Hand, Foot and Mouth Disease in Vietnam: Epidemiology, Heart Rate Variability and Economic Burden

Thesis

HAND, FOOT AND MOUTH DISEASE IN VIETNAM: EPIDEMIOLOGY, HEART RATE VARIABILITY AND ECONOMIC BURDEN

by

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A thesis submitted to the Open University U.K

For the degree of Doctor of Philosophy in the field of Life Science

Oxford University Clinical Research Unit

Hospital for Tropical Diseases

Ho Chi Minh City, Viet Nam

December, 2019

Abstract

Over the last two decades, hand, foot and mouth disease (HFMD) has become a major clinical problem in Vietnam and the Asia-Pacific region. HFMD affects children, especially those under 5 years old, and has pandemic potential. Since 1997, there have been several outbreaks with severe clinical phenotypes, including brain stem encephalitis, attacking millions of children and causing thousands of deaths. Synthesizing data on epidemiology, etiology, disease pathophysiology and economic burden of this emerging infection remains essential to inform clinical management and health policy makers in prioritizing the development of intervention strategies.

Using data from >56,000 hospitalized cases over an 11 year period, I described the spatial and temporal distribution of HFMD in Ho Chi Minh City, the main hotspot of HFMD in Vietnam. I found that the disease started in the west and then moved to the south-east and finally came back the west of the city.

Results from a prospective multi-hospital based study conducted during 2015–2018 showed that of ~1200 enrolled patients, enterovirus A71 (EV-A71) was the most common HFMD pathogen detected, while coxsackievirus A6 (CV-A6) has emerged and replaced CV-A16 to become the second most common virus causing HFMD in Vietnam during the study period. Despite the emergence of other pathogens and the diversity of enterovirus serotypes (~20 serotypes) detected in HFMD patients, EV-A71 was the main cause of severe HFMD. Using long-term data synthesized as part of the research program, I also demonstrated for the first time that compared to EV-A71 subgenogroup B5, subgenogroup C4 was associated with more severe clinical phenotypes. Moreover, the predominance of subgenogroup C4 coincided with large, severe HFMD outbreaks in Vietnam (e.g. in 2011-12 and 2018). Collectively, the data suggest that an EV-A71 vaccine would be likely to substantially reduce the burden of HFMD, but a multivalent vaccine should be developed to control the ongoing HFMD

epidemic because CV-A6, CV-A10 and CV-A16 were responsible for approximately 12% of severe HFMD cases and cross-reaction between these CV-As and EV-A71 is poor.

In order to improve our knowledge of HFMD pathophysiology, I used ECG signal recorded by a wearable device (e-Patch) to depict the distribution of heart rate variability (HRV) indices by severity and by detected pathogens and found that compared to mild disease HRV parameters reflecting parasympathetic nervous system activation in the severe group decreased whereas those mirroring sympathetic activity and autonomic nervous system imbalance increased. In a similar trend, compared to HFMD associated with non-EV-A71, HRV indices reflecting the imbalance between sympathetic and parasympathetic activation in HFMD associated with EV-A71 were significantly higher. This suggests that children with EV-A71 infection were more likely to have ANS imbalance. Alongside with these findings, the feasibility of this wearable device in children has brought promising applications in HFMD case management by early detection of severe disease in future.

To inform health policy makers in Vietnam about the burden of HFMD, I also estimated its economic burden. I showed that the total cost per case for mild and severe disease was $245.8 and $1326.7, respectively. Additionally, I also found that compared to CV-A infections, EV-A71 infection resulted in higher illness costs. At nationwide level, the total economic burden in Vietnam was estimated at >US$90 million for two-year period of 2016 – 2017.

Co-Authorship

The work presented in this thesis was mostly carried out by me, under the supervision by Dr Le Van Tan, Dr C Louise Thwaites and Dr H. Rogier van Doorn during the course of my PhD projects. Moreover, colleagues from Children's Hospital 1, Children's Hospital 2 and Hospital for Tropical Diseases in Ho Chi Minh City, Emerging Infections (EI) group, Mathematical Modeling and Health Economics group of the Oxford University Clinical Research Unit in Ho Chi Minh City (OUCRU-HCMC) and Institute of Biomedical Engineering from the University of Oxford have contributed to my work as follows:

For the hospital-based epidemiology study in chapter two, my colleagues from the aforementioned hospitals provided the available data from the health information systems. I designed the protocol and did mapping analysis and wrote the report with advice from Dr Matthew Graham and Dr Hannah Clapham from the Mathematical Modeling group at OUCRU.

Regarding the epidemiological, clinical and etiological study described in chapter three, the protocol, case report form (CRF) and data-entry procedure were already established and recruitment was ongoing, while laboratory work was done by staff of EI group. During my PhD research, I continued to run the clinical study, performed data analysis and produced the first draft of scientific papers arising as part of the project.

In relation to economic burden study, I developed the protocol and adapted an available CRF from Dr Angela Devine (a researcher from the Mahidol Oxford Research Unit, Bangkok, Thailand). Data collection and data entry were integrated into the ongoing study and were performed by study nurses. I performed data analysis and prepared the manuscript for publication, while receiving input from Dr Hugo Turner, head of the Health Economics group at OUCRU.

As part of the heart rate variability (HRV) study, I developed the protocol and CRF. Data collection was carried out by me and a study nurse who also did data entry. For the ECG signal data, I performed analysis with guidance from Dr Girmaw Abebe, a Postdoctoral researcher in machine learning, Institute of Biomedical Engineering, University of Oxford.

Acknowledgements

I would like to say thank you to all patients and their relatives who voluntarily gave consent to participate in the studies presented in this thesis. Without their participations, the thesis could not be finished.

I would like to express my sincere thankfulness to my supervisors - Dr Le Van Tan, Dr C Louise Thwaites and Associate Professor H. Rogier van Doorn for their tremendous support and help. All three supervisors have given invaluable guidance and kept in very close touch with the progress of my studies and thesis. Particularly, they sacrificed their free time on weekends to correct my thesis. Once again, I really appreciate their supervision.

I especially wish to thank all members of EI group who helped me to collect data, specimens and performed laboratory work. All of them have had significant contribution to make this thesis possible.

Special thanks I would like to send to Associate Professor Nguyen Thanh Hung, Dr Le Bich Lien, Dr Dao Trung Hieu, Dr Ngo Ngoc Quang Minh, Dr Truong Huu Khanh, Dr Pham Van Quang, Dr Du Tuan Quy and Nurse Do Duong Kim Han for their support and help during the course of my PhD thesis.

I would like to take this opportunity to say thank you very much Dr Leigh Jones and Ms Le Thi Kim Yen from OUCRU Training Committee and Mr Ho Van Hien and his IT team for their valuable support in administrative work and data management.

I am particularly grateful to all members of Outreach and International Department, Children's Hospital 1, especially Dr Le Thi Cam who often covered my work when I got stuck in my PhD work.

Finally, I would like to send my deepest thanks to my parents, my wife, my daughters, my brothers and my sisters who are always by my side and motivate me to overcome difficulties during this PhD course.

Publications

A. Publications arising as part of my PhD research

1. **Le Nguyen Thanh Nhan**, Hugo Turner, Truong Huu Khanh, Nguyen Thanh Hung, Le Bich Lien, Nguyen Thi Thu Hong, Le Nguyen Truc Nhu, Nguyen Thi Han Ny, Lam Anh Nguyet, Tran Tan Thanh, Hoang Minh Tu Van, Ho Lu Viet, Trinh Huu Tung, Tran Thi Lan Phuong, Angela Devine, Guy Thwaites, Nguyen Van Vinh Chau, Louise Thwaites, H. Rogier van Doorn and Le Van Tan. Economic burden attributed to children presenting to hospitals with hand, foot and mouth disease in Vietnam. *Open Forum Infectious Diseases*. 2019 Jul 1; 6(7).doi: 10.1093/ofid/ofz284.

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Abbreviations

WHO	World Health Organization
MOH	Ministry of Health
CH1	Children's Hospital 1
CH2	Children's Hospital 2
HTD	Hospital for Tropical Diseases
OUCRU	Oxford University Clinical Research Unit
OxTREC	Oxford Tropical Research Ethics Committee
HCMC	Ho Chi Minh City
UK	United Kingdom
US	United States
CDC	Center for Diseases Control and Prevention
NESID	National Epidemiological Surveillance of Infectious Diseases
HFMD	Hand, foot and mouth disease
EVs	Enteroviruses
CVs	Coxsackie viruses
EV-A71	Enterovirus A serotype 71
CV-A6	Coxsackievirus serotype A6
CV-A10	Coxsackievirus serotype A10
CV-A16	Coxsackievirus serotype A16
CNS	Central nervous system
ANS	Autonomic nervous system
PNS	Parasympathetic nervous system

SNS	Sympathetic nervous system
HRV	Heart rate variability
T	Temperature
AT	Average temperature
H	Humidity
RH	Relative humidity
RF	Rainfall
SH	Sunshine hours
P	Precipitation
WS	Wind speed
SR	Solar radiation
AQI	Air quality index
NO2	Nitride dioxide
TS	Time series analysis
DLNLM	Distributed lag non-linear models
GLM	Generalized linear regression models
SARIMA	Autoregressive integrated moving average
CART	Classification and regression trees
SIR	Susceptible-Infected-Recovered
SD	Standard deviation
IQR	Interquartile range
OR	Odd ratio
LOS	Length of stay
IVIG	Intravenous immunoglobulin
ICU	Intensive Care Unit

CSF	Central spinal fluid
CT	Computerized tomography
MRI	Magnetic resonance imaging
IV	Intravenous
CVP	Central venous pressure
SpO2	Saturation of peripheral oxygen
BP	Blood pressure
GCS	Glasgow coma scale
P.O	By mouth/Orally
ECG	Electrocardiogram
SBP	Systolic blood pressure
DBP	Diastolic blood pressure
HR	Heart rate
SDNN	Standard deviation of intervals of successive normal N-N intervals
RMSSD	Square root of the mean of the sum of the squares of differences between adjacent NN intervals
Ptot	Total power
VLF	Very low frequency
LF	Low frequency
HF	High frequency
pNN50	Number of pairs of adjacent NN intervals differing by more than 50 ms divided by the total number of all NN intervals in the entire recording
VP	Viral protein
PCR	Polymerase chain reaction
RT-PCR	Reverse transcription polymerase chain reaction
ELISA	Enzyme-linked immunosorbent assay

CSF	Cerebrospinal fluid
COI	Cost of illness
CRF	Case report form
bpm	Beats per minute
&	And
µg	Microgram
mg	Milligram
RNA	Ribonucleic acid
µM	Micromole
µL	Micro liter
ml	Milliliter
rpm	Rotation per minute
A	Alanine
L	Lysine
AFLP	Amplified fragment length polymorphism
AMP	Ampicillin
API20E	Analytical profile index 20 enterobacteria
ASO	Allele - specific oligo
ATP	Adenosine tri-phosphate
AZM	Azithromycin
BLAST	Basic local alignment search tool
bp	Base pairs
cat	Chloramphenicol acetyl transferase
CFU	Colony forming unit
CHL	Chloramphenicol
CIP	Ciprofloxacin

CLSI	Clinical and laboratory standards institute
CRO	Ceftriaxone
CTAB	Cetyl-trimethylammonium bromide
D	Aspartic acid
ddNTP	Dideoxyribonucleotide triphosphate
DNA	Deoxyribose nucleic acid
dNTP	Deoxyribonucleotide triphosphate
DTCS	Dye terminator cycle sequencing
ESBL	Extended spectrum beta lactamase
F	Phenylalanine
G	Glycine
g	Gram
GAT	Gatifloxacin
I	Isoleucine
ID	Identification
IR	Inverted repeat
IVI	International vaccine institute
Kb	Kilo base pairs
kV	Kilovolt
ms	Millisecond
WBC	White blood cell count
CRP	C reactive protein

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Chapter 1

Introduction

1.1. Epidemiology

1.1.1 Case definition

The term "hand, foot and mouth disease" (HFMD) was first used in 1959, in an article describing a cluster of cases centred around Birmingham (UK), characterized by fever, stomatitis and a vesicular rash. The cases were detected after a pediatrician noted an unfamiliar cluster of these signs in a child and initiated a referral to the specialist viral unit. When 3 other similarly affected children were identified, an alert was sent to general practitioners to report similar cases. Over the next 3 months, a further 82 patients with similar signs and symptoms were identified. The clinical features of the 24 cases which occurred in Birmingham, were described by Alsop *et al* [1]. The authors described presence of fever, mouth ulcers and skin lesions in 8%, 96% and 92% of cases, respectively. They noted that these features were similar to those noted in an outbreak in Toronto (Canada) in 1957, and similar to the Toronto outbreak, coxsackievirus A16 was detected in one patient [2].

Since then, HFMD has continued to occur throughout the world, notably causing large outbreaks particularly in the Asia-Pacific Region. Precise definitions of what constitutes HFMD have varied. According to some authors, an HFMD case is defined as any patient presenting with a typical rash (papular or vesicular rash) on hands, feet, mouth or buttocks with or without fever [3–5].

In other words, the term “HFMD” includes the syndrome of herpangina (HA) and does not require the presence of fever. Others, however, have employed a case definition for HFMD which does include fever as well as the typical skin rash [6,7]. In 2011, the World Health Organization (WHO) proposed a definition of clinical HFMD of “febrile illness with papular/vesicular rash on palms and soles, with or without vesicles/ulcers in the mouth”. Similarly, they defined herpangina as fever, mouth ulcers and no skin rash [8].

1.1.2 **Historical outbreaks**

Following the report of HFMD in Birmingham, described above [1], other small outbreaks occurred in New York (USA), Sweden, Bulgaria and Hungary during the 1970’s. These comprised of 11, 195, 705 and 1550 cases, respectively [9]. Notably, the epidemic in Bulgaria had a high incidence of paralysis and killed 44 people, while the outbreak in Hungary was characterized by a mixture of aseptic meningitis, encephalitis and flaccid paralysis and caused 27 deaths in young children [9,10].

Whilst in recent years, HFMD has been sporadically reported in European countries such as the UK, Norway, the Netherlands and Denmark [8,11,12], it has come to global renown due to outbreaks occurring in the Asia-Pacific region.

Globally, outbreaks have totaled more than 16 million cases and 4 thousand deaths [4,6,8,13–18]. Most of these have occurred in the Asia-Pacific region [4,8].

Japan experienced two large outbreaks in 1973 and 1978, with a total of 39,597 patients and a high proportion of patients with complications. Of the affected patients, 24% and 8% had central nervous system (CNS) involvement (localized encephalitis, aseptic meningitis and polio-like paresis), respectively [15]. In the 1980s, there were two notable outbreaks in Australia and Hong Kong. In Australia, a high proportion of cases presented with CNS involvement, and 51% (33/65) of hospitalized patients progressed to severe stages [8,16]. In 1985, 5 children in Hong Kong were reported to have HFMD and neurological complications. The next two decades witnessed many large outbreaks in Malaysia, Singapore, Japan, Taiwan, Vietnam and China [4,8,14]. The 1997 outbreak in Malaysia claimed lives of 29 young children and affected at least 2628 other patients [8,19]. One year later, Taiwan suffered from an unprecedented outbreak of HMFD, with an estimated 1.5 million cases, 405 hospitalized young children with severe neurological complications and 78 fatal cases [6,8,20]. In 2005, Tu *et al* described the first outbreak of HFMD in Vietnam through a study of 764 HFMD cases admitted to a referral pediatric hospital in Ho Chi Minh City [21]. Six years later, Vietnam experienced a large-scale outbreak with 113,121 cases and 170 deaths [3]. From 2008 to 2014, several large HFMD outbreaks occurred in China, affecting approximately 11 million children and with a total of 3046 fatal cases [22]. In addition to these countries, HFMD was also observed in other countries,

including South Korea, Brunei, Thailand, Laos and Cambodia. In Cambodia, at least 54 children died in a 2012 outbreak of severe HFMD [8,14,23–25].

1.1.3 **Morbidity, mortality and patterns of distribution**

Formal reports detailing the prevalence, incidence or mortality rate of HFMD at national levels mostly come from Asian countries where HFMD is often a notifiable disease.

In 1997, an estimated 7,253 HFMD cases and 40 deaths were recorded in two outbreaks occurring in Sarawak and Peninsular Malaysia [26,27]. Accordingly, the estimated incidence and mortality rate were around 33.6/100,000 and 0.185/100,000, respectively (population of Malaysia in 1997 was around 21,570,000). Between 1998 and 2005, a total of 2950 clinical cases and 10 fatal cases were reported to a sentinel surveillance program [27,28]. On average, the incidence rate was approximately 1.54/100,000 for each year in the period (average population of Malaysia for the 8 year-period was around 23,922,500) [29]. In following years, the number of reported HFMD cases was 210 in 2006, 5380 in 2007, 450 in 2008, 381 in 2009 and 1647 in the first 33 weeks of year 2010. Fatal cases from 2006 to 2010 were not documented. From 2011 onwards, data on HFMD in Malaysia has not been formally published.

In Taiwan, a study reported that there were a total of 1.5 million cases and 78 deaths among a population of approximately 21,178,000 in the 1998 outbreak, resulting an incidence rate of 609.6/100,000 and mortality rate of 0.368/100,000 [20]. From 2000 to 2005, an analysis from the national sentinel

surveillance revealed that there were a total of 12,236 laboratory-confirmed cases, with annual average numbers of roughly between 1300 and 2500 cases [30]. Moreover, using the database of National Health Insurance, an investigation estimated that the yearly episodes of HFMD from 2006 to 2010 were between 9823 and 133,461, with an annual incidence varying from 0.9% to 13.6% in children under 5 years old [31]. This study also provided the numbers of HFMD or herpangina for every 1000 emergency visits, with details in Figure 1.1. Regarding mortality, a total of 101 deaths were documented between 1999 and 2012, with the highest at 27 cases in 2001 [31].

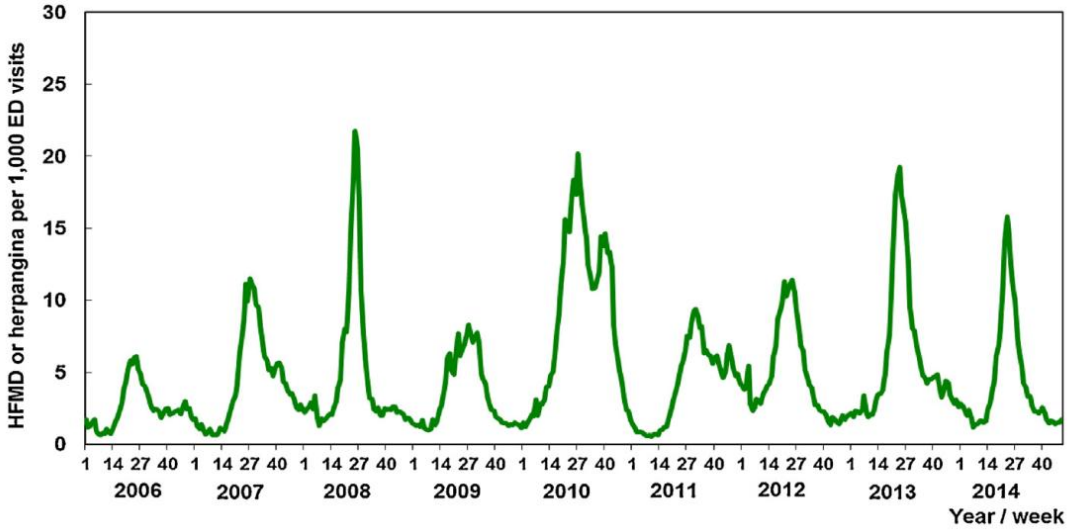


Figure 1.1: Proportion of HFMD or herpangina per 1,000 emergency visits in Taiwan from 2006 to 2014 [31]**.**

In China, HFMD became a notifiable disease in May 2008. Data from China CDC revealed that the incidence rate of HFMD significantly increased from 36.9 per 100,000 in 2008 to 161.6 per 100,000 in 2012. In addition, there was an upward trend in the mortality rate of HFMD in China between 2008 and

2012. The mortality rate remarkably increased from 0.01 in 2008 to 0.03 per 100,000 in 2009 and reached a peak of 0.07 per 100,000 in 2010 before it leveled off at 0.04 per 100,000 in 2011 and 2012 [4]. From 2013 onwards, China reported hundreds of thousands of cases to WHO on a monthly basis, as shown in Figure 1.2 (WHO biweekly report).

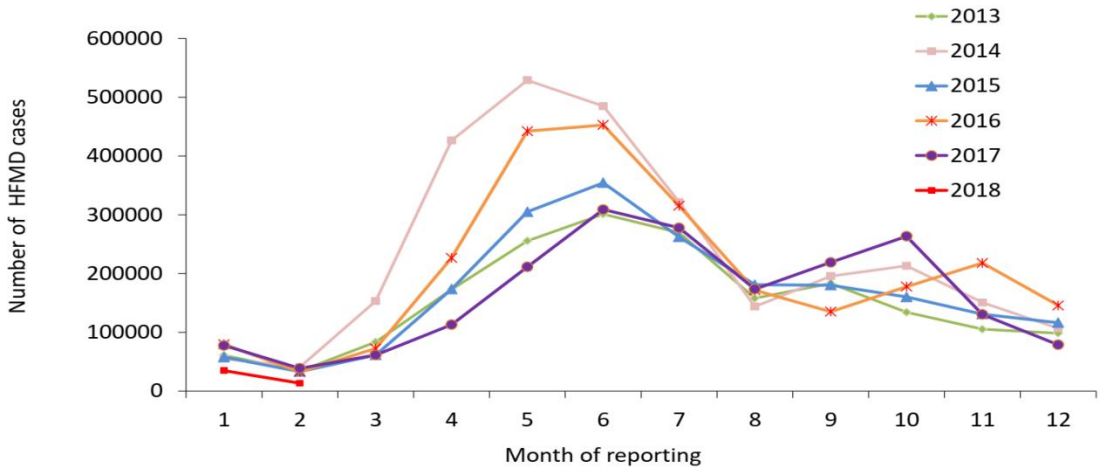


Figure 1.2: Distribution of HFMD by month in China [32]

In Singapore, nationwide sentinel surveillance was officially established in April 1998. Since then, reporting of HFMD cases has become compulsory for all health care facilities and child-care centers. In 2000, Singapore experienced a large-scale outbreak of HFMD. The number of cases and deaths in this epidemic were 3790 and 3, respectively, making an incidence and mortality rate at approximately 94 per 100,000 and 0.074 per 100,000 [8], respectively (population of Singapore in 2000 was 4,027,900). For the next seven years, the incidence rates varied from 125.5 per 100,000 in 2001 to 435.9 per 100,000 in 2007. From March to May 2008, Singapore witnessed another large outbreak.

The annual accumulative number of HMFD cases in 2008 was 29,686, producing an incidence rate of 613.5 per 100,000. From 2009 to 2017, average incidence rate was roughly 545 per 100,000. In terms of mortality, there were three cases reported in 2001 and one case reported in 2008. Besides these years, no deaths have been reported to the Ministry of Health in Singapore since 2001 [33,34].

Table 1.1: Number of cases, population and incidence rate by year in Singapore [35]

Year	Number of cases	Population	Incidence rate (per 100,000)
2009	17278	4,988,000	346.4
2010	30878	5,077,000	608.2
2011	20687	5,184,000	399.1
2012	37125	5,312,000	698.9
2013	31779	5,339,000	595.2
2014	21797	5,470,000	398.5
2015	28216	5,535,000	509.8
2016	42154	5,607,000	751.8
2017	33710	5,610,000	600.9
2018 (first 19 weeks)	15505	5,783,000	268.1

In Japan, the National Epidemiological Surveillance of Infectious Diseases (NESID) started in 1981. Since then, data on HFMD from thousands of sentinel sites have been reported to the NESID in accordance with the law. From 1982 to 1997, a total of 1,294,638 HFMD cases were reported, with annual numbers

ranging from 21,000 in 1989 to 159,000 cases in 1995. For the next 12-year period, between 1999 and 2010, the average annual number of HFMD cases was 115,265, with the lowest and highest number of cases recorded in 1999 (n=50,814) and 2000 (n=205,365), respectively. In addition, the annual reported herpangina cases ranged from 75,666 cases in 2009 to 154,802 cases in 1999. Totally, the incidence rates of HFMD and herpangina varied from 112.69 per 100,000 in 2009 to 278.11 per 100,000 in 2000, with an average incidence rate of 189.76 per 100,000. Details are shown in Figure 1.3 [36].

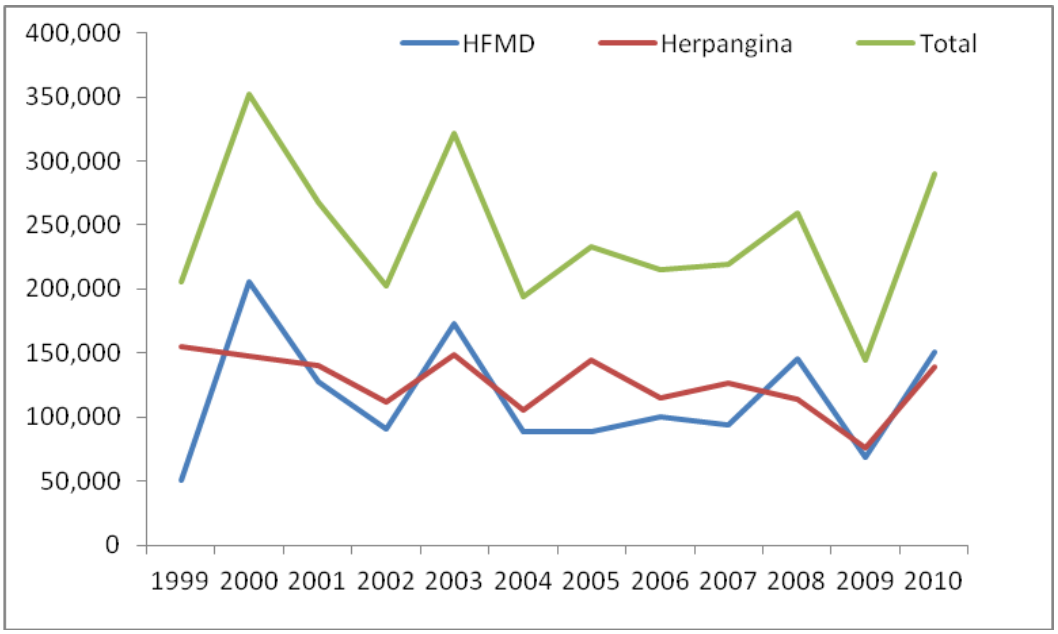


Figure 1.3: Distribution of HFMD/herpangina in Japan by years

From 2013 onwards, data on HFMD was reported to WHO, with details being depicted in Figure 1.4 (WHO biweekly report). Generally, outbreaks of HFMD occurred every two years between 2013 and 2017 with the highest number of cases reported during weeks 27 and 33.

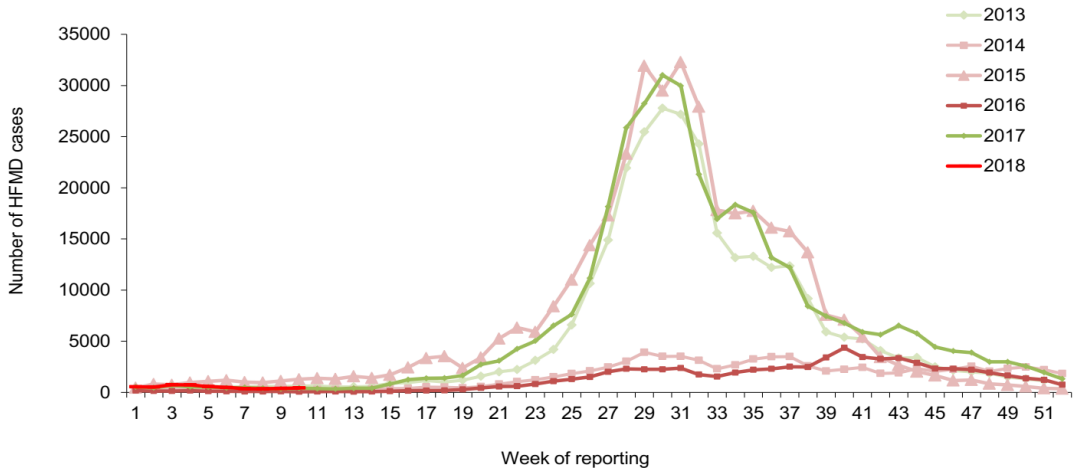


Figure 1.4 Distribution of HFMD by weeks between 2013 and 2018 in Japan [32]

In Vietnam, the first case of HFMD was reported in 2003. Seven hundred and sixty four hospitalized cases were recorded in 2005. From 2005 onwards, the number of HFMD cases significantly increased to 5719 in 2007, then to 157,391 cases in 2012. The incidence rate of HFMD in 2016 was noted at 51.17 per 100,000 people [37].The number of HFMD cases and deaths are described in Table 1.2. Of note, Ho Chi Minh City has continually had the highest number of HFMD cases among 63 provinces of Vietnam (7816 cases in 2014 and 5276 in 2015) [3,38].

Table 1.2: Reported cases of HFMD in Vietnam between 2007 and 2017

Year	Number of reported cases	Number of deaths
2007	5719	23
2008	10958	25
2009	10632	23
2011	113121	170
2012	157391	45
2013	79495	23
2014	77296	9
2015	56471	5
2016	47428	2
2017	46885	1

1.1.4 **Patterns of HFMD**

In Asian countries reporting HFMD, differing seasonal patterns have been noted in different countries or territories. For example, higher incidence between May and July in Japan, from February to May and September to October in Taiwan, between February and September in Malaysia, but year round (from January to December) in Singapore, Thailand and Hong Kong. China is a very large country, and different patterns were noted in different regions. For example, in Northeast China, HFMD was often observed from May to August while in the Southwest region HFMD mostly circulated between April and June [39].

In southern Vietnam, according to a study of 764 children with HFMD admitted to Children's Hospital 1, a large tertiary referral hospital for southern

Vietnam, there were two peaks of HFMD activity during 2005. The first peak occurred between March and May and the second was observed from September to December [21]. Between 2006 and 2012, the HFMD pattern was not described in any formal publication, but a high number of HFMD admissions were recorded between February 2011 and July 2012 [40]. From 2013 to the first 33 weeks of 2018, HFMD was mostly observed from September to November although it was reported throughout the year (Figure 1.5) [38].

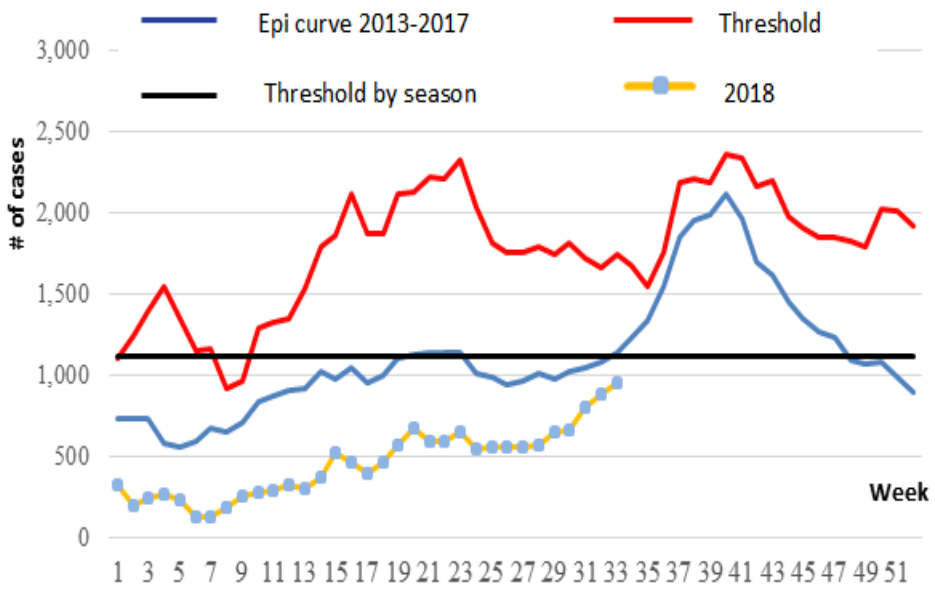


Figure 1.5: Distribution of HFMD in Vietnam between 2013 and 2018 [38]

1.1.5 **Climatic factors and HFMD occurrence**

As I mentioned before, different distributions of HFMD have been observed in different parts of the world. The exact reasons for this remain unknown, but weather variation has been considered as a possible explanation. Dozens of studies focusing on association between climatic factors (e.g. rainfall,

humidity, sunshine hours, wind speed, precipitation and temperature) and HFMD incidence have been reported in recent years, with different statistical methods and inconsistent results (Table 1.2). In a systematic review published in 2016, Koh *et al* reported that mean temperature and absolute humidity positively correlated with HFMD incidence in Singapore and Tokyo whereas these correlations were not observed in Taiwan and Hong Kong. Moreover, the correlation between mean relative humidity with HFMD cases was only observed in Singapore. In addition to studies included in this review, several other studies from Japan, South Korea and China also show an association between ambient temperature, relative humidity and HFMD incidence. However, characteristics of this association were different among these studies. For example, the correlation phenotypes between HFMD incidence and temperature were both linear and non-linear. Moreover, the cut-off points of temperature and relative humidity were different, corresponding to distinctive weather of study settings [41–47]. Besides temperature and relative humidity, other climatic factors such as rainfall, precipitation, sunshine hours and wind speed were mentioned as the risk factors for HFMD in some studies. In Vietnam, there have been two studies reporting the effect of weather variables on HMFD incidence. The first study using daily cases from the sentinel surveillance in Can Tho – a city in Mekong Delta River region in the south of Vietnam revealed that the HFMD transmission was positively associated with temperature, humidity and rainfall. More specifically, HFMD incidence

increased 5.6, 1.7 and 0.5 percent for every unit increase of temperature, humidity and rainfall, respectively [43]. The other study utilizing data at nation-wide level for the period between 2011 and 2014, showed that at provincial level, the HFMD transmission in Vietnam was positively associated with humidity and temperature, but negatively associated with rainfall [43]. In summary, these two studies had consistent results in terms of the correlation between temperature, humidity and HFMD incidence, but inconsistent results with respect to rainfall. This may, in part, be due to the differing climates in Vietnam, with the southern region experiencing a tropical climate (wet and dry seasons) and the north more temperate (4 seasons). Moreover, this data from the studies, was of relatively short duration and included the large-scale outbreaks in 2011-2012 hence may not depict the whole story of HFMD in Vietnam.

Table 1.3: Climatic factors and HFMD occurrence

Year	Location	Data	Climatic variables	Methods	Key findings
2012	Fukuoka, Japan	Weekly cases 2000-2010	Temperature (T) and humidity (H)	Time-series analyses (TS) and distributed lag non-linear models (DLNLM)	Average temperature (AT) and relative humidity (RH) associated with HFMD incidence. Every 1°C or 1% increase of AT or RH HFMD increases 11.2% or 4.7% respectively [41].
2017	Japan	Weekly cases 2010-2015	T, RH and total rainfall (RF)	Maximum entropy method	Mean temperature < 6^{o}C or >30^{0} C was associated with growth in incidence rate [48]
2016	Korea	Weekly cases 2010-2013	T, RH, sunshine hours (SH), precipitation (P), and wind speed (WS)	Generalized additive model with smoothing splines	HFMD incidence increased by growth of mean temperature and humidity [42]
2017	Can Tho, Vietnam	Daily cases 2012-2014	T, RH, and RF	Generalized linear model	A 1°C increase in AT was associated with 5.6% increase in HFMD rate. A 1% increase in H had equal influence of 1.7% increases on HFMD rate. An increase in 1 unit of RF was associated with a

					0.5% increase of HFMD rate [43]
2018	Vietnam	Monthly cases 2011 - 2014	T, RF and H	Spatial autoregressive model	RR increased by 3.1% for 1 °C increase in T above 26 °C and 1% increase in H above 76% whereas HFMD decreased 3.1% associated with 1 mm increase in RF [49]
2016	Hong Kong China	Daily cases 2008-2011	T, RH, WS, solar radiation (SR) and RF	Negative binomial generalized additive models and DLNLM	T and RH associated with HFMD incidence. Moderate RF and stronger wind and SR were associated with more admissions [50]
2013	Guangdong China	Monthly cases 2008 - 2011	T, RH and SH	Kulldorff scan statistic and spatial paneled model	AT, RH, the proportion of population 5 years, male-to-female ratio, and SH were the risk factors for HFMD [45]
2014	Mainland China	Daily cases 2008 - 2009	T, RH, WS, P, atmospheric pressure and SH	Logistic and auto-logistic regression model	Average P, AT, WS, the number of industrial enterprises above designated size, the population density and the proportion of student population were the risk factors for HFMD [46]
2015	Jiangsu China	Monthly cases 2009-2013	No mentioned	Space-time scan statistic and Bayesian model	AT and RF were positively correlated with HFMD incidence, while the number of days with RF, low T, high T and SH were negatively related. Particularly,

					RH had no relationship [51]
2015	Beijing China	Individual cases 2008-2011	P and AT, RH and WS	Geographically weighted regression model	(AT had the greatest effect among the four weather factors. The influence of AT and WS speed were greater in the summer than the winter, the influence of P was positive in the summer and negative in the winter. RH was negatively correlated with HFMD [44]
2015	Suzhou China	Daily cases 2008-2013	T, RH, RF, SH and WS	Stepwise regression and TS	AT and RF were strongly correlated with probable HFMD. Only EV-A71 cases were associated with AT during the study period of 2012–2013 [52]
2016	Mainland China	Weekly cases 2008-2013	T, RH, RF, SH and WS	The extra-Poisson multilevel spatial polynomial model	T was highly associated with HFMD incidence. AT and T difference approximate inverse “V” shape and “V” shape relationships were associated with HFMD incidence. T can be used to explain most of variation of HFMD incidence [53]
2016	Qingdao China	Weekly cases 2007-2014	T, P, RH and SH	Descriptive statistical and correlation analysis	Positively correlation between HFMD incidence and T, RH and P was observed [54]

2016	China	Monthly cases 2008- 2012	T, RH, vapor pressure, atmospheric pressure (AP), RF, P, WS and SH	Descriptive analysis, spatial statistic and spatial panel data models	The effect of different meteorological factors including atmospheric pressure, vapor pressure, SH, T, WS, R and rain day count on HFMD incidence varied, depending on distinctive climatic zones across China [55]
2016	Guangdong China	Daily data 2009–2013	T, RH, P, AP, WS and SH	Mixed generalized additive models	Significant non-linear positively effects of high RH were observed with a 13 % increase in the risk of HFMD. The effect geographically varied among the cities having different latitudes and longitudes [56]
2017	Beijing China	Daily data 2013	AT, P, SH	SaTScan analysis and spatial paneled model	T, RH and WS were positively associated with the incidence rate, while P and SH had a negative association [57]
2017	Gansu China	Individual data 2010	T, RH and RF	Poisson regression model	T was identified as a risk factor for HFMD. However, the cut-off point of T was not mentioned [58]
2017	Anhui China	Daily cases 2009-2014	T, RH, RF, and AP	(DLNM) and SARIMA	Relative risk for HFMD increased once AT was between 25 and 35^{o}C or RH lied between 65 and 80% [59]

2018	Asia	22, 2017 for articles	T and RH	Systematic review and meta analysis	Every one corresponding unit increase in T and RH were significantly associated with increased HFMD incidence. People living in subtropical and middle income areas had a higher risk for HFMD [60]
2018	Shanghai China	Daily cases 2009-2015	T, RH, WS, RF and SH	DLNM	HFMD incidence increased alongside with increasing average RH and WS and with decreasing daily RF and SH [61]
2016	Hong Kong, Taiwan, Tokyo and Singapore	Daily data 1957-2014	T, absolute H and RH	Auto-regression model	AT and absolute H positively statistically correlated with HFMD incidence in Singapore and Tokyo whereas these correlations were not observed in Taiwan and Hong Kong. The correlation between mean RH with HFMD cases was only observed in Singapore [39]
2018	Henna China	Monthly data 2012 -2013	T, RH, WS, P, SH and air pressure	GeoDetector and Bayesian space-time hierarchy model	1 °C rise in T was related to an increase of 4.09% in the HFMD incidence, a 1% increment in RH was associated with a 1.77% increase of the disease. 1% increment in ratio of urban to rural population was associated with a 0.16% increase of the disease [62].
2018	Beijing	Daily data	T,	Bayesian	Mean temperature, relative humidity, wind velocity

	China	2010-2012	RH, atmospheric pressure, P, SH and WS	conditional auto regressive (CAR) model approach	and sunshine hours were all positively associated with HFMD. The effect of wind velocity was significant with a relative risk (RR) of 3.30 per meter per second increase, as was sunshine hours with a RR of 1.20 per 1 hour increase [63]
2018	Gansu China	Weekly data 2010-2014	T , RH and RF	Generalized linear regression models (GLM) and classification and regression trees (CART)	Every 1 °C increase in AT was associated with a growth of 1.8%, 5.9% and 8% in HFMD incidence in Jiuquan, Tianshui and Lanzhou, respectively. 1% increase of RH could increase weekly HFMD of 2.47% in Lanzhou and 1.11% in Tianshui. AT and RH were the first two important determinants of HFMD [64]
2019	Guangdong China	Daily data 2009-2012	Air quality index (AQI) and T and RH	DLNLM and TS	Compared with low level T(≤23.5°C), high level T (>23.5 °C) had a RR of 1.486 on days with "good" air quality (AQI ≤ 46), and RR of 1.013 on days with "moderate" air quality (AQI>46). For RH, the high level (>77%) had a RR of 1.082 on days with "good" air quality, and RR of 1.039 on days with "moderate" air quality [65]

2019	Henan, Anhui and Chongqing China	Bi-weekly data 2008-2011	T, RH, P and SH	Dynamical stochastic SIR model	Meteorological factors can not solely explain the seasonality in HFMD transmission in mainland China. However, they may have combined effects with school terms and the highway passenger traffic on the transmission rate in Anhui during the fall semester [66]
2019	Shenzhen China	Daily data 2009 - 2017	Air pressure, T, RH, RF, WS and SH	DLNLM	There was a facilitating on HFMD once RH varied 46.0% to 88.8%. Short daily SH promoted HFMD. The positive correlation between RF and HFMD reversed when it exceeded 78.3mm. Ozone suppressed HFMD when it exceeded 104g/m3. NO2 promoted HFMD among infants[67]

1.2. **Virology**

HFMD is caused by several different enteroviruses. Enteroviruses (EV) are non-enveloped, single-stranded, positive-sense RNA viruses belonging to the *Enterovirus* genus within the *Picornaviridae* family. They are small, approximately 20-30nm in diameter and icosahedral in shape. The viral genome is about 7.5 Kbp in length, encoding for structural and non-structural proteins called P1, P2 and P3. The P1 region encodes four structural proteins, including viral protein 1 (VP1), VP2, VP3 and VP4. A virion is comprised of 60 protomers and each protomer contains one VP1, VP2, VP3 and VP4 protein. VP1 is the major capsid protein on the surface of a virion and a major antigen, whereas VP4 is located internally, and is not exposed on the viral surface [5].

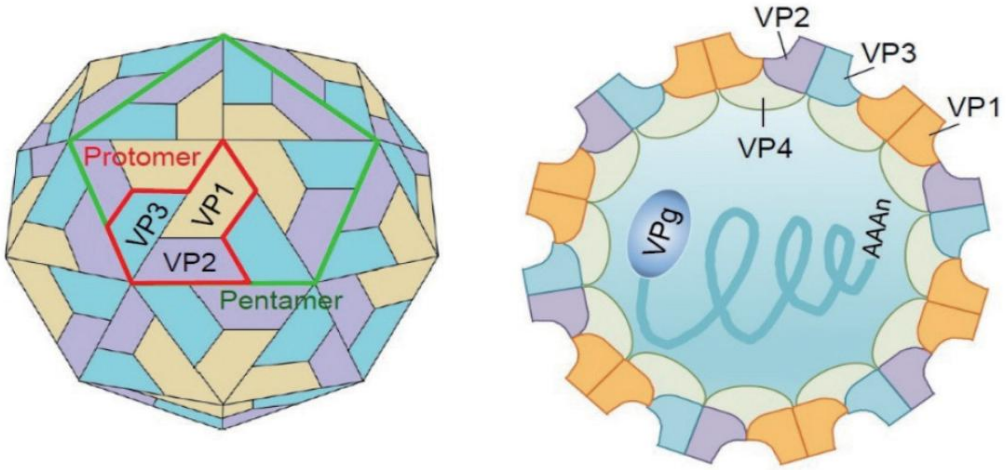


Figure 1.6: Structure of the enterovirus virion [5]

Based on molecular and serological characteristics, the genus *Enterovirus* is classified into 15 different species, namely enterovirus A-L and rhinovirus A-C [68]. Among these species, enterovirus A-D and rhinovirus A-C are human pathogens. Enterovirus A serotypes have been noted as the main causes of HFMD and this species contains coxsackievirus A (CV-A) 2-8, 10, 12, 14, 16 and enterovirus A71 (EV-A71) serotypes. In the first report of HFMD pathogens from Canada in 1958, Robinson C.R. and Rhodes A.J revealed that coxsackievirus A was isolated from 71% (22/31) of tested patients and CV-A16 was detected in 3 out of 4 cases [2]. Since then, the etiology of HFMD has changed significantly, with various EV serotypes circulating across the world, especially in the Asia-Pacific region. Overall, almost all severe outbreaks with high incidence of severe cases and fatalities have been associated with EV-A71, while epidemics with mild HFMD have been attributed to different CV-As such as CV-A16, CV-A10 and CV-A6 [8,69–74].

As for EV-A71, genetically, a complete VP1 sequence-based phylogenetic analysis conducted in 1999 reveals that there are three genogroups of EV-A71, namely A, B and C [5]. Genogroup A was first isolated in California (USA) in 1969. Genogroup B and C are divided into five subgenogroups, from B1 to B5 and C1-C5, respectively. Viruses of genogroup B and C have been widespread and caused major outbreaks worldwide. However, the distribution of EV-A71 subgenogroups differed from country to country in the last two decades, from

1997 to 2014. In particular, C4 was the major subgenogroup reported in China, with B5 sporadically reported, while other countries reported at least two subgenogroups circulating in the same period and/or replacing each other over time [8,69] (Figure 1.7). In Vietnam, C5 was responsible for the first outbreak in 2005. However, in recent years, there has been a replacement between C4 and B5 with C4 being responsible for the major outbreak during 2011 and 2012. Subgenogroup C4 also caused the severe outbreak in 2012 in Cambodia [14]. The distribution of EV-A71 subgenogroups from 2013 to 2018 is shown in Figure 1.8 [18]

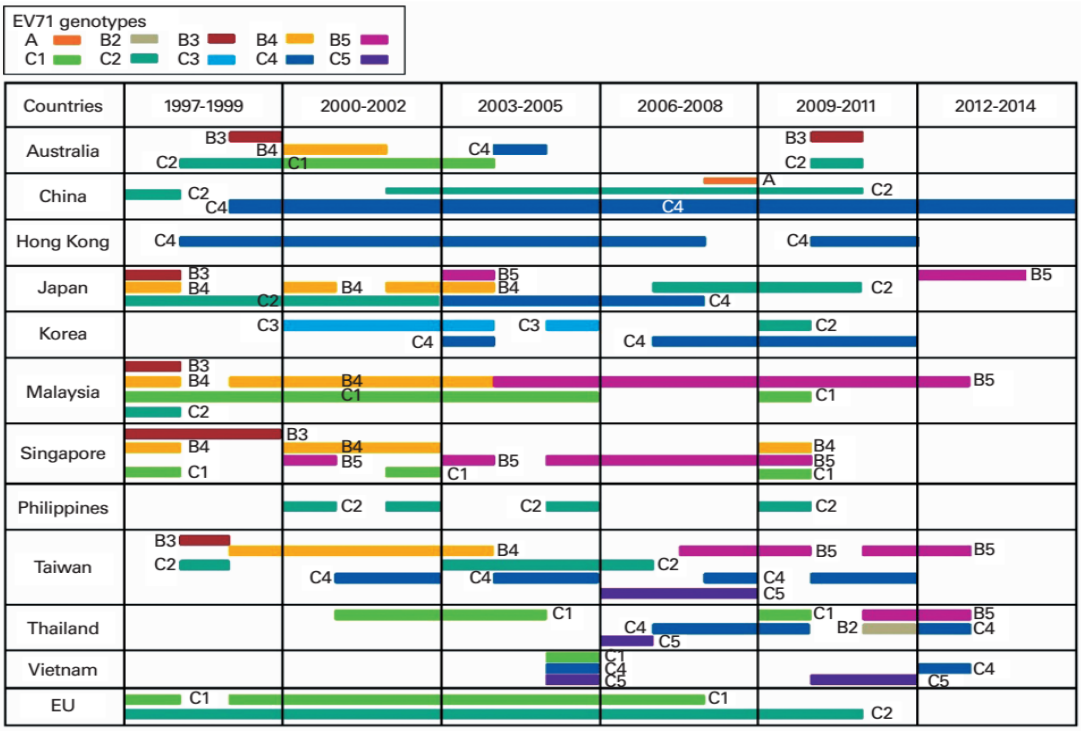


Figure 1.7: Distribution of EV-A71 subgenogroups worldwide [73]

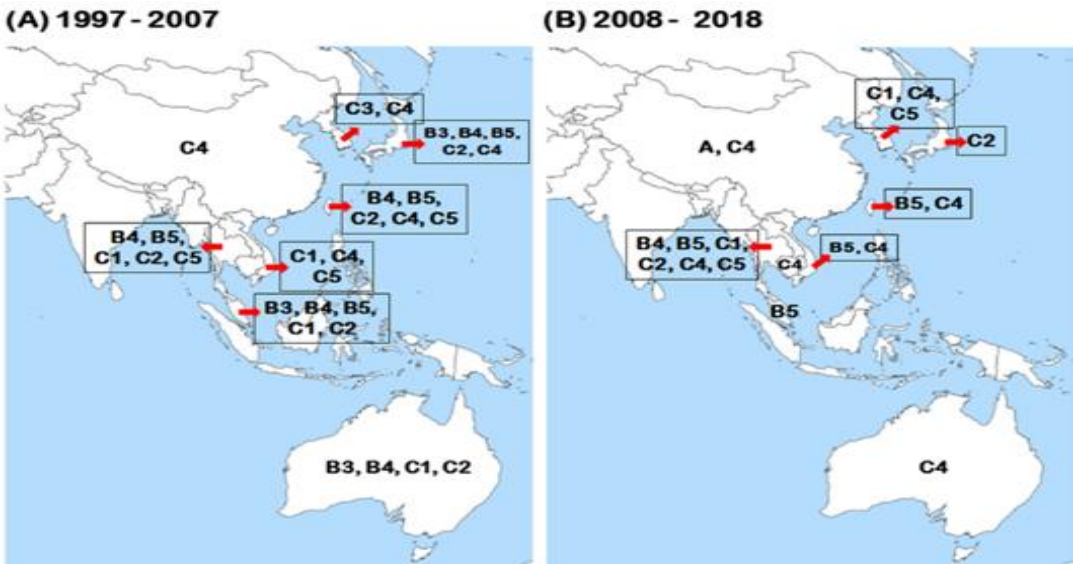


Figure 1.8: Distribution of EV-A71 subgenogroups in Asia Pacific region [18]

As for non-EV-A71 causes of HFMD, other EV A serotypes, especially CV-A6, A10 and A16 were reported as the most common non-EV-A71 pathogens of HFMD in Europe (Finland, Spain, Hungary, France, Denmark and the UK), the Americas (Cuba, USA) and Asia (China, India, Japan, South Korea, Malaysia, Singapore, Taiwan, Thailand and Vietnam) [75]. Of note, CV-A6 was mentioned as the predominant pathogen in Taiwan (2007-2008), China (2013), Japan (2005, 2011) and Vietnam (2014-2015) [75,76].

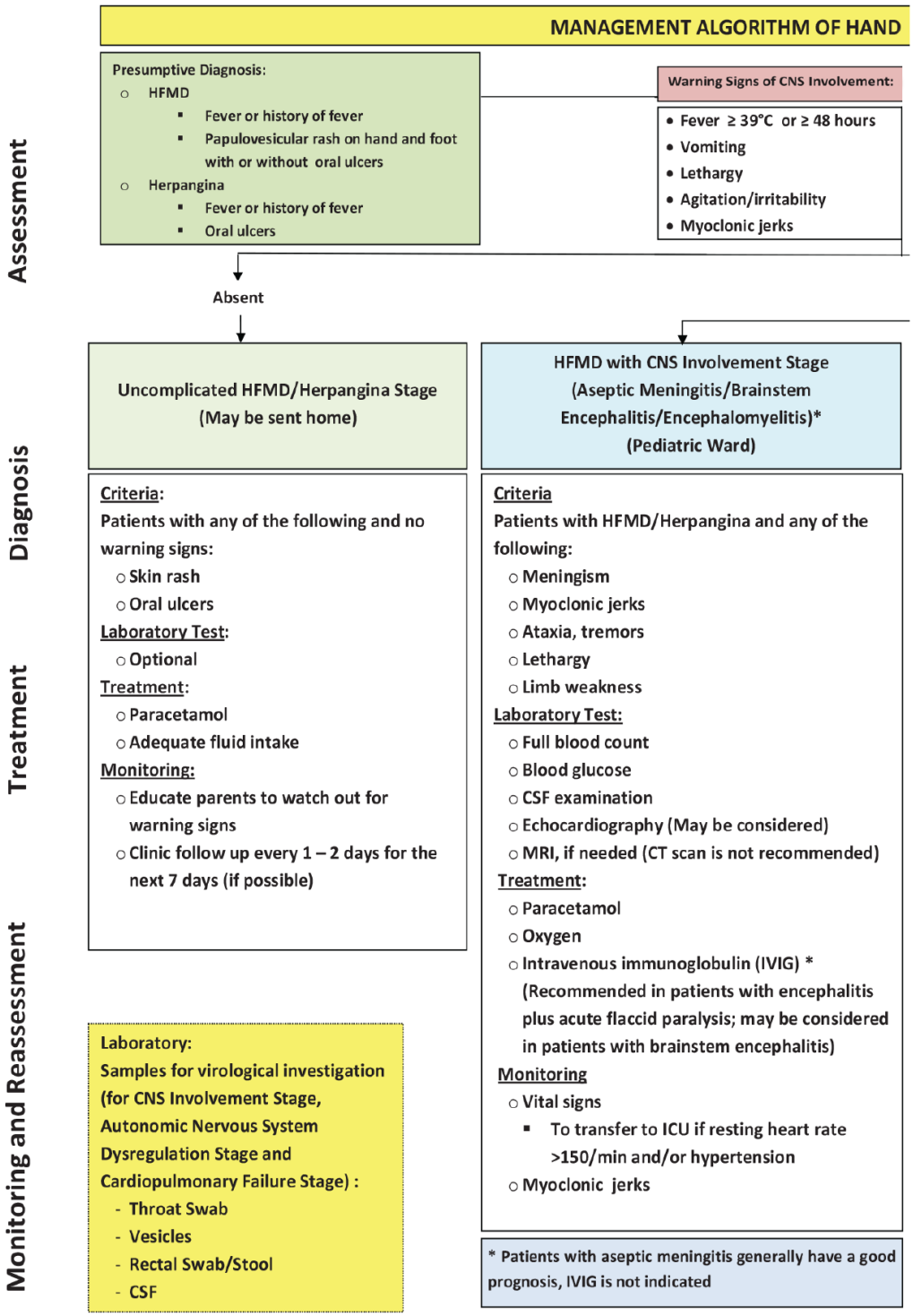
1.3. **Clinical management**

1.3.1 **Clinical features and case management**

HFMD is clinically characterized by a triad including fever, typical skin lesions on hands and feet and vesicle ulcers in the mouth, and is commonly benign and self-limited. However, 10%-30% of hospitalized patients with EV-A71-related HFMD

may progress to severe complications, including central nervous system involvement and autonomic nervous system imbalance leading to cardiopulmonary failure, which may lead to severe sequelae or even death [8]. In addition, a wide range of other signs or symptoms have been described, from mild to severe grades such as: diarrhea, vomiting, myoclonic jerks, ataxia, lethargy, convulsion, paralysis, fast breathing, eye-wandering, hypertension, profuse sweating, shock and pulmonary edema [13].

In 2011, WHO proposed guidelines on HFMD case management [8], giving a wide range of information on epidemiology, virology, pathology, clinical features, laboratory diagnosis, classification/clinical diagnosis and treatment. In brief, a patient is clinically diagnosed as HFMD when he/she has fever, rash on palms or feet and vesicles in the mouth. To guide case management, the disease is divided into four grades, namely uncomplicated HFMD or herpangina, HFMD with CNS involvement, HFMD with ANS imbalance and HFMD with cardiopulmonary failure (Figure 1.9).


MANAGEMENT ALGORITHM OF HAND
Assessment
Diagnosis
Treatment
Monitoring and Reassessment
Presumptive Diagnosis:
HFMD
Fever or history of fever
Papulovesicular rash on hand and foot with or without oral ulcers
Herpangina
Fever or history of fever
Oral ulcers
Warning Signs of CNS Involvement:
Fever ≥ 39°C or ≥ 48 hours
Vomiting
Lethargy
Agitation/irritability
Myoclonic jerks
Absent
Uncomplicated HFMD/Herpangina Stage (May be sent home)
HFMD with CNS Involvement Stage (Aseptic Meningitis/Brainstem Encephalitis/Encephalomyelitis)* (Pediatric Ward)
Criteria:
Patients with any of the following and no warning signs:
Skin rash
Oral ulcers
Laboratory Test:
Optional
Treatment:
Paracetamol
Adequate fluid intake
Monitoring:
Educate parents to watch out for warning signs
Clinic follow up every 1 – 2 days for the next 7 days (if possible)
Criteria
Patients with HFMD/Herpangina and any of the following:
Meningism
Myoclonic jerks
Ataxia, tremors
Lethargy
Limb weakness
Laboratory Test:
Full blood count
Blood glucose
CSF examination
Echocardiography (May be considered)
MRI, if needed (CT scan is not recommended)
Treatment:
Paracetamol
Oxygen
Intravenous immunoglobulin (IVIG) * (Recommended in patients with encephalitis plus acute flaccid paralysis; may be considered in patients with brainstem encephalitis)
Monitoring
Vital signs
To transfer to ICU if resting heart rate >150/min and/or hypertension
Myoclonic jerks
Laboratory:
Samples for virological investigation (for CNS Involvement Stage, Autonomic Nervous System Dysregulation Stage and Cardiopulmonary Failure Stage) :
- Throat Swab
- Vesicles
- Rectal Swab/Stool
- CSF
* Patients with aseptic meningitis generally have a good prognosis, IVIG is not indicated

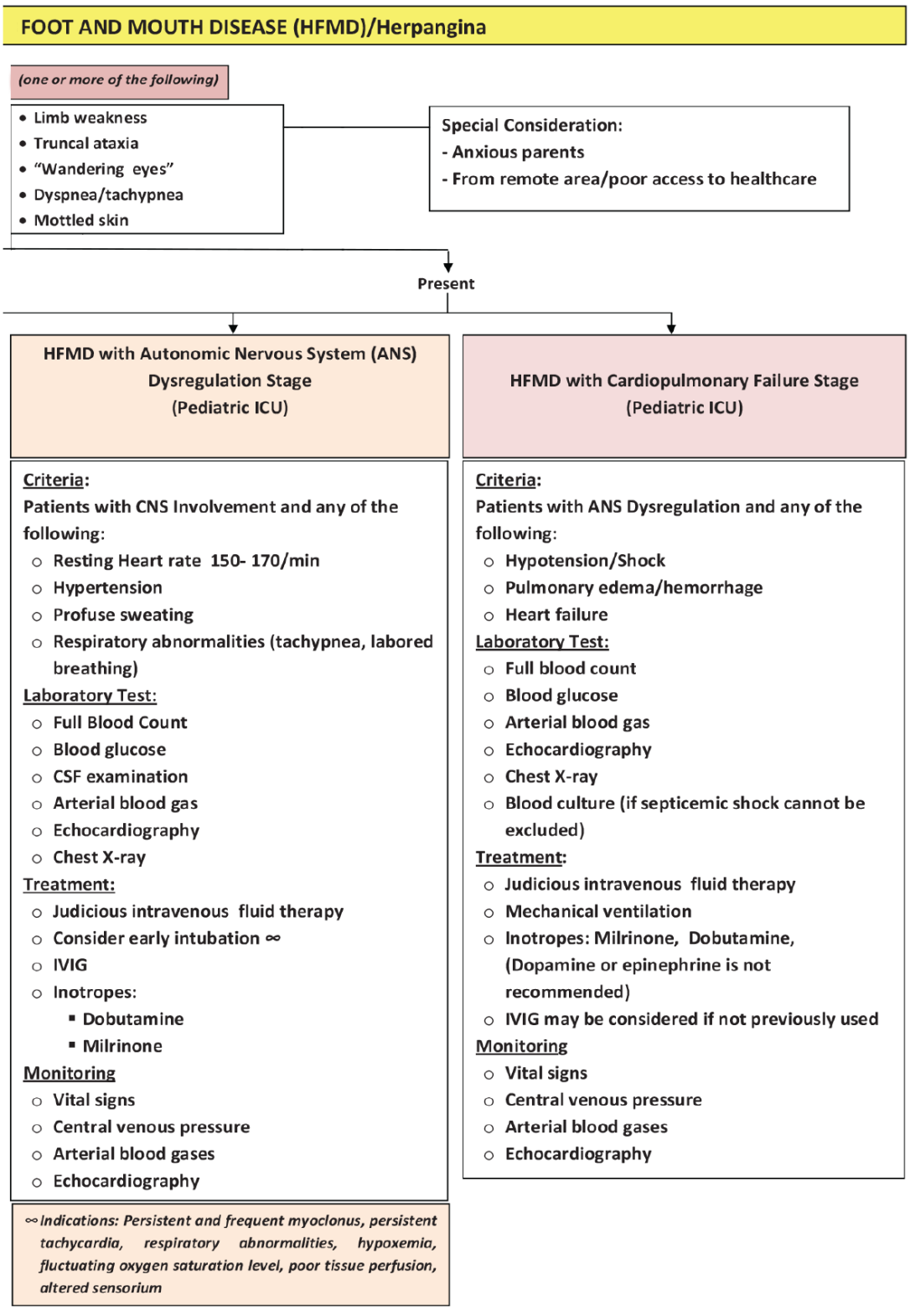


Figure 1.9: WHO guidelines for HFMD case management [8]

In response to the 2011 outbreak, an adapted version of the Taiwanese HFMD classification was issued by the Vietnamese Ministry of Health (MOH) after consulting with experts in the field to help clinicians in their daily practice. Compared to WHO guideline, the Vietnamese guideline also has 4 grades, with increasing severity from grade 1 to grade 4. In detail, grade 1, grade 3 and grade 4 are correspondingly similar to uncomplicated, ANS dysregulation and cardiopulmonary failure. However, grade 2, which corresponds to HFMD with CNS involvement, is divided into grade 2A, grade 2B1 and grade 2B2. In terms of treatment, the Vietnamese guideline showed a practical approach with comprehensive details to guide the management approaches (Table 1.4). More importantly, the guideline included hemofiltration for critically ill patients with the four following indications: grade 4 disease and hemodynamic instability within the first hour whose mean blood pressure could be maintained at at least 50 mmHg; coma, mechanic ventilation and uncontrolled high fever; coma, mechanic ventilation but hemodynamically unstable after 1-2 hours active resuscitation; ANS dysregulation for example fluctuation of arterial oxygen saturation, profuse sweating, pallor and heart rate ≥180 bpm (in a patient who is calm and not crying). In Vietnam, hemofiltration is not readily available in many centres and is considered the last-line treatment for HFMD patients with cardiopulmonary failure, although there is no evidence from randomized controlled trials.

Table 1.4: Summary of Vietnamese guideline for HFMD case management

Clinical signs/symptoms	Classification	Key treatments
One of the following signs/symptoms: • No breathing • Cyanosis or SpO2<92% • Pulmonary oedema • Shock	**Grade 4** **Severe cardiopulmonary failure**	• Transfer to ICU • Do intubation and mechanic ventilation • Give dobutamin • Do IV fluid test if shock and no cardiac failure while waiting for CVP measurement. • Measure CVP and give treatments by clinical responses and corresponding CVP values • Give Phenobarbital 10-20mg/kg/ slow IV • Give antipyretic if having high fever. • Give IVIG if mean BP ≥50 mmHg • Give Furosemide 1mg/kg/IV

One of the following signs/symptoms:
• Resting heart rate>170 times/min
• Profuse sweating
• Hypertension
• Fast breathing
• Coma (GCS<10)
• Abnormal breathing: Apnea, shallow breathing, chest indrawing, wheezing and stridor.
• Un-respond pyretic fever and one of the following signs:
  ○ Ataxia
  ○ Nystagmus
  ○ Cranial nerve paralysis
  ○ Limb weakness
	Grade 3
	
	Cardiopulmonary failure
		• Transfer to ICU
		• Oxygen 1-3l/min
		• Do intubation if having abnormal breathing or fast breathing (>70 times/min) or ANS imbalance or coma
		• Give Phenobarbital 10-20mg/kg/IV in 30 minutes
		• Give IVIG 1g/kg/IV in 6-8 hours (2 doses).
		• Give Dobutamin if BP is normal and heart rate is >170 times/minute.
		• Give Milrinone 0.4mg/kg/minute IV
		• Give antipyretic if high fever exists
		• Give midazolam or diazepam if convulsion
		• Prepare for hemofiltration if get worse in the first few hours and having one of following signs:
		  ○ Unstable blood pressure
		  ○ ANS imbalance exists
		  ○ High fever and un-respond to active antipyretic therapies.

Group 2: One of the following signs • Ataxia	**Grade 2B2**	• Hospitalized into inpatient wards • If having persistent high fever,

• Nystagmus or squint-eye • Limb weakness or flaccid paralysis • Cranial nerve paralysis • High fever and difficult to respond to active antipyretic therapies. • Heart rate >150 times/minute (calm and no fever).	**Severe CNS involvement**	give treatments as grade 3. • Give oxygen 1-3l/minute • Give Phenobarbital 10-20mg/kg/IV for 30 minutes and repeat after 6 hours if myoclonic jerk still exists. • Give IVIG 1g/kg/IV for 6-8 hours and repeat the second dose if not better.
Group 1: One of the following signs • Myoclonic jerk identified by health staff • Myoclonic jerk reported by caretakers (historical jerk) ≥ 2times/30 minutes. • Historical jerk and lethargy or heart rate >130 times/minute (calm and no fever).	**Grade 2B1** **Severe CNS involvement**	• Give Phenobarbital 10-20mg/kg/IV for 30 minutes and repeat after 6 hours if any myoclonic jerk exists. • Give IVIG if patient gets worse or did not respond to Phenobarbital therapy. Re-assess after 24 hours and consider the second dose as group 2B2. • Do lumbar puncture if having high fever or any meningitis possibility.
One of the following signs: • Historical jerk <2 times/30 minutes • Drowsiness or agitation or difficult to sleep • Fever ≥ 3 days or fever ≥39^0C • Often vomiting	**Grade 2A** **Mild CNS involvement**	• Hospitalized into inpatient wards • Give Phenobarbital 5-7mg/kg/day (P.O) • Counsel caretakers on warning signs
Skin lesion and/or mouth ulcers only	**Grade 1**	• Ambulatory care • Counsel caretakers on warning signs • Follow-up every day

1.3.2 **Indicators of ANS dysfunction: Heart rate variability**

1.3.2.1 Overview:

Heart rate variability (HRV), time variation between adjacent heartbeats or R-R intervals [77] was first described in 1960's when Hon and Lee observed alterations in R-R intervals followed by fetal distress [78]. Heartbeats originate from the sinoatrial node which is regulated by sympathetic (SNS) and parasympathetic nervous systems (PNS). These two divisions of nervous system are also called autonomic nervous system. The interplay of PNS and SNS is dynamic, with PNS

dominating at rest or during digestion time whereas SNS predominates in ‘flight, fright, fight’ situations [78,79]. By measuring the corresponding HRV indices reflecting overall ANS, SNS and PNS activity, one could quantify the relationship between the two branches of ANS and HRV has been suggested as a noninvasive marker of ANS balance [78,80]. Many measures of heart rate variability have been suggested (see below) and by observing the effects on these following the application of parasympathetic or sympathetic agonists or antagonists, some have been suggested as particular markers of autonomic nervous system activation [78–80]. It should be noted however that the autonomic nervous system’s regulation of heart rate is highly complex and occurs at many levels and affected by many variables. Nevertheless, there is broad consensus to approach to analysis and which metrics to measure [78,79,81,82]. HRV is traditionally analysed either as time-domain variables or as frequency-domain variables. Time domain variables are calculated from the distribution of R-R interval variation and include measures such as standard deviation, or root mean square of the standard deviation. These time domain measures depend upon the total number of R-R intervals and therefore the overall recording time. Frequency domain variables are calculated following Fast Fourier Transformation of the distribution resulting in a spectrogram of component frequencies, and arbitrary cut-offs applied to distinguish low, high, very low and ultra low frequencies. More recently other methods of analysis have been cited, for empirical mode decomposition has also

been advocated, where similar to Fourier Transformation, the waveform is broken down into component parts, although without leaving the time-domain [78,83].

In order to standardize and allow comparison between HRV variables, guidelines as to which variables should be used and the optimal recording time have been published [77,78]. Guidelines state that frequency domain variables should be measured from 5-minute recordings whereas time-domain should be calculated from 24-hour recordings [77]. Different HRV measures thus represent underlying ANS activation and/or relative activity of parasympathetic and sympathetic components. It should be remembered that interpretation is complex and depends upon many factors, such as sinus node sensitivity, age, fitness and cardiovascular system compliance. Table 1.4 contains the most commonly cited and generally accepted interpretations of HRV variables.

Table 1.5.: Definition of HRV indices and normal range in children up to 24 months [78]

HRV indices	**Definition**	**Median** [82] **(3^{rd}-97^{th} percentile)**
Time domain		
HR (beats/minute)	Heart rate	105.3 (88.5-130.6)
Standard deviation of NN intervals -SDNN (ms)	Standard deviation of all NN intervals, a global of R-R intervals variability, reflects an estimate of short-term beat-to-beat variability [78,83]. It represents the variability over the entire recording period, giving the overall autonomic modulation regardless of sympathetic or parasympathetic arm [84]. However there are limitations interpreting this in short duration recordings.	73.4 (32.7-118.5)
Square root of the mean squared differences of successive NN intervals - RMSSD (ms)	The square root of the mean of the sum of the squares of differences between adjacent NN intervals. This index measures short-term beat-to-beat variability and is associated with parasympathetic activity [83].	14.6 (41.4-127.3)
Frequency domain		
Total power - Ptot (ms^2)	Five- minute total power is the variance of R-R intervals over the	4716 (1061-19692)

	temporal segment (<4Hz)	
Very low frequency power - VLF (ms^2)	Power in very low frequency range (≤0.04 Hz). Activity is abolished with atropine and therefore likely to represent parasympathetic activity.	938 (267-2517)
Low frequency power-LF (ms^2)	Power in low frequency range (0.04 Hz-0.15 Hz) Modulated by the baroreflex and representing sympathetic and parasympathetic activity.	648 (122-2723)
LF nu (%)	LF power in normalized units; LF/(Total power –VLF)*100	
High frequency power-HF (ms^2)	Power in high frequency range (0.15-0.4 Hz) representing parasympathetic (vagal activity), although can be affected by very high or low respiratory rates.	700.1 (73-8508.2)
HF nu (%)	HF power in normalized units; HF/(Total power –VLF)*100	
LF/HF	Ratio LF/HF	1.30 (0.50-3.33)
Poincaré plot		
Standard deviation 1 (Width of ellipse) - SD1 (ms)	Standard deviation of the distance of each point from the y = x axis. This is an index from Poincaré plot that represents short-term RR intervals variability [82]. It is mediated by parasympathetic nervous system via controlling the sinus node[85]	26.51 (8.98-77.60)

Standard deviation 2 (Length of ellipse) - SD2 (ms)	Standard deviation of the distance of each point from the $y = x +$ average R–R interval. This is an index from Poincaré plot that represents long-term RR intervals variability. It reflects both parasympathetic and sympathetic activity	123.84 (61.92-198.6)
SD1/SD2 (%)	Ratio of SD1 and SD2	0.222 (0.107-0.456)
Empirical mode decomposition		
pLF1 (ms^2)	Power of low frequency band (0.1- 0.15 Hz) derived from empirical mode decomposition method.	418.4 (79.5-1562.5)
pLF2 (ms^2)	Power of low frequency band (0.04- 0.1 Hz) derived from empirical mode decomposition method.	425.3 (96.0-1176.1)
pHF1 (ms^2)	Power of high frequency band (>0.15 Hz) derived from empirical mode decomposition method.	396.4 (57.8-3211.2)
pHF2 (ms^2)	Power of high frequency band (>0.15 Hz) derived from empirical mode decomposition method.	734.1 (74.3-9171.2)
IMAI1	Ratio between pLF1 to total high frequency power (pHF1+pHF2) derived from empirical mode decomposition method.	0.479 (0.189-1.113)
IMAI2	Ratio between pLF2 to total high frequency power (pHF1+pHF2) derived from empirical mode decomposition method [82,84,86]	0.551 (0.172-1.526)

Over the last two decades, an increasing number of HRV studies have been published, reporting potential applications of HRV in different fields such as physiology, psychology, meditation, sport training and healthcare[77,87–89]. In medicine, heart rate variability has been used as a non-invasive marker of ANS imbalance in several diseases in both adults and children[78,90–92]. There have been many studies citing the value of HRV in predicting outcome in ischaemic heart disease but in adults, recent studies also report that HRV is a predictor for survival in patients with cancer [92], sepsis [82]. sudden death [93] or diabetes mellitus [94]. It also correlated with inflammation markers and albumin levels in patients with systemic lupus erythematosus [95].

1.3.2.2 HRV in pediatric population

In children, several studies of HRV have been conducted, focusing on different aspects such as neurodevelopment [96,97], ANS maturation [82], inflammation [98], immunization [99], obesity [100] and infectious diseases [80,101–104]. There were a limited number of studies on HRV in children with infectious diseases. Thus, I will focus the literature review on HRV in children with infectious diseases including sepsis, dengue and HFMD.

In terms of sepsis, HRV indices were identified as a predictor for mortality in adult patients [80]. However, in the paediatric population, few studies have looked at the relationship between sepsis and changes in HRV indices although a lot of studies have reported the association between heart rate characteristics and severe outcomes of sepsis [105,106]. A study enrolling 30 children aged 1 month to 18

years old, who were treated in a pediatric Intensive Care Unit in the US, revealed that LF and HF values of the sepsis group were significantly lower than that of septic shock while the difference in LF/HF ratio between the two groups was not statistically significant [107].

With respect to dengue fever, in 35 Thai children with mean age 11.7 years, changes in HRV indices between the convalescent stage and recovery were described. The results showed that there was no statistically significant difference in the averaged RR interval, the time-domain HRV (SDNN, RMSSD, pNN50) or frequency-domain HRV (LF, HF, LF/HF ratio) between the convalescent stage and recovery [101]. However, the sample size of this study was small, thereby affecting power of paired t-test. Another study conducted in 27 Thai children with dengue fever aged from 6 months to 15 years old, which collected ECG signal at Day 0, 1 and 2 after admission, revealed that means of HF, LF significantly increased from 194, 249 (ms^2) on Day 0 to 824 and 1060 ms^2 on Day 2, respectively. However, LF/HF ratio reduced from 8.2 to 2.4 on corresponding days [102]. In summary, in children with Dengue, there was imbalance of ANS and it steadily decreased after 2 days admission while ANS activation slightly changed between convalescent stage and 14 day-follow up.

In children with HFMD, HRV indices were first described in 40 Taiwanese children in 2006 in comparison with 20 healthy controls [104]. HFMD patients were divided into three groups with different severities. The results showed that there was a downward trend of SDNN, RMSSD, Ptot, LF and HF corresponding to

the higher severity of HFMD and the difference among these groups was statistically significant. In contrast, LF/HF ratio increased from 2.17 in stage I to 4.96 in stage III, but their significant difference among these groups was not observed. Although potential implications for prediction of severe disease have been noted, the number of severe patients with autonomic imbalances was small, which may pose considerable bias to HRV indices. Moreover, in this study HRV indices were taken from a single 5 minute Holter-monitor recording and from a population with HFMD but not differentiated according to pathogen. Since then, no additional studies have been reported. Nevertheless further knowledge regarding HRV may help clinicians to improve their ability in early detection of severe HFMD, thereby improving patient management and outcome.

1.4. **Laboratory diagnosis**

As for other viral infections, virus isolation is traditionally considered as the 'gold standard'. However, because of its low sensitivity, virus isolation is not considered for routine diagnosis nowadays. Serological testing is not recommended for HFMD diagnosis because second blood sample is needed to demonstrate the seroconversion, and the specificity of commonly used methods such as ELISA is low [8]. Meanwhile, PCR detection of viral nucleic acids in clinical specimens is more sensitive and high throughput. It is therefore a method of choice for both outbreak situation and clinical setting [8,108,109]. Detection of HFMD causing enteroviruses can be achieved by generic PCR targeted at the 5'-untranslated

region (5’ UTR), while viral protein 1 (VP1) region is more variable and is therefore more useful for serotyping purpose (i.e. detection of specific enterovirus serotypes in clinical specimens and subgenogroup determination in case of EV-A71). The clinical specificity and sensitivity of PCR results are clinical specimen type dependent [109,110]. Due to the likelihood of coincidental carriage in gastrointestinal tract, virus detection in throat and rectal swabs is less reliable than that from sterile sites, such as plasma, cerebrospinal fluid (CSF) and vesicular fluid. However, because swabs are easily obtained, they are still the recommended samples for HFMD diagnosis [108,109].

1.5. **Vaccines**

With the success of the ongoing poliomyelitis elimination program, vaccine is considered as the best effective way to control HFMD. There are currently several approaches to produce vaccines, based on characteristics of antigens, such as inactivated whole cell vaccine, attenuated live vaccine, synthetic peptide vaccine, recombinant vaccine, virus like particle – based vaccine.

Because EV-A71 is the main cause of severe HFMD, especially in recent outbreaks in the Asia-Pacific region, attention has focused on the development of EV-A71 vaccines. To date, only inactivated whole EV-A71 vaccines have been studied in humans while vaccines for other HFMD pathogens such as CV-A6, CV-A10 and CV-A16 have not gone beyond animal models [111].

Currently, there are a total of five inactivated whole EV-A71 vaccines produced by different companies from China, Taiwan and Singapore. Of these, three vaccines using subgenogroup C4 as virus seeds have been tested in three different phase 3-trials in China. The overall efficacy against EV-A71-associated HFMD of the three vaccines varied from 89% to 97% [112,113] and no serious adverse event was reported. These vaccines were approved by the China Food and Drug Administration in 2015 and have been used in only some parts of mainland China [114,115]. In contrast, the other two inactivated whole EV-A71 vaccines using viruses of subgenogroup B4 produced in Taiwan have completed phase 2 trials and one phase 3-trial has been conducted in southern provinces of Vietnam [115,116].

With high efficacy against the most important pathogen of HFMD, it is likely that these inactivated whole EV-A71 vaccines will be implemented in Vietnam and the region. However, to provide baseline data before introducing vaccines, synthesizing data on economic burden of HFMD is essential to inform health policy makers in these countries.

1.6. **Economic burden**

HFMD has become a public health concern in the Asia-Pacific region. It poses a significant burden on the community when large outbreaks occur. Parents of affected children have to take time off from work in order to take care of their child due to hospitalization and/or school/kindergarten closure. Uncertainty over

which children will progress to severe disease means many children must be admitted to hospital for observation, placing even more pressure on already strained resources. Cost of illness (COI) is one measure of burden of a disease, measuring impact of the disease on a community in terms of prevalence, incidence, morbidity, mortality and various aspects of health economics [117–119]. A critical aim of a cost of illness study is usually to estimate direct costs, indirect costs, premature deaths and productivity losses. A COI study can be conducted using different approaches and/or under various perspectives. Consequently, the study results greatly vary on the methods employed and the designs of such studies are dependent on researchers' perspectives [120].

There are currently various approaches to calculate cost of illness of HFMD, using different methods and from different perspectives [121–124]. For example, from the society perspective, a Taiwanese study revealed that annual expenditures incurred by national health insurance was $526 to $557 million ($475-567 per capita), constituting 1.1% to 3.7% of national health insurance spending. For indirect costs which include travel cost, productivity cost and productivity losses due to premature mortality, total cost of the first two was $37.1 million per year, of which, costs of EV-A71 accounted for 5% of total costs due to enteroviruses associated with HFMD. The productivity losses from premature deaths were $0.8 million a year, with EV-A71 being in charge of 96.3% [31]. Another study in which productivity losses from premature deaths were not included in indirect

costs reported that the economic burden of HFMD incurred by a population of 1.42 million children was $7.66 million and the mean cost per case was $208.2. In outpatients, the direct mean costs were $56.7 and indirect mean costs were $108.6. In hospitalized patients with mild disease, mean direct costs were $550.3 and indirect costs were $309.5. In severe HFMD, the direct mean cost was more than double that of the indirect one, at around $1500 and indirect costs were $650 [124].

Despite the high incidence of HFMD in Vietnam, there are no reports about the associated cost of illness posed by HFMD. Such knowledge is essential for health policy makers for planning and budgeting of resources for disease management, prioritizing vaccine development and implementation of interventional strategies in response to disease outbreaks.

1.7. **Hypotheses and research questions**

From the literature review as aforementioned, I have identified several important knowledge gaps, where a better understanding could lead to improved preventative measures and treatments of HFMD. Whilst these focus on Ho Chi Minh City, where there is a significant burden of HFMD, better understanding is of value to HFMD management throughout the world.

Specifically, I hypothesize that:

1. The ongoing epidemic of HFMD in southern Vietnam, especially in Ho Chi Minh City, is complex and is driven the emergence of pathogens.

2. HRV indices are different between HFMD patients infected with different viruses and/or presenting with different clinical severities.
3. HFMD causes high economic burden in Vietnam.

Accordingly, my research questions are:

Epidemiology:

- What is the spatial-temporal distribution of HFMD in Ho Chi Minh City between 2005 and 2015?
- What are the most common pathogens associated with HFMD during 2015 – 2018?

Heart rate variability:

- Is it possible to use wearable devices such as E-patch to identify HRV indices in children with HFMD?
- Is there any statistically significant difference in values of HRV indices among disease severities or between the most common detected pathogens?

Economic burden:

- What are the cost units of different grades of HFMD in Vietnam?
- What are the costs of HFMD caused by the most common HFMD pathogens in Vietnam?
- What is the total economic burden that HFMD posed to society in Vietnam during 2016 – 2017?

Chapter 2

Epidemiology, and temporal and spatial distributions of hospitalised hand, foot and mouth disease cases in Ho Chi Minh City, Vietnam between 2005 and 2015

2.1 **Background**

Vietnam has a rapidly urbanizing population. Ho Chi Minh City is the most populous city in Southern Vietnam with a population of approximately 13 million. The city is divided into 24 administrative districts, which are further subdivided into 322 wards. On average, each ward has around 40,000 residents. Ho Chi Minh City is one of the most densely populated cities in the world with an average population density of over 6200 inhabitants per square kilometers [125]. HFMD is a communicable disease, transferred through fecal oral transmission and managing HFMD outbreaks in such dense urban populations has proved extremely challenging. Furthermore, there are limited data describing geographical spread HFMD in HCMC which could be used to guide public health policy. The limited existing data were collected in outbreak-related studies and are therefore inherently biased because of the time and resources available for the studies [21,40]. Disease notification data is also limited as it does not contain data about residency, and potentially duplicates data in HFMD cases to initially admit to local health care centers and then transferred to major hospitals. As such a better understanding of epidemiology and spatial and temporal distribution of HFMD in this dynamic setting remains fundamental to understanding this emerging infection and guiding further research in coming years. Herein, I aim to provide a general picture about these aspects of HFMD in HCMC over 11 years (from 2005 to

2015), preceding the prospective hospital-based study I conducted during the period of my PhD study (Chapter 3).

2.2 **Materials and Methods**

2.2.1 **Hospitalized HFMD data and study settings**

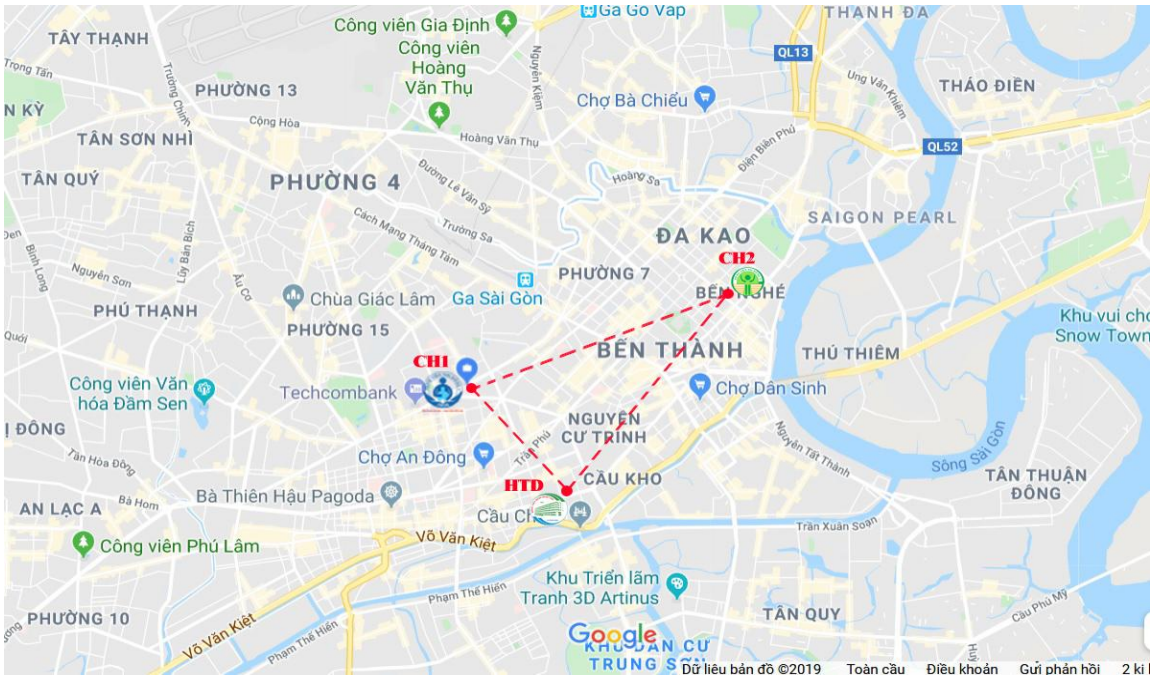


Figure 2.1: Map of a part of HCMC locating CH1, CH2 and HTD in red.

HFMD data were derived from hospitalized cases at 3 major hospitals in Ho Chi Minh City: Children's Hospital 1 (CH1), Children's Hospital 2 (CH2) and Hospital for Tropical Diseases in Ho Chi Minh City (HTD). These three hospitals have been responsible for treatment of the vast majority of hospitalized HFMD cases from Ho Chi Minh City and severe cases from the southern provinces of Vietnam.

CH1 was established in 1956 and is the highest level of pediatrics in South of Vietnam, with 1400 beds and 23 clinical departments in inpatient area and 63 consultation rooms in outpatient clinic. Although located in Ho Chi Minh City, CH1 provides child healthcare services for around 20 provinces (from Binh Thuan to Ca Mau) in south of Vietnam with an estimated population of 42.3 million people. On average, CH1 receives 1.8 million outpatients and 100.000 inpatient admissions a year. Of these, 60% of outpatients come from Ho Chi Minh City and 40% from southern provinces whereas these figures for inpatients are approximately 70% and 30%, respectively. All studies in this thesis were conducted in Outpatient Clinic, Infectious Disease Department (IDD) and Intensive Care Unit (ICU) of this hospital. According to CH1's guidelines, IDD is responsible for treating HFMD patients with grade 1, grade 2 and grade 3 while ICU is in charge of receiving critically severe HFMD cases including patients needing mechanical ventilation and/or hemofiltration.

CH2 was established in 1978 and is the referral pediatric hospital at the same level with CH1. The catchment area of CH2 is mainly provinces located in south-east of Ho Chi Minh City and Central Vietnam. CH2 has similar pediatric specialties to those available at CH1 and also a comparable number of outpatients and inpatients to CH1.

HTD is the highest level hospital in terms of infectious diseases in Vietnam's southern provinces, providing health care for both children and adults with tropical diseases. The hospital has a total of 550 beds including 200 beds for children

under 16 years old. On average, there are 1700 patients seen in the hospital outpatient department each day. Approximately 600,000 patients are admitted each year.

2.2.2 **Study design**

This is a retrospective study using available data from the health informatics systems of CH1, CH2 and HTD.

2.2.3 **Data collection**

Individual data of HFMD patients, who were under 16 years of age, lived in Ho Chi Minh City and admitted to Children's Hospital 1 (CH1), Children's Hospital 2 (CH2) and the Hospital for Tropical Diseases (HTD) between 1 January 2005 and 31 December 2015 were extracted from the health information database of the three hospitals. Extraction of the HFMD data was based on the 10^{th} revision of the International Statistical Classification of Diseases and Related Health Problems (ICD-10), with codes of HFMD being B08.4 or B08.4-1 or B08.4-2A or B08.4-2B or B08.4-3 or B08.4-4. Information extracted consisted of date of birth, date of admission, patients' address provided by accompanying relatives, gender, diagnostics on admission and at discharge, and discharge outcomes.

Although the aforementioned hospitals are located in urban districts of HCMC and only 10 kilometers apart, the three hospitals are not only responsible for treatment of patients from HCMC but also receive sick children with HFMD, especially those with severe disease, from other provinces in south of Vietnam. For high

resolution of the mapping analysis, here we focused our analysis on data from patients coming from Ho Chi Minh City only, defined as living within one of the 24 districts of Ho Chi Minh City.

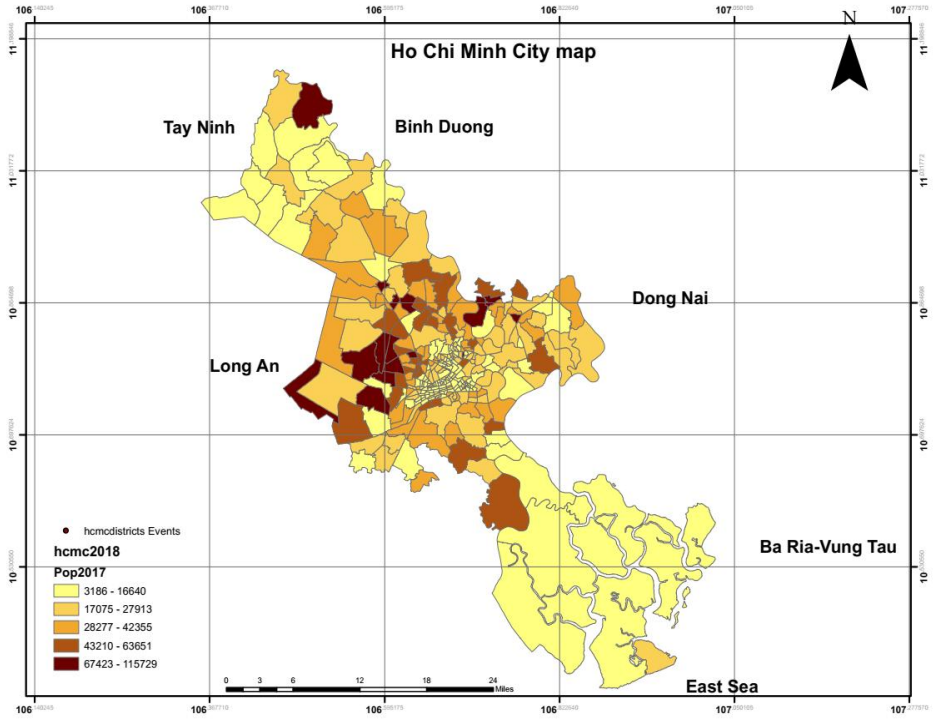


Figure 2.2: Ho Chi Minh City map and population density at commune level in 2017

2.2.4 **Methodology for mapping analysis**

Mean center, standard distance and directional distribution (standard deviational ellipse) were employed to depict the characteristics of spatial distribution of HFMD in Ho Chi Minh City, using ArcGIS software version 10.3. To understand the patterns of disease spread maps were used to illustrate the distribution of HFMD cases by wards and years.

In this study, each ward is represented by a point located in the centre of ward. This central point has its own coordinate including X and Y. The mean center is a point generated by taking the average of X and Y coordinates of all central points. While a mean center is similar to a geometric mean in traditional statistics, a standard distance is equivalent to a standard deviation (SD) describing to what extent points disperse around a mean center. A standard distance is displayed by a circle having inside 68% (1SD), 95% (2SD) or 99% (3SD) of total points in a map. By looking at the mean center and standard distance, characteristics of a spatial distribution can be assessed. In addition to this, a standard deviational ellipse which measures the pattern for a set of points is used to determine a directional distribution of a feature. Here is the orientation spread of HFMD in HCMC [126,127].

2.2.5 **Ethics**

The Institutional Review Board of CH1, CH2 and HTD, and the Oxford Tropical Research Ethics Committee (OxTREC) approved the study.

2.3 **Results**

2.3.1 **Characteristics of all hospitalized HFMD patients**

Over an 11-year period, between January 2005 and December 2015, there were a total of 56,048 patients living in HCMC admitted to the three aforementioned hospitals, including 25,057 (44.7%) admitted to CH1, 16,575 (29.6%) admitted to CH2 and 14,416 (25.7%) admitted to HTD. Male patients accounted for 60.8% (34,072/56,048). The predominance of males was relatively consistent during the

study period (i.e. before, during and after the major outbreak occurring in 2011-12). A total of 74 patients were recorded as decease, accounting for 0.13% of the total hospitalized cases, with the peak number of 35 cases recorded in 2011 – 2012 during the large-scale outbreak [40]. There were 23,017 cases without a specific grade recorded. Of the remaining patients, 5,650 patients had grade 1, 25,608 had grade 2A, 1225 had grade 2B, 489 had grade 3 and 59 had grade 4. While the majority of hospitalized patients during 2005-2010 have no clinical grade available for comparison, the distribution of clinical grades was relatively similar between the periods 2011-12 and 2013-15. The number of patients under 1, from >1 to 5 and > 5 years old was 11998 (21.4%), 42997 (76.7%) and 959 (1.7%), respectively (Table 2.1). The age structure of hospitalized cases was comparable during the study period (i.e. before, during and after the major outbreak occurring during 2011-12).

Table 2.1: Characteristics of study population

Variables	**All (n=56048)**	**2005-2010 (n=10918)**	**2011-2012 (n=23467)**	**2013-2015 (n=21663)**
Age (months) Median (IQR)	18 (12-25.2)	18 (12-25.2)	18 (12-26.4)	16.8 (12-24)
Age groups				
- < 12 months	11998 (21.4)	2105 (19.3)	4971 (21.2)	4922 (22.7)
- 12-24	28944 (51.6)	5942 (54.4)	11599 (49.4)	11403 (52.6)
- 25-36	9833 (17.5)	1929 (17.7)	4437 (18.9)	3467 (16.0)
- 37-60	4220 (7.5)	760 (7.0)	1987 (8.5)	1473 (6.8)
- >60	959 (1.7)	119 (1.1)	456 (1.9)	384 (1.8)

- Unknown	94 (0.2)	63 (0.6)	17 (0.1)	14 (0.1)
Gender (n, %)				
- Male	34072 (60.8)	6816 (62.4)	14200 (60.5)	13056 (60.3)
- Female	21976 (39.2)	4102 (37.6)	9267 (39.5)	8607 (39.7)
Clinical grade				
- 1	5650 (10.1)	1358 (12.4)	3022 (12.9)	1270 (5.9)
- 2A	25608 (45.7)	753 (6.9)	10151 (43.3)	14704 (67.9)
- 2B	1225 (2.2)	0	824 (3.5)	401 (1.9)
- 3	490 (0.9)	1	261 (1.1)	227 (1)
- 4	58 (0.1)	0	47 (0.2)	12 (0.1)
- Non specific	23017 (41.1)	8806 (80.7)	9162 (39)	5049 (23.3)
Fatal outcome	74 (0.1)	36 (0.3)	35 (0.1)	3

2.3.2 **Temporal distribution of hospitalized HFMD cases in Ho Chi Minh City**

The total number of hospitalized HFMD cases observed increased each year from 2005 to 2012, beginning at 294 in 2005, reaching 2,930 in 2010, and then increasing to 12,112 in 2011 and 11,355 in 2012 (Figure 2.1). From 2013 to 2015, an average of around 7,200 hospitalized HFMD cases was recorded. The peak number of cases a month occurred in September – December in 9 out of the 11 years of the data. In 2008 and 2011, the peak occurred in May and June respectively.

2.3.3 **Spatial distribution of HFMD cases in Ho Chi Minh City**

The Figure 2.2 shows annual cases in each district over the 11-year period. In general, it is obvious that there was an upward trend in the number of HFMD hospitalized cases in all districts over the study period, with the highest peaks in

2011 and 2012. Compared to the overall average data for the entire 11-year period, 24 districts of the city could be classified into three groups. The first group including Binh Chanh, District 8 and Binh Tan had significant greater number of cases than that of other districts. The second group consisting of District 6, District 12, Go Vap, Tan Binh, Tan Phu and Hoc Mon had slight higher number of cases than that of the third group.

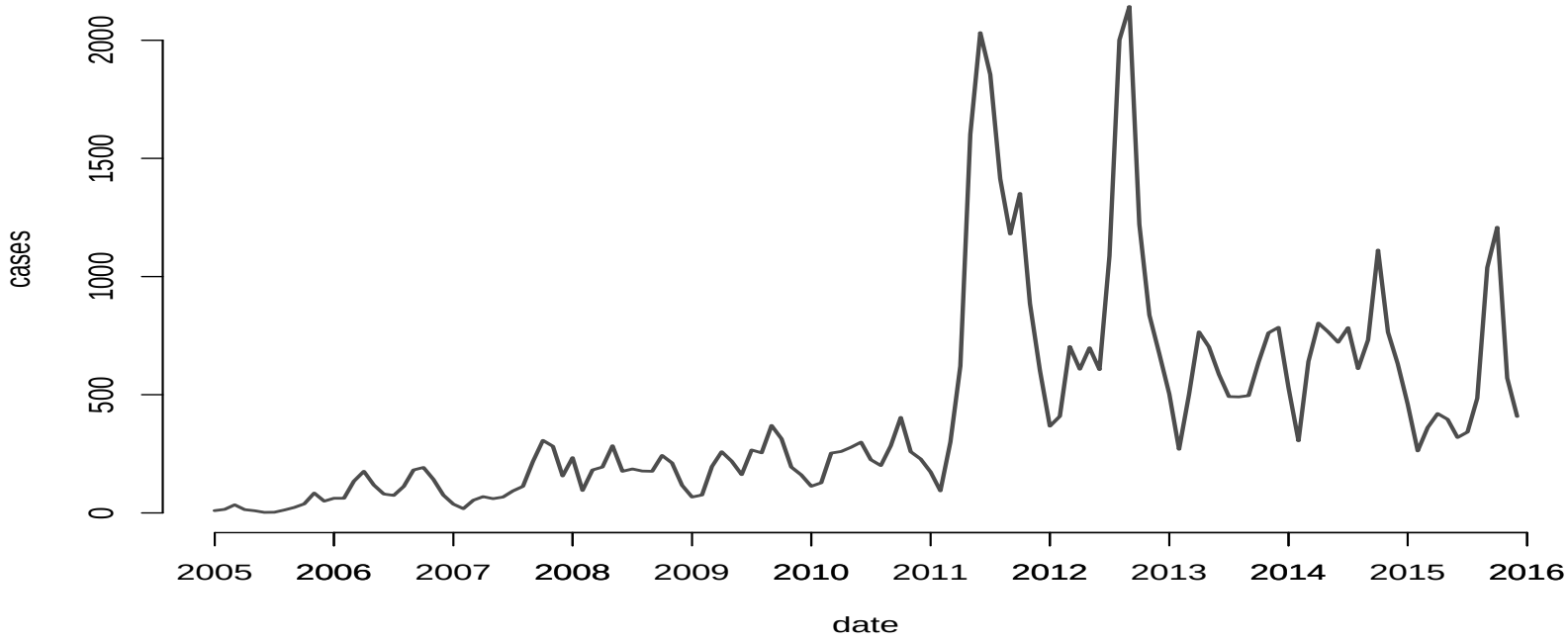


Figure 2.3: Temporal distribution of HFMD in HCMC, Vietnam from 2005 to 2015

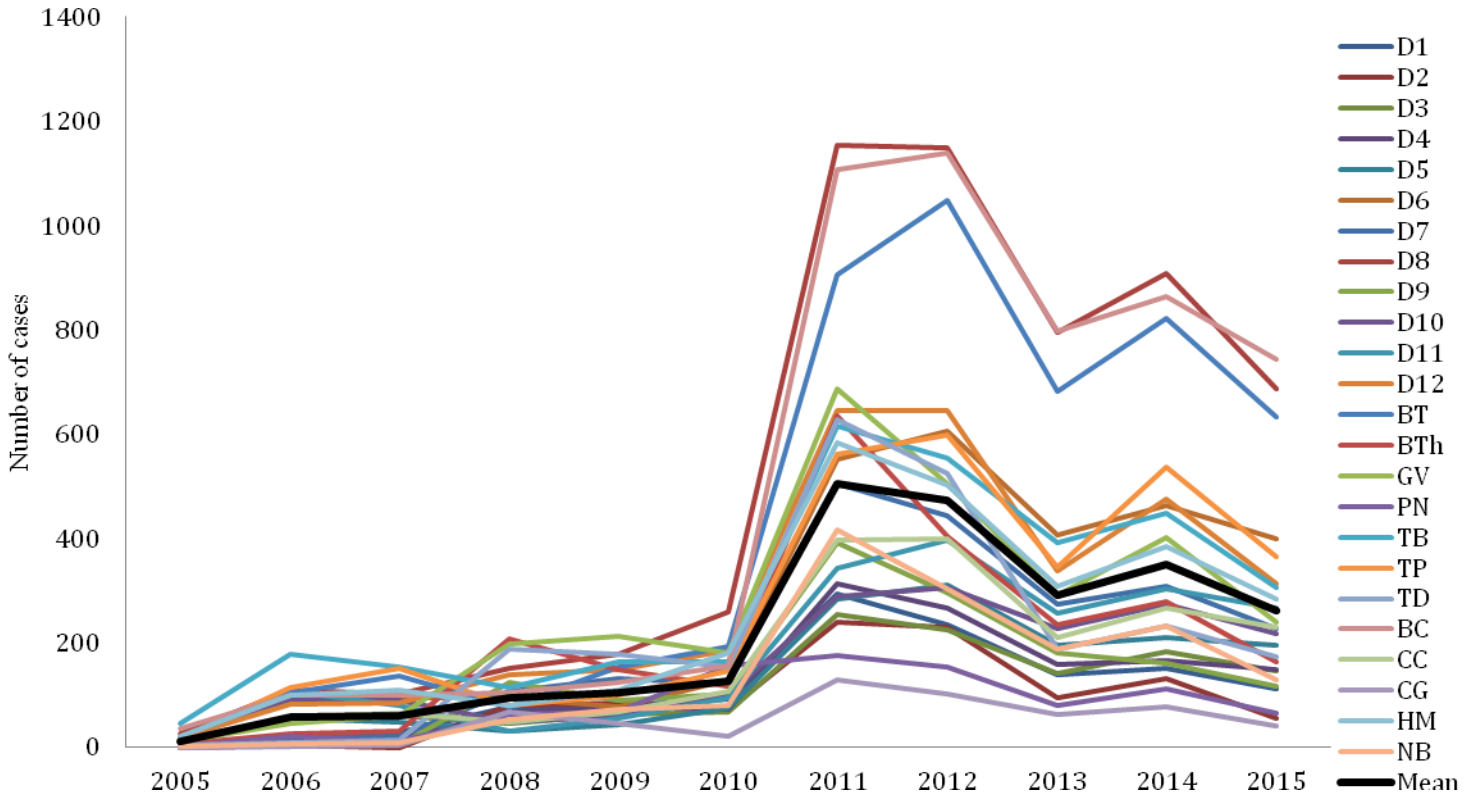
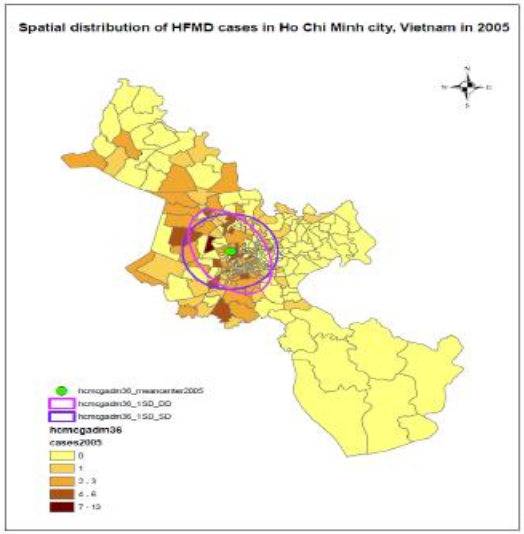
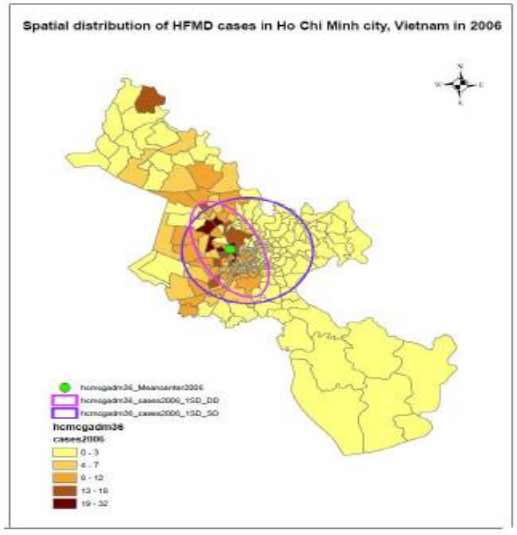


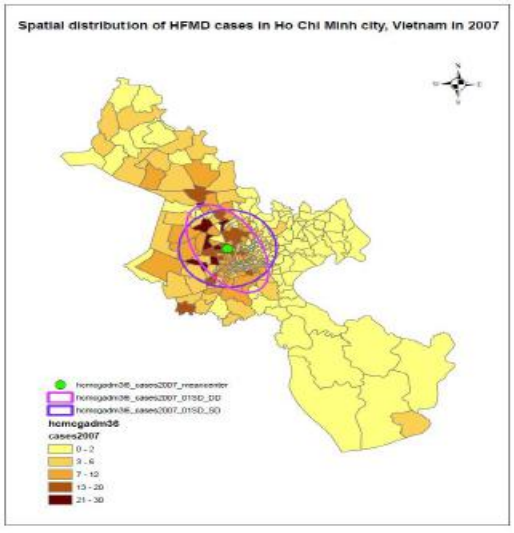
Figure 2.4: Distribution of HFMD cases by district from 2005 to 2015

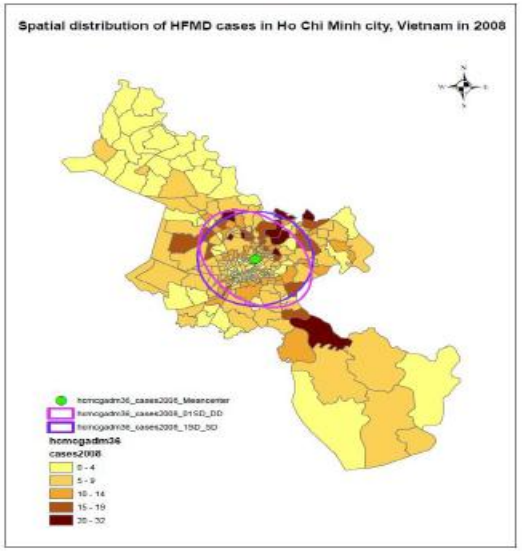
Note to Figure 2.4: D1: District 1, D2: District 2, D3: District 3, D4: District 4, D5: District 5, D6: District 6, D7: District 7, D8: District 8, D9: District 9, D10: District 10, D11: District 11, D12: District 12, BT: Binh Tan District, BTh: Binh Thanh District, GV: Go Vap District, PN: Phu Nhuan District, TB: Tan Binh District, TP: Tan Phu District, TD: Thu Duc District, BC: Binh Chanh District, CC: Cu Chi District, CG: Can Gio District, HM: Hoc Mon District, NB: Nha Be District

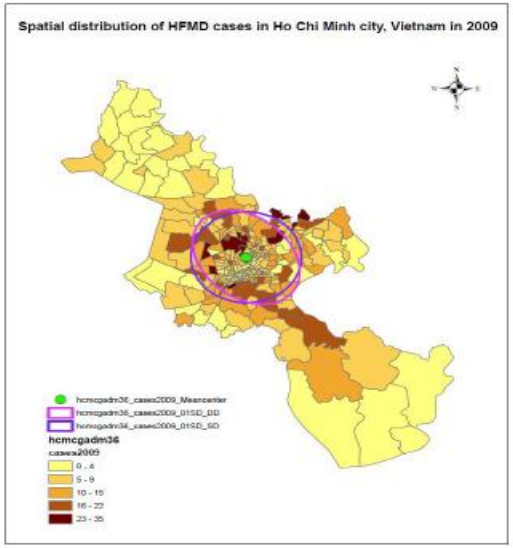
In terms of mapping analysis, the Figure 2.3 shows the distribution of HFMD hospitalized cases in Ho Chi Minh City over the 11-year period, with location unit being ward. It is clear that the turning point of the HFMD trend was 2008 when the disease spread to the south-east of the city where there were several wards with high numbers of cases (Figure 2.3). As a result, the mean center moved to the east at the coordinate of 106.682, 10.791. For the next two years, HFMD cases moved to the west again, coinciding with the unprecedented outbreak in 2011 and 2012 with the vast majority of wards in the city, especially those in the west, affected. After 2012 the disease mostly existed in the west of the city, with a steady decrease in the number of cases for the last three years of the study period.

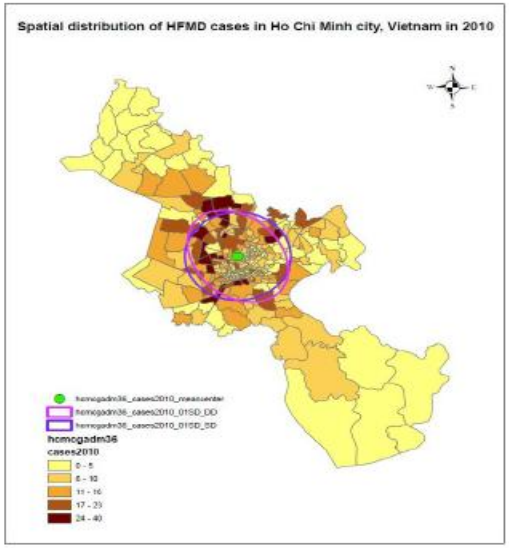

Spatial distribution of HFMD cases in Ho Chi Minh city, Vietnam in 2005
hcmcgadm36
cases2005
0
1
2 - 3
4 - 6
7 - 13


Spatial distribution of HFMD cases in Ho Chi Minh city, Vietnam in 2006
hcmcgadm36
cases2006
0 - 3
4 - 7
8 - 12
13 - 18
19 - 32


Spatial distribution of HFMD cases in Ho Chi Minh city, Vietnam in 2007
hcmcgadm36
cases2007
0 - 2
3 - 6
7 - 12
13 - 20
21 - 30


Spatial distribution of HFMD cases in Ho Chi Minh city, Vietnam in 2008
hcmcgadm36
cases2008
0 - 4
5 - 9
10 - 14
15 - 19
20 - 32


Spatial distribution of HFMD cases in Ho Chi Minh city, Vietnam in 2009
hcmcgadm36
cases2009
0 - 4
5 - 9
10 - 15
16 - 22
23 - 35


Spatial distribution of HFMD cases in Ho Chi Minh city, Vietnam in 2010
hcmcgadm36
cases2010
0 - 5
6 - 10
11 - 16
17 - 23
24 - 40

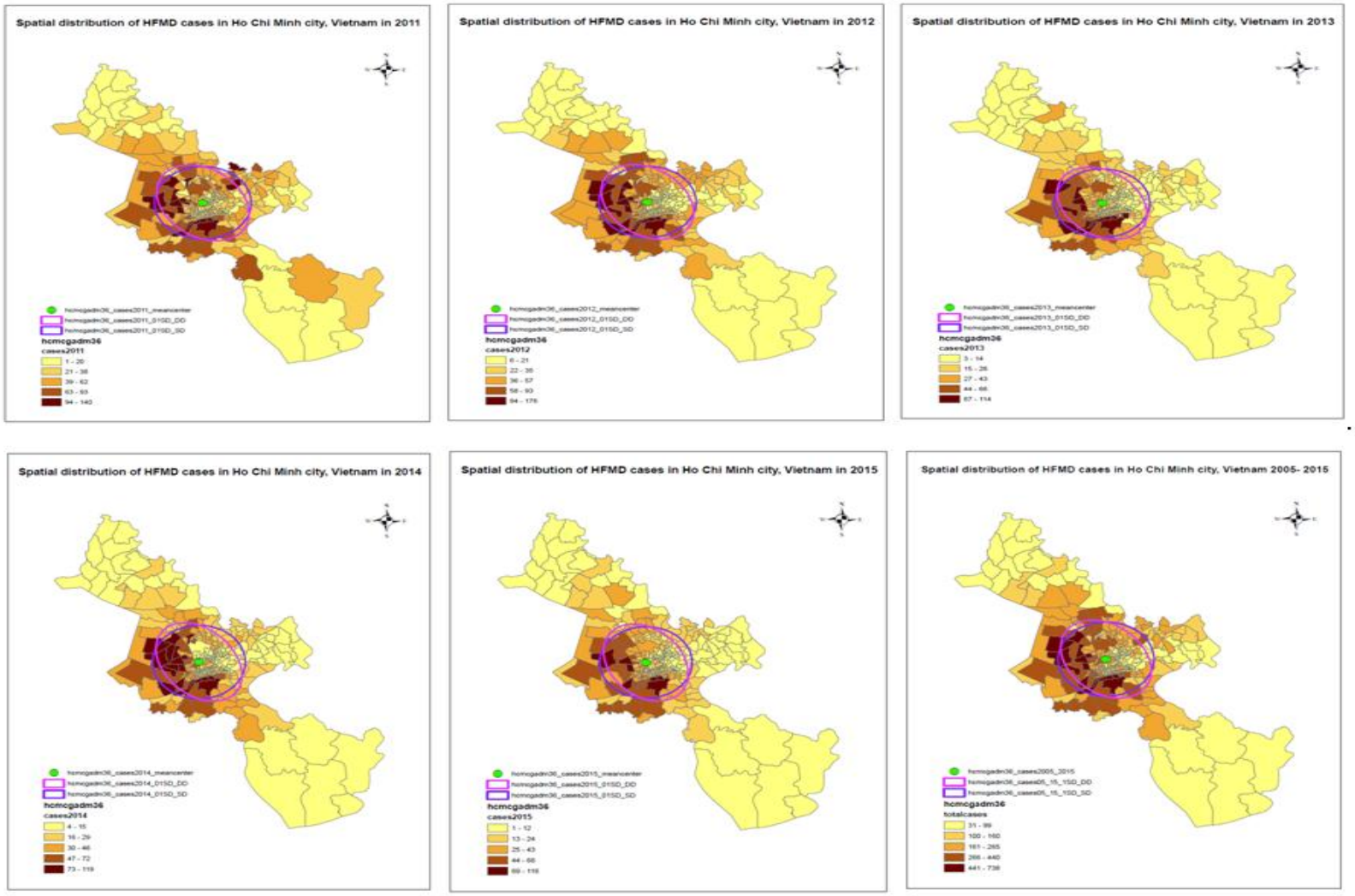


Figure 2.5: Spatial-temporal distribution of HFMD cases in HCMC, Vietnam, from 2005 to 2015.

Note to Figure 2.5: The red point, violet circle and pink ellipse represented the mean center, 01SD distance deviation and directional distribution, respectively.

2.4 **Discussion**

HFMD has been a notifiable disease in Vietnam since 2005, primarily being observed in southern provinces with HCMC being the major hotspot. Here I described the epidemiology and reconstructed the temporal and spatial distributions of hospitalized HFMD cases in HCMC over an 11-year period in HCMC.

2.4.1 **Characteristics of HFMD hospitalized cases**

Our study found that HFMD was more likely to be observed in males, with proportion at around 60%, consistent with previous reports [21,39,128], and findings from other infectious diseases (e.g. dengue) in Vietnam and the region. It may suggest that besides host immune status and hygienic condition, behavior may be the main factor to explain why males had a higher risk of HFMD infection [128]. It has been suggested that boys, in Vietnam particularly, may have more interactions with others (e.g. adults) because presumably they often receive more attention from relatives and others, and tend to be more active than girls, proposing higher exposure to contaminated surfaces, toys, or fomites [5,128]. In relation to age distribution, our finding consistently showed that 98% of cases were under 5 years old with 21% were under 1 year. This finding is agreement with previous reports [21,22,128,129]. However, the proportion of infants in our study was nearly double that of the study conducted in Henan-China in which this

figure was reported at around 10% [130]. This discrepancy may be because of different host and/or community immune status, socioeconomic factors and climatic factors, and/or the difference in design of the respective studies [41,49]. In terms of fatal outcome, there were a total of 74 deaths over 11-year period, with the majority of cases occurring before 2013 (71/74) and half of the cases attributed to the 2011-12 outbreak. This may be related to three factors. First, health staff may lack experience in identification and treatment of HFMD, especially physicians working in primary health facilities. As a consequence, this may lead to delayed admission to the three referral hospitals or presentation at these hospitals in severe stages of disease [3]. Secondly, there may have been changes in the virulence of pathogens causing HFMD over the study period. It was reported that EV-A71 and CV-A16 were the most common pathogens of HFMD before 2011 and EV-A71 was responsible for 3 deaths among 764 hospitalized cases in CH1 [21]. After that EV-A71 was detected in a high proportion of hospitalized cases and deaths during the large-scale outbreak in 2011-2012 [3,40]. From 2013 to 2015 alongside with EV-A71 (identified in 24.4% of cases), CV-A6, CV-A10 and CV-A16 were detected in 21.8%, 7.9% and 10.8% of cases respectively. The third possible explanation for the lower mortality is that, the healthcare system itself has been strengthened after the large outbreak and activities such as staff training and updated guidelines have led to improved outcomes.

2.4.2 **Temporal and spatial distribution of HFMD hospitalized cases**

Our results showed that the HFMD epidemic in HCMC started in wards located in the west of the city. The directional spread of the disease from the north-west to the south-east between 2005 and 2007, followed by the spread to the west and the south of the city in 2008 may be attributable to several factors. Firstly, the wards in the west of the city may have had higher numbers of vulnerable subjects (i.e. young children) than the other wards (the 2017 population of the west, east, north, south and central districts was approximately 3.7, 0.62, 2.48, 0.7 and 0.84 million, respectively)[131]. Secondly, these wards have borders with Long An province, which is part of the Mekong Delta River Region, which has been the source of several circulating infectious disease outbreaks such as hand, foot and mouth disease, dengue, typhoid, Japanese encephalitis and others[49,131]. Due to the living cost and job opportunities, the immigrants from this region may tend to choose the nearer districts to live when they come to HCMC for the first time. Lastly, people, including adults who also contribute to the transmission chains of HFMD, are perhaps more likely to move towards the center of the city for their work and education, possibly making HFMD spread to the eastern districts.

The exact reasons for the spread of HFMD in 2009 and 2010 are unknown. At this time HFMD spread back to the west and attacked several new wards in the north and centre of the city. This may have been a consequence of public health

response, herd immunity or pathogen emergence combined. Of note, we have recently report the emergence of CV-A6 in Vietnam [23,76], which was identified to be circulating in the southern Vietnam some time in 2010 prior to emergence in 2012-2013.

The large-scale outbreak occurred at a citywide level in Ho Chi Minh City in 2011 and 2012, with more than 23,000 admissions and 35 deaths. The main cause of the outbreak was EV-A71[3,23,40,132], which also caused various severe outbreaks in Taiwan, Malaysia, Korea, Japan and China [5,21]. The epidemic cycle pattern of HFMD in HCMC however differs from that in Taiwan, Japan and Malaysia. While Taiwan, Japan and Malaysia had a pattern of 2-3 yearly outbreaks, HFMD has been observed annually in Vietnam [5,21]. The occasionally observed two peaks per year of HFMD may have been attributed to the circulation and replacement of dominant HFMD causing enterovirus serotypes with CV-A being dominant in the first part- and EV-A71 in the second part of the year [21,76].

2.4.3 **Limitations and future plans**

Despite a holistic effort looking at the epidemiology of HFMD over an 11-year period in the most populous city in Vietnam, our study has some limitations. First, only admission data from 3 hospitals were used and it is likely that the total number of cases is much higher than reported, especially that of mild disease. However by confining our study to hospitalized cases we ensured more accurate

diagnoses and also concentrated on the disease severity that presents the greatest public health burden. Furthermore the hospitals we included are specialist hospitals with experienced staff making the accurate diagnosis of HFMD more likely. This study also did not explore effect of population movement on HFMD transmission because it is currently estimated that there are at least two million non-residents who enter and leave HCMC every day. These people may bring HFMD to the city or spread the disease to other provinces, but focusing our analysis only on HCMC residents may have reduced sensitivity to detect important patterns here. Further limitations are that due to the unavailability of the data, I did not taken into account factors such as climatic variables, socioeconomic factors, the pathogens and co-variables (population size), especially the proportion of children less than 5 years old, for the spatial analysis. Ideally these would be included in future studies if data can be obtained.

2.5 **Conclusion**

The present study highlights a significant burden posed by the ongoing HFMD epidemic in HCMC, a metropolitan city of Vietnam over 11 years, with an increasing number of hospitalized cases, especially during the last 5 years of the study period (2011-15). Despite the upward trend of the reported cases, it was relatively consistent that districts located in the west of the city often acted as the main ‘hotspots’ of the epidemic, which merits further research. Likewise, because

of its burden, research to unravel important aspects on etiology, clinical characteristics, economic burden and disease pathophysiology of this emerging infection remains essential for outbreak response, the development of intervention strategies (e.g. vaccines) and clinical management.

Chapter 3

Clinical, etiological and epidemiological investigation of hand, foot and mouth disease in southern Vietnam during 2015 – 2018

3.1 **Background**

Given the high endemicity and the complex epidemic dynamics of HFMD, synthesizing clinical, epidemiology and etiology of this emerging infection remains essential to inform the development of appropriate interventions (including vaccines) and design public health measures in order to reduce its global burden. As such since 2013, a comprehensive hospital based study has been conducted at three major hospitals in Ho Chi Minh City aiming at capturing long-term data on these aspects of this emerging infection [21,76,133,134]. Herein, I report the results of my investigations for the period during 2015 – 2018, covering the most recent severe HFMD outbreak recently documented in Vietnam [135].

3.2 **Materials and Methods**

3.2.1 **Settings**

The clinical and patient data used in the present study were derived from an ongoing clinical study of HFMD, which has been conducted at the Hospital for Tropical Diseases (HTD), Children's Hospital 1 (CH1) and Children's Hospital 2 (CH2) in Ho Chi Minh City, Vietnam since 2013 [134,135]. The results of the first part of the clinical study for the period from July 2013 to July 2015 have previously been reported elsewhere [134]. Here, I focus on analysis for the period from August 2015 to December 2018.

3.2.2 **Patient enrollment and data collection**

Any patient ≤12 years of age presenting to outpatient departments or admitted to inpatient wards of the three participating hospitals with a clinical diagnosis of HFMD and, if outpatients, an illness day of ≤3 days for enrolment in our study was eligible to participate in the study. Patients were excluded if the attending physician believed another diagnosis was more likely. Because of the waning of the epidemic in 2017 and the availability of the resources, from January to December 2018, patient recruitment focus was reduced to CH1 site.

Collected information included demographic, clinical signs/symptoms (including clinical grades) on enrolment to the study and (if inpatients) daily until discharge or day seven of hospitalization (whichever came first), treatments, laboratory tests, length of hospital stay (inpatients only), and outcomes. Additionally, for virological assessments, an acute throat swab and rectal swab were collected at enrolment from each participant.

3.2.3 **Determination of enterovirus serotype and EV-A71 subgenogroup**

Enterovirus serotypes determination was carried out using a combination of PCR and sequencing approaches [109,136,137] (Figure 3.1). In brief, viral RNA was first extracted from throat swabs collected from the study participants using a QIAamp viral RNA kit (Qiagen GmbH, Hilden, Germany). Extracted viral RNA was then analysed using a one-step multiplex real-time RT-PCR assay to simultaneously detect enteroviruses and EV-A71 in the extracted NA materials

[109]. All specimens positive for enterovirus serotypes or EV-A71 were then tested to further identify specific enterovirus serotypes or EV-A71 subgenogroups, respectively, using a combination of VP1 PCR amplification and sequencing of the obtained PCR amplicon [40,136,137]. Finally, the obtained VP1 sequences were then analyzed using a previously described online tool to determine enterovirus serotype or EV-A71 subgenogroup [138]. In case the one-step multiplex real-time RT-PCR analysis of the throat samples gave a negative result, rectal swabs were further analyzed, and if positive, the same subsequent steps were repeated to identify enterovirus serotypes or EV-A71 subgenogroups [109]. All primers and probes used are listed in Table 3.1, and the detailed PCR and sequencing procedures are presented in Appendix 1.

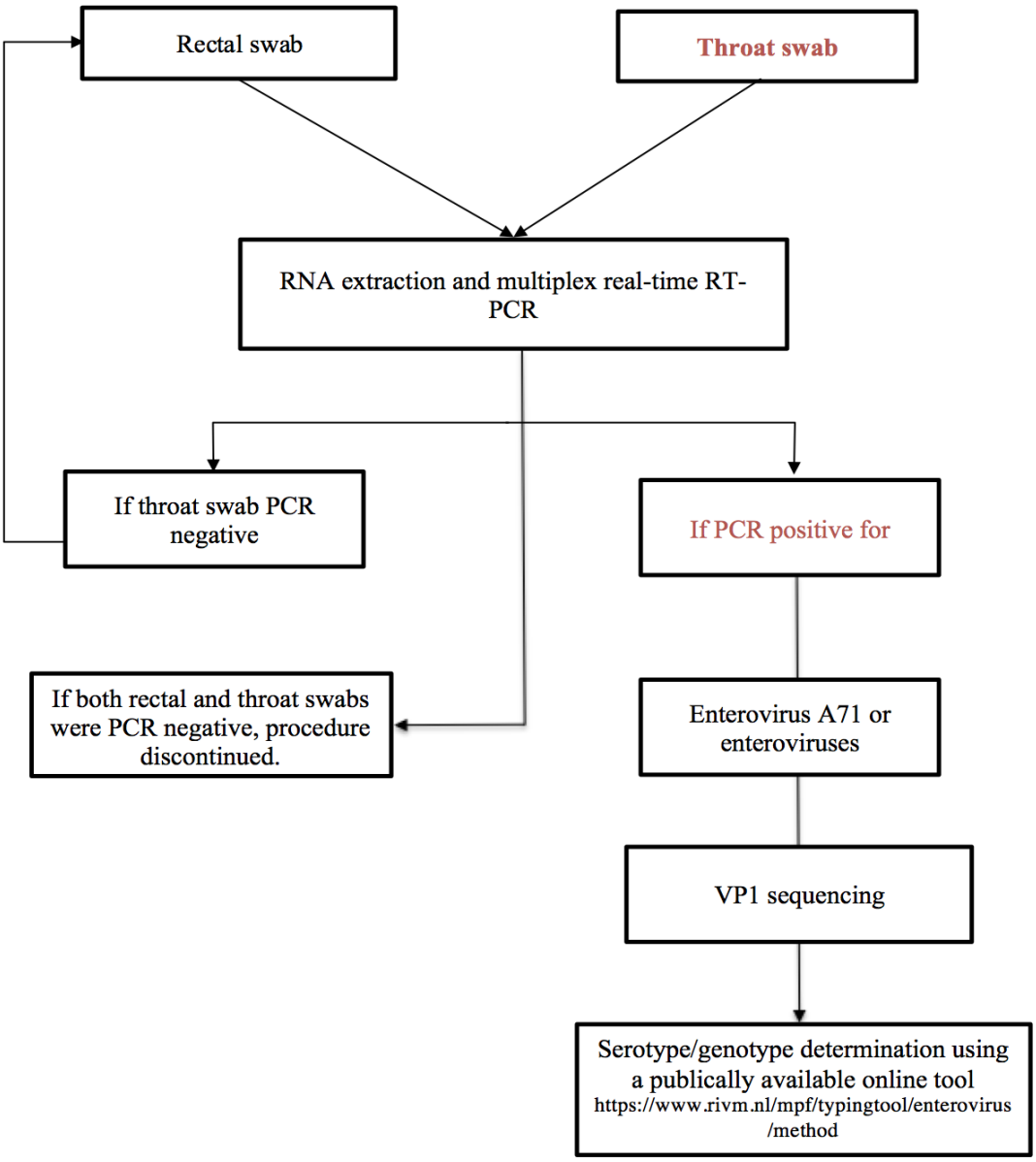


Figure 3.1: Flow chart outlining the procedure applied for enterovirus detection in clinical samples and serotyping of enteroviruses.

Table 3.1: List of oligo sequences used for simultaneous detection, serotyping of EVs or genotyping of EV-A71

Primers and probes used for primary screening of clinical samples [109]			
Name	**Sequence (5'→3')**	**Position**	**Note**
ENT-F	CCCTGAATGCGGCTAAT	452 - 468	
ENT-R	ATTGTCACCATAAGCAGCC	579 - 597	
ENTr-probe	Cy5-ACCCAAAGTAGTCGGTTCCG -BHQ3	531 - 551	
EAV-F primer	CATCTCTTGCTTTGCTCCTTAG	1847–1868	
EAV-R primer	AGCCGCACCTTCACATTG	1980–1997	
EAV-probe	FAM-CGCTGTCAGAACAACATTATTGCCCAC-BHQ1	1926–1964	
EV-A71-634 F	GGAGAACACAARCARGAGAAAGA	634 - 656	
EV-A71-743R	ACYAAAGGGTACTTGGAYTTVGA	721 - 743	
EV-A71-probe	Cyan500-TGATGGGCACDTTCTCRGTGCG-BHQ1	686 - 707	
Primers used for enterovirus serotyping [136]			
Name	**Sequence (5'→3')**	**Position**	**Note**
SO224	GCIATGYTIGGIACICAYRT	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	2602 - 2627	2nd round PCR (forward)
AN88	TACTGGACCACCTGGNGGNAYRWACAT	2977 - 2951	2nd round PCR (reverse)
AN232	CCAGCACTGACAGCA	2602 - 2616	Sequencing
AN233	TACTGGACCACCTGG	2977 - 2963	Sequencing
Primers used for enterovirus A71 genotyping [40]			
Name	**Sequence (5'→3')**	**Position**	**Note**
EV71-VP1-3F	AGAYAGGGTGGCRGATGT	2462 - 2445	
EV71-VP1-703R	CTGAGAACGTGCCCATCA	3125 3142	

Note to Table 3.1: EAV: Equine arteritis virus, which serves internal control; *(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)*

3.2.4 **Phenotypic comparison between major subgenogroups of EV-A71**

The abovementioned clinical study has been sampling HFMD cases since July 2013. As such, this gave me a unique opportunity to capture the transition between predominant subgenogroups of EV-A71 in Vietnam over a period of 6 years (2013 - 2018). To assess the differences in clinical phenotypes between subgenogroups of EV-A71, I used a combined dataset of the first- and the second phases of the clinical study (i.e. during July 2013-July 2015 and August 2015-December 2018, respectively).

3.2.5 **Statistical analysis**

Data was analysed using IBM Statistical Package for Social Sciences (SPSS) Version 23.0. A P value of less than 0.05 ($P < 0.05$) was considered to be statistically significant.

3.2.6 **Ethical statement**

The Institutional Review Board of CH1, CH2 and HTD, and the Oxford Tropical Research Ethics Committee (OxTREC) approved the study. Written informed consent was obtained from a parent or legal guardian of each enrolled patients.

3.3 **Results**

3.3.1 **Baseline characteristics of all patients, and in- and outpatients**

During August 2015 – December 2018, a total of 1196 children presenting with clinically suspected HFMD were enrolled in the clinical study. The majority of the participants were young children, with median age of 18.7 months (range 2.4 – 118.2), and predominantly males (male/female: 746/450). Nearly half (n=568, 47.5%) of the patients were from Ho Chi Minh City. In terms of clinical phenotypes, 281 (23.5%) patients had severe HFMD, comprised of 71 cases with grade 2B1, 68 cases with grade 2B2, 136 cases with grade 3 and six cases with grade 4. Milrinon, magnesium sulfate and intravenous immunoglobulin were given to 69 (5.8%), 1 (0.1%) and 215 (18.0%) patients, respectively (Table 3.2). At discharge, unfavorable outcomes were recorded in 15 patients (1.25%), including three deaths (0.25%) and 12 with complications or underlying diseases (1%). Additional details about the study participants are presented in Table 3.2.

Table 3.2: Baseline characteristics of the enrolled patients

	Total (n=1196)	Outpatients (n=262)	Inpatients (n=934)	P value	Mild* (n=915)	Severe (n=281)	P value
Demographics							
Median age (months) (range)	18.7 (2.4-118.2)	20.6 (4.1-111.1)	18.1 (2.4-118.2)	0.008	17.77 (2.4-111.1)	21.2 (4.1-118.2)	0.0001^
Male/female	746/450	170/92	576/358	0.345	564/351	182/99	0.361
HCMC origin (n, %)	568 (47.5)	162 (61.8)	406 (43.5)	0.0001	448 (49.0)	120 (42.7)	0.076
Day from onset to enrolment (days)	1 (0-10)	1 (0-3)	1 (0-10)	0.167	1 (0-10)	2 (0-7)	0.0001^
Length of stay (days)	4 (1-31)	-	4 (1-31)	-	3 (1-31)	5 (1-26)	0.0001^
Clinical features (n, %)							
Fever	936 (78.3)	110 (42.0)	826 (88.4)	0.0001	660 (72.1)	276 (98.2)	0.0001
Skin lesions	1034 (86.5)	234 (89.3)	800 (85.7)	0.126	777 (84.9)	257 (91.5)	0.005
Cough	291 (24.3)	82 (31.3)	209 (22.4)	0.003	228 (24.9)	63 (22.4)	0.393
Runny nose	237 (19.8)	78 (29.8)	159 (17.0)	0.0001	186 (20.4)	51 (18.1)	0.493
Diarrhea	117 (9.8)	10 (3.8)	107 (11.5)	0.0001	85 (9.3)	32 (11.4)	0.308
Drowsiness	263 (22.0)	19 (7.3)	244 (26.1)	0.0001	116 (12.7)	147 (52.3)	0.0001
Sweating	17 (1.4)	2 (0.7)	15 (1.6)	0.391	6 (0.7)	11 (3.9)	0.0001

Vomiting	525 (43.9)	61 (23.3)	464 (49.7)	0.0001	349 (38.1)	176 (62.6)	0.0001
Irritability	357 (29.8)	12 (4.6)	345 (36.9)	0.0001	184 (20.1)	173 (61.6)	0.0001
Myoclonus	568 (47.5)	0	568 (60.8)	0.0001	323 (36.0)	245 (87.2)	0.0001
Lethargy	27 (2.3)	3 (1.1)	24 (2.6)	0.239	7 (0.8)	20 (7.1)	0.0001
Tremor	111 (9.3)	2 (0.8)	109 (11.7)	0.0001	9 (1.0)	102 (36.3)	0.0001
Ataxia	3 (0.3)	0	3 (0.3)	ND	0	3 (1.1)	0.042
Nystagmus	1 (0.1)	0	1 (0.1)	ND	0	1 (0.4)	0.235
Limb weakness	11 (0.9)	0	11 (1.2)	ND	0	11 (3.9)	0.0001
Hypertension	69 (5.8)	0	69 (7.4)	ND	0	69 (24.6)	0.0001
Blood tests							
WBC (1000 cells/mm^3)	12.3 (2.5-76.0)	11.3 (5.6-26.9)	12.6 (2.5-76.0)	0.001	12.2 (3.9-64)	12.4 (2.5-76.0)	0.559^
Platelet (1000 cells/mm^3)	321 (29-849)	325 (29-741)	321 (31-849)	0.421	314 (29-788)	347 (38-849)	0.0001^
Blood glucose (mg/L)	101 (42-223)	121 (42-212)	96 (42-223)	0.0001	102 (42-223)	98 (42-176)	0.028^
CRP (mg/L)	9.8 (0-620)	7.8 (0-79)	11.0 (0-620)	0.025	12.0 (0-620)	3.6 (0-87.5)	0.0001^
Highest grade (n, %)							
1	251 (21.0)	249 (95.0)	2 (0.2)	0.0001	257 (27.8)	NA	NA
2A	664 (55.5)	13 (5.0)	651 (69.7)	0.0001	668 (72.2)	NA	NA

2B1	71 (5.9)	NA	71 (7.6)	0.0001	NA	71 (25.3)	NA
2B2	68 (5.7)	NA	68 (7.3)	0.0001	NA	68 (24.2)	NA
3	136 (11.4)	NA	136 (14.6)	0.0001	NA	136 (48.4)	NA
4	6 (0.5)	NA	6 (0.6)	0.349	NA	6 (2.1)	NA
Treatment (n, %)							
Milrinon	69 (5.8)	0	69 (7.4)	ND	0	69 (5.8)	0.0001
Magnesium sulfate	1 (0.1)	0	1 (0.1)	ND	0	1 (0.4)	ND
IVIg	215 (18.0)	0	215 (23.0)	ND	0	215 (76.5)	0.0001
Outcome (n, %)							
Full recovery	1181 (98.7)	262 (100)	919 (98.4)	0.052	913 (99.8)	268 (95.4)	0.0001
Recovery with complication or underlying diseases#	12 (1.0)	0	12 (1.3)	0.08	2 (0.2)	10 (3.6)	0.0001
Death	3 (0.25)	0	3 (0.3)	1	0	3 (1.1)	0.013

**Note to Table 3.2: ***Grade 1 and 2A; ND: not done; (^): Mann- Whitney U test

[#]Limb weakness and squint-eyed (n=2), limb weakness (n=5), "slow communication" (n=1), cranial nerve VII paralysis (n=1), unconsciousness (n=1), Guillain Barre syndrome (n=1), prolonged fever and paralyzed diaphragm (n=1), severe anemia (n=2) and subacute encephalitis (n=1).

Of 1196 patients enrolled in the study, there were a total of 262 outpatients (21.9%) and 934 inpatients (78.1%). There were considerable differences in demographics, clinical presentation and results of blood biochemistry parameters between out- and inpatients. Notably, inpatients were younger than outpatients and were more likely to come from other provinces. Inpatients had a slightly higher while blood cell count and C reactive protein, but a lower level of blood glucose (Table 3.2).

Compared with mild patients (Grade 1 or 2A), severe patients (Grade 2B1 or above) were admitted to the hospital later. Additionally, severe patients were slightly older, had a higher platelet count, but a lower level blood glucose and C reactive protein level than those with mild disease. However, the differences between out-and inpatients as well as between mild and severe patients in clinical presentations merely reflect the differences in clinical criteria utilized to classify HFMD grades.

3.3.2 **Results of enteroviral investigation: an overview**

Evidence of enterovirus was identified in 977 (81.6%) of 1196 study participants, including 339 EV-A71 (28.3%) and 638 non-EV-A71 enteroviruses (53.3%). Of the 339 EV-A71 positive cases, subgenogroup determination was successful in 124 (36.5%) cases (including 52 subgenogroup B5 and 72 subgenogroup C4). Of the non-EV-A71 enterovirus positive cases, sequencing of VP1 fragment was

successful in 494/638 (77.4%), which consisted of 18 different enterovirus serotypes, with CV-A6, CV-A10 and CV-A16 being the major serotypes (Figure 3.2). The frequencies of the remaining 15 enterovirus serotypes are presented in Table 3.3.

Table 3.3: Frequency of other enterovirus serotypes detected in HFMD cases enrolled in the clinical study

Enterovirus serotypes	All	Inpatient	Outpatient	Severe patient	Mild patient
Coxsackievirus A8	22	18	4	0	22
Coxsackievirus A2	12	12	0	2	10
Coxsackievirus A4	8	7	1	0	8
Coxsackievirus A5	7	7	0	0	6
Coxsackievirus A1	2	2	0	2	0
Coxsackievirus B1	1	1	0	0	1
Coxsackievirus B2	1	1	0	0	1
Coxsackievirus B3	1	1	0	0	1
Coxsackievirus B4	1	0	1	0	1
Echovirus 18	0	4	0	0	4
Echovirus 5	0	3	0	1	2
Echovirus 6	0	0	1	0	1
Echovirus 9	0	1	0	0	1
Poliovirus 2	0	1	0	0	1
Rhinovirus A	0	1	0	0	1

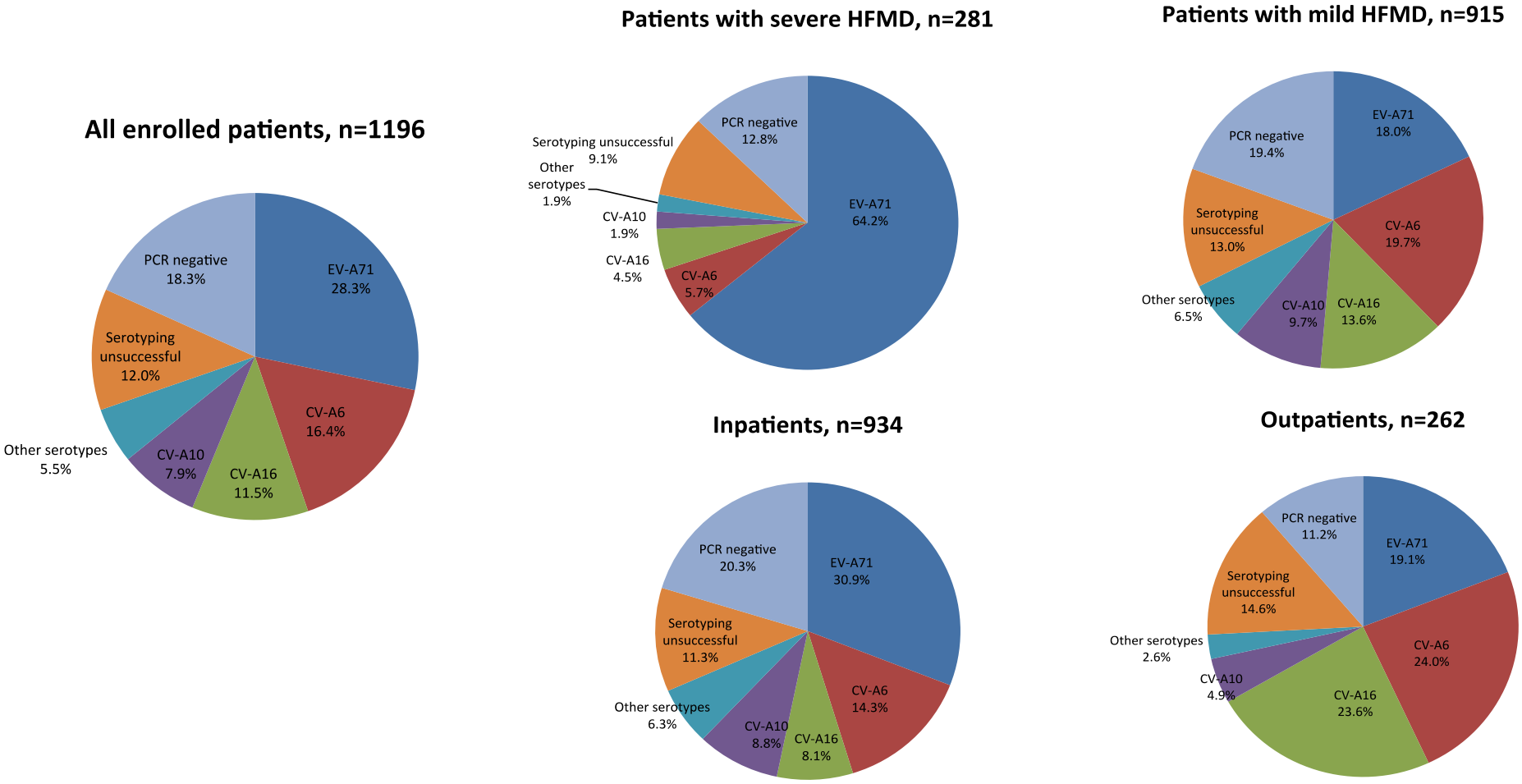


Figure 3.2: Pie charts showing the detection rates of enterovirus serotypes in all HFMD patients enrolled in the study and groups of patient with severe and mild HFMD, and in- and outpatients.

3.3.3 **The frequency of enterovirus serotypes detected in outpatients and inpatients**

The overall PCR diagnosis yield of the inpatient group was lower than that of the outpatient group. Likewise, there were some differences in the frequency of the four predominant enterovirus serotypes detected in these two groups, with EV-A71 being the major viruses detected in the inpatient group, followed by CV-A6, CV-A10 and CV-A16. In contrast with the inpatient group, CV-A6 and CV-A16 were the two leading viruses detected at comparable frequencies in the outpatient group, followed by EV-A71 and CV-A10 (Figure 3.2).

3.3.4 **The frequency of enterovirus serotypes detected in patients with mild and severe HFMD**

There were considerable differences in the overall diagnostic yield and the detection rates of predominant enterovirus serotypes (EV-A71, CV-A6, CV-A10 and CV-A16) between patients with severe and mild HFMD (Figure 3.1). More specifically, of the 281 patients presenting with severe HFMD, EV-A71 was the virus detected in 64.2% of the patients, followed by sporadic detections of CV-A6 (5.7%), CV-A16 (4.5%) and CV-A10 (1.9%) and other serotypes (1.9%)(Figure 3.2 and Table 3.3). Meanwhile, of the 915 patients with mild HFMD, the detections were comparable between EV-A71 (18%) and CV-A6 (19.7%), followed by CV-A16 (13.6%) and CV-A10 (9.7%). The frequency of the

remaining serotypes detected in out/inpatients and patients with mild/severe HFMD are presented in Table 3.3.

3.3.5 **Associated demographics, clinical and outcome of predominant enterovirus serotypes**

Detailed comparisons between patients positive for predominant enterovirus serotypes (EV-A71, CV-A6, CV-A10 and CV-A16) are presented in Table 3.4. Generally, there were remarkable similarities between patients infected with CV-A6, CV-A10 and CV-A16, while the clinical signs/symptoms as myoclonous, tremor, hypertension, vomiting and irritability, reflecting the characteristics of severe HFMD, were more often observed in EV-A71 infected patients. Likewise, unfavorable outcomes, especially death, were almost exclusively recorded in patients with EV-A71 infections. Other notable differences included age, the frequency of skin lesions, and blood biochemistry results. More specifically, patients with CV-A6 and CV-A10 infection were younger than those infected with EV-A71 and CV-A16. Skin lesions were recorded in only 61.1% (58/95) of CV-A10 positive patients, but in almost all (>91%) EV-A71/CV-A6/CV-A16 infected patients. Patients with CV-A10 and CV-A6 infection had a higher level of C reactive protein and a slightly lower level of platelet count than EV-A71/CV-A16 infected patients.

Table 3.4: Characteristics of HFMD patients positive for predominant pathogens

	EV-A71 (n=340)	CV-A6 (n=196)	CV-A10 (n=95)	CV-A16 (n=137)	P value
Demographics					
Median age **(**months**)** (range)	22.07 (4.2-118.2)	15.5 (4.4-55.4)	15.23 (2.4-77.2)	20.7 (9.0-106.1)	0.0001(*)
Sex (male/female)	215 /125	142/54	55/40	80/57	0.107
HCMC origin (n, %)	158 (46.5)	91 (46.4)	52/43	73/64	0.023
Day from onset to enrolment	2 (0-7)	1 (0-10)	1 (0-5)	1 (0-5)	0.0001*
Length of stay (days)	4 (1-26)	3 (1-31)	3 (1-8)	3 (1-12)	0.0001*
Clinical features (n, %)					
Skin lesions	324 (95.3)	182 (92.9)	57 (60.0)	132 (96.4)	0.0001
Mouth ulcers	295 (86.8)	172 (87.8)	94 (98.9)	131 (95.6)	0.0001
Fever	304 (89.4)	136 (69.4)	75 (78.9)	78 (56.9)	0.0001
Cough	76 (22.4)	39 (19.9)	26 (27.4)	31 (22.6)	0.560
Runny nose	54 (15.9)	32 (16.3)	22 (23.2)	36 (26.3)	0.032
Diarrhea	25 (7.4)	10 (5.1)	11 (11.6)	11 (8.0)	0.263
Drowsiness	143 (42.1)	22 (11.2)	9 (9.5)	8 (5.8)	0.0001
Sweating	8 (2.4)	4 (2.0)	1 (1.1)	2 (1.5)	0.833

Vomiting	175 (51.5)	85 (43.4)	30 (31.6)	43 (31.4)	0.0001
Irritability	164 (48.2)	38 (19.4)	11 (11.6)	24 (17.5)	0.0001
Myoclonus	233 (68.5)	64 (32.7)	33 (34.7)	38 (27.7)	0.0001
Lethargy	17 (5.0)	2 (1.0)	2 (2.1)	1 (0.7)	0.015
Tremor	88 (25.9)	6 (3.1)	3 (3.2)	1 (0.7)	0.0001
Ataxia	3 (0.9)	1 (0.5)	0	0	0.557
Nystagmus	1 (0.3)	0	0	0	0.739
Limb weakness	5 (1.5)	1 (0.5)	0	1 (0.7)	0.484
Hypertension	47 (13.8)	5 (2.6)	1 (1.1)	2 (1.5)	0.0001
Blood tests					
WBC (x1000 cells/mm^3)	12.5 (3.2-31.8)	12.9 (4.2-76.0)	14.55 (5.0-34.7)	11.6 (2.5-45.5)	0.0001*
Platelet (x1000 cells/mm^3)	350.5 (29-844)	324 (35-849)	308.5 (68-590)	324 (74-741)	0.0001*
Blood glucose (mg/L)	100 (42-198)	102 (42-174)	96 (48-212)	104.5 (42-195)	0.596*
CRP (mg/L)	3.25 (0-74)	19.9 (1.0-620)	25.1 (1.0-187.0)	8 (0.4-79.8)	0.0001*
Highest grade (n, %)					
1	50 (14.7)	55 (28.1)	14 (14.7)	63 (46.0)	0.0001
2	113 (33.2)	125 (63.8)	76 (80.0)	60 (43.8)	0.0001

2B1	32 (9.4)	5 (2.6)	3 (3.2)	8 (5.8)	0.007
2B2	50 (14.7)	4 (2.0)	0	1 (0.7)	0.0001
3	89 (26.2)	7 (3.6)	2 (2.1)	5 (3.6)	0.0001
4	6 (1.8)	0	0	0	0.055
Treatments (n, %)					
Milrinon	49 (14.4)	4 (2.0)	1 (1.1)	2 (1.5)	0.0001
Magnesium sulfate	1 (0.3)	0	0	0	0.285
IVIg	135 (15.6)	10 (5.1)	2 (2.1)	6 (4.4)	0.0001
Outcome (n, %)					
Full recovery	331 (97.4)	195 (99.5)	95 (100)	135 (98.5)	0.137
Recovery with complication	7 (2.1)	1 (0.5)	0	2 (1.5)	0.290

Note to Table 3.4: *Kruskal-Wallis test

3.3.6 **Temporal distribution of predominant enterovirus serotypes**

Temporally, there were remarkable fluctuations in the detection rates of predominant enterovirus serotypes (EV-A71, CV-A6, CV-A10 and CV-A16) over the study period. Generally, EV-A71 activity was consistently high during the second half of the years, while CV-A6, CV-A10 and CV-A16 predominantly circulated in the first half of the years (Figure 3.3). Of particular note also was the almost absence of EV-A71 during 2017 and the first half of 2018 followed by its emergence in late 2018.

3.3.7 **Temporal distribution of EV-A71 subgenogroup C4 and B5 and phenotypic comparison between them**

Together with the previous phase of the study [133,134,139], information about EV-A71 subgenogroups was available in a total of 273 patients (including 190 subgenogroup B5 and 83 subgenogroup C4). Temporally, these two subgenogroups replaced each other over six years in southern Vietnam. Specifically, from July 2013 to December 2016, B5 was dominant. After a period of low EV-A71 activity in 2017 and early 2018, C4 became the major virus detected between July and October 2018, followed by co-circulation of C4 and B5 during the last two months of 2018 (Figure 3.4).

In terms of phenotype, patients infected with subgenogroup C4 were more likely to have severe HFMD than those infected with subgenogroup B5. The results were consistent for the whole study period (i.e. during 2013 and 2018), (Table 3.5).

Table 3.5: Phenotypic comparison between EV-A71 subgenogroup C4 and subgenogroup B5

	2013 – 2017		2018		Total (2013 – 2018)	
	C4 (n=17)	B5 (n=164)	C4 (n=66)	B5 (n=26)	C4 (n=83)	B5 (n=190)
Mild, n%	5 (29)	139 (85)	15 (23)	12 (46)	20 (24)	151 (79)
Severe, n%	12 (71)	25 (15)	51 (77)	14 (54)	63 (76)	39 (21)
2B1	4	12	4	1	8	13
2B2	3	9	12	4	15	13
3	5	4	32	9	37	13
4	0	0	3	0	3	0
P value*	<0.001		0.04		<0.001	
OR (95%CI)	13 (3.9 – 51.5)		2.9 (0.99 – 8.5)		12.1 (6.3 – 23.7)	

Note to Table 3.5: mild: grade 1 or 2A, severe: grade 2B1 or above, *comparison between mild and severe groups

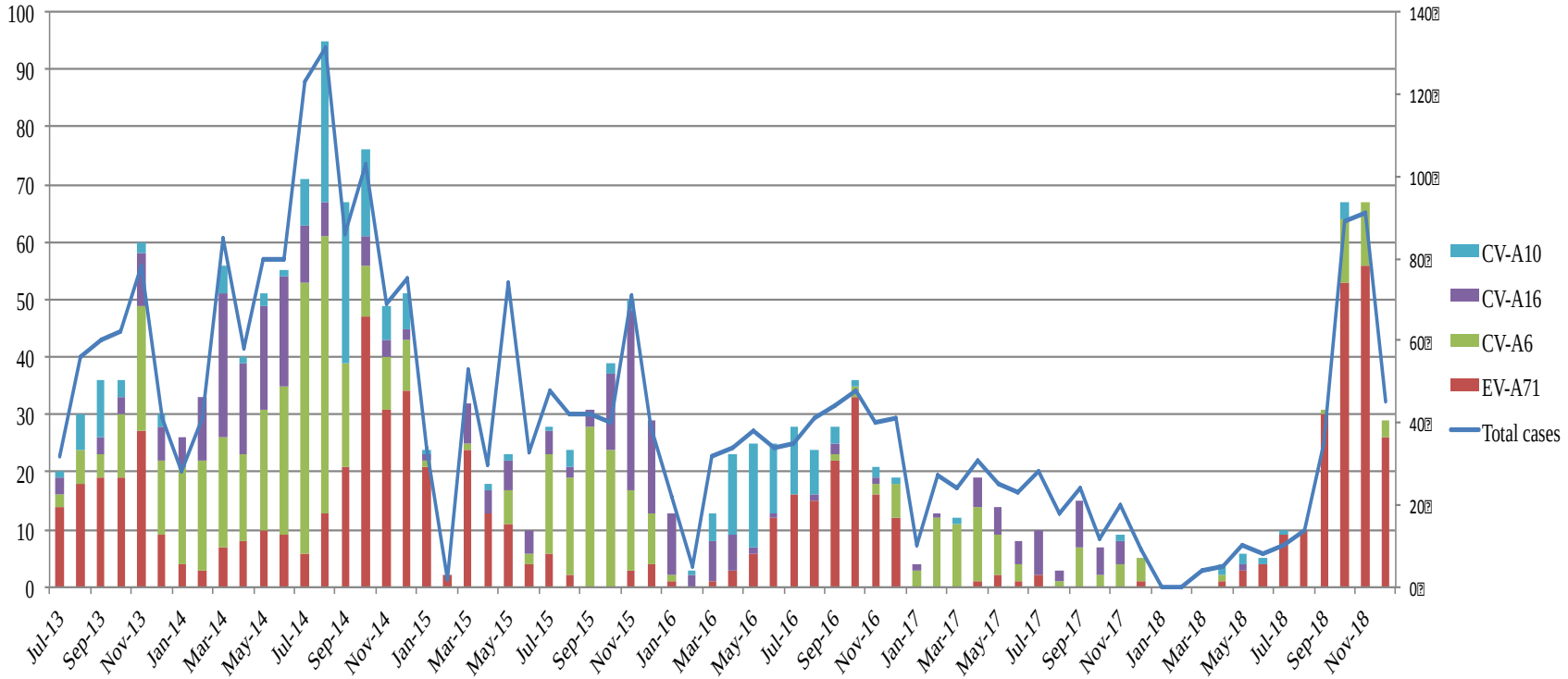


Figure 3.3: Temporal distribution of four major enterovirus serotypes during the study period and the period from July 2013 to July 2015.

Note: The Y-axis shows the number of cases. The synthesized data also covers the period from July-2013 to July 2015 which was previously reported.

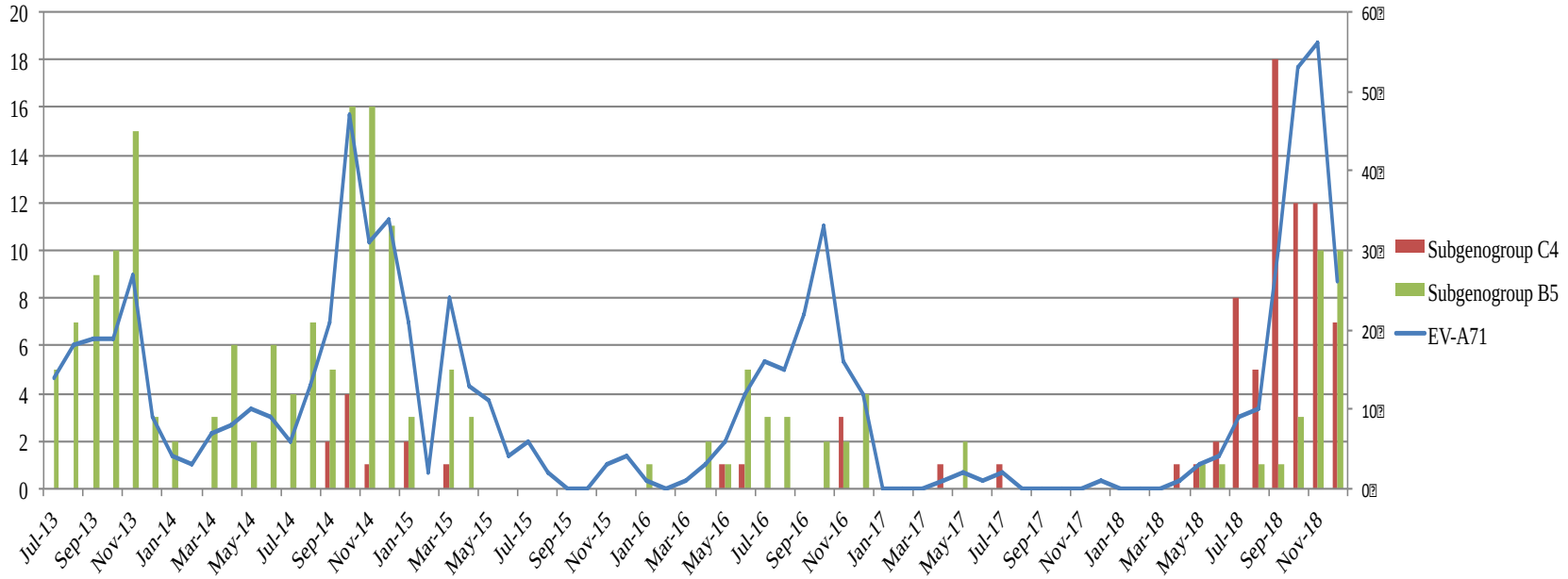


Figure 3.4: Reconstructed temporal distribution of EV-A71 subgenogroup C4 and B5 during 2013 – 2018.

Note: The left Y-axis shows the number of subgenogroup C4/B5 cases. The right Y-axis shows the number of EV-A71 cases. Data were synthesized for the period during July 2015 and December 2018 which was also derived from our previous reports for the period from July 2013 to July 2015 [134].

3.4 **Discussion**

Here I report the results of clinical, etiological and epidemiological investigations of patients presenting with clinically suspected HFMD, who were admitted to or sought for medical treatment at outpatient departments at the major hospitals in Ho Chi Minh City Vietnam, where the majority of HFMD cases in Vietnam has been reported to date. Together with previous reports from Vietnam [134,135], the data have thus provided significant insights into important aspects of this emerging infection over six years (2013 – 2018). More specifically, I have demonstrated the emergence CV-A6 as a major cause of HFMD in Vietnam, consistent with the situation worldwide [76,140], and the temporal replacement between main causes of HFMD over time, hugely important for vaccine development and implementation. Yet, despite the emergence of CV-A6 and to some extend CV-A10, EV-A71 remains the leading cause of severe HFMD in Vietnam and that EV-A71 activity was high in the second part of the calendar years during the study period. Additionally, adding to the existing data about dominance of EV-A71 subgenogroup C4 in Vietnam between July and October 2018, for which I led the investigation as the outbreak occurred [135], was the co-circulation of C4 and B5 during the last two months of the 2018 outbreak.

The underlying mechanism determining the temporal replacement between predominant HFMD pathogens throughout the year in Vietnam, as well as the emergence/disappearance of specific EV-A71 subgenogroup remain to be

determined [21,134]. Notably, in contrast to the continuous circulation of EV-A71 during the first two years of the study (2013- 2015) [134], results of the present study show that the emergence of EV-A71 during the second half 2018 was preceded by the low detection rate of EV-A71 in HFMD cases enrolled in the clinical study during 2017 and the first half of 2018, suggesting that EV-A71 endemicity in Vietnam was low during this period, which may have resulted in the accumulation of a sufficient number of susceptible young children leading to the emergence of EV-A71 in late 2018 [135,141]. Collectively, the data point out that active surveillance for pathogen circulation, especially viruses that are known to cause severe outbreaks, e.g. EV-A71, remains essential to inform the local public health authorities in response to HFMD outbreaks and the development of appropriate/effective HFMD vaccines.

My data has demonstrated that, compared with subgenogroup B5, C4 was more likely to cause severe HFMD, suggesting that they may be different in pathogenicity. This finding is consistent with a recent study conducted in northern parts of Vietnam between 2015 and 2016 [142]. In this study, Chu S.T. et al suggested that EV-A71 subgenogroup C4 might be more neuro-virulent than subgenogroup B5 based on mouse models and the proportion of hospitalized cases. However, the number of EV-A71 subgenogroup C4 in this study was significantly lower than in my study (10 vs 83) and 90% (38/40) of inpatients in this study had a clinical diagnosis of grade 2 illness, but the number of cases with grade 2A, 2B1 or 2B2 was not clearly reported and therefore, it is uncertain

whether inpatients had severe disease, defined as grade 2B1, 2B2, grade 3 and grade 4 in my study. Collectively, in terms of the association between EV-A71 subgenogroup C4 and severe HFMD, my study provided more evidence in clinical perspective while Chu S.T. *et al* proved that C4 strains of EV-A71 might cause severe disease in mice.

Viruses of subgenogroups C and B, especially C4 and B5, have been predominantly detected in recent outbreaks in the Asia-Pacific region, including China, Taiwan and Vietnam, with frequent switches in predominant subgenogroups overtime. To this end, existing data fails to link the association between specific (sub)genogroups and severe HFMD. In Vietnam, historically subgenogroup C4 has been linked with major outbreaks during 2011–2012 [40] and most recently 2018 [135]. Additionally, data of the present study show that C4 infected patients were more likely to have severe HFMD than B5 patients. As such, the results parallel observational data from Thailand and China, where C4 has been responsible for major HFMD outbreaks [4,143]. In contrast, elsewhere in Taiwan, subgenogroup C2 and B4 were responsible for the massive outbreak involving 1.5 million infections and 78 death in 1998 [6], while C4 and B5 have both been linked with recent outbreaks [144,145]. Collectively, further research should address the underlying factors that determine the scales and the severity of HFMD in specific localities, paramount importance for development of intervention strategies.

HFMD is generally a mild infection, with severe disease occurring in only a small proportion of the affected patients. It remains unresolved regarding the factors determining the clinical consequences of the affected individuals, although EV-A71 is often associated with severe HFMD. The observed data on the late hospital admission among EV-A71 infected patients and among patients presenting with severe HFMD suggests that the time since onset of illness to hospital admission may play a role.

The overall PCR diagnostic yield of 81.6% obtained in the present study is within the ranges of previous reports. The higher PCR detection rate of the outpatient group as compared to that of the inpatient group may be attributed to the longer duration of illness at enrolment of the inpatients, albeit not statistically significant. Likewise, similar to previous reports [146,147], the results show that HFMD is diverse in etiology with over 19 enterovirus serotypes being linked with the current HFMD epidemic in Vietnam. Given the weak/lack of cross-neutralization between them, especially among predominant serotypes [148–151], the data thus point out that multivalent vaccines are needed to control this ongoing epidemic. Albeit arguably challenging to develop, the requirement of such multivalent vaccines are essential due to their potential benefit [147,151–153], and the high burden posed by HFMD [31,124,133]. Nevertheless, because of the high prevalence of EV-A71 in both mild and severe HFMD patients, EV-A71 vaccines, which have been successfully developed elsewhere [113,151], could potentially reduce the major impact of HFMD in the region where EV-A71 endemic is high.

The major strength of my study is that it was based at tertiary referral hospitals in Chi Minh City, which are responsible for receiving HFMD from southern Vietnam with a population of over 40 million. Nevertheless, owing the limitation of resource, I was not able to expand my sampling to the central and northern Vietnam. Therefore, the epidemic picture in these respective areas remains to be unlocked. Additional limitation includes the adjustment in sampling approach from three to one study site (CH1) in 2018. Nevertheless, given that CH1 is the largest tertiary center for CH1 in southern Vietnam, the results have uniquely reflected the ongoing epidemic patterns of HFMD in southern Vietnam over six years (2013 – 2018).

3.5 **Conclusion**

To summarize, the obtained results of this chapter showed a complex dynamics of the ongoing HFMD epidemic in Vietnam, which have in part been driven by the emergence of pathogens, especially EV-A71 and CV-A6. The results pointed out that there is an urgent need to develop multivalent vaccine in order to control this emerging infection and that active surveillance for pathogen circulation remains essential to inform the local public health authorities in development of appropriate intervention strategies in order to reduce the overall burden of unprecedented HFMD outbreaks.

Chapter 4

Heart rate variability in children with hand, foot and mouth disease in Vietnam between 2017 and 2018

4.1 **Background**

Whilst the vast majority of HFMD cases are mild and self-limited, approximately 10% of admitted cases progress to severe and potentially fatal conditions including central nervous system involvement, autonomic nervous system (ANS) dysregulation and cardiopulmonary failure [8]. Early identification of those likely to deteriorate or with more severe diseases would be beneficial both for patient management and resource allocation, and methods of detecting autonomic nervous system activity in real-time may be valuable in enabling this.

HRV has been investigated in many diseases associated with autonomic nervous system imbalance: it is predictive of poor outcome following myocardial infarction and in diabetes mellitus, and was recently shown to be an indicator of autonomic nervous system disturbance in tetanus [78,94,107,154]. It is therefore possible that HRV is a valuable marker of autonomic nervous system imbalance and therefore disease severity in HFMD. Some preliminary data in HFMD has been published, reporting HRV metrics in 40 Taiwanese children with HFMD, which indicated relative sympathetic nervous system activation. However, in this study indices were taken from a single 5 minute Holter-monitor recording and from a population with HFMD but not differentiated according to pathogen. It is unclear how HRV measures relate to the different causes of HFMD (ie EV-A71 vs non EV-A71 pathogens) and whether there are more scalable ways of obtaining

ECG recordings, potentially allowing real-time analysis, vital in outbreak situations.

I therefore carried out the present pilot study, using wearable devices to record the ECG signal for up to 24 hours in a large sample of children with different severities of HFMD and known aetiology.

4.2 **Methods**

4.2.1 **Setting and study design**

This study was prospectively conducted at CH1 during 2017–2018. Hospitalized patients aged 16 years old or younger, who were clinically diagnosed with HFMD at different severities, from grade 2a to grade 4, and had an illness day of 7 or less were eligible for enrollment. After informed consent was obtained, patients were enrolled and followed up until discharge.

4.2.2 **Inclusion and exclusion criteria:**

Inclusion criteria: Patients who met all following criteria were eligible for enrolment in this study: (1) child with a clinical diagnosis of HFMD grade 2A or above; (2) need to be monitored at the Emergency room of Infectious Disease Department, Children's Hospital 1; (3) their day of illness from the onset is 7 or less; (4) their legal representative consented to participate in the study.

Exclusion criterion: Any patient for whom the attending physician believed another diagnosis was more likely.

4.2.3 **Sample size**

This was a pilot study and I aimed to collect at least 120 patients in order to include at least 30 very severe patients (Grade 3 or 4). Additionally one of the aims of this study was to test the feasibility of a wearable device to measure in an outpatient setting, thus we also wanted at least 30 cases presenting with grade 1.

4.2.4 **Data collection**

ECG signal was collected for up to 24 hours, using e-Patch (see below). Information about demographics, weight, body temperature, breath rate, blood pressure, signs/symptoms, disease grades on admission and disease grades at discharge were also collected using an adapted version of the CRF of our on-going HFMD program. Throat swabs were collected from all participants at enrolment in order to identify pathogens of the disease using methods described in chapter 3 and Appendix 1.

4.2.5 **E-Patch**

The ECG signal is generated by electrical activity of the sinoatrial node which controls the expansion and contraction of the heart. It can be recorded using an ECG machine with electrodes placed at various positions on the surface of the chest whereas the e-Patch ECG signal is measured by a small patch firmly adhered to the skin of the sternum area. Each e-Patch consists of two parts: a sensor and a single electrode.

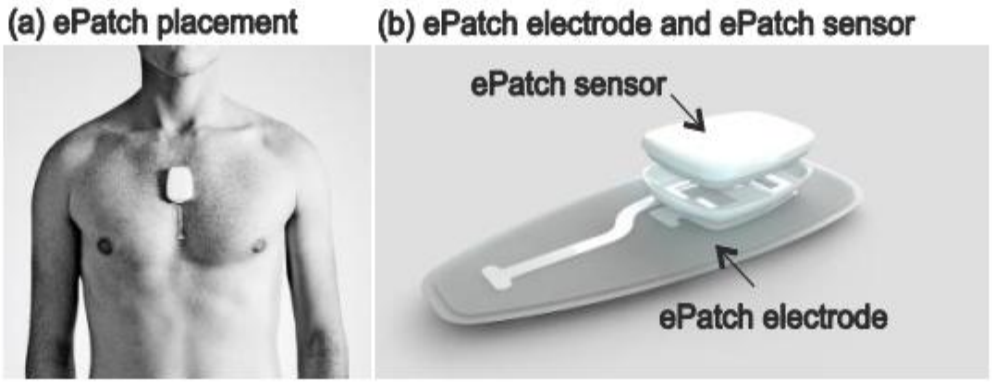


Figure 4.1: Elements and position of E-patch in patients' chest

The e-Patches used in this study are produced by DELTA Danish Electronics, with the serial number AMS3000.

4.2.6 **HRV analysis and statistical consideration**

Before HRV analysis was performed, the ECG signal was manually edited to exclude noise and artefacts. Recordings were divided into 5-minute windows. All 5-minute windows with artefacts or noise were excluded. The remaining recordings were analysed using the sequential function of HRV analysis software version 1.1, produced by St. Etienne University, France dated May 2017 [84]. For severe and very severe patients who had to stay in the emergence room or intensive care unit the ECG signal was recorded for up to 24 hours and averages of HRV indices derived from night time windows (between 10 pm to 4 am) were used for analysis. For children with mild grades who were observed in other rooms in the Infectious Disease Department, recording durations fluctuated at around six hours. For these patients, means of indices from three consecutive five-minute

HRV windows when patients were calm (based on regularity of heart rates) were selected.

The linear and non-linear HRV indices were calculated with the reference being standard and a re-sampling rate of 2 Hz. Linear methods include time domain and frequency domain analysis while non-linear approach consists of Poincare´ plot analysis and detrended fluctuation analysis (DFA) [83]. These HRV indices are clearly defined in Table 1.4 and were selected as most commonly used and those recommended by consensus guidelines. Additionally, as this is an initial exploratory analysis, by using different analytic methods we aimed to identify consistent trends that might indicate autonomic nervous system activity.

Median and inter-quartile range (IQR) are given for skewed variables. Statistical differences in HRV indices among severities of disease and groups of detected pathogens were tested using the Mann-Whitney U- test with a P-value of less than 0.05 considered as a statistically significant difference.

4.3 **Results**

4.3.1 **Characteristics of hospitalized patients with HFMD**

From April 2017 to December 2018, a total of 142 hospitalised patients were enrolled in this study. Of these, the number of grade 2A, 2B1, 2B2, grade 3 and grade 4 was 40 (28.2%), 16 (11.3%), 32 (22.5%), 50 (35.2%) and 4 (2.8%), respectively. Demographic characteristics and clinical features at enrollment of 142 enrolled patients were described in Table 4.1. In general, the vast majority of

cases (97.2%) were younger than 60 months with a median age of 21.7 (IQR: 2.4-154.8) months. Male and HCM city origin accounted for 57.7% and 42.3%, respectively. Clinical features on admission are given in Table 4.1. In terms of clinical presentation, the proportion of high fever, lethargy, myoclonus, trembling and mottled skin in severe and very severe group was significantly higher than in the mild group. Regarding detected pathogens, the most common enterovirus serotype was EV-A71 (56.3% of cases), followed by CV-A16 (4.9%), CV-A10 (4.2%) and CV-A6 (3.5%). In the severe group, the proportion of children with EV-A71 (64.6%) was almost double that of those in the mild group (30%). Conversely, CV-A6, CV-A10 and CV-A16 were more common in the mild group.

4.3.2 **Data collection from e-Patch**

E-Patch recordings were taken in all subjects. In all of these recordings, the ECG signal was obtained and no adverse events associated with using the wearable occurred. A total of 5526 epoches of 5-minute ECG recordings were available for analysis. Of these, the number of qualified epoches from grade 2a, 2b1, 2b2, grade 3 and grade 4 were 120, 863, 1783, 2627 and 133, respectively.

Table 4.1: Baseline characteristics of patients with HFMD on admission

Variables	Mild (grade 2A) n=40	Severe (2B1&2B2) n=48	Very severe (grade 3&4) n=54	Total n= 142
Demographics				
Male/female	21/19	26/22	35/19	82/60
Median age (months) (range)	16 (2.4-43.2)	25.6 (6.8-154.8)	19 (4.1-118.2)	21.7 (2.4-154.8)
HCM city origin Other provinces	18 (45) 22 (55)	19 (39.6) 29 (60.4)	23 (42.6) 31 (57.4)	60 (42.3) 82 (57.7)
Length of stay (days) Median (range)	3 (2-10)	4 (2-10)	7 (1-43)	5 (1-43)
Clinical features (n, %)				
Skin lesion	37 (92.5)	45 (93.8)	54 (100)	135 (95.1)
Mouth ulcer	38 (95)	45 (93.8)	46 (85.2)	129 (90.8)
High fever	25 (62.5)	36 (75)	50 (92.6)	111 (78.2)
Cough	4 (10)	2 (4.1)	6 (11.1)	12 (8.5)
Runny nose	13 (32.5)	1 (2.1)	3 (5.5)	17 (12)
Vomiting	11 (27.5)	11 (22.9)	8 (14.8)	30 (21.1)
Diarrhea	4 (10)	6 (12.5)	8 (14.8)	18)12.7)
Lethargy	9 (22.5)	34 (70.8)	41 (75.9)	84 (59.2)
Irritable	35 (87.5)	39 (81.3)	38 (70.4)	111 (78.2)
Myoclonus (history)	8 (20)	35 (72.9)	33 (61.1)	75 (52.8)
Sweating	2 (5)	1 (2.1)	5 (9.2)	8 (5.6)
Drowsiness	1 (2.5)	3 (6.3)	10 (18.5)	14 (9.9)
Trembling	0	18 (37.5)	16 (29.6)	34 (23.9)
Mottled skin	0	2 (4.1)	5 (9.2)	7 (4.9)
Pulse (times/minute) Median (range)	140 (110-168)	140 (106-187)	148 (118-190)	143.5 (106-190)

SBP (mmHg) Median (range)	92.5 (80-110)	100 (90-160)	105 (85-160)	100 (80-160)
DBP (mmHg) Median (range)	60 (45-68)	60 (50-95)	60 (52-100)	60 (45-100)
Respiratory rate (times/minute) Median(range)	32 (22-60)	34 (22-46)	38 (22-70)	34 (22-70)
Pathogens (n, %)				
- EV-A71	12 (30)	31 (64.6)	37 (68.5)	80 (56.3)
- CV-A6	2 (5)	2 (4.3)	1 (1.8)	5 (3.5)
- CV-A10	6 (15)	0	0	6 (4.2)
- CV-A16	2 (5)	3 (6.4)	2 (3.6)	7 (4.9)
- Other enterovirus serotypes (*)	8 (20)	4 (8.3)	8 (14.8)	20 (14.1)
- PCR negative	10 (25)	7 (14.6)	7 (13)	24 (16.9)

(*): Consists of CV-A5 (1 case), CV-A8 (1 case) and not serotyped enteroviruses (18 cases)

4.3.3 **Distribution of HRV indices by severity of HFMD**

The distribution of HRV indices groups by disease severity is shown in Table 4.2. In general, the overall autonomic system activity as indicated by Total Power and SDNN showed was with increasing severity of HFMD between mild and more severe cases, although values for very severe were lower than severe.

In terms of parasympathetic activity all indices show a consistent trend to reduced parasympathetic activity with increasing grades of disease (Table 4.2). The time domain index (RMSSD) gradually declined by disease severity, with median decreasing from 8.46 milliseconds (ms) in mild group, 6.45 ms in severe group to 5.43 ms in the very severe group. A similar trend was seen in the frequency

domain variables, where HFnu in mild patients was 1.8 and 2.2 times higher than among severe and very severe patients, respectively (p = 0.0001). SD1 and SD1nu also gradually declined by severity of the disease, with the highest value being observed in mild patients, followed by severe and then very severe ones.

Indices indicative of mixed parasympathetic and sympathetic activity however showed the opposite pattern, with a trend towards increased values in more severe grades of disease. Low frequency (LF), normalized low frequency (LFnu) and LF/HF ratio of severe and very severe group were significantly higher than in the mild group (p= 0.0001). Poincaré-derived variables of SD2 and SD2nu, were significantly higher among mild patients than in the severe and very severe group (P=0.044 and P=0.017, respectively). Indices reflecting relative high and low frequency activity of IMA1, IMA2, LF:HF and SD1:SD2 showed increase in lower frequency ranges, indicative of relative increase in sympathetic activity.

Table 4.2: Heart rate variability indices in children with HFMD by severity

HRV indices Median (IQR)	Mild Grade 2a (n=40)	Severe Grade 2b1/2b2 (n=48)	Very severe Grade 3/4 (n=53)	P1 (*)	P2 (*)	P3 (*)
Heart rate (beats/minute)	125.53 (117.76-143.42)	137.23 (124.25-142.99)	137.88 (127.61-147.56)	0.159	0.011	0.256
SDNN (ms)	12.37 (9.50-20.97)	16.82 (12.78-21.84)	14.38 (9.31-21.77)	0.059	0.571	0.262
RMSSD (ms)	8.46 (4.93-12.88)	6.45 (4.69-9.25)	5.43 (4.72-8.61)	0.248	0.026	0.314
Ptot (ms^2)	128.00 (72.20-351.28)	256.38 (142.97-486.49)	189.13 (76.34-445.02)	0.019	0.288	0.199
VLF (ms^2)	71.78 (30.44-188.96)	158.97 (99.91-306.12)	116.27 (41.11-266.51)	0.002	0.091	0.083
LF ((ms^2))	29.76 (15.17-63.80)	48.41 (25.59-104.53)	42.53 (18.37-113.15)	0.009	0.088	0.572
HF ((ms^2))	6.38 (2.96-24.60)	6.35 (2.60-18.01)	3.59 (1.78-11.89)	0.814	0.049	0.081
LFnu (%)	48.28 (37.70-60.27)	63.76 (53.11-69.30)	64.38 (54.72-78.65)	0.0001	0.0001	0.399
HFnu (%)	13.30 (9.09-19.79)	7.35 (5.73-11.82)	6.02 (4.30-8.71)	0.0001	0.0001	0.009
LF/HF	3.85 (2.56-6.15)	11.31 (5.86-15.66)	12.56 (9.09-18.58)	0.0001	0.0001	0.088
SD1 (ms)	5.99 (3.49-9.11)	4.51 (3.31-6.54)	3.73 (3.33-6.09)	0.209	0.025	0.373
SD2 (ms)	15.75 (12.33-27.51)	22.53 (16.90-29.95)	19.95 (12.74-29.52)	0.044	0.344	0.331
SD1/SD2	0.34 (0.27-0.49)	0.25 (0.20-0.32)	0.24 (0.18-0.31)	0.0001	0.0001	0.550
SD1nu (%)	1.24 (0.89-1.83)	1.04 (0.76-1.37)	0.94 (0.79-1.33)	0.147	0.038	0.629

SD2nu (%)	3.71 (2.56-5.57)	4.97 (3.90-6.06)	5.04 (3.06-6.46)	0.017	0.103	0.591
pLF1 (ms^2)	19.72 (9.28-42.84)	34.80 (18.53-60.27)	26.98 (12.21-63.15)	0.013	0.185	0.327
pLF2 (ms^2)	30.35 (13.22-60.48)	55.02 (31.91-101.74)	43.40 (15.32-87.65)	0.002	0.192	0.146
pHF1 (ms^2)	11.09 (4.66-19.18)	13.45 (8.61-29.69)	8.24 (4.10-22.73)	0.131	0.571	0.031
pHF2 (ms^2)	16.44 (3.80-75.44)	10.86 (3.55-32.13)	6.32 (2.90-24.15)	0.412	0.063	0.231
IMAI1	0.79 (0.38-1.30)	1.94 (1.11-2.37	1.53 (1.13-2.53)	0.0001	0.0001	0.739
IMAI2	1.08 (0.58-2.13)	3.22 (1.70-4.32)	2.49 (1.52- 4.49)	0.0001	0.0001	0.454

(*) Mann-Whitney U test; P1: Mild vs Severe; P2: Mild vs Very Severe; P3: Severe vs Very Severe

3.2 Distribution of HRV indices by pathogens

Table 4.3 depicts the distribution of HRV indices by detected pathogens (EV-A71 and non EV-A71). In general, HRV indices for patients with HFMD caused by EV-A71 were lower than those caused by other pathogens. Compared to patients with non EV-A71 pathogens, patients with EV-A71 associated HFMD had increased HRV indices associated with sympathetic activity and decreased HRV indices associated with parasympathetic activity. When markers of autonomic balance were specifically looked at those with EV-A71 had a significantly higher LF/HF ratio, IMAI1 and IMAI2 than those with non EV-A71 HFMD, indicating relative activation of the sympathetic nervous system compared to parasympathetic nervous system. The SD1:SD2 ratio was also non-significantly reduced (also indicating relative parasympathetic activation to sympathetic activation).

Table 4.3: Heart rate variability indices in children with HFMD by pathogen

HRV indices	EV-A71 (n=80) Median (IQR)	Non-EV-A71 (n=38) Median (IQR)	P-value
Heart rate (times/minute)	137.88 (128.01 - 144.59)	130.08 (118.91 - 145.99)	0.188
SDNN (ms)	14.38 (9.24 - 21.29)	17.38 (12.64 - 23.51)	0.070
RMSSD (ms)	5.72 (4.67 - 8.50)	8.77 (5.26 - 13.76)	0.001
Ptot (ms^2)	194.57 (85.99 - 476.32)	286.15 (122.62 - 544.19)	0.186
VLF (ms^2)	116.27 (44.11 - 313.64)	147.89 (52.52 - 260.00)	0.609
LF (ms^2)	40.71 (19.29 - 86.86)	59.11 (28.87 - 115.62)	0.103
HF (ms^2)	4.31 (1.74 - 10.89)	12.31 (2.75 - 21.49)	0.004

LFnu (%)	64.32 (54.10 - 69.36)	55.03 (42.79 - 64.86)	0.008
HFnu (%)	7.17 (5.54 - 9.71)	11.62 (5.60 - 17.65)	0.012
LF/HF	10.67 (7.57 - 15.08)	5.00 (2.79 - 13.89)	0.001
SD1 (ms)	4.02 (3.22 - 5.98)	6.20 (3.71 - 9.74)	0.001
SD2 (ms)	19.95 (12.59 - 27.82)	23.32 (16.22 - 31.90)	0.090
SD1/SD2	0.25 (0.20 - 0.32)	0.31 (0.21 - 0.44)	0.130
SD1nu (%)	0.93 (0.76 - 1.26)	1.32 (0.98 - 1.90)	0.001
SD2nu (%)	4.55 (2.98 - 5.81)	5.23 (3.85 - 6.46)	0.122
pLF1 (ms^2)	26.36 (12.21 - 53.78)	37.17 (17.00 - 66.91)	0.157
pLF2 (ms^2)	46.31 (17.89 - 83.50)	49.13 (22.27 - 92.81)	0.492
pHF1 (ms^2)	9.76 (5.02 - 20.22)	18.98 (8.60 - 36.34)	0.015
pHF2 (ms^2)	6.32 (2.95 - 21.71)	23.97 (4.05 - 83.42)	0.002
IMAI1	1.83 (1.07 - 2.39)	0.80 (0.53 - 1.54)	0.0001
IMAI2	3.19 (1.63 - 4.42)	1.17 (0.63 - 2.26)	0.0001

Figures 4.2-5 show HRV indices according to both disease severity and pathogen. Again, consistent patterns can be seen in terms of HRV indices. Patients with EV-A71 associated HFMD have consistently lower HRV indices at every severity grade than other patients with HFMD. In terms of parasympathetic-related indices, most clear are HF and HFnu, the most commonly cited indicators of parasympathetic activity, and they show a clear decrease with increasing severity of disease for both EV-A71 and non EV-A71 cases. Similarly for HRV indices associated with sympathetic activity, patients with EV-A71 HFMD have consistently lower HRV variables at each disease severity level, but both groups of

patient show a trend to higher sympathetic activation. This is further reflected in LF/HF and SD1/SD2 ratios and IMA1 and IMA2 values.

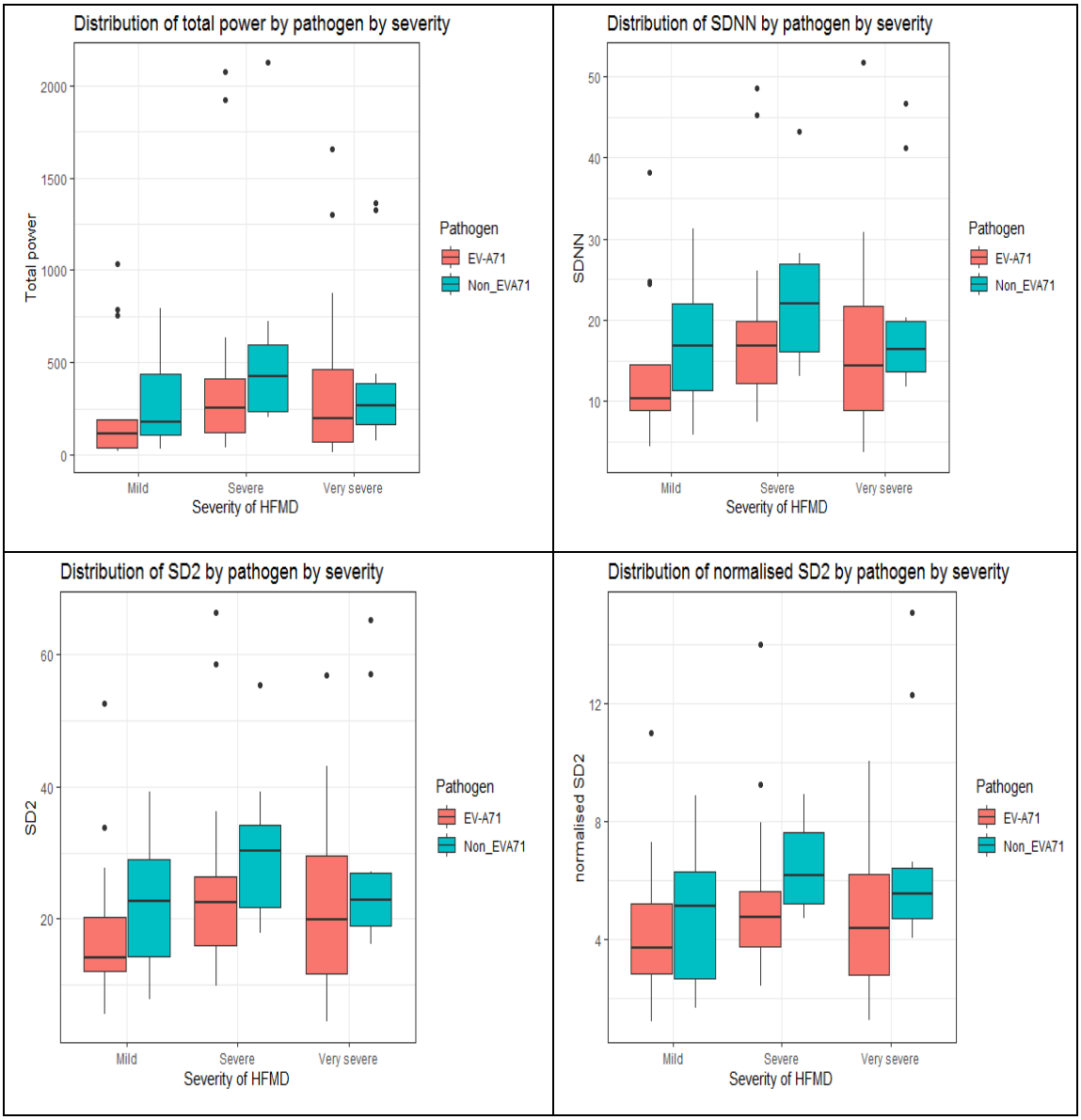


Figure 4.2: Distribution of HRV indices reflecting the general activity of ANS by pathogens in different severities

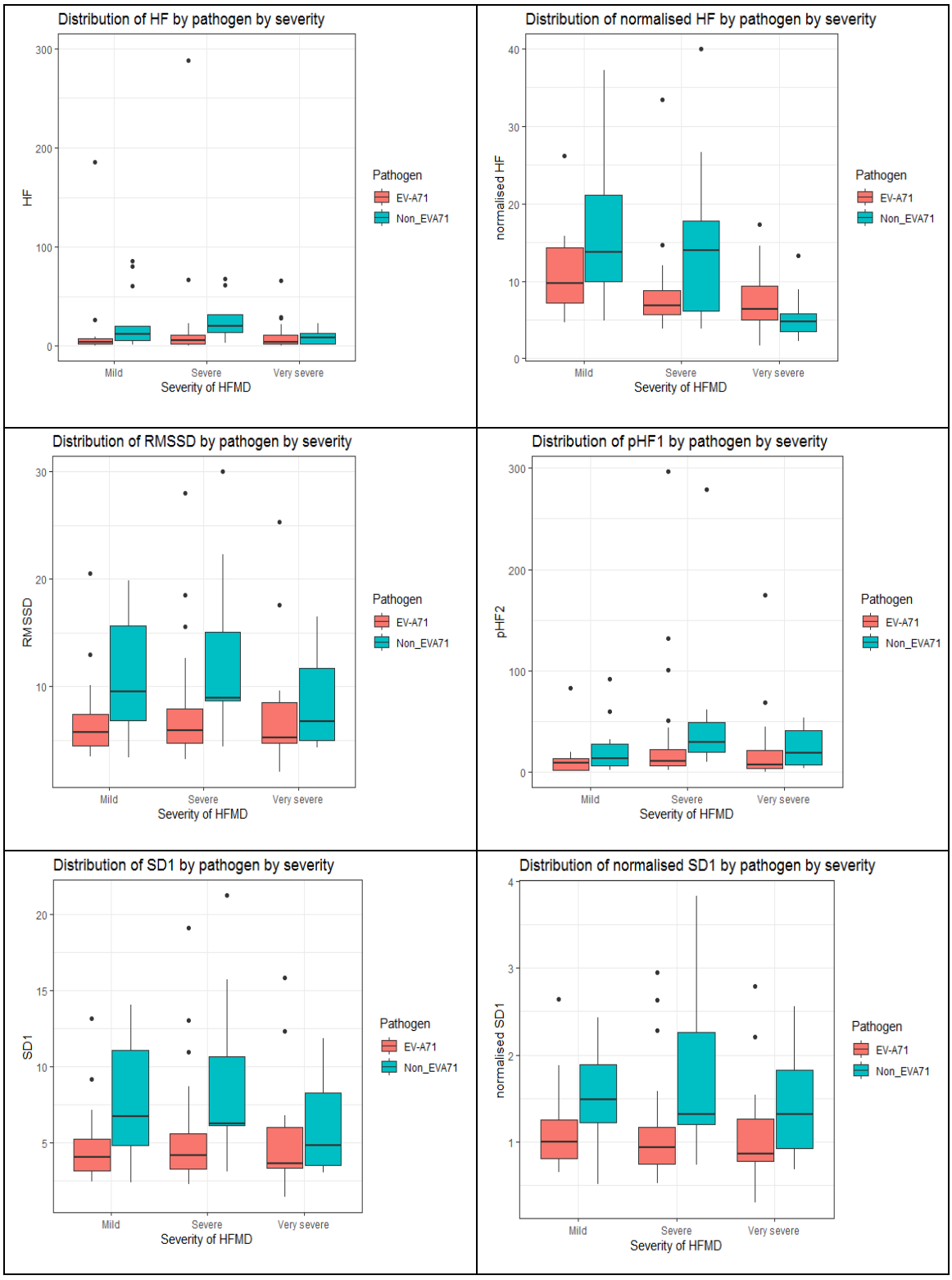


Figure 4.3: Distribution of HRV indices reflecting parasympathetic activity by pathogens in different severities

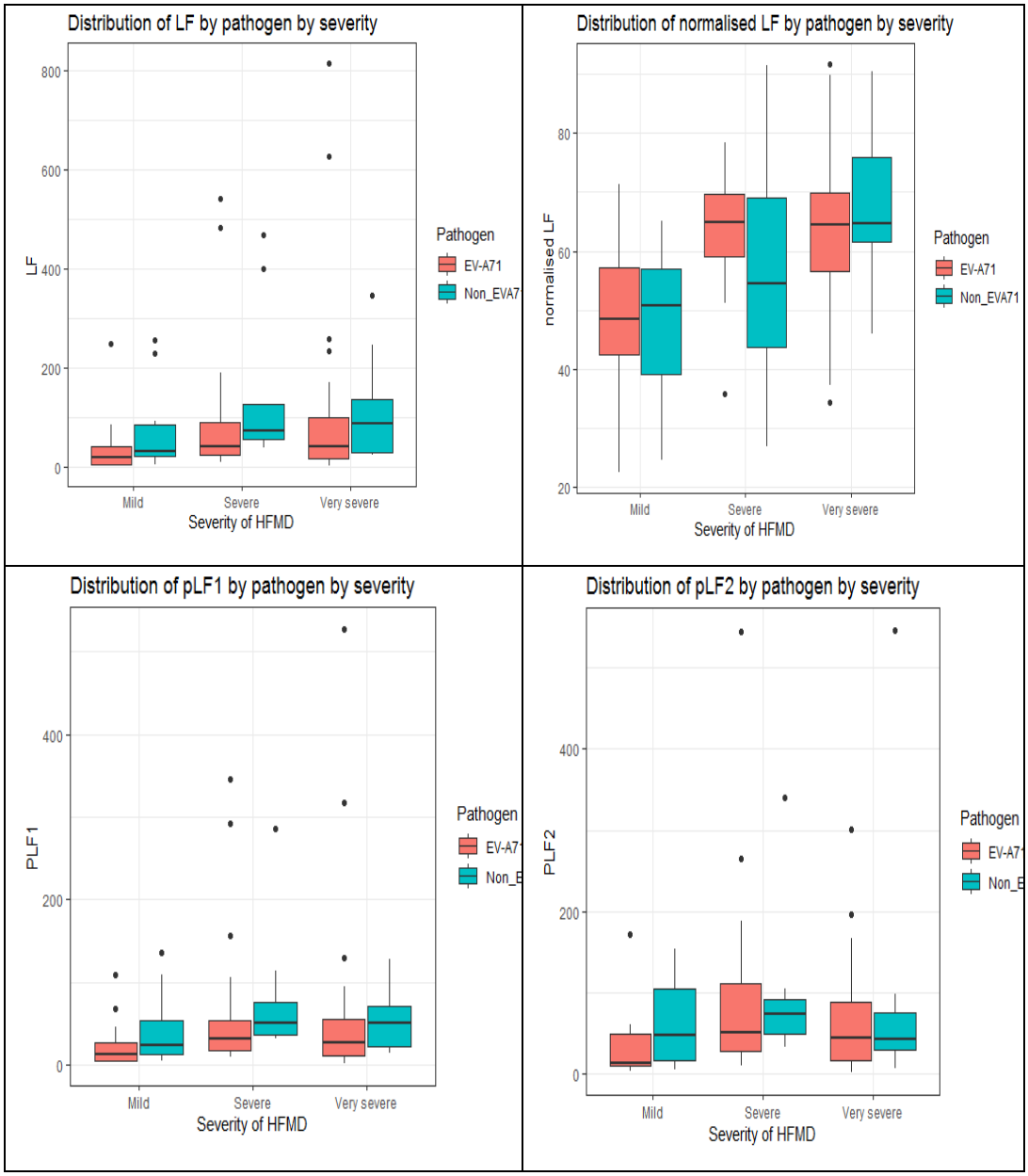


Figure 4.4: Distribution of HRV indices reflecting sympathetic activity by pathogens in different severities

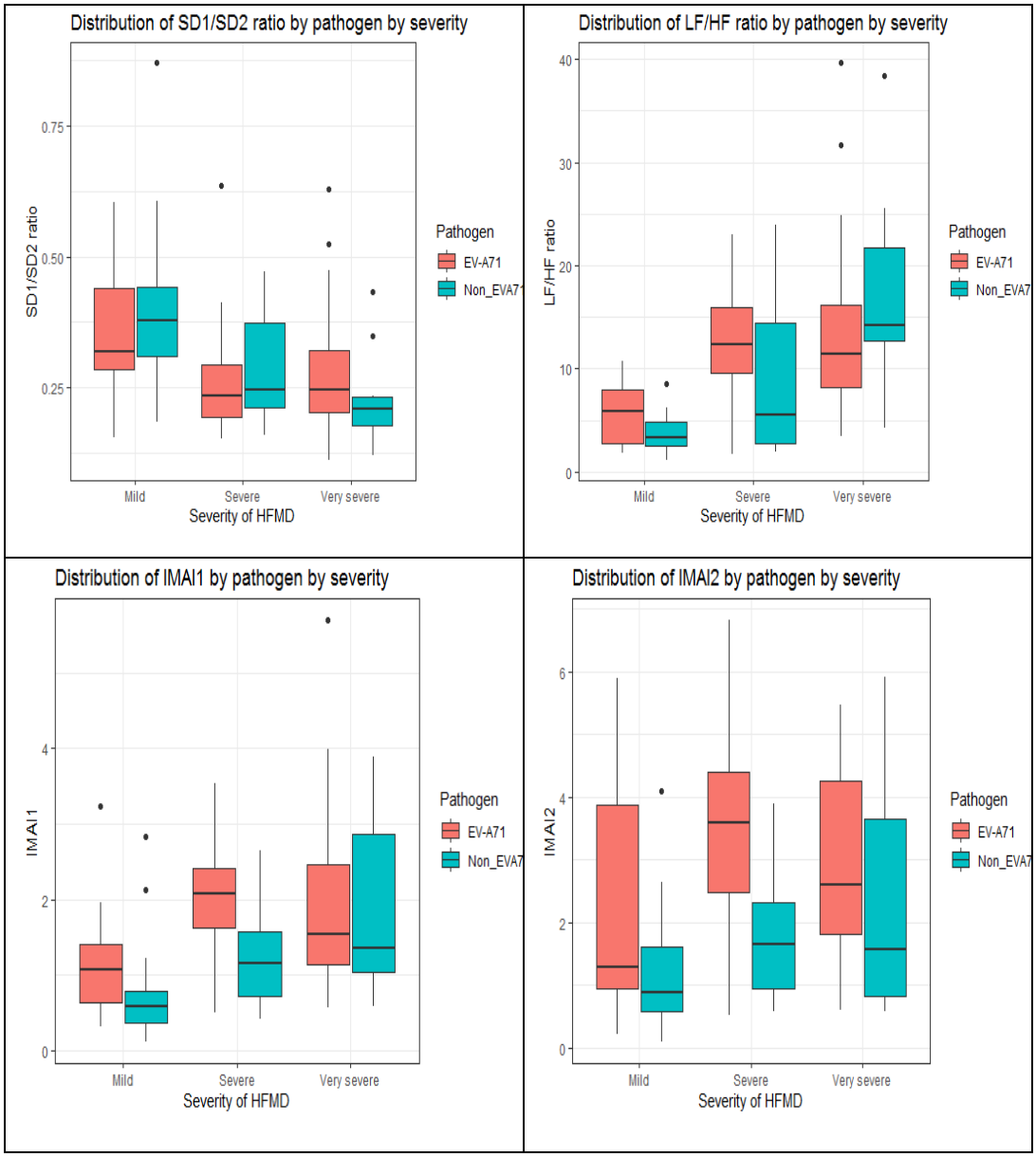


Figure 4.5: Distribution of HRV indices reflecting the autonomic imbalance by pathogens in different severities

4.4 **Discussion**

This study was designed as a pilot study to explore HRV indices in children with HFMD of known aetiology and to investigate whether HRV might be of value in severity prediction in HFMD. This is the first study providing a wide range of HRV indices in hospitalized children with HFMD and the largest reported series of HRV data in HFMD.

My study was also a pilot study to establish the feasibility of using a wearable device to accurately record ECG signal (and hence HRV) in young children with different degrees of disease severity. As such, I have established the feasibility of this method, which may therefore be suitable for a monitoring large numbers of children, for example in outbreak situations.

In general, the values of these HRV indices were significantly lower than in normal range of healthy children [82]. Studies in other diseases have indicated reduced HRV with increasing disease severity and poor outcome [154]. A previous study in Taiwanese children with HFMD also showed similar findings of reduced HRV parameters with increasing disease severity [104]. Mechanisms for this overall reduction in HRV are likely to be complex but several theories have been proposed to explain the reduced HRV in cardiovascular disease. Principal theories include a reduction in vagal tone, ‘uncoupling’ of regulatory mechanisms and changes in sino-atrial node responsiveness. It should also be noted that reduced power of HRV represents reduced fluctuation within the autonomic

nervous system and therefore may occur due to lowering of autonomic input or, conversely, a ceiling effect with high sympathetic input.

HRV indices in this study were generally lower than those reported in the study in children with HFMD in Taiwan. There are many possible reasons for this, however, the Taiwanese study used a single recording only which may have introduced bias, in this study noisy recordings with movement artefacts (which falsely increase the HRV indices measured) were removed. Children in the Taiwanese study were also older, which may be associated with increased HRV. Other reasons include differences in sample size and other treatments administered.

It is also however possible that there are technical reasons for this difference. Firstly, it is possible that we obtained different results as we recorded the ECG signal by a different device to the standard Holter monitor used in most other studies. The present study used e-Patch with one electrode while [82] used a Holter monitor with several electrodes. With more leads, Holter monitors were more likely to capture heart beat signal at higher power compared to a single electrode-cardiac wearable device. Nevertheless, the main measure here is R-R interval from which all the indices in this chapter are described and it is unlikely that such a significant difference occurs in measuring this. An alternative explanation is that the present study minimized effect of noise and artifacts on HRV features (i.e. improving the signal quality) allowing more accurate measurement. It was noted that much higher variability was detected

(inaccurately) when noise or movement artefacts were present. By excluding this before analysis we have removed this as a source of error in variability measurements [82].

4.4.1 **HRV indices and severity of HFMD**

The interplay between parasympathetic and sympathetic nervous systems in control of heart rate is highly complex and a simple interpretation of a reciprocal relationship between the two is insufficient. Nevertheless, in order to understand the pathophysiology of HFMD, a relatively simple interpretation may be helpful.

In my study, the relationship between HRV indicators of overall autonomic modulation and pathogen and disease severity is not clear, however, the complex nature of HRV modulation and confounding factors, such as medication, diurnal rhythm and position, are possible explanations. My data, however, does show much more clear relationships between indicators associated with sympathetic or parasympathetic nervous system modulation and both disease severity and pathogen.

Consistent trends in HRV in both time frequency and Poincaré domains are seen, implying autonomic imbalance varying with disease severity. Moreover, HRV indices could be considered as potential factors/indicators in classifying the severity of HFMD alongside existing clinical signs and symptoms.

A novel and important finding in this study is that consistently lower HRV metrics are seen in patients with EV-A71 associated HFMD, at each level of disease severity, compared to those with non EV-A71 associated disease. This finding is

novel and supports the view that EV-A71 associated HFMD disease is different to that caused by other enteroviruses. It is known that there are differences in clinical features depending on aetiology, and that EV-A71 associated disease is often more severe. Mortality rates in EV-A71 associated disease are increased compared to non EV-A71 disease and our study suggests that this may be due to fundamental differences in cardiopulmonary or neural pathophysiology. One possible hypothesis is that EV-A71 disease is associated with a different inflammatory phenotype than non EV-A71 disease. Observational studies in cardiovascular disease have shown a relationship between HRV and inflammatory markers with reduced HRV associated with increased pro-inflammatory markers of C-reactive protein (CRP), interleukin-1, interleukin-6 and tumour necrosis factor alpha. Inflammatory markers were not measured in this study, but existing data reports EV-A71 associated disease to be associated with lower CRP than other causes of HFMD [134,135].

An alternative explanation is that EV-A71 HFMD results in different central nervous system pathology. EV-A71 associated HFMD is associated with MRI hyperintense signals in the medulla, pons and midbrain, with the pontine tegmentum being the commonest lesion. As this region contains sympathetic neural projections, it has been suggested that lesions here are central in pathophysiology of complications of severe HFMD. There are few published data concerning MRI findings in non-EV-A71 HFMD [155,156], but it is possible that these are different, hence differential effect on the nervous system.

4.4.2 **Limitation of the study**

To my best knowledge, this is the first time e-Patch was used to record ECG signal in children with HFMD for at least six hours. However, the quality of ECG signal was not so good for whole recording duration because it was hard to keep sick young children calm for hours, especially in mild patients. The movement of patients may increase their heart rates, affecting HRV indices, although short-term HRV indices produced from the ECG signal recording at rest or sleep were used to minimize that affect. A further significant limitation is that more severe patients received more sedative drugs and were less mobile, which may have also affected HRV. A larger study or a more complex modeling approach may be able to address this issue.

4.5 **Conclusion**

My data reveals that children with EV-A71 infection were more likely to have ANS imbalance and HRV indices may have a potential application in differentiating disease severity and early detection of disease progression. Modern machine learning techniques may allow more in-depth analysis of ECG signal and more accurate classification and prediction of disease severity. Moreover, algorithms extracted from models could be utilized to create an app which may be installed in smart phones or other devices for clinical use in future.

Chapter 5

Economic burden attributed to children presenting to hospitals with hand, foot and mouth disease in Vietnam

5.1 **Background**

In view of recent advances in HFMD vaccine development and the success of EV-A71 vaccine research as well as its potential benefit [157], it is anticipated that HFMD vaccines, will be widely implemented in the near future. Therefore, knowledge about the economic burden of HFMD in Vietnam is essential to inform local policy makers in planning and prioritizing resources for vaccine development and implementation, and outbreak response. However, such data are currently unavailable for Vietnam. Here, I report the results of a prospective descriptive hospital-based study aiming at estimating costs attributed to HFMD for all clinical severities, specific pathogens and geographic locations in Vietnam.

5.2 **Methods**

5.2.1 **Study design and Settings**

The present study was conducted at Children's Hospital 1 (CH1), CH2 and Hospital for Tropical Diseases (HTD) in Ho Chi Minh City, Vietnam during April 2016–December 2017. Direct and indirect costs have been collected from patients with HFMD participating in an ongoing prospective observational HFMD study. Regarding estimation of indirect costs, I excluded productivity losses due to premature deaths [117].

5.2.2 **Patient enrollment and data collection**

Patient enrollment was carried out at the aforementioned hospitals during April 2016–December 2017. I screened any patient ≤16 years of age presenting to outpatient departments or admitted to inpatient wards of CH1, CH2 or HTD with a

clinical diagnosis of HFMD and, if outpatients, an illness day of ≤3 days for enrolment in our study.

Information regarding demographic, clinical signs or symptoms, clinical grades of the disease, treatments, laboratory tests, length of hospital stay, disease outcomes, and associated economic costs from the participants was collected from each study participant. For enterovirus serotype determination, acute throat and rectal swabs were also collected at enrolment.

Information about associated economic burden was captured through hospital invoices and face-to-face interview of the accompanying relatives. The former included information about hospital fees attributed to hospitalization including both costs covered by health insurance and patients' relative out-of-pocket payments. The remaining information was collected for the period before hospital admission, during hospitalization and 7 days after discharge, including costs associated with medicine, transportation (e.g. visiting private clinics and hospital admission) and accommodation for relatives during the course of their child being hospitalized, and work loss of relatives due to caregiving.

5.2.3 **Enterovirus detection and serotype determination**

A combination of PCR and sequencing were employed to identify enterovirus serotypes causing HFMD. Because of the focus of this chapter, EV-A71 positive samples were not subjected to genotype determination, otherwise the PCRs and sequencing procedure were carried out as previously described in Chapter 3 [109] and Appendix 1.

5.2.4 **Components of economic costs and data analysis**

An overview about economic burden components of HFMD is presented in Figure 5.1. The total illness costs consist of direct costs and productivity costs (Figure 5.1). The former includes direct medical and non-medical costs. Direct medical costs consist of the costs associated with the goods and resources directly related to medical services over the course of illness (e.g. before hospital admission, during hospitalization and seven days after discharge), and direct non-medical costs consist of the costs associated non-medical resources (such as transportation, food and accommodation). The productivity costs (also known as indirect costs) include the costs attributed to work loss of relatives due to caregiving before hospital admission, during hospitalization and seven days after discharge. The estimation of productivity costs was based on the numbers of work-off days, collected during the face-to-face interview, and average salary for those with paid-work or average minimum income for those with unpaid work [158,159]. I retrieved average salary and minimum income of Vietnamese people for the period of the study duration from [160,161]. The productivity loss of children (such as from missed school days) was not valued.

The analysis took the societal perspective, which quantity all of the costs associated with an intervention/healthcare, regardless of whom they are incurred by. It was not possible to stratify the costs depending on if they were incurred by the health care provider or the patients themselves. No excess mortality was considered in the calculation of productivity costs.

I summarized all values of illness costs as means and 95% confident intervals (95%CI) in US dollars with an average conversion rate of 1 US$ equivalent to 22,000 Vietnamese Dong for the duration of the study period (2016 – 2017) [162]. All statistical analyses were done using IBM SPSS Statistics for Window version 23 (Armonk, NY: IBM Corp.)

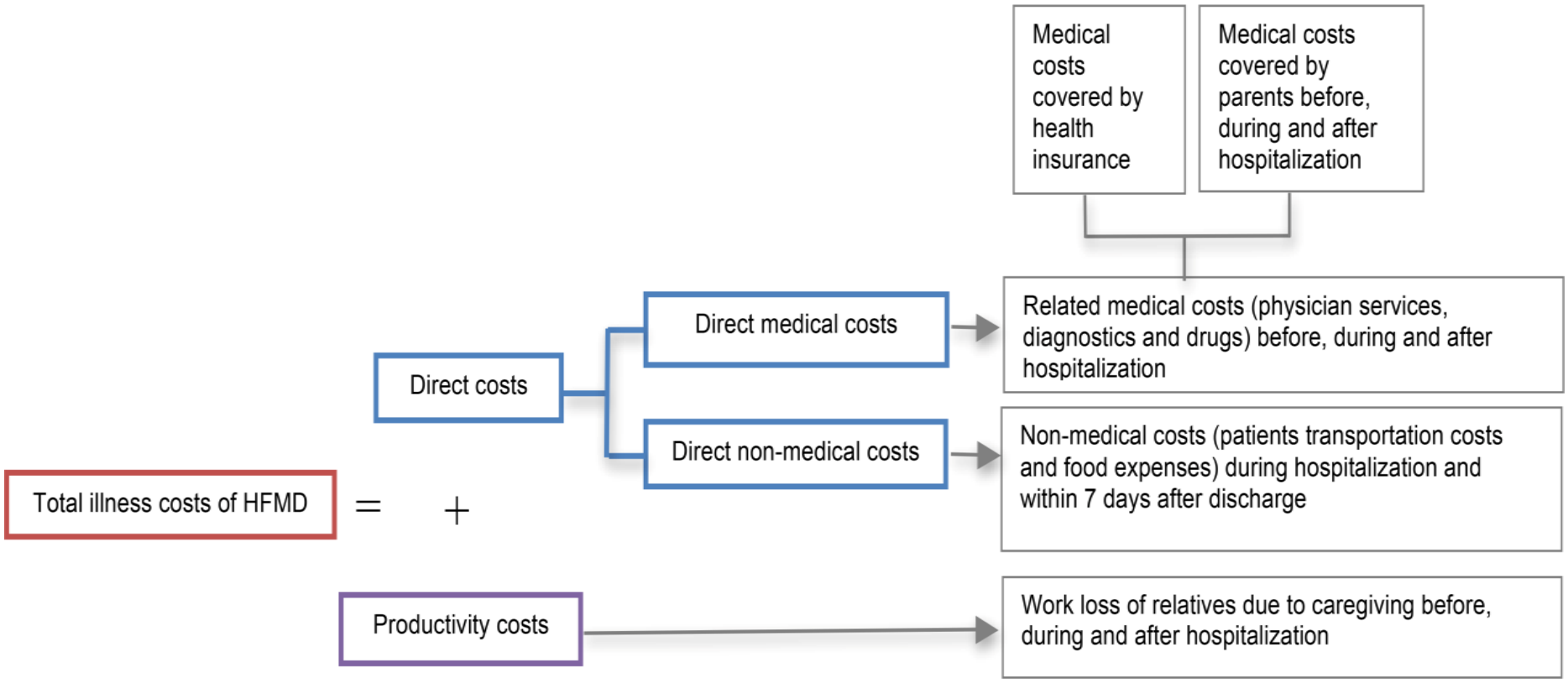


Figure 5.1: Individual components of total costs attributed to HFMD

5.2.5 **Estimation of total economic burden of HFMD at nationwide level during 2016–2017**

To estimate the economic burden of HFMD at nation-wide level in Vietnam during the study period, I first calculated the number HFMD outpatients seeking for medical treatment at CH1 during 2016–2017 using the formula:

The number of outpatients = the number of outpatient visits⁄2 − α×the number of outpatient visits⁄2

Where 2 is the average numbers of outpatient department visits per HFMD episode as per the medical practice in Vietnam [163]; α is the proportion of outpatients progressing to inpatients calculated based on the data collected from the present clinical study; and the number of outpatient visits were the data for 2016–2017 period, and were retrieved from the database record of CH1.

Using the numbers of hospitalized HFMD cases at CH1 during 2016–2017, which were retrieved from the hospital database record, and the number of estimated outpatients at CH1 obtained using the above formula, I calculated the ratio of inpatient and outpatients (β). With this obtained β value, I extrapolated the number of outpatients at nation-wide level during 2016–2017 by dividing the number of hospitalized cases across Vietnam by β. Finally, the total economic burden of HFMD in Vietnam is the sum of illness costs posed by inpatients and outpatients, which were calculated using the estimated illness costs for respective patient groups obtained in the present study and the estimated number of inpatients and outpatients across Vietnam.

5.2.6 **Ethical statement**

The Institutional Review Board of CH1, CH2 and HTD, and the Oxford Tropical Research Ethics Committee (OxTREC) approved the study. Written informed consent was obtained from a parent or guardian of each enrolled patients.

5.3 **Results**

5.3.1 **Baseline characteristics of the patients**

During April 2016–December 2017, a total of 466 patients were enrolled in the clinical study, including 427 inpatients and 39 outpatients. The baseline characteristics and clinical outcome are presented in Table 5.1. The majority (203/466, 43.6%) of the patients lived in Ho Chi Minh City, and only 25/466 (5.4%) were transferred from other hospitals. 436 patients (93.6%) had between 2 to 4 caregivers during the course of illness. The number of patients with severe HFMD (i.e. Grade 2B1 or above) accounted for 15.5%. Of 39 outpatients (Grade 1), two were subsequently progressed to Grade 2A and were then admitted to the hospital without additional deterioration. These two patients were treated as inpatients in subsequent analyses. Compared with mild patients, patients with severe HFMD were admitted to the hospitals later (Table 5.1) and had a longer duration of hospitalization; median number of days (range): 2 (0–7) vs. 1 (0–7), P=0.009 and 5 (1–11) vs. 3 (1–31), P<0.001, respectively (Table 6.1). In terms of clinical outcome, 463 (99.4%) patients recovered without complication, while unfavourable outcomes were recorded in three, including pneumonia (n=1),

paralysis (n=1) and death (n=1). The fatal case had no enterovirus found in the collected swabs by the diagnostic work up of the present study.

Table 5.1: Baseline characteristics and etiology of patients enrolled in the study

Characteristics	Total (n=466)	Mild* (n=394)	Severe# (n=72)	Outpatients (n=37)	Inpatients (n=429)	P[1]	P[2]
Sex ratio, male/female	271/195	226/168	45/27	16/21	255/174	0.42	0.055
Median age in months (range)	17.5 (4–103)	17 (4–103)	19 (4–58)	22(7–85)	17(4–103)	0.29	0.11
Transferred from other hospitals	25 (5.4%)	10 (2.5%)	15 (20.8%)	0	25 (5.8%)	<0.001	0.25
Geographic locations							
Ho Chi Minh City (n, %)	203 (43.6)	171 (43.4)	32 (44.4)	29(78.4)	174(40.6)	0.87	0.0001
Other provinces (n, %)	263 (56.4)	223 (56.6)	40 (55.6)	8(21.6)	255(59.4)		
Median illness days before admission (range)	1 (0–7)	1 (0–7)	2 (0–7)	1 (0-3)	1(0–7)	0.009	ND[Ψ]
Median length of stay (range)	3 (1–31)	3 (1–31)	5 (1–11)	NA	2(1–30)	<0.001	NA
Number of caregivers per child							
1	30 (6.4)	25 (6.3)	5 (6.9)	13 (35.1)	18 (4.2)		
2	374 (80.3)	316 (80.2)	58 (80.6)	23 (62.2)	348 (81.1)	0.85	<0.001
3	60 (12.9)	51 (12.9)	9 (12.5)	1 (2.7)	61 (14.2)		
4	2 (0.4)	2 (0.5)	0	0	2 (0.5)		
Occupation of caregivers (n, %)							
Paid	639 (66.1)	538 (65.7)	101(68.2)	34 (54.8)	601 (66.6)		
Unpaid	328 (33.9)	281 (34.3)	47 (31.8)	28 (35.2)	302 (33.4)	0.55	0.06
Median number of days off from work (range)	12.5 (1.25–44)	12 (1.25–44)	15 (3.5–40)	8(1.25-15.5)	13 (2.5–44)	<0.001	0.004

Highest clinical grade (n, %)							
Grade 1	39 (8.3)	39 (8.3)	NA	37 (100)	2 (0.5)	NA	NA
Grade 2A	355 (76.2)	355 (76.2)	NA	0	355 (82.8)	NA	NA
Grade 2B1	24 (5.2)	NA	24 (5.2)	0	24 (5.6)	NA	NA
Grade 2B2	12 (2.6)	NA	12 (2.6)	0	12 (2.8)	NA	NA
Grade 3	36 (7.7)	NA	36 (7.7)	0	36 (8.4)	NA	NA
Outcome at discharge (n, %)							
Full recovery	463 (99.4)	392 (95.5)	71 (98.6)	37 (100)	426 (99.3)		
Recovery with complication**	2 (0.4)	2(0.5)	0	0	2 (0.5)	0.054	0.88
Death[$]	1 (0.2)	0	1 (1.4)	0	1 (0.2)		
Etiology (n, %)							
EV-A71	90 (19.3)	58 (4.7)	32 (44.4)	9 (24.3)	81 (18.9)		
CV-A6	71 (15.2)	67 (17.0)	4 (5.6)	6 (16.2)	65 (15.2)		
CV-A10	43 (9.2)	40 (10.2)	3 (4.2)	1 (2.7)	42 (9.8)	<0.001	0.079
CV-A16	32 (6.9)	28 (7.1)	4 (5.6)	1 (2.7)	31 (7.2)		
Other EVs	109 (23.4)	98 (24.9)	11 (15.3)	14 (37.8)	95 (22.1)		
PCR negative	121 (26)	103 (26.1)	18 (25)	6 (16.2)	115 (26.8)		

Note to Table 5.1: *grade 1/2A, [#]grade 2B1 or above; **pneumonia (n=1), and prolonged fever and diaphragm paralysis (n=1), [$]the fatal case was unknown etiology; [Ψ]ND: not done (because of enrolment bias, see patient enrolment for more details); [1]comparison between severe and mild cases; [2]comparison between out and in patients; EVs: enteroviruses.

Table 5.2: Baseline characteristics of patients infected with different EV serotypes

Characteristics	EV-A71 (n=90)	CV-A6/10/16 (n=146)	Other EVs (n=109)	P[1]	P[2]
Sex ratio, male/female	54/36	94/52	63/46	0.50	0.75
Median age in months (range)	21.5 (7–68)	16 (5–77)	19 (4–63)	<0.001	0.10
Transferred from other hospitals (n, %)	2 (2.2)	11 (7.5)	6 (5.5)	0.14	0.30
Geographic locations					
Ho Chi Minh City (n, %)	43 (47.8)	66 (45.2)	52 (47.7)	0.7	0.99
Other provinces (n, %)	47 (52.2)	80 (54.8)	57 (52.3)		
Median illness days before admission (range)	2 (0–7)	1 (0–5)	1 (0-6)	<0.001	0.054
Median days of hospitalization (range)	3 (1–10)	2 (1–30)	2 (1–9)	0.005	0.008
Number of caregivers per child					
1	12 (13.3)	6 (4.1)	2 (1.8)	0.004	0.0001
2	60 (66.7)	119 (81.5)	97 (89)		
3	18 (20)	21 (14.4)	9 (8.3)		
4	0	0	1 (0.9)		
Occupation of caregivers (n, %)					
Paid	121 (65.1)	197 (64)	160 (70.2)	0.81	0.27
Unpaid	65 (34.9)	111 (36)	68 (29.8)		
Median number of days off from work (range)	14 (2-30)	12 (4.5–44)	11 (2.5–27)	0.057	0.004

Highest clinical grade (n, %)					
Grade 1	9 (10.0)	9 (6.2)	15 (13.8)	<0.001	<0.001
Grade 2A	49 (54.4)	126 (86.3)	83 (76.1)		
Grade 2B1	7 (7.8)	6 (4.1)	5 (4.6)		
Grade 2B2	6 (6.7)	1 (0.7)	3 (2.8)		
Grade 3	19 (21.1)	4 (2.7)	3 (2.8)		
Outcome at discharge (n, %)					
Full recovery	90 (100)	145 (99.3)	109 (100)	1	NA
Recovery with complication	0	1 (0.7)**	0		
Death	0	0	0		

5.3.2 **Results of etiological investigations**

The results of enteroviral diagnostic are presented in the Table 5.1 & Table 5.2. Enteroviruses were identified in 74% of the 466 enrolled patients with EV-A71, CV-A6, CV-A10 and CV-A16 being the most common viruses identified, accounting for 90/466 (19.3%), 71/466 (17%), 43/466 (9.2%) and 32/466 (6.9%), respectively (i.e. 50.6% of total enrolled cases). While the detection rates of these common causes were similar among inpatients and outpatient groups, the detection rates were statistically different between hospitalised patients with severe and mild HFMD, with EV-A71 being more commonly detected in patients with severe clinical phenotypes (Table 5.2), supporting previous reports linking EV-A71 infection to severe disease [164]. Notably, EV-A71 PCR positive patients were older than those infected with CV-A6/10/16, and had a longer duration of hospitalization and later hospital admissions (Table 5.2).

5.3.3 **Illness costs attributed to HFMD: a general overview**

The detailed economic burden associated with HFMD for all enrolled patients and for different patient groups are presented in Table 5.3. Overall, there were considerable differences in direct medical costs between groups of patients with different clinical severities and patients infected with different pathogens (i.e. EV-A71 vs. non-EV-A71). In contrast, the direct non-medical and productivity costs were relatively similar between patient groups. The mean total economic burden per case was estimated to be US$400.8 (95%CI: 353.8–448.9), of which the total direct costs accounted for 69.7% (mean: US$279.3, 95%CI: 235.3–328.9), while

direct medical costs accounted for 51.1% (mean: US$204.7, 95%CI: 162.1–253.1).

Table 5.3: Economic costs associated with HFMD in Vietnam

Patient groups[#]	Direct medical costs (US$)	Direct non-medical costs (US$)	Total direct costs (US$)	Productivity costs (US$)	Total economic costs (US$)**	P values[Ψ]
All patients (n=466)	204.7 (162.1–253.1)	74.6 (68.8–80.9)	279.3 (235.3–328.9)	121.5 (116.4–126.2)	400.8 (353.8–448.9)	NA
Geographic locations						
HCMC (n=203)	221.9 (145.5–300.0)	46.3 (41.7–50.8)	268.2 (190.1–346.7)	113.5 (106.7–120.4)	381.7 (302.9–464.5)	0.50
Other provinces (n=263)	191.4 (134.9–254.6)	96.5 (88.0–105.6)	287.9 (230.7–351.6)	127.6 (121.1–134.4)	415.5 (355.8–480.9)	
In/outpatients						
Out patients (n=37)	16.8 (13.5 –19.5)	14.5 (8.2–22.3)	31.2 (24.9–38.4)	63.6 (54.1–73.9)	94.8 (81.7-108.7)	<0.001
Inpatients (n=429)	220.9 (173.3–271.4)	79.8 (73.5–86.7)	300.7 (250.7–351.2)	126.5 (121.8–131.6)	427.2 (376.8–478.6)	
Disease severities[$]						
Mild (n=357)	50.6 (41.7–63.5)	74.8 (69.7–80.0)	125.4 (113.9–139.9)	120.3 (115.5–125.7)	245.8 (231.9–261.3)	<0.001
Severe* (n=72)	1065.2 (883.5–1269.4)	104.6 (83.7–133.6)	1169.8 (986.1–1368.0)	157.0 (143.7–171.0)	1326. 7 (1144.6–1528.9)	
Grade 2B1 (n=24)	342.8 (142.3–578.5)	109.1 (71.9–152.8)	451.9 (241.2–702.5)	145.3 (123.3–167.2)	597.2 (396.3–839.8)	ND
Grade 2B2 (n=12)	795.2 (521.5–1100.7)	86.3 (66.9–104.0)	881.4 (609.1–1185.5)	163.3 (133.2–189.8)	1045.0 (772.6–1341.9)	ND
Grade 3 (n=36)	1,636.7 (1412.3–1,865.2)	107.7 (77.2–157.2)	1744.5 (1,539.7–1,965.4)	162.4 (140.7–183.7)	1861.0 (1,687.8–2,088.9)	ND
Pathogens						
EV-A71 (n=90)	502.2 (349.4–669.8)	74.8 (62.1–90.7)	577.0 (422.8–745.8)	128.9 (117.7–140.6)	706.0 (544.1–878.3)	<0.001
CV-A6/10/16 (n=146)	96.5 (53.3–150. 2)	72.6 (64.6–81.6)	169.1 (125.3–225.6)	118.1 (109.1–125.9)	287.1 (238.9–348.3)	
Other EVs (n=109)	118.2 (62.7–194.4)	70.2 (56.9–87.1)	188.4 (128.3–268.5)	113.1 (104.1–122.7)	301.5 (238.0–382.0)	<0.001

Note to Table 5.3: [#]Data are presented as mean (95%CI); *grade 2b1 or above; **see Figure 1; EVs: enteroviruses; [$]inpatients only; ND: not done; NA: non applicable; [Ψ]comparison made for total economic costs

5.3.4 **Economic burden of HFMD by geographic locations, disease severity and pathogens**

In terms of geographic locations, albeit not statistically significant, the associated economic costs of patients coming from other provinces were slightly higher than that of patients living in HCMC (mean: US$415.5, 95%CI: 355.8–480.9 vs. mean: US$381.7, 95%CI: 302.9 –464.5), which was attributed to the higher direct non-medical costs (such as travel costs) of patients coming from other provinces rather than HCMC (Table 5.3).

As expected, patients with severe HFMD were associated with a significantly higher total illness costs compared to those with mild disease (including outpatients and inpatients with Grade 2A) (Table 5.3), of which the majority were attributed to direct medical costs. Additionally, of the severe patients (i.e. Grade 2B1 or above), the higher clinical grades, the more illness costs were; estimated mean of total illness costs (95%CI): US$597.2 (396.3–839.8) for Grade 2B1, 1045.0 (772.6–1341.9) for Grade 2B2 and 1861.0 (1,687.8–2,088.9) for Grade 3.

In terms of enterovirus serotypes and associated costs, because EV-A71 was predominantly found in patients with severe clinical phenotypes, as a consequence the estimated mean illness costs attributed EV-A71 were higher than that of other HFMD causing enterovirus serotypes (including CV-A6, CV-A10 and CV-A16) (Table 5.3).

5.3.5 **Total economic burden of HFMD at nation-wide level in Vietnam during 2016–2017**

During the study period, Vietnam recorded a total of 94,313 hospitalized HFMD cases (47,428 cases in 2016 and 46,885 case in 2017) [139]. Accordingly, the estimated number of outpatients in Vietnam during 2016–2017 is 532,397. This would be equivalent to a nation-wide total economic burden posed by HFMD cases presenting to hospitals in Vietnam of US$90,761,749 (95%CI: 79,033,973–103,009,756) (Table 5.4).

Table 5.4: Estimated total economic burden of HFMD cases presenting to hospitals in Vietnam during 2016–2017

HFMD cases	**Statistics**
Reported outpatient visits at CH1*	95,405
Estimated number of outpatients at CH1**	45,256
Hospitalized cases at CH1*	8,017
Hospitalized cases in Vietnam	94,313
Estimated number of outpatients in Vietnam[#]	532,397
Illness costs in US$	**Mean (95%CI)**
Outpatients	50,471,235 (43,496,835 – 57,871,554)
Inpatients	40,290,514 (35,537,138 – 45,138,202)
Total	90,761,749 (79,033,973 – 103,009,756)

Note to Table 5.4: *derived from database record of CH1, **the proportion of outpatients progressing to inpatients (α): 2/39=0.05128; [#]the ratio of inpatient and outpatients (β):8,017/45,256=0.17715;

5.4 **Discussion**

Despite the public health threat of HFMD, scarce information about its economic burden is available to support policy makers in endemic countries in prioritizing

the resources for the development and implementation of intervention strategies, especially vaccines. Likewise, no data exists for the economic burden of HFMD in Vietnam. In this chapter, I describe the results of a prospective multi-hospital based study during 2016–2017, aiming at estimating the economic burden of HFMD in Vietnam, where HFMD has become a major clinical problem since 2005, representing the first report about illness costs of HFMD in Vietnam. The results show that HFMD caused substantial economic burden in Vietnam, corresponding to estimated costs of US$90,761,749 (95%CI: 79,003,973–103,009,756) for the period between 2016 and 2017, and that illness costs varied between enteroviruses and disease severities. EV-A71 caused the highest economic burden, especially if the disease was severe, and the higher costs were mostly attributed to the direct medical costs. In contrast to the fluctuations of the direct medical costs, the in-direct medical costs and productivity losses were similar across the clinical phenotype groups. Around 50% of the HFMD economic burden is out-of-pocket payments. This is a substantial burden at a national level, especially for a developing country like Vietnam.

Although comparison of the illness costs between studies conducted in different countries has limitations due to the differences in study designs and social/economic statuses, the obtained results in this chapter are in agreement with previous reports including those from China and Taiwan [31,164,165]. A recent study from China reported the total costs for outpatients and inpatients with mild

and severe HFMD were US$196 (95%CI: 75–318), US$990 (95%CI: 431–1549) and US$3084 (95%CI: 813–5354). While the data from China was retrospectively obtained through telephone interview of the participants, and may therefore not accurately estimate the disease burden, the higher estimated costs for respective disease groups in China may be due to the difference in social economic statuses between Vietnam and China [166]. Additionally, in line with a recent report from Taiwan [31], our results showed that EV-A71 infection resulted in higher illness costs than other HFMD causing enterovirus serotypes did, attributable to the predominance of EV-A71 found among patients with severe HFMD. The productivity costs were similar between groups of patients infected with different enterovirus serotypes.

The obtained results of etiological investigation showed, although HFMD is diverse in etiology, EV-A71 was predominantly found in patients presenting with Grade 2B1 or above (i.e. severe HFMD). As such, my findings are consistent with previous etiological studies in the region [135,167]. Of note, existing serological data obtained from vaccine trials and in vitro experiments suggest cross-neutralization against heterogeneous serotypes of HFMD causing enteroviruses are absent and/or insufficient to provide long-term protection [113,148,157]. Collectively, my and others data indicate that multivalent vaccines are needed to control HFMD [157]. However, while the development of such multivalent vaccines might be highly challenging because of the unprecedented emergence of

HFMD causing viruses [76], effective EV-A71 vaccine(s) are urgently needed as such interventions would substantially reduce the major (~50%) burden caused by severe HFMD.

The strengths of my study include that it was prospectively conducted at major hospitals in Ho Chi Minh City, Vietnam, with a catchment population of over 40 million and that the data on illness costs was comprehensively captured from both in- and outpatients for the periods before hospital admission, during hospitalization and one week after discharge. Few economic burden studies in this area have reported such level of details [168].

My study has some limitations. First, I only based the economic burden estimations at nationwide level on cases presenting to hospitals for medical treatments, but did not take into account those seeking for medical treatments at private clinics or being managed at home by parents. Therefore, I may have under-estimated the economic burden of HFMD in Vietnam. Second, I extrapolated the total costs using our estimated illness costs data obtained from major hospitals in Ho Chi Minh City, an urban setting, while around 50% of HFMD patients are managed at provincial hospitals, which may result in lower hospital costs. I may therefore have overestimated the economic burden of HFMD at nationwide level. Third, despite our holistic approach, it is not always straightforward to accurately value productivity losses, which is subjected to variations in income of the population, particularly those with unpaid work. Collectively, the results,

especially the data on economic burden at the nationwide level, should be interpreted with caution.

5.5 **Conclusion**

To summarize, for the first time, I show that HFMD causes substantial annually economic burden in Vietnam. An effective EV-A71 vaccine could substantially reduce the majority of severe cases, thereby significantly reducing the illness costs from severe disease. My results will be helpful for policy makers in Vietnam in prioritizing resources for the development and implementation of intervention strategies, including vaccines, to reduce the burden of HFMD.

Chapter 6

General discussion and future directions

Since 1997, HFMD has become a major public health concern in Asia and beyond, affecting mostly children under 5 years old. The disease poses a substantial threat to society, especially when outbreaks occur. It is caused by a group of enteroviruses, especially EV-A71, CV-A6, CV-A10 and CV-A16. Although the vast majority of cases are mild and self-limiting, a small proportion of patients may progress to severe diseases, which can result in long-term sequelae or fatality. There are currently no clinically proven effective antiviral. EV-A71 vaccines have been used in selected population in China [169], while phase three trials of EV-A71 vaccines have also been conducted in Vietnam [111]. Despite this progress, there remain significant gaps in knowledge about important aspects of this emerging infection, especially in settings like Vietnam where HFMD has periodically overwhelmed the healthcare systems since 2005. As such, I set out to conduct a comprehensive research program aiming at addressing unanswered questions about epidemiology, disease pathophysiology, and economic burden of HFMD in Vietnam, which I will discuss herein.

My specific hypotheses were

1. The ongoing epidemic of HFMD in southern Vietnam, especially in Ho Chi Minh City, is complex and is driven the emergence of pathogens.
2. HRV indices are different between HFMD patients infected with different viruses and/or presenting with different clinical severities.
3. HFMD causes a substantial economic burden in Vietnam that needs to be assessed to inform decisions about vaccination

6.1 **Epidemiology**

To investigate the patterns of HFMD and the factors driving this, using hospital data, I have described the patterns of spread of HFMD in Ho Chi Minh City over an 11-year period (2005 – 2015). I identified that the spreading patterns of HFMD in HCMC were as follows: starting in the north-west of HCMC, HFMD moved south-east and then returned to the western part of the city. Additionally, over this 11 year period, there were more than 56,000 hospitalized cases due to HFMD in HCMC. My data also consistently showed that the affected patients were young children. Because the majority of families in HCMC are nuclear families, meaning that parents may have had to take time off from work to care for their children, my data thus emphasize that HFMD has posed a significant burden on the society as a whole in Vietnam. These findings have stimulated me to conduct prospective studies to gain further insights into the epidemiology, pathophysiology and economic burden of this emerging infection in Vietnam.

One of the main findings of my prospective hospital based study was that among 19 enterovirus serotypes detected in 80% of ~1200 patients with HFMD; EV-A71, CV-A6, CV-A10 and CV-A16 were the most common pathogens circulating and causing HFMD in Vietnam during 2015 – 2018, supporting findings from the region [169,170]. Moreover, together with data synthesized during 2013 – 2015 as part of the ongoing HFMD research program in the collaboration between OUCRU, CH1, CH2 and HTD, for the first time, long-term etiological data of HFMD in southern Vietnam were generated. Fascinatingly, unlike observational data from Malaysia, Taiwan and Singapore, where EV-A71 infection exhibited a 2-3 yearly cyclic pattern [19,171], in Vietnam EV-A71 activity has generally been documented annually, and EV-A71 cases were more often observed in the second half of the year. In contrast, CV-A6/10/16 were more frequently detected in the first half of the year. In other words, several serotypes of enteroviruses were responsible for the HFMD burden in Vietnam. Therefore, although a monovalent vaccine could significantly reduce the burden of (severe) HFMD, multivalent vaccines should be developed in order to control this emerging infection.

Another key finding was the emergence of CV-A6, and the relative absence of EV-A71 in 2017 and the first half of 2018, potentially causing an EV-A71 immune window that allowed the large outbreak occurring in southern provinces of Vietnam in late 2018 [135]. Using data obtained from the clinical study conducted as part of my PhD research, I was the first to identify and report the

unprecedented upward trend of severe HFMD cases and the reemergence of subgenogroup C4 in Vietnam in 2018 [135]. This put my hospital (CH1) in a very good position to handle last year's HFMD outbreak.

Despite the emergence of coxsackieviruses, especially CV-A6, EV-A71 remains the major cause of severe HFMD in Vietnam. Additionally, I showed for the first time that compared to subgenogroup B5, C4 caused more severe disease and the dominance of C4 coincided with large, severe HFMD outbreaks in Vietnam (2011-12 and 2018). As such, I propose that active hospital-based surveillance should be continuously conducted so as to promptly detect subgenogroups with potential of causing severe outbreaks and the sudden increase of cases with severe HFMD. This would enable us to inform local health authorities and clinicians about potential HFMD outbreaks in a timely manner, allowing appropriate public health staff preparations. For example enhancing practice of hand washing, training staff how to recognize a suspected case of HFMD in kindergartens, re-training health staff on the key signs/symptoms associated with severe HFMD and preparing sufficient drugs for treatment of critically cases (e.g intravenous immunoglobulin, milrinon, mechanic ventilators and hemofiltration machines).

6.2 **Heart rate variability**

In terms of HFMD pathophysiology, my thesis focused on describing the HRV indices in children with differing severities and aetiologies of HFMD in order to address my second hypothesis described above. HRV has been mentioned as a

non-invasive marker for detecting ANS imbalance, which may be present in severe HFMD. Differing from [104], the present study used E-patches to record the ECG signal for hours instead of just 5 minutes from specific monitors so that we could have more data to produce time series of HRV indices for further analysis.

One of the striking findings was that there was association between EV-A71 infection and ANS imbalance including both elevation of sympathetic activity and reduction of parasympathetic activity among all levels of severity of illness. This finding improves and supports our understanding of the mechanism of severe HFMD. MRI studies have indicated that EV-A71 invades the CNS, causing brainstem encephalitis and especially damages medulla oblongata, the region that modulates ANS balance [5,19,172]. In severe disease ANS dysfunction is thought to be a major cause of cardiorespiratory instability and death. The finding from this study is consistent with several studies [170,171] which have reported that children with HFMD caused by EV-A71 were more likely to develop severe disease with ANS dysfunction compared to those infected by other EV serotypes.

Another finding was that there were significant differences in HRV indices reflecting activation of both sympathetic and parasympathetic arm of ANS between different groups of disease severity. This suggests that the HRV indices (RMSSD, HF, LF, HFnu, LFnu, LF/HF ratio, SD1, SD2, SD1nu, SD2nu, pLF1, pLF2, pHF1, pHF2, IMAI1 and IMAI2) may have potential applications in

discriminating severity of the disease. Moreover, as aforementioned, these HRV indices were produced from ECG signal recorded by wearable devices (E-patches) for up to 24 hours. These data could be further analyzed using different methods in future. For example, from time series of these HRV indices an algorithm could be built and extracted, using machine learning, potentially developing a classification method for HFMD based on an ECG signal. The algorithm could be incorporated into an app which could be created and installed to smart phones of nurses and doctors for monitoring patients in their daily work. Every time, the selected HRV indices are over their threshold a massage or alarm noise will be sent to medical staff's phones.

6.3 **Economic burden**

My final hypothesis was that HFMD causes a major economic burden in Vietnam. It is widely acknowledged in the international literature that HFMD has become a major public health issue in Asia and other parts of the world because of its health-care associated burden, and my study provides evidence of its economic burden. Vaccination has been considered as a possible solution to control this emerging infection and three inactivated EV-A71 vaccines have been used in China while two others are being evaluated in Vietnam. Therefore, estimating the economic burden of HFMD is very useful for health policy makers in Vietnam in prioritizing to the introduction of vaccine. From the societal perspective, my thesis aimed to estimate the economic costs of HFMD in children presenting to outpatient clinics

and inpatient wards of the three major hospitals in Ho Chi Minh City through a prospective observational study. The economic costs of HFMD at national wide level were then also extrapolated.

My PhD research showed that the mean total costs of a HFMD case presenting in outpatient and inpatient ward was roughly US$95 and US$427, respectively. More specifically, direct costs, direct non-medical costs, indirect costs accounted for 17.7% (16.8/94.8), 15.3% (14.5/94.8) and 67% (63.6/94.8) of the mean total cost of outpatient group, respectively whereas the corresponding figures in inpatient group were 51.7% (220.9/427.2), 18.7% (79.8/427.2) and 29.6% (126.5/427.2). Among inpatients, patients with severe clinical phenotypes posed higher economic costs than those with mild diseases did. Likewise, because EV-A71 was the major cause of severe HFMD, economic costs attributed to EV-A71 infections were higher than that of other pathogens (e.g. CV-A6, CV-10 and CV-10). Overall, the estimated total economic costs of outpatients and inpatients at national level in Vietnam were around $50.47 million and $40.29 million, respectively for the period between 2016 and 2017 (Table 5.4). In summary, for the first time I quantified the substantial economic burden of HFMD in Vietnam during 2016 and 2017. Together with the obtained virological data, the results showed that although EV-A71 vaccines could significantly reduce the economic burden of HFMD, multivalent vaccines should be developed to control the ongoing HFMD epidemic.

6.4 **Perspective and future directions**

Considered as part of the global field of HFMD research, my thesis has contributed significantly in many areas. Despite recent advances, several important gaps in knowledge about this emerging infection remain to be unraveled and should, from my perspective, be addressed in future studies. In particular, it is unknown why HFMD pathogens such as EV-A71, CV-A6, CV-A10 and CV-A16, which were isolated over six decades ago, have explosively emerged since 1997. Additionally, although the epidemic of HFMD, especially severe outbreaks, was originally confined to the Asia-Pacific region, over the last years, there have been numerous HFMD outbreaks reported elsewhere in Europe and most recently in the United States in 2018 [8,173]. Collectively, I hypothesize that population immunity and/or host genetics might play a role. As such, comprehensive serosurveillance combined with modeling approach assessing the association between population immunity and disease emergence, and studies focusing on identifying potential genetic makers associated with severe HFMD are of particular interest from both clinical management and public health perspectives. Notably, previous studies from China have shown that HLA-A33 is associated with EV-A71 susceptibility [5,16,174].

As a clinician, I am also particularly interested in identifying how to best diagnose and manage patients with severe HFMD, especially those at risk of deterioration. Findings on HRV in Chapter 4 are obviously just the beginning of a new story,

and have opened up a new opportunity to look into the utility potential of portable devices like E-patches and the information they offer, which can potentially be translated into patient management. Indeed, work is currently ongoing with collaborators in Oxford to develop machine learning based approaches that can accurately predict disease progression and classify HFMD according to disease severity.

Although it is highly challenging to carry out, the clinical benefit of intravenous immunoglobulin should be tested in the future. Likewise, because my PhD research has pointed out that there are currently around 20 enterovirus serotypes responsible for the ongoing HFMD epidemic in Vietnam, assessing the potential impact of EV-A71 monovalent vaccine on the epidemic dynamic of HFMD as a whole are essential to inform future directions on the development and implementation of interventions, especially EV-A71 vaccine. Last but not least, I believe that in relation to vaccine development and implementation, my data on economic burden have offered valuable information, which can serve as the basis for studies aiming at estimating the benefit that EV-A71 vaccine may offer, which should also be one of the focuses of a future HFMD research program.

Appendices

Appendix 1: Laboratory procedures used for the detection and serotype/genogroup determination of enteroviruses

Viral RNA extraction

Viral RNA was extracted using QIAamp® Viral RNA Mini Kits (QIAgen GmbH, Hilden, Germany) performed according to the manufacturer's instruction and as following:

1. Pipette 560 µl of buffer AVL containing carrier RNA into RNA free 1.5 ml microcentrifuge tube.
2. Add 140 µl of throat/rectal swabs in viral transport medium to the tube.
3. Add 20 µl of EAV (equine arteritis virus) to the mixture.
4. Incubate at room temperature for 10 minutes then spin the tube for a few seconds to remove drops from the lid.
5. Add 560 µl of absolute ethanol to the mixture, mix by pulse vortexting for 15 seconds, and centrifuge briefly for a few seconds to remove drops.
6. Careful transfer 600 µl of the lysate to QIAamp spin column.
7. Spin at 13,200 rpm for 1 minute.
8. Place the column onto a 2ml clean collection tube. Transfer the rest of lysate to the column.

9. Add 500 µl of buffer AW1 (provided in the kit). Centrifuge at 13,200 rpm for 1 minute.
10. Place the QIAamp spin column in a new 2ml collection tube.
11. Add 500 µl of buffer AW2 (provided in the kit). Centrifuge at 13,200 rpm for 3 minutes.
12. Place the QIAamp spin column into a new 1.5ml collection tube and centrifuge for 1 minute to get rid of all AW2 buffer.
13. Place the QIAamp spin column into an RNAse free 1.5 microcentrifuge tube. Carefully load 50 µl of buffer AVE (provided in the kit).
14. Incubate at room temperature for 1 minute. Centrifuge at 14,000 rpm for 1 minute.
15. Load another 50 µl of buffer AVE to the column. Centrifuge at 13,200 rpm for 1 minute
16. Transfer the eluted RNA (100 µl in total) into a clean 1.5ml Eppendorf tube and store at -80^{o}C until analysis.

Multiplex real time PCR for simultaneous detection of enterovirus and enterovirus A71

The detection of enterovirus and enterovirus A71 associated with hand, foot and mouth disease, was performed by real-time RT-PCR using SuperScript® III Platinum® One-Step qRT-PCR Kit (Invitrogen) and in the LightCycler480 (Roche diagnostics). The real-time RT-PCR reaction was carried-out in total volume of 25 µl containing 12.5 µl of reaction mix (included in the kit), 1 µl of enzyme (included in the kit), 1 µl of each primer pair and 1 µl of each probe (Supplementary Table 1) [109], and 5 µl of viral RNA.

The thermal cycling condition: 1 cycle at 60^{o}C for 3 minutes, 53^{o}C for 15 minutes and 95^{o}C for 2 minutes, followed by 45 cycles at 95^{o}C for 15 seconds, 53^{o}C for 1 minute with fluorophore reading, and 72^{o}C for 15 seconds, and final extension at 72^{o}C for 10 seconds.

Valid result interpretation when negative controls were negative and internal and positive controls were positive with Cp values within expected ranges (32-35).

Supplementary Table 1: List of primers and probes used for the multiplex real time RT-PCR [109]

Name	Sequence (5'→3')	Working concentration (µM)	Note
EAVF primer	CAT CTC TTG CTT TGC TCC TTA G	10	Internal control
EAVR primer	AGC CGC ACC TTC ACA TTG	10	
EAV-probe	FAM-5'-CGCTGTCAGAACAACATTATTGCCCAC-3'-BHQ1	2.5	
ENT-F	5'-CCCTGAATGCGGCTAAT-3'	10	Enterovirus
ENT-R	5'- ATTGTCACCATAAGCAGCC-3'	10	
ENTr-probe	Cy5-ACCCAAAGTAGTCGGTTCCG -BHQ3	5	
EV71-634F3	GGAGAACACAARCARGARAAAGA	10	Enterovirus A71
EV71-743R3	ACYAAAGGGTACTTGGAYTTBGA	10	
EV71-probe	Cyan500-TGATGGGCACDTTCTCRGTGCG-BHQ1	1	

Enterovirus serotype determination

Enterovirus serotype determination was carried out using a nested RT-PCR targeted at VP1 coding region.

1. First round RT-PCR

First round RT-PCR was performed using the SuperSript OneStep RT-PCR Platinum Taq (Invitrogen) in an Eppendorf PCR machine (Cat. 5333). The reaction was carried-out in a total volume of 20 µl containing 10 µl of 2X reaction mix (included in the kit), 1 µl of a 4 primer (AN32, AN33, AN34 and AN34; Supplementary Table 2) mixture (10 µM each), 0.5 µl enzyme (provided in the kit), 5 µl of viral RNA (extracted by QIAamp® kit) and 3.5 µl of water.

The thermo cylcing condition: one cycle at 55^{o}C for 3 minutes, 22^{o}C for 10 minutes, and 42^{o}C for 30 minutes. Then amplification was performed in the same PCR machine at the following conditions: 95^{o}C for 1 minute followed by 40 cycles of 94^{o}C for 30 seconds, 42^{o}C for 30 seconds and 60^{o}C for 1 minute, and final extension at 72^{o}C for 7 minutes.

2. Second round PCR

The second round PCR was performed using the Platinum PCR Super mix (Invitrogen) and each reaction contains 23 µl of the Platinum Super mix (included in the kit), 0.5 µl of each of the AN89 and AN88 primers (Supplementary Table 2), and 1 µl of the first round RT-PCR product in total volume of 25 µl. The thermocyling condition was as following: 95^{o}C for 1 minute followed by 40 cycles

of 94°C for 20 seconds, 55°C for 20 seconds, and 72°C for 10 seconds, and final extension at 72°C for 7 minutes. PCR products were then analysed in 2% agarogel and positive samples (PCR band at the expected size ~370bp) were purified using ethanol as following:

a. To each tube of PCR product, add 2 µl of Sodium Acetate 3M.
b. Add 60 µl of 95% cold ethanol to the tube and then transfer the mixture to 1.5ml Eppendorf.
c. Spin at stop speed (13,000 rpm) at 4°C for 10 minutes.
d. Discard the aqueous phase and add 300 µl of 70% cold ethanol.
e. Spin at stop speed for 5 minutes and discard the aqueous phase.
f. Repeat the washing with 70% cold ethanol one more time.
g. Air dry the pellet for 15 minutes and resolve the pellet in 25 µl of molecular grade water
h. Store the purified PCR product at 20°C until use

3. VP1 sequencing

Sequencing was performed using BigDye Terminator Cycle Sequencing Kit (ABI, Cat. 4336917) in the 3130XL ABI sequencer platform. Each sequencing reaction contained 1 µl sequencing mix (included in the kit), 2 µl buffer (included in the kit), 11 µl water, 1 µl primer (AN232 or AN233; Supplementary Table 2), and 5 µl purified DNA. The reaction was performed at 95°C for 1 minute, followed by

30 cycles at 94^{o}C for 20 seconds, 60^{o}C for 2 minutes and finally hold at 10^{o}C until ending. Products were purified with cold ethanol as following:

a. To each tube of dye termination product, add 4 µl of STOP solution (containing 2 volume of EDTA, 2 volume of Sodium Acetate, and 1 volume of Glycogen).
b. Add 60 µl of 95% cold ethanol and then transfer the mixture to 1.5ml Eppendorf tube.
c. Spin at stop speed (13,000 rpm) at 4^{o}C for 15 minutes.
d. Discard the aqueous phase and add 300 µl of 70% cold ethanol.
e. Spin at stop speed for 5 minutes and discard the aqueous phase.
f. Repeat the washing with 70% cold ethanol one more time.
g. Dry the pellet in a vacuum dryer for 25 minutes. Then elute the pellet in 25 µl of HiDi solution (included in the kit).
h. The DNA was then transferred to a 96 ABI sequencing plate and sequenced in the ABI 3130XL sequencer using standard condition.

4. Serotype determination

Viral sequence data was assembled and edited using ContigExpress software, a component of the Vector NTI version 7.0 (Thermo Fisher Scientific). Viral sequence was then subjected to an online enterovirus genotyping tool available at https://www.rivm.nl/mpf/typingtool/enterovirus/ for serotype determination.

Supplementary Table 2: Primer sequences and working concentration [136]

Name	Sequence (5'→3')	Application	Working concentration (µM)
AN32	GTYTGCCA	1st round RT-PCR	10 µM of each
AN33	GAYTGCCA	1st round RT-PCR	
AN34	CCRTCRTA	1st round RT-PCR	
AN35	RCTYTGCCA	1st round RT-PCR	
AN89	CCAGCACTGACAGCAGYNGARAYNGG	2nd round PCR (forward)	10
AN88	TACTGGACCACCTGGNGGNAYRWACAT	2nd round PCR (reverse)	10
AN232	CCAGCACTGACAGCA	Sequencing	10
AN233	TACTGGACCACCTGG	Sequencing	10

Enterovirus A71 subgenogroup determination

Enterovirus A71 PCR positive samples were sequenced to determine subgenogroup. The PCR condition (including the thermo cycling program) and the primers used are listed as below:

RT-PCR

Component	Voume per reaction
2X buffer	12.5 ul
EV71-VP1-3F (10uM)	1 ul
EV71-VP1-703R (10uM)	1 ul
Superscript III one-step RT PCR	0.5 ul
Viral RNA	5ul
Water	5 ul

RT-PCR conditions:

50^{o}C for 30 minutes, 95 ^{o}C for 2 minutes, and followed by 45 cycles of 94 ^{o}C for 20 seconds, 50 ^{o}C for 30 seconds, and 72 ^{o}C for 1 minute, and final extension step at 72 ^{o}C for 7 minutes.

Supplementary Table 3: Primers sequences used for EV-A71 subgenogroup determination [40]

EV71 VP1 sequencing	EV71-VP1-3F	AGAYAGGGTGGCRGATGT	701 bp
	EV71-VP1-703R	CTGAGAACGTGCCCATCA	

Sequencing of the obtained PCR amplicon was carried out as described in the aforementioned VP1 procedure for serotype determination of enteroviruses using EV-A71 PCR primers.

Appendix 2: Publication from chapter three

Appendix 3: Publication from chapter five

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