blurr [ ] New lesion [ ] Describe [ ]	Unchanged [ ] Disappear [ ] Blurr [ ] New lesion [ ] Describe [ ]	Unchanged [ ] Disappear [ ] Blurr [ ] New lesion [ ] Describe [ ]	Unchanged [ ] Disappear [ ] Blurr [ ] New lesion [ ] Describe [ ]
Irritability	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N
Vomiting	Y/ N	Y/ N	Y/ N	Y/ N	Y/ N	Y/ N	Y/ N
Myoclonus	Y/ N	Y/ N	Y/ N	Y/ N	Y/ N	Y/ N	Y/ N
Lethargy	Y/ N	Y/ N	Y/ N	Y/ N	Y/ N	Y/ N	Y/ N
Ataxia	Y/ N	Y/ N	Y/ N	Y/ N	Y/ N	Y/ N	Y/ N
Nystagmus	Y/ N	Y/ N	Y/ N	Y/ N	Y/ N	Y/ N	Y/ N
Limb weakness	Y/ N	Y/ N	Y/ N	Y/ N	Y/ N	Y/ N	Y/ N
GRADING							

DAILY EXAMINATION	**-----**
Participant number 03EI-[__|__]-[__|__|__|__]	Initials [__|__|__|__|__|__]

GRADE as guidelines	○ 1 ○ 2a ○ 2b (1) ○ 2b (2) ○ 3 ○ 4 ○ no progress	○ 1 ○ 2a ○ 2b (1) ○ 2b (2) ○ 3 ○ 4 ○ no progress	○ 1 ○ 2a ○ 2b (1) ○ 2b (2) ○ 3 ○ 4 ○ no progress	○ 1 ○ 2a ○ 2b (1) ○ 2b (2) ○ 3 ○ 4 ○ no progress	○ 1 ○ 2a ○ 2b (1) ○ 2b (2) ○ 3 ○ 4 ○ no progress	○ 1 ○ 2a ○ 2b (1) ○ 2b (2) ○ 3 ○ 4 ○ no progress	○ 1 ○ 2a ○ 2b (1) ○ 2b (2) ○ 3 ○ 4 ○ no progress
If no change in grade: describe clinical situation in relation to previous assessment	○Stable ○deteriorate ○impovement	○Stable ○deteriorate ○impovement	○Stable ○deteriorate ○impovement	○Stable ○deteriorate ○impovement	○Stable ○deteriorate ○impovement	○Stable ○deteriorate ○impovement	○Stable ○deteriorate ○impovement

DAILY EXAMINATION	-----
Participant number 03EI-[__|__]-[__|__|__|__]	Initials [__|__|__|__|__|__]

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
MEDICATIONS								
Midazolam	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N
Phenobarbitone oral	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N
Phenobarbitone IV	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N
Milrinone	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N
Inotropics (dopamine/dobutamine/(nor)epinephrin)	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N
Antibiotics	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N
Methylprednisolone	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N
Magnesium	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N
Nasal cannulla	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N
CPAP	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N
Intubation	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N
Resuscitated/CPR	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N
IVIg	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N
CVVH	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N
Completed by (Signature)								

Discharge	**DISC**
Participant number 03EI-[__|__]-[__|__|__|__]	Initials [__|__|__|__|__|__]

Discharge
1. Date of discharge: [__|__]/[__|__]/[__|__] (dd/mm/yy) 2. Discharge Diagnosis HFMD: ○ Yes ○ No If No, specify discharge diagnosis: [_____] 3. Highest grade during admission ○ Grade 1 ○ Grade 2a ○ Grade 2b(1) ○ Grade 2b(2) ○ Grade 3 ○ Grade 4
4. Outcome at discharge: ○ Full recovery without complication ○ Incomplete recovery ○ Transferred to another hospital ○ Death ○ Taken home without approval ○ Other, specify: _____
5. If Incomplete recovery ☐ Seizures ☐ Hypertonicity ☐ Paralysis ☐ Discharged with tracheostomy ☐ Swallowing difficulties ☐ Other, specify: [_____]

Sampling, if discharged at day 8 or later (Day of enrolment is day 1)
1. EDTA taken ○ Yes ○ No

Completed by: _____ *Date completed:* _____

Follow up	**FU**
Participant number 03EI-[__|__]-[__|__|__|__]	Initials [__|__|__|__|__|__]

Follow-up at day 8-15, if discharged before day 8 (Day of enrolment is day 1).

2. Date of follow-up [__|__]/[__|__]/[__|__] (dd/mm/yy)

3. Outcome at follow-up:

- ○ Full recovery without complication
- ○ Incomplete recovery
- ○ Death
- ○ Other, specify: _____

4. If Incomplete recovery

- ☐ Seizures
- ☐ Hypertonicity
- ☐ Paralysis
- ☐ Swallowing difficulties
- ☐ Other, specify: [_____]

Sampling
5. EDTA taken ○ Yes ○ No

Completed by: _____ *Date completed:* _____

Appendix for Grading Disease

Grade 1	
Vesicular rash on hands	1
Vesicular rash on feet	2
Vesicular rash on buttocks	3
Ulcers in mouth	4
Grade 2a	
History of myoclonus (<2 times/30 minutes but not seen at the time of examination)	5
Sluggishness	6
Insomnia	7
Irritable	8
Fever for more than 2 days	9
Temperature > 39oC,	10
Vomiting	11
Grade 2b (1)	
Myoclonus on examination	12
History of myoclonus>2times/30minutes	13
Lethargy	14
HR>130 (afebrile and at rest)	15
Grade 2b (2)	
Ataxia	16
Nystagmus	17
Weak limbs (<4/5)	18
Cranial nerve paralysis	19
Temp >39 °C and unresponsive to antipyretics	20
HR >150 (afebrile and at rest)	21
Grade 3	
HR >170beats/min (afebrile and at rest)	22
Mottled skin	23
High systolic Blood pressure • <1year >110mmHg • 1-2 years >115mmHg • >2 years >120mmHg	24
Raised respiratory rate	25
GCS <10	26
apnea	27
Abdominal respiration	28
Shallow breathing	29
Recession	30
Wheezing	31
Laryngeal stridor	32
Grade 4	

apnea	33
spO2 <92% (with 6L O_2 nasal cannulae)	34
Shock: Undetectable bp	35
Low systolic blood pressure: • <12 months <70mmHg • >12 months <80mmHg	36
Pulse pressure ≤25mmHg	37
Respiratory arrest	38
Cardiac arrest	39
Acute pulmonary oedema	40

8.4. CASE REPORT FORM FOR OUTPATIENT WHO NEED TO ADMIT TO HOSPITAL – OBSERVATIONAL STUDY

Inclusion and Exclusion Criteria	**INEX**
Participant number 03EI-[__|__]-[__|__|__|__]	Initials [__|__|__|__|__|__]

General information	
1. Date of enrolment:	[__|__]/[__|__]/[__|__] (dd/mm/yy)
2. Hospital Site:	○ HTD ○ CH1 ○ CH2
3. Hospital number:*	[_____]
4. Ward of enrolment:	○OPC ○ ID Ward ○ Emergency ward ○PICU

** Information are not entered into the database*

Completed by: _____ *Date completed:* _____

History	**HIST**
Participant number 03EI-[__|__]-[__|__|__|__]	Initials [__|__|__|__|__|__]

History

1. Date of admission to this hospital [__|__]/[__|__]/[__|__|__|__] (dd/mm/yyyy)
2. Transfer from another hospital ○Yes ○ No

   If yes: Which hospital [_____]

   When was the patient admitted there: [__|__]/[__|__]/[__|__|__|__] (dd/mm/yyyy)
3. What grade of HFMD did the child have on the day s/he was first admitted to hospital

○ Grade 1 ○ Grade 2a ○ Grade 2b(1) ○ Grade 2b(2) ○ Grade 3 ○ Grade 4

4. Did the patient get any of the following, since onset of illness prior to enrolment?
   a. Cardiac arrest ○Yes ○ No
   b. Mechanical Ventilation ○Yes ○ No
   c. Hemofiltration ○Yes ○ No
   d. Intravenous Immunoglobulin ○Yes ○ No
   e. Milrinone ○Yes ○ No
   f. IV Magnesium ○Yes ○ No
   g. Phenobarbital ○Yes ○ No
   h. Corticosteroids / Methylprednisolone ○Yes ○ No
   i. Antibiotics ○Yes ○ No

Completed by: _____ *Date completed:* _____

Laboratory tests **LAB**

Participant number
03EI-[__|__]-[__|__|__|__]

Initials
[__|__|__|__|__|__]

ADMISSION NEUROLOGICAL EXAMINATION

1. Has the child had Phenobarbital within the last 12 hours?
   ..... ○ Yes: oral ○ Yes: intravenous ○ No

Blantyr Coma Score

A state of unrousable coma is reached at a score of <3
Rub knuckles on patient's sternum Firm pressure on thumbnail bed with horizontal pencil

2. Best motor response	Localises painful stimulus	2		○
	Withdraws limb from pain	1		○
	Nonspecific or absent response	0		○
3. Verbal response	Appropriate cry	2		○
	or inappropriate cry	1		○
	None	0		○
4. Eye movements	Directed (e.g follows mother's face)	1		○
	Not directed	0		○
5. Total		0-5		[__]

GLASGOW COMA SCORE

6. Admission GCS documented ○Yes ○ No
   If Yes, state components of GCS:
   E: [__] / 4 V: [__] / 5 M: [__] / 6
7. Pupils react to light **Right**○Yes ○ No **Left**○Yes ○ No
8. Nystagmus ○Yes ○ No ○Not documented
9. Tremor ○Yes ○ No ○Not documented
10. Ataxia ○Yes ○ No ○Not documented
11. Limb weakness ○Yes ○ No
    a. If Yes, specify: Upper limb ○Right ○Left
    b. Lower limb ○Right ○Left

Completed by: _____ *Date completed:* _____

DAILY EXAMINATION	-----
Participant number 03EI-[__|__]-[__|__|__|__]	Initials [__|__|__|__|__|__]

	Record highest value from the hospital chart recorded since the time of last measurement						
DAY	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**	**Day 8**
Date of assessment	___/___/___	___/___/___	___/___/___	___/___/___	___/___/___	___/___/___	___/___/___
Temperature (°C)	[__|__].[__]	[__|__].[__]	[__|__].[__]	[__|__].[__]	[__|__].[__]	[__|__].[__]	[__|__].[__]
Pulse (/min)	[__|__|__]	[__|__|__]	[__|__|__]	[__|__|__]	[__|__|__]	[__|__|__]	[__|__|__]
Blood pressure (mmHg)	_____/_____	_____/_____	_____/_____	_____/_____	_____/_____	_____/_____	_____/_____
Respiratory rate (/min)	[__|__]	[__|__]	[__|__]	[__|__]	[__|__]	[__|__]	[__|__]
Skin/mouth lesion	Unchanged [ ] Disappear [ ] Blurr [ ] New lesion [ ] Describe [ ]	Unchanged [ ] Disappear [ ] Blurr [ ] New lesion [ ] Describe [ ]	Unchanged [ ] Disappear [ ] Blurr [ ] New lesion [ ] Describe [ ]	Unchanged [ ] Disappear [ ] Blurr [ ] New lesion [ ] Describe [ ]	Unchanged [ ] Disappear [ ] Blurr [ ] New lesion [ ] Describe [ ]	Unchanged [ ] Disappear [ ] Blurr [ ] New lesion [ ] Describe [ ]	Unchanged [ ] Disappear [ ] Blurr [ ] New lesion [ ] Describe [ ]
Irritability	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N
Vomiting	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N
Myoclonus	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N
Lethargy	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N
Ataxia	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N
Nystagmus	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N
Limb weakness	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N
GRADING							
GRADE as guidelines	○ 1	○ 1	○ 1	○ 1	○ 1	○ 1	○ 1

DAILY EXAMINATION	**-----**
Participant number 03EI-[__|__]-[__|__|__|__]	Initials [__|__|__|__|__|__]

	○ 2a ○ 2b (1) ○ 2b (2) ○ 3 ○ 4 ○ no progress	○ 2a ○ 2b (1) ○ 2b (2) ○ 3 ○ 4 ○ no progress	○ 2a ○ 2b (1) ○ 2b (2) ○ 3 ○ 4 ○ no progress	○ 2a ○ 2b (1) ○ 2b (2) ○ 3 ○ 4 ○ no progress	○ 2a ○ 2b (1) ○ 2b (2) ○ 3 ○ 4 ○ no progress	○ 2a ○ 2b (1) ○ 2b (2) ○ 3 ○ 4 ○ no progress	○ 2a ○ 2b (1) ○ 2b (2) ○ 3 ○ 4 ○ no progress
If no change in grade: describe clinical situation in relation to previous assessment	○ Stable ○ deteriorate ○ impovement	○ Stable ○ deteriorate ○ impovement	○ Stable ○ deteriorate ○ impovement	○ Stable ○ deteriorate ○ impovement	○ Stable ○ deteriorate ○ impovement	○ Stable ○ deteriorate ○ impovement	○ Stable ○ deteriorate ○ impovement

DAILY EXAMINATION	**-----**
Participant number 03EI-[__|__]-[__|__|__|__]	Initials [__|__|__|__|__|__]

	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**	**Day 8**
Time of Assessment	14:00	14:00	14:00	14:00	14:00	14:00	14:00	14:00
MEDICATIONS								
Midazolam	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N
Phenobarbitone oral	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N
Phenobarbitone IV	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N
Milrinone	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N
Inotropics (dopamine/dobutamine/(nor)epinephrin)	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N
Antibiotics	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N
Methylprednisolone	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N
Magnesium	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N
Nasal cannulla	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N
CPAP	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N
Intubation	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N
Resuscitated/CPR	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N
IVIg	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N
CVVH	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N
Completed by (Signature)								

Discharge	**DISC**
Participant number 03EI-[__|__]-[__|__|__|__]	Initials [__|__|__|__|__|__]

Discharge

1. Date of discharge: [__|__]/[__|__]/[__|__] (dd/mm/yy)
2. Discharge Diagnosis HFMD: ○ Yes ○ No
   If No, specify discharge diagnosis: [_____]
3. Highest grade during admission

○ Grade 1 ○ Grade 2a ○ Grade 2b(1) ○ Grade 2b(2) ○ Grade 3 ○ Grade 4

4. Outcome at discharge:
   - ○ Full recovery without complication
   - ○ Incomplete recovery
   - ○ Transferred to another hospital
   - ○ Death
   - ○ Taken home without approval
   - ○ Other, specify: _____

5. If Incomplete recovery
   - ☐ Seizures
   - ☐ Hypertonicity
   - ☐ Paralysis
   - ☐ Discharged with tracheostomy
   - ☐ Swallowing difficulties
   - ☐ Other, specify: [_____]

Sampling, if discharged at day 8 or later (Day of enrolment is day 1)

1. EDTA taken ○ Yes ○ No

Completed by: _____ *Date completed:* _____

Follow up	**FU**
Participant number 03EI-[__|__]-[__|__|__|__]	Initials [__|__|__|__|__|__]

Follow-up at day 8-15, if discharged before day 8 (Day of enrolment is day 1).

2. Date of follow-up [__|__]/[__|__]/[__|__] (dd/mm/yy)

3. Outcome at follow-up:

   ○ Full recovery without complication

   ○Incomplete recovery

   ○Death

   ○Other, specify: _____

4. If Incomplete recovery

   ☐Seizures
   ☐Hypertonicity
   ☐Paralysis
   ☐Swallowing difficulties
   ☐Other, specify: [_____]

Sampling
5. EDTA taken ○Yes ○ No

Completed by: _____ *Date completed:* _____

Appendix for Grading Disease

1	apnea	
2	spO2<92% (with 6L O2 nasal cannulae)	
3	Shock: Undetectable bp	
4	Low bp: systolic <12 months :<70mmHg >12 months: <80mmHg	
5	Pulse pressure (systolic – diastolic)≤25mmHg	
6	Respiratory arrest	
7	Cardiac arrest	
8	Acute pulmonary oedema	
	Grade 4	
9	HR>170beats/min (afebrile and at rest)	
10	Mottled skin	
11	Systolic Blood pressure • <1year >110mmHg • 1-2 years >115mmHg • >2 years >120mmHg	
12	Raised respiratory rate	
13	*GCS<10*	
14	apnea	
15	Abdominal respiration	
16	Shallow breathing	
17	Recession	
18	Wheezing	
19	Laryngeal stridor	
	Grade 3	
20	Ataxia	
21	Nystagmus	
22	Weak limbs (<4/5)	
23	Cranial nerve paralysis	
24	Temp>39 and unresponsive to antipyretics	
25	HR>150 (afebrile and at rest)	
	Grade 2b (2)	
26	Myoclonus on examination	
27	History of myoclonus>2times/30minutes	
28	Lethargy	
29	HR>130 (afebrile and at rest)	
	Grade 2b (1)	
30	Medical history of infrequent jump (<2 times/30 minutes and it is not seen at the time of examination)	
31	Sluggishness	
32	Insomnia	
33	Irritable	
34	Fever for more than 2 days	
35	Temperature > 39oC,	
36	Vomiting	
	Grade 2a	
37	Vesicular rash on hands	
38	Vesicular rash on feet	
39	Vesicular rash on buttocks	
40	Ulcers in mouth	
	Grade 1	

8.5. CASE REPORT FORM FOR RETROSPECTIVE STUDY

DATA COLLECTION FORM 1 (FOR ALL CASES)

1. Subject ID (1-2400):
2. Hospital number:
3. Gender: Male/ Female
4. Date of birth:
5. Date of admission:
6. Grade at admission:

   Grade 1 Grade 2A Grade 2B1 Grade 2B2
   Grade 3 Grade 4 Other diagnosis
7. Grade at discharge:

   Grade 1 Grade 2A Grade 2B1 Grade 2B2 Grade 3 Grade 4
8. Time prior to admission: days NA
9. Admission ward

   ED ID Transfer from other hospital
10. Ward of discharge/death

    ED ID PICU
11. Fever: Yes No NA
12. Highest degree:
13. Total duration of fever

    Vomiting Yes No NA
    Erythema Yes No NA
    Mouth ulcers Yes No NA
    Myoclonus Yes No NA
    Irritability Yes No NA
    Tremor Yes No NA
14. Date of discharge/dead/transfer
15. Outcome:

    Complete recovery Incomplete recovery
    Dead Discharge to die
16. Lab result
    a. WBC (k/ul) NA
    b. Neutrophiles (%) NA
    c. Lymphocytes (%) NA
    d. Platelets (k/ul) NA
    e. Blood glucose (mg/dl) NA
    f. CRP (g/l) NA

DATA COLLECTION FORM 2 (FOR SEVERE CASES)

1. Clinical signs in the 1st 72 hours

Day 1

Temp			
Pulse			
SBP			
DBP			
RR			
Irritability	Yes	No	NA
Myoclonus	Yes	No	NA
Vomit	Yes	No	NA
Lethargy	Yes	No	NA
Ataxia	Yes	No	NA
Nystagmus	Yes	No	NA
Tremor	Yes	No	NA
Limb weakness	Yes	No	NA

Day 2

Temp			
Pulse			
SBP			
DBP			
RR			
Irritability	Yes	No	NA
Myoclonus	Yes	No	NA
Vomit	Yes	No	NA
Lethargy	Yes	No	NA
Ataxia	Yes	No	NA
Nystagmus	Yes	No	NA
Tremor	Yes	No	NA
Limb weakness	Yes	No	NA

Day 3

Temp				
Pulse				
SBP				
DBP				
RR				
Irritability	Yes	No	NA	
Myoclonus	Yes	No	NA	
Vomit	Yes	No	NA	
Lethargy	Yes	No	NA	
Ataxia	Yes	No	NA	
Nystagmus	Yes	No	NA	
Tremor	Yes	No	NA	
Limb weakness	Yes	No	NA	

2. Treatment

IVIg	Yes	No	NA	Date
Grade when receiving IVIg:	2B1	2B2	3	4
Milrinone	Yes	No	NA	Date
Inotropes	Yes	No	NA	Date
Intubation	Yes	No	NA	Date
CRRT	Yes	No	NA	Date
CPR	Yes	No	NA	Date

3. PCR result

EV	Positive	Neg	NA
EV71	Positive	Neg	NA

8.6. PUBLICATION PLAN AND PAPERS FROM THIS PROJECT

PUBLICATION PLAN (FIRST AUTHOR) AND LIST OF PUBLICATIONS

1. **Clinical and aetiological study of hand, foot and mouth disease in in- and outpatients in the three main referral centres in southern Vietnam, 2013-2015**

Authors: Hoang Minh Tu Van[1,3], Nguyen To Anh[1], Tran Tan Thanh[1], Vu Thi Ty Hang[1], Le Nguyen Truc Nhu[1], Nguyen Thi Han Ny[1], Le Thanh Hoang Nhat[1], Nguyen Thi Thu Hong[1], Nguyen Thanh Hung[2], Le Nguyen Thanh Nhan[2], Truong Huu Khanh[2], Du Tuan Quy[2] , Ha Manh Tuan[3], Ho Lu Viet[3], Do Chau Viet[3], Nguyen Tran Nam[3], Nguyen Thi My Thanh[4], Saraswathy Sabanathan[1], Phan Tu Qui[1,4], Nguyen Van Vinh Chau[4], Guy E. Thwaites[1,5], Bridget Wills[1,5], C. Louise Thwaites [1,5], Le Van Tan[1], and H. Rogier van Doorn[1,5]

[1]Oxford Clinical Research Unit, Ho Chi Minh City, Vietnam

[2]Children's Hospital 1, Ho Chi Minh City, Vietnam

[3]Children's Hospital 2, Ho Chi Minh City, Vietnam

[4]Hospital of Tropical Diseases, Ho Chi Minh City, Vietnam

[5]Centre for Tropical Medicine, Nuffield Department of Medicine, University of Oxford, Oxford, UK

Paper submitted to Clinical Microbiology and Infection and under peer review.

2. **Longitudinal description of clinical features, management and outcome of Hand, Foot and Mouth Disease in Children's Hospital 2, Ho Chi Minh City from 2009 to 2015.**

Authors: Hoang Minh Tu Van[1,2], Ha Manh Tuan[1], Ho Lu Viet[1], Le Van Tan[2], C.Louise Thwaites[2,4] and H.Rogier van Doorn[3,4].

[1] Children's Hospital 2, Ho Chi Minh City, Vietnam

[2] Oxford Clinical Research Unit, Ho Chi Minh City, Vietnam

[3] Oxford Clinical Research Unit, Ha Noi, Vietnam

[4]Centre for Tropical Medicine, Nuffield Department of Medicine, University of Oxford, Oxford, UK

Paper in preparation, expected to be submitted in June.

3. Evolution of Enterovirus A71-associated Hand Foot and Mouth Disease in Southern Vietnam from 2013 to 2015

Authors: Hoang Minh Tu Van[1,3], Nguyen To Anh[1], Tran Tan Thanh[1], Vu Thi Ty Hang[1], Le Nguyen Truc Nhu[1], Nguyen Thi Han Ny[1], Nguyen Thi Thu Hong[1], Nguyen Thanh Hung[2], Le Nguyen Thanh Nhan[2], Truong Huu Khanh[2], Ha Manh Tuan[3], Ho Lu Viet[3], Do Chau Viet[3], Nguyen Thi My Thanh[4], Saraswathy Sabanathan[1], Phan Tu Qui[1,4], Nguyen Van Vinh Chau[4], Guy E. Thwaites[1,5], C. Louise Thwaites [1,5], Le Van Tan[1], and H. Rogier van Doorn[5,6]

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Culture results added, paper in preparation and expected to be submitted by the end of June.

Nguyen et al. Virology Journal (2016) 13:125
DOI 10.1186/s12985-016-0580-9

Virology Journal

METHODOLOGY Open Access

Development and evaluation of a non-ribosomal random PCR and next-generation sequencing based assay for detection and sequencing of hand, foot and mouth disease pathogens

Anh To Nguyen[1*], Thanh Tan Tran[1], Van Minh Tu Hoang[2], Ngoc My Nghiem[3], Nhu Nguyen Truc Le[1], Thanh Thi My Le[3], Qui Tu Phan[3], Khanh Huu Truong[4], Nhan Nguyen Thanh Le[4], Viet Lu Ho[2], Viet Chau Do[2], Tuan Manh Ha[2], Hung Thanh Nguyen[4], Chau Van Vinh Nguyen[3], Guy Thwaites[1,5], H. Rogier van Doorn[1,5] and Tan Van Le[1]

Abstract

Background: Hand, foot and mouth disease (HFMD) has become a major public health problem across the Asia-Pacific region, and is commonly caused by enterovirus A71 (EV-A71) and coxsackievirus A6 (CV-A6), CV-A10 and CV-A16. Generating pathogen whole-genome sequences is essential for understanding their evolutionary biology. The frequent replacements among EV serotypes and a limited numbers of available whole-genome sequences hinder the development of overlapping PCRs for whole-genome sequencing.
We developed and evaluated a non-ribosomal random PCR (rPCR) and next-generation sequencing based assay for sequence-independent whole-genome amplification and sequencing of HFMD pathogens. A total of 16 EV-A71/CV-A6/CV-A10/CV-A16 PCR positive rectal/throat swabs (Cp values: 20.9–33.3) were used for assay evaluation.

Results: Our assay evidently outperformed the conventional rPCR in terms of the total number of EV-A71 reads and the percentage of EV-A71 reads: 2.6 % (1275/50,000 reads) vs. 0.1 % (31/50,000) and 6 % (3008/50,000) vs. 0.9 % (433/50,000) for two samples with Cp values of 30 and 26, respectively. Additionally the assay could generate genome sequences with the percentages of coverage of 94–100 % of 4 different enterovirus serotypes in 73 % of the tested samples, representing the first whole-genome sequences of CV-A6/10/16 from Vietnam, and could assign correctly serotyping results in 100 % of 24 tested specimens. In all but three the obtained consensuses of two replicates from the same sample were 100 % identical, suggesting that our assay is highly reproducible.

Conclusions: In conclusion, we have successfully developed a non-ribosomal rPCR and next-generation sequencing based assay for sensitive detection and direct whole-genome sequencing of HFMD pathogens from clinical samples.

Keywords: Hand, foot and mouth disease, Enterovirus A, Random PCR, FR26RV-Endoh primer, Next-generation sequencing

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Background

Hand, foot and mouth disease (HFMD) is a common and usually mild disease of children worldwide. The disease is caused by different genotypes of the species Enterovirus A, genus *Enterovirus*, family *Picornaviridae* (including coxsackievirus A (CV-A) 6, 10 and 16 and particularly EV-A71). However, EV-A71 has emerged and caused large and sometimes severe/fatal HFMD outbreaks [1] across the Asia-Pacific region since 1997. Of note, the frequent replacements between EV-As have been observed over the last decade in the regions where HFMD is endemic [2–6]. In recent years CV-A6 has emerged and replaced CV-A16 to become the dominant EV-A detected in HFMD patients [7, 8]. While the underlying mechanism of this phenomenon remains unknown, the data highlight the importance of continued effort to monitor the evolution of the causative agents of HFMD.

Currently, there is no clinically proven antiviral drug available to treat severe disease. Likewise, although phase III trials of three monovalent inactivated EV-A71 vaccines have been completed in China with an efficacy of over 95 %, routine use is still far away. Moreover, to what degree the implementation of a monovalent vaccine for EV-A71 may influence the epidemic patterns of HFMD and the evolution of the causative agents in endemic countries is a subject that merits follow-up research.

Collectively, the ability to generate viral whole-genome sequences is essential for understanding the evolutionary biology and epidemiology of HFMD. It is also important for the development of intervention strategies, especially vaccines. While the availability of relatively large numbers of EV-A71 whole-genome sequences (n = ~524) deposited in GenBank has facilitated the development of a sensitive overlapping PCR based whole-genome sequencing assay [9], smaller numbers of whole-genome sequences of other EV-As are available (CV-A16; n = 61, A6; 35, A10; 11) from limited localities. This is problematic for the selection of specific PCR primers that can amplify diverse EV-As. Additionally, one of the major drawbacks of specific-PCR based sequencing assays is that due to the nature of quick evolution rates of RNA viruses, selected primers may need to be adjusted regularly to be able to amplify newly emerging viral variants or genotypes. As a consequence, a sequence-independent approach is thus attractive to overcome such obstacles.

Developed by Froussard in 1992 [10], random PCR (rPCR) primer (FR26RV-N6: 5′-GCCGGAGCTCTGCA GATATCNNNNNN-3′) consists of a fixed 20 nucleotides (FR20RV: GCCGGAGCTCTGCAGATATC) at the 5′-end and a random hexanucleotides at 3′ end (N6: NNNNNN). In 2005 Endoh and his colleagues designed a set of 96 hexanucleotides for specific amplification of viral sequences called non-ribosomal hexanucleotides [11]. For sequence-independent whole-genome amplification and sequencing of HFMD pathogens, herein we describe the development and evaluation of a non-ribosomal random amplification assay utilizing the 96 non-ribosomal hexanucleotide oligos designed by Endoh [11] and the 5′-end fixed oligo of the conventional random PCR primers (FR20RV) [10]. When combined with next-generation sequencing, our assay showed that it could generate full-genome sequences of HFMD pathogens directly from clinical specimens.

Methods

Samples

The clinical samples used included two residual throat swabs from anonymous HFMD patients with EV-A71 infection admitted to the Hospital for Tropical Diseases in Ho Chi Minh City in 2012. Additionally, 13 throat/rectal swabs of diverse viral load (including CV-A6; n = 4, CV-A10; n = 4, CV-A16; n = 3 and EV-A71; n = 2) derived from patients enrolled into an on-going prospective observational HFMD study of all severities in three referral hospitals in Ho Chi Minh City, Vietnam since 2013 were also used [9]. The clinical samples were collected in viral transport medium, divided into three aliquots and stored at -80 °C until use. Viral detection and serotype identification were done as per the study protocol using previous described assays [12, 13].

Development and preparation of non-ribosomal random PCR primers

For selective amplification of viral sequences, we replaced the random hexanucleotide motif at the 3′-end of the primer FR26RV-N6 by those 96 hexanucleotides designed by Endoh. This resulted in a set of 96 separate primers consisting of an FR20RV sequence at 5′-end plus one of the 96 Endoh's hexanucleotides at the 3′-end (Additional file 1: Table S1).

Each individual primer was synthesized at a concentration of 100 μM, and an equal amount of each synthesized oligo was pooled together to make working solution (~1 μM). This primer mixture was named FR26RV-Endoh.

Sample pretreatment and nucleic acid extraction

An overview of the whole procedure is described in Fig. 1. Sample pretreatment was carried out as previously described [14]. In short, prior to nucleic acid isolation 110 μl of clinical samples was centrifuged at 10,000 g for 10 min. The resulting 100 μl of supernatants were collected and treated with 2U/ul of turbo DNase (Ambion, Life Technology, Carlsbad, CA, USA) at 37 °C for 30 min. Viral RNA was then extracted from the treated material using QIAamp viral RNA kit

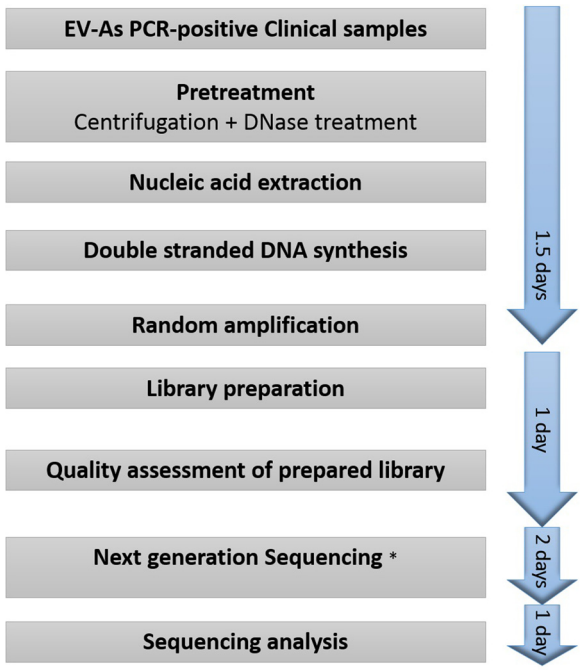


Fig. 1 Flowchart showing an overview of the whole procedure of rPCR-Miseq based assay. Note: * the turn-around time may vary, especially when using service platform, which may take more than 2 days

(QIAgen GmbH, Hilden, Germany), following the manufacturer's instructions, and finally eluted in 50 μl of elution buffer (provided with the extraction kit).

cDNA and double stranded DNA synthesis

Double stranded (ds) DNA was synthesized from the extracted RNA using either FR26RV-N6, FR26RV-Endoh, random hexanucleotides or non-ribosomal \hexanucleotides primer. Firstly, 10 μl of extracted RNA was mixed with 0.1 μM of the primer and 0.5nM of dNTPs (Roche Diagnostics GmbH, Mannheim, Germany). The mixture was incubated at 65 °C for 5 min, and was then immediately chilled on ice for 1 min. Secondly, 7 μl of a reaction mix containing 200U of Super Script III, 40 U of RNase OUT, 0.1 M DTT and 1X first strand buffer (Invitrogen, Carlsbad, CA, USA) was added into the first reaction mixture. The reaction was continued at 25 °C for 10 min, 37 °C for 1 min and 94 °C for 2 min, and then immediately chilled on ice for 2 min. Next, 5U of exo-Klenow fragment (Ambion) and 10U of Ribonuclease H (Ambion) were added into the reaction mixture, which was finally subjected to a double-stranded (ds) DNA synthesis step consisting of 25 °C for 5 min, 37 °C for 1 h and 75 °C for 10 min.

Random amplification

The resulting dsDNA products generated by FR26RV-N6 and FR26RV-Endoh primers were amplified using FR20RV primer (5′-GCCGGAGCTCTGCAGATATC-3′). PCR amplification was carried out in a total reaction volume of 50 μl consisting of 3 μl of dsDNA, 0.4 μM of primer FR20RV and 45 μl of Platinum PCR supermix high fidelity (Invitrogen). The thermal cycling condition consisted of 94 °C for 2 min and followed by 40 cycles of 94 °C for 30s, 55 °C for 30s and 72 °C for 3 min and 1 cycle of 72 °C for 2 min.

Next generation sequencing library preparation and sequencing

The resulting dsDNA generated by hexanucleotides or non-ribosomal hexanucleotides and rPCR products were purified with use of QIAquick PCR purification kit (QIAgen GmbH, Hilden, Germany). DNA concentration of the purified products was measured by Qubit dsDNA HS kit (Invitrogen). One nanogram of the purified DNA was then subjected to library preparation steps by using Nextera XT DNA library preparation kit (Illumina, San Diego, CA, USA), according to manufacturer's instructions. Prior to sequencing, the quantity of the prepared library was measured by using KAPA Library Quant Kit (Kapa Biosystems, Wilmington, MA, USA), following manufacturer's instructions.

The prepared library was sequenced using MiSeq reagent kit V2 in an Illumina Miseq platform (Illumina). For each run, tested samples were multiplexed and differentiated by double indexes using Nextera XT Index Kit (Illumina).

Sequence analysis

The sequences generated by Illumina Miseq were analyzed using Geneious 8.1.5 (Biomatters, San Francisco, CA, USA). The obtained sequences were processed to remove primer sequences. Sequence assembly was carried out by using a reference-based mapping strategy available in Geneious (CV-A10, HQ728262; CV-A6, JN582001; CV-A16, JX481738; EV-A71 B5, DQ341363; EV-A71 C4, AB550338), followed by manual editing of the obtained consensus.

Representatives of viral protein 1 (VP1) sequences of CV-A16 ($n = 39$), A6 (38), A10 (29) and EV-A71 (36) of different subgenotypes and from various localities worldwide were used for phylogenetic inference. Pairwise alignment was performed using Geneious alignment tool. Phylogenetic reconstructions were performed using maximum likelihood method (ML) with general time reversible (GTR) nucleotide substitution model available in Geneious package, and support for individual nodes was assessed using a bootstrap procedure (1000 replicates).

The sequences obtained in this study were submitted to NCBI (GenBank) and assigned accession numbers KX430795-KX430824.

Results

Non-ribosomal rPCR vs. conventional rPCR

To test whether our modified rPCR, which we named non-ribosomal rPCR, can selectively amplify viral sequences in clinical specimen as compared to the conventional rPCR, two EV-A71 positive swabs with Cp values of 26 (ID.13) and 30 (ID.14) (i.e. high and low viral load) were selected and subjected to random amplification procedures utilizing either FR26RV-N6 or FR26RV-Endoh, and followed by Illumina Miseq sequencing. The total- and percentage of EV-A71 reads, genome coverage and sequencing depth/coverage (i.e. the number of times a single nucleotide was sequenced) were taken into account for comparison.

In order to avoid the potential biases introduced by variable number of reads between barcodes, a total of 50,000 reads were randomly taken from each index for the analysis. In both tested EV-A71 positive samples, the total number of EV-A71 reads and the percentage of EV-A71 reads generated by non-ribosomal rPCR based assay was higher than the corresponding outputs generated by the conventional rPCR-based assay; 2.6 % (1275/50,000 reads) vs. 0.1 % (31/50,000 reads) for the sample ID14 with Cp value of 30 and 6 % (3008/50,000 reads) vs. 0.9 % (433/50,000 reads) for the sample ID13 with Cp value of 26 (Fig. 2). Additionally, a higher EV-A71 genome coverage and sequencing depth were also observed in both samples sequenced by non-ribosomal rPCR-based assay (Fig. 3). Taken together, the data indicated that our non-ribosomal rPCR is more viral specific and efficient than the conventional rPCR.

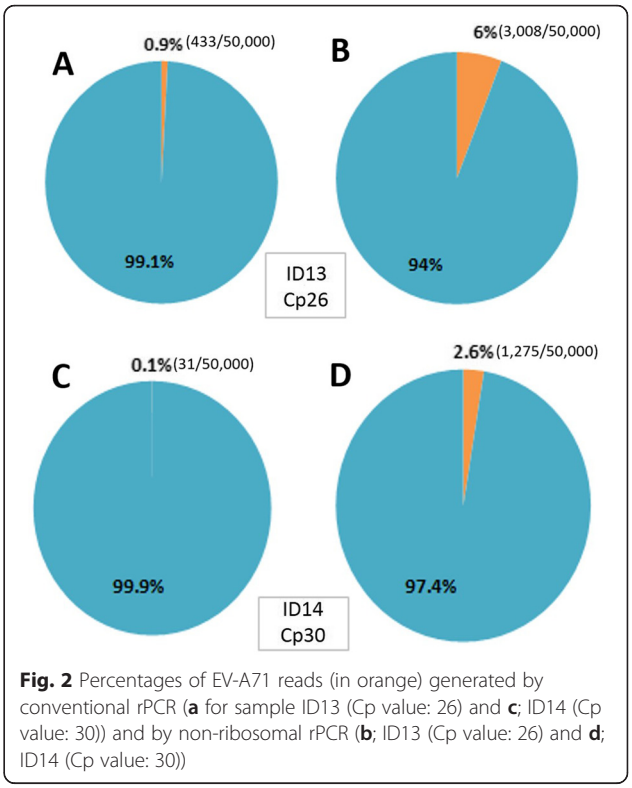


Fig. 2 Percentages of EV-A71 reads (in orange) generated by conventional rPCR (**a** for sample ID13 (Cp value: 26) and **c**; ID14 (Cp value: 30)) and by non-ribosomal rPCR (**b**; ID13 (Cp value: 26) and **d**; ID14 (Cp value: 30))

Non-ribosomal rPCR vs. direct sequencing

Previous studies shown that viral load enrichment by random amplification step resulted in biases in genome coverage [15, 16]. We therefore further evaluated our non-ribosomal rPCR by comparing its performance against that of direct sequencing of dsDNA library generated by hexanucleotide or non-ribosomal hexanucleotide primers. An EV-A71 positive throat swab (sample ID15) with a Cp value of 31 was used. After normalization, the obtained reads of each DNA library were map to an EV-A71 genome (DQ341363.1). Despite biases in terms of sequencing depth across the genome, non-ribosomal rPCR based workflow could generate nearly complete EV-A71 genome sequence (KX430823), while dsDNA library produced by hexanucleotide and non-ribosomal hexanucleotide primers could not (Additional file 1: Figure S1).

Detection and sequencing of HFMD pathogens: assessment of assay sensitivity and reproducibility

To further evaluate the performance of our non-ribosomal rPCR assay in terms of sensitivity and reproducibility a series of 12 swabs that were EVs real time PCR positive with different common HFMD pathogens (including CV-A6, CV-A10, CV-A16 and EV-A71) and with a wide range of Cp values from 20.8 to 33.3 [12] (i.e. from high to low viral load) (described in Methods section) were included for testing (Table 1). The included samples were tested in duplicate from sample pretreatment to nucleic acid isolation, random amplification by FR26RV-Endoh primers and sequencing by Illumina Miseq, resulting a total of 24 MiSeq datasets (Table 1).

Assay sensitivity

Illumina Miseq sequencing results showed that in addition to successfully providing correct serotype information (i.e. diagnostic results) in 100 % (24/24) of the tested samples, the assay could generate 17/24 (71 %) genome sequences of HFMD pathogens with the percentages of coverage of between 94 and 100 % (Table 1).

Collectively, of 24 tested samples, whole-genome sequencing success rates of 100 % (8/8), 93 % (13/14) and 71 % (17/24) with genome coverage of 94-100 % without internal gap were achieved among samples with Cp values of ≤25, ≤30 and ≤33.3, respectively (Table 1).

Assay reproducibility

To investigate the reproducibility of the assay, we compared the level of sequence identity between the obtained consensuses of the tested sample and its replicate. In 9/12 tested samples the consensuses of both

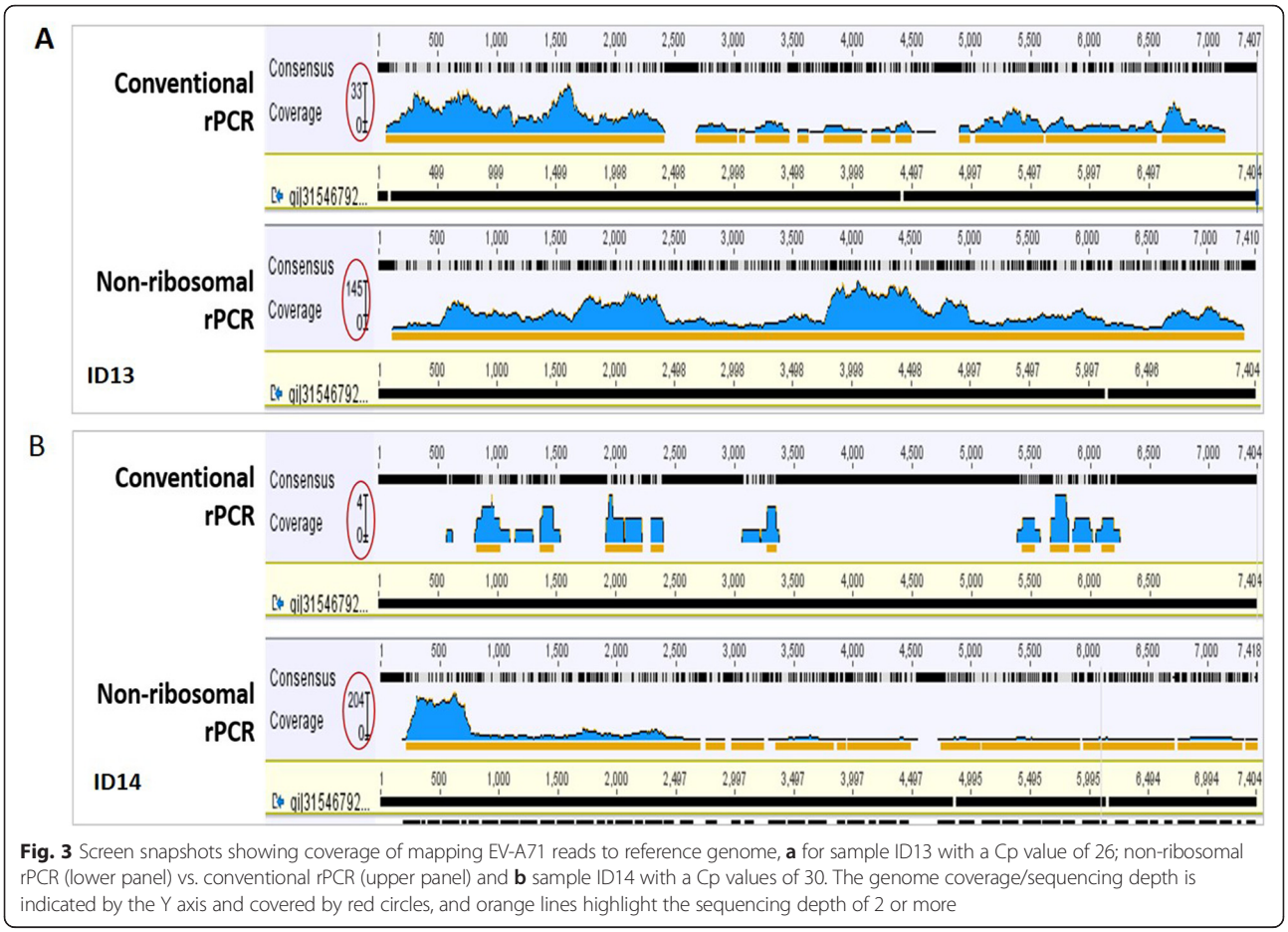


Fig. 3 Screen snapshots showing coverage of mapping EV-A71 reads to reference genome, **a** for sample ID13 with a Cp value of 26; non-ribosomal rPCR (lower panel) vs. conventional rPCR (upper panel) and **b** sample ID14 with a Cp values of 30. The genome coverage/sequencing depth is indicated by the Y axis and covered by red circles, and orange lines highlight the sequencing depth of 2 or more

replicates were 100 % identical (Table 1). In the remaining 3 samples, the differences of between 0.01 - 0.04 % were recorded (Additional file 1: Table S2). Additionally, the level of genome coverage, mean coverage (i.e. the numbers of times that a single nucleotide was sequenced) and the percentage of viral reads were comparable between two replicates (Table 1).

Phylogenetic analysis

Currently there are relatively few whole-genome sequences of CV-A6, CV-A10 and CV-A16 from limited geographical localities available in GenBank. To make more meaningful phylogenetic inference, we therefore first focused our analysis on representative VP1 sequences collected from different geographic locations worldwide.

Phylogenetic analysis of VP1 sequences suggested that the EV-A71 strains obtained in the present study sampled in 2012 belonged to subgenogroup C4, whereas the viruses collected in 2013 belonged to subgenogroup B5 (Additional file 1: Figure S1), which reconfirmed our previous finding about the replacement between these two subgenogroups occurring in Vietnam around 2012 [17]. All CV-A16 sequences belonged to genogroup B1a. In Vietnam, this B1a genogroup was first detected in the 2005 outbreak [18] and showed a close relatedness to the viruses circulating in the Asia-Pacific region (e.g. China, Japan, Thailand and Malaysia) (Additional file 1: Figure S2). In contrast, the analysis of CV-A6 sequences indicated that our CV-A6 belonged to genogroup A, which consists of CV-A6 strains sampled from United Kingdom and others viruses from China and Taiwan (Fig. 4b). Likewise, the CV-A10 strains sequenced in the present study belonged to genogroup C consisting of viral trains originating from various parts of the world and associated with HFMD outbreaks in Europe and Asia including in Spain, France and China (Fig. 4a). Similar results in terms of phylogenetic clustering of the sequences were obtained when whole-genome sequences were analyzed separately (data not shown).

Discussion

Traditionally, obtaining whole-genome sequence of a pathogen requires the design of several overlapping specific PCR primers based on the basis of sequence alignment of the published genome sequences. Although such strategies have been successfully applied for sequencing of HFMD pathogens including EV-A71 and other EV-As [9, 19–21], except for EV-A71, these

Table 1 Result summary of non-ribosomal rPCR and Miseq run

Virus[a]	Sample ID	Sample type	Cp values	% of enteroviral read	% Genome coverage	Internal gap length (bp)	Mean coverage	Accession numbers	Pairwise identity (%)
CV-A6	1	RS	22.69	90.2	99.5	0	26542	KX430795	100
				85.1	100	0	22630	KX430796	
	2	TS	28.34	11.2	97.5	0	1173	KX430797	100
				10.9	95.3	0	1119	KX430798	
	3	RS	30.5	7.9	97.7	0	1822	KX430799	100
				8.3	97.3	57	2244	KX430800	
	4	TS	32.06	7.1	75	1625	1328	KX430801	99.96
				13.3	96.8	41	3061	KX430802	
CV-A10	5	RS	20.92	40.7	99.2	0	11189	KX430803	100
				53.8	98	0	12725	KX430804	
	6	RS	23.59	53.1	97.5	0	17439	KX430805	100
				51.9	97.6	0	14086	KX430806	
	7	RS	26.71	18.2	98	0	4299	KX430807	99.99
				14.5	97	0	3820	KX430808	
	8	TS	33.2	42.4	94	0	12216	KX430809	99.99
				30.5	97.2	26	8412	KX430810	
CV-A16	9	TS	24.97	83.8	99.7	0	3161	KX430811	100
				80.1	99	0	20597	KX430812	
	10	TS	26.72	2	91	71	475	KX430813	100
				2.1	96	0	509	KX430814	
	11	TS	33.26	4.5	96	0	971	KX430815	100
				4.2	86	712	1101	KX430816	
EV-A71	12	TS	31.1	0.2	72.5	1447	5.3	KX430817	100
				0.3	94	0	52.6	KX430818	

[a]Miseq run was multiplexed. Only run output of relevant samples were shown here; CV-A6: coxsackievirus A6, CV-A10: coxsackievirus A10, CV-A16: coxsackievirus A16 and EV-A71 B5: enterovirus A71 subgenogroup B5; TS: Throat swab; RS: rectal swab

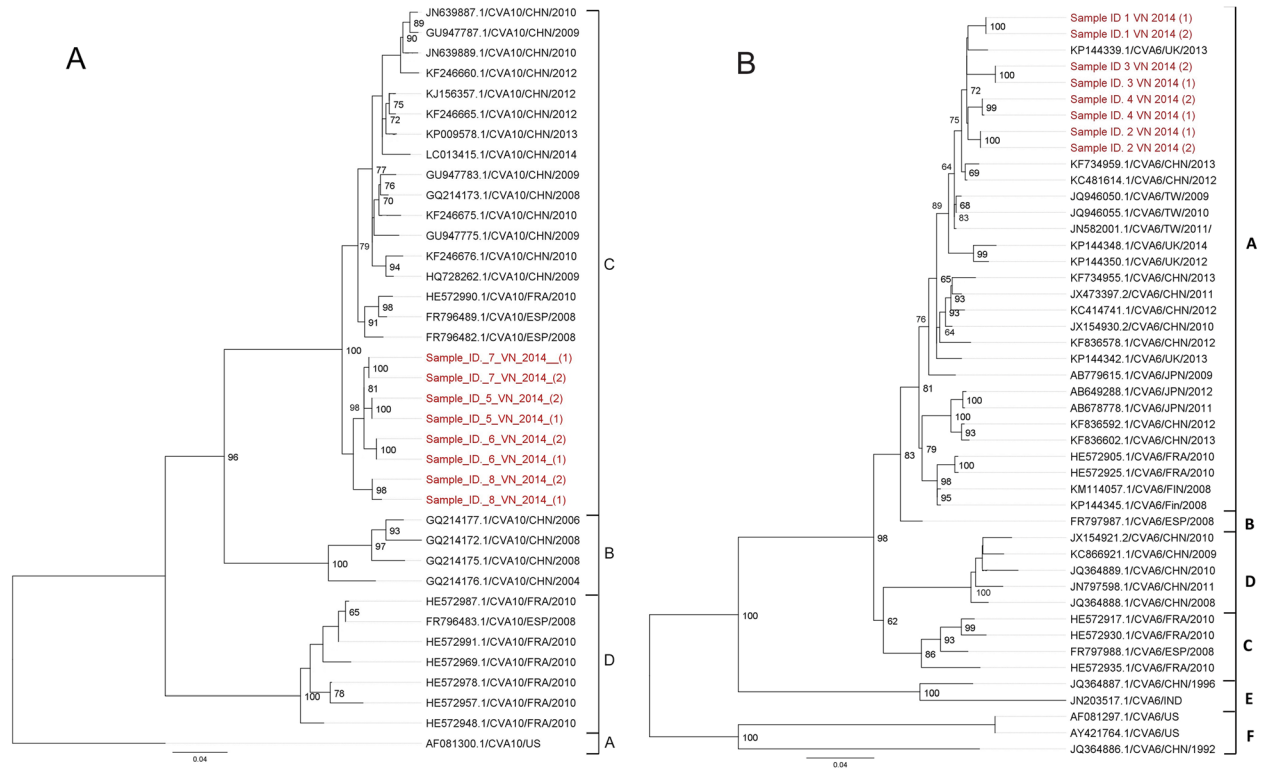


Fig. 4 The Maximum likelihood phylogenetic trees based on completed VP1 nucleotide sequences obtained in this study and representatives of VP1 sequences retrieved from GenBank. **a** ML phylogeny of VP1 sequences (894 nt) of CV-A10 strains ($n = 54$); **b** ML phylogeny of VP1 sequences (915 nt) of CV-A6 strains ($n = 60$). Scale bars indicated numbers of nucleotide substitution per site. CHN, China; FRA, France; ESP, Spain; US, United states; IND, India; Fin, Finland; JPN, Japan; TW, Taiwan; UK, United Kingdom; VN, Vietnam

overlapping primers were designed based on a limited numbers of sequences of EV-As and therefore may not function properly on diverse circulating viral strains whose complete genomes are yet to be sequenced. In addition, to be able to amplify emerging outbreak/novel strain, such viral specific PCR primers often need to be updated regularly, which is always challenging.

There have been several reports regarding the use of random primers, e.g. FR26RV-N6 primer, to generate whole-genome sequence of viral pathogens [22, 23]. However, as FR26RV-N6 primer contains a random hexamer motif at the 3′ end, which is not viral specific, assays may therefore lack specificity when used on materials such as rectal/throat swabs, which contain high amounts of host genetic materials and low concentrations of targeted virus. Meanwhile, Endoh's non-ribosomal hexanucleotide oligos have recently been successfully used as an alternative to random hexamers for selective amplification of viral RNA in the field of viral pathogen discovery [24–26]. For specific amplification and sequencing of viral pathogens in particular HFMD viruses (which were the focus of the present study) in clinical specimens, we adapted the fixed 5′ end oligo of the normal random PCR and Endoh's non-ribosomal hexanucleotides to create a novel 96 viral specific rPCR primer set (Additional file 1: Table S1).

When compared back-to-back using EV-A71 positive swabs, our non-ribosomal rPCR evidently outperformed the normal rPCR utilizing FR26RV-N6 primers and direct sequencing of dsDNA libraries generated by either hexanucleotides or non-ribosomal hexanucleotides. In subsequent testing we showed that without the requirement of viral specific PCR, our assay could generate whole-genome sequences of 4 different common HFMD pathogens (including CV-A6, CV-A10, CV-A16 and EV-A71) in either rectal or throat swabs with diverse viral load. Of 24 tested samples with Cp values between 20.9 and 33.2, (nearly) complete genomes were obtained in 17/24 (71 %) samples, representing the first whole-genome sequences of CV-A6, CV-A10 and CV-A16 from Vietnam. In three tested swabs and their replicates, the obtained consensuses occupied between 0.01–0.04 % of differences. This is however below the reported error rate of next generation sequencing (0.1 %). Of note, 2 out of the 4 EV-A71 genomes sequenced in the present study (sample IDs: 13 and 15) were previously recovered (KJ686266 and KX430824) using an overlapping PCRs and deep sequencing based workflow [9, 17]. And

pairwise comparisons of the obtained consensuses generated by both workflows revealed only 0.03 % and 0.04 % of variations without amino acid substitution observed (data not shown). Collectively, the data points to the fact that potential biases (if any) introduced by enrichment steps as 40-cycle PCR amplification by FR20RV primer of the present workflow is negligible and that our non-ribosomal rPCR and next-generation based assay is reproducible and sensitive.

Despite the use of non-ribosomal primers and the employment of a sample pretreatment step incorporating centrifugation and DNase treatment to enrich for enteroviral content in the swabs, the percentage of enteroviral reads in the obtained MiSeq libraries ranged between 0.2 and 90.2 %. This might have been attributed to the difference in terms of the compositions of non-enteroviral contents between the samples and/or the viral load of the tested viruses. Meanwhile there have been other reports about alternative sequence-independent whole-genome next-generation sequencing based assays including those incorporating sample pretreatment steps as physical virion enrichment and RNase digestion [27–29]. It is therefore of interest to evaluate the usefulness of those sample pretreatment steps when combined with our non-ribosomal rPCR. Likewise, comparing the performance of our non-ribosomal rPCR with those existing sequence-independent assays warrants further research, which is however beyond the scope of the present study.

For clinical diagnostics, obtaining partial viral genome sequence is sufficient for establishment of the diagnostic result. Exploring the use of next-generation sequencing based assay as a diagnostic tool was an objective in many recent reports [30–32]. In addition, next-generation sequencing has been shown to be able to establish the diagnostics in swabs from HFMD patients that were enterovirus specific PCR negative [33]. Similarly, our assay could sequence and provide correct serotype information of the targeted enteroviruses in all tested samples with Cp values between 20.9 and 33.2, although we did not test our assay on samples with lower viral load (i.e. Cp value of >33.2). Assuming that a Cp value of 33.2 is the assay limit of detection, and a Cp value of <30 is required for the purpose of whole-genome sequencing; among a sample collection from over 1300 HFMD patients enrolled in our ongoing HFMD study in Ho Chi Minh City, Vietnam (data not shown), we can conservatively extrapolate that our assay can detect enterovirus in 97 % and generate complete or nearly complete genome sequence of enteroviruses in 62 % of the RT-PCR positive clinical samples, respectively.

The advantages of random amplification and NGS based assay include: i) there is no requirement for several pathogen specific assays to diagnose diseases caused by multiple pathogens as HFMD, and ii) in addition to providing diagnostic information, the obtained sequencing result is informative for study of viral evolution and identification of the source of an outbreak. Indeed, by analyzing the obtained sequences we were able to reveal interesting insights into the evolution and origin of the CV-A6, CV-A10, CV-A16 and EV-A71 in Vietnam, albeit the sample size was small. As a consequence, further effort to obtain full genome sequences of HFMD causing pathogens is currently ongoing as part of our HFMD research program, which ultimately would lay the foundation for future research focusing on genetic diversity and evolutionary dynamics of HFMD in Vietnam and beyond, and can now be facilitated by our viral specific rPCR and next-generation sequencing based assay.

Our study has some limitations: i) we only evaluated our assay performance on rectal/throat swabs, whereas in HFMD, viral detection in vesicle swab, blood, CSF and urine has been reported, albeit at a lower frequency in the latter 3 sample types. Evaluation of the assay on these sample types is therefore needed, ii) similar to other reports [15, 16], we observed that the level of sequencing depth varied across the genomes of the tested viruses generated in the present study. While in silico investigation did not reveal any biases in terms of binding preference of the non-ribosomal hexanucleotides to specific genomic regions of the targeted viruses, it is attempting to speculate that such biases were attributed to the transposome-based library workflow as previously reported [34], iii) the current high cost (~$USD 75 per sample as compared to $USD 5–8 per one monoplex PCR reaction), low throughput (total operation time is about 5 days to complete) and bioinformatics requirements remain major barriers for next-generation sequencing-based assays to be widely applied in a diagnostic setting, in particular in less developed countries in Asia where HFMD is endemic, iv) the capacity of rPCR and next generation sequencing based assay to detect mixed infection and to identify novel/new viral variants [27, 35, 36] was not explored as it is beyond the scope of this study. For the latter, de novo assembly approach followed by metagenomic analysis using appropriate bioinformatics tool is recommended. Likewise, evaluating the viability of the 96 non-ribosomal hexanucleotides on new viral species discovered from 2005 onward is needed.

Conclusion

We have successfully developed a non-ribosomal rPCR and next-generation sequencing based assay for sensitive detection and whole-genome sequencing of HFMD pathogens in clinical samples. Our assay can be used to study the genetic diversity and evolutionary biology of HFMD pathogens, which may aid the development of intervention strategies (including vaccines), and guide

public health plans in response to future HFMD outbreaks. As next-generation sequencing associated cost has been going down quickly, and once the bioinformatics challenge becomes less burden, one would expect the expanding use of next-generation sequencing based methodologies in clinical research and routine care, both in developed and less developed countries.

Additional file

Additional file 1: Table S1. List of 96 FR26RV-Endoh and FR20RV primer sequences. **Table S2.** Result summary of consensus sequence variations recorded between 2 replicates of 3 tested swabs. Note: NA: not applicable. **Figure S1.** Screen snapshots showing the mapping results of EV-A71 MiSeq reads to an EV-A71 reference genome of sample ID15; non-ribosomal rPCR assay (bottom panel), non-ribosomal hexanucleotide primers assay (middle panel) and hexanucleotide assay (top panel); the genome sequencing depth is indicated by the Y axis and covered by red circles. **Figure S2.** Maximum likelihood phylogenetic tree based on completed VP1 nucleotide sequences (891 nt) of EV-A71 strains obtained from this study (in bold red) and representatives retrieved from GenBank. Scale bars indicated numbers of nucleotide substitution per site. CHN, China; USA, United states; TW, Taiwan; NL, Netherlands; MY, Malaysia; KOR, Korean; VN, Vietnam. **Figure S3.** Maximum likelihood phylogenetic tree based on completed VP1 nucleotide sequences (891 nt) of CV-A16 strains obtained from this study (in bold red) and representatives retrieved from GenBank. Scale bars indicated numbers of nucleotide substitution per site. CHN, China; US, United states; TL, Thailand; JPN, Japan; AUS, Australia; MY, Malaysia; KOR, Korean; VN, Vietnam. (PDF 783 kb)

Acknowledgements
We thank Mrs. Le Kim Thanh from Oxford University Clinical Research Unit in Ho Chi Minh City, Vietnam for her logistic assistance. We are indebted to patients and their parents for their participation in this study, and all the nursing and medical staff at the Children's Hospital 1 and 2, and the Hospital for Tropical Diseases who provided care for the patients and helped collect clinical data.

Funding
The research leading to these results has received funding from the Wellcome Trust (101104/Z/13/Z and 089276/Z/09/Z).

Authors' contributions
NTA and LVT: designed the study, did laboratory testing, analysed the test results, performed statistical analysis and drafted the manuscript. TTT, HMTV, NMN, LNTN, LTMT, PTQ, THK, LNTN, HLV, DCV, HMT, NTH, NWC, GT: enrolled patients, took samples and did laboratory testing. RHvD: designed the study and involved in drafting the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Ethics approval and consent to participate
The clinical samples used in this study were derived from an on-going HFMD study in three referral hospitals in Ho Chi Minh city, Vietnam. The study was reviewed and approved by the local Institutional Review Boards and the Oxford Tropical Research Ethics Committee (OxTREC), University of Oxford, Oxford, United Kingdom. The institutional review board of HTD in HCMC, Vietnam approved the whole-genome sequencing of residual swabs of anonymous HFMD patients. Written informed consent was obtained from a parent or guardian of each enrolled patients.

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Received: 24 November 2015 Accepted: 29 June 2016
Published online: 07 July 2016

References
1. Brown BA, Oberste MS, Alexander JP, Kennett ML, Pallansch MA. Molecular epidemiology and evolution of enterovirus 71 strains isolated from 1970 to 1998. J Virol. 1999;73:9969–75.
2. Li L, He Y, Yang H, Zhu J, Xu X, Dong J, Zhu Y. Genetic Characteristics of Human Enterovirus 71 and Coxsackievirus A16 Circulating from 1999 to 2004 in Shenzhen, People' s Republic of China Genetic Characteristics of Human Enterovirus 71 and Coxsackievirus A16 Circulating from 1999 to 2004 in Shenzhe. Society. 2005;43:3835–9.
3. Bin LQ, Zhang XA, Wo Y, Xu HM, Li XJ, Wang XJ, Ding SJ, Chen XD, He C, Liu LJ, Li H, Yang H, Li TY, Liu W, Cao WC. Circulation of Coxsackievirus A10 and A6 in Hand-Foot-Mouth Disease in China, 2009-2011. PLoS One. 2012;7:e52073.
4. Gopalkrishna V, Patil PR, Patil GP, Chitambar SD. Circulation of multiple enterovirus serotypes causing hand, foot and mouth disease in India. J Med Microbiol. 2012;61:420–5.
5. Puenpa J, Chieochansin T, Linsuwanon P, Korkong S, Thongkomplew S, Vichaiwattana P, Theamboonlers A, Poovorawan Y. Hand, foot, and mouth disease caused by Coxsackievirus A6, Thailand, 2012. Emerg Infect Dis. 2013; 19:641–3.
6. Hu Y-Q, Xie G-C, Li D-D, Pang L-L, Xie J, Wang P, Chen Y, Yang J, Cheng W-X, Zhang Q, Jin Y, Duan Z-J. Prevalence of Coxsackievirus A6 and Enterovirus 71 in Hand, Foot, and Mouth Disease in Nanjing, China in 2013. Pediatr Infect Dis J. 2015;34:951–7.
7. Bian L, Wang Y, Yao X, Mao Q, Xu M, Liang Z. Coxsackievirus A6: a new emerging pathogen causing hand, foot and mouth disease outbreaks worldwide. Expert Rev Anti Infect Ther. 2015;13:1061–71.
8. Lu J, Zeng H, Zheng H, Yi L, Guo X, Liu L. Hand, foot and mouth disease in Guangdong, China, in 2013 : new trends in the continuing epidemic. Clin Microbiol Infect. 2014;20:7.
9. Van Tan L, Tuyen NTK, Thanh TT, Ngan TT, Van HMT, Sabanathan S, Van TTM, Thanh LTM, Nguyet LA, Geoghegan JL, Ong KC, Perera D, Hang VTT, Ny NTH, Anh NT, Ha DQ, Qui PT, Viet DC, Tuan HM, Wong KT, Holmes EC, Chau NW, Thwaites G, van Doorn HR. A generic assay for whole-genome amplification and deep sequencing of enterovirus A71. J Virol Methods. 2015;215–216:30–6.
10. Froussard P. A random-PCR method (rPCR) to construct whole cDNA library from low amounts of RNA. Nucleic Acids Res. 1992;20:2900.
11. Endoh D, Mizutani T, Kirisawa R, Maki Y, Saito H, Kon Y, Morikawa S, Hayashi M. Species-independent detection of RNA virus by representational difference analysis using non-ribosomal hexanucleotides for reverse transcription. Nucleic Acids Res. 2005;33:1–11.
12. Thanh T, Anh N, Tham N, Van H, Sabanathan S, Qui P, Ngan T, Van T, Nguyet L, Ny N, Thanh L, Chai O, Perera D, Viet D, Khanh T, Ha D, Tuan H, Wong K, Hung N, Chau N, Thwaites G, van Doorn H, Van Tan L. Validation and utilization of an internally controlled multiplex Real-time RT-PCR assay for simultaneous detection of enteroviruses and enterovirus A71 associated with hand foot and mouth disease. Virol J. 2015;12:85.
13. Allan Nix W, Oberste MS, Pallansch MA. Sensitive, seminested PCR amplification of VP1 sequences for direct identification of all enterovirus serotypes from original clinical specimens. J Clin Microbiol. 2006;44:2698–704.
14. Van Tan L, van Doorn HR, van der Hoek L, Hien VM, Jebbink MF, Ha DQ, Farrar J, van Vinh Chau N, de Jong MD. Random PCR and ultracentrifugation increases sensitivity and throughput of VIDISCA for screening of pathogens in clinical specimens. J Infect Dev Ctries. 2011;5:142–8.
15. Rosseel T, Van Borm S, Vandenbussche F, Hoffmann B, van den Berg T, Beer M, Höper D. The Origin of Biased Sequence Depth in Sequence-Independent Nucleic Acid Amplification and Optimization for Efficient Massive Parallel Sequencing. PLoS One. 2013;8:1–9.
16. Karlsson OE, Belák S, Granberg F. The effect of preprocessing by sequence-independent, single-primer amplification (SISPA) on metagenomic detection of viruses. Biosecur Bioterror. 2013;11:S227–34.
17. Geoghegan JL, Van Tan L, Kühnert D, Halpin RA, Lin X, Simenauer A, Akopov A, Das SR, Stockwell TB, Shrivastava S, Ngoc NM, Uyen LTT, Tuyen NTK, Thanh TT, Hang VTT, Qui PT, Hung NT, Khanh TH, Thinh LQ, Nhan LNT, Van HMT, Viet DC, Tuan HM, Viet HL, Hien TT, Chau NVV, Thwaites G,

Grenfell BT, Stadler T, Wentworth DE, Holmes EC, Van Doorn HR. Phylodynamics of Enterovirus A71-Associated Hand, Foot and Mouth Disease in Viet Nam. J Virol. 2015;89:8871–9.
18. Perera D, Yusof MA, Podin Y, Ooi MH, Thao NTT, Wong KK, Zaki A, Chua KB, Malik YA, Tu PV, Tien NTK, Puthavathana P, McMinn PC, Cardosa MJ. Molecular phylogeny of modern coxsackievirus A16. Arch Virol. 2007;152:1201–8.
19. Hu YF, Yang F, Du J, Dong J, Zhang T, Wu ZQ, Xue Y, Jin Q. Complete genome analysis of coxsackievirus A2, A4, A5, and A10 strains isolated from hand, foot, and mouth disease patients in China revealing frequent recombination of human enterovirus A. J Clin Microbiol. 2011;49:2426–34.
20. Liu W, Wu S, Xiong Y, Li T, Wen Z, Yan M, Qin K, Liu Y, Wu J. Co-circulation and genomic recombination of coxsackievirus A16 and enterovirus 71 during a large outbreak of hand, foot, and mouth disease in Central China. PLoS One. 2014;9:e96051.
21. Feng X, Guan W, Guo Y, Yu H, Zhang X, Cheng R, Wang Z, Zhang Z, Zhang J, Li H, Zhuang Y, Zhang H, Lu Z, Li M, Yu H, Bao Y, Hu Y YZ. Genome Sequence of a Novel Recombinant Coxsackievirus A6 Strain. Genome Announc. 2015;3:e01347–14.
22. Djikeng A, Halpin R, Kuzmickas R, Depasse J, Feldblyum J, Sengamalay N, Afonso C, Zhang X, Anderson NG, Ghedin E, Spiro DJ. Viral genome sequencing by random priming methods. BMC Genomics. 2008;9:5.
23. Rosseel T, Lambrecht B, Vandenbussche F, van den Berg T, Van Borm S. Identification and complete genome sequencing of paramyxoviruses in mallard ducks (Anas platyrhynchos) using random access amplification and next generation sequencing technologies. Virol J. 2011;8:463.
24. De Vries M, Deijs M, Canuti M, van Schaik BDC, Faria NR, van de Garde MDB, Jachimowski LCM, Jebbink MF, Jakobs M, Luyf ACM, Coenjaerts FEJ, Claas ECJ, Molenkamp R, Koekkoek SM, Lammens C, Leus F, Goossens H, Ieven M, Baas F, van der Hoek L. A sensitive assay for virus discovery in respiratory clinical samples. PLoS One. 2011;6:e16118.
25. Van Tan L, Van Doorn HR, Trung D, Hong T, Phuong T, De Vries M. Identification of a New Cyclovirus in Cerebrospinal Fluid of Patients with Acute Central Nervus System Infections. MBio. 2013;4:1–10.
26. De Groof A, Guelen L, Deijs M, van der Wal Y, Miyata M, Ng KS, van Grinsven L, Simmelink B, Biermann Y, Grisez L, van Lent J, de Ronde A, Chang SF, Schrier C, van der Hoek L. A Novel Virus Causes Scale Drop Disease in Lates calcarifer. PLoS Pathog. 2015;11:e1005074.
27. Li L, Deng X, Mee ET, Collot-Teixeira S, Anderson R, Schepelmann S, Minor PD, Delwart E. Comparing viral metagenomics methods using a highly multiplexed human viral pathogens reagent. J Virol Methods. 2014;213C:139–46.
28. Rosseel T, Ozhelvaci O, Freimanis G, Van Borm S. Evaluation of convenient pretreatment protocols for RNA virus metagenomics in serum and tissue samples. J Virol Methods. 2015;222:72–80.
29. Conceição-Neto N, Zeller M, Lefrère H, De Bruyn P, Beller L, Deboutte W, Yinda CK, Lavigne R, Maes P, Van Ranst M, Heylen E, Matthijnssens J. Modular approach to customise sample preparation procedures for viral metagenomics: a reproducible protocol for virome analysis. Sci Rep. 2015;5:16532.
30. Raman R, Thomas RG, Weiner MW, Jack CR, Ernstrom K, Aisen PS, Tariot PN, Quinn JF. Actionable Diagnosis of Neuroleptospirosis by Next-Generation Sequencing. N Engl J Med. 2014;23:333–6.
31. Prachayangprecha S, Schapendonk CME, Koopmans MP, Osterhaus ADME, Schurch AC, Pas SD, van der Eijk AA, Poovorawan Y, Haagmans BL, Smits SL Exploring the Potential of Next-Generation Sequencing in Detection of Respiratory Viruses. J Clin Microbiol. 2014;52:3722–30.
32. Thorburn F, Bennett S, Modha S, Murdoch D, Gunson R, Murcia PR. The use of next generation sequencing in the diagnosis and typing of respiratory infections. J Clin Virol. 2015;69:96–100.
33. Linsuwanon P, Poovorawan Y, Li L, Deng X, Vongpunsawad S, Delwart E. The Fecal Virome of Children with Hand, Foot, and Mouth Disease that Tested PCR Negative for Pathogenic Enteroviruses. PLoS One. 2015;10:e0135573.
34. Aird D, Ross MG, Chen W-S, Danielsson M, Fennell T, Russ C, Jaffe DB, Nusbaum C, Gnirke A. Analyzing and minimizing PCR amplification bias in Illumina sequencing libraries. Genome Biol. 2011;12:R18.
35. Blomström AL, Widén F, Hammer AS, Belák S, Berg M. Detection of a novel astrovirus in brain tissue of mink suffering from shaking mink syndrome by use of viral metagenomics. J Clin Microbiol. 2010;48:4392–6.
36. Allander T, Emerson SU, Engle RE, Purcell RH, Bukh J. A virus discovery method incorporating DNase treatment and its application to the identification of two bovine parvovirus species. Proc Natl Acad Sci U S A. 2001;98:11609–14.

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Thanh et al. Virology Journal (2015) 12:85
DOI 10.1186/s12985-015-0316-2

VIROLOGY JOURNAL

METHODOLOGY Open Access

Validation and utilization of an internally controlled multiplex Real-time RT-PCR assay for simultaneous detection of enteroviruses and enterovirus A71 associated with hand foot and mouth disease

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Abstract

Background: Hand foot and mouth disease (HFMD) is a disease of public health importance across the Asia-Pacific region. The disease is caused by enteroviruses (EVs), in particular enterovirus A71 (EV-A71). In EV-A71-associated HFMD, the infection is sometimes associated with severe manifestations including neurological involvement and fatal outcome. The availability of a robust diagnostic assay to distinguish EV-A71 from other EVs is important for patient management and outbreak response.

Methods: We developed and validated an internally controlled one-step single-tube real-time RT-PCR in terms of sensitivity, linearity, precision, and specificity for simultaneous detection of EVs and EV-A71. Subsequently, the assay was then applied on throat and rectal swabs sampled from 434 HFMD patients.

Results: The assay was evaluated using both plasmid DNA and viral RNA and has shown to be reproducible with a maximum assay variation of 4.41 % and sensitive with a limit of detection less than 10 copies of target template per reaction, while cross-reactivity with other EV serotypes was not observed. When compared against a published VP1 nested RT-PCR using 112 diagnostic throat and rectal swabs from 112 children with a clinical diagnosis of HFMD during 2014, the multiplex assay had a higher sensitivity and 100 % concordance with sequencing results which showed EVs in 77/112 (68.8 %) and EV-A71 in 7/112 (6.3 %). When applied to clinical diagnostics for 322 children, the assay detected EVs in throat swabs of 257/322 (79.8 %) of which EV-A71 was detected in 36/322 (11.2 %) children. The detection rate increased to 93.5 % (301/322) and 13.4 % (43/322) for EVs and EV-A71, respectively, when rectal swabs from 65 throat-negative children were further analyzed.

Conclusion: We have successfully developed and validated a sensitive internally controlled multiplex assay for rapid detection of EVs and EV-A71, which is useful for clinical management and outbreak control of HFMD.

Keywords: Hand foot and mouth disease, Enteroviruses, Enterovirus A71, Real-time RT-PCR, Diagnosis

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Introduction

Hand foot and mouth disease (HFMD) is a commonly benign and self-limiting viral infection of infants and young children worldwide. The disease is caused by acute infection with *Enterovirus A* of the family *Picornaviridae* including coxsackievirus A6 (CV-A6), CV-A10, CV-A16, and enterovirus A71 (EV-A71) [1–5]. However, in contrast to the common mild patterns of previous sporadic epidemics, since 1997 fulminant outbreaks of HFMD involving millions of children with neurological involvement and sometimes fatal cardiopulmonary complications have occurred across the Asia-Pacific region with EV-A71 being the most frequent pathogen isolated from patients with clinical complications and fatal cases [6–9]. Despite the public health burden of (EV-A71-associated) HFMD, currently, no clinically proven effective antiviral agents or vaccines are available for routine use.

The availability of a rapid, high-throughput and accurate diagnostic assay that can simultaneously detect and distinguish between EVs and EV-A71 is an ideal aid to patient management and may help outbreak response with regards to predicting the possible level of severity of the outbreak and thereby initiating appropriate public health interventions. Although EVs/EV-A71 diagnosis can be achieved by virus isolation, cell culture based methods are time-consuming and insensitive [10]. Molecular assays based on specific amplification viral nucleic acids, in particular real-time PCR, are sensitive, specific and high-throughput and are therefore methods of choice [11, 12]. Herein, we describe the development and validation of an internally controlled single-step multiplex real-time RT-PCR for simultaneous detection of EVs and EV-A71 in clinical specimens from patients with HFMD.

Results

Analytical specificity and 95 % LLOD

The analytical specificity of the assay was evaluated on extracted RNA derived from EV strains belonging to 9 different EV serotypes and isolates belonging to 8 different (sub)genogroups of EV-A71 (Table 1). The fluorescent signal of the EV probe was detected from all reactions containing viral RNA extracted from diverse EV serotypes belonging to different species including A (including EV-A71), B and C, whereas the signal of the EV-A71 probe was detected only from reactions containing viral RNA extracted from EV-A71 of all 8 tested subgenogroups, but not from other EV A-C types.

The analytical sensitivity (95 % LLOD) of the assay was evaluated using both plasmid DNA and EV-A71 RNA. The detection limit of the assay for EVs and EV-A71 was 7.1 and 5.3 copies of plasmid DNA per reaction, and was 2.9 and 1.1 $TCID_{50}$ (equivalent to 9.1 and 3.2 cDNA copies per reaction, respectively) of EV-A71, respectively.

Linearity and precision

The linearity of the multiplex assay was calculated from amplification of serial ten-fold dilutions of plasmid DNA

Table 1 Virus isolate and origin/source

Strain	Serotypes	Species	Subgeno-group	Accession number	Location/source	Year of isolation
9522	EV-A71	A	C1	AY258300	Malaysia	2003
8 M/6/99	EV-A71	A	C2	AY126012	Australia	1999
001-KOR-00	EV-A71	A	C3	AY125966	Korea	2000
VN152	EV-A71	A	C4	NA	Vietnam	2011
VN5559	EV-A71	A	C4	AM490152	Vietnam	2005
VN5784	EV-A71	A	C5	AM490158	Vietnam	2005
13903	EV-A71	A	B3	AY207648	Malaysia	1997
A10/4	EV-A71	A	B4	AF376067	Malaysia	2000
18431	EV-A71	A	B5	NA	Malaysia	2006
H08214350	CV-A4	A	NA	NA	[a]	NA
H07314334	CV-A16	A	NA	NA	[a]	NA
H06418058	CV-A21	C	NA	NA	[a]	NA
H06474277	E-6	B	NA	NA	[a]	NA
H08306574	E-30	B	NA	NA	[a]	NA
H05062273	CV-B5	B	NA	NA	[a]	NA
Gdula	CV-A6	A	NA	AY42164	[b]	NA
Texas 12	CV-A12	A	NA	AY42168	[b]	NA
G-14	CV-A14	A	NA	AY421769	[b]	NA

Note: [a]Public Health England, UK, [b]American Type Culture Collection, *NA*: Not available

and EV-A71 RNA. The linear regression of plasmid DNA and viral RNA was 0.993 and 0.995, and 0.996 and 0.995 for the EV and EV-A71, respectively (Figs. 1 and 2).

The precision of the assay was assessed by measuring variations within (intra-assay variation) and between runs (inter-assay variation) at high, medium, and low concentrations of template. The maximum intra- and inter-assay variations using plasmid DNA and viral RNA were 3.12 % and 4.41 %, respectively (Table 2).

Comparison with nested RT-PCR and sequencing confirmation

To assess the clinical specificity of the assay, a subset of 112 swabs (45 throat and 67 rectal swabs from 112 children with clinically suspected HFMD) was randomly selected for comparison with a published nested RT-PCR assay [13]. Among 102 specimens positive for EVs using multiplex real-time RT-PCR, 21 were not detected by the nested RT-PCR assay, while none of the 10 multiplex RT-PCR negative specimens were positive by the nested RT-PCR assay (Table 3). Further confirmation for the accuracy of the multiplex RT-PCR results was achieved by sequencing of amplified products generated by the VP1 assay. Of the 81 nested RT-PCR positive specimens, VP1 sequences were successfully obtained from 75 (71 non-EV-A71 and 4 EV-A71). Sequence analyses revealed that all 71 viruses detected by the EVs generic primers/probe but not by the EV-A71 assay belonged to 11 different EV serotypes of which CV-A6 accounted for 60 %, whereas all 4 EV-A71 positive specimens detected by the multiplex assay were confirmed as EV-A71 (Fig. 3).

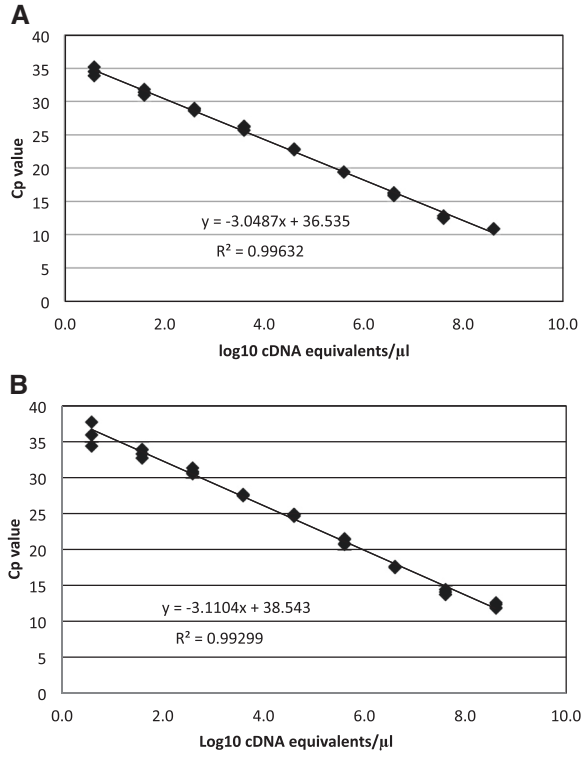


Fig. 1 Linearity of the multiplex RT-PCR assay using 10-fold dilution series of plasmid DNA containing the PCR templates of EV (**a**) and EV-A71 (**b**), Cp = crossing point

Assay application in clinical studies

The assay was applied in clinical samples (rectal and throat swabs) obtained from 322 children enrolled in our on-going HFMD research program. Multiplex RT-PCR analysis of throat swab specimens revealed an overall detection rate of 79.8 % (257/322) for EVs, of which EV-A71 was detected in 11.2 % (36/322). Further diagnostic effort on rectal-swab specimens from 65/322 (20.2 %) children whose throat swabs were negative by the multiplex assay showed that 44/65 (56.9 %) rectal swabs were EVs positive, of which 7(10.8 %) were EV-A71. No virus was detected in 21 paired throat and rectal swabs, corresponding to 6.5 %. Thus, the overall detection rates of EVs and EV-A71 in 322 clinically suspected HFMD children were 93.5 % (301/322) and 13.4 % (43/322), respectively.

Discussion

We have successfully developed and validated a single-tube multiplex one-step real-time RT-PCR for high-throughput, sensitive, and specific detection of EVs and EV-A71 in clinical samples. The performance of the assay is internally controlled by adding an optimal amount of EAV to the tested specimens allowing for quality assessment of the whole procedure from nucleic acid extraction to amplification. Furthermore, this assay is rapid (turnaround time of less than 3 h), cost-effective (reagent associated cost of $ 8 per sample) and resource efficient (both EVs and EV-A71 are tested simultaneously) and is therefore useful for clinical management and outbreak response of HFMD.

Although primers and probes used in this study were derived from previous studies [8, 14, 15], different reporters and quenchers were selected and combined to ensure efficiency of assay performance (i.e. no cross-fluorescence). In addition, because when combined with the new reporter (Cy5) and quencher (BHQ3), the original EVs probe [14] produced a high baseline signal. The sequence of the EVs probe was therefore changed into its reverse complement to reduce high baseline signal (data not shown), while maintaining sensitivity and specificity as shown by the outcome of the specificity experiments.

We originally developed the EV-A71 assay that was successfully used for diagnosis of HFMD caused by EV-A71 in previous studies [8] and during the outbreak of severe HFMD in Cambodia in 2012 [16]. However, this

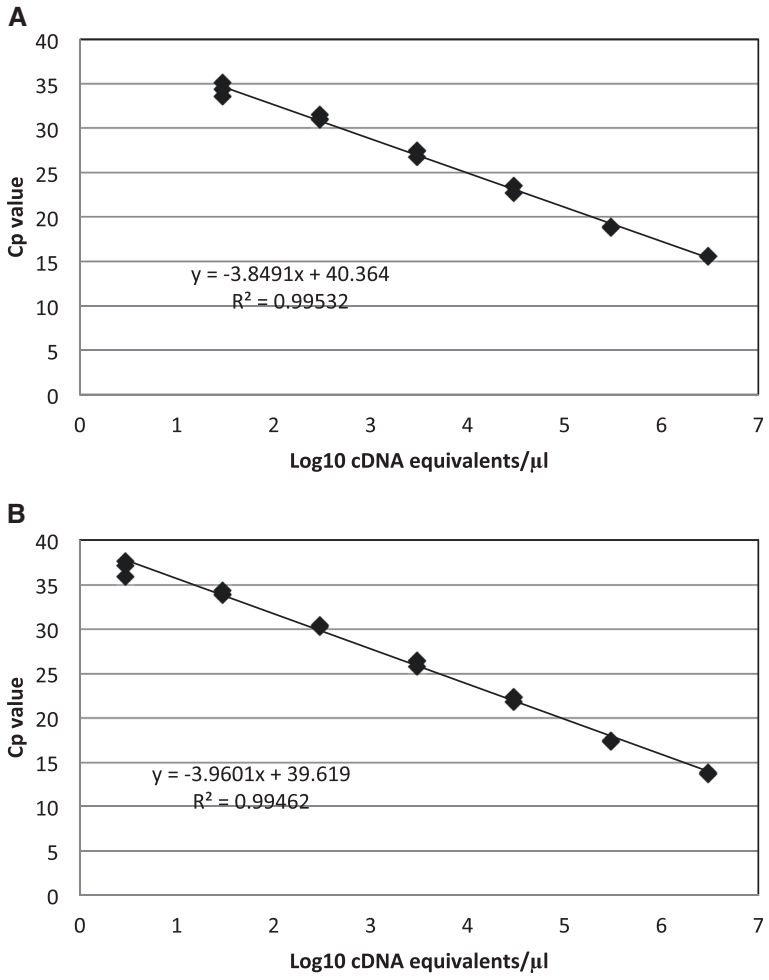


Fig. 2 Linearity of the multiplex RT-PCR assay using 10-fold dilution series of RNA extracted from an EV-A71 isolate; EV (**a**) and EV-A71 (**b**), Cp: crossing point. Concentration used were equivalent to 3×10^1 to 3×10^6 cDNA copies per reaction for EV and 3×10^0 to 3×10^6 cDNA copies per reaction for EV-A71

assay was specifically designed for the detection of subgenogroups C4 and C5 that were circulating at that time [8, 16]. Our recent assessment of EV-A71 sequences available in the GenBank suggested that modification by incorporation degenerated bases in the primer sequences was required to ensure a detection of all EV-A71 subgenogroups (Additional file 1: Figure S1). Indeed, the EV-A71 assay with modified primers was able to detect EV-A71 isolates of subgenotypes B3–B5 and C1–C5 that have been circulating in the Asia-Pacific region, while maintaining specificity. Although the assay has not been evaluated for subgenotypes B1 and B2, *in silico* analysis showed that the primers and probes used in this assay would enable their detection (Additional file 1: Figure S1).

The multiplex RT-PCR assay has been fully evaluated in terms of linearity, analytical sensitivity, precision and specificity. When evaluated on a wide range of template

Table 2 Maximum intra and inter assay variations in different concentrations of plasmid DNA and viral RNA

Concentration		Plasmid DNA (copies)			Viral RNA ($TCID_{50}$)		
		4×10^6	4×10^3	4×10^1	9.6×10^5	9.6×10^3	9.6×10^1
Maximum EV assay co-variation	Intra-assay	0.53	0.93	1.37	1.49	0.84	0.77
	Inter-assay	3.13	1.36	2.18	4.41	3.01	2.54
Maximum EV-A71 assay co-variation	Intra-assay	0.52	1.00	2.18	1.34	0.73	0.73
	Inter-assay	3.19	1.37	1.85	2.66	1.56	1.53

Table 3 Comparison with VP1 nested RT-PCR and serotype confirmation by sequencing

Tests		Multiplex real-time RT-PCR			Total (n)
		EVs positive		EVs negative	
		Non-EV-A71	EV-A71		
Nested RT-PCR	Positive (n)	77	4	0	81
	Negative (n)	18	3	10	31
	Total (n)	95	7	10	112
VP1 sequencing (n)		71	4	0	75

concentrations, the assay showed a good analytical linearity, thus fulfilling the requirements of a clinical diagnostic molecular assay [17]. The 95 % LLOD was less than 10 copies RNA/DNA per reaction, and thereby compliant with MIQE (Minimum Information for publication of Quantitative real-time PCR Experiments) guidelines [18].

All tested EV serotypes, representing a broad range of types belonging to EV A-C were detected by the EVs generic PCR, which is in accordance with the published report [14]. The EV-A71 primer-probe was specific for EV-A71 viruses since cross-reactivity with other EV serotypes was not observed when the assay was tested on cultured isolates of different non-EV-A71 serotypes.

The assay performance was further compared against a VP1 nested RT-PCR by testing both assay performance on 112 rectal and throat swabs from 112 children with HFMD. The VP1 nested RT-PCR was selected as an assay for comparison because: 1) it can function as a 'reference' assay for assessment of our assay performance in terms of both sensitivity and specificity as it also allows for serotype determination of HFMD pathogens by sequencing of the obtained PCR amplicon [13], 2) it is a WHO recommended assay for diagnostics of HFMD patients [19], and 3) it has been widely implemented in diagnostic laboratories [20, 21]. In these clinical specimens, the assay described here was more sensitive than the VP1 nested RT-PCR. Indeed, the multiplex RT-PCR could detect the presence of EVs RNA in 102 specimens as compared to 81 by the nested one. In addition, the specificity of the diagnostic result was confirmed by VP1 sequencing of 71 specimens that were non-EV-A71 PCR positive and 4 EV-A71 positive specimens, which further confirms our initial sequence analyses including BLAST and sequence alignment comparison (data not shown) that showed not potential cross reactivity of EV-A71 assay with other EV serotypes.

Following our proposed diagnostic workflow, the assay was applied on samples from 322 children enrolled to our on-going HFMD studies between May and September 2014. Using throat swab alone for initial diagnosis, 80 % (257/322) of the children had EVs/EV-A71 detected in throat swabs. The detection rate increased with 13.5 % (i.e. an overall diagnostic rate of 93.5 % (301/322) was achieved) when rectal swabs from 65 patients who had a negative throat swab test were further analyzed, indicating that the use of throat swab alone would result in false negative results for a substantial proportion of cases.

It is worth to note that throat and rectal swabs have been used for diagnostics of HFMD patients, although throat swab is more sensitive by virus isolation [19, 22]. However, parallel testing of both throat and rectal swabs would not be cost-effective. Therefore, following our

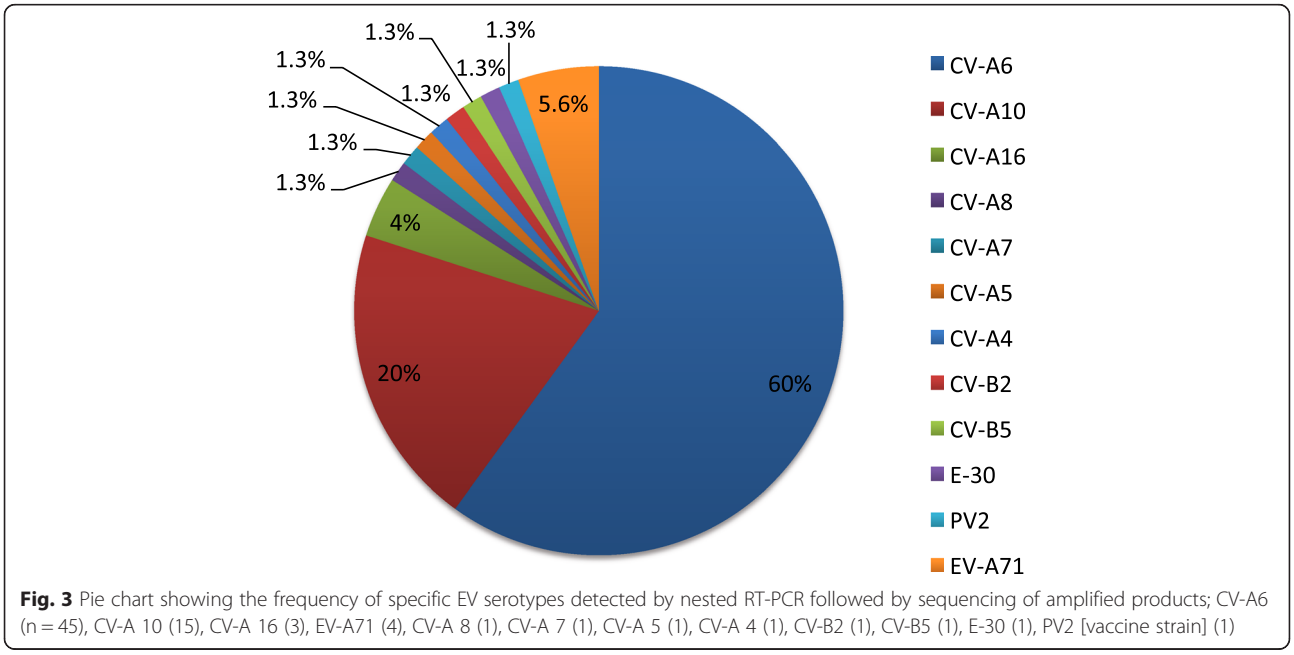


Fig. 3 Pie chart showing the frequency of specific EV serotypes detected by nested RT-PCR followed by sequencing of amplified products; CV-A6 (n = 45), CV-A 10 (15), CV-A 16 (3), EV-A71 (4), CV-A 8 (1), CV-A 7 (1), CV-A 5 (1), CV-A 4 (1), CV-B2 (1), CV-B5 (1), E-30 (1), PV2 [vaccine strain] (1)

proposed flowchart, the rate of EVs/EV-A71 detection was maximized, while maintaining cost-effectiveness (i.e. only 20 % of the rectal swabs needed to be tested), and retaining the usefulness of throat swabs for subsequent analyses (such as virus isolation [22]).

Although detection and differentiation of EV-A71 can be achieved with the existing real-time RT-PCR assays [11, 12, 14, 23–29], some of these assays are not multiplexed, targeting either EVs [14, 29] or EV-A71 [12, 24, 26, 27] separately. Multiplex RT-PCRs for EV-A71 and other EVs have been recently developed [11, 23, 25, 28]. However, these assays did not include internal controls [11, 25, 28] or were not fully validated in terms sensitivity, linearity, precision, and specificity. Although a comparison of our assay with other published methods is desirable, the absence of a true gold standard limits the usefulness of this. It should however be noted that the 95 % LLOD of our assay was less than 10 copies per reaction, in agreement with MIQE guidelines (as mentioned above) and within the detection limit ranges of between 5 and 90 copies per reaction of those previously published assays. Our paper represents another option for internally controlled rapid and simultaneous detection of EVs and EV-A71.

Conclusions

We have successfully developed and validated a single-tube multiplex one-step real-time RT-PCR for high-throughput, sensitive, and specific detection of EVs and EV-A71 in clinical samples, which can be a great aid to clinical management and outbreak response of HFMD in the future.

Materials and methods

Clinical samples

Diagnostic throat and rectal swab specimens used in this study were collected prospectively from 434 children with a clinical diagnosis of HFMD presenting or admitted to three referral hospitals for southern Vietnam in Ho Chi Minh City: Hospital for tropical Diseases (HTD) and Children's Hospital 1 (CH1) and 2 (CH2), between May and September 2014. Swabs were collected in viral transport medium, divided into three aliquots and stored at -80 °C until analysis.

Virus isolates

An EV-A71 subgenogroup C4 isolate from a child with HFMD admitted to HTD during the 2011 outbreak and 17 other different isolates belonging to different EV-A71 subgenogroups and non-EV-A71 serotypes obtained from the Department of Biomedical Science, University of Malaya, Malaysia, Public Health England, UK and American Type Culture Collection, USA were used for assay validation (Table 1).

Infective titers of 50 % tissue culture infectious doses ($TCID_{50}$) were titrated on human rhabdomyosarcoma cells using a standard method [22]. The concentration of viral stock was converted to cDNA copies per μl by the multiplex real-time RT-PCR assay with plasmid DNA serving as standard control.

Equine Arteritis Virus (EAV) was used as an internal control and was prepared as described previously [15, 30]. The concentration of internal control virus was selected using RT-PCR analysis of serial tenfold dilutions and the amount of virus used in the PCR assay had expected crossing point (Cp) values of between 30 and 33 [15, 30].

Primers and probes

Primers and probes for EVs, EV-A71 and EAV assays used in this study were adapted from previous studies [8, 14, 15, 31] with slight modifications and are listed in Table 4. Briefly, for the EVs assay, the original probe was replaced by its reverse-complement. Modification of EV-A71 primers and probe was done by incorporation of degenerate bases to allow detection of all known subgenotypes of EV-A71.

Table 4 Primer and probe sequences and concentration used in single reaction

Name	Sequence (5′→3′)	Final concentration	Note
EAV-F primer	CATCTCTTGCTTTGCTCCTTAG	400 nM	Internal control [15]
EAV-R primer	AGCCGCACCTTCACATTG	400 nM	
EAV-probe	FAM-CGCTGTCAGAACAACATTATTGCCCAC-BHQ1	100 nM	
ENT-F	CCCTGAATGCGGCTAAT	400 nM	Enterovirus specific primers and probe [14]
ENT-R	ATTGTCACCATAAGCAGCC	400 nM	
ENTr-probe	Cy5-ACCCAAAGTAGTCGGTTCCG -BHQ3	200 nM	
EV-A71-634 F	GGAGAACACAARCARGAGAAAGA	400 nM	Enterovirus 71 specific primers and probe [8]
EV-A71-743R	ACYAAAGGGTACTTGGAYTTVGA	400 nM	
EV-A71-probe	Cyan500-TGATGGGCACDTTCTCRGTGCG-BHQ1	40 nM	

Note: FAM = Carboxyfluorescein; *Cy5* = Cyanine 5; Cyan500 = Cyan 500 NHS ester; *BHQ* = black hole quencher; R = A and G; Y = T and C; V = A, C, and G; D = A, G, and T

Nucleic acid extraction and RT-PCR

Viral RNA was extracted from clinical specimens and culture materials using the QIAamp Viral RNA Mini kit (QIAgen GmbH, Hilden, Germany). In brief, 140 μl of culture supernatant/throat/rectal swabs in viral transport medium was first mixed with 20 μl of EAV and total nucleic acid was then extracted according to the manufacturer's instruction, eluted in 100 μl elution buffer and stored at -80 °C until used.

Real-time RT-PCR was done using the SuperScript^R III One-Step qRT-PCR System with Platinum^R Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA) and was performed in a LightCycler 480 II machine (Roche Diagnostics GmbH, Mannheim, Germany). The reaction was performed in a final volume of 25 μl containing 12.5 μl 2X RT-PCR reaction Mix, primers and probes at appropriate concentrations (Table 4), 0.5 μl Enzyme Mix, and 2 μl viral RNA or plasmid DNA. The cycling conditions included one cycle of 60 °C for 3 min, followed by 15 min at 53 °C and 2 min at 95 °C, and 45 cycles of 15 s at 95 °C, 1 min at 53 °C (including fluorescence acquisition) and 15 s at 72 °C.

Preparation of plasmid controls

Amplified PCR products generated by RT-PCR amplification of viral RNA derived from an EV-A71 subgenogroup C4 isolate mentioned above utilizing EV and EV-A71 specific primers (Table 1) were purified and cloned into a pCR 2.1-TOPO plasmid using the pCR 2.1-TOPO TA Cloning kit (Invitrogen), following the manufacturer's instruction. Constructed plasmid containing amplified product was purified using the QIAprep Spin Miniprep Kit (Qiagen). Plasmid concentration was determined by NanoDrop spectrometer (Thermo-Scientific, Loughborough, UK). An equal amount of plasmids containing the cloned EV and EV-A71 target genes was mixed together and stored at -80 °C until analysis.

Lower limit of 95 % detection, linearity and precision

Analytical evaluation of the multiplex assay was done as previously described [17, 18] using both plasmid DNA controls and extracted RNA from an EV-A71 isolate (VN152, Table 1).

To obtain the lower limit of 95 % detection (95 % LLOD), the mixed EV/EV-A71 plasmid DNA controls were serially diluted two-fold to obtain a series of concentrations ranging from 20 copies/μl to 0.6 copies/μl. Dilution series of extracted viral RNA from an EV-A71 virus with concentrations ranging from 0.3-9.6 $TCID_{50}$/μl equivalent to 0.9 – 30.1 cDNA copies/μl were used. The experiment was performed on these dilution series once a day on five consecutive days with 10 replicates for each concentration. The 95 % LLOD was estimated using the Probit regression model in SPSS 19 (SPSS Inc, NY, USA) as described previously [15].

The linearity was assessed by performing the assay on 10-fold serial dilutions of plasmid DNA controls (ranging from 10^8 to 10^1 copies/μl) or EV-A71 RNA (ranging from 9.6×10^5 to 9.6 $TCID_{50}$ /μl equivalent to 3.1×10^6 to 30.1 cDNA copies/μl). For each material, the experiment was carried out in a single run with 5 replicates for each dilution point. Linearity was calculated using linear regression analysis in Microsoft Excel and a range of input with an R^2 value of 0.99 was accepted.

The precision was determined using both plasmid DNA and viral RNA controls at high, intermediate and low concentrations. The experiments were performed daily on five consecutive days in quintuplicate for each concentration. The intra- and inter-assay variability were calculated by assessing the Cp value deviation.

Assay interpretation

The procedure was monitored by including an optimal amount of EAV (Cp value between 30 and 33) functioning as internal control for nucleic acid extraction and amplification steps. A sample was considered positive if negative controls were negative and the tested sample was positive with a Cp value equal to or less than 40. A sample was considered negative if negative controls were negative, positive controls were positive with Cp values of about 30 and internal control showed a positive result with a Cp value between 30 and 33. A result was considered as inconclusive if negative controls were positive and/or the internal control showed a result outside of

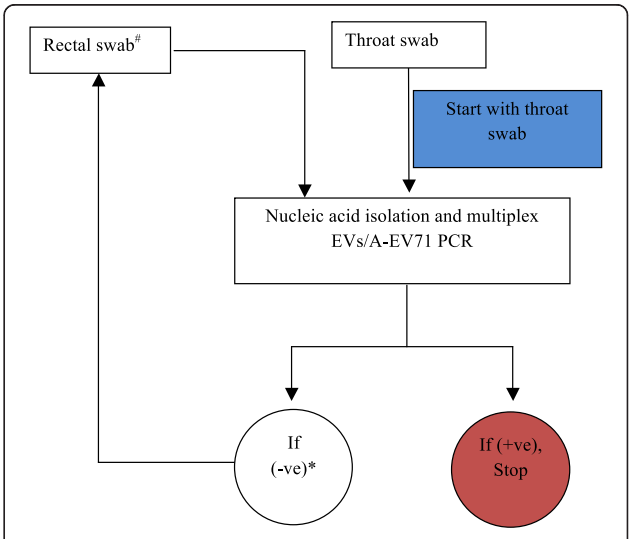


Fig. 4 A flow chart showing the work-flow diagnosis. Note: +ve = positive; -ve = negative # Rectal swab analysis only when patients had throat-swab PCR negative *The analysis ended when rectal swab PCR is completed

the expected range and no amplified signal was observed for EVs/EV-A71 [30].

Nested RT-PCR, sequencing and sequence analysis

The nested RT-PCR targeting viral protein 1 (VP1) gene sequence was carried out as previously described [13], and served as a reference for evaluation of the multiplex RT-PCR as there is currently no gold standard assay for diagnosis of HFMD. For this purpose, 112 clinical specimens (45 throat and 67 rectal swabs from 112 children) were used. Sequencing of VP1 PCR products was done using the BigDye Terminator V3.1 Cycle Sequencing kit in an ABI 3130XL DNA sequencer (Applied Biosystems, Carlsbad, CA, USA). The sequences were assembled using ContigExpress software - a component of Vector NTI Suit 7 (Informax Inc., NY, USA). Viral serotype was determined as described previously [13], e.g. by BLASTing the obtained sequences against the sequence available in GenBank.

Assay application

The assay was applied on clinical samples obtained from 322 children with clinically suspected HFMD enrolled to our ongoing HFMD research program. The analysis was carried out using the diagnostic work-flow as described in Fig. 4, which includes an initial screening for EVs/EV-A71 in throat swabs and, to maximize the yield of the detection, rectal swabs from children who had a negative throat swab (Fig. 4) was further tested.

Ethical approval

Clinical specimens used in the present study were derived from our ongoing HFMD research program conducted at HTD, CH1 and CH2. These studies were reviewed and approved by the local Institutional Review Boards and the Oxford Tropical Research Ethical Committee (OxTREC). Informed consent was obtained from parents or legal guardians of all enrolled children.

Additional file

Additional file 1: Figure S1. A snapshot showing multiple sequence alignment and EV-A71 primer/probe binding sites.

Competing interests

The authors declare that they have no competing interests.

Author's contributions

TTT and LVT: designed the study, did laboratory testing, analysed the test results, and drafted the manuscript. NTA, NTT, HMTV, SS, PTQ, TTN, TTMV, LAN, NTHN, LTMT, OKC, DP, DCV, THK, DQH, HMT, KTW, NTH, NVVC, GT: enrolled patients, took samples and did laboratory testing. RHvD: designed the study and involved in drafting the manuscript. All authors read and approved the final manuscript.

Acknowledgements

We are indebted to Ms Le Kim Thanh for providing supports in sample storage. The research leading to these results has received funding from the Wellcome Trust of Great Britain [101104/Z/13/Z and 089276/Z/09/Z] and the Li Ka Shing Foundation–University of Oxford Global Health Program strategic award (LG23). OKC and WKT are supported by a High Impact Research Grant (H20001-E00004) from the Ministry of Education, Malaysia Government and University of Malaya Research Grant (RG480/12HTM). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. Le Van Tan is supported by a Wellcome-Trust Training Fellowship.

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Received: 20 March 2015 Accepted: 30 May 2015
Published online: 09 June 2015

References

1. Fujimoto T et al. Hand, foot, and mouth disease caused by coxsackievirus A6, Japan, 2011. Emerg Infect Dis. 2012;18(2):337–9.
2. Li JL et al. Epidemic characteristics of Hand, Foot, and Mouth Disease in Southern China, 2013: Coxsackievirus A6 has emerged as the predominant causative agent. J Infect. 2014;69(3):299–330.
3. Lu QB et al. Circulation of Coxsackievirus A10 and A6 in hand-foot-mouth disease in China, 2009-2011. PLoS One. 2012;7(12):e52073.
4. Wu, P.C., et al., An outbreak of coxsackievirus A16 infection: comparison with other enteroviruses in a preschool in Taipei. J Microbiol Immunol Infect. 43(4): p. 271-7
5. Cabrerizo M et al. Molecular epidemiology of enterovirus 71, coxsackievirus A16 and A6 associated with hand, foot and mouth disease in Spain. Clin Microbiol Infect. 2014;20(3):O150–6.
6. Ho M et al. An epidemic of enterovirus 71 infection in Taiwan. Taiwan Enterovirus Epidemic Working Group. N Engl J Med. 1999;341(13):929–35.
7. Xing W et al. Hand, foot, and mouth disease in China, 2008-12: an epidemiological study. Lancet Infect Dis. 2014;14(4):308–18.
8. Khanh TH et al. Enterovirus 71-associated Hand, Foot, and Mouth Disease, Southern Vietnam, 2011. Emerg Infect Dis. 2012;18(12):2002–5.
9. Solomon T et al. Virology, epidemiology, pathogenesis, and control of enterovirus 71. Lancet Infect Dis. 2010;10(11):778–90.
10. Bourlet T et al. Comparison of a rapid culture method combining an immunoperoxidase test and a group specific anti-VP1 monoclonal antibody with conventional virus isolation techniques for routine detection of enteroviruses in stools. J Med Virol. 1998;54(3):204–9.
11. Tan EL et al. Development of multiplex real-time hybridization probe reverse transcriptase polymerase chain reaction for specific detection and differentiation of Enterovirus 71 and Coxsackievirus A16. Diagn Microbiol Infect Dis. 2008;61(3):294–301.
12. Tan EL et al. Rapid detection of enterovirus 71 by real-time TaqMan RT-PCR. J Clin Virol. 2008;42(2):203–6.
13. Nix WA, Oberste MS, Pallansch MA. Sensitive, seminested PCR amplification of VP1 sequences for direct identification of all enterovirus serotypes from original clinical specimens. J Clin Microbiol. 2006;44(8):2698–704.
14. Beld M et al. Highly sensitive assay for detection of enterovirus in clinical specimens by reverse transcription-PCR with an armored RNA internal control. J Clin Microbiol. 2004;42(7):3059–64.
15. Hue KD et al. Validation of an internally controlled one-step real-time multiplex RT-PCR assay for the detection and quantitation of dengue virus RNA in plasma. J Virol Methods. 2011;177(2):168–73.
16. Seiff A. Cambodia unravels cause of mystery illness. Lancet. 2012;380(9838):206.
17. Burd EM. Validation of laboratory-developed molecular assays for infectious diseases. Clin Microbiol Rev. 2010;23(3):550–76.

18. Bustin SA et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem. 2009;55(4):611–22.
19. WHO, A Guide to clinical management and public health response for hand, foot and mouth disease (HFMD). WHO Library Cataloguing in Publication Data, 2011. http://www.wpro.who.int/publications/docs/GuidancefortheclinicalmanagementofHFMD.pdf.
20. Kim KH. Enterovirus 71 infection: An experience in Korea. Korean J Pediatr. 2009;53(5):616–22.
21. Yang F et al. Survey of enterovirus infections from hand, foot and mouth disease outbreak in China, 2009. Virol J. 2011;8:508.
22. Ooi MH et al. Evaluation of different clinical sample types in diagnosis of human enterovirus 71-associated hand-foot-and-mouth disease. J Clin Microbiol. 2007;45(6):1858–66.
23. Zhang S et al. A one-step, triplex, real-time RT-PCR assay for the simultaneous detection of enterovirus 71, coxsackie A16 and pan-enterovirus in a single tube. PLoS One. 2014;9(7), e102724.
24. Tan EL et al. Specific detection of enterovirus 71 directly from clinical specimens using real-time RT-PCR hybridization probe assay. Mol Cell Probes. 2006;20(2):135–40.
25. Xiao XL et al. Simultaneous detection of human enterovirus 71 and coxsackievirus A16 in clinical specimens by multiplex real-time PCR with an internal amplification control. Arch Virol. 2009;154(1):121–5.
26. Dubot-Peres A et al. SYBR Green Real-Time PCR for the Detection of All Enterovirus-A71 Genogroups. PLoS One. 2014;9(3), e89963.
27. Chen Q et al. Development and evaluation of a real-time method of simultaneous amplification and testing of enterovirus 71 incorporating a RNA internal control system. J Virol Methods. 2014;196:139–44.
28. Cui A et al. The development and application of the two real-time RT-PCR assays to detect the pathogen of HFMD. PLoS One. 2013;8(4), e61451.
29. Piqueur MA et al. Improvement of a real-time RT-PCR assay for the detection of enterovirus RNA. Virol J. 2009;6:95.
30. Dung TT et al. The validation and utility of a quantitative one-step multiplex RT real-time PCR targeting rotavirus A and norovirus. J Virol Methods. 2013;187(1):138–43.
31. Scheltinga SA et al. Diagnosis of human metapneumovirus and rhinovirus in patients with respiratory tract infections by an internally controlled multiplex real-time RNA PCR. J Clin Virol. 2005;33(4):306–11.

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Phylodynamics of Enterovirus A71-Associated Hand, Foot, and Mouth Disease in Viet Nam

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ABSTRACT

Enterovirus A71 (EV-A71) is a major cause of hand, foot, and mouth disease (HFMD) and is particularly prevalent in parts of Southeast Asia, affecting thousands of children and infants each year. Revealing the evolutionary and epidemiological dynamics of EV-A71 through time and space is central to understanding its outbreak potential. We generated the full genome sequences of 200 EV-A71 strains sampled from various locations in Viet Nam between 2011 and 2013 and used these sequence data to determine the evolutionary history and phylodynamics of EV-A71 in Viet Nam, providing estimates of the effective reproduction number (R_e) of the infection through time. In addition, we described the phylogeography of EV-A71 throughout Southeast Asia, documenting patterns of viral gene flow. Accordingly, our analysis reveals that a rapid genogroup switch from C4 to B5 likely took place during 2012 in Viet Nam. We show that the R_e of subgenogroup C4 decreased during the time frame of sampling, whereas that of B5 increased and remained >1 at the end of 2013, corresponding to a rise in B5 prevalence. Our study reveals that the subgenogroup B5 virus that emerged into Viet Nam is closely related to variants that were responsible for large epidemics in Malaysia and Taiwan and therefore extends our knowledge regarding its associated area of endemicity. Subgenogroup B5 evidently has the potential to cause more widespread outbreaks across Southeast Asia.

IMPORTANCE

EV-A71 is one of many viruses that cause HFMD, a common syndrome that largely affects infants and children. HFMD usually causes only mild illness with no long-term consequences. Occasionally, however, severe infection may arise, especially in very young children, causing neurological complications and even death. EV-A71 is highly contagious and is associated with the most severe HFMD cases, with large and frequent epidemics of the virus recorded worldwide. Although major advances have been made in the development of a potential EV-A71 vaccine, there is no current prevention and little is known about the patterns and dynamics of EV-A71 spread. In this study, we utilize full-length genome sequence data obtained from HFMD patients in Viet Nam, a geographical region where the disease has been endemic since 2003, to characterize the phylodynamics of this important emerging virus.

Understanding the evolution and epidemiological dynamics of an infectious disease within and between countries where this disease is endemic is critical for predicting its emergence in new locations and to inform an effective public health response. Enterovirus A71 (EV-A71)-associated hand, foot and mouth disease (HFMD) is endemic in large parts of Southeast Asia, with a cyclical 2-to 3-year outbreak pattern (1–3). Notable outbreaks have occurred in 1998 in Taiwan (1,500,000 cases) (4), in 2008 in China (490,000 cases) (5), and in 2011 in Viet Nam (110,000 cases) (6). More recently, EV-A71 was detected in Cambodia, where it caused a major epidemic of severe HFMD with a 90% mortality rate (7). The increasing number of EV-A71 cases and the spread of the virus across Asia has raised major concerns about its pandemic potential (4, 7–10).

EV-A71 belongs to the species enterovirus A of the *Enterovirus* genus within the family *Picornaviridae*. Its genome comprises a single-stranded, positive-sense RNA molecule ~7.5 kb in length. EV-A71 is thought to have diverged from coxsackievirus A16 around 1940 (11), and sequence analysis of the structural viral protein 1 (VP1) coding sequence indicates that it can be divided into three genogroups (A, B, and C). The latter two are further divided into several different subgenogroups, denoted B0 to B5 and C1 to C5.

EV-A71-related HFMD is most common in children and infants and is often associated with mild illness characterized by papulovesicular lesions on the hands, feet, orophyaryngeal mucosa, and buttocks (11). Occasionally, severe infections may arise, associated with neurological involvement, autonomic dysregulation, and often fatal cardiopulmonary complications. EV-A71 was first isolated from a patient with an infection of the central ner-

vous system in California in 1969. Soon after, large outbreaks of EV-A71-associated encephalitis, with high mortality, were reported in Europe, North America, and Australia (12–16). However, it was not until 1973 that the first clear association between HFMD and EV-A71 was obtained during a large outbreak in Japan (17, 18). Outbreaks of EV-A71 have often been associated with switches between (sub)genogroups, occasionally accompanied by recombination events (8, 11, 19). For example, sequence data from Viet Nam obtained between 2005 and 2011 have shown that a subgenogroup switch from C5 to C4 occurred in 2011 (9, 20). Notably, this C4 subgenogroup has been associated with the ongoing epidemic of HFMD in China since 2008, as well as with the recent outbreak in Cambodia (7, 21).

Currently, there is no specific antiviral treatment available for EV-A71. Phase III trials of three different inactivated EV-A71 vaccines from China have recently been completed and have shown 95% protection rates against EV-A71-associated HFMD. Antibodies induced by natural infection with subgenogroup C4 or by vaccination with inactivated C4 induce broad cross-neutralizing activity for different EV-A71 subgenogroups, including genogroup B virus (22). Nevertheless, these vaccines are yet to be implemented, and it is unclear whether they also provide protection against the severe form of the disease (5, 23, 24) and to what extent they would shape the dynamics of (EV-A71-related) HFMD. In addition, enteroviruses commonly undergo extensive recombination. Such genetic exchanges can occur in, and even between, different serotypes, meaning that EV-A71 is highly variable both genetically and antigenically (25, 26).

Analysis of global VP1 sequences over a 4-decade period has shown that HFMD epidemics are likely to be driven in part by the migration of viruses between locations rather than continuous transmission within a locality (11). However, little is known about the patterns and dynamics of EV-A71 transmission at a local scale. We utilized full-length genome sequence data obtained from HFMD patients in Viet Nam to characterize viral phylodynamics. EV-A71-associated HFMD was first identified to be the source of an outbreak causing acute neurological disease in Viet Nam in 2003 (20) and still represents a major disease burden with an average of some 100,000 cases reported per year since 2011 (27). We report the population dynamics of EV-A71 within Viet Nam between 2011 and 2013, providing an estimate of temporal changes in the effective reproduction number (R_e) of the infection through time as a more quantitative reflection of epidemiological dynamics. In addition, we examine the epidemiological dynamics of EV-A71 throughout Southeast Asia, where epidemics of EV-A71-associated HFMD have been established since 1973. Overall, these data provide a unique opportunity to investigate the phylodynamics of EV-A71 within a geographical region where it is endemic.

Received 16 March 2015 **Accepted** 7 June 2015

Accepted manuscript posted online 17 June 2015

Citation Geoghegan JL, Tan LV, Kühnert D, Halpin RA, Lin X, Simenauer A, Akopov A, Das SR, Stockwell TB, Shrivastava S, Ngoc NM, Uyen LTT, Tuyen NTK, Thanh TT, Hang VTT, Qui PT, Hung NT, Khanh TH, Thinh LQ, Nhan LNT, Van HMT, Viet DC, Tuan HM, Viet HL, Hien TT, Chau NVV, Thwaites G, Grenfell BT, Stadler T, Wentworth DE, Holmes EC, Doorn HRV. 2015. Phylodynamics of enterovirus A71-associated hand, foot, and mouth disease in Viet Nam. J Virol 89:8871–8879. doi:10.1128/JVI.00706-15.

Editor: A. García-Sastre

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Supplemental material for this article may be found at http://dx.doi.org/10.1128/JVI.00706-15.

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doi:10.1128/JVI.00706-15

MATERIALS AND METHODS

Settings: studies, patients, and clinical samples. The studies described here were conducted at three primary, secondary, and tertiary referral hospitals for infectious diseases, covering all provinces in Viet Nam. These included the Hospital for Tropical Diseases (HTD), Children's Hospital 1 (CH1), and Children's Hospital 2 (CH2). All of the hospitals are located in Ho Chi Minh City (HCMC) and serve a population of 42 million people. Patient enrollment was undertaken between August 2011 and December 2012 and between July 2013 and December 2013 as part of two different studies.

The first study was conducted at the Pediatric Intensive Care Unit (PICU) at HTD between August 2011 and December 2012, and at the Infectious Diseases Department and PICU at CH1 between September and December 2011. These departments only admitted HFMD patients with a clinical diagnosis of moderate to severe HFMD with neurological involvement. Swabs (including rectal/throat/vesicle swabs) were collected from anonymous HFMD children for routine diagnostics as part of standard patient management and placed in viral transport medium (28). After routine diagnostic analysis, any remaining swab material was stored at 4°C and transferred to the Oxford University Clinical Research Unit in HCMC, Viet Nam (OUCRU), where EV-A71 detection was performed using an in-house real-time reverse transcription-PCR targeting a VP1 fragment (9). Samples with a crossing point (Cp) value of ≤32 were then selected for whole-genome amplification and sequencing. Demographic and basic clinical information (including clinical grading on admission and at discharge) were also retrieved from the electronic database systems of the collaborating hospitals. In total, 250 samples (including 201 throat swabs, 47 rectal swabs, and 2 vesicle swabs) were included.

The second cohort was part of an ongoing observational study on HFMD patients of all severities conducted at the three hospitals described above. Patient enrollment began in July 2013 and is ongoing. This study reported the analysis of whole-genome sequencing efforts on 22 throat swabs collected at the Outpatient Clinic of CH2 and HTD, as well as the Infectious Diseases Department and PICU of CH2, between July and November 2013.

Ethics statement. The institutional review boards (IRBs) of HTD and CH1 in HCMC, Viet Nam, approved the whole-genome sequencing of residual swabs of anonymous HFMD patients. The observational study on HFMD was approved by the IRB of the collaborating hospitals and by the Oxford University Tropical Research Ethics Committee.

RNA extraction, whole-genome amplification, and sequencing. Whole-genome amplification and sequencing of the viral genomes were done either at the J. Craig Venter Institute (Rockville, MD) for the first cohort or at OUCRU for the second cohort.

First study. A total of 200 μl of viral clinical material was combined with 600 μl of Zymo research viral RNA buffer containing 0.1% 2-mercaptoethonol. Viral RNA was extracted using a ZR-96 viral RNA kit (Zymo Research Corp., Irvine, CA). Full-length cDNA was generated using the SuperScript III First-Strand Synthesis SuperMix (Thermo Fisher Scientific, Waltham, MA) and oligo(dT) (45°C for 15 min, 50°C for 50 min, and 55°C for 15 min). Due to the complex structure of the EV71 internal ribosome site, two DNA fragments (short and long) were PCR amplified for each virus. A short fragment (~640 bp) was generated with a Q5 Hot Start high-fidelity DNA polymerase reaction system (New England BioLabs, Ipswich, MA) and the primers EV71-1F (TTAAAACAGCC TGTGGGTT) and EV71-641R (GGCCAATCCAATAGCTATATG) at

concentrations of 10 μM each. Next, 3 μl of cDNA was added to 7 μl of Q5 reaction mixture and subjected to the following thermal cycling program: 98°C for 3 min (98°C for 20 s, 62°C for 30 s, and 72°C for 1 min + 5 s/cycle) for 40 cycles, followed by 72°C for 10 min and then a 4°C hold. A long fragment (~7 kb) was generated using a Phusion high-fidelity PCR kit (New England BioLabs, Ipswich, MA) and the primers EV71-473F (AATGCGGCTAATCCYAACT) and EV71-7405R (GCTATTCTGGTTA TAACAAATTTACC) at concentrations of 10 μM each. Then, 4 μl of cDNA was added to a 21-μl reaction mixture, which was then subjected to the following thermal cycler program: 98°C for 3 min (98°C for 30 s, 62°C for 30 s, and 72°C for 6 min + 5 s/cycle) for 35 cycles, followed by 72°C for 10 min and then a 4°C hold. The short amplicons were detected and quantitated on a QIAxcel capillary electrophoresis platform, while the long amplicons were detected via agarose gel electrophoresis and quantitated using Sybr green and Gen5 software.

For each virus sample with two amplicons, 15 ng of the short amplicon and 150 ng of the long amplicon were pooled. Illumina libraries were prepared from the pooled amplicons using the Nextera DNA sample preparation kit (Illumina, Inc., San Diego, CA) with half reaction volumes. Briefly, 25 ng of pooled DNA amplicons were tagmented at 55°C for 5 min. Tagmented DNA was cleaned with a ZR-96 DNA Clean & Concentrator kit (Zymo Research Corp., Irvine, CA) and eluted in 25 μl of resuspension buffer. Illumina sequencing adapters and barcodes were added to tagmented DNA via PCR amplification: 20 μl of tagmented DNA was combined with 7.5 μl of Nextera PCR master mix, 2.5 μl of Nextera PCR Primer Cocktail, and 2.5 μl of each index primer (Integrated DNA Technologies, Coralville, IA) for a total volume of 35 μl per reaction mixture. Thermal cycling was performed with 5 cycles of PCR according to the Nextera DNA sample preparation kit protocol (3 min at 72°C, denaturation for 10 s at 98°C, annealing for 30 s at 63°C, and extension for 3 min at 72°C) to create a dual indexed library for each sample. After PCR amplification, 10 μl of each library was pooled into a 1.5-ml tube, and the pool was cleaned twice with Ampure XP reagent (Beckman Coulter, Inc., Brea, CA) to remove all leftover primers and small DNA fragments. The first cleaning used a 1.2× volume Ampure reagent, while the second cleaning used a 0.6× volume Ampure reagent. The two pools were sequenced on an Illumina MiSeq v2 instrument with 300-bp paired-end reads.

The sequence reads from the Illumina data were sorted by barcode, trimmed, and *de novo* assembled using CLC Bio's (Qiagen, Hilden, Germany) the clc_novo_assemble program, and the resulting contigs were searched against custom full-length EV-A71 nucleotide databases to find the closest reference sequence for each virus. All sequence reads were then mapped to the selected reference EV-A71 virus using CLC Bio's clc_ref_assemble_long program.

Second study. Total nucleic acid was isolated from 140 μl of clinical material using the QIAamp viral RNA kit (Qiagen), recovered in 50 μl of elution buffer (provided with the kit), and was immediately stored at −80°C for the subsequent whole-genome amplification step. Whole-genome amplification and sequencing was undertaken as previously described using in-house designed PCR primers and the Miseq system (Illumina), respectively (29). The reads obtained were processed to remove PCR primers using CLC Genomics Workbench (Qiagen). Sequence assembly was performed using the Genieous 7.1.3 software package utilizing a reference-based mapping tool (i.e., the consensus sequence was obtained by mapping individual reads of each sample to a reference sequence).

Recombination analysis. Because enteroviruses are known for their ability to undergo extensive recombination (30), we determined the frequency and occurrence of this process in the 200 whole genomes sequenced in this study by using the genetic algorithm recombination detection (GARD) method available at the Datamonkey webserver (31). This analysis used the Hasegawa-Kishino-Yano (HKY) model of nucleotide substitution and default parameters in all other cases.

Phylogeny and phylogeography of EV-A71. The phylogenetic relationships of EV-A71 within Southeast Asia were estimated from a total of 1,176 complete VP1 sequences (891 nucleotides [nt]), including the 200 sequences from Viet Nam obtained in the present study. The data set also comprised 976 randomly selected sequences downloaded from GenBank, sampled between 1997 and 2013. Sequences were aligned in Geneious (v7.1.3) using the multiple-sequence alignment tool, MAFFT. Phylogenetic inference utilized the maximum-likelihood (ML) method available in RAxML (v7.2.8), applying the general time reversible (GTR) nucleotide substitution model with a gamma (Γ) distribution of among-site rate variation. Support for individual nodes was assessed using a bootstrap procedure with 100 replicates.

This EV-A71 data set was also used to investigate the phylogeographic history of EV-A71 in Southeast Asia. To this end, the data were grouped into 10 discrete country (or autonomous regions) locations within Southeast Asia (China, Hong Kong, Japan, South Korea, Malaysia, Philippines, Singapore, Taiwan, Thailand, and Viet Nam). For this analysis we used a time-aware coalescent Bayesian skyride model available in BEAST (32) (v1.8), which utilizes Gaussian Markov random field smoothing prior to estimate the changes in relative genetic diversity over time (33). The HKY model of nucleotide substitution was used (determined by JModelTest [version 2.1.4] to be the best-fit model), along with an uncorrelated relaxed molecular clock and a uniform distributed clock rate prior of 0.004. Using the Bayesian Markov Chain Monte Carlo framework, 100 million steps were run, sampling every 10,000 and removing 10% as burn-in. Convergence was assessed using Tracer (v1.4) (34), and effective sample size (ESS) values above 200 were accepted. A maximum clade credibility (MCC) tree was summarized with TreeAnnotator (available in the BEAST package) and visualized in Figtree (v1.4).

To determine whether there was more clustering of EV-A71 by place of sampling than expected by chance alone, we utilized two phylogeny-trait association tests: the association index (AI) and parsimony score (PS), both of which are available in the BaTS package (35). The maximum-clade statistic was also used to determine which individual locations showed the strongest spatial clustering. Phylogenetic uncertainty in the data was incorporated through the use of the posterior distribution of trees (determined from the BEAST analysis described above) with 1,000 random permutations of sampling locations undertaken to create a null distribution for each statistic.

We also investigated phylogeography at a local level within Viet Nam. Of the 200 whole-genome samples successfully sequenced, 190 included the place (province) in which the patient was registered (and we assume this was where the disease was contracted). We analyzed these samples from 18 discrete provinces in Viet Nam, using the phylogeny-trait association tests described above (using both whole genomes and VP1). Unfortunately, the relatively small sample size, together with the collection methods of the different cohorts, prevented us from exploiting clinical information associated with the disease in our analyses.

Phylodynamics. To determine the population dynamics of EV-A71 in Viet Nam, we analyzed whole-genome (7,360 nt) sequences, together with their exact date (day) of sampling, for the two main viral lineages circulating in Viet Nam, representing subgenogroup B5 ($n = 38$) and subgenogroup C4 ($n = 154$) (these sequences are marked with an asterisk in Fig. 1A). Lineages were compared using the same Bayesian skyride method described above (using both whole-genome sequences and VP1), which enables us to reveal changes in relative genetic diversity over time.

In addition, we estimated temporal changes in R_c for subgenogroups C4 and B5 in Viet Nam using the birth-death skyline model (BDSKY) (36) available in BEASTii (v2.1.3). For each analysis, we utilized the same methods as described above: the HKY model of nucleotide substitution, an uncorrelated relaxed molecular clock, and a uniform distributed clock rate prior of 0.004. We also assumed that the proportion of individuals being sampled was <5% of the total infected population. This assumption was based on the assessment that <5% of the total number of symptomatic cases were subjected to virological diagnosis and that the vast majority

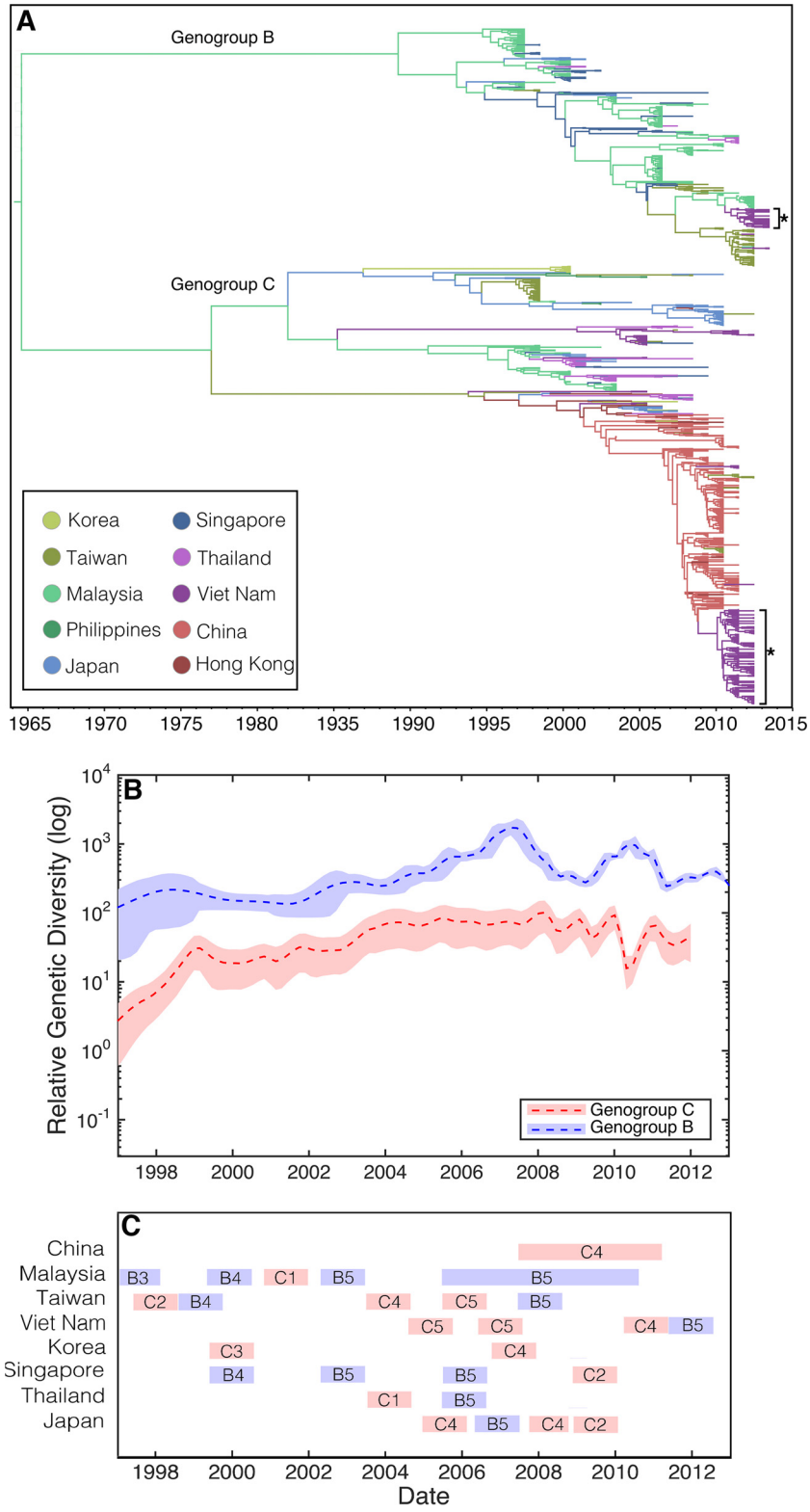


FIG 1 (A) MCC tree of 1,176 complete VP1 sequences. The phylogeny includes 200 sequences from Viet Nam, as well as 976 randomly selected sequences from GenBank, sampled from 10 discrete locations in Southeast Asia (China, Hong Kong, Japan, South Korea, Malaysia, Philippines, Singapore, Taiwan, Thailand, and Viet Nam) between 1997 and 2013. Branches are color-coded according to the location of sampling. Asterisks indicate the two major subgenogroups from Viet Nam sampled in the present study. (B) Bayesian skyride plots, illustrating the relative genetic diversity of genogroups B (blue) and C (red) through time. A dashed line indicates the mean while shaded areas show the upper and lower 95% HPD values. (C) The temporal distribution of the different subgenogroups circulating in the region (partially adapted from Fig. 2.2 in reference 54). The data are from countries with enhanced surveillance since 1997.

of EV-A71 infections are asymptomatic (37). We allowed the sampling rate to change over time, reflecting the heterogeneity of collections in the study, while assuming that the rate at which patients recover remained constant. For the rate at which individuals recover, which we assume to coincide with becoming noninfectious, we used a lognormal distribution prior [logN(3.415,0,2)], which translates to the sum of the incubation (~5 days) and infectious period (~7 days) (38) to center around 12 days.

GenBank accession numbers. The genome sequences of EV-A71 obtained in the present study have been deposited on GenBank under accession numbers KJ686127 to KJ686308 and KP691643 to KP691664.

RESULTS

EV-A71 genome sequences. In total, 200 full (consensus) genome sequences of EV-A71 were successfully generated as part of this project: 178 by sequencing efforts undertaken at the J. Craig Venter Institute funded by a U.S. National Institutes of Health/National Institute of Allergy and Infectious Disease Genome Sequencing Center contract and an additional 22 complete genome sequences through an in-house sequencing pipeline at OUCRU.

Recombination in Vietnamese EV71. Our recombination analysis (GARD) detected a single breakpoint ($P < 0.01$) in the 2C gene sequence (nucleotide position 4554). Accordingly, ML phylogenetic trees were then inferred on either side of this breakpoint, and these revealed that sequence ID:0_023 (collected from Binh Duong province on 21 August 2013) had experienced a minor change of topological position within the subgenogroup B5 clade. Because of the minimal phylogenetic movement, this sequence was retained in the data set. No other recombination events were detected, which is also supported by the very tight clustering within each of the C4, C5 and B5 subgenogroups.

Phylogeny and phylogeography. Phylogenetic analysis of 1,176 complete VP1 gene sequences sampled between 1997 and 2013 revealed the existence of two major genogroups, B and C (see Fig. S1 in the supplemental material). The majority of samples obtained from Viet Nam in this study fell into two lineages: 154 in subgenogroup C4 and 38 in subgenogroup B5, while the remaining 8 sequences clustered in subgenogroup C5.

Phylogeographic analysis revealed significant spatial clustering by sampling location (i.e., country or autonomous region): AI ($P = 0$) and PS ($P = 0$). These results were summarized using an MCC tree in which branches were colored according to sampling location (Fig. 1A). Notably, both genogroups exhibited a varied, multicountry distribution. Subgenogroup C4 viruses in Viet Nam were closely related to those from China and Hong Kong and, given these data, likely originated from there. In contrast, genogroup B predominantly contained samples from Malaysia and Taiwan, to which the small subgenogroup B5 samples from Viet Nam were closely related. Several viruses from Viet Nam sampled first in 2005 (20) and then in 2011 and 2012 (the present study) were found to be members of subgenogroup C5.

The phylogeography of EV-A71 within Viet Nam was similarly examined by considering the 18 discrete provinces from which the samples originated. Although we found more spatial clustering by sampling location than expected by chance (AI, $P = 0$; PS, $P < 0.01$), the relatively small sample size in certain provinces, as well the intrinsic biases in sampling protocols, prohibited further spatial analysis (Fig. 2). Nevertheless, this analysis showed some fluidity within Viet Nam, as is evident from the phylogenetic tree, compared to relatively infrequent movement of viruses at the international level. This may in part be due to the small geographic region in southern Viet Nam from which the majority of samples were taken (only two sequences originated from northern provinces, Thai Nguyen and Thanh Hoa, with the remainder sampled from southern Viet Nam). To account for the possible impact of recombination on our phylogeographic inferences, we repeated this analysis on the VP1 gene alone, which resulted in very similar patterns to observed with the complete genome data (see Fig. S2A in the supplemental material).

EV-A71 phylodynamics. Bayesian skyride plots of genogroups B and C depict changes in relative genetic diversity over time for EV-A71 across Southeast Asia as a whole (Fig. 1B). Although these results need to be treated with caution because of the occurrence of population subdivision by country, it is clear from this analysis that irregular cycles of relative genetic diversity were observed, often sharply increasing then decreasing. Some of these irregular cycles coincided with large EV-A71 epidemics, most notably the 2006 B5 outbreak that affected Malaysia, Thailand, and Singapore (39) (Fig. 1C).

Two distinct lineages from Viet Nam—subgenogroups B5 and C4—were analyzed in more detail (sequences indicated on Fig. 1A with asterisks). In particular, we again used the Bayesian skyride analysis to reveal temporal changes in genetic diversity (Fig. 3A). Notably, the skyride plot captures the rapid increase in genetic diversity of subgenogroup C4 at the end of 2011 before peaking at the beginning of 2012, followed by a brief decline from May to June and eventually a plateau. In contrast, the relative genetic diversity of subgenogroup B5 increased markedly over the same time period, seemingly replacing subgenogroup C4 at the end of 2012, and continued to increase during 2013. This observation is consistent with a subgenogroup switch from C4 to B5 during 2012. Indeed, all samples collected during 2013 in Viet Nam were exclusively subgenogroup B5 (Fig. 3B). This analysis was also undertaken using the VP1 gene sequences and captured a very similar pattern, indicating that it has not been adversely affected by recombination (see Fig. S2B in the supplemental material).

Finally, we obtained R_e estimates for subgenogroup C4 in Viet Nam using a phylogenetic (birth-death) approach. Notably, the BDSKY analysis provided clear evidence for temporal changes of R_e during the 2011-2013 outbreak. Specifically, there was a decrease in the R_e of subgenogroup C4 in Viet Nam in 2012 (Fig. 3C). Concomitantly, we observed a slight increase in R_e of subgenogroup B5 during 2012 and, importantly, R_e remained >1 at the end of the study period (Fig. 3D). The total duration of incubation and infectiousness estimated under this approach was 17.9 days (95% HPD, 11.7 to 25.9) for C4 and 19.8 days (95% HPD, 13.3 to 28.0) for B5. Again, we repeated this analysis using the VP1 gene sequences, which resulted in a similar trend (see Fig. S2C and D in the supplemental material).

DISCUSSION

This study documented the evolutionary history of the main genogroups of EV-A71 circulating in Viet Nam and throughout Southeast Asia. Our analysis of the overall population genetic history of EV-A71 revealed major fluctuations in genetic diversity through time, coinciding with large and varied epidemics, thereby supporting previous observations (11). Notably, EV-A71 genetic diversity remained relatively high even between large outbreaks, suggesting that the virus was likely sustained in the local population.

Our phylogenetic analysis revealed the presence of two main genogroups, B and C, in the Southeast Asian region. While both

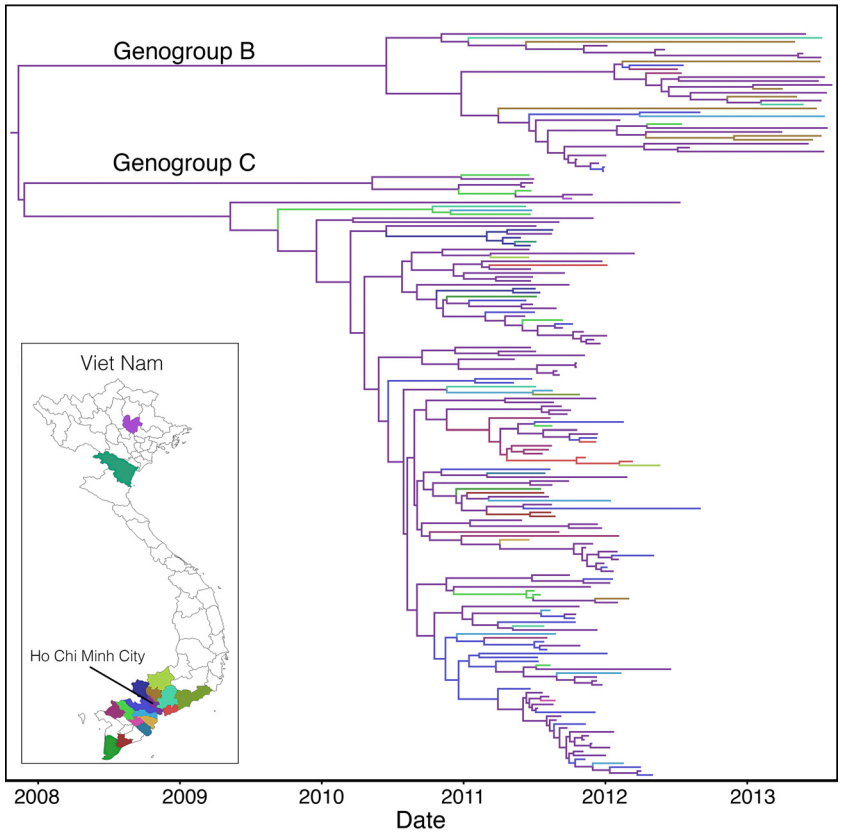


FIG 2 MCC tree of 190 whole-genome EV-A71 sequences sampled from 18 provinces in Viet Nam between 2011 and 2013. Branches are color-coded according to location of sampling.

genogroups exhibited a multicountry distribution, there was also strong clustering by sampling location. More refined analysis revealed that genogroups C4 and B5 were the two major viruses present in Viet Nam during the course of this study. Our phylogeographic analysis revealed that subgenogroup C4 likely originated from the large and ongoing outbreak in China, while B5 is closely related to sequences sampled from Malaysia and Taiwan, and likely entered Viet Nam circa 2010 (2 to 3 years after that of subgenogroup C4). Indeed, genogroup B5 was the cause of the major Malaysian (Sarawak) outbreak in 2006 (40), and its emergence in Taiwan in 2008 resulted in the region's largest outbreak of HFMD in 11 years (41). We also found a small lineage of subgenogroup C5 viruses in Viet Nam that were closely related to a previous small outbreak in 2005 (also in Viet Nam) (20), suggesting that C5 virus may have been in continual circulation at low prevalence over this period.

During 2011-2012, a large outbreak of EV-A71 associated HFMD occurred in Viet Nam followed by ongoing circulation, but with fewer (severe) cases, in 2013-2014 (42). By examining population dynamics at a local level within Viet Nam, our data suggested that a rapid genogroup switch from C4 to B5 likely took place at the end of 2012. Genogroup switching has been seen elsewhere during previous outbreaks of EV-A71-associated HFMD (8, 11, 19, 43, 44), including in Malaysia, Japan, Viet Nam, and Taiwan (8, 20). For example, between 2009-2012, the dominant genogroup in Taiwan appeared to have changed twice: from B to C and then later from C to B (41).

EV-A71 exists as a single serotype, as measured by hyperimmune animal antiserum, yet results from human studies on antigenic variations among subgenogroups have been complex and inconsistent. Antigenic cartography has revealed clear antigenic differences among (sub)genotypes, with B5 isolates from the 2008 outbreak in Taiwan comprising a distinct group on the antigenic map and possessing an Asp164 amino acid residue in VP1 that distinguished it from other genogroup B viruses (41, 45, 46). In contrast, antibodies from C4 vaccines, as well as from children naturally infected with a variety of EV-A71 subgenogroups, cross-neutralized a broad range of genogroup B and C viruses, including B5 (22, 47). These sera were all taken just weeks after vaccination or infection and it is possible that the initial wide spectrum of the cross-neutralizing immune response narrows over time to become more focused on the vaccine/infecting strain, as previously described in dengue and influenza virus infections (48, 49). Interestingly, however, sera from healthy individuals in Japan, where EV-A71 is endemic and caused outbreaks every 3 years, also showed a broad spectrum of neutralizing activity (50), implying that cross-neutralizing antibodies are long lasting but may also be caused by (multiple) infections with different closely related enterovirus serotypes and/or EV-A71 subgenogroups.

The genogroup switch from subgenogroup C4 to B5 in Viet Nam may imply that the circulation of C4 did not provide lasting cross-protection to B5 infection and tentatively suggests that host immunity may drive genogroup switching. However, this idea clearly needs to be tested experimentally, and it was notable that

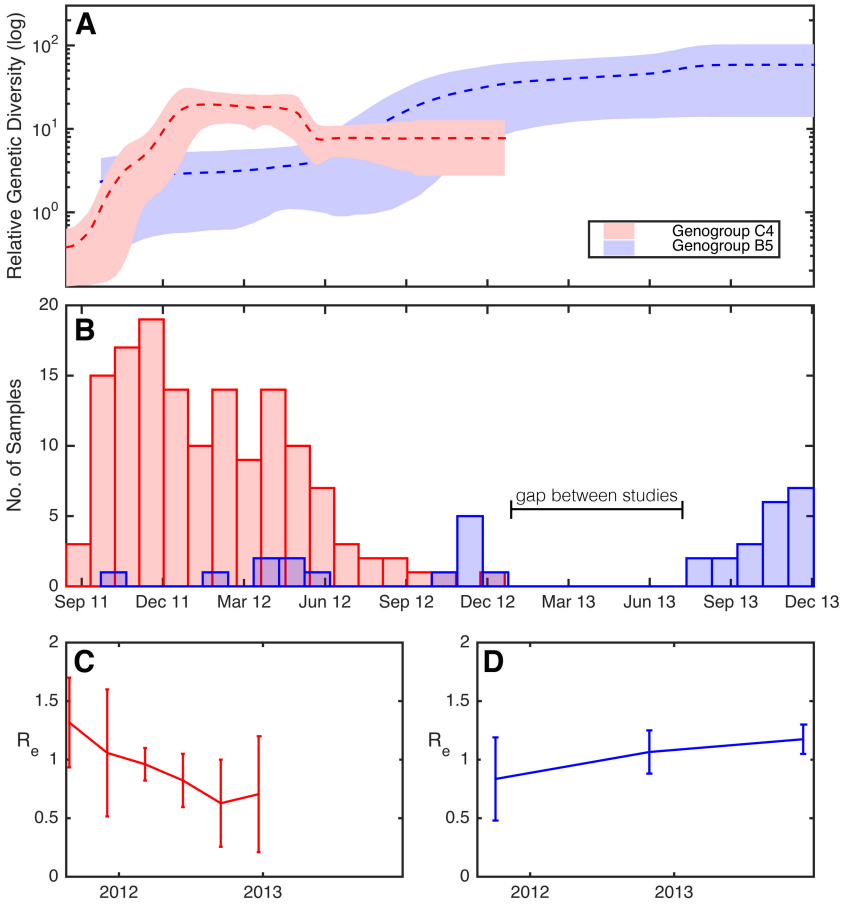


FIG 3 (A) Bayesian skyride plots showing changing levels of relative genetic diversity over time of the two Viet Nam lineages indicated by asterisks in Fig. 1A. Two skyride plots are shown: one from subgenogroup B5 (blue), and the other from subgenogroup C4 (red). A dashed line indicates the mean while the shaded area shows the upper and lower 95% HPD values. (B) Sampling prevalence of subgenogroup C4 (red) and B5 (blue) over the study period. Changing values of R_e over time for EV-A71 subgenogroups C4 (C) and B5 (D), estimated using a serial-sampled birth-death model. The line plot shows the median estimate of R_e, while 95% HPD values are given by error bars.

the switch to B5 was not associated with another large outbreak as in 2011 and 2012. This may be explained by partial residual herd immunity against B5 after C4 circulation, by depletion of the susceptible population of children under five after 2 years of hyperendemicity of C4, or by other phenotypic changes in the virus that have accumulated as a consequence of its ongoing genetic evolution. Alternatively, it is possible that the C4 and B5 subgenogroups are in fact cross-reactive but were infecting different population groups within Viet Nam. Further research is evidently needed to study the spectrum and duration of cross-neutralizing activity of EV-A71 antibodies among vaccines and recovered patients, and to determine to what extent the implementation of a single subgenogroup vaccine would shape the dynamics of HFMD.

Our estimate of R_e through time showed a decrease in the transmission of subgenogroup C4 in Viet Nam between 2011 and 2012. This pattern is consistent with the reduction in C4 cases, as well as with the decrease in genetic diversity observed during the latter half of this study. In contrast, we observed a rise in the reproductive number of the B5 subgenogroup, which remained >1 at the end of 2013. Previously, R_e for EV-A71 has been determined using incidence data obtained from laboratory-confirmed HFMD outbreaks, with median estimates ranging from 1.4 (51) to 5.5 (38) and sometimes higher (up to 10 to 15 [52]). Our temporal and gene sequence-based estimates are at the lower end of this range, which may be due to the fact that our analyses are focused on separate subgenogroups, instead of the overall EV-A71 virus population as the case with incidence-based measures. The relatively short sampling time frame used here may also have produced an incomplete picture. For instance, we have apparently captured data concerning only the decline phase in C4 transmission, as well as data concerning only the initial phase of the B5 introduction into Viet Nam. Of note, it has been hypothesized that emerging subgenogroups may cryptically circulate in the community at low prevalence for years and cause mild infection prior to emergence (11). However, it is surprising that our estimates of R_e are low (albeit with the range previously reported for EV-A71), given the seemingly rapid and large epidemic experienced in Viet Nam, although they are robust to a variety of prior distributions for the input parameters. Nonetheless, our observational data on patients enrolled into the ongoing cohort showed that the overall prevalence of EV-A71 is currently low (data not shown). Although a major advantage of these phylodynamic methods is that they allow population dynamic estimations from sequences when other data are lacking, further research, such as

subgenogroup-specific prevalence data, will be valuable in confirming these results.

It is worth noting that sampling time frame, dissimilarities in recruitment protocols, and the brief hiatus between studies may have had some affect on the results presented here. In particular, our observation of subgenogroup switching was concomitant with major changes in study design. Despite this short-coming, Bayesian inference of past evolutionary events using genome sequence data is capable of accounting for such discrepancies in sampling frequency. In addition, this study utilizes data from an ongoing recruitment effort and thus paves the way for further, more in-depth analysis on the phylodynamics of EV-A71 in Viet Nam and throughout Southeast Asia.

Finally, our estimates of the infectious period were slightly higher than previously estimated. It is generally assumed that infectiousness lasts for approximately 7 days (38). However, several studies suggest a much longer period, lasting several weeks to months (53), albeit without clear evidence of virus transmission. Accurately predicting the number of contacts and the probability of transmission is often difficult to establish from case data alone, in which case our estimates of a longer infectious period may be plausible.

ACKNOWLEDGMENTS

This research was supported by the Wellcome Trust (101104/Z/13/Z and 089276/Z/09/Z) and the Li Ka Shing Foundation–University of Oxford Global Health Program strategic award (LG23). J.L.G. is supported by the Judith and David Coffey fellowship from the Charles Perkins Centre, University of Sydney, and ECH by an NHMRC Australia Fellowship (AF30). D.K. is supported by an ETH Zurich postdoctoral fellowship, T.S. was supported in part by the European Research Council under the 7th Framework Programme of the European Commission (PhyPD; grant agreement 335529), and B.T.G. was supported by grants from the Research and Policy for Infectious Disease Dynamics program of the Science and Technology Directorate, the U.S. Department of Homeland Security, the Fogarty International Center, and the National Institutes of Health and by the Bill and Melinda Gates Foundation. This project has been funded in whole or part with federal funds from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services under contract HHSN272200900007C.

We thank Nadia Fedorova, Vishal Thovarai, Vinita Puri, and Susmita Shrivastava at JCVI for informatics support.

REFERENCES

1. **Sabanathan S, Tan LV, Thwaites L, Wills B, Qui PT, van Doorn HR.** 2014. Enterovirus 71 related severe hand, foot and mouth disease outbreaks in South-East Asia: current situation and ongoing challenges. J Epidemiol Community Health **68:**500–502. http://dx.doi.org/10.1136/jech-2014-203836.
2. **Podin Y, Gias ELM, Ong F, Leong Y-W, Yee S-F, Yusof MA, Perera D, Teo B, Wee T-Y, Yao S-C, Yao S-K, Kiyu A, Arif MT, Cardosa MJ.** 2006. Sentinel surveillance for human enterovirus 71 in Sarawak, Malaysia: lessons from the first 7 years. BMC Public Health **6:**180–190. http://dx.doi.org/10.1186/1471-2458-6-180.
3. **National Institutes of Health.** 2015. IDWR surveillance data table. National Institutes of Health, Bethesda, MD. http://www.nih.go.jp/niid/en/survaillance-data-table-english.html.
4. **Ho M, Chen ER, Hsu KH, Twu SJ, Chen KT, Tsai SF, Wang JR, Shih SR.** 1999. An epidemic of enterovirus 71 infection in Taiwan. N Engl J Med **341:**929–935. http://dx.doi.org/10.1056/NEJM199909233411301.
5. **Zhu FC, Meng FY, Li JX, Li XL, Mao QY, Tao H, Zhang YT, Yao X, Chu K, Chen QH, Hu YM, Wu X, Liu P, Zhu LY, Gao F, Jin H, Chen YJ, Dong YY, Liang YC, Shi NM, Ge HM, Liu L, Chen SG, Ai X, Zhang ZY, Ji YG, Luo FJ, Chen XQ, Zhang Y, Zhu LW, Liang ZL, Shen XL.** 2013. Efficacy, safety, and immunology of an inactivated alum-adjuvant enterovirus 71 vaccine in children in China: a multicentre, randomised, double-blind, placebo-controlled, phase 3 trial. Lancet **381:**2024–2032. http://dx.doi.org/10.1016/S0140-6736(13)61049-1.
6. **World Health Organization.** 2012. WPRO hand, foot and mouth disease situation update. World Health Organization, Geneva, Switzerland.
7. **Seiff A.** 2012. Cambodia unravels cause of mystery illness. Lancet **380:**206. http://dx.doi.org/10.1016/S0140-6736(12)61201-X.
8. **Solomon T, Lewthwaite P, Perera D, Cardosa MJ, McMinn P, Ooi MH.** 2010. Virology, epidemiology, pathogenesis, and control of enterovirus 71. Lancet Infect Dis **10:**778–790. http://dx.doi.org/10.1016/S1473-3099(10)70194-8.
9. **Khanh TH, Sabanathan S, Thanh TT, le Thoa PK, Thuong TC, Hang V, Farrar J, Hien TT, Chau N, van Doorn HR.** 2012. Enterovirus 71-associated hand, foot, and mouth disease, Southern Vietnam, 2011. Emerg Infect Dis **18:**2002–2005. http://dx.doi.org/10.3201/eid1812.120929.
10. **Zhang Y, Zhu Z, Yang W, Ren J, Tan X, Wang Y, Mao N, Xu S, Zhu S, Cui A, Zhang Y, Yan D, Li Q, Dong X, Zhang J, Zhao Y, Wan J, Feng Z, Sun J, Wang S, Li D, Xu W.** 2010. An emerging recombinant human enterovirus 71 responsible for the 2008 outbreak of hand foot and mouth disease in Fuyang city of China. Virology **7:**94–103. http://dx.doi.org/10.1186/1743-422X-7-94.
11. **Tee KK, Lam TT, Chan YF, Bible JM, Kamarulzaman A, Tong CY, Takebe Y, Pybus OG.** 2010. Evolutionary genetics of human enterovirus 71: origin, population dynamics, natural selection, and seasonal periodicity of the VP1 gene. J Virol **84:**3339–3350. http://dx.doi.org/10.1128/JVI.01019-09.
12. **Blomberg J, Lycke E, Ahlfors K, Johnsson T, Wolontis S, von Zeipel G.** 1974. New enterovirus type associated with epidemic of aseptic meningitis and-or hand, foot, and mouth disease. Lancet **ii:**112–113.
13. **Kennett ML, Birch CJ, Lewis FA, Yung AP, Locarnini SA, Gust ID.** 1974. Enterovirus type 71 infection in Melbourne. Bull World Health Organ **51:**609–615.
14. **Chumakov M, Voroshilova M, Shindarov L, Lavrova I, Gracheva L, Koroleva G, Vasilenko S, Brodvarova I, Nikolova M, Gyurova S, Gacheva M, Mitov G, Ninov N, Tsylka E, Robinson I, Frolova M, Bashkirtsev V, Martiyanova L, Rodin V.** 1979. Enterovirus 71 isolated from cases of epidemic poliomyelitis-like disease in Bulgaria. Arch Virol **60:**329–340. http://dx.doi.org/10.1007/BF01317504.
15. **Shindarov LM, Chumakov MP, Voroshilova MK, Bojinov S, Vasilenko SM, Iordanov I, Kirov ID, Kamenov E, Leshchinskaya EV, Mitov G, Robinson IA, Sivchev S, Staikov S.** 1979. Epidemiological, clinical, and pathomorphological characteristics of epidemic poliomyelitis-like disease caused by enterovirus 71. J Hyg Epidemiol Microbiol Immunol **23:**284–295.
16. **Nagy G, Takatsy S, Kukan E, Mihaly I, Domok I.** 1982. Virological diagnosis of enterovirus type 71 infections: experiences gained during an epidemic of acute CNS diseases in Hungary in 1978. Arch Virol **71:**217–227. http://dx.doi.org/10.1007/BF01314873.
17. **Hagiwara A, Tagaya I, Yoneyama T.** 1978. Epidemic of hand, foot and mouth disease associated with enterovirus 71 infection. Intervirology **9:**60–63. http://dx.doi.org/10.1159/000148922.
18. **Ishimaru Y, Nakano S, Yamaoka K, Takami S.** 1980. Outbreaks of hand, foot, and mouth disease by enterovirus 71: high incidence of complication disorders of central nervous system. Arch Dis Child **55:**583–588.
19. **McWilliam Leitch EC, Cabrerizo M, Cardosa J, Harvala H, Ivanova OE, Koike S, Kroes AC, Lukashev A, Perera D, Roivainen M, Susi P, Trallero G, Evans DJ, Simmonds P.** 2012. The association of recombination events in the founding and emergence of subgenogroup evolutionary lineages of human enterovirus 71. J Virol **86:**2676–2685. http://dx.doi.org/10.1128/JVI.06065-11.
20. **Tu PV, Thao NT, Perera D, Huu TK, Tien NT, Thuong TC, How OM, Cardosa MJ, McMinn PC.** 2007. Epidemiologic and virologic investigation of hand, foot, and mouth disease, southern Vietnam, 2005. Emerg Infect Dis **13:**1733–1741. http://dx.doi.org/10.3201/eid1311.070632.
21. **Tan X, Huang X, Zhu S, Chen H, Yu Q, Wang H, Huo X, Zhou J, Wu Y, Yan D, Zhang Y, Wang D, Cui A, An H, Xu W.** 2011. The persistent circulation of enterovirus 71 in People's Republic of China: causing emerging nationwide epidemics since 2008. PLoS One **6:**e25662. http://dx.doi.org/10.1371/journal.pone.0025662.
22. **Zhang H, An D, Liu W, Mao Q, Jin J, Xu L, Sun S, Jiang L, Li X, Shao J, Ma H, Huang X, Guo S, Chen H, Cheng T, Yang L, Su W, Kong W, Liang Z, Jiang C.** 2014. Analysis of cross-reactive neutralizing antibodies

in human HFMD serum with an EV71 pseudovirus-based assay. PLoS One **9:**e100545. http://dx.doi.org/10.1371/journal.pone.0100545.
23. **Li R, Liu L, Mo Z, Wang X, Xia J, Liang Z, Zhang Y, Li Y, Mao Q, Wang J, Jiang L, Dong C, Che Y, Huang T, Jiang Z, Xie Z, Wang L, Liao Y, Liang Y, Nong Y, Liu J, Zhao H, Na R, Guo L, Pu J, Yang E, Sun L, Cui P, Shi H, Wang J, Li Q.** 2014. An inactivated enterovirus 71 vaccine in healthy children. N Engl J Med **370:**829–837. http://dx.doi.org/10.1056/NEJMoa1303224.
24. **Zhu F, Xu W, Xia J, Liang Z, Liu Y, Zhang X, Tan X, Wang L, Mao Q, Wu J, Hu Y, Ji T, Song L, Liang Q, Zhang B, Gao Q, Li J, Wang S, Hu Y, Gu S, Zhang J, Yao G, Gu J, Wang X, Zhou Y, Chen C, Zhang M, Cao M, Wang J, Wang H, Wang N.** 2014. Efficacy, safety, and immunogenicity of an enterovirus 71 vaccine in China. N Engl J Med **370:**818–828. http://dx.doi.org/10.1056/NEJMoa1304923.
25. **Oberste MS, Maher K, Pallansch MA.** 2004. Evidence for frequent recombination within species human enterovirus B based on complete genomic sequences of all thirty-seven serotypes. J Virol **78:**855–867. http://dx.doi.org/10.1128/JVI.78.2.855-867.2004.
26. **Simmonds P, Welch J.** 2006. Frequency and dynamics of recombination within different species of human enteroviruses. J Virol **80:**483–493. http://dx.doi.org/10.1128/JVI.80.1.483-493.2006.
27. **World Health Organization.** 2014. 2011-2014: Emerging disease surveillance and response: hand, foot and mouth disease updates by year. World Health Organization, Geneva, Switzerland.
28. **Johnson FB.** 1990. Transport of viral specimens. Clin Microbiol Rev **3:**120–131.
29. **Tan LV, Tuyen NT, Thanh TT, Ngan TT, Van HM, Sabanathan S, Van TT, Thanh LT, Nguyet LA, Geoghegan JL, Chai OK, Perera D, Hang VT, Ny NT, Anh NT, Ha DQ, Qui PT, Viet DC, Tuan HM, Wong KT, Holmes EC, Chau NV, Thwaites G, van Doorn HR.** 2015. A generic assay for whole-genome amplification and deep sequencing of enterovirus A71. J Virol Methods **215:**30–36. http://dx.doi.org/10.1016/j.jviromet.2015.02.011.
30. **Liu W, Wu S, Xiong Y, Li T, Wen Z, Yan M, Qin K, Liu Y, Wu J.** 2014. Co-circulation and genomic recombination of coxsackievirus A16 and enterovirus 71 during a large outbreak of hand, foot, and mouth disease in Central China. PLoS One **9:**e96051. http://dx.doi.org/10.1371/journal.pone.0096051.
31. **Delport W, Poon AFY, Frost SDW, Pond SLK.** 2010. Datamonkey 2010: a suite of phylogenetic analysis tools for evolutionary biology. Bioinformatics **26:**2455–2457. http://dx.doi.org/10.1093/bioinformatics/btq429.
32. **Drummond AJ, Suchard MA, Xie D, Rambaut A.** 2012. Bayesian phylogenetics with BEAUti and the BEAST 1.7. Mol Biol Evol **29:**1969–1973. http://dx.doi.org/10.1093/molbev/mss075.
33. **Minin VN, Bloomquist EW, Suchard MA.** 2008. Smooth skyride through a rough skyline: Bayesian coalescent-based inference of population dynamics. Mol Biol Evol **25:**1459–1471. http://dx.doi.org/10.1093/molbev/msn090.
34. **Rambaut A, Drummond A.** 2007. Tracer version 1.4. http://beast.bio.edac.uk/Tracer.
35. **Parker J, Rambaut A, Pybus OG.** 2008. Correlating viral phenotypes with phylogeny: accounting for phylogenetic uncertainty. Infect Genet Evol **8:**239–246. http://dx.doi.org/10.1016/j.meegid.2007.08.001.
36. **Stadler T, Kühnert D, Bonhoeffer S, Drummond AJ.** 2013. Birth-death skyline plot reveals temporal changes of epidemic spread in HIV and hepatitis C virus (HCV). Proc Natl Acad Sci U S A **110:**228–233. http://dx.doi.org/10.1073/pnas.1207965110.
37. **Tran CBN, Nguyen HT, Phan HTT, Tran NV, Wills B, Farrar J, Santangelo JD, Simmons CP.** 2011. The seroprevalence and seroincidence of enterovirus71 infection in infants and children in Ho Chi Minh City, Viet Nam. PLoS One **6:**e21116. http://dx.doi.org/10.1371/journal.pone.0021116.
38. **Ma E, Fung C, Yip SHL, Wong C, Chuang SK, Tsang T.** 2011. Estimation of the basic reproduction number of enterovirus 71 and coxsackievirus A16 in hand, foot, and mouth disease outbreaks. Pediatr Infect Dis J **30:**675–679. http://dx.doi.org/10.1097/INF.0b013e3182116e95.
39. **AbuBakar S, Sam IC, Yusof J, Lim MK, Misbah S, MatRahim N, Hooi P-S.** 2009. Enterovirus 71 outbreak, Brunei. Emerg Infect Dis **15:**79–82. http://dx.doi.org/10.3201/eid1501.080264.
40. **Chua KB, Kasri AR.** 2011. Hand foot and mouth disease due to enterovirus 71 in Malaysia. Virol Sin **26:**221–228. http://dx.doi.org/10.1007/s12250-011-3195-8.
41. **Huang S-W, Hsu Y-W, Smith DJ, Kiang D, Tsai H-P, Lin K-H, Wang S-M, Liu C-C, Su I-J, Wang J-R.** 2009. Reemergence of enterovirus 71 in 2008 in Taiwan: dynamics of genetic and antigenic evolution from 1998 to 2008. J Clin Microbiol **47:**3653–3662. http://dx.doi.org/10.1128/JCM.00630-09.
42. **WHO.** 2015. Emerging disease surveillance and response: hand, foot and mouth disease (HFMD). World Health Organization, Geneva, Switzerland. http://www.wpro.who.int/emerging_diseases/HFMD/en/.
43. **Huang Y-P, Lin T-L, Lin T-H, Wu H-S.** 2013. Antigenic and genetic diversity of human enterovirus 71 from 2009 to 2012, Taiwan. PLoS One **8:**e80942. http://dx.doi.org/10.1371/journal.pone.0080942.
44. **Sanden Svd Avoort Hvd Lemey P, Uslu G, Koopmans M.** 2010. Evolutionary trajectory of the VP1 gene of human enterovirus 71 genogroup B and C viruses. J Gen Virol **91:**1949–1958. http://dx.doi.org/10.1099/vir.0.019695-0.
45. **Huang YP, Lin TL, Hsu LC, Chen YJ, Tseng YH, Hsu CC, Fan WB, Yang JY, Chang FY, Wu HS.** 2010. Genetic diversity and C2-like subgenogroup strains of enterovirus 71, Taiwan, 2008. Virology **7:**277–288. http://dx.doi.org/10.1186/1743-422X-7-277.
46. **Lee MS, Lin TY, Chiang PS, Li WC, Luo ST, Tsao KC, Liou GY, Huang ML, Hsia SH, Huang YC, Chang SC.** 2010. An investigation of epidemic enterovirus 71 infection in Taiwan, 2008: clinical, virologic, and serologic features. Pediatr Infect Dis J **29:**1030–1034. http://dx.doi.org/10.1097/INF.0b013e3181e52945.
47. **Huang ML, Chiang PS, Chia MY, Luo ST, Chang LY, Lin TY, Ho MS, Lee MS.** 2013. Cross-reactive neutralizing antibody responses to enterovirus 71 infections in young children: implications for vaccine development. PLoS Negl Trop Dis **7:**e2067. http://dx.doi.org/10.1371/journal.pntd.0002067.
48. **Sabin AB.** 1952. Research on dengue during World War II. Am J Trop Med Hyg **1:**30–50.
49. **Kreijtz JH, Fouchier RA, Rimmelzwaan GF.** 2011. Immune responses to influenza virus infection. Virus Res **162:**19–30. http://dx.doi.org/10.1016/j.virusres.2011.09.022.
50. **Mizuta K, Aoki Y, Suto A, Ootani K, Katsushima N, Itagaki T, Ohmi A, Okamoto M, Nishimura H, Matsuzaki Y, Hongo S, Sugawara K, Shimizu H, Ahiko T.** 2009. Cross-antigenicity among EV71 strains from different genogroups isolated in Yamagata, Japan, between 1990 and 2007. Vaccine **27:**3153–3158. http://dx.doi.org/10.1016/j.vaccine.2009.03.060.
51. **Wang Y, Feng Z, Yang Y, Self S, Gao Y, Longini IM, Wakefield J, Zhang J, Wang L, Chen X, Yao L, Stanaway JD, Wang Z, Yang W.** 2011. Hand, foot, and mouth disease in China: patterns of spread and transmissibility during 2008-2009. Epidemiology **22:**781–792. http://dx.doi.org/10.1097/EDE.0b013e318231d67a.
52. **Fine PEM, Carneiro IAM.** 1999. Transmissibility and persistence of oral polio vaccine viruses: implications for the global poliomyelitis eradication initiative. Am J Epidemiol **150:**1001–1021. http://dx.doi.org/10.1093/oxfordjournals.aje.a009924.
53. **Chung P-W, Huang Y-C, Chang L-Y, Lin T-Y, Ning H-C.** 2001. Duration of enterovirus shedding in stool. J Microbiol Immunol Infect **34:**167–170.
54. **World Health Organization.** 2011. A guide to clinical management and public health response for hand, foot and mouth disease (HFMD). World Health Organization, Geneva, Switzerland.

Journal of Virological Methods 215–216 (2015) 30–36

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

A generic assay for whole-genome amplification and deep sequencing of enterovirus A71

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Article history:
Received 22 October 2014
Received in revised form 24 January 2015
Accepted 11 February 2015
Available online 19 February 2015

Keywords:
Enterovirus A71
Picornavirus
Hand foot and mouth disease
Deep sequencing
Phylogeny

A B S T R A C T

Enterovirus A71 (EV-A71) has emerged as the most important cause of large outbreaks of severe and sometimes fatal hand, foot and mouth disease (HFMD) across the Asia-Pacific region. EV-A71 outbreaks have been associated with (sub)genogroup switches, sometimes accompanied by recombination events. Understanding EV-A71 population dynamics is therefore essential for understanding this emerging infection, and may provide pivotal information for vaccine development. Despite the public health burden of EV-A71, relatively few EV-A71 complete-genome sequences are available for analysis and from limited geographical localities. The availability of an efficient procedure for whole-genome sequencing would stimulate effort to generate more viral sequence data. Herein, we report for the first time the development of a next-generation sequencing based protocol for whole-genome sequencing of EV-A71 directly from clinical specimens. We were able to sequence viruses of subgenogroup C4 and B5, while RNA from culture materials of diverse EV-A71 subgenogroups belonging to both genogroup B and C was successfully amplified. The nature of intra-host genetic diversity was explored in 22 clinical samples, revealing 107 positions carrying minor variants (ranging from 0 to 15 variants per sample). Our analysis of EV-A71 strains sampled in 2013 showed that they all belonged to subgenogroup B5, representing the first report of this subgenogroup in Vietnam. In conclusion, we have successfully developed a high-throughput next-generation sequencing-based assay for whole-genome sequencing of EV-A71 from clinical samples.

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1. Introduction

Enterovirus A71 (EV-A71) belongs to the Enterovirus A species of the family *Picornaviridae*, and is genetically divided into three genogroups (A, B, and C). The latter two are further divided into subgenogroups, denoted B0 – 5 and C1 – 5, respectively. Since 1997, EV-A71 has emerged as the most important cause of large outbreaks of severe and sometimes fatal hand, foot and mouth disease (HFMD) across the Asia-Pacific region (Solomon et al., 2010; Xing et al., 2014). In Vietnam, EV-A71-related HFMD was first described in 2003, and became a notifiable illness in 2008. Between 2011 and 2012, more than 200,000 hospitalized cases due to HFMD were reported in Vietnam, of which 207 died as a consequence of clinical complications (including cardio-pulmonary compromise with

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http://dx.doi.org/10.1016/j.jviromet.2015.02.011
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pulmonary edema or hemorrhage) (Khanh et al., 2012; Nguyen et al., 2014). There was a strong association between the detection of EV-A71 and severity and death in this outbreak (Khanh et al., 2012; Nguyen et al., 2014). Although phase III trials of three different inactivated EV-A71 vaccines from China were recently completed with 95% protection rates against EV-A71 associated HFMD (Zhu et al., 2014; Zhu et al., 2013; Li et al., 2014), there are currently no vaccines or specific antiviral drugs available for EV-A71.

Large EV-A71 outbreaks have been associated with (sub)genogroup switches, sometimes accompanied by recombination events (Solomon et al., 2010; McWilliam Leitch et al., 2012; Tee et al., 2010). Similarly, sequence data from Vietnam obtained between 2005 and 2011 show a subgenogroup switch from C5 to C4 in 2011 (Khanh et al., 2012; Tu et al., 2007). Of note, this C4 subgenogroup was also associated with the epidemic in China in 2008 and with a recent outbreak in Cambodia (Xing et al., 2014; Tan et al., 2011; Seiff, 2012). Interestingly, phylogenetic analyses and molecular clock dating suggest that strain replacement commonly occurs in EV-A71 (Tan et al., 2011), and that emerging subgenogroups may circulate cryptically in the community at low prevalence for years and cause mild infection prior to outbreak emergence (Tee et al., 2010). Critically, it is unclear whether viral evolution is the driver of emergence and large outbreaks of HFMD in Southeast Asia, or a consequence of the greater number of infected hosts. The former would have profound implications for vaccine development, necessitating active surveillance and periodic vaccine updates. Taken together the available data highlight the importance of understanding EV-A71 evolution and population dynamics within and between endemic countries, which may be essential for understanding and controlling this emerging infection, including vaccine development.

Despite the public health burden of EV-A71, relatively few (~500) EV-A71 complete genome sequences are available for analysis and from limited geographical localities, compared to ~4500 sequences of A/H3N2 human influenza virus alone (Viboud et al., 2013). Hence, the availability of an efficient procedure for whole-genome sequencing would be an important step toward stimulating scientific effort to generate more viral sequence data. Herein, we report the development of a complete genome sequencing protocol for EV-A71 directly from clinical specimens using RT-PCR amplification of three overlapping amplicons and next-generation sequencing technology.

2. Materials and methods

2.1. Patients and Clinical specimens

Samples used in this study were derived from patients enrolled in an ongoing three-year prospective observational study of HFMD patients of all severities (including out- and in patients) admitted to the outpatient clinics, infectious diseases wards, and the paediatric intensive care units in three major referral hospitals in Ho Chi Minh City, Vietnam. This study utilized 65/82 consecutive EV-A71 RT-PCR positive throat swabs collected in viral transport medium from patients enrolled between July 2013 and December 2013 at the Hospital for Tropical Diseases (HTD) and Children's Hospital 2. For the purpose of assay evaluation, the swabs with a wide range of viral loads were selected based on real time RT-PCR crossing point (Cp) values of between 24 and 36 (i.e. from high to low viral load).

2.2. Virus isolates

An EV-A71 subgenogroup C4 isolate from a child with HFMD admitted to HTD during the 2011 outbreak, and eight different isolates each belonging to a different subgenogroup (Table 2) obtained from the Department of Biomedical Science, University of Malaya, Malaysia were used for the initial assessment of assay performance.

2.3. Primer design

Overlapping primer pairs were designed for the amplification of three amplicons spanning the entire EV-A71 genome. The primers were derived either from previous publications (Khanh et al., 2012) or newly designed based on the alignment of 279 complete genome sequences of all EV-A71 subgenogroups available in GenBank (Genogroup A, $n = 1$; B0, 2; B1, 14; B2, 4; B3, 8; B4, 11; B5, 18; C1, 17; C2, 33; C3, 3; C4, 165; C5, 2, and undefined, 1; accessed in April 2013). Sequence alignment was performed using MUSCLE available within the Geneious 7.1.3 software package (http://www.geneious.com). The resulting alignment was used to identify conserved regions for selection of PCR primers. The location of primers was chosen to generate PCR fragments with an overlap of 400 nucleotides (nt) or more, allowing for proper sequence assembly. To avoid compromise of PCR sensitivity, a maximum of two degenerate nucleotides were introduced per primer. As a consequence, in some occasions, two or more primers targeting one genomic region were selected for amplification of diverse EV-A71 subgenogroups. Primer sequences, genomic locations, amplified fragment lengths, and alignment profiles of primer binding sites are shown in Table 1 and Supplementary Appendix Fig. 1.

2.4. Nucleic acid isolation

Viral RNA was extracted from 140 μl of culture supernatant or clinical material using the QIAamp viral RNA kit (QIAgen GmbH, Hilden, Germany), recovered in 50 μl of elution buffer (provided with the kit) and was either immediately subjected to RT-PCR amplification or stored at −80 °C for subsequent use.

2.5. RT-PCR

RT-PCR was performed using SuperScript III One-Step RT-PCR system with Platinum *Taq* DNA High Fidelity (Invitrogen, Carlsbad, CA, USA) in a 25 μl reaction consisting of 800 nM of each primer (Table 1), 12.5 μl of 2× reaction buffer (provided with the kit), 0.5 μl of SuperScriptIII RT/Platinum *Taq* High Fidelity mix and 4 μl of template RNA. RT-PCR reaction was performed in a Mastercycler (Eppendorf, Hamburg, Germany). Cycling conditions are specified in Table 1.

2.6. Genome sequencing, sequence assembly and minor variation detection

For each sample, PCR products were quantified by a fluorescence-based dsDNA quantification method using the Quant-iT dsDNA Assay Kit in a Qubit fluorometer (Invitrogen) and then pooled with an equal quantity of each individual PCR amplicon. One nanogram of pooled DNA from individual samples was then subjected to library preparation using the Nextera XT DNA sample preparation kit (Illumina, San Diego, CA, USA), in which each sample was assigned to a unique barcode sequence using the Nextera XT Index Kit (Illumina). Sequencing of the prepared library was carried out using the Miseq reagent kit v2 (300 cycles, Illumina) in an Illumina Miseq platform. A total of 24 samples were sequenced in a single run. The reads obtained were processed to remove PCR primers using CLC Genomics Workbench (QIAgen).

Sequence assembly was performed using the Geneious 7.1.3 software package utilizing a reference-based mapping tool (i.e. the consensus sequence was obtained by mapping individual reads of each sample to a reference sequence). Finally, screening of minor (sub-consensus) variants was performed using the SNP detection

Table 1
Primer sequences, location and size of amplified products.

RT-PCR[a]	Primer	Oligo sequence (5′–3′)	Orientation	Start–end[b]	Genomic locations	Product size (Kb)
1	FL5F	TTAAAACAGCCTGTGGGTTGYACC	Forward	1–24	5′UTR	3.2
	FL9R	ACTAAAGGGTACTTGGACTTVGA	Reverse	3180–3158	VP1	
2	FL15F	GGATTRGTWGGGGAGATAGACCT	Forward	2699–2721	VP1	
	FL16F	GGATTRGTWGGAGAGATAGACCT	Forward	2699–2721	VP1	
	FL17F	GGATTRGTWGGGGAGATAGATCT	Forward	2699–2721	VP1	2.1
	FL18F	GGATTRGTWGGAGAGATAGATCT	Forward	2699–2721	VP1	
	FL19R	GTCACTTCAATRTCRCAGTCCAT	Reverse	4827–4805	2C	
	FL20R	GTCACTTCAATRTCRCAATCCAT	Reverse	4827–4805	2C	
3	FL10F	CAAACACCGWATTGAACCTGT	Forward	4423–4443	2C	2.9
	FL3R2	GCTATTCTGGTTATAACAAATTTACC	Reverse	7405–7380	3′UTR	

Note:
[a] Thermal cycling conditions of RT-PCR 1: 1 cycle of 60 °C, 3 min, 53 °C, 30 min, 94 °C, 2 min, 45 cycles of 94 °C, 15 s, 53 °C, 30 s, 68 °C, 4 min and 1 cycle of 68 °C, 5 min; and RT-PCR 2 and 3: 1 cycle of 60 °C, 3 min, 50 °C, 30 min, 94 °C, 2 min, 45 cycles of 94 °C, 15 s, 50 °C, 30 s, 68 °C, 3.5 min and 1 cycle of 68 °C, 5 min.
[b] Positions of primers are given based on the genomic sequence of an EV-A71 strain from Vietnam, Vietnam/540 V/VNM/05/C4 (GenBank accession JQ965759.1); Y = C/T, V = A/C/G, R = A/G, W = A/T;

tool available in Geneious. A minimum variant frequency of 5% and 500-fold coverage were chosen as cut-off values.

2.7. Sequence analysis of the obtained consensuses

Sequence alignment of the consensus genomes obtained in this study was performed using MUSCLE (Edgar, 2004), and the phylogenetic relationships among these data and 25 representative sequences taken from GenBank were estimated using the maximum likelihood (ML) PhyML method available within the Geneious package. The ML phylogenetic analysis utilized the general time reversible (GTR) nucleotide substitution model (Guindon and Gascuel, 2003), and support for individual nodes was assessed using a bootstrap procedure (1000 replicates).

2.8. Sequence accession numbers

The sequences of EV-A71 obtained in this study were submitted to NCBI (GenBank) and assigned accession numbers KP691643–KP691666.

2.9. Ethical statement

The studies from which clinical specimens were selected for use in this study were reviewed and approved by the Institutional Review Boards of the sites of enrolment in Ho Chi Minh City, Vietnam and the Oxford Tropical Research Ethics Committee (OxTREC), University of Oxford, Oxford, United Kingdom.

3. Results

3.1. Whole-genome RT-PCRs

After examining the complete genome sequence alignment of EV-A71, primer pairs for three overlapping RT-PCRs spanning the whole genome were designed. To assess their performance, we first tested the assays on RNA derived from culture supernatants of different EV-A71 subgenogroups. Notably, all three RT-PCRs were able to amplify viral RNA from each sample (Table 2).

The overlapping PCRs were further tested on 65 EV-A71 RT-PCR-positive throat swab samples collected from children with HFMD. All three fragments were successfully amplified in 50/65 (77%) samples (Fig. 1). Eleven out of 15 samples that were not amplified by the three overlapping PCRs were due to failure of the 3.2Kb PCRs (details in Supplementary Appendix Table 1). A significantly higher viral load (as suggested by Cp values generated by an EV-A71 real-time RT-PCR (Khanh et al., 2012)) was observed among samples that were amplified by all three RT-PCRs compared to samples that were not (Fig. 2). Accordingly, clinical samples with a Cp value of 30 or less allowed the successful amplification of the three genomic fragments.

3.2. Genome sequencing using the Illumina platform

Amplified products from 23 clinical samples and an EV-A71 isolate were sequenced in one batch. The run resulted in an output of 4.9 Gb, of which 60% could be successfully assembled. Over 92% of the assembled bases had Phred quality scores of ≥30 (i.e. a probability of ≥99.9% that the single bases were correctly sequenced). The remaining bases had Phred quality scores of between >10 and 29 (i.e. >90% of accuracy). Twenty-three complete genome sequences (including 22 from clinical samples and one from virus culture material) and one near-complete genome sequence of EV-A71 were successfully assembled. A coverage of ≥500-fold was achieved in 22 samples (including 21 clinical samples and the virus isolate) and

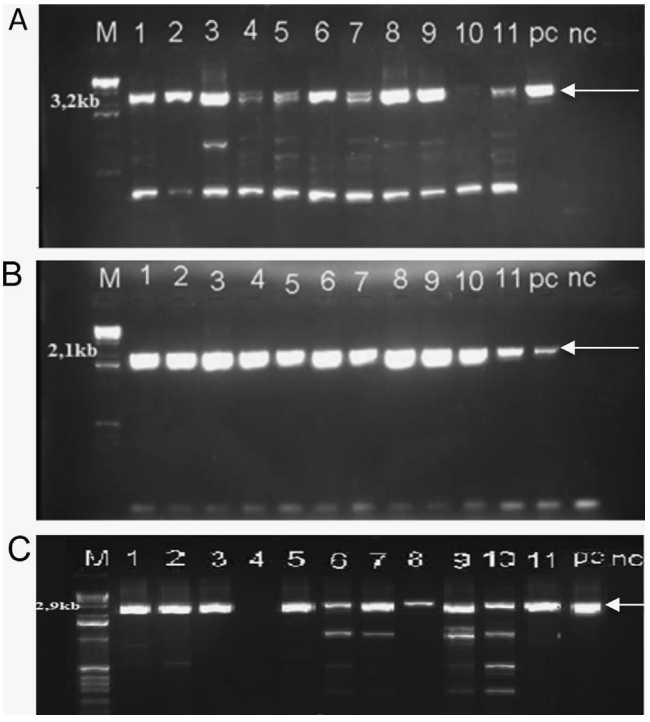


Fig. 1. Agarose gel electrophoresis analysis of amplified products from 11 throat swabs from HFMD patients; (A) PCR 1; (B) PCR 2; (C) PCR 3; lanes 1-11: clinical samples, PC: positive control; NC: negative control; amplified products of expected sizes are indicated by arrows.

Table 2
Virus isolates and results of overlapping RT-PCRs.

EV-A71 strain	Subgeno-group	Accession number	Location	Year of isolation	Overlapping RT-PCRs	Subjected to Miseq sequencing
9522	C1	AY258300	Malaysia	2003	Positive	No
8M/6/99	C2	AY126012	Australia	1999	Positive	No
001-KOR-00	C3	AY125966	Korea	2000	Positive	No
VN152	C4	Not available	Vietnam	2011	Positive	Yes
VN5559	C4	AM490152	Vietnam	2005	Positive	No
VN5784	C5	AM490158	Vietnam	2005	Positive	No
13903	B3	AY207648	Malaysia	1997	Positive	No
A10/4	B4	AF376067	Malaysia	2000	Positive	No
18431	B5	Not available	Malaysia	2006	Positive	No

>80-fold in one other. Details of sequencing output per sample are presented in Supplementary Appendix Table 2.

Phylogenetic analysis suggested that all viruses derived from throat swabs collected in 2013 belonged to EV-A71 subgenogroup B5 (Fig. 3), while the virus from culture material belonged to subgenogroup C4. This is in agreement with our initial typing result based on sequencing of VP1 region (data not shown).

3.3. Minor variant detection

Using the criteria described in the Methods section, we identified a total of 107 positions in the EV-A71 genome carrying minor variants (ranging from 0 to 15 variants per sample), of which 15 (14%) were nonsynonymous. Interestingly, 73% (11/15) of the nonsynonymous variants were present in the nonstructural protein coding region, which also contained 65% of the total 107 variants observed, with mutations in the 2C protein being the next most frequent (38/107, 35.5%). Most variants (101) represented minor variants. However six variants were found to be either minor or dominant (i.e. >50%) variants in different samples, suggesting that they may impact viral fitness in some way, and hence merit additional investigation. More details on the minor variants are presented Supplementary Appendix Table 3, including the frequency of the six variants shared between viruses found in the data set of 279 genome sequences used in the present study.

4. Discussion

We have successfully developed a high-throughput Illumina Miseq-based generic assay for direct whole-genome sequencing of EV-A71 from clinical samples. Although primers for whole-genome amplification of EV-A71 have been proposed previously (Shih et al., 2000; Cordey et al., 2012; Huang et al., 2009; Yoke-Fun and AbuBakar, 2006; Chang et al., 2012; Zhang et al., 2013), these primer sets were either large (i.e. at least eight combinations were used) and/or not tested on diverse EV-A71 subgenogroups. The procedure described here only requires three overlapping PCRs to amplify the entire virus genome directly from clinical specimens. In addition, the entire procedure from nucleic acid extraction and PCR amplification to obtaining a total of between 24 and 96 virus genomes takes approximately 5 days (Flowchart 1). The total reagent-associated cost is $120 USD per sample if 24 samples are multiplexed in one run (date of cost assessment: July 2014). This can be reduced to $70 USD if 96 samples are combined. In addition, with the great sequencing depth (i.e. a single nucleotide is sequenced at least 500 times), the extent

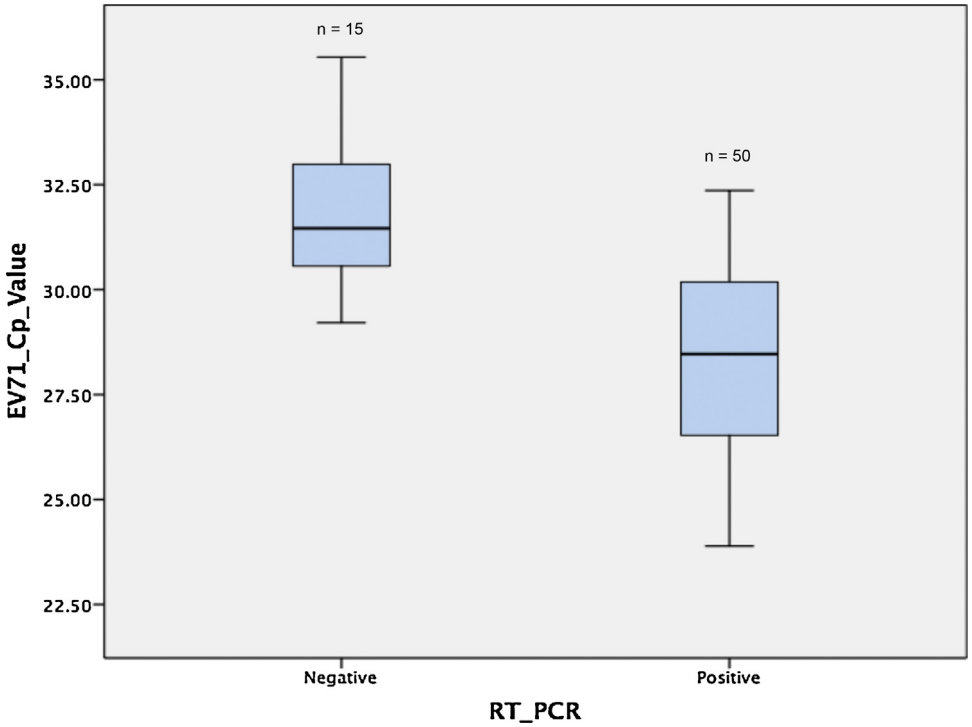


Fig. 2. Boxplots showing Cp values generated by an EV-A71 real time RT-PCR of RT-PCR positive and negative groups, Cp value median; range: 28.2; 23.9–32.4 versus 31.2; 29.2–35.5; $P < 0.001$.

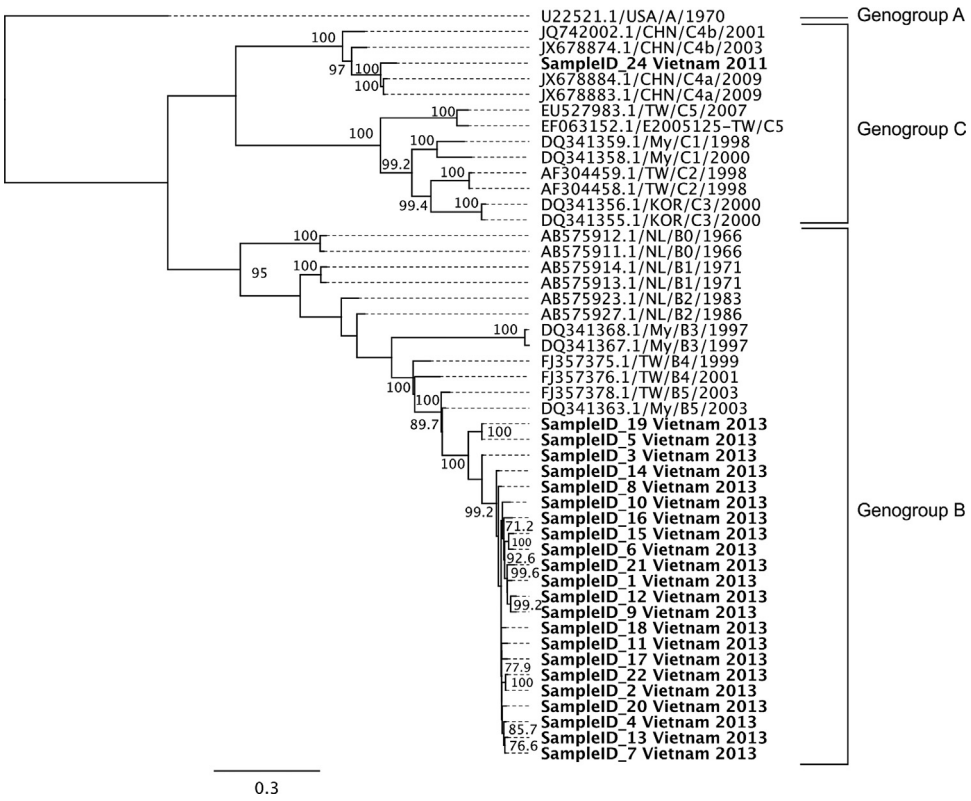
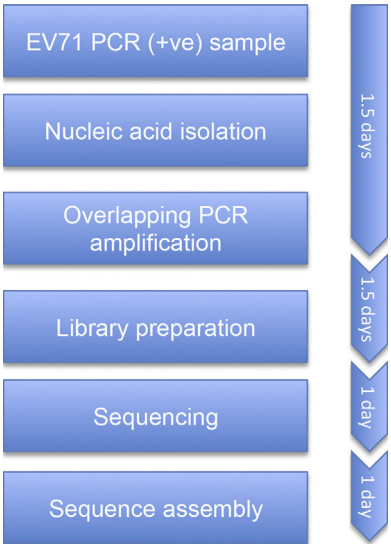


Fig. 3. ML phylogeny based on complete genomes (in bold) obtained in this study and those of representatives of EV-A71 downloaded from the GenBank. A similar result was obtained when VP1 sequences were analyzed separately (data not shown). The tree is rooted on a single genogroup A sequence (USA/A/1970) and all horizontal branch lengths are drawn to a scale of nucleotide substitutions per site. Bootstraps >70% are also shown. My: Malaysia, TW: Taiwan, NL: the Netherlands, KOR: Korea, CHN: China.



Flowchart 1. Chart showing analysis procedure for obtaining the complete genome of EV-A71 from clinical samples.

and pattern of intra-host genetic diversity data can be explored in addition to the consensus sequence. It should however be noted that although the current cost per genome is affordable, the total cost per run (~$3000 USD) together with hardware-associated cost combined with bioinformatics challenges may remain the major obstacles preventing Illumina based whole-genome sequencing assay from being widely used, in particular in developing countries including parts of Southeast Asia where EV-A71 and HFMD are endemic. Alternatively, generating full-length virus sequence can be achieved using Sanger-sequencing-based strategies (e.g. DNA walking of the obtained PCR amplicons described in the present study and/or multiple small overlapping PCRs spanning the whole virus genome). While the associated cost per genome of such strategies might be comparable with that of Illumina Miseq one, generating full-length virus genome using Sanger sequencing technology is a laborious procedure as it not possible to multiplex between 24 and 96 samples per run. Likewise, with Sanger-sequencing data, exploring the pattern of intra-host diversity of the pathogen at a level of frequency of 5% is unachievable.

Using our procedure we were able to sequence viruses of subgenogroup C4 and B5. Likewise, RNA from culture materials of diverse EV-A71 subgenogroups belonging to both genogroup B and C (Table 2) that have been associated with outbreaks of HFMD in the Asia-Pacific region since the 1990s was successfully amplified. Assuming that a Cp value of ~30 is the threshold of assay limit of detection (Fig. 2), our observational data on Cp values obtained from 824 throat swabs from HFMD patients in Vietnam (data not shown) show that 75% of viruses in RT-PCR positive throat swabs can be sequenced by our assay. Of note, among 65 tested swabs, in the majority of cases the procedure failure was attributed to the RT-PCRs that generated the largest amplicon (RT-PCR 1) and/or targeted at the genetically variable regions of the virus genome (RT-PCR 2) (Supplementary Appendix Table 1), which may suggest that fragment size and/or sequence variations play a part in addition to the viral-load factor.

We conservatively chose cut-off values of 500-fold coverage and a frequency of 5% for detection of minor variants. This is well above the next-generation sequencing error rate of 0.1% (Archer et al., 2012), while a previous study suggested that a coverage of >400-fold is required for reliable detection of minor variants present at 1% in next generation sequencing data (Wang et al., 2007).

Although an association between EV-A71 subgenogroups and disease severity has not been identified, previous studies have shown potential associations between single nucleotide variants and clinical severity (Cordey et al., 2012; Zhang et al., 2014). In addition, a recent study of C4, B1, B2 and B5 sequences found that nonsynonymous and synonymous substitutions occurred more frequently in the nonstructural than the structural protein-coding region, suggesting that the former was a major fitness determinant (Huang et al., 2014). We similarly observed a majority of minor variants (including nonsynonymous ones) in the nonstructural protein-coding region, although the function of these mutations is uncertain. As a consequence, these data further emphasize the need to study viral diversity and evolution at the whole-genome level, rather than in the VP1 protein alone which has been the main focus to date. Likewise, exploring the association between specific viral genotypes/single nucleotide polymorphisms and clinical phenotype is clearly an area of importance, although beyond the scope of the present study.

Our analysis of EV-A71 strains sampled in 2013 showed that they all belonged to subgenogroup B5, representing the first report of this subgenogroup in Vietnam, while subgenogroup C4 caused the large outbreak of HFMD that occurred in Vietnam in 2011–12 (Khanh et al., 2012). Subgenogroup switches commonly occur in other countries of Asia-Pacific region where EV-A71 and HFMD are endemic (Solomon et al., 2010), although the evolutionary and epidemiological processes responsible for these switches are still unclear. Taken together, these data highlight the importance of studying spatial and temporal dynamics of EV-A71 across endemic countries, which may in turn inform the development of intervention strategies, including vaccine development and implementation, and can now be facilitated by the availability of a high throughput/cost-effective whole-genome sequencing procedure.

In conclusion, we have successfully developed a high-throughput next-generation sequencing-based generic assay for direct whole-genome sequencing of EV-A71 from clinical samples that provides important insights into viral diversity and evolution.

Funding

The research leading to these results has received funding from the Wellcome Trust of Great-Britain (101104/Z/13/Z and 089276/Z/09/Z), and the Li Ka Shing Foundation–University of Oxford Global Health Program strategic award (LG23). ECH is supported by an NHMRC Australia Fellowship. OKC is supported by a High Impact Research Grant (H20001-E00004) from the Ministry of Education, Malaysia Government and University of Malaya Research Grant (RG480/12HTM). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Conflict of interest

No conflict of interest.

Acknowledgements

We thank Ms Le Kim Thanh from Oxford University Clinical Research Unit in Ho Chi Minh City, Vietnam for her logistic assistance, Ms Ho Bao Khuyen and her colleagues at Biomedic company for their help with the Miseq run, and Truong Duy for the figure of the ML tree. We are indebted to patients and their parents for their participation in this study, and all the nursing and medical staff at the paediatric ICU, infectious diseases wards and outpatient clinics at Children's Hospital 2 and the Hospital for Tropical Diseases who provided care for the patients and helped collect clinical data. Saraswathy Sabanathan and Phan Tu Qui are registered for a PhD at the Open University UK, and Hoang Minh Tu Van at the Oxford University UK.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jviromet.2015.02.011.

References

Archer, J., Baillie, G., Watson, S.J., Kellam, P., Rambaut, A., Robertson, D.L., 2012. Analysis of high-depth sequence data for studying viral diversity: a comparison of next generation sequencing platforms using Segminator II. BMC Bioinformatics 13, 47.

Chang, S.C., Li, W.C., Chen, G.W., Tsao, K.C., Huang, C.G., Huang, Y.C., Chiu, C.H., Kuo, C.Y., Tsai, K.N., Shih, S.R., Lin, T.Y., 2012. Genetic characterization of enterovirus 71 isolated from patients with severe disease by comparative analysis of complete genomes. J. Med. Virol. 84 (6), 931–939.

Cordey, S., Petty, T.J., Schibler, M., Martinez, Y., Gerlach, D., van Belle, S., Turin, L., Zdobnov, E., Kaiser, L., Tapparel, C., 2012. Identification of site-specific adaptations conferring increased neural cell tropism during human enterovirus 71 infection. PLoS Pathog. 8 (7), e1002826.

Edgar, R.C., 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32 (5), 1792–1797.

Guindon, S., Gascuel, O., 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst. Biol. 52 (5), 696–704.

Huang, S.W., Cheng, H.L., Hsieh, H.Y., Chang, C.L., Tsai, H.P., Kuo, P.H., Wang, S.M., Liu, C.C., Su, I.J., Wang, J.R., 2014. Mutations in the non-structural protein region contribute to intra-genotypic evolution of enterovirus 71. J. Biomed. Sci. 21, 33.

Huang, S.W., Hsu, Y.W., Smith, D.J., Kiang, D., Tsai, H.P., Lin, K.H., Wang, S.M., Liu, C.C., Su, I.J., Wang, J.R., 2009. Reemergence of enterovirus 71 in 2008 in taiwan: dynamics of genetic and antigenic evolution from 1998 to 2008. J. Clin. Microbiol. 47 (11), 3653–3662.

Khanh, T.H., Sabanathan, S., Thanh, T.T., Thoa le, P.K., Thuong, T.C., Hang, V., Farrar, J., Hien, T.T., Chau, N., van Doorn, H.R., 2012. Enterovirus 71-associated hand, foot, and mouth disease, Southern Vietnam, 2011. Emerg. Infect. Dis. 18 (12), 2002–2005.

Li, R., Liu, L., Mo, Z., Wang, X., Xia, J., Liang, Z., Zhang, Y., Li, Y., Mao, Q., Wang, J., Jiang, L., Dong, C., Che, Y., Huang, T., Jiang, Z., Xie, Z., Wang, L., Liao, Y., Liang, Y., Nong, Y., Liu, J., Zhao, H., Na, R., Guo, L., Pu, J., Yang, E., Sun, L., Cui, P., Shi, H., Wang, J., Li, Q., 2014. An inactivated enterovirus 71 vaccine in healthy children. N. Engl. J. Med. 370 (9), 829–837.

McWilliam Leitch, E.C., Cabrerizo, M., Cardosa, J., Harvala, H., Ivanova, O.E., Koike, S., Kroes, A.C., Lukashev, A., Perera, D., Roivainen, M., Susi, P., Trallero, G., Evans, D.J., Simmonds, P., 2012. The association of recombination events in the founding and emergence of subgenogroup evolutionary lineages of human enterovirus 71. J. Virol. 86 (5), 2676–2685.

Nguyen, N.T., Pham, H.V., Hoang, C.Q., Nguyen, T.M., Nguyen, L.T., Phan, H.C., Phan, L.T., Vu, L.N., Tran Minh, N.N., 2014. Epidemiological and clinical characteristics of children who died from hand, foot and mouth disease in Vietnam, 2011. BMC Infect. Dis. 14, 341.

Seiff, A., 2012. Cambodia unravels cause of mystery illness. Lancet 380 (9838), 206.

Shih, S.R., Ho, M.S., Lin, K.H., Wu, S.L., Chen, Y.T., Wu, C.N., Lin, T.Y., Chang, L.Y., Tsao, K.C., Ning, H.C., Chang, P.Y., Jung, S.M., Hsueh, C., Chang, K.S., 2000. Genetic analysis of enterovirus 71 isolated from fatal and non-fatal cases of hand, foot and mouth disease during an epidemic in Taiwan, 1998. Virus Res. 68 (2), 127–136.

Solomon, T., Lewthwaite, P., Perera, D., Cardosa, M.J., McMinn, P., Ooi, M.H., Virology, 2010. epidemiology, pathogenesis, and control of enterovirus 71. Lancet Infect. Dis. 10 (11), 778–790.

Tan, X., Huang, X., Zhu, S., Chen, H., Yu, Q., Wang, H., Huo, X., Zhou, J., Wu, Y., Yan, D., Zhang, Y., Wang, D., Cui, A., An, H., Xu, W., 2011. The persistent circulation of enterovirus 71 in People's Republic of China: causing emerging nationwide epidemics since 2008. PLoS ONE 6 (9), e25662.

Tee, K.K., Lam, T.T., Chan, Y.F., Bible, J.M., Kamarulzaman, A., Tong, C.Y., Takebe, Y., Pybus, O.G., 2010. Evolutionary genetics of human enterovirus 71: origin, population dynamics, natural selection, and seasonal periodicity of the VP1 gene. J. Virol. 84 (7), 3339–3350.

Tu, P.V., Thao, N.T., Perera, D., Huu, T.K., Tien, N.T., Thuong, T.C., How, O.M., Cardosa, M.J., McMinn, P.C., 2007. Epidemiologic and virologic investigation of hand, foot, and mouth disease, southern Vietnam, 2005. Emerg. Infect. Dis. 13 (11), 1733–1741.

Viboud, C., Nelson, M.I., Tan, Y., Holmes, E.C., 2013. Contrasting the epidemiological and evolutionary dynamics of influenza spatial transmission. Philos. Trans. R. Soc. Lond. B: Biol. Sci. 368 (1614), 20120199.

Wang, C., Mitsuya, Y., Gharizadeh, B., Ronaghi, M., Shafer, R.W., 2007. Characterization of mutation spectra with ultra-deep pyrosequencing: application to HIV-1 drug resistance. Genome Res. 17 (8), 1195–1201.

Xing, W., Liao, Q., Viboud, C., Zhang, J., Sun, J., Wu, J.T., Chang, Z., Liu, F., Fang, V.J., Zheng, Y., Cowling, B.J., Varma, J.K., Farrar, J.J., Leung, G.M., Yu, H., 2014. Hand,

foot, and mouth disease in China, 2008–12: an epidemiological study. Lancet Infect. Dis. 14 (4), 308–318.

Yoke-Fun, C., AbuBakar, S., 2006. Phylogenetic evidence for inter-typic recombination in the emergence of human enterovirus 71 subgenotypes. BMC Microbiol. 6, 74.

Zhang, B., Wu, X., Huang, K., Li, L., Zheng, L., Wan, C., He, M.L., Zhao, W., 2014. The variations of VP1 protein might be associated with nervous system symptoms caused by enterovirus 71 infection. BMC Infect. Dis. 14, 243.

Zhang, Y., Tan, X., Cui, A., Mao, N., Xu, S., Zhu, Z., Zhou, J., Shi, J., Zhao, Y., Wang, X., Huang, X., Zhu, S., Zhang, Y., Tang, W., Ling, H., Xu, W., 2013. Complete genome analysis of the C4 subgenotype strains of enterovirus 71: predominant recombination C4 viruses persistently circulating in China for 14 years. PLoS ONE 8 (2), e56341.

Zhu, F.C., Meng, F.Y., Li, J.X., Li, X.L., Mao, Q.Y., Tao, H., Zhang, Y.T., Yao, X., Chu, K., Chen, Q.H., Hu, Y.M., Wu, X., Liu, P., Zhu, L.Y., Gao, F., Jin, H., Chen, Y.J., Dong, Y.Y., Liang, Y.C., Shi, N.M., Ge, H.M., Liu, L., Chen, S.G., Ai, X., Zhang, Z.Y., Ji, Y.G., Luo, F.J., Chen, X.Q., Zhang, Y., Zhu, L.W., Liang, Z.L., Shen, X.L., 2013. Efficacy, safety, and immunology of an inactivated alum-adjuvant enterovirus 71 vaccine in children in China: a multicentre, randomised, double-blind, placebo-controlled, phase 3 trial. Lancet 381 (9882), 2024–2032.

Zhu, F., Xu, W., Xia, J., Liang, Z., Liu, Y., Zhang, X., Tan, X., Wang, L., Mao, Q., Wu, J., Hu, Y., Ji, T., Song, L., Liang, Q., Zhang, B., Gao, Q., Li, J., Wang, S., Hu, Y., Gu, S., Zhang, J., Yao, G., Gu, J., Wang, X., Zhou, Y., Chen, C., Zhang, M., Cao, M., Wang, J., Wang, H., Wang, N., 2014. Efficacy, safety, and immunogenicity of an enterovirus 71 vaccine in China. N. Engl. J. Med. 370 (9), 818–828.