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Epidemiology and dynamic of Hand, Foot and Mouth Disease in Vietnam

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LIST OF ABBREVIATIONS

ANS	Autonomic nervous system
cDNA	Complementary Deoxyribonucleic acid
CPBS	Center Agents Pathogens and Biotechnology for Health study
CNRS	The French National Centre for Scientific Research
CNS	Central nervous system
CSF	Cerebrospinal fluid
CODEHOP	Consensus-degenerate hybrid oligonucleotide primer
CV-A	Coxsackievirus A
DNA	Deoxyribonucleic acid
EV-A	Human Enterovirus A
EV-B	Human Enterovirus B
EV-C	Human Enterovirus C
EV-D	Human Enterovirus D
HFMD	Hand, Foot and Mouth Disease
HRV	Human rhinovirus
HIV	Human immunodeficiency virus
HLA	Human leucocyte antigen
HA	Herpangina
IL	Interleukin
IP	Interferon induced protein
IgG	Immunoglobulin G
I/V	Isoleucine/Valine
MoH	Ministry of Health
MRI	Magnetic resonance imaging
NIHE	National Institute of Hygien and Epidemiology
ORF	Open reading frame
PV	Poliovirus
RD cell	Human rhabdomyosarcoma cell
RNA	Ribonucleic Acid
RT- PCR	Reverse Transcriptase Polymerase Chain Reaction

Real-time PCR	Real-time Polymerase Chain Reaction
UTR	Untranslate region
USA	United state of America
US-CDC	US - Centers for Disease Control and Prevention
Vero cells	African green monkey kidney cells
VLP	Virus-like particle
VPg	Virus protein, genome linked
VP 1	Capsid protein of Enterovirus 1
VP 2	Capsid protein of Enterovirus 2
VP 3	Capsid protein of Enterovirus 3
VP 4	Capsid protein of Enterovirus 4
WHO	World Health Organizaion
WPRO	Western Pacific Regional Office

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GENERAL INTRODUCTION

In the recent decades, our world has been facing a series of communicable diseases threats such as avian influenza, SARS, MERS-CoV, Ebola, Zika… Beside of the that, the re-emergence of other communicable diseases still remains major burden for human. Hand, foot and mouth disease (HFMD) is the ones that reported for long time ago. This disease is an acute febrile illness in children with a papulovesicular skin rash at the palms or soles of the feet, or both. Presentation can be with or without inclusion of mouth ulcers. HFMD can result in severe complications such as encephalitis, aseptic meningitis, pulmonary edema, myocarditis, and death (1). HFMD is caused by types of Enterovirus A species which includes some Coxsackievirus A (CV-A) and Enterovirus A71 (EV-A71) (2), (3). The transmission can be faecal-oral and respiratory secretions route by direct person-to-person contact, droplets or fomites. Recent, the widespread community outbreaks of HFMD have occurred mostly in Asia Pacific region with notable amount of cases and deaths. The disease still has neither no specific treatment or vaccines so far.

It was thought that the disease attacks mostly in the poor hygiene areas because of transmission mode. However, HFMD has been hitting both developing and developed countries since reported. In 1969, in California, USA, EV-A71 strain firstly was isolated from a 9 months aged child with encephalitis diagnostic (1). There are then small and large outbreaks of HFMD have been reported throughout the world (2). In the early 1970s, several countries in different continent including Sweden, Australia, USA and Japan had reported small HFMD outbreaks with some tens of cases and almost of them are children whose clinical aspects are mostly typical of HFMD, sometimes aseptic meningitis (3), (4), (5), (6). After that, HFMD was only reported in Europe in late half of the 1970s with two large outbreaks in Bulgaria (year 1975, 451 cases and 44 deaths), Hungary (year 1978, 1550 cases and 47 deaths) and a small number of cases in France in 1979 (7), (8), (9). The 1980s there are also some small outbreaks in Hong Kong, Australia, USA (10), (11), (12) and no reported cases from other countries

In the late 1990s, many country members of Asia Pacific region have experienced large HFMD outbreaks. It began in 1997 with two large widespread community outbreaks

in Sarawak, Malaysia and Taiwan, with 2628 and 129,106 cases reported, respectively (13), (14). Following that, a series of small and large outbreaks happen throughout the region in which Japan, Australia, China, Malaysia, Singapore, Taiwan (China), Korea, Mongolia, Vietnam, Brunei has been the hotpots of epidemic with cycle of every 2–3 years period (15), (16), (17). So far, the latest large outbreak in the region was in one province named Anhui of China in 2008 with around 490,000 cases and 126 deaths in children were reported, the case-fatality rate is around 0.03% but in certain local outbreaks, such as in Fuyang City of Anhui Province, this rate can reach up to 0.3% (18), (19). During those outbreaks, almost of the cases are under 5 years old children and although clinical manifestations were mostly typical of HFMD, a cluster of deaths among young children was identified. Moreover, cases involving the central nervous system complication and/ or pulmonary oedema have also been observed for the first time (20), (21). There were several small outbreaks or sporadic HFMD cases outside the Asia-Pacific region.

Vietnam is located in South Eastern Asia and shares the border with China, Laos, and Cambodia. The climate is tropical in south; monsoonal in north with 4 seasons are spring, summer, autumn and winter. Although EV-A71 was isolated for the first time in Vietnam in 2003, the first outbreak of HFMD was not reported in the southern provinces until 2005. The 2005 outbreak was associated with EV-A71 C1, C4 and C5 subgenogroups and CV-A16 (22), (23). For the southern part of the country, in the periods of 2007 - 2009, the numbers of reported cases and deaths were 5,719 and 23; 10,958 and 25, and 10,632 and 23, respectively. In contrast, there were a few sporadic HFMD cases in the northern. In 2005 - 2007 periods, seven cases were identified. In 2008, 88 cases were reported from 13/28 provinces. No severe or fatal cases were reported. Since 2011, Vietnam have experienced continuously large outbreaks of HFMD and the disease became a notifiable one in the national communicable disease surveillance system. According to data of Viet Nam Ministry of Health (MoH) from 2011 to 2015, number of reported cases and deaths were 113,121 and 170 (2011), 157,391 and 45 (2012), 78,818 and 23 (2013), 77,296 and 9 (2014), 56,329 and 5 (2015), respectively which have been reported from across all 63/63 provinces. HFMD outbreaks have continuously occurred nationwide. Responding to HFMD outbreaks, MoH issued two specific guidelines applied nationwide. The first one published on the February 24, 2012 concerned surveillance, prevention and control of

HFMD. The second guideline was issued on March 30, 2012 were about diagnosis and treatment.

Indeed, in Viet Nam, HFMD is now important public health concern. Recent studies mainly observe for basic epidemiology and etiology characteristics (23), (24), (22), (25). Besides, the relationship between etiological agents and clinical epidemiological behavior, temporal and spatial analysis, pathogenicity-related mutations of virus, modeling approach for prediction, evaluation of countermeasures has not been reported. In order to more comprehensive understanding which contribute to prevention and control of HFMD in Vietnam, This work analyzed all HFMD cases reported in Hai Phong city by both the community and hospitals in 2011 and 2012. Since it was the very first outbreak to occur in Hai Phong city, it was a good model for investigating the dynamic of the disease without interference and potential remains from previous outbreaks or patient immunological adaptation and it provide findings related to influence of HFMD guidelines during the outbreak period too. This work is also an integrative analysis including spatial analysis and social evolution as well as genetic evolution to describe the dynamic of HFMD in a well delimited area. We will also analyze the VP1 gene of strains isolated throughout Northern Vietnam during the 2011-2012 outbreak and develop the modeling the dynamic of a multiphase disease. This part of the study is coordinated with Montpellier University 2, Montpellier, France.

The specific objective of the research aims:

1. To describe the epidemiological and etiological characteristics during 2011-2012 outbreak in Hai Phong City, Vietnam.
2. To monitor influence of HFMD new guidelines on patients care during 2011-2012 outbreak in Hai Phong City, Vietnam.
3. To spatial analyze the HFMD dynamic during 2011-2012 outbreak in Hai Phong City, Vietnam
4. To analyze pathogenicity-related mutations in the VP1 sequence of EV-A71 strains isolated throughout Northern Vietnam during the 2011-2012 outbreak.

5. To better understand the monitor HFMD throughout modeling the HFMD dynamic during 2011-2012 outbreak in Hai Phong City, Vietnam.

The outline will be displayed in form of chapters with results presented as a form of in press, submitted or in preparation articles. The articles included in this thesis are listed below:

FIRST PART

Nghia Ngu Duy, Tran Nhu Duong, Christian Devaux, Roger Frutos. Emergence of EV-A71 infection in Asia Pacific region. Asia Pacific Jounal of Tropical Medicine

SECOND PART

CHAPTER 1

Nghia Ngu Duy, Le Thi Thanh Huong, Patrice Ravel, Le Thi Song Huong, Ankit Dwivedi, October Michael Sessions, Yan'An Hou, Robert Chua, Guilhem Kister, Aneta Afelt, Laurent Gavotte, Catherine Moulia, Christian Devaux, Duane J Gubler, Vu Dinh Thiem, Nguyen Thi Hien Thanh, Tran Nhu Duong, Nguyen Tran Hien, Emmanuel Cornillot, Roger Frutos. Monitoring influence of Hand, Foot and Mouth Disease new guidelines on patients care during 2011-2012 outbreak in Hai Phong City, Vietnam. Asian Pacific Journal of Public Health.

CHAPTER 2

Nghia Ngu Duy, Le Thi Thanh Huong, Patrice Ravel, Le Thi Song Huong, Ankit Dwivedi, October Michael Sessions, Yan'An Hou, Robert Chua, Guilhem Kister, Aneta Afelt, Catherine Moulia, Duane J Gubler, Vu Dinh Thiem, Nguyen Thi Hien Thanh, Christian Devaux, Tran Nhu Duong, Nguyen Tran Hien, Emmanuel Cornillot, Laurent Gavotte, Roger Frutos*. Valine/isoleucine variants drive selective pressure in the VP1 sequence of EV-A71 enteroviruses. BMC Infectious Diseases

CHAPTER 3

Patrice Ravel, **Nghia Ngu Duy**, Aneta Afelt, Guilhem Kister, Le Thi Thanh Huong, Le Thi Song Huong, Ankit Dwivedi, October Michael Sessions, Yan'An Hou, Robert

Chua, Catherine Moulia, Duane J Gubler, Vu Dinh Thiem, Nguyen Thi Hien Thanh, Christian Devaux, Tran Nhu Duong, Nguyen Tran Hien, Laurent Gavotte, Emmanuel Cornillot, Roger Frutos. Modelling the dynamic of a multiphase disease: the example of HFMD. In preparation.

TÓM TẮT

Những thập kỷ gần đây, thế giới đang đối mặt với hàng loạt các bệnh truyền nhiễm mới nổi như cúm gia cầm, dịch SARS, MERS-CoV, Ebola, Zika…Bên cạnh đó, sự tái nổi trội của các bệnh truyền nhiễm khác cũng là gánh nặng bệnh tật rất lớn cho con người. Bệnh Tay chân miệng (TCM) là một trong số đó, TCM là bệnh sốt cấp tính, thường xảy ra ở trẻ em với biểu hiện chủ yếu là các ban dạng phỏng nước, thường xuất hiện ở gan bàn chân, bàn tay, mông, đầu gối và niêm mạc miệng. Bệnh đa số ở thể nhẹ, có thể tự khỏi trong vòng 1 tuần. Tuy nhiên có một số trường hợp có biến chứng thần kinh có thể dẫn tới tử vong. Căn nguyên gây bệnh là vi rút đường ruột, trong đó quan trọng nhất là EV-A71 và CA-16. Bệnh lây qua đường tiêu hoá, tiếp xúc với bề mặt bị ô nhiễm. Hiện nay chưa có vắc xin và thuốc điều trị đặc hiệu.

Bệnh TCM do EV-A71 lần đầu tiên ghi nhận tại California, Mỹ. Sau đó bệnh lưu hành và gây các vụ dịch nhỏ tại châu Mỹ, Châu Âu, Úc… Đến cuối những năm 1990s, TCM bùng phát và gây dịch lớn tại các quốc gia thuộc khu vực Châu á Thái bình dương, biến chứng thần kinh viêm thân não lần đầu tiên được ghi nhận. Tại Việt Nam, mặc dù EV-A71 được ghi nhận từ 2003, nhưng từ năm 2011, hàng năm, bệnh TCM gây dịch trên phạm vi cả nước với số mắc và tử vong cao. Thực vậy, TCM hiện nay là mối quan tâm về y tế công cộng. Một số nghiên cứu gần đây mới chỉ tập trung vào các đặc điểm dịch tễ, căn nguyên cơ bản nhất. Bên cạnh đó các mối liên hệ giữa tác nhân gây bệnh và đặc điểm dịch tễ học lâm sàng; các phân tích về phân bố bệnh theo không gian, thời gian; các biến đổi của vi rút liên quan đến độc lực; mô hình hoá sự biến động của bệnh dịch; đánh giá các biện pháp can thiệp kiểm soát bệnh dịch… vẫn chưa được nghiên cứu đầy đủ. Để cung cấp các kiến thức, thông tin toàn diện hơn về bệnh TCM góp phần phòng chống bệnh dịch này hiệu quả hơn, Nghiên cứa này phân tích toàn bộ các ca bệnh TCM được báo cáo trong vụ dịch đầu tiên tại Hải Phòng năm 2011-2012 tại cả cộng đồng và bệnh viện. Vụ dịch lớn đầu tiên này là một thực địa rất tốt cho điều tra , nghiên cứu bệnh dịch này. Nghiên cứu này tập trung phân tích các đặc điểm dịch tễ học, tác nhân gây bệnh, tác động của những hướng dân kiểm soát bệnh dich, biến động bệnh theo không gia, thời gian; phân tích các bieens đổi về di truyền liên quan đến độc lực; mô hình sự biến động bệnh dịch…

Một số khuyến cáo đối với lĩnh vực giám sát: Sự đa dạng về I/V variants là cách tốt để giám sát và xác định sự lưu hành của các chủng EV-A71; Chẩn đoán EV-A71 với bộ mồi MAS nên đuọc thực hiện một cách hệ thống từ sản phẩm của mồi SO. Cần chú ý tới

những mẫu âm tính khi thực hiện giám sát do có thể mồi phát hiện không phù hợp. Bộ mồi AN89 nên được thiết kế lại để phát hiện EVs tốt hơn. Đối với kiểm soát bệnh: Tiếp tục áp dụng các hướng dẫn giám sát và kiểm soát của Bộ Y tế. Các biện pháp can thiệp nên tập trung vào nhóm nguy cơ cao (trẻ < 5 tuổi) và gia đình, người chăm sóc trẻ ở cả khu vực đô thị và nông thôn. Giai đoạn ưu tiên can thiệp là tháng 3 – 5 và tháng 9-10 hàng năm. Quan tâm đặc biệt đến trẻ dưới 1 tuổi do có nguy cơ diễn biến nặng. Mô hình biến động nên được áp dụng để quản lsy tốt các đợt dịch trong tương lai. Đối với nghiên cứu: Phân tích toàn bộ gen của tác nhân gây bệnh để thấy rõ hơn mối liên hệ giữa các biến đổi di truyền và độc lực, nghiên cứu nguyên nhân bùng phát dịch nên tập trung them vào các yếu tố khác ngoài tác nhân gây bệnh. Sau cùng, nghiên cứu này đã đóng góp những kết quả cần thiết và quan trọng giúp phần phát triển các công cụ giám sát và kiểm soát bệnh TCM tốt hơn.

PART I. LITERATURE REVIEW

1. Picornaviridae

1.1. Classification

Picornaviruses are viruses which belonging to the family *Picornaviridae*. The name is derived from *pico*, meaning small, and *RNA*, referring to the ribonucleic acid genome, so "*picornavirus*" means small RNA virus (26). According to International committee on Taxonomy of Viruses (27), Picornaviruses are separated into 12 genera which consisting of *Enterovirus, Cardiovirus, Aphthovirus, Hepatovirus, Parechovirus, Erbovirus, Kobuvirus, Teschovirus, Sapelovirus, Senecavirus, Tremovirus, Avihepatovirus*. There are many important pathogens of humans in the family *Picornaviridae* (28), (Figure 1).

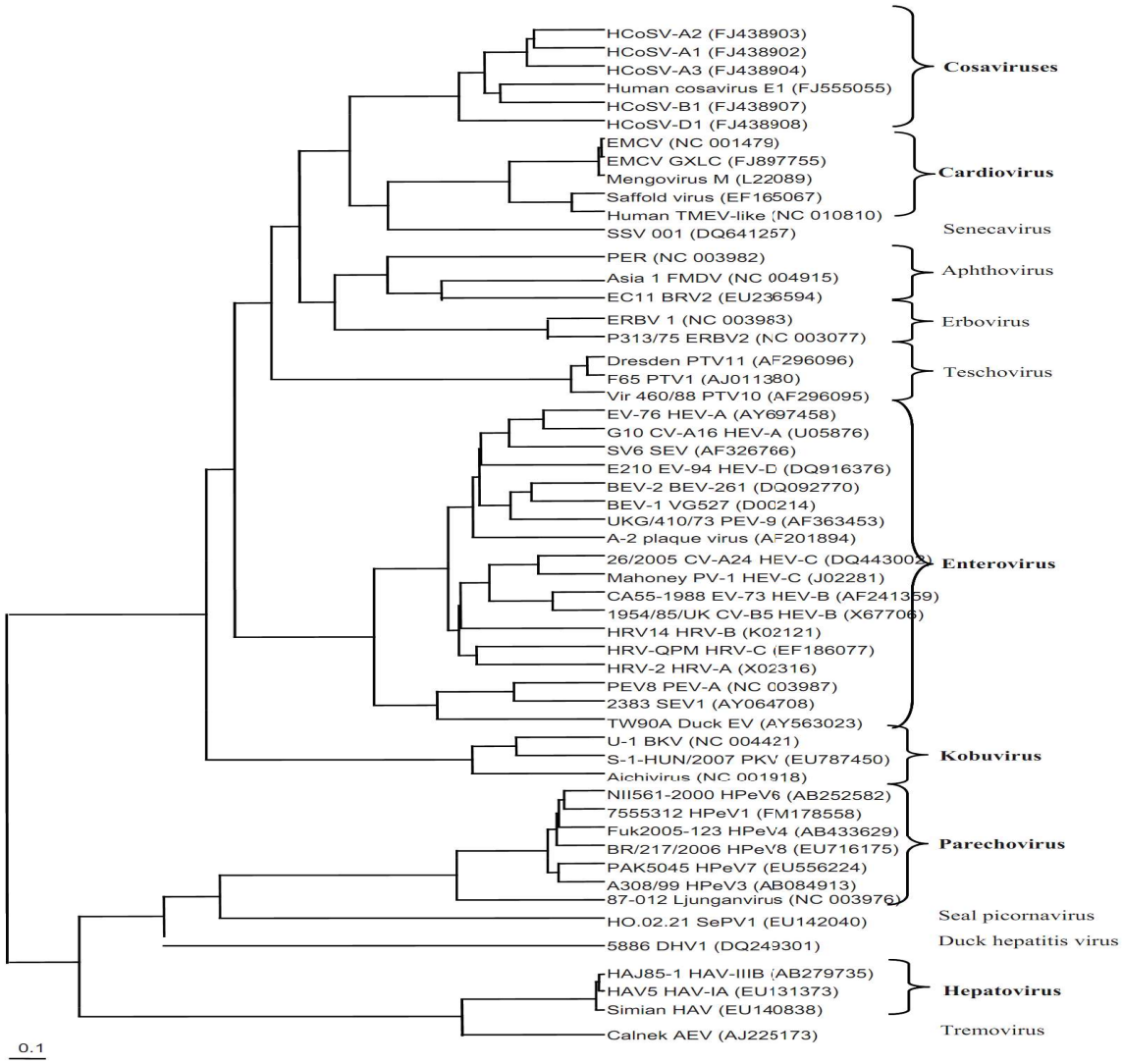

Figure 1: Phylogenetic tree based on the deduced amino-acid sequence of the structural P1 region of representatives of the different genera within Picornaviridae. Within the genera, types infecting humans among other species are shown in bold, (28).

For the disease manifestation, Picornaviruses can cause diseases in both human and animal. They are grouped by genus based on their pathogenic properties and/or route of infection. For example, the *enterovirus* genus includes poliovirus and coxsackieviruses based upon the natural oral route of entry into the host and replication in gut tissue (29), (Table 1).

Genus	Virus	Principal Species Affected	Disease
Aphthovirus	Foot-and-mouth disease viruses Equine rhinitis A virus Bovine rhinitis B virus	Cattle, sheep, swine, ruminant species Horses, camelids Cattle	Foot-and-mouth disease Systemic infection Mild respiratory
Cardiovirus	Encephalomyocarditis virus Theiler's mouse encephalomyelitis virus	Rodents, swine, elephants, primates, mammals in contact with rodents Mice	Encephalomyelitis and myocarditis in swine and elephants; rarely in other species Murine poliomyelitis
Enterovirus	Human enteroviruses A, B, C, and D Human rhinoviruses A, B, and C Bovine enteroviruses Simian enteroviruses Porcine enterovirus B	Humans Humans Cattle Primates Swine	Aseptic meningitis, poliomyelitis, myocarditis Respiratory disease Vesicular disease Mild enteric and respiratory disease Usually asymptomatic
Erbovirus	Equine rhinitis B virus	Horses	Mild rhinitis
Kobuvirus	Bovine kobuvirus	Cattle	Possible enteritis
Teschovirus	Porcine teschovirus 1 Porcine teschoviruses	Swine	Encephalomyelitis mild diarrhea, pericarditis
Tremovirus	Avian ncephalomyelitis virus	Chickens	Encephalomyelitis
Avihepatovirus	Duck hepatitis A virus	Ducks	Hepatitis

Table 1: Importance of Piconaviruses of Humans and Animals (29)

However, Base upon serology aspect (neutralized antibody against to capsid antigens), the Picornaviruses are further subclassified on the basis of serotype. This can range from a single serotype in the case of hepatitis A virus (HAV), or, in the case of rhinovirus, to about 100 identified serotypes (26) (Table 2).

Genus	Species	Serotypes
Enterovirus	*Bovine enterovirus*	2 types: bovine enterovirus 1-2
	Human enterovirus A	21 types including some coxsackie A viruses and enteroviruses
	Human enterovirus B	59 types: enteroviruses, coxsackie B, echoviruses, swine vesicular disease virus
	Human enterovirus C	19 types including poliovirus 1-3, some coxsackie A viruses, enteroviruses
	Human enterovirus D	3 types: EV-68, EV-70, EV-94
	Porcine enterovirus B	2 types: porcine enterovirus 9-10
	Simian enterovirus A	1 type: simian enterovirus A1
	Human rhinovirus A	75 types
	Human rhinovirus B	25 types
	Human rhinovirus C	7+ types
Cardiovirus	*Encephalomyocarditis virus*	1 type: encephalomyocarditis virus.
	Theilovirus	12 types: Theiler's murine encephalomyelitis virus, Vilyuisk human encephalomyelitis virus, Thera virus, Saffold virus 1-9
Aphthovirus	*Foot-and-mouth disease virus*	7 types: O, A, C, Southern African Territories 1 - 3 and Asia 1
	Equine rhinitis A virus	1 type: equine rhinitis A virus
	Bovine rhinitis B virus	1 type: bovine rhinitis B virus
Hepatovirus	*Hepatitis A virus*	1 type: Hepatitis A virus

Parechovirus	*Human parechovirus*	14 types: Human parechovirus 1-14
	Ljungan virus	4 types: Ljungan virus 1-4
Erbovirus	*Equine rhinitis B virus*	3 types: equine rhinitis B virus 1-3
Kobuvirus	*Aichi virus*	1 type: Aichi virus
	Bovine kobuvirus	1 type: bovine kobuvirus
Teschovirus	*Porcine teschovirus*	11 serotypes: porcine teschovirus
Sapelovirus	*Porcine sapelovirus*	1 type: porcine sapelovirus
	Simian sapelovirus	3 types: simian sapleovirus 1-3
	Avian sapelovirus	1 type: avian sapelovirus
Senecavirus	*Seneca Valley virus*	1 type: Seneca Valley virus
Tremovirus	*Avian encephalomyelitis virus*	1 type: avian encephalomyelitis virus
Avihepatovirus	*Duck hepatitis A virus*	3 types: duck hepatitis A virus 1-3

Table 2: Picornaviruses: Genera, Species and Serotypes

1.2. Virion

Picornaviruses have a simple structure with a small single-stranded RNA inside. The RNA is covered by a capsid, which is spherical shape with diameter of about 25–30 nm (30), (Figure 2).

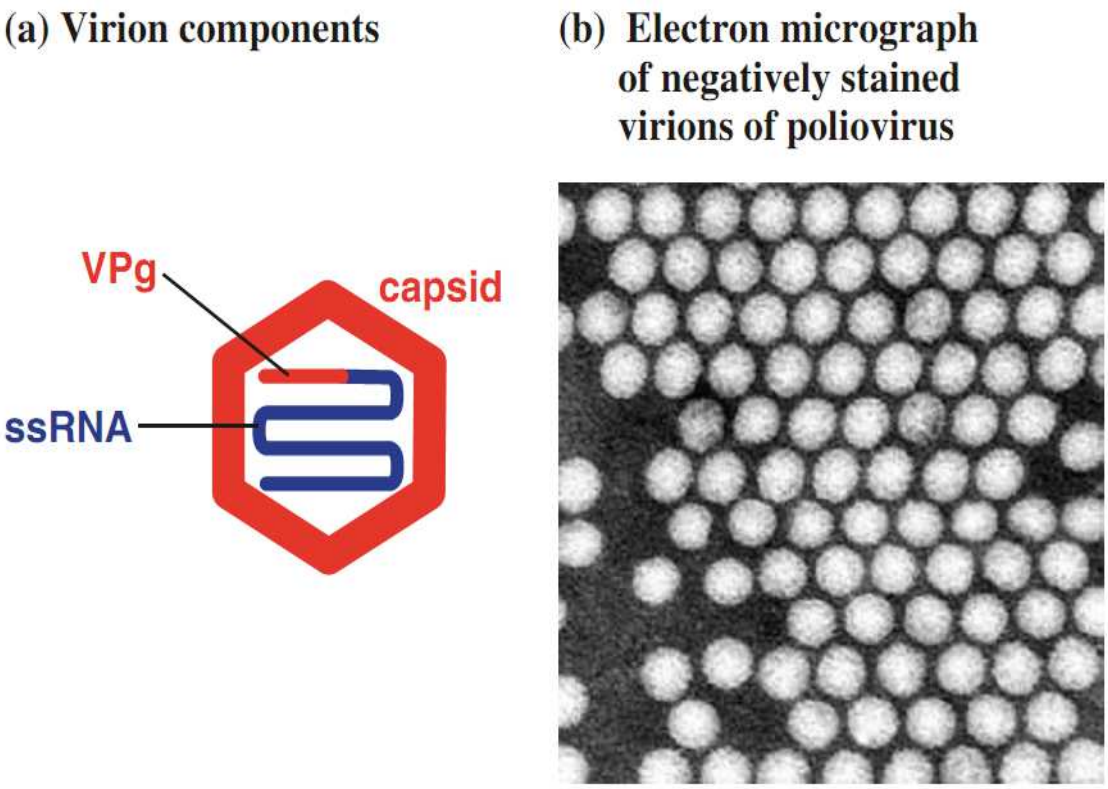

Figure 2: The picornavirus virion.VPg: virus protein, genome linked. Electron micrograph courtesy of J. Esposito (US-CDC) and Professor Frederick A. Murphy (University of Texas Medical Branch) (30).

1.3 Capsid

According to (30), There are 4 capsid proteins named VP1-VP4 which numbered from the largest to smallest one. 60 copies each of these four proteins create the virus capsid with icosahedral symmetry. Each of the proteins VP1–3 contains an eight-stranded β-barrel like many virus capsid proteins. For human rhinovirus 14, the N termini of VP1–3 and all 60 VP4 molecules are completely internal while all the rest parts of VP1–3 are at the surface of the virion. Moreover, in many picornaviruses, there is an approximately 2 nm deep groove as canyon around each of the 12 vertices of the icosahedron (Figure 3). The canyons which contain the virus attachment sites are lined by the C termini of VP1 and VP3 molecules. Evolution of picornaviruses has generated a lot of variability in the capsid proteins, some of which is reflected in the existence of distinct serotypes such as poliovirus with 3 serotyps or rhinovirus with more than 100 serotypes.

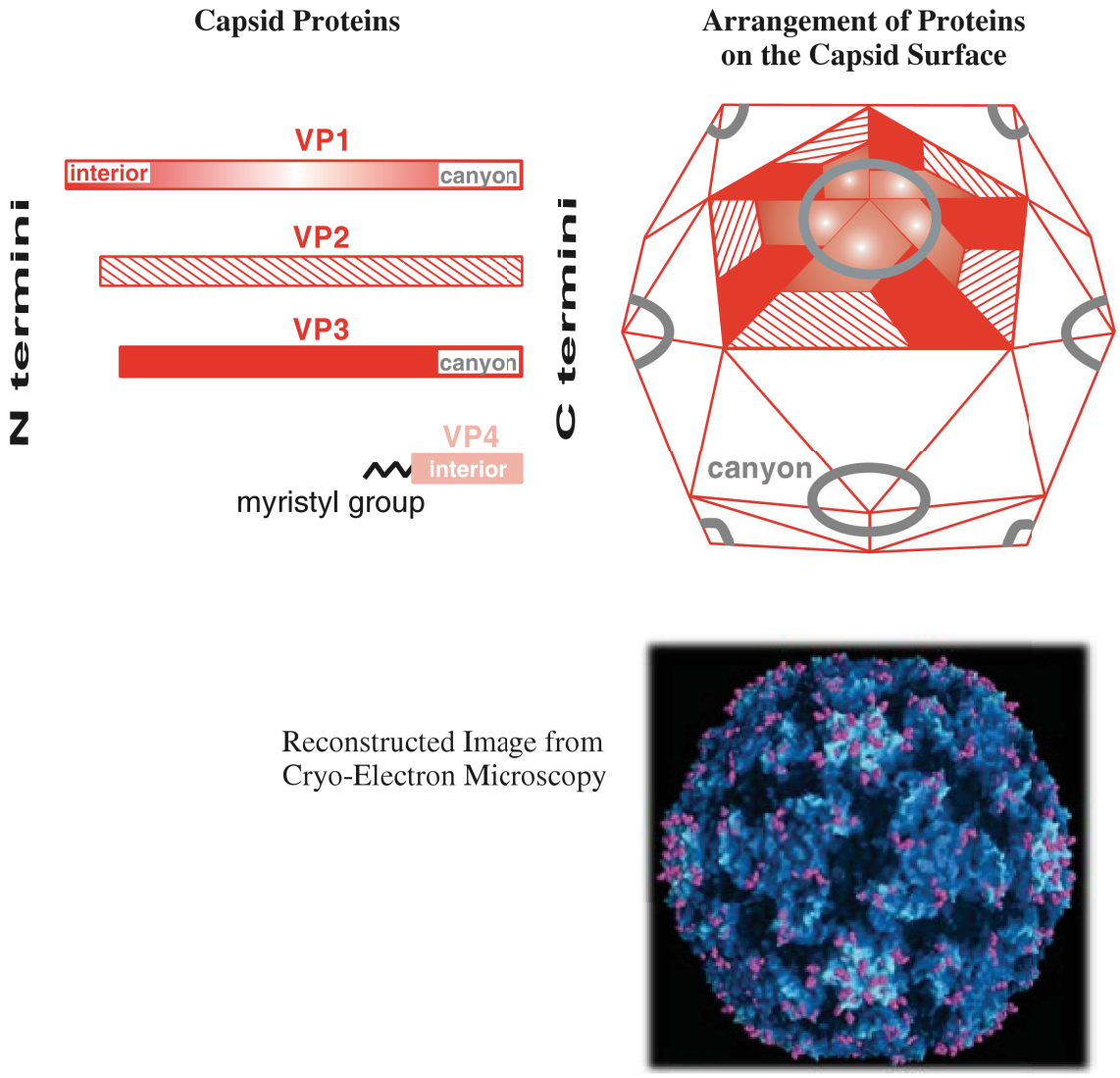

Figure 3: The capsid of human rhinovirus 14. The canyons are visible in the reconstructed image, courtesy of J.Y. Sgro, University of Wisconsin – Madison (30).

1.4. Genome structure and features

All picornaviruses have a similar genomic organization that is conserved in some but varies in other regions (31) (32). According to (26), picornaviruses have a quite short genome which containing a single molecule of positive-sense RNA within the capsid. The length of the RNA genome ranges from 6500 to 9500 nucleotides for whole. The genome consists of one single open reading frame (ORF) preceded by a long 5'-UTR and followed by a much smaller 3'UTR and a genetically encoded poly-(A) tail (Figure 4).

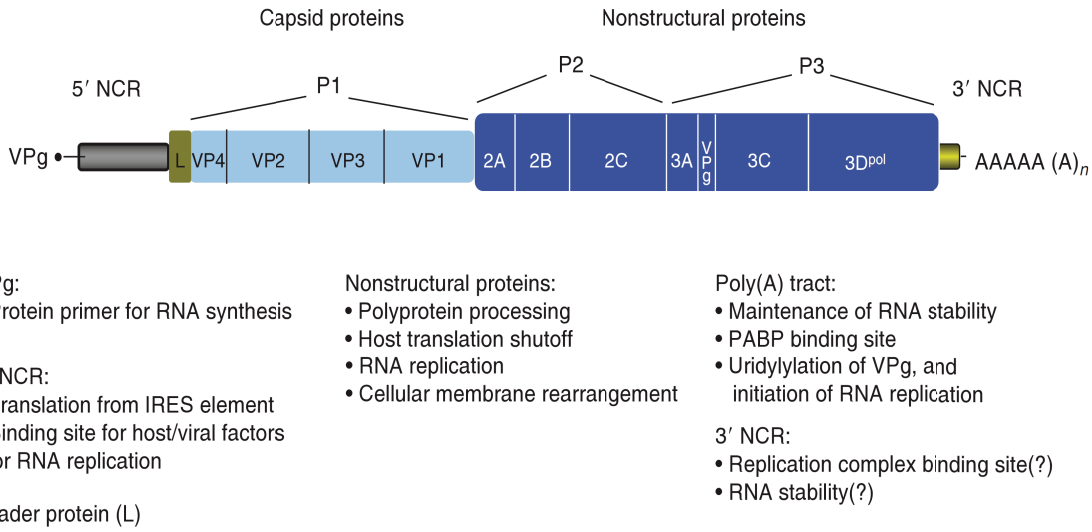

Figure 4: Organization of a typical picornavirus RNA genome. (26)

The 5’ end of the viral genome is covalently linked to the viral polypeptide VPg (virus protein, genome linked). The ORF which is divided into three consecutive regions, P1, P2 and P3 which translated into a single large polyprotein with approximately 2200 amino acid residues size. It is subsequently cleaved by viral protease(s) into mature proteins and their intermediates. For details, the P1 region encodes for the structural (capsid-forming) proteins 1A–1C which also known as VP4, VP2, VP3, and VP1, respectively. VP4 may not be formed in every picornavirus. The P2 and P3 region encodes for the nonstructural replication proteins 2A–2C and 3A–3D, respectively, as well as cleavage intermediates. In some picornaviruses, two or three unrelated 2A proteins may be formed while in others, two or three paralogous copies of 3B (also known as VPg) are (predicted to be) produced. The L region encodes for a leader protein in viruses of five genera. The L and 2A proteins may not be homologous, thus performing lineage-specific functions in different picornaviruses (33), (28), (27), (Figure 5), (Table 3).

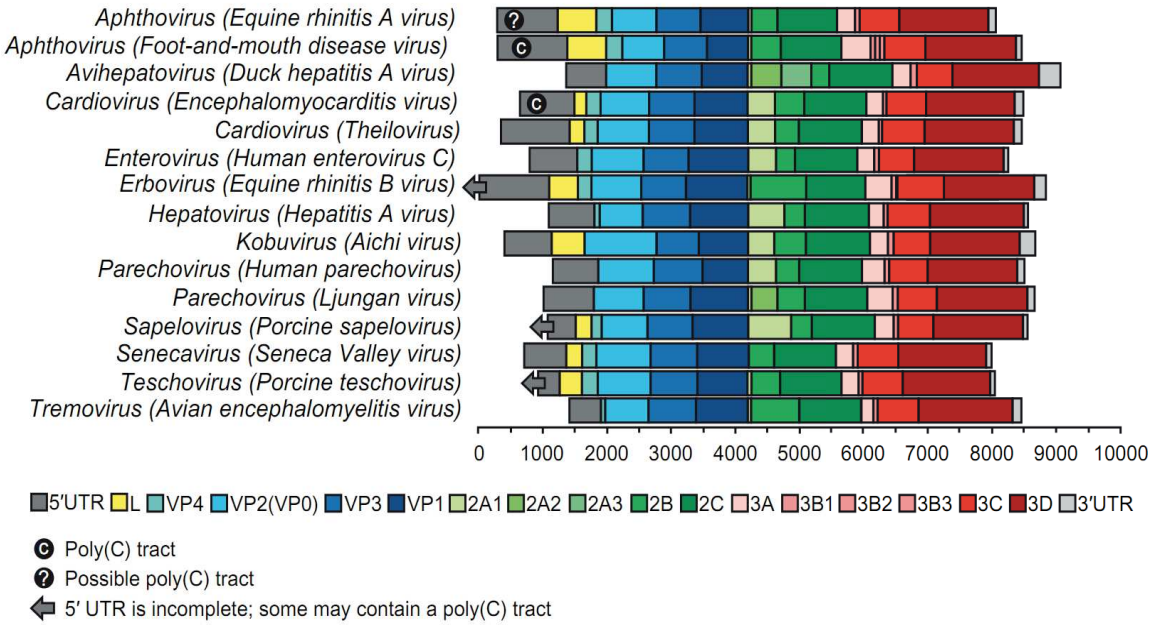

Figure 5: Genome structure and gene organization of members of the family Picornaviridae. Each of the 12 genera is represented, as are species where there is a significant difference within a genus (27).

Genomic region	Protein designation	Genus	Protein function
L	Leader	Aphthovirus, Erbovirus	Papain-like cysteine protease implicated in virus–host interaction
L	Leader	Cardiovirus	Involved in internal ribosome entry site-mediated translation of viral RNA
L	Leader	Kobuvirus	No protease activity; involved in both viral RNA replication and encapsidation
P1	VP2, VP3, VP1, VP4	All majority	Major capsid proteins Small capsid protein implicated in virion uncoating that is present in viruses of most genera
P2	2A	Enterovirus	Chymotrypsin-like cysteine protease releasing capsid precursor from the

			nascent polypeptide; implicated in the control of RNA synthesis
	2A	Cardiovirus Aphthovirus Parechovirus Senecavirus Erbovirus Teschovirus	Small protein whose synthesis is accompanied by termination and reinitiation of translation to separate 2A and 2B proteins
	2A	Hepatovirus	Structural protein
	2A	Parechovirus Tremovirus	Putative acyltransferase implicated in virus–host interaction
	2B	All	Membrane–anchoring protein for the virus replication complex
	2C	All	Multifunctional protein with ATPase and predicted helicase activity implicated in capsid assembly, virion uncoating, and RNA synthesis
P3	3A	Enterovirus and likely all	Membrane–anchoring protein for the virus replication complex; inhibits ER to Golgi membrane and secretory traffic
	3B, VPg	All	Protein primer for the initiation of RNA-synthesis
	3C	All	Chymotrypsin-like cysteine protease mediating most cleavages in polyprotein
	3D	All	RNA-dependent RNA polymerase
	3CD	Enterovirus	Responsible for processing capsid P1 precursor and regulation of RNA synthesis through binding to two RNA cis signals

Table 3: Major demonstrated and predicted functions of picornavirus proteins (28).

1.5. Replication

Attachment

The cell receptors for many picornaviruses have been characterized as the glycoprotein which is glycoprotein CD155 for poliovirus (30). It is the molecules with three immunoglobulin-like domains; the virus attachment site is located in the outermost domain. CD155 is found only in humans and some primate species. There are the major changes to capsid structure when virion binds to receptors which are the N termini of VP1 move to the exterior capsid surface and VP4 move out from virus capsid (Figure 7).

Entry

There are two modes of genome entry into the host cell have been proposed for picornaviruses. The first one is directly transfer of the RNA from the virion into the cytoplasm at the plasma membrane, leaving the capsid at the cell surface and the other is endocytosis. The VPg is removed from the 5'end by a cell enzyme as the virus genome is free in the cytoplasm (Figure 7).

Translation and post-translational modifications

The 40S ribosomal subunit binds internally at the IRES of the RNA. After a single polyprotein is created it is firstly cut to yield P1, P2 and P3. P1 becomes myristylated at the N terminus before being cleaved to VP0, VP3 and VP1, the proteins that will form procapsids; VP0 will later be cleaved to produce VP2 and VP4. Other cleavage products include 3B (VPg), 2C (an ATPase) and 3D (the RNA polymerase) (Figure 6).

Transcription/genome replication

RNA synthesis occurs in replication complexes are associated with membranous vesicles that created in the cytoplasm of infected cells and also contain cell proteins, as well as virus proteins and RNA. Multiple copies of (−) RNA are transcribed from infecting (+) RNA and then used as templates for (+) RNA synthesis. The small VPg is primer for both strands synthesis. Many RNA in the replication complexes play a role as replicative intermediates (RIs). Indeed, some RIs consist of a (+) RNA associated with growing (−) RNAs, while other RIs consist of a (−) RNA associated with growing (+) RNAs. Also

associated with RIs are copies of the RNA polymerase. The poly (A) sequence at the 3'end of the plus strand is transcribed and synthesized from a poly (U) sequence at the 5'end of the minus strand (Figure 7).

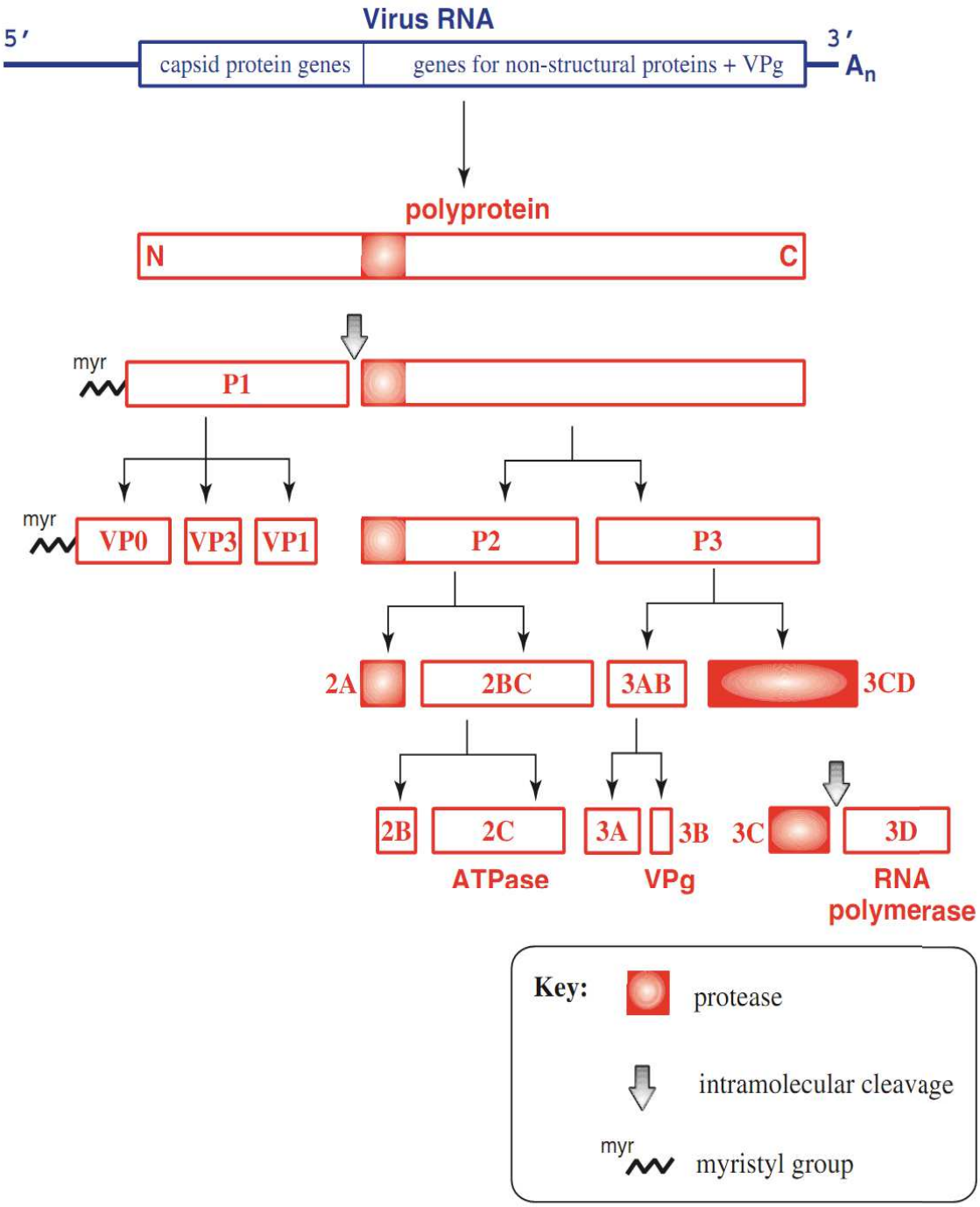

Figure 6: Poliovirus translation and post-translational modifications (30).

Assembly and exit

A procapsid is created by 12 pentamer which assembled from 5 copies each of VP0, VP3 and VP1. Each procapsid acquires a copy of the virus genome, with VPg still attached at the 5'end. Then the 60 copies of VP0 are cut into VP4 and VP2. The virions are released as cell lysis (Figure 7).

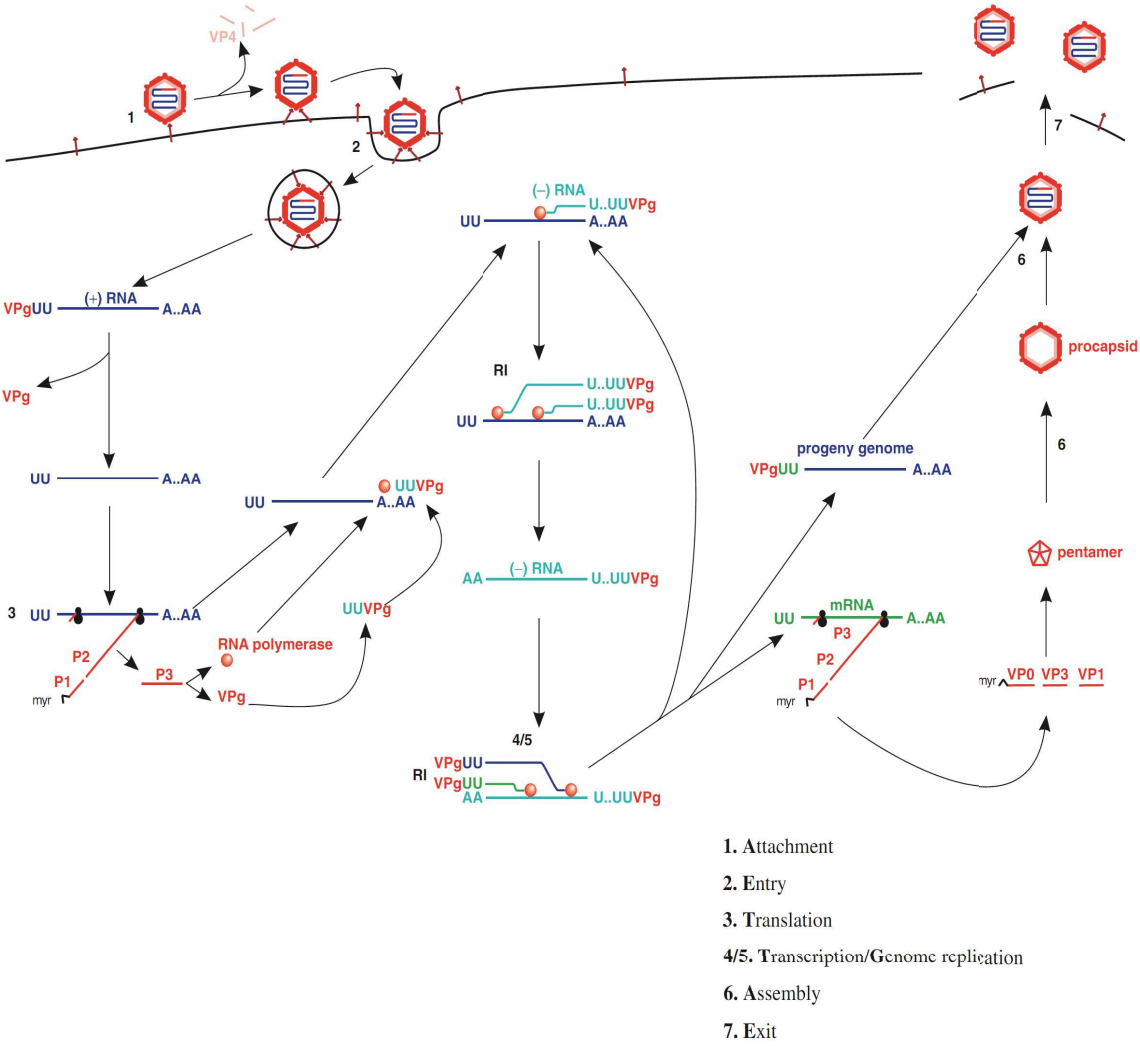

Figure 7: The picornavirus replication cycle. Note that translation gets under way before transcription, and that transcription and genome replication involve a single process (synthesis of (+) RNA). myr: myristyl group. RI: replicative intermediate (30).

2. Enterovirus

2.1. Classification

According to International Committee on Taxonomy of Viruses (27), enteroviruses are classified into 10 species and based on their sequence homologies, each species contains numerous viruses that are pathogenic to humans and animals (Table 4).

Species	Subspecies
Bovine enterovirus	Bovine enterovirus 1 Bovine enterovirus 2
Human enterovirus A	Coxsackievirus A2 [Fleetwood] Other types: CV-A3 to A8, CV-A10, CV-A12, CV-A14, CV-A16, EV-A71, EV-A76, EV-A89 to EV-A92, EV-A114, SV19, SV43, SV46, baboon enterovirus A13
Human enterovirus B	Coxsackievirus B1 Other types: CV-B2 to CV-B6, CV-A9, EV-B1, EV-B2 to 7, EV-B9, EV-B11 to 21, EV-B24 to 27, EV-B29 to 33, EV-B69, EV-B73 to EV-B74, EV-B75, EV-B77 to EV-B88, EV-B93, EV-B97 to 101, EV-B106 to 107, EV-B110, SA5
Human enterovirus C	Poliovirus 1 Other types: PV-2, PV-3, CV-A1, CV-A11, CV-A13, CV-A17, CV-A19, CV-A20, CV-A21, CV-A22, CV-A24, EV-C95, EV-C96, EV-C99, EV-C102, EV-C104, EV-C105, EV-C109, EV-C113, EV-C116
Human enterovirus D	Enterovirus D68 Other types: EV-D70, EV-D94, EV-D111
Porcine enterovirus B	Porcine enterovirus 9 Porcine enterovirus 10
Simian enterovirus A	Simian enterovirus A1 Other types: SV28, SA4
Human rhinovirus A	Human rhinovirus A1

	Other types: HRV-A2, HRV-A7, HRV-A8 to HRV-A13, HRV-A15, HRV-A16, HRV-A18 to HRV-A25, HRV-A28 to HRV-A34, HRV-A36, HRV-A38 to HRV-A41, HRV-A43 to HRV-A47, HRV-A49 to HRV-A51, HRV-A53 to HRV-A68, HRV-A71, HRV-A73 to HRV-A78, HRV-A80 to HRV-A82, HRV-A85, HRV-A88 to HRV-A90, HRV-A94 to HRV-A96, HRV-A98, HRV-A100 to HRV-A103
Human rhinovirus B	Human rhinovirus B3 [FEB] Other types: HRV-B4 to HRV-B6, HRV-B14, HRV-B17, HRV-B26, HRV-B27, HRV-B35, HRV-B37, HRV-B42, HRV-B48, HRV-B52, HRV-B69, HRV-B70, HRV-B72, HRV-B79, HRV-B83, HRV-B84, HRV-B86, HRV-B91 to HRV-B93, HRV-B97, HRV-B99
Human rhinovirus C	Human rhinovirus C1 [NAT001] Other types: HRV-C2 to HRV-C49

Table 4: Enterovirus genus and species, abbreviated as follows: bovine enterovirus (BEV); coxsackievirus (CV); echovirus (E); enterovirus (EV); human rhinovirus (HRV); poliovirus (PV); porcine enterovirus (PEV); simian agent (SA); simian virus (SV); simian enterovirus (SEV) (27).

2.2. Human Enteroviruses cause Hand, Foot and Mouth Disease

Origin, gene groups, evolution, and geographical distribution of EV-A71

Human enteroviruses have been classified into four species, HEV-A, HEV-B, HEV-C, and HEV-D. HFMD is caused by types of Enterovirus A species which includes some Coxsackievirus A (CV-A) and Enterovirus A71 (EV-A71) (34). The EV-A71 viruses are genetically related to CV-A; indeed, it has been suggested that these viruses may have diverged as recently as the 1920s. EV-A71 isolates are classified on the basis of VP1 gene into six independent genogroups: A, B, C, D, E and F. The EV-A71 B and C genogroups are each further subdivided into subgenogroups, B0 to B5 and C1 to C5 (Bessaud et al.,

2014). CV-As are subdivided into serotypes CV-A2-8, CV-A10, CV-A12, CV-A14, CV-A16. Both EV-A71 and CV-A infection has been associated with severe HFMD in young children, sometimes resulting in death. But EV-A71 strain frequently causes severe HFMD as neurological forms and death in large epidemics while mild disease with other serotypes (35), (36), (37).

There is only one member in Group A, the prototype BrCr strain of EV-A71, which was first isolated in California, USA, in 1969. So far, the secondly isolates were reported in a HFMD epidemic among children in Anhui province of central China (38). Surveillance for HFMD by the Chinese Center for Disease Control and Prevention which do not seem to indicate any group A viruses (19).

Group B viruses were firstly separated into B1 and B2 subgenogroups with 12% divergence at the nucleotide level. These subgenogroups were the predominant circulating strains in the 1970s and 1980s. In details, B1 was circulating in USA, Europe, Japan and Australia in 1970s and B2 mainly were isolated in USA in the 1980s. After that subgenogroup B3 was emerged from 1997 in Sarawak, Malaysia in several large outbreaks of Asia Pacific region. These viruses were also found in Singapore (39), (40), (41). Subgenogroup B4 were circulating and associated with the large outbreaks through Asia Pacific region in the 1990s but were also sampled by 2000. In 2003, subgenogroup B5 replaced subgenogroup B4 in Sarawak, Malaysia and circulate as the important viruses in Japan, Malaysia, Singapore and Taiwan (42), (43), (44), (15), (45), (Figure 6).

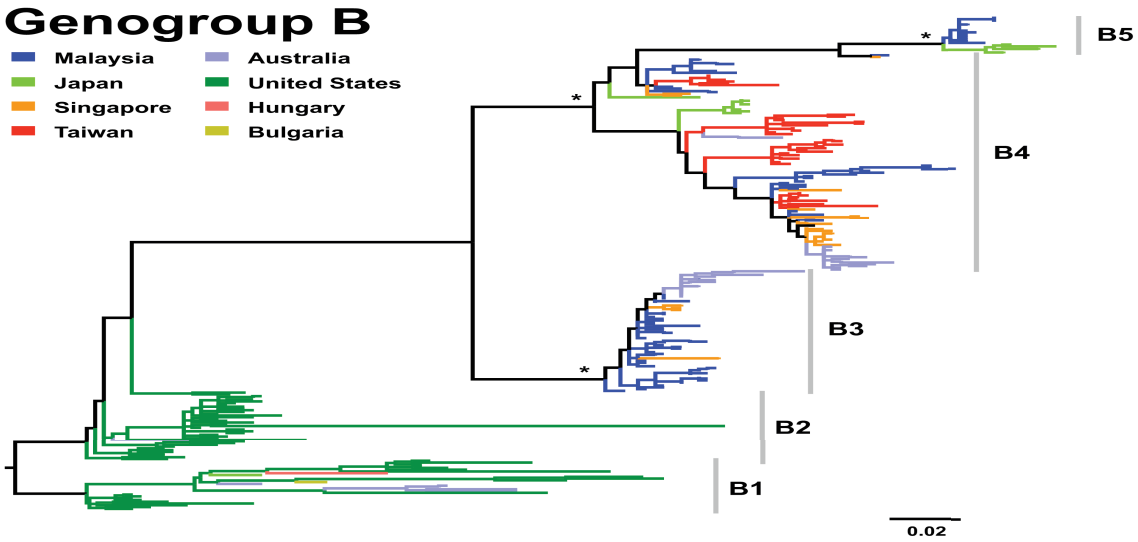

Figure 8: Genogroupe B of Enterovirus 71(EV-A71)

Group C viruses were initially separated into the C1 and C2 subgroups. Subgenogroup C1 viruses were first reported in USA and Australia in the 1970s, 1980s. From its initial detection, up to now these viruses have been identified continuously in many countries throughout Europe, Asia Pacific region. But they have not caused any large epidemics since the major community outbreak in Sydney in 1986 (46), (44). Subgenogroups C2, C4, and C5 is the novel subgenogroups which divers from genogroup C in the Asia Pacific region in the late 1990s. They cause fatal HFMD cases in Taiwan (14), China (47), and Vietnam (22), respectively. Subgenogroup C3 was isolated in Japan in 1994, and in Korea in 2000 (40), (48), (49). According to (45), subgenogroups C1, C2, and C4 globally distribute in many parts of the world while the C3 and C5 lineages are restricted to Korea and Vietnam, respectively (Figure 7).

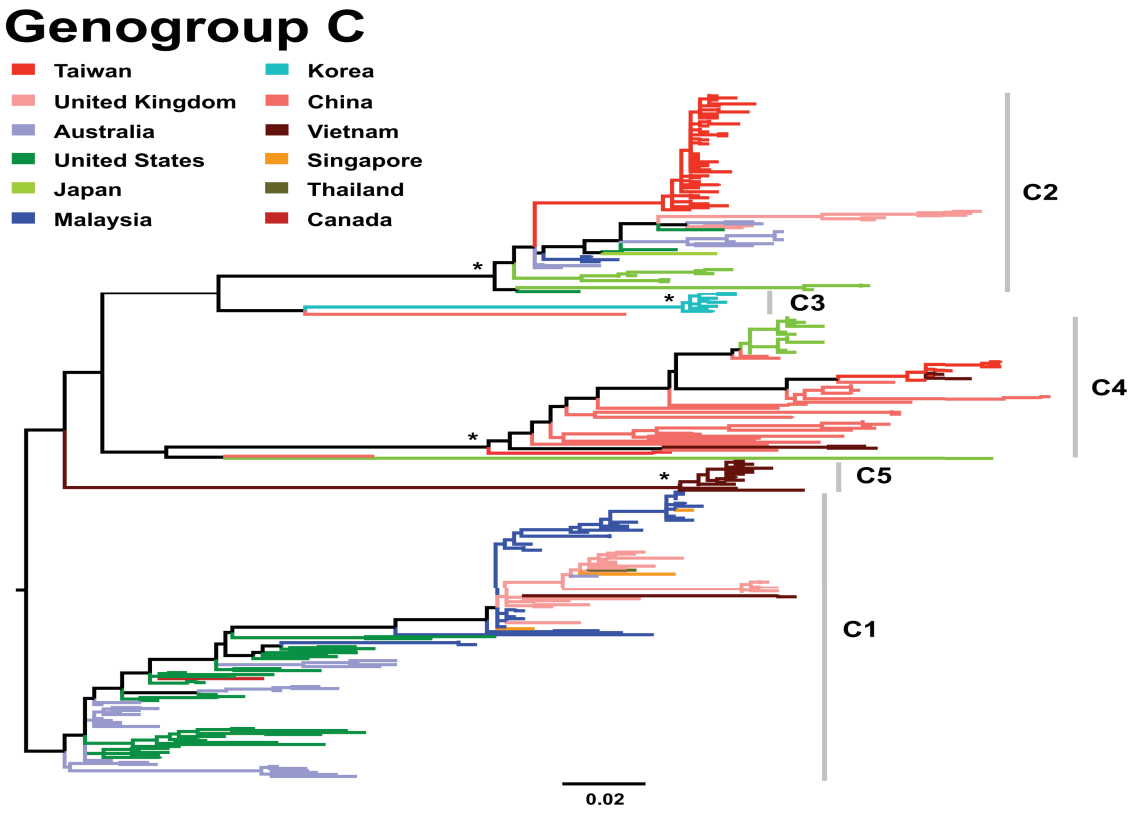

Figure 9: Genogroupe C of Enterovirus 71(EV-A71).

Recombination and reservoir of EV-A71

The recombination of EV-A71 has been observed most frequently in the 5'UTR and 3'UTR regions. This rarely happens in the structural protein gene region (50), (51), (52). So far, the effect of EV71 recombination on the virulence or transmissibility is unknown. But some studies observe the replacement of the 3' half of the genome of a non-recombinant EV-A71 field isolate with a EV-B species virus which improves growth in cell culture compared with a non-recombinant strain (53). Reservoir of EV-A71 is human and replication happens both in the intestinal tract and upper respiratory tract. The virion typically shed from 2 to 4 weeks and especially up to 12 weeks after infection. The transmission mode is through direct person-to-person contact, droplets or fomites by faecal-oral and respiratory secretions (54).

3. Pathogenesis

3.1. Virus entry and spread

The main transmission mode of HFMD is via the faces-oral route. It can also get infection through contact with virus contaminated surfaces, secretions, fluid or fomites. The virus can spread by respiratory droplets as well (55). According to (56), the enteroviruses initially replicate in the lymphoid tissues of the oropharyngeal cavity (tonsils) and small bowel resulting a mild viraemia. Most infections are asymptomatic at this time. The viruses follow bloodstream to other organs as the reticuloendothelial system (liver, spleen, bone marrow, and lymph nodes), heart, lung, pancreas, skin, mucous membranes, and center nervous system (CNS) at the onset time of disease with given clinical symptom. For virus shedding, it is up to 2 weeks from throat or 11 weeks from intestine after an acute EV71 infection (57).

3.2. Pathological findings

Clinical manifestations for HFMD widely vary from asymptomatic, mild to neurologic cases. But CNS infection is the features most frequently seen of EV-A71 infection (58).

For uncomplicated HFMD, regarding (59), the experiment on a mouse model shows the virus infect to skin which suggest the same results with human skin or oral mucosa lesions. But the information for skin or oral mucosa biopsies is still lacking.

For neurologic case, there is no inflammatory in cerebella cortex, basal ganglia, thalamus, peripheral nerve or autonomic ganglia. In contrast, the grey matter of the spinal cord and the whole medulla oblongata is affected predominantly and the hypothalamus, subthalamic, dentate nuclei as well. The motor cortex is also influenced but lesser. However, virus, viral antigen or RNA in the brainstem has not been seen or very rare for some cases in neuronal processes and phagocytic cells (20), (60).

For severe pulmonary oedema and cadiac dysfunction, It is supposed that explosive pulmonary oedema especially associated with neurogenic mechanisms secondary to brainstem inflammation, cardiac dysfunction, increased vascular permeability, and cytokine storm but its exact mechanism is still unclear and the pathogenesis is not completely understood (2), (Figure 8). In-vivo models, including those in mice and non-human primates, have replicated some of the features of severe EV-A71 disease, such as neuroinvasion with inflammatory changes, but none has yet been able to reproduce the severe systemic features, such as pulmonary oedema (59), (61), (62).

3.3. Virus virulence and host factors

So far, there is still not clear evidence from genetic analysis on virus-virulence factor. But data from some HFMD outbreaks show that the difference of genogroup may contribute to virulence aspect. Subgenogroup C2 from the epidemic in Taiwan (1998) was almost exclusively isolated from children with severe neurological disease. In contrast, subgroup B3 from the epidemic in Sarawak, Malysia (1997) was isolated mainly from children with mild HFMD (41), (44). As well, to date, still lack of information about infective virus doses related to the illness severity. It should be investigated further. In EV71 infection with explosive pulmonary oedema, there is a storm of cytokines and chemokines in cerebrospinal fluid and serum such as interleukin IL-6, IL-1beta, IL-10, IL-8, interferon induced protein (IP-10) and monokine induced by interferon gamma (MIG) (63), (64), (65). In contrast, number of T lymphocytes and NK cells reduce (66).

According to (67), HLA-A33, which is a common phenotype in Asian populations but is rare in white populations, was most significantly associated with EV-A71 infection and HLA-A2 was significantly related to cardiopulmonary failure. Receptors on the host target cell were identified as scavenger receptor B2 (68), and P-selectin glycoprotein ligand-1 (69), which expressed or limited to leukocytes, respectively.

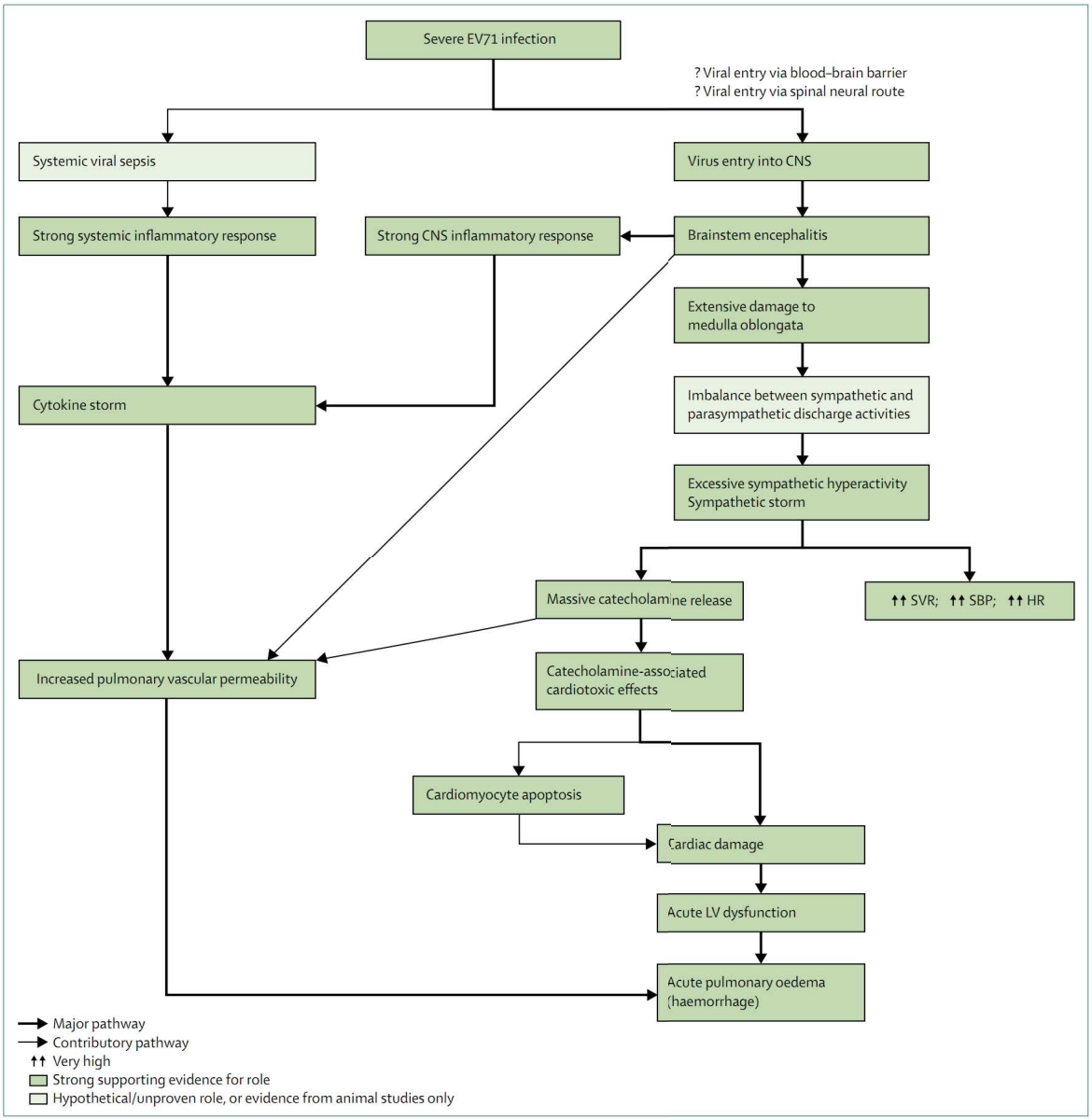

Figure 10: The postulated pathogenesis of enterovirus-71-associated acute pulmonary oedema (2). EV71=enterovirus 71. CNS=central nervous system. SVR=systemic vascular resistance. SBP=systemic blood pressure. HR=heart rate. LV=left ventricular.

Age and gender may play a role on susceptibility as a higher incidence of disease has been reported in male children. The relative immaturity of the innate and adaptive immune system in children aged between one and five years could contribute as well (70), (71), (25).

3.4. Protective immunity

According to (72), the cellular and humoral immune responses are both essential for decreasing the viral load and mortality in mice. In details, by mouse model, the disease severity, mortality, and tissue viral loads of mice lack of B, CD4 T, or CD8 T cells were significantly higher than those of wild-type mice after infection. Further, use a virus-specific antibody as therapy before or after infection significantly reduced the disease severity, mortality, and tissue viral loads of mice deficient in B cells. On the other hand, cellular immunity in human plays an important role of prevention of serious complications from HEV-71 infection (73), (74). Beside that there are some evidences of the protective immunity against infection by neutralizing antibodies from the humoral response (75), (76), (77). In humans, presence of maternal anti-EV-A71 antibodies has also been demonstrated in neonates, the prevalence and titer of which correlate with those levels in the mothers (78), (25). In mice, transplancental transfer of antibodies following maternal immunization against EV-A71 protects against lethal infection of newborn mice (79). Thus, it appears that the seroprevalence of neutralizing antibodies in women of childbearing age is important in protecting infants aged less than 6 months.

4. Diagnosis

4.1. Case definition and classification

For outbreaks, there are several kinds of clinical manifestation. It could be HFMD or Herpangina (HA) which usually is self-limiting and benign illness. But there is also a small number of cases can develop to neurological and systemic complication that may lead to death (Figure 9). According to (80), Case definition and classification is defined as below.

HFMD is a brief febrile illness in children with skin rash which typically is papulovesicular at the palms or soles of the feet, or both. This can be with or without mouth ulcers. Normally in younger children and infants the rash may be maculopapular without vesicles, and may also involve the buttocks, knees or elbows. HA is also fever and typically with multiple, painful mouth ulcers at mainly the posterior oral cavity (the anterior pharyngeal folds, uvula, tonsils and soft palate). In some cases, the mouth ulcers also affect other parts of the mouth such as the buccal mucosa and tongue.

For neurological and systemic complication, the enteroviruses, in general, can cause central nervous system complications as aseptic meningitis, acute flaccid paralysis, encephalitis. In particular, brainstem encephalitis is typically by EV-A71 infection and is frequently together with severe cardiorespiratory symptoms that have been attributed to neurogenic pulmonary oedema (21), (2). This typically clinical syndrome starts normally after 3–5 days of fever by a sudden deterioration as shock and pulmonary oedema or haemorrhages resulting from an acute and rapidly progressing cardiorespiratory failure. Finally, the children will die before reaching hospital or in the first day of admission, despite intensive care support (13), (20).

Fatal case firstly undergoes a short period of febrile illness with subtle neurological signs. Then dramatically suffer an acute refractory myocardial dysfunction and fulminant pulmonary oedema within hours of developing tachycardia, poor peripheral perfusion and tachypnea (80).

Disease	Proposed Case Definition
HFMD	Febrile illness with papulovesicular rash on palms and soles, with or without mounth vesicles/ulcers. Rash may be maculopapular without vesicular lesion, and may also involve the buttocks, knees or elbows, particularly in younger children and infants.
Herpangina	Febrile illness with multiple oral ulcers on the posterior parts of the oral cavity.

Aseptic meningitis	Febrile illness with headache, vomiting and meningism associated with presence of more than 5 – 10 white cells per cubic millimeter in cerebrospinal (CSF) fluid, and negative results on CSF bacterial culture.
Brainstem Encephalitis	Myoclonus, ataxia, nystagmus, oculomotor palsies, and bulbar palsy in various combinations, with or without MRI. In resource-limited settings, the diagnosis of brainstem encephalitis can be made in children with frequent myoclonic jerks and CSF pleocytosis.
Encephalitis	Impaired consciousness, including lethargy, drowsiness or coma, or seizures or myoclonus.
Encephalomyelitis	Acute onset of hyporeflexic flaccid muscle weakness with myoclonus, ataxia, nystagmus, oculomotor palsies and bulbar palsy in various combinations.
Acute flaccid paralysis	Acute onset of flaccid muscle weakness and lack of reflexes.
ANS dysregulation	Presence of cold sweating, mottled skin, tachycardia, tachypnea, and hypertension.
Pulmonary oedema/ haemorrhage	Respiratory distress with tachycardia, tachypnea, rales, and pink frothy secretion that develops after ANS dysregulation, together with a chest radiograph that shows bilateral pulmonary infiltrates without cardiomegaly.
Cardiorespiratory failure	Cardiorespiratory failure is defined by the presence of tachycardia, respiratory distress, pulmonary oedema, poor peripheral perfusion requiring inotropes, pulmonary congestion on chest radiography and reduced cardiac contractility on echocardiography.

Table 5: Proposed clinical case definitions for HFMD/HA and associated complications (80).

4.2. Laboratory diagnosis

Clinical samples

Throat swab, vesicle swab and stool, all can be used for virus detection. However, in the rate of detection point, throat and vesicle swab are considered to be the most useful specimens for both inpatients and outpatients. Especially, vesicle isolates always represent current systemic infection. It can also detect the virus from cerebrospinal fluid of central nervous system complication-patient, but the detection rate is very low (less than 5%). Serum sample from the patient is used in serological test as well (80) (81). The detection of the virus from the throat swabs was more frequent than from stool specimens; the time to positivity by viral culture was also shorter (82). In practice, it should be taken both kinds of sample as throat and vesicle swab or throat swab and stool in order to increase the possibility of virus detection.

Virus isolation

As usual for enterovirus isolation, some specimen pre-treatment such as complete mixing, filtration, chloroform treatment which needs to be implemented before inoculation. Two cell lines have been widely used for EV-A71 and CV-A16 virus detection are RD cells and Vero cells because of their relatively high sensitivity and the apparent cytopathic effect. For more, there are also some cell lines which are MRC-5, HEL, HeLa, L20B can be used as well (83). In addition, mouse cell lines are considered to give functional cellular receptors for enterovirus 71 which can be used for the selective isolation of EV-A71 or CV-A16 from clinical specimens (68), (69). Virus isolation and identification using suckling mice are still useful for some EV-A isolates, particularly for clinical samples from herpangina cases, but requires equipment, human resources and time (84).

Identification of virus isolates

Neutralization test recently gives confidence for virus identification. From virus isolation in cultured cells, conventional neutralization test which use a qualified type specific anti serum to identified the serotype of EV-A71 and CV-A16 virus, but these antisera are still not commercially available and it normally wait for a week to get the test result (80).

Reverse transcription - polymerase chain reaction (RT-PCR) and sequencing always has advantage of universal detection and amplification of gene targets, with any serotypes and genotypes, even though it is newly emerged variants or new serotypes of enteroviruses (85), (86), (39). With virus isolation from cultured cells, various gene targets which are 5'untranslated region, VP1 and VP4/VP2 genes have been widely used for molecular identification through by RT-PCR amplification of viral RNA and sequencing of the DNA amplicons (80). Indirect immunofluorescence assay (IFA) tests can provide quick, technically simple and reliable EV-A7 identification. It uses anti-EV-A71 monoclonal antibodies that are commercially available (80).

Rapid diagnosis directly from clinical samples

Nested RT-PCR: According to (87), use consensus-degenerate hybrid oligonucleotide primer (CODEHOP) on the VP1 region has enabled partial VP1 sequencing with a high sensitivity and broader specificity for all known enterovirus serotypes. Indeed, these viruses will be identified by VP1 sequences derived from the CODEHOP PCR products directly from clinical samples. It makes the advantage of quicker identification than virus culture. EV-A71-specific primers can be revised based on the sequences of newly emerging EV-A71 genogroups and variants for ensuring reliability of the test (88), (89). Multiplex RT-PCR methods are already developed for combined EV-A71and CV-A16 identification (90) and for the specific detection of EV-A71and CV-A16 (91).

Real-time PCR can reduce the risk of cross-contamination when compared with conventional RT-PCR, particularly nested PCR systems. The reliability of recently EV-A71-specific real-time RT-PCR systems needs to be addressed by using different genogroups of EV-A71, CV-A16, EV-A strains, and clinical samples. In general, EV-A71-specific primers can be revised based on the sequences of newly emerging EV-A71 genogroups and variants for ensuring reliability of the test (80).

Molecular epidemiological analysis (genotyping) of EV-A71 strains

Determination of geographic and evolutional origin of a virus is normally today by genotyping technique (the molecular characterization genomes). For EV-A71 trains, based

upon a capsid VP1 sequence database, it can develop a quick way to tracking the regional transmission and current prevalence of EV-A71strains by comparing the extent of genetic changes that are observed between strains. For detailed molecular epidemiological analysis of EV-A71 strains, the entire VP1 sequence should be determined and analyzed, beside partial VP1 sequencing would be enough to genotype each EV71isolate (80).

Serological analysis

Testing for neutralizing antibodies against enteroviruses is not recommended for routine use in the diagnosis of enterovirus infections because of interpretation of serum antibody titers is sometimes difficult. In contrast, this test is useful for evaluating of immunity levels for EV-A71 infection within communities (54), also for monitoring the cross-reactivity of serum among different genogroups of EV71 (92) Serum samples from inpatients could be useful for the rapid immunoglobulin M (IgM) detection of EV71 (93), but the specificity and sensitivity of serological tests for EV-A71 infection remains difficulties in evaluation.

5. Epidemiology of Hand, Foot and Mouth Disease

5.1. History and epidemiological features of HFMD

In 1969, in California, USA, EV-A71 strain firstly was isolated from a 9 months aged child with encephalitis diagnostic (1). There are then small and large outbreaks of HFMD have been reported throughout the world (2), (Figure 10).

In the early 1970s, several countries in different continent including Sweden, Australia, USA and Japan had reported small HFMD outbreaks with some tens of cases and almost of them are children whose clinical aspects are mostly typical of HFMD, sometimes aseptic meningitis (3), (4), (5), (6). After that, HFMD was only reported in Europe in late half of the 1970s with two large outbreaks in Bulgaria (year 1975, 451 cases and 44 deaths), Hungary (year 1978, 1550 cases and 47 deaths) and a small number of cases in France in 1979 (7), (8), (9).

The 1980s there are also some small outbreaks in Hong Kong, Australia, USA (10), (11), (12) and no reported cases from other countries.

In the late 1990s, many country members of the Asia Pacific region have experienced large HFMD outbreaks. It began in 1997 with two large widespread community outbreaks in Sarawak, Malaysia and Taiwan, with 2628 and 129,106 cases reported, respectively (13), (14). Following that, a series of small and large outbreaks happen throughout the region in which Japan, Australia, China, Malaysia, Singapore, Taiwan (China), Korea, Mongolia, Vietnam, Brunei has been the hotpots of epidemic with cycle of every 2–3 years period (15), (16), (17). So far, the latest large outbreak in the region was in one province named Anhui of China in 2008 with around 490,000 cases and 126 deaths in children were reported, the case-fatality rate is around 0.03% but in certain local outbreaks, such as in Fuyang City of Anhui Province, this rate can reach up to 0.3% (18), (19). The explosive emergence of the disease in the region may be related to the association between HLA-A33 (which is higher prevalence in Asian populations than white population) and susceptibility to HEV-71 infection (67). During those outbreaks, almost of the cases are under 5 years old children and although clinical manifestations were mostly typical of HFMD, a cluster of deaths among young children was identified. Moreover, cases involving the central nervous system complication and/ or pulmonary oedema have also been observed for the first time (20), (21). The major pathogens of Hand, Foot and Mouth Disease (HFMD) are the EV-A in which some main serotypes are coxsackievirus A6, coxsackievirus A10, particularly coxsackievirus A16 (CV-A16) and enterovirus 71 (EV-A71). In the Asia Pacific region, EV-A71 strain frequently causes severe HFMD as neurological forms and death in large epidemics while mild disease with other serotypes (81), (36). Moreover, some EV71 subgenotypes can co-circulate in the same epidemic, as well as other non-EV-A71enteroviruses, such as Coxsackievirus-A. There are evidences show that the co-infection is possible in HFMD and it is not a primary cause leading to severe form (94), (21), (Table 10). Recurrent epidemics could be due to both of the accumulation of susceptible individuals in the community and introduction of new genotypes or strains in the Asia Pacific region (42). For instances, the outbreaks in Taiwan in 1998 and 2000 are caused by C2 and B4 strains, respectively (95), and by

sentinel surveillance in Sarawak, Malaysia, the emergence of a new subgenotype C1 is the cause of 2003 epidemic (15).

There were several small outbreaks or sporadic HFMD cases outside the Asia-Pacific region. In The Netherlands, there are only 58 cases of EV-A71 infection requiring hospitalization were reported in 2007 during 21 year period of very low endemicity by surveillance (96). In the United Kingdom, there were also only 32 patients, 01 death in 2003 with EV-A71 infection accompanied by neurological complications and/or cutaneous manifestations during that 1998 to 2006 period (97). 20 children with EV-A71 were admitted to a tertiary hospital in Canada in 1998, but no symptoms were severe and all improved rapidly (98). Similarly, in 2003 and 2005 in Denver, CO, USA, 16 children aged 4 weeks to 9 years get EV-A71 infection; one child died (99). A longitudinal study from Norway (2001- 2003), indicated asymptomatic circulation of EV71. Prevalence of EV-A71 in stool samples showed that EV-A71was circulating widely. But no clinical cases during this same period (100). In Nairobi, Kenya, two small institutional outbreaks of EV-A71 infection were reported in an HIV orphanage in 1999 and 2000 (101).

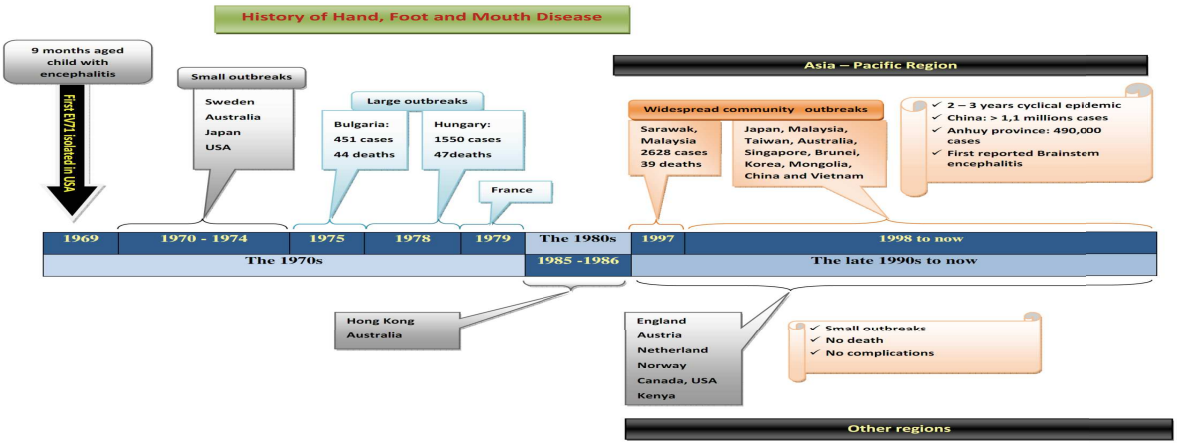

Figure 10: History of Hand, Foot and Mouth Disease (1969 – 2011)

Year	Location	Genotype of EV-A71 and other co-circulating viruses
1997	Peninsular and Sarawak, Malaysia	EV-A71 (B3, B4, C1, C2); CV-A (16, 2, 4, 6, 9); CV-B5; EV (1,4,5,7)
1998	Taiwan	EV-A71 (C2); CV-A16; CV-B (1, 2, 3, 5); EV(6, 7, 11, 22, 27).
1999	Australia	EV-A71 (C2).
2000	Australia	EV-A71 (B4).
2000	Singapore	EV-A71 (B4); CV-A(16, 3, 4, 5, 6, 10, 23); HEV-18.
2000	Taiwan	EV-A71 (B4), CV(A16, A9, A24); CV-B (1, 3, 4); EV (4, 9).
2001	Taiwan	EV-A71 (B4); CV-A(16, 6, 9, 24); CV-B(4, 5); EV (4, 6).
2000	Sarawak, Malaysia	EV-A71 (B4); CV-A16.
2000	Peninsular Malaysia	EV-A71 (C1, B4)
2003	Sarawak, Malaysia	EV-A71 (B4, B5, C1); CV-A16.
2005	Peninsular, Malaysia	EV-A71 (B5, C1)
2006	Singapore	EV-A71 (B5); CV-A16
2008, 2009	China	EV-A71 (C4)

Table 6: Recent outbreaks of HFMD due to in the Asia Pacific region (102).

5.2. Sources, Transmission and Susceptibility of HFMD

Sources and transmission modes

Human beings now are the only known natural hosts of EV-A71. This pathogen can replicates in both the intestinal tract and the upper respiratory tract. It is typically shed for from 2 to 12 weeks and two weeks post-infection, respectively (57). So disease transmission can be faecal-oral and respiratory secretions route by direct person-to-person

contact, droplets or fomites. Indeed, there are evidences of intra-familial transmission of EV-A71 which can be up to 84% among family member contact in Taiwan and attendance at childcare settings was significant risk factors associated with EV-A71 infection (103), (70). For more factors related personal hygiene which associated to getting disease also suggested that the faeco-oral route is probably important (104). It thought that in developing countries, hygiene maybe an important role for disease transmission by the faeco-oral route. In contrast for developed countries with much better sanitation, respiratory transmission may become more significant. Further, faeco-oral transmission may contribute more to an endemic disease in the community and the respiratory tract may contribute more to the epidemic spread of the viruses during outbreak situations.

Susceptibility

Almost of HFMD cases occur in preschool-aged children, especially less than 3 years old group, boys more than girls (105). A seroepidemiology study in Singapore shows that EV71 infection outside preschool years is rare. This finding is supported by the following observations: 1) the small proportion of children <2 years old who were seropositive; 2) the proportion of seropositive children reached a steady state after 5 years of age; and 3) The geometric mean titer of anti-HEV71 antibody declined with age (106). In addition, seroprevalance study among children in South Vietnam in 2007 shows the EV71 neutralizing antibody seroconversion rate was 5.6% in the first year and 14% in the second year of life. In children 5–15 yrs of age, seroprevalence of EV71 neutralizing antibodies was 84% and in cord blood it was 55% (25). Studies in Taiwan showed that the age-specific seroprevalence are correlated with the incidence of severe disease and mortality rates (70).

5.3. HFMD in Vietnam

Vietnam is located in South Eastern Asia and shares the border with South China Sea, China, Laos, and Cambodia. The climate is tropical in south; monsoonal in north with 4 seasons are spring, summer, autumn and winter. Although EV-A71 was isolated for the first time in Vietnam in 2003, the first outbreak of HFMD was not reported until 2005. The 2005 outbreak in the southern was associated with EV-A71 C1, C4 and C5 subgenogroups and CV-A16 (22), (23). For the southern part of the country, in the periods of 2007 - 2009,

the numbers of reported cases and deaths were 5,719 and 23; 10,958 and 25, and 10,632 and 23, respectively. In contrast, there were a few sporadic HFMD cases in the northern. In 2005 - 2007 periods, seven cases were identified. In 2008, 88 cases were reported from 13/28 provinces. No severe or fatal cases were reported.

Since 2011, Vietnam have experienced continuosly large outbreaks of HFMD and the disease became a notifiable one in the national communicable disease surveillance system. According to data of Viet Nam Ministry of Health (MoH) from 2011 to 2015, number of reported cases and deaths were 113,121 and 170 (2011), 157,391 and 45 (2012), 78,818 and 23 (2013), 77,296 and 9 (2014), 56,329 and 5 (2015), respectively which have been reported from across all 63/63 provinces. HFMD outbreaks have continuously occurred nationwide. Responding to HFMD outbreaks, MoH issued two specific guidelines applied for whole country. The first one published on the February 24, 2012 concerned surveillance, prevention and control of HFMD. The second guideline was issued on March 30, 2012 were about diagnosis and treatment.

This study (22), shows information as most of the cases (96.2%) were less than five years of age. 173 specimens (42.1%) were identified as EV-A71, and 214 (52.1%) as CV-A16. Of those patients with EV-A71 infections, 51(29.3%) were complicated by acute neurological disease and three (1.7%) were fatal. EV-A71 was isolated throughout the year but peak of occurrence is from October to November. Phylogenetic analysis of 23 EV-A71 isolates showed that during the first half of 2005, viruses belonging to 3 subgenogroups, C1, C4, and a previously undescribed subgenogroup, C5, cocirculated in southern Vietnam. In the second half of the year, viruses belonging to subgenogroup C5 predominated during a period of higher EV-A71 activity. Another study (25), pointed out seroprevalance study among children the EV-A71 neutralizing antibody seroconversion rate was 5.6% in the first year and 14% in the second year of life. In children 5–15 yrs of age, seroprevalence of EV-A71 neutralizing antibodies was 84% and in cord blood it was 55%. The first peak of the disease seemed to be in April and September for the second one (Figure 11). During those outbreaks, almost of the cases are under 5 years old children, severe cases and deaths was identified that involving the central nervous system complication and/ or pulmonary oedema and most of them are under 3 years old (24).

Gender of the HFMD cases seem trend to boys than girls. The etiology are both CV and EV-A71. Moreover, some EV-A71 subgenotypes can co-circulate in the same epidemic, as well as other non-EV-A71 enteroviruses, such as Coxsackievirus-A. 39.7% positive with EV-A71. EV-A71 infection is primary cause of the death by HFMD (76%).

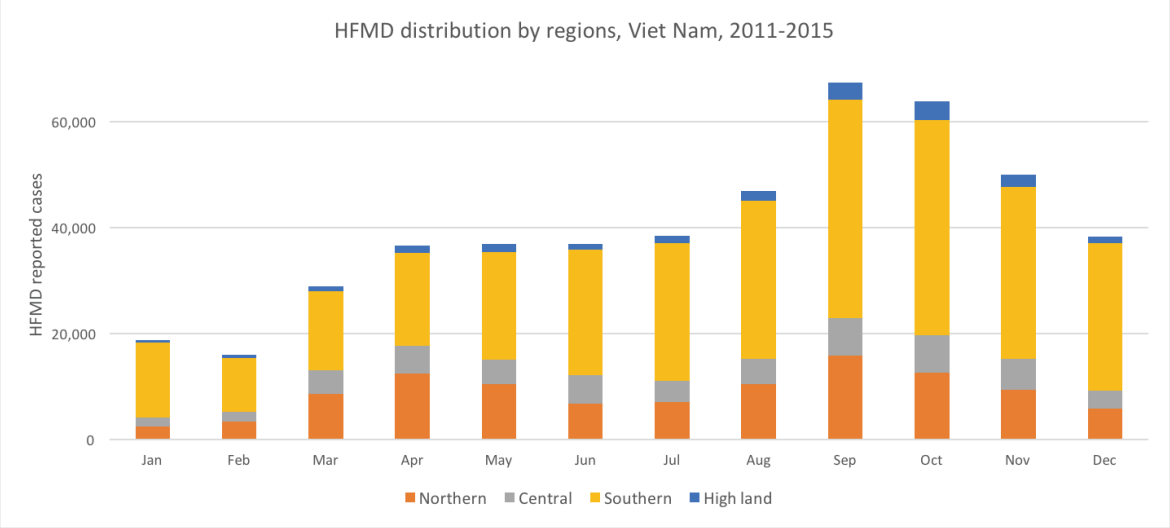

Figure 11: Monthly distribution of HFMD in Vietnam, 2011-2015

6. Prevention and control measures of HFMD

6.1. Options for prevention and control

So far, the public health approaches are only measures for disease prevention and control with the aim is to interrupt the chain of virus transmission. In addition, by the heightened surveillance activities, the outbreaks will be detected early and especially early recognition and intervention of severe cases are very important to minimize the impact of the disease. There are many measures can be implemented to effectively reduce transmission but the key is a combination of interventions. The main measures (80), currently being taken to manage HFMD outbreaks include:

1. Establishing and strengthening surveillance;
2. Conducting information and education campaigns on good hygiene and basic sanitation;
3. Providing assistance to kindergartens, day-care facilities and schools during outbreaks

4. Strengthening infection-control measures in both health care facilities and the community;
5. Improving clinical case-management services, particularly for severe manifestations requiring intensive medical care;
6. Exchanging information and disseminating best practices related to the preparedness, response and management of HFMD, particularly during outbreaks;
7. Providing an administrative framework to national agencies/bodies to implement prevention and control options, including the:
   - Delegation of powers to act to key ministries, including through supportive legislation;
   - Development of mechanisms to establish and support interagency/intersectoral collaboration; and
   - Strengthening of coordinated risk communication.
8. Monitoring and evaluation.

At the individual level, the risk of infection can be lowered by following good hygiene practices which include:

1. Washing hands frequently and correctly and especially after changing diapers and after using the toilet;
2. Cleaning dirty surfaces and soiled items, including toys, first with soap and water and then disinfecting them by cleansing with a solution of chlorine bleach (made by adding 1 tablespoon of bleach to 4 cups of water); and
3. Avoiding close contact (kissing, hugging, sharing eating utensils or cups, etc.) with persons with HFMD.

6.2. Vaccines

For communicable disease, especially without antiviral therapies, vaccine is always powerful in term of prevention and disease control. To date, No available vaccines against EV71 exist but there are several ones are currently under development in the preclinical stage. In which, there still have been some limitations need to improve before application. Moreover, the cost and availability of the vaccine plays very important role in term of the

vaccine widely dissemination in poor Asian countries. It needs to be concerned as the cheap, easily produced, and readily available vaccine.

Inactivated virus vaccine

There are several evidences of the inactivated virus vaccine for the effective control of EV71 (75), (107). In which formalin-inactivated and heat inactivated virus vaccine was used to immunize adult mice. As the result neonatal mice were protected from EV-A71 deathly infection and also for suckling mice it can prolong the survival. In the past, an EV-A71 strain named YN3-4a was developed in laboratory which shows the advantage on rapid growth rate in Vero cells with a larger plaque size and a lower lethal dose 50 in newborn mice. It also induced to produce mouse antiserum that can neutralize a wide range of EV-A71 strains circulating around the word (108). This strain has potential for development as an inactivated vaccine strain.

Attenuated strain vaccine

The EV71 (S1-3') strain with mutations in the 5'UTR, 3D polymerase and 3'UTR 5'non-translated same as the attenuation determinants of poliovirus have been developed as candidate strain for attenuated vaccine (109), (110), has attenuated neurovirulence and limited spread features. In experiment on cynomolgus monkeys with EV-A71 (S1-3'), after lethal challenge with the prototype virulent strain EV-A71 (BrCr-TR), these monkeys survived without severe symptoms and their sera can neutralized against many different genotypes of EV-A71 (111). But it still causes mild neurological symptoms when inoculated via the intravenous route and the genetic stability need to concern of the attenuated strain before application.

Subunit vaccine (recombinant vaccine)

For enteroviruses, the VP1, VP2, and VP3 capsid protein are responsible for the antigenic diversity, especially in the VP1 there is cluster of neutralization epitopes which become potentially the focused area of antiviral subunit vaccine (112). Indeed, 2001, an experiment have showed that the VP1 protein with a complete adjuvant is able to induce a neutralizing antibody response, enhance T helper cell proliferation, and induce high levels of interleukin (IL)-10 and interferon (IFN)-g in mice. These findings provide the

possibility of developing subunit vaccines against EV71 with direct evidence that the VP1 protein contains neutralizing epitopes independent of other viral capsid proteins (107).

DNA vaccine

There are two experiments (107), (113), of development an EV-A71 DNA vaccine in mice. The results showed that the neutralization of the sera of mice immunized with the VP1 DNA vaccine was much lower when compare to effects of EV-A71-infected human serum and the anti-VP1 IgG level declined after boosting immunization. Indeed, strategies to increase the immune stimulation ability of DNA vaccines need to be concerned because the DNA vaccine contains fewer antigenic epitopes and it induces a weaker immune stimulation than the whole virus particles.

Virus-like particle (VLP) vaccine

According to (114), the VLP immunization of mother mice conferred protection to neonatal mice against the lethal viral challenge inducing both Th1 and Th2 immune responses. Their VPLs is assembled from VP1, VP3, and VP0 capsid protein through the CD3 protein by using a recombinant baculovirus expression system. They also found that the denatured VLPs elicited significantly lower levels of neutralizing antibodies and conferred lower protection level against virus challenge when compared to the intact VLPs (115). These findings provide evidence for preserving the conformation-dependent epitopes in preventing EV-A71 infection.

Moreover, by optimizing the process parameters, the resultant VLPs not only resembled the VLPs produced from Bac-P1-3CD infection in density, size and shape, but also induced potent antibody responses in mouse models. The antibodies neutralized EV-A71 strains of homologous and heterologous genogroups, implicating the potential of the VLPs to confer cross-protection for the prevention of future epidemics. Summary, Bac-P1-C3CD and the bioprocess render mass production more economical obviate the need for cell lyses and hold promise for future industrial vaccine production (116).

The results of this work are summarized in the article entitled “Emergence of EV-A71 infection in the Asia Pacific region”. This article submitted to “Asian Pacific Journal of Tropical Medicine” is presented thereafter.

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Emergence of EV-A71 infection in the Asia Pacific region

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Running title: Emerging HFMD in the Asia Pacific region.

Key words: HFMD, Enterovirus, EV-A71, Asia Pacific region

Abstract

EV-A71 was first isolated in 1969 in California, USA. Since the late 1990s, many country members of the Asia Pacific region have experienced large widespread HFMD outbreaks including Malaysia, Taiwan, Japan, Korea, Singapore, China, Thailand, Vietnam or Cambodia, with hundred thousand HFMD cases and deaths. For the first time, brainstem

encephalitis was observed. EV-A71 strain frequently causes severe HFMD as neurological forms and death in large epidemics while mild symptoms are usually associated with other serotypes. Moreover, the co-circulation of different serotypes and alternation between EV-A71 and Coxsackievirus (CV-A) has been commonly observed during HFMD epidemics. HFMD epidemics could be due to both the accumulation of susceptible individuals in the community and introduction of new genotypes or strains in the Asia Pacific region. HFMD due to EV-A71 has become a major public health concern in the Asia Pacific region, which has been a hotspot for outbreaks with a 2–3 year period and show two picks around April and October. No available vaccines against EV-A71 exist but there are several currently under development. Public health approaches are therefore the best measures for disease prevention and control with the aim to interrupt the chain of virus transmission.

1. Introduction

Enterovirus A71 (EV-A71) belong to Enterovirus genus in the family Picornaviridae. It is first reported in 1969 in California, USA. Since, EV71has emerged and caused hand, foot and mouth disease outbreaks worldwide especially across Asia Pacific region. In which, the EV-A71 was frequently linked to severe neurological complication leading to death in large outbreaks while mild forms were usually associated other serotypes. EV-A71 is classified into three independent genogroups: A, B, and C. The EV-A71 B and C genogroups are each further subdivided into five genotypes, B1 to B5 and C1 to C5 [1]. Recently decades the disease mostly has been reported in the Asia Pacific region with unclear explanation for the reason. In this Review we discuss epidemiology, etiology, genetics, evolution and pathogenicity and prospects for control.

2. Epidemiology

EV-A71 strain of enterovirus was first isolated in 1969 in California, USA, from a 9-month child diagnosed with encephalitis [2]. Since then many HFMD outbreaks of differing magnitude have been reported throughout the world [3], (Figure 1).

In the early 1970s, several countries in different continent including Sweden, Australia, USA and Japan had reported small HFMD outbreaks with some tens of cases for most of which clinical aspects typical to HFMD and sometimes aseptic meningitis [4], [5], [6], [7]. HFMD was then reported in Europe in Bulgaria in 1975 (451 cases and 44 deaths), in 1978 (1550 cases and 47 deaths) and in France in 1979 [8], [9], [10]. In the 1980s small outbreaks were reported in Hong Kong, Australia and USA [11], [12], [13]. Since the late 1990s, many countries in the Asia-Pacific region experienced large HFMD outbreaks. In 1997 two large widespread community outbreaks occurred in Sarawak, Malaysia and in

Taiwan, with 2628 and 129,106 cases reported, respectively [14], [15]. Following these two initial episodes, a series of small and large HFMD outbreaks happen throughout the region with Japan, Australia, China, Malaysia, Singapore, Taiwan, Korea, Mongolia, Vietnam, Brunei as epidemic hotspots with cycles of 2–3 years period [16], [17], [18]. The most recent large outbreak in the Asia-Pacific region occurred in the province of Anhui in China in 2008 with around 490,000 cases and 126 deaths. The case-fatality rate was around 0.03% but in certain local outbreaks, such as in the city of Fuyang, this rate was up to 0.3% [19], [20].

3. Etiology

The explosive emergence of the disease in the region might have been related to the association between HLA-A33 (which display a higher prevalence in Asian populations than white populations) and susceptibility to Enterovirus A71 infection [21]. During those outbreaks, almost all cases were children under 5 and although clinical manifestations were mostly typical of HFMD, a cluster of deaths among young children was identified. Moreover, cases involving central nervous system complications and/ or pulmonary oedema have also been observed for the first time [22], [23]. The causative agents of HFMD are Enterovirus A which include Coxsackievirus A (CV-A) and Enterovirus A71 (EV-A71). In the Asia-Pacific region, the EV-A71 serotype was frequently linked to severe neurological forms leading to death in large epidemics while mild symptoms were usually associated with the other serotype [24][25]. The co-circulation of different serotypes and alternation between EV-A71 and CV-A were commonly observed during HFMD epidemics [26], [27], [28], [19]. However, this co-infection was not a primary cause of severe forms [29], [23], (Table 1). Epidemics could be due to both the accumulation of

susceptible individuals in the community and introduction of new genotypes or strains into the Asia-Pacific region [30]. For instances, the outbreaks in Taiwan in 1998 and 2000 were caused by EV-A71 C2 and B4 strains, respectively [31]. Sentinel surveillance in Sarawak, Malaysia, demonstrated that the emergence of the novel subgenotype C1 of EV-A71 was the cause of the 2003 outbreak [16].

Outside the Asia-Pacific region, several EV-A71 small outbreaks or sporadic HFMD cases were reported. In The Netherlands, only 58 cases of EV-A71 infection requiring hospitalization were reported, in 2007, over a 21-year period of very low endemicity [32]. In UK, only 32 EV-A71 patients, including 1 death in 2003, displaying neurological complications and/or cutaneous manifestations were reported during the 1998 to 2006 period [33]. 20 children infected with EV-A71 but with no severe symptoms were admitted to a tertiary hospital in Canada in 1998 and all improved rapidly [34]. Similarly, in 2003 and 2005 in Denver, CO, USA, a total of 16 children from 1 month to 9 years old were infected resulting in one death [35]. A longitudinal study conducted in Norway from 2001 to 2003, indicated a wide asymptomatic circulation of EV-A7 but with no clinical cases during this same period [36]. In Nairobi, Kenya, two small institutional outbreaks of EV-A71 infection were reported in an HIV orphanage in 1999 and 2000.[37].

4. Transmission

Human beings are currently the only known natural hosts of EV-A71. This virus can replicate in both the intestinal tract and the upper respiratory tract. It is typically shed for from 2 to 12 weeks and two weeks post-infection, respectively. [38]. EV-A71 can therefore be transmitted through faecal-oral and respiratory secretions route by direct person-to-person contact, droplets or fomites. Indeed, there is evidence of intra-familial transmission

of EV-A71 which can be up to 84% among family members while attendance at childcare settings was another significant risk factor associated with HEV-71 infection [39], [40]. The faeco-oral route was however considered the most important one [41] in particular in developing countries where hygiene-related factors may play a more important role in the transmission. In contrast, in developed countries with higher sanitation, respiratory transmission may become more significant. Faeco-oral transmission may contribute more to an endemic disease in the community while the respiratory route may contribute more to the epidemic spread of viruses during outbreak situations. Almost of HFMD cases occur in preschool-aged children, especially below 3 years old with boys being more infected than girls [42]. A seroepidemiology study conducted in Singapore showed that EV-A71 infection outside preschool years was rare. The proportion of seropositive children below 2 was very small and reached a steady state after 5 while the geometric mean titer of anti-HEV-A71 antibody declined with age [43]. In addition, seroprevalance studies among children in southern Vietnam in 2007 demonstrated that the EV-A71 neutralizing antibody seroconversion rate was 5.6% in the first year and 14% in the second year of life [44]. In children of 5 to 15 years old, seroprevalence of EV-A71 neutralizing antibodies was 84% while it was 55% in cord blood [44]. Age-specific seroprevalence was also correlated with the incidence of severe forms and mortality rates [40].

Most outbreaks showed two picks of occurrence around April and October with a sharp decrease of the total number of cases during dry periods in January and July. These fluctuations were observed in Taiwan [45], China [46], [47], Singapore [48] and Malaysia [23]. The reasons for this cyclic variation is still unclear but a study conducted in Singapore [49] showed that the risk of HFMD incidence significantly increased with short term variability of weekly temperature difference (above 7°C), maximum temperature

(above 32°C), and moderate weekly cumulative rainfall (below 75 mm) at time lag of 1 to 2 weeks. Higher air temperature and humidity as well as lower rainfall and duration of daylight increased incidence of HFDM [50] [51]. Epidemiological surveillance and prevention efforts should thus be focused at the end of the dry period to anticipate large outbreaks.

Several kinds of clinical manifestation have been described which correspond to Hand, foot and mouth disease (HFMD) or Herpangina (HA), a usually self-limiting and benign illness. However, in a small number of cases, neurological and systemic complication could develop and lead to death. HFMD is a brief febrile illness in children with skin rash which typically is papulovesicular at the palms or soles of the feet, or both. These symptoms can be accompanied or not mouth ulcers. Usually, in younger children and infants the rash is maculopapular without vesicles, and may also involve the buttocks, knees or elbows. HA is also characterized by fever and typically with multiple, painful mouth ulcers mainly at the posterior oral cavity (anterior pharyngeal folds, uvula, tonsils and soft palate). In some cases, the mouth ulcers also affect other parts of the mouth such as the buccal mucosa and tongue. Neurological and systemic complications usually correspond to aseptic meningitis, acute flaccid paralysis and encephalitis. In fatal cases, there is first a short period of febrile illness with subtle neurological signs followed then by an acute refractory myocardial dysfunction, fulminant pulmonary oedema ithin hours of developing tachycardia, poor peripheral perfusion and tachypnea [52]. Brainstem encephalitis was also observed as a typical sign EV-A71 infection and was frequently associated with severe cardiorespiratory symptoms attributed to neurogenic pulmonary oedema [22], [23], [23], [3]. This specific clinical syndrome begins usually 3–5 days after onset and and is accompanied by a sudden deterioration such as shock and pulmonary

oedema or haemorrhages resulting from an acute and rapidly progressing cardiorespiratory failure. Finally, children die before reaching hospital or in the first day of admission, despite intensive care support [14], [22]. Clinical manifestations of HFMD vary widely from asymptomatic, mild to neurologic cases. But central neurological system infection is the feature most frequently seen in EV-A71 infection [53]. Uncomplicated HFMD, is suspected, as shown on a mouse model, to infect skin or oral mucosa [54]. However, information on human skin or oral mucosa biopsies is still lacking.

Neurologic cases are not associated with inflammatory in cerebella cortex, basal ganglia, thalamus, peripheral nerve or autonomic ganglia. In contrast, the grey matter of the spinal cord and the whole medulla oblongata are predominantly affected along with the hypothalamus, subthalamic and dentate nuclei. The motor cortex is also affected but to a lower extend. However, virus, viral antigen or RNA have not been seen in the brainstem except in some very rare cases in neuronal processes and phagocytic cells [22], [55]. It is supposed that explosive pulmonary oedema is associated with neurogenic mechanisms secondary to brainstem inflammation, cardiac dysfunction, increased vascular permeability, and cytokine storm but the exact mechanism is still unclear and the pathogenesis is not completely understood [3]. In-vivo models, including mice and non-human primates, have replicated some of the features of severe EV-A71 disease, such as neuroinvasion with inflammatory changes, but none has yet been able to reproduce the severe systemic features, such as pulmonary oedema [54], [56], [57].

5. Etiology

Human enteroviruses have been classified into four species, HEV-A, HEV-B, HEV-C, and HEV-D. HFMD is caused by several genotypes of Enterovirus A species which

include some Coxsackievirus A (CV-A) and Enterovirus A71 (EV-A71) [48]. EV-A71 viruses are genetically related to CV-A; indeed, it has been suggested that these viruses may have diverged as recently as the 1940s [1]. EV-A71 isolates are classified on the basis of the VP1 gene sequence into six independent genogroups: A, B, C, D, E and F. The EV-A71 B and C genogroups are each further subdivided into subgenogroups, B0 to B5 and C1 to C5 [58]. CV-As are subdivided into serotypes CV-A2-8, CV-A10, CV-A12, CV-A14, CV-A16. Both EV-A71 and CV-A infections have been associated with severe HFMD in young children, sometimes resulting in death while other genotypes are usually associated with milder forms of the disease [59], [25], [47], [24].

Group A comprises only one member, the prototype BrCr strain of EV-A71, which was first isolated in California, USA, in 1969. BrCr secondly isolates were reported in a HFMD epidemic among children in Anhui province of central China [60]. Surveillance for HFMD by the Chinese Center for Disease Control and Prevention did not indicate the presence of group A viruses in China [20].

Group B viruses were first separated into B1 and B2 subgenogroups with 12% divergence at the nucleotide level. These subgenogroups were the predominant circulating strains in the 1970s and 1980s. In details, B1 was circulating in USA, Europe, Japan and Australia in 1970s and B2 viruses were mainly isolated in USA in the 1980s. The subgenogroup B3 emerged in 1997 in Sarawak, Malaysia in relation with several large outbreaks in the Asia-Pacific region. This subgenogroup was also found in Singapore [1], [61], [62]. Subgenogroup B4 was circulating and associated with the large outbreaks through the Asia-Pacific region in the 1990s but was also sampled in 2000. In 2003, subgenogroup B5 replaced subgenogroup B4 in Sarawak, Malaysia and became the most

important group circulating in Japan, Malaysia, Singapore and Taiwan [30], [63], [64], [16], [65], (Figure 2).

Group C viruses were initially separated into the C1 and C2 subgroups. Subgenogroup C1 viruses were first reported in USA and Australia in the 1970s, 1980s. Since the initial detection of the group, these viruses have been continuously isolated in many countries throughout Europe and the Asia-Pacific region. However, they have not caused any large epidemics since the major community outbreak in Sydney in 1986 [66], [64]. Subgenogroups C2, C4, and C5 emerged within genogroup C in the Asia-Pacific region in the late 1990s. They caused fatal HFMD cases in Taiwan [15], China [67], and Vietnam [26]. Subgenogroup C3 was isolated in Japan in 1994, and in Korea in 2000 [61], [68], [69]. According to [65], subgenogroups C1, C2, and C4 are present worldwide while the C3 and C5 lineages are restricted to Korea and Vietnam, respectively, (Figure 3).

6. Genetics, Evolution and Pathogenicity

Recombination of EV-A71 has been observed most frequently in the 5'UTR and 3'UTR regions. This rarely happens in the structural protein gene region [70], [71], [72]. So far, the effect of EV-A71 recombination on the virulence or transmissibility is unknown. But some studies observe the replacement of the 3' half of the genome of a non-recombinant EV-A71 field isolate with a HEV-B species virus which improves growth in cell culture compared with a non-recombinant strain [73]. The reservoir of EV-A71 is human and replication happens both in the intestinal tract and upper respiratory tract. The virion typically shed from 2 to 4 weeks and especially up to 12 weeks after infection. The transmission mode is through direct person-to-person contact, droplets or fomites by faecal-oral and respiratory secretions [74].

Although there is still no clear genetic evidence of virus-virulence factor, data from HFMD outbreaks suggest that the difference of genogroup may contribute to virulence aspect. Subgenogroup C2 from the epidemic in Taiwan (1998) was almost exclusively isolated from children with the severe neurological form. In contrast, subgroup B3 viruses from the 1997 outbreak in Sarawak, Malaysia, were isolated mainly from children with mild HFMD [62], [64]. As per today, there is still a lack of information about the relation between viral load and illness severity. In the case of EV-A71, infection with explosive pulmonary oedema, there is a storm of cytokines and chemokines in cerebrospinal fluid and serum such as interleukin (IL)-6, IL-1beta, IL-10, IL-8, interferon induced protein (IP-10) and monokine induced by interferon gamma (MIG) [75], [76], [77]. In contrast, the number of T lymphocytes and NK cells is lower [78]. HLA-A33, which is a common phenotype in Asian populations, but rare in white populations, was most significantly associated with enterovirus 71 infection and HLA-A2 was significantly related to cardiopulmonary failure [21], Receptors on the host target cell were identified as the scavenger receptor B2 [79], and the P-selectin glycoprotein ligand-1 [80], which are associated with leukocytes.

7. Prevention and control measures of HFM

Current public health approaches are only measures for disease prevention and control with the aim OF interrupting the chain of virus transmission. In addition, by the heightened surveillance activities, the outbreaks will be detected early and especially early recognition and intervention of severe cases are very important to minimize the impact of the disease. There are many measures can be implemented to effectively reduce transmission but the

key is a combination of interventions. The main measures [52], currently being taken to manage HFMD outbreaks include: Establishing and strengthening surveillance; Conducting information and education campaigns on good hygiene and basic sanitation; Providing assistance to kindergartens, day-care facilities and schools during outbreaks; Strengthening infection-control measures in both health care facilities and the community; Improving clinical case-management services, particularly for severe manifestations requiring intensive medical care; Exchanging information and disseminating best practices related to the preparedness, response and management of HFMD, particularly during outbreaks; Providing an administrative framework to national agencies/bodies to implement prevention and control options; Monitoring and evaluation.

At the individual level, the risk of infection can be lowered by following good hygiene practices which include: Washing hands frequently and correctly and especially after changing diapers and after using the toilet; Cleaning dirty surfaces and soiled items, including toys, first with soap and water and then disinfecting them by cleansing with a solution of chlorine bleach (made by adding 1 tablespoon of bleach to 4 cups of water); and Avoiding close contact (kissing, hugging, sharing eating utensils or cups, etc.) with persons with HFMD.

8. Vaccines

For communicable disease, especially without antiviral therapies, vaccine is always powerful in term of prevention and disease control. To date, no vaccine against EV-A71 exists but there are several ones are currently under development in the preclinical stage. In which, there still have been some limitations need to improve before application. Moreover, the cost and availability of the vaccine plays very important role in term of the

vaccine widely dissemination in poor Asian countries. It needs to be concerned as the cheap, easily produced, and readily available vaccine.

8.1 Inactivated virus vaccine

There are several evidences of the inactivated virus vaccine for the effective control of EV-A71 [81], [82]. In which formalin-inactivated and heat inactivated virus vaccine was used to immunize adult mice. As the result neonatal mice were protected from EV-A71 deathly infection and also for suckling mice it can prolong the survival. In the past, an EV-A71 strain named YN3-4a was developed in laboratory which shows the advantage on rapid growth rate in Vero cells with a larger plaque size and a lower lethal dose 50 in newborn mice. It also induced to produce mouse antiserum that can neutralize a wide range of EV-A71 strains circulating around the word [83]. This strain has potential for development as an inactivated vaccine strain.

8.2 Attenuated vaccine

The EV-A71 (S1-3') strain with mutations in the 5'UTR, 3D polymerase (3Dpol) and 3'UTR 5'non-translated same as the attenuation determinants of poliovirus have been developed as a candidate strain for an attenuated vaccine [84], [85]. It has attenuated neurovirulence and limited spread features. In experiment on *Cynomolgus* monkeys with EV-A71 (S1-3'), after lethal challenge with the prototype virulent strain EV-A71 (BrCr-TR), these monkeys survived without severe symptoms and their sera could neutralize many different genotypes of EV-A71 [86]. But it still causes mild neurological symptoms when inoculated via the intravenous route and the genetic stability need to concern of the attenuated strain before application.

8.3.Subunit vaccine (recombinant vaccine)

Enteroviruses VP1, VP2, and VP3 capsid proteins are responsible for the antigenic diversity, especially in the VP1 there is cluster of neutralization epitopes which become potentially the focused area of antiviral subunit vaccine [87]. Indeed, 2001, an experiment have showed that the VP1 protein with a complete adjuvant is able to induce a neutralizing antibody response, enhance T helper cell proliferation, and induce high levels of interleukin (IL)-10 and interferon (IFN)-g in mice. These findings provide the possibility of developing subunit vaccines against EV-A71 with direct evidence that the VP1 protein contains neutralizing epitopes independent of other viral capsid proteins [82].

8.4. DNA vaccine

Two experiments [82, 88] of development an EV-A71 DNA vaccine in mice were conducted. Results showed that the neutralization of the sera of mice immunized with the VP1 DNA vaccine was much lower when compare to effects of EV-A71-infected human serum and the anti-VP1 IgG level declined after boosting immunization. Indeed, strategies to increase the immune stimulation ability of DNA vaccines need to be concerned because the DNA vaccine contains fewer antigenic epitopes and it induces a weaker immune stimulation than the whole virus particles.

8.5. Virus-like particle (VLP) vaccine

According to [89], the VLP immunization of mother mice conferred protection to neonatal mice against the lethal viral challenge inducing both Th1 and Th2 immune responses. Their VPLs is assembled from VP1, VP3, and VP0 capsid protein through the CD3 protein by using a recombinant baculovirus expression system. They also found that the denatured VLPs elicited significantly lower levels of neutralizing antibodies and conferred lower protection level against virus challenge when compared to the intact VLPs [90]. These findings provide evidence for preserving the conformation-dependent epitopes

in preventing EV-A71 infection. Moreover, by optimizing the process parameters, the resultant VLPs not only resembled the VLPs produced from Bac-P1-3CD infection in density, size and shape, but also induced potent antibody responses in mouse models. The antibodies neutralized EV-A71 strains of homologous and heterologous genogroups, implicating the potential of the VLPs to confer cross-protection for the prevention of future epidemics. Summary, Bac-P1-C3CD and the bioprocess render mass production more economical obviate the need for cell lyses and hold promise for future industrial vaccine production [91].

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10. References

[1] B. A. Brown, M. S. Oberste, J. P. Alexander Jr, M. L. Kennett, and M. A. Pallansch, "Molecular epidemiology and evolution of enterovirus 71 strains isolated from 1970 to 1998," *J. Virol.*, vol. 73, no. 12, p. 9969, 1999.

[2] N. J. Schmidt, E. H. Lennette, and H. H. Ho, "An apparently new enterovirus isolated from patients with disease of the central nervous system," *J. Infect. Dis.*, vol. 129, no. 3, p. 304, 1974.

[3] T. Solomon, P. Lewthwaite, D. Perera, M. J. Cardosa, P. McMinn, and M. H. Ooi, "Virology, epidemiology, pathogenesis, and control of enterovirus 71," *Lancet Infect. Dis.*, vol. 10, no. 11, pp. 778–790, 2010.

[4] D. R, F. TD, and S. V, "Central nervous system infections in New York State. Etiologic and epidemiologic observations, 1974.," *N. Y. State J. Med.*, vol. 75, no. 13, p. 2337.

[5] A. Hagiwara, I. Tagaya, and T. Yoneyama, "Epidemic of hand, foot and mouth disease associated with enterovirus 71 infection," *Intervirology*, vol. 9, no. 1, pp. 60–63, 1978.

[6] J. Blomberg, E. Lycke, K. Ahlfors, T. Johnsson, S. Wolontis, G. Von Zeipel, and others, "Letter: New enterovirus type associated with epidemic of aseptic meningitis and-or hand, foot, and mouth disease.," *Lancet*, vol. 2, no. 7872, p. 112, 1974.

[7] M. L. Kennett, C. J. Birch, F. A. Lewis, A. P. Yung, S. A. Locarnini, and I. D. Gust, "Enterovirus type 71 infection in Melbourne," *Bull. World Health Organ.*, vol. 51, no. 6, pp. 609–615, 1974.

[8] M. Chumakov, M. Voroshilova, L. Shindarov, I. Lavrova, L. Gracheva, G. Koroleva, S. Vasilenko, I. Brodvarova, M. Nikolova, S. Gyurova, and others, "Enterovirus 71 isolated from cases of epidemic poliomyelitis-like disease in Bulgaria," *Arch. Virol.*, vol. 60, no. 3, pp. 329–340, 1979.

[9] G. Nagy, S. Takátsy, E. Kukán, I. Mihály, and I. Dömök, "Virological diagnosis of enterovirus type 71 infections: experiences gained during an epidemic of acute CNS diseases in Hungary in 1978," *Arch. Virol.*, vol. 71, no. 3, pp. 217–227, 1982.

[10] WHO weekly epidemiology report, "World Health Organization: Enterovirus type 71 surveillance. Weekly Epidemiol Rec 1979;54:219." 1979.

[11] G. M. SAMUDA, W. A. I. K. CHANG, C. Y. YEUNG, and P. A. K. S. TANG, "Monoplegia caused by enterovirus 71: an outbreak in Hong Kong," *Pediatr. Infect. Dis. J.*, vol. 6, no. 2, p. 206, 1987.

[12] G. L. GILBERT, K. E. DICKSON, M. J. O. WATERS, M. L. KENNETT, S. A. LAND, and M. SNEDDON, "Outbreak of enterovirus 71 infection in Victoria, Australia, with a high incidence of neurologic involvement," *Pediatr. Infect. Dis. J.*, vol. 7, no. 7, p. 484, 1988.

[13] J. C. Hayward, S. M. Gillespie, K. M. Kaplan, R. Packer, M. Pallansch, S. Plotkin, L. B. Schonberger, and others, "Outbreak of poliomyelitis-like paralysis associated with enterovirus 71.," *Pediatr. Infect. Dis. J.*, vol. 8, no. 9, p. 611, 1989.

[14] L. Chan, U. D. Parashar, M. Lye, F. Ong, S. R. Zaki, J. P. Alexander, K. Ho, L. L. Han, M. A. Pallansch, A. B. Suleiman, and others, "Deaths of children during an outbreak of hand, foot, and mouth disease in Sarawak, Malaysia: clinical and pathological characteristics of the disease," *Clin. Infect. Dis.*, vol. 31, no. 3, p. 678, 2000.

[15] M. Ho, E. R. Chen, K. H. Hsu, S. J. Twu, K. T. Chen, S. F. Tsai, J. R. Wang, and S. R. Shih, "An epidemic of enterovirus 71 infection in Taiwan," *N. Engl. J. Med.*, vol. 341, no. 13, pp. 929–935, 1999.

[16] Y. Podin, E. L. M. Gias, F. Ong, Y.-W. Leong, S.-F. Yee, M. A. Yusof, D. Perera, B. Teo, T.-Y. Wee, S.-C. Yao, S.-K. Yao, A. Kiyu, M. T. Arif, and M. J. Cardosa, "Sentinel surveillance for human enterovirus 71 in Sarawak, Malaysia: lessons from the first 7 years," *BMC Public Health*, vol. 6, p. 180, 2006.

[17] K. Mizuta, C. Abiko, T. Murata, Y. Matsuzaki, T. Itagaki, K. Sanjoh, M. Sakamoto, S. Hongo, S. Murayama, and K. Hayasaka, "Frequent importation of enterovirus 71 from surrounding countries into the local community of Yamagata, Japan, between 1998 and 2003," *J. Clin. Microbiol.*, vol. 43, no. 12, p. 6171, 2005.

[18] M. Hosoya, Y. Kawasaki, M. Sato, K. Honzumi, A. Kato, T. Hiroshima, H. Ishiko, and H. Suzuki, "Genetic diversity of enterovirus 71 associated with hand, foot and mouth disease epidemics in Japan from 1983 to 2003," *Pediatr. Infect. Dis. J.*, vol. 25, no. 8, p. 691, 2006.

[19] F. Yang, L. Ren, Z. Xiong, J. Li, Y. Xiao, R. Zhao, Y. He, G. Bu, S. Zhou, J. Wang, and others, "Enterovirus 71 outbreak in the People's Republic of China in 2008," *J. Clin. Microbiol.*, vol. 47, no. 7, p. 2351, 2009.

[20] Y. Zhang, Z. Zhu, W. Yang, J. Ren, X. Tan, Y. Wang, N. Mao, S. Xu, S. Zhu, A. Cui, and others, "An emerging recombinant human enterovirus 71 responsible for the 2008 outbreak of Hand Foot and Mouth Disease in Fuyang city of China," *Virol. J.*, vol. 7, no. 1, p. 94, 2010.

[21] L. Y. Chang, I. Chang, W. J. Chen, Y. C. Huang, G. W. Chen, S. R. Shih, J. L. Juang, H. M. Shih, C. A. Hsiung, T. Y. Lin, and others, "HLA-A33 is associated with susceptibility to enterovirus 71 infection," *Pediatrics*, vol. 122, no. 6, p. 1271, 2008.

[22] C. C. Huang, C. C. Liu, Y. C. Chang, C. Y. Chen, S. T. Wang, and T. F. Yeh, "Neurologic complications in children with enterovirus 71 infection," *N. Engl. J. Med.*, vol. 341, no. 13, pp. 936–942, 1999.

[23] M. H. Ooi, S. C. Wong, Y. Podin, W. Akin, S. del Sel, A. Mohan, C. H. Chieng, D. Perera, D. Clear, D. Wong, and others, "Human enterovirus 71 disease in Sarawak, Malaysia: a prospective clinical, virological, and molecular epidemiological study," *Clin. Infect. Dis.*, vol. 44, no. 5, p. 646, 2007.

[24] M. H. Ooi, T. Solomon, Y. Podin, A. Mohan, W. Akin, M. A. Yusuf, S. Del Sel, K. M. Kontol, B. F. Lai, D. Clear, and others, "Evaluation of different clinical sample types in the diagnosis of human enterovirus 71 associated hand-foot-and-mouth disease," *J. Clin. Microbiol.*, p. JCM–01394, 2007.

[25] M. H. Ooi, S. C. Wong, P. Lewthwaite, M. J. Cardosa, and T. Solomon, "Clinical features, diagnosis, and management of enterovirus 71," *Lancet Neurol.*, vol. 9, no. 11, pp. 1097–1105, 2010.

[26] P. V. Tu, N. T. T. Thao, D. Perera, T. K. Huu, N. T. K. Tien, T. C. Thuong, O. M. How, M. J. Cardosa, and P. C. McMinn, "Epidemiologic and virologic investigation of hand, foot, and mouth disease, southern Vietnam, 2005," *Emerg. Infect. Dis.*, vol. 13, no. 11, pp. 1733–1741, Nov. 2007.

[27] T. H. Khanh, S. Sabanathan, T. T. Thanh, L. P. K. Thoa, T. C. Thuong, V. thi T. Hang, J. Farrar, T. T. Hien, N. van V. Chau, and H. R. van Doorn, "Enterovirus 71-associated hand, foot, and mouth disease, Southern Vietnam, 2011," *Emerg. Infect. Dis.*, vol. 18, no. 12, pp. 2002–2005, Dec. 2012.

[28] C. Xiang, "Detection of human enterovirus 71 and coxsackievirus A16 in children with hand, foot and mouth disease in China," *Mol. Med. Rep.*, Jan. 2012.

[29] K. P. Chan, K. T. Goh, C. Y. Chong, E. S. Teo, G. Lau, and A. E. Ling, "Epidemic hand, foot and mouth disease caused by human enterovirus 71, Singapore," *Emerg. Infect. Dis.*, vol. 9, no. 1, p. 78, 2003.

[30] H. Shimizu, A. Utama, N. Onnimala, C. Li, Z. Li-Bi, M. Yu-Jie, Y. Pongsuwanna, and T. Miyamura, "Molecular epidemiology of enterovirus 71 infection in the Western Pacific Region," *Pediatr. Int. Off. J. Jpn. Pediatr. Soc.*, vol. 46, no. 2, pp. 231–235, 2004.

[31] J. R. Wang, Y. C. Tuan, H. P. Tsai, J. J. Yan, C. C. Liu, and I. J. Su, "Change of major genotype of enterovirus 71 in outbreaks of hand-foot-and-mouth disease in Taiwan between 1998 and 2000," *J. Clin. Microbiol.*, vol. 40, no. 1, p. 10, 2002.

[32] S. Van Der Sanden, M. Koopmans, G. Uslu, H. Van Der Avoort, and others, "Epidemiology of enterovirus 71 in the Netherlands, 1963 to 2008," *J. Clin. Microbiol.*, vol. 47, no. 9, p. 2826, 2009.

[33] J. M. Bible, M. Iturriza-Gomara, B. Megson, D. Brown, P. Pantelidis, P. Earl, J. Bendig, and C. Tong, "Molecular epidemiology of human enterovirus 71 in the United Kingdom from 1998 to 2006," *J. Clin. Microbiol.*, vol. 46, no. 10, p. 3192, 2008.

[34] L. Merovitz, A. M. Demers, D. Newby, and J. McDonald, "Enterovirus 71 infections at a Canadian center," *Pediatr. Infect. Dis. J.*, vol. 19, no. 8, p. 755, 2000.

[35] C. M. Pérez-Vélez, M. S. Anderson, C. C. Robinson, E. J. McFarland, W. A. Nix, M. A. Pallansch, M. S. Oberste, and M. P. Glodé, "Outbreak of neurologic enterovirus type 71 disease: a diagnostic challenge," *Clin. Infect. Dis.*, vol. 45, no. 8, p. 950, 2007.

[36] E. Witso, G. Palacios, K. S. Ronningen, O. Cinek, D. Janowitz, M. Rewers, B. Grinde, and W. I. Lipkin, "Asymptomatic circulation of HEV71 in Norway," *Virus Res.*, vol. 123, no. 1, pp. 19–29, 2007.

[37] R. Chakraborty, M. Iturriza-Gómara, R. Musoke, T. Palakudy, A. D'Agostino, and J. Gray, "An epidemic of enterovirus 71 infection among HIV-1-infected orphans in Nairobi," *Aids*, vol. 18, no. 14, p. 1968, 2004.

[38] P. W. Chung, Y. C. Huang, L. Y. Chang, T. Y. Lin, H. C. Ning, and others, "Duration of enterovirus shedding in stool," *J. Microbiol. Immunol. Infect.*, vol. 34, no. 3, pp. 167–170, 2001.

[39] L. Y. Chang, K. C. Tsao, S. H. Hsia, S. R. Shih, C. G. Huang, W. K. Chan, K. H. Hsu, T. Y. Fang, Y. C. Huang, and T. Y. Lin, "Transmission and clinical features of enterovirus 71 infections in household contacts in Taiwan," *JAMA J. Am. Med. Assoc.*, vol. 291, no. 2, p. 222, 2004.

[40] L. Y. Chang, C. C. King, K. H. Hsu, H. C. Ning, K. C. Tsao, C. C. Li, Y. C. Huang, S. R. Shih, S. T. Chiou, P. Y. Chen, and others, "Risk factors of enterovirus 71 infection and associated hand, foot, and mouth disease/herpangina in children during an epidemic in Taiwan," *Pediatrics*, vol. 109, no. 6, p. e88, 2002.

[41] M. Kuramitsu, C. Kuroiwa, H. Yoshida, M. Miyoshi, J. Okumura, H. Shimizu, L. Narantuya, and D. Bat-Ochir, "Non-polio enterovirus isolation among families in Ulaanbaatar and Tov province, Mongolia: prevalence, intrafamilial spread, and risk factors for infection," *Epidemiol. Infect.*, vol. 133, no. 6, pp. 1131–1142, 2005.

[42] Q. Shao, G. Lou, and J. Yan, "[The clinical epidemiology characteristics of HFMD in 2010 of Hangzhou]," *Zhonghua Shi Yan He Lin Chuang Bing Xue Za Zhi Zhonghua Shiyan He Linchuang Bingduxue Zazhi Chin. J. Exp. Clin. Virol.*, vol. 25, no. 4, pp. 289–291, Aug. 2011.

[43] E. E. Ooi, M. C. Phoon, B. Ishak, and S. H. Chan, "Seroepidemiology of human enterovirus 71, Singapore," *Emerg. Infect. Dis.*, vol. 8, no. 9, p. 995, 2002.

[44] C. B. N. Tran, H. T. Nguyen, H. T. T. Phan, N. Van Tran, B. Wills, J. Farrar, J. D. Santangelo, and C. P. Simmons, "The seroprevalence and seroincidence of enterovirus71 infection in infants and children in Ho Chi Minh City, Viet Nam," *PLoS One*, vol. 6, no. 7, p. e21116, 2011.

[45] K.-T. Chen, H.-L. Chang, S.-T. Wang, Y.-T. Cheng, and J.-Y. Yang, "Epidemiologic features of hand-foot-mouth disease and herpangina caused by enterovirus 71 in Taiwan, 1998–2005," *Pediatrics*, vol. 120, no. 2, pp. e244–e252, 2007.

[46] H. Ni, B. Yi, J. Yin, T. Fang, T. He, Y. Du, J. Wang, H. Zhang, L. Xie, and Y. Ding, "Epidemiological and etiological characteristics of hand, foot, and mouth disease in Ningbo, China, 2008–2011," *J. Clin. Virol.*, 2012.

[47] M. Zeng, Y. F. Li, X. H. Wang, G. P. Lu, H. G. Shen, H. Yu, and Q. R. Zhu, "Epidemiology of hand, foot, and mouth disease in children in Shanghai 2007–2010," *Epidemiol. Infect.*, vol. 140, no. 6, p. 1122, 2012.

[48] L. W. Ang, B. K. Koh, K. P. Chan, L. T. Chua, L. James, and K. T. Goh, "Epidemiology and control of hand, foot and mouth disease in Singapore," *Ann Acad Med Singap.*, vol. 38, pp. 106–112, 2009.

[49] Y. L. Hii, J. Rocklöv, and N. Ng, "Short term effects of weather on hand, foot and mouth disease," *PLoS One*, vol. 6, no. 2, p. e16796, 2011.

[50] M. Urashima, N. Shindo, and N. Okabe, "Seasonal models of herpangina and hand-foot-mouth disease to simulate annual fluctuations in urban warming in Tokyo," *Jpn. J. Infect. Dis.*, vol. 56, no. 2, pp. 48–53, 2003.

[51] D. Onozuka and M. Hashizume, "The influence of temperature and humidity on the incidence of hand, foot, and mouth disease in Japan," *Sci. Total Environ.*, vol. 410–411, pp. 119–125, Dec. 2011.

[52] WHO WPRO, "A Guide to Clinical Management and Public Health Response for Hand, Foot and Mouth Disease (HFMD)," *WHO WPRO*, 2011. [Online]. Available: http://webcache.googleusercontent.com/search?q=cache:Dtg4fDp6fqcJ:www.wpro.who.int/publications/PUB_9789290615255.htm+A+guide+to+clinical+management+and+public+health+response+for+Hand,+Foot+nad+Mouth+Disease&cd=2&hl=en&ct=clnk&gl=fr&client=firefox-a.

[53] S. Li-mei, Z. Huan-ying, Z. Hui-zhen, G. Xue, H. Jian-feng, G. Da-wei, K. Min, L. Zheng, K. Chang-wen, L. Jian-sen, and others, "An enterovirus 71 epidemic in Guangdong Province of China, 2008: epidemiological, clinical, and virogenic manifestations," *Jpn J Infect Dis*, vol. 64, no. 1, pp. 13–18, 2011.

[54] Y. C. Chen, C. K. Yu, Y. F. Wang, C. C. Liu, I. J. Su, and H. Y. Lei, "A murine oral enterovirus 71 infection model with central nervous system involvement," *J. Gen. Virol.*, vol. 85, no. 1, p. 69, 2004.

[55] K. T. Wong, B. Munisamy, K. C. Ong, H. Kojima, N. Noriyo, K. B. Chua, B. B. Ong, and K. Nagashima, "The distribution of inflammation and virus in human enterovirus 71 encephalomyelitis suggests possible viral spread by neural pathways," *J. Neuropathol. Exp. Neurol.*, vol. 67, no. 2, pp. 162–169, Feb. 2008.

[56] Y. F. Wang, C. T. Chou, H. Y. Lei, C. C. Liu, S. M. Wang, J. J. Yan, I. J. Su, J. R. Wang, T. M. Yeh, S. H. Chen, and others, "A mouse-adapted enterovirus 71 strain causes neurological disease in mice after oral infection," *J. Virol.*, vol. 78, no. 15, p. 7916, 2004.

[57] M. Arita, H. Shimizu, N. Nagata, Y. Ami, Y. Suzaki, T. Sata, T. Iwasaki, and T. Miyamura, "Temperature-sensitive mutants of enterovirus 71 show attenuation in cynomolgus monkeys," *J. Gen. Virol.*, vol. 86, no. 5, p. 1391, 2005.

[58] M. Bessaud, R. Razafindratsimandresy, A. Nougairède, M.-L. Joffret, J. M. Deshpande, A. Dubot-Pérès, J.-M. Héraud, X. de Lamballerie, F. Delpeyroux, and J.-L. Bailly, "Molecular comparison and evolutionary analyses of VP1 nucleotide sequences of new African human enterovirus 71 isolates reveal a wide genetic diversity," *PloS One*, vol. 9, no. 3, p. e90624, 2014.

[59] S. AbuBakar, H.-Y. Chee, M. F. Al-Kobaisi, J. Xiaoshan, K. Bing Chua, and S. Kit Lam, "Identification of enterovirus 71 isolates from an outbreak of hand, foot and mouth disease (HFMD) with fatal cases of encephalomyelitis in Malaysia," *Virus Res.*, vol. 61, no. 1, pp. 1–9, May 1999.

[60] H. Yu, W. Chen, H. Chang, R. Tang, J. Zhao, L. Gan, B. Liu, J. Chen, and M. Wang, "Genetic analysis of the VP1 region of enterovirus 71 reveals the emergence of genotype A in central China in 2008," *Virus Genes*, vol. 41, no. 1, pp. 1–4, 2010.

[61] M. J. Cardosa, D. Perera, B. A. Brown, D. Cheon, H. M. Chan, K. P. Chan, H. Cho, and P. McMinn, "Molecular epidemiology of human enterovirus 71 strains and recent outbreaks in the Asia-Pacific region: comparative analysis of the VP1 and VP4 genes," *Emerg. Infect. Dis.*, vol. 9, no. 4, pp. 461–468, Apr. 2003.

[62] P. McMinn, K. Lindsay, D. Perera, H. M. Chan, K. P. Chan, and M. J. Cardosa, "Phylogenetic analysis of enterovirus 71 strains isolated during linked epidemics in Malaysia, Singapore, and Western Australia," *J. Virol.*, vol. 75, no. 16, pp. 7732–7738, Aug. 2001.

[63] S.-W. Huang, Y.-W. Hsu, D. J. Smith, D. Kiang, H.-P. Tsai, K.-H. Lin, S.-M. Wang, C.-C. Liu, I.-J. Su, and J.-R. Wang, "Reemergence of enterovirus 71 in 2008 in taiwan: dynamics of genetic and antigenic evolution from 1998 to 2008," *J. Clin. Microbiol.*, vol. 47, no. 11, pp. 3653–3662, Nov. 2009.

[64] S. Sanders, L. Herrero, K. McPhie, S. Chow, M. Craig, D. Dwyer, W. Rawlinson, and P. McMinn, "Molecular epidemiology of enterovirus 71 over two decades in an Australian urban community," *Arch. Virol.*, vol. 151, no. 5, pp. 1003–1013, 2006.

[65] K. K. Tee, T. T. Y. Lam, Y. F. Chan, J. M. Bible, A. Kamarulzaman, C. Tong, Y. Takebe, and O. G. Pybus, "Evolutionary genetics of human enterovirus 71: origin, population dynamics, natural selection, and seasonal periodicity of the VP1 gene," *J. Virol.*, vol. 84, no. 7, p. 3339, 2010.

[66] J. P. Alexander, L. Baden, M. A. Pallansch, and L. J. Anderson, "Enterovirus 71 infections and neurologic disease—United States, 1977–1991," *J. Infect. Dis.*, vol. 169, no. 4, p. 905, 1994.

[67] "Outbreak news. Enterovirus, China," *Relevé Épidémiologique Hebd. Sect. Hygiène Secrétariat Société Nations Wkly. Epidemiol. Rec. Health Sect. Secr. Leag. Nations*, vol. 83, no. 19, pp. 169–170, May 2008.

[68] Y. Jee, D. S. Cheon, K. Kim, J. Cho, Y. S. Chung, J. Lee, S. Lee, K. Park, J. H. Lee, E. C. Kim, and others, "Genetic analysis of the VP1 region of human enterovirus 71 strains isolated in Korea during 2000," *Arch. Virol.*, vol. 148, no. 9, pp. 1735–1746, 2003.

[69] M. Iwai, A. Masaki, S. Hasegawa, M. Obara, E. Horimoto, K. Nakamura, Y. Tanaka, K. Endo, K. Tanaka, J. Ueda, and others, "Genetic changes of coxsackievirus A16 and enterovirus 71 isolated from hand, foot, and mouth disease patients in Toyama, Japan between 1981 and 2007," *Jpn J Infect Dis*, vol. 62, no. 4, pp. 254–259, 2009.

[70] X. Chen, Q. Zhang, J. Li, W. Cao, J.-X. Zhang, L. Zhang, W. Zhang, Z.-J. Shao, and Y. Yan, "Analysis of recombination and natural selection in human enterovirus 71," *Virology*, vol. 398, no. 2, pp. 251–261, Mar. 2010.

[71] S.-C. Huang, Y.-W. Hsu, H.-C. Wang, S.-W. Huang, D. Kiang, H.-P. Tsai, S.-M. Wang, C.-C. Liu, K.-H. Lin, I.-J. Su, and J.-R. Wang, "Appearance of intratypic recombination of enterovirus 71 in Taiwan from 2002 to 2005," *Virus Res.*, vol. 131, no. 2, pp. 250–259, Feb. 2008.

[72] C. Yoke-Fun and S. AbuBakar, "Phylogenetic evidence for inter-typic recombination in the emergence of human enterovirus 71 subgenotypes," *BMC Microbiol.*, vol. 6, no. 1, p. 74, 2006.

[73] P. Phuektes, "Development of a reverse genetic system for Human enterovirus 71 (HEV71) and the molecular basis of its growth phenotype and adaptation to mice," 2009.

[74] E.-E. Ooi, M.-C. Phoon, B. Ishak, and S.-H. Chan, "Seroepidemiology of human enterovirus 71, Singapore," *Emerg. Infect. Dis.*, vol. 8, no. 9, pp. 995–997, Sep. 2002.

[75] T. Lin, L. Chang, Y. Huang, K. Hsu, C. Chiu, K. Yang, and others, "Different proinflammatory reactions in fatal and non-fatal enterovirus 71 infections: implications for early recognition and therapy," *Acta Paediatr.*, vol. 91, no. 6, pp. 632–635, 2002.

[76] S. M. Wang, H. Y. Lei, L. Y. Su, J. M. Wu, C. K. Yu, J. R. Wang, and C. C. Liu, "Cerebrospinal fluid cytokines in enterovirus 71 brain stem encephalitis and echovirus meningitis infections of varying severity," *Clin. Microbiol. Infect.*, vol. 13, no. 7, pp. 677–682, 2007.

[77] S. M. Wang, H. Y. Lei, C. K. Yu, J. R. Wang, I. J. Su, and C. C. Liu, "Acute chemokine response in the blood and cerebrospinal fluid of children with enterovirus 71-associated brainstem encephalitis," *J. Infect. Dis.*, vol. 198, no. 7, p. 1002, 2008.

[78] S. M. Wang, H. Y. Lei, K. J. Huang, J. M. Wu, J. R. Wang, C. K. Yu, I. J. Su, and C. C. Liu, "Pathogenesis of enterovirus 71 brainstem encephalitis in pediatric patients: roles of cytokines and cellular immune activation in patients with pulmonary edema," *J. Infect. Dis.*, vol. 188, no. 4, p. 564, 2003.

[79] S. Yamayoshi, Y. Yamashita, J. Li, N. Hanagata, T. Minowa, T. Takemura, and S. Koike, "Scavenger receptor B2 is a cellular receptor for enterovirus 71," *Nat. Med.*, vol. 15, no. 7, pp. 798–801, 2009.

[80] Y. Nishimura, M. Shimojima, Y. Tano, T. Miyamura, T. Wakita, and H. Shimizu, "Human P-selectin glycoprotein ligand-1 is a functional receptor for enterovirus 71," *Nat. Med.*, vol. 15, no. 7, pp. 794–797, 2009.

[81] C.-K. Yu, C.-C. Chen, C.-L. Chen, J.-R. Wang, C.-C. Liu, J.-J. Yan, and I.-J. Su, "Neutralizing antibody provided protection against enterovirus type 71 lethal challenge in neonatal mice," *J. Biomed. Sci.*, vol. 7, no. 6, pp. 523–528, Nov. 2000.

[82] C.-N. Wu, Y.-C. Lin, C. Fann, N.-S. Liao, S.-R. Shih, and M.-S. Ho, "Protection against lethal enterovirus 71 infection in newborn mice by passive immunization with subunit VP1 vaccines and inactivated virus," *Vaccine*, vol. 20, no. 5–6, pp. 895–904, Dec. 2001.

[83] Y.-C. Lin, C.-N. Wu, S.-R. Shih, and M.-S. Ho, "Characterization of a vero cell-adapted virulent strain of enterovirus 71 suitable for use as a vaccine candidate," *Vaccine*, vol. 20, no. 19–20, pp. 2485–2493, Jun. 2002.

[84] M. Arita, H. Shimizu, N. Nagata, Y. Ami, Y. Suzaki, T. Sata, T. Iwasaki, and T. Miyamura, "Temperature-sensitive mutants of enterovirus 71 show attenuation in cynomolgus monkeys," *J. Gen. Virol.*, vol. 86, no. 5, p. 1391, 2005.

[85] M. Arita, Y. Ami, T. Wakita, and H. Shimizu, "Cooperative effect of the attenuation determinants derived from poliovirus Sabin 1 strain is essential for attenuation of enterovirus 71 in the NOD/SCID mouse infection model," *J. Virol.*, vol. 82, no. 4, p. 1787, 2008.

[86] M. Arita, N. Nagata, N. Iwata, Y. Ami, Y. Suzaki, K. Mizuta, T. Iwasaki, T. Sata, T. Wakita, and H. Shimizu, "An attenuated strain of enterovirus 71 belonging to genotype a showed a broad spectrum of antigenicity with attenuated neurovirulence in cynomolgus monkeys," *J. Virol.*, vol. 81, no. 17, pp. 9386–9395, 2007.

[87] M. Ho, “Enterovirus 71: the virus, its infections and outbreaks,” *J. Microbiol. Immunol. Infect.*, vol. 33, no. 4, pp. 205–216, 2000.

[88] W. S. Tung, S. A. Bakar, Z. Sekawi, and R. Rosli, “DNA vaccine constructs against enterovirus 71 elicit immune response in mice,” *Genet Vaccines Ther*, vol. 5, no. 6, pp. 1–13, 2007.

[89] Y.-C. Chung, J.-H. Huang, C.-W. Lai, H.-C. Sheng, S.-R. Shih, M.-S. Ho, and Y.-C. Hu, “Expression, purification and characterization of enterovirus-71 virus-like particles,” *World J. Gastroenterol. WJG*, vol. 12, no. 6, pp. 921–927, Feb. 2006.

[90] Y. C. Chung, M. S. Ho, J. C. Wu, W. J. Chen, J. H. Huang, S. T. Chou, and Y. C. Hu, “Immunization with virus-like particles of enterovirus 71 elicits potent immune responses and protects mice against lethal challenge,” *Vaccine*, vol. 26, no. 15, pp. 1855–1862, 2008.

[91] C.-Y. Chung, C.-Y. Chen, S.-Y. Lin, Y.-C. Chung, H.-Y. Chiu, W.-K. Chi, Y.-L. Lin, B.-L. Chiang, W.-J. Chen, and Y.-C. Hu, “Enterovirus 71 virus-like particle vaccine: Improved production conditions for enhanced yield,” *Vaccine*, vol. 28, no. 43, pp. 6951–6957, Oct. 2010.

[92] S. Wong, C. Yip, S. Lau, K. Yuen, and others, “Human enterovirus 71 and hand, foot and mouth disease,” *Epidemiol. Infect.*, vol. 138, no. 8, pp. 1071–1089, 2010.

1. Figures and table legends

Figure 1: History of Hand, Foot and Mouth Disease (1969 – 2011)

Figure 2: Genogroup B of Enterovirus 71(EV-A71)

Table 1: Recent outbreaks of HFMD due to in the Asia Pacific region [92]

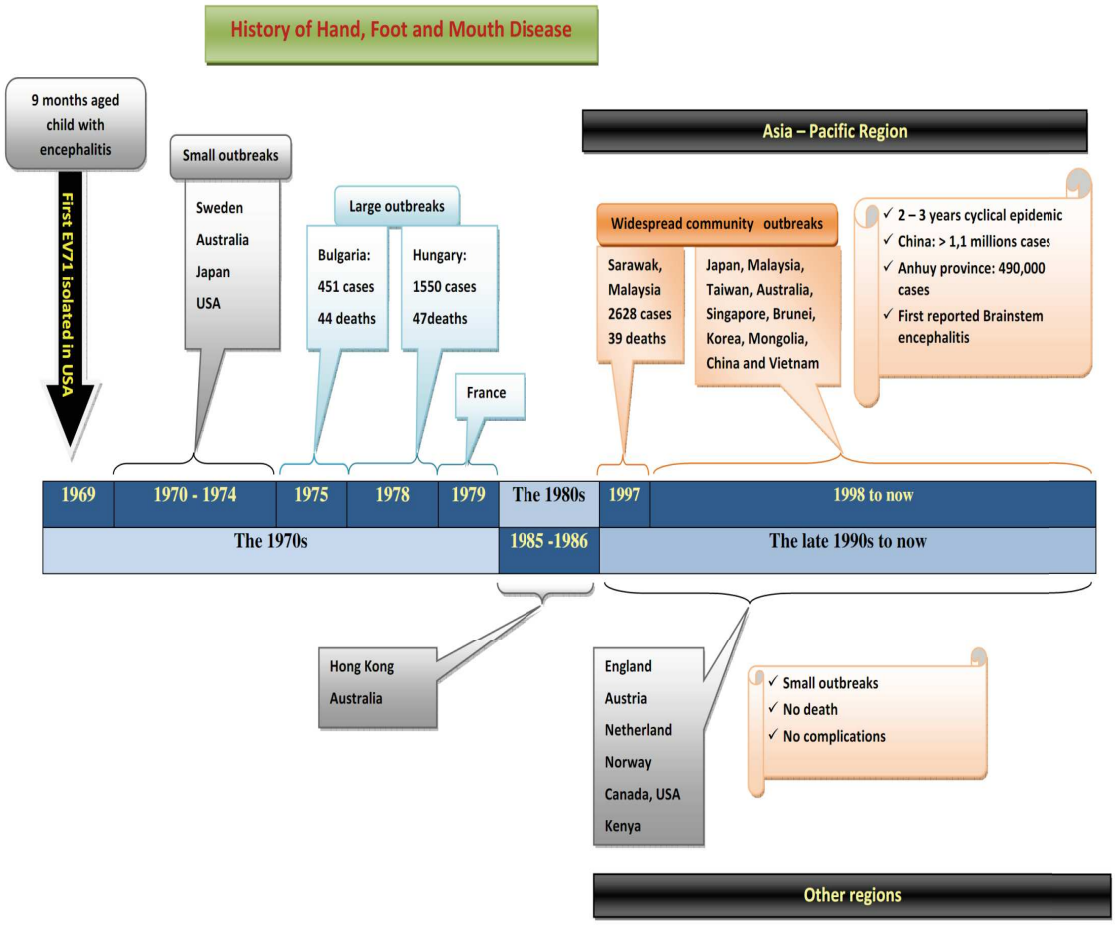

Figure 1: History of Hand, Foot and Mouth Disease (1969 – 2011)

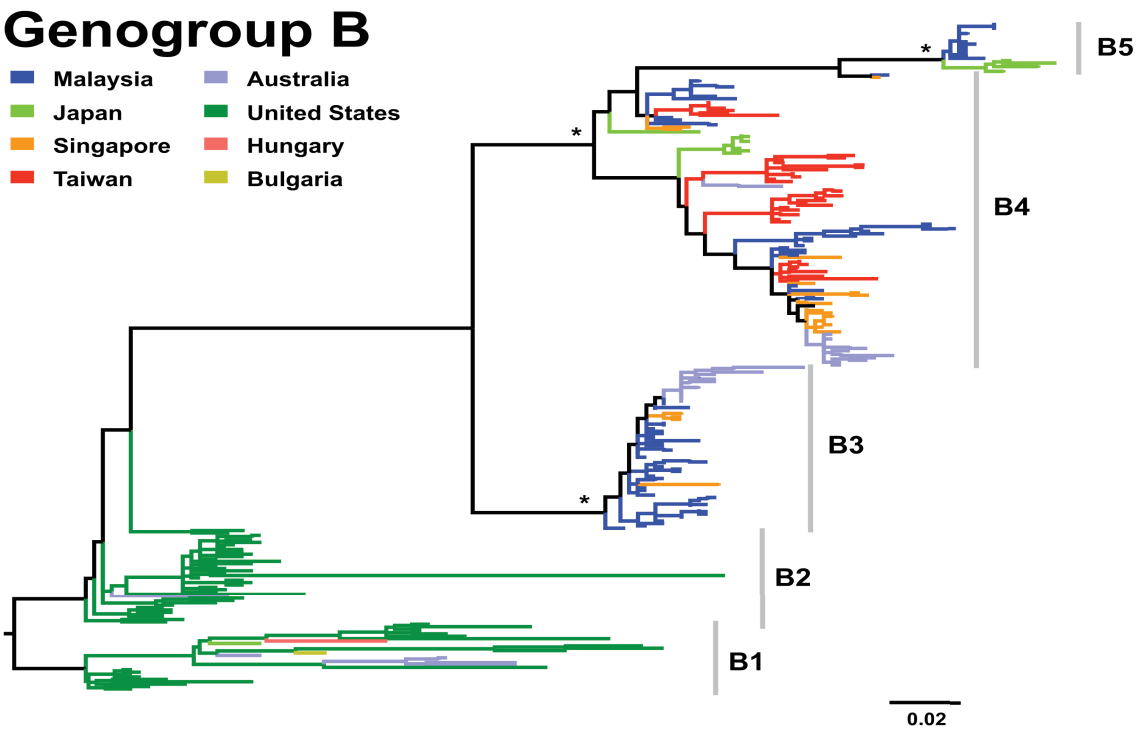

Figure 2: Genogroupe B of Enterovirus 71(EV-A71)

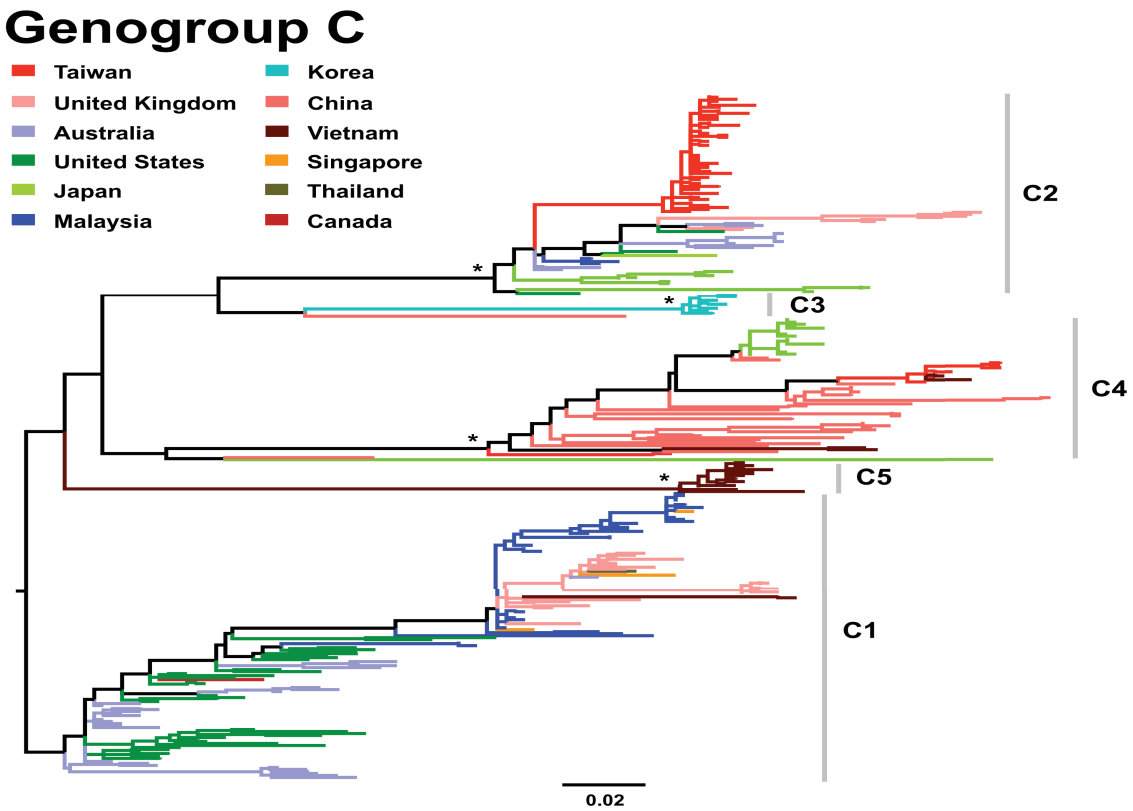

Figure 3: Genogroupe C of Enterovirus 71(EV-A71)

Table 1. Recent outbreaks of HFMD due to in the Asia Pacific region

Year	Location	Genotype of EV-A71 and other co-circulating viruses
1997	Peninsular and Sarawak, Malaysia	EV-A71 (B3, B4, C1, C2); CV-A (16, 2, 4, 6, 9); CV-B5; EV (1,4,5,7)
1998	Taiwan	EV-A71 (C2); CV-A16; CV-B (1, 2, 3, 5); EV (6, 7, 11, 22, 27).
1999	Australia	EV-A71 (C2).
2000	Australia	EV-A71 (B4).
2000	Singapore	EV-A71 (B4); CV-A (16, 3, 4, 5, 6, 10, 23); HEV-18.
2000	Taiwan	EV-A71 (B4), CV (A16, A9, A24); CV-B (1, 3, 4); EV (4, 9).
2001	Taiwan	EV-A71 (B4); CV-A (16, 6, 9, 24); CV-B(4, 5); EV (4, 6).
2000	Sarawak, Malaysia	EV-A71 (B4); CV-A16.
2000	Peninsular Malaysia	EV-A71 (C1, B4)
2003	Sarawak, Malaysia	EV-A71 (B4, B5, C1); CV-A16.
2005	Peninsular, Malaysia	EV-A71 (B5, C1)
2006	Singapore	EV-A71 (B5); CV-A16
2008, 2009	China	EV-A71 (C4)

PART II

DYNAMIC AND RE-EMERGING OF HAND, FOOT AND MOUTH DIEASE IN VIETNAM

CHAPTER 1. EPIDEMIOLOGY AND ETIOLOGY OF HAND, FOOT AND MOUTH DISEASE, VIETNAM

1.1. Context of study

Hai Phong, the biggets seaport city of the Northern, Viet Nam. It is a large urban city with a population estimated at 2 million, encompassing an area of 1,507.57 km² for the seven urban and six countryside districts and one large island.

Figure 12: Location of Hai Phong city, Vietnam.

Hai Phong is also a major economic center. Industry is a key sector including food processing, light industries and heavy industries. Despite its status as a city, around one third of Hai Phong's area are used for agriculture. Hai Phong is also a regional junction with international airport, hydro transportation with major seaport and large river system, many national highways and railway as well. The city features a humid subtropical climate, with hot, humid summers and warm, dry winters.

Before 2011, there were no reported HFMD cases in Hai Phong city. The first large outbreak in Hai Phong city started in 2011 and was followed until the end of year

2012. The first reported case was a 6 years old girl admitted in an urban district hospital in week 16th (17, Apr 2011). Later weeks (weeks 17 - 38), HFMD occurred sporadically in almost all part of the city with low incidence (8 cases per week in average). The number of HFMD cases increased suddenly in middle of September (week 39th), 2011. The incidence peaked at 472 cases per week on early December 2011. The second period of activity of the virus is between February and May 2012. A third peak of illness appears in early October 2012. Then it decreased to 18 cases per week by last week of December 2012. The disease has been occurring continuously until now but with lower burden. So far there is no studies published on the epidemiology of HFMD in Northern Vietnam, just are only few for the southern part of the country. All based on hospital based data with limited number of HFMD cases. These studies mainly observe for basic epidemiology and etiology characteristics. Besides, the relationship between etiological agents and clinical epidemiological behavior, temporal and spatial analysis, evaluation of countermeasures has not been reported. In order to more comprehensive understanding which contribute to prevention and control of HFMD in Vietnam, In this work we analyzed all HFMD cases reported in Hai Phong city by the community and hospitals in 2011 and 2012. Since it was the very first outbreak to occur in Hai Phong city, it was a good model for investigating the dynamic of the disease without interference and potential remains from previous outbreaks or patient immunological adaptation and provide findings related to influence of HFMD guidelines during the outbreak period. This work is also an integrative analysis including spatial analysis and social evolution as well as genetic evolution to describe the dynamic of HFMD in a well delimited area.

1.2. Objective

To describe the epidemiological and etiological characteristics during 2011-2012 outbreak in Hai Phong City, Vietnam. To monitor influence of HFMD new guidelines on patients care during 2011-2012 outbreak in Hai Phong City, Vietnam. To spatial analyse the HFMD dynamic during 2011-2012 outbreak in Hai Phong City, Vietnam.

1.3. Discussion and conclusions

This study analyzed all HFMD cases reported in Hai Phong city by the community and hospitals in 2011 and 2012. The 2011-2012 HFMD epidemic was the largest to have ever occurred in Vietnam and the first recorded in the northern part of the country while Hai Phong city experienced the highest HFMD incidence in North Vietnam. However, no fatal cases were reported in Hai Phong unlike what was observed in South Vietnam (9). Age-specific incidence was the highest in the 1-2 year age group. This would be in agreement with both the persistence of maternally-derived neutralizing antibodies for up to 6 months and the kinetics of seroprevalence of EV-A71 virus neutralizing antibodies which increases with age (15,16). However, this was the very first recorded outbreak of HFMD in northern Vietnam questioning thus the existence of maternally-derived neutralizing antibodies or pre-existing immunity. Children under 3 represented 85.85% of cases. They are in Vietnam traditionally cared for at home by family members. The high HFMD incidence in this population may thus have resulted from contact with adults and older children acting as asymptomatic carriers of the virus (17,18).

The first guideline released related to surveillance, prevention and control and gave clear HFMD case definition, reporting procedure and strategy for collecting clinical samples. The first effect of the guideline release was a significant increase of the severity score. The number of moderate and severe cases admitted to Hai Phong pediatric hospital increased significantly after guidelines publication while the proportion of the mild cases decreased sharply. Another positive effect was the reduced delay between onset and admission after guidelines publication. It decreased during the second period and remained very homogeneous. The most important feature of the second guideline was the decentralization and transfer of responsibility to health care facilities. A more homogeneous spatial distribution of patients visiting pediatric hospitals was visible. Mild cases were treated at the commune level whereas districts were in charge of mild and moderate cases. At the province level, all cases were addressed. All patients recorded as severe went to province hospitals during period 2 while local health facilities hosted patients unable to go to main hospitals. Patients who remained at home only displayed mild symptoms. This positive effect of guidelines is also of an increased awareness and precautious approach from parents and physicians leading to patients being majoritarily

declared with severe symptoms in order to ensure a better treatment and surveillance. This could explained why a higher disease severity scores was observed in CV-A-infected patients (wave 3) than in EV-A71 cases ($p< 0.01$). Awareness led to the modification of guidelines but changes occurred only after publication, suggesting that the legal framework created by the guidelines is needed for implementation eventhough awareness is present. Public and professional awareness are not sufficient for implementing changes. Furthermore, emergence of CV-A (wave 3) during the second period did not lead to variation in severity and time to admission. Conversely, the publication of guidelines during phase 2 led to different patient patterns although the virus was the same. Evolution of clinical patterns should not be considered only in the light of the evolution or replacement of pathogens or host-pathogen interactions but also according to the evolution of behavior and social perception.

During the Hai Phong outbreak, circulation of both EV-A71 and CV-A was recorded, a feature already reported (8,19,20,21,22). EV-A71 virus is considered to be the most frequent cause of severe HFMD disease (10,23,24) although CV-A have been shown to cause severe infections with meningitis (25). An uncharacterized virus might also have circulated during wave 1 and mostly wave 2. The high number of EV non-positive PCR reactions on clinically positive samples suggests that the set of primers used for enterovirus detection might not have been discriminative enough. The ratio of non-positive tests was similar to those previously reported (26,27,28,29). EV-A71 detection with MAS primers should thus be systematically performed on SO primers products and SO222 primer should be redesigned to match with the 5' part of the AN88 primer used for EVs detection. More attention should be therefore paid to the PCR negative patients.

Nguyen et al. (9) have shown the presence of HFMD in provinces west of Hai Phong after the outbreak started in South Vietnam, making the northwestern side of Hai Phong is the most likely route of entry. Despite, the main economical role of Hai Phong no early cases occurred along or at the end of the main highway linking Hai Phong to the rest of the country, indicating that major industrial and export commercial movements are not linked to the dynamic of the disease. Instead the disease seems to have expanded following the eastbound river system to reach densely populated settlements from where it

secondarily expanded through local roads. Disease expansion might thus have followed secondary local commercial routes. This commercial route allows time for the disease to be transmitted and involves a lot of favorable human to human contacts. The presence of early cases in the island and in isolated coastal localities in the southern part of the city also illustrates the role of sea transportation and role of the local trade and occupational activities in the spread of the disease. The southern part may have been affected later due to fragmentation of the territory and isolation of the communes by the complex river system. The early occurrence of the disease in northwestern communes not connected to the main local road might be related to a specific occupational activities. Considering the average age of the patients, around 2, the source of contamination must be sought for within asymptomatic adults being contaminated during their occupational activities and in local and regional movements.

The results of this work are summarized in the article entitled “Monitoring influence of Hand, Foot and Mouth Disease new guidelines on patients care during 2011-2012 outbreak in Hai Phong City, Vietnam”. This article in Press in “Asian Pacific Journal of Public Health” is presented thereafter.

Monitoring influence of Hand, Foot and Mouth Disease new guidelines on patients care during 2011-2012 outbreak in Hai Phong City, Vietnam

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Abstract

In 2011-2012, Northern Vietnam suffered its first large scale hand foot and mouth disease (HFMD) epidemic. Two sets of official guidelines were issued during the outbreak to handle the HFMD crisis. The city of Hai Phong was therefore used as a model to analyze the impact of the guidelines release. 9621 HFMD cases were reported in Hai Phong city from April 2011 to December 2012. Three distinct waves of HFMD occurred. Enterovirus A71, Coxackievirus A16 and an unknown virus were associated to the epidemics. The incidence of the disease was the highest in the 0.5-3 year age group. Two periods, before and after guidelines realease, could be distinguished which were characterized by different patient patterns time to admission and severity changed notably. Guidelines publication improved the system. The main routes of infection were rivers and local secondary roads most likely through local trade and occupational movements of people.

Running title: Monitoring impact of HFMD guidelines in Hai Phong
Key words: HFMD, Enterovirus, EV-A71, CV-A16, Hai Phong, Vietnam

Introduction

Hand, foot and mouth disease (HFMD) is an acute febrile illness in children with a papulovesicular skin rash at the palms or soles of the feet, or both. Presentation can be with or without inclusion of mouth ulcers. HFMD can result in severe complications such as encephalitis, aseptic meningitis, pulmonary edema, myocarditis, and death (1). HFMD is caused by types of Enterovirus A species which includes some Coxsackievirus A (CV-A) and Enterovirus A71 (EV-A71) (2,3). The EV-A71 viruses are genetically related to CV-A, and have diverged as recently as the 1920s (4). Both EV-A71 and CV-A infections have been associated with severe HFMD in young children, sometimes resulting in death (1,5,6,7).

Although EV-A71 was isolated for the first time in Vietnam in 2003, the first outbreak of HFMD was reported in the South in 2005 (8). The 2011 HFMD epidemics which caused fatal cases in South Vietnam (9) was the first one to occur in the North.

Materials and methods.

Epidemiological information and specimen collection. All HFMD cases in Hai Phong city were reported to the National Institute of Hygiene and Epidemiology (NIHE) through the national communicable disease surveillance system since 2011. HFMD patients that were present to health centers or hospitals were diagnosed and classified in 4 severity levels. The evaluation of the disease was performed according to the guidelines specifically published by the Vietnamese Ministry of Health (Supplementary table 1) which are based on but slightly differ from WHO (1) and Taiwanese guidelines (10).

PCR amplification and nucleotide sequencing. Molecular analyses were done on 257 throat swabs collected at the main pediatric hospital in Hai Phong city from HFMD-diagnosed patients from 14 out of the 15 districts. From February 2012 through August 2012, following authority requirements, samples were collected only on patients presenting severe symptoms (severity level 2b up). Enterovirus-positive and EV-A71-positive samples were identified according to Nix et al (11) using *SO*, *AN* and *MAS* primers (12). Samples collected in November 2011, December 2011, March 2012 and from September

2012 to December 2012 were subjected to Sanger sequencing and analyzed with the Enterovirus Genotyping Tool (http://www.rivm.nl/mpf/enterovirus/typingtool).

Statistical analysis and hierarchical classification. Population size was estimated using 2009 census data for comparative analysis (13). Hierarchical classification was used to cluster the clinical data set from 8585 cases (children older than 5 were excluded). Incomplete data (less than 5%) were excluded. Each patient was described by four parameters: age, severity, time between onset of the disease and admission to hospital, and date of onset. Patients were clustered according to the first three parameters (clinical parameters) using an un-weighted average Euclidean distance. The three clinical parameters used for clustering analysis were combined into a scoring formula to generate a parameter called Global score. Scores from 1 to 4 were set for each value. The age of patients (S1) was scored according to the following rule: age $\in$ [0,2]=> S1 = 4, age $\in$ [2,3] => S1 = 3, age $\in$ [3,4]=> S1 = 2 and age $\in$ [4,5] => S1 = 1. The severity score (S2) was kept as established by physicians. The score of the delay of onset to admission (S3) was based on the time lapse between onset of disease and admission of 1 day or less, 2 days, 3 days or 4 days or more. The values for S3 were 2, 4, 3 and 1, respectively. The sum S of all scores was S=S1+S2+S3. All patients displaying the same values for the first three parameters were grouped together into a 'type case' category. A histogram of occurrence over time was computed for all individuals in each type case using the fourth parameter (date of onset). A Mann-Whitney U test was applied to each cluster and for all clinical parameters. Correlations between the distribution of the total number of patients obtained by hierarchical classification and date of occurrence were assessed by a chi-square test. Clusters of median time were tested with a non-parametric Mann-Whitney U test. Type I error was set at 5% for all statistical tests. Hierarchical classification was conducted using MATLAB v5.07. Statistical tests on clinical data were performed using Stata 9.0 for Windows. Mean comparison was implemented by a Student's T-test. A Chi-square test was used for proportion comparison of Hai Phong city population and a one-way ANOVA test was used for the variance analysis.

Bias and Ethics. Training session HFMD cases definition and reporting were organized for the staff of the routine surveillance system to enhance quality and consistency of case

report. This work was conducted following the requirements of the Vietnamese Ministry of Health and under the Law of Communicable Diseases Prevention and Control passed in 2007.

Spatial and landscape analysis. GoogleEarth Pro satellite images were downloaded from January 14 to January 20, 2015 (initial satellite data from May 2014). LandSat CNS/Astrum images were used as source data for roads and main river networks. Multispectral images with a 30-meter resolution were obtained from GlobeLand30 operated by the National Geomatics Center of China (14). Initial data were produced in 2010 with an update in 2014. Spatial analyses were conducted with Quantum GIS, version 2.6.1. Brighton. All spatial data are in the WGS 84 coordinate system. Six classes of land cover out of ten possible were found in Hai Phong city: cultivated land, forest, grassland, wetland, water bodies and artificial aera (settlement area). Figures were done with CorelDRAW Graphics Suite X5. The period of occurrence of the index case is shown as a cartogram.

Results

Monitoring of the HFMD burden during the 2011-2012 epidemic. The large HFMD epidemic of 2011-2012 was the first such outbreak to occur in northern Vietnam (65,039 cases). However, the number of cases was higher in the Southern part where epidemic HFMD has been observed since 2005 (157,975 cases). Hai Phong was the hardest hit among the 28 northern Vietnam provinces during the 2011-2012 HFMD epidemic with an average prevalence of 524/100,000 persons. A total of 9621 cases were collected during this period from health centers and the main pediatric hospital of Hai Phong city. The city of Hai Phong is composed of 7 urban districts, 6 countryside districts and 1 large island. HFMD cases were reported throughout the entirety of the city (Supplementary Table 2) and the epidemic was slightly delayed in 2011 when compared to the rest of northern Vietnam (Figures 1a and 1b). The HFMD epidemic could be subdivided into three separate waves of infection: the first one stretching from August 2011 to January 2012 (Wave 1), the second from February 2012 to July 2012 (Wave 2) and the third one from August 2012 to January 2013 (Wave 3). Before the first wave started HFMD occurred sporadically in all

parts of the city with low incidence (8 cases per week on average). The number of cases increased suddenly in mid-September, 2011. The outbreak peaked at 472 cases per week on early December 2011 followed by two smaller peaks in April and October of 2012 (Figure 1b). Two periods, corresponding to different epidemiological patterns, could be distinguished: from August 2011 to March 2102 and from March 2012 to January 2013. The limit between the two periods is marked by the publication of two specific guidelines by the Ministry of Health (MoH). The first one published on the February 24, 2012 concerned surveillance, prevention and control of HFMD. The second guideline was issued on March 30, 2012 were about diagnosis and treatment. The evaluation process of the disease burden was therefore changed during wave 2. Moderate forms (severity level 2a) were reported for the majority of cases (5262 cases, 54.92%), but 218 patients were encountering severe symptoms (2.28%). Among this group, only 9 patients had severity score of 3 and no case with the highest level of 4 (Supplementary Table 2). Gender was not associated with severity. Moderate forms of HFMD were particularly pronounced in children below 2 years old ($p< 0.01$, Supplementary Table 4). The level of moderate cases was significantly lower during wave 1 ($p< 0.01$, Supplementary Table 5 and 6). The level of moderate cases was significantly higher during the second period. Conversely, the level of mild cases decreased notably to significantly lower after the first period of the epidemic (Figure 1b), ($p< 0.01$, Supplementary Table 7). The age of patients ranged from 24 days to 15 years (median at 2 years, IQR of 2 years, Supplementary Table 2). Out of 9142 cases, 8857 (96.9%) were under the age of 5 with the age-specific incidence highest in the 1-2 year age group (3067 cases, 33.6%) and remained very low for older children. The lowest incidence was observed in infants <5 months (1.88%) and children above 10 years old (0.4%). Boys had a significantly higher prevalence rate (59.74%). Wave 1 was associated to an increase of the number of children between 2 and 5. Variations in patient age after guideline release were noticeable (Figure 1c). The proportion of cases below 2 was significantly higher to the end of second period ($p<0.01$, Supplementary Table 7) and during wave 3 ($p<0.01$, Supplementary Table 3). The time after onset to admission was the third epidemiological parameter followed in the present study. It varied greatly over the first period (Figure 1d, Supplementary Table 6). The curves for time to admission after onset to admission corresponding to 1-day and 2-days crossed in March 2012 (Figure 1d) concomitantly with those representing mild and moderate level of severity (Figure 1b).

INSERT FIGURE 1

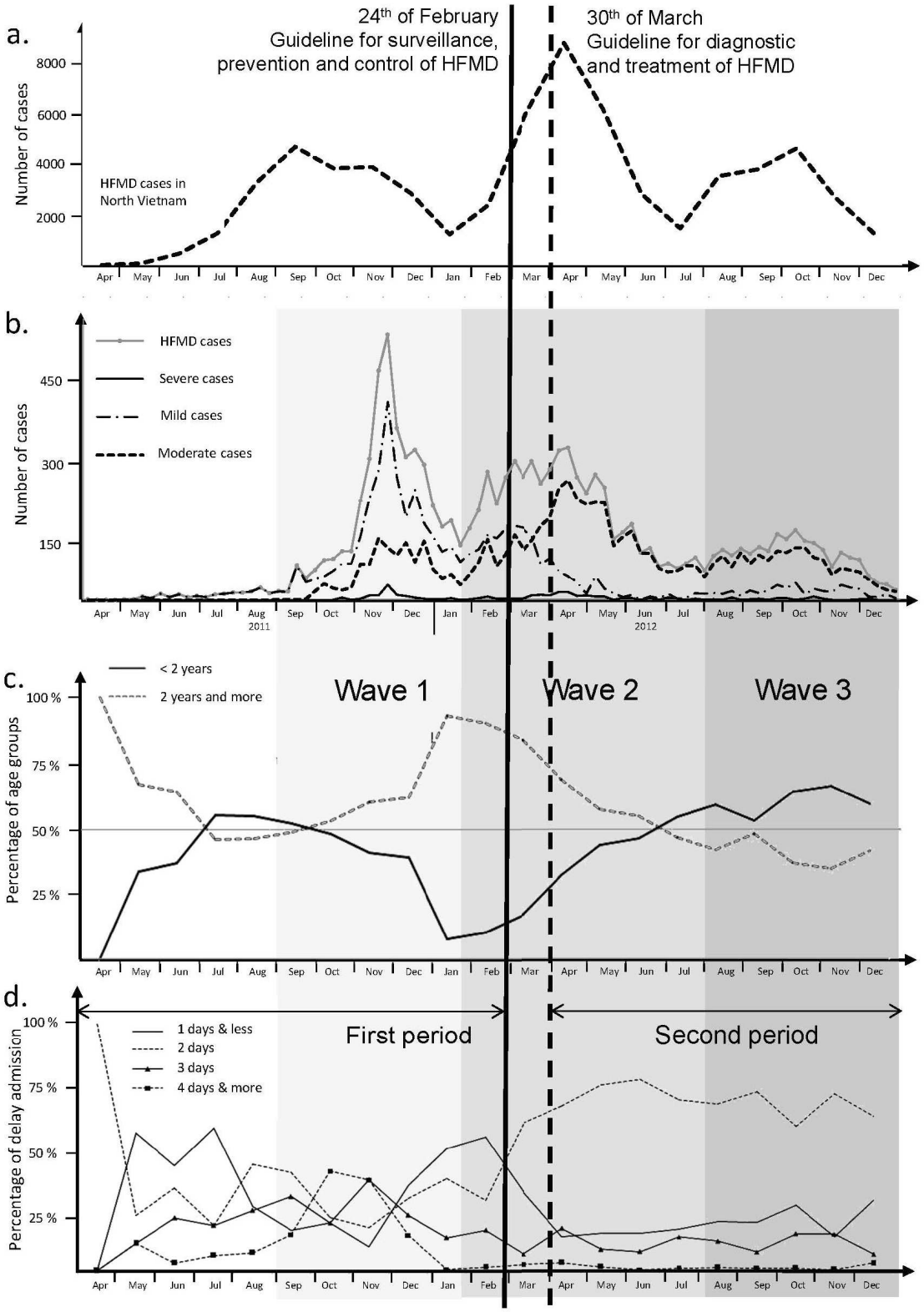


Figure 1

Evolution of HFMD admission at Hai Phong city pediatric hospital. The proportion of the moderate and severe cases admitted at the pediatric hospital increased significantly during the second period (Supplementary Table 8). Concomitantly, the number of HFMD admissions increased in district hospitals. The number of outpatients (treatment at home) also increased notably with mild level of severity during the second period. The ratio between young (below 2) and old patients admitted at the pediatric hospital was reversed after March 2012, but not in district hospitals and local health stations (Supplementary Table 9). The share of patients between the pediatric hospital and the local health facilities clearly improved during the second period (Supplementary Table 10a). The number of patient admited at the pediatric hospital coming from non-urban districts compared to urban ones remained the same over the two periods. This ratio was similar for the total number of cases (Supplementary Table 10b), but the number of non-urban district patients with severe symptoms admitted at the pediatric hospital increased (Supplementary Table 10c).

Variation in disease assessment. Severity score, age of patient and time from onset to admission were combined together in a single analysis to evaluate the effect of guidelines recommendations on surveillance and diagnosis. Ten groups (clusters) of patients (clusters) were identified through hierarchical classification (Supplementary Figure 1) and each cluster was associated to specific presentations according to average values of quantitative parameters (Supplementary Table 11). New guidelines publication modified drastically the distribution of patients among the different clusters. The diversity of presentations in patients decreased sharply after March 2012. From 60% to 100% of patients were declared during the first period of the study in 8 clusters. A large majority of patients associated with extreme presentations (old patients, high severity or long time from onset to admission from clusters 5 to 9) were diagnosed before guidelines release. Patients with low severity and long onset to admission delay (cluster 6) were no longer seen after guideline publications whereas clusters 4 and 10 emerged as the two main ones after guidelines release. These two groups differed on delay and severity. Cluster 4, the largest, corresponded to young children (1.3 y in average) with moderate symptoms admitted in average two days after onset. A similar group of patient was also present before guidelines publications (cluster 2). Children from cluster 10 were presented after only one day but with a severity from moderate to severe. Patients from clusters 3 and 10 were mostly from non urban areas.

INSERT FIGURE 2

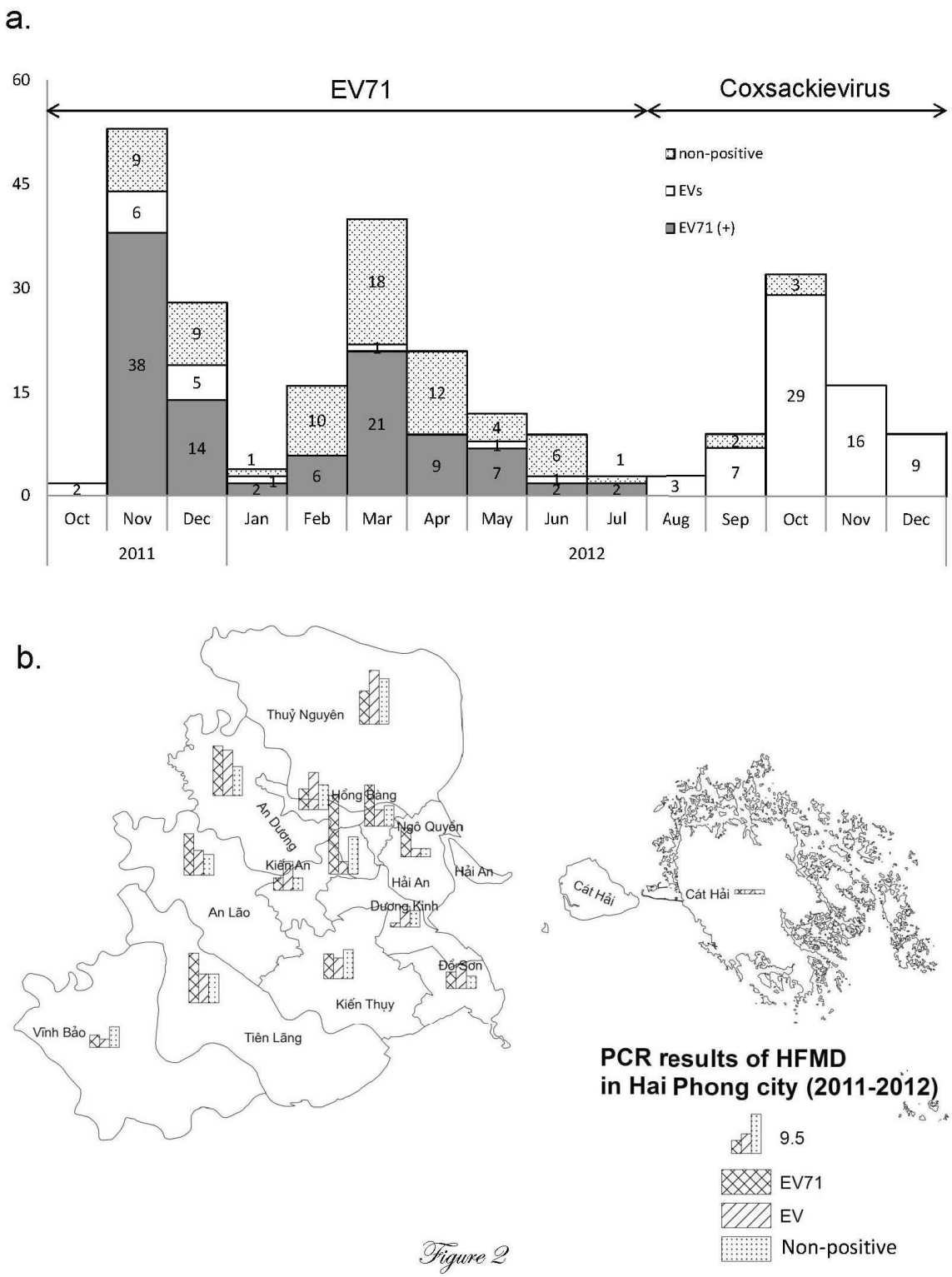


Figure 2

Both EV-A71 and CV-A were present during the epidemic. Molecular diagnostic confirmation was conducted by PCR on 257 samples from cases clinically identified as HFMD. Nearly 71% were positive for Human Enterovirus (182/257). Of the 182 positives, 101 (55%) were EV-A71 and 81 (45%) corresponded to other enteroviruses (EVs) (Figure 2a). The identified EV-A71 isolates belonged to subgenogroup C4 present on northern and central city and C5 present also in northern, central and southern city (Figure 2b). 75 patients diagnosed as HFMD during waves 1 and 2 (Figure 2a) were not positive for enterovirus. EV-A71 coincided with wave 1 and wave 2 (Figure 2a). Wave 3 was associated with the co-circulation of CV-A6 and CV-A16 (Figure 2b). The rate of EV negative samples started to increase in December, 2011 to reach a maximum in March, 2012 and was very low during wave 3.

INSERT FIGURE 3

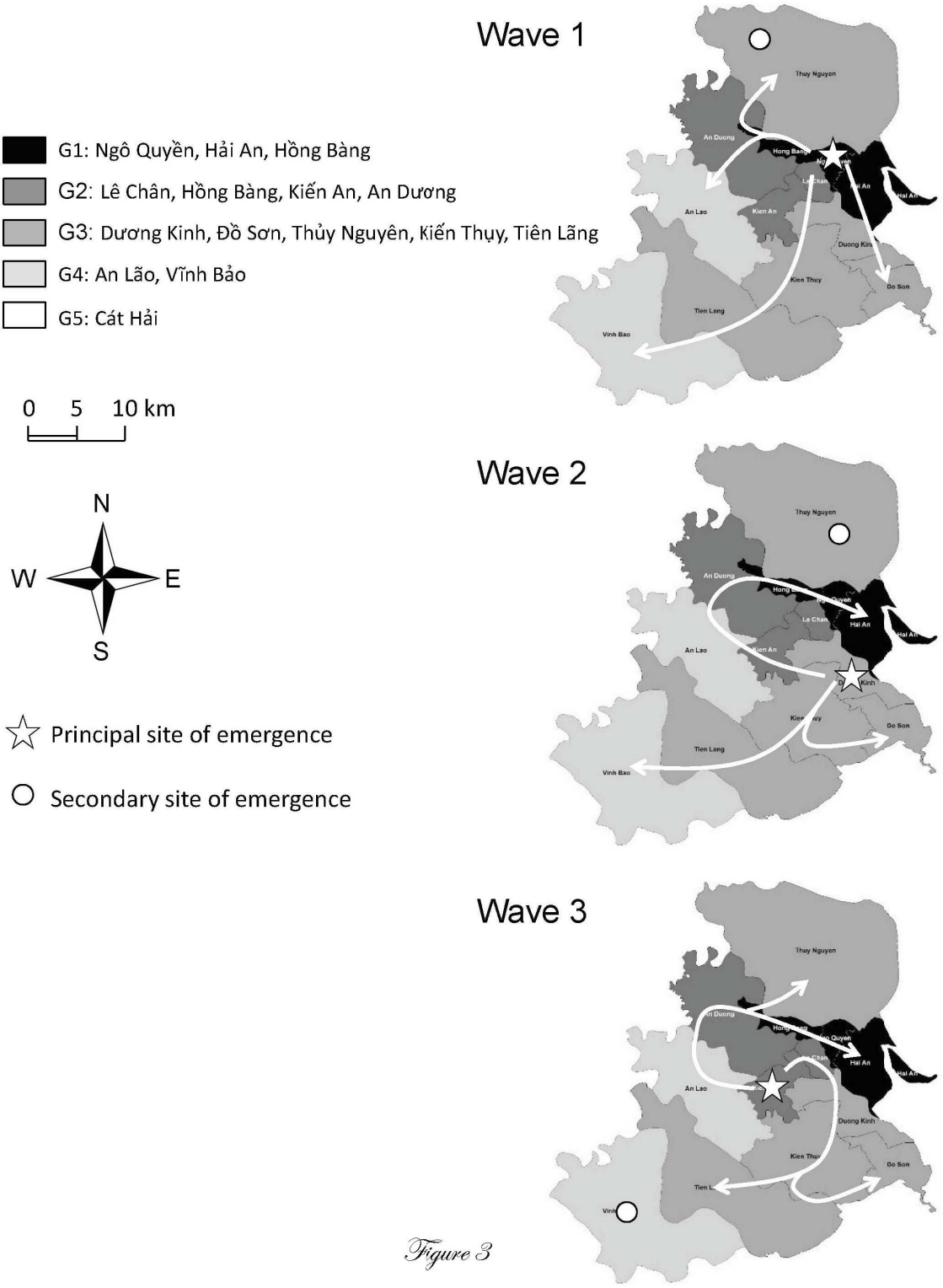


Figure 3

Spread of the disease in Hai Phong city. With one exception (commune 214), no early case was found along the main motorway (Supplementary Figure 2a). Early cases appeared in the northern and urban zone of the city (Supplementary Figure 3) and expanded to the west and to the south (Figure. 3). Each waves displayed a different main site of emergence (Figure 3, Supplementary Table 12). Wave 1 started in the city center whereas waves 2 and 3 emerged at the periphery. The order of occurrence defined five groups of districts (Supplementary Figure 3): Hai Phong city center (Group 1), New urban areas in the southern part of the City and a western rural district (Group 2), peripheric districts and Do Son (Group 3), two rural districts not connected to the main road netwok (Group 4) and Cat Hai islands (Group 5). The diffusion of the disease to the south followed the axis supported by two main roads allowing to cross rivers and canals. No direct transmission of the disease was observed between the city center and these new urban areas during wave 2 and 3. Patterns of transmission among groups were similar for the three waves. Re-emergence of the disease during wave 2 and 3 shows similarity despite the presence of different etiological agent.

Discussion

The first HFMD outbreak in North Vietnam. The 2011-2012 HFMD epidemic was the largest to have ever occurred in Vietnam and the first recorded in the northern part of the country while Hai Phong city experienced the highest HFMD incidence in North Vietnam. However, no fatal cases were reported in Hai Phong unlike what was observed in South Vietnam (9). The age of the patient was the single most important variable. Age-specific incidence was the highest in the 1-2 year age group. This would be in agreement with both the persistence of maternally-derived neutralizing antibodies for up to 6 months and the kinetics of seroprevalence of EV-A71 virus neutralizing antibodies which increases with age (15,16). However, this was the very first recorded outbreak of HFMD in northern Vietnam questioning thus the existence of maternally-derived neutralizing antibodies or pre-existing immunity. Children under 3 represented 85.85% of cases. They are in Vietnam traditionally cared for at home by family members. The high HFMD incidence in this population may thus have resulted from contact with adults and older children acting as asymptomatic carriers of the virus (17,18).

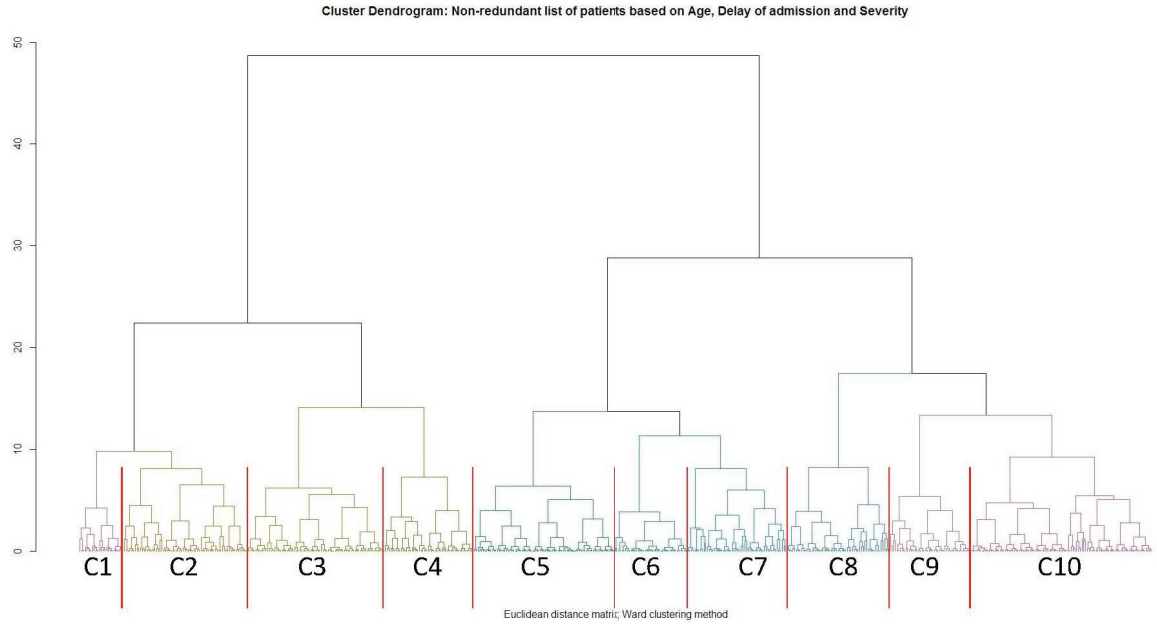
Guidelines positively influence disease management. The first guideline released related to surveillance, prevention and control and gave clear HFMD case definition, reporting procedure and strategy for collecting clinical samples. The first effect of the guideline release was a signifcant increase of the severity score. Indeed, 73.41% of patients were scored 2a after guidelines publication but only 25.59% before. The number of moderate and severe cases admitted to Hai Phong pediatric hospital increased significantly after guidelines publication while the proportion of the mild cases decreased sharply. Another positive effect was the reduced delay between onset and admission after guidelines publication. It decreased during the second period and remained very homogeneous. The presence of 9 out of 10 clusters in the first half of the outbreak supports this conclusion. The most important feature of the second guideline was the decentralization and transfer of responsibility to health care facilities. A more homogeneous spatial distribution of patients visiting pediatric hospitals was visible. Mild cases were treated at the commune level whereas districts were in charge of mild and moderate cases. At the province level, all cases were addressed. All patients recorded as severe went to province hospitals during period 2 while local health facilities hosted patients unable to go to main hospitals. Patients who remained at home only displayed mild symptoms.

Awareness and legal framework. This positive effect of guidelines is not only the consequence of the publication of guidelines but also of an increased awareness and precautious approach from parents and physicians leading to patients being majoritarily declared with severe symptoms in order to ensure a better treatment and surveillance. This could explained why a higher disease severity scores was observed in CV-A-infected patients (wave 3) than in EV-A71 cases ($p< 0.01$). Awareness led to the modification of guidelines but changes occurred only after publication, suggesting that the legal framework created by the guidelines is needed for implementation eventhough awareness is present. Public and professional awareness are not sufficient for implementing changes. Furthermore, emergence of CV-A (wave 3) during the second period did not lead to variation in severity and time to admission. Conversely, the publication of guidelines during phase 2 led to different patient patterns although the virus was the same. Evolution of clinical patterns should not be considered only in the light of the evolution or replacement of pathogens or host-pathogen interactions but also according to the evolution of behavior and social perception.

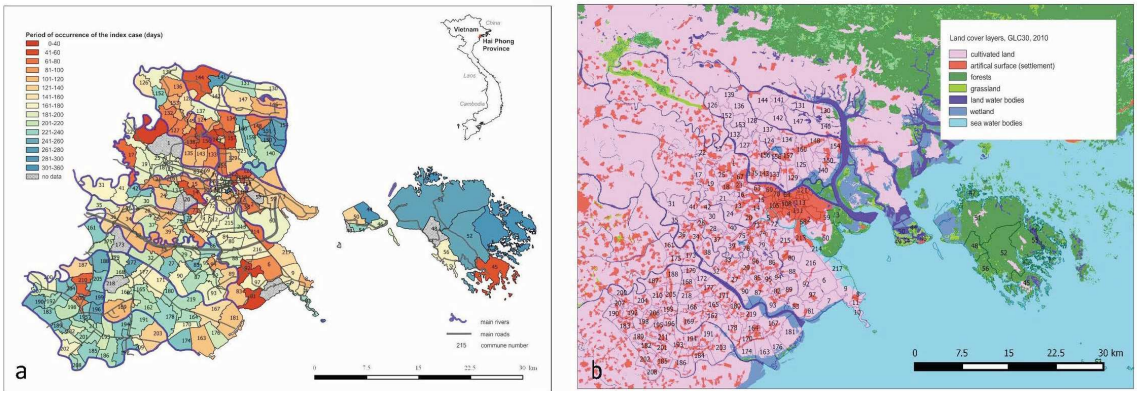
Shift of etiology. Improvement of molecular diagnostic was not considered by the new guidelines and they therefore had no impact on the detection of etiological agents in patients. During the Hai Phong outbreak, circulation of both EV-A71 and CV-A was recorded, a feature already reported (8,19,20,21,22). EV-A71 virus is considered to be the most frequent cause of severe HFMD disease (10,23,24) although CV-A have been shown to cause severe infections with meningitis (25). An uncharacterized virus might also have circulated during wave 1 and mostly wave 2. The high number of EV non-positive PCR reactions on clinically positive samples suggests that the set of primers used for enterovirus detection might not have been discriminative enough. The ratio of non-positive tests was similar to those previously reported (26,27,28,29). EV-A71 detection with MAS primers should thus be systematically performed on SO primers products and SO222 primer should be redesigned to match with the 5' part of the AN88 primer used for EVs detection. More attention should be therefore paid to the PCR negative patients.

Spatio-temporal dynamic and disease control. Nguyen et al. (9) have shown the presence of HFMD in provinces west of Hai Phong after the outbreak started in South

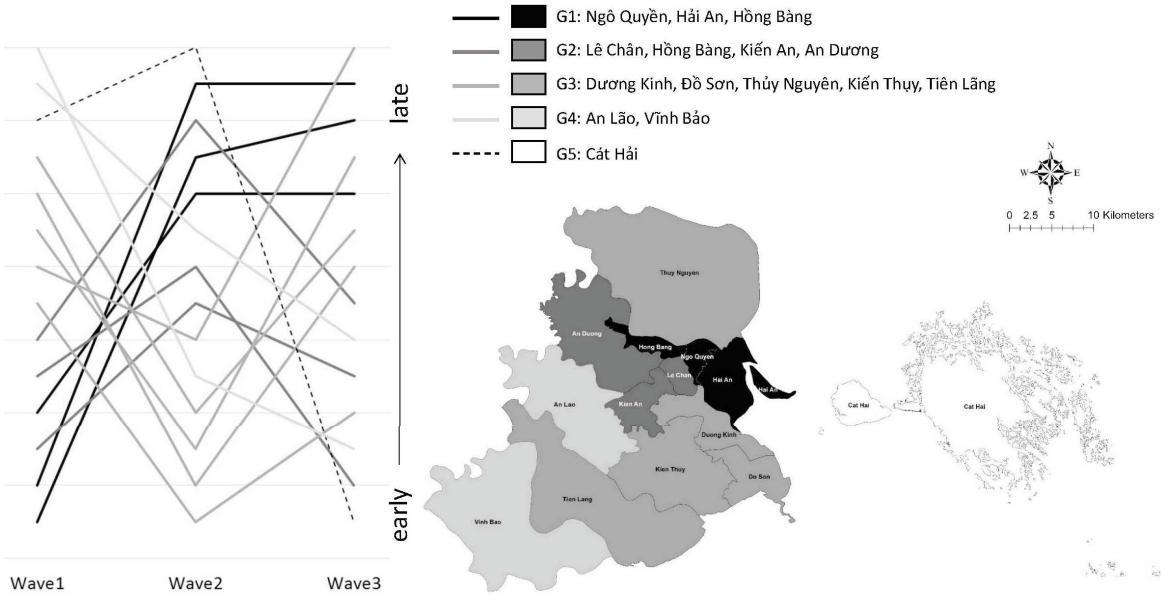
Vietnam, making the northwestern side of Hai Phong is the most likely route of entry. Despite, the main economical role of Hai Phong no early cases occurred along or at the end of the main highway linking Hai Phong to the rest of the country, indicating that major industrial and export commercial movements are not linked to the dynamic of the disease. Instead the disease seems to have expanded following the eastbound river system to reach densely populated settlements from where it secondarily expanded through local roads. Disease expansion might thus have followed secondary local commercial routes. This commercial route allows time for the disease to be transmitted and involves a lot of favorable human to human contacts. The presence of early cases in the island and in isolated coastal localities in the southern part of the city also illustrates the role of sea transportation and role of the local trade and occupational activities in the spread of the disease. The southern part may have been affected later due to fragmentation of the territory and isolation of the communes by the complex river system. The early occurrence of the disease in northwestern communes not connected to the main local road might be related to a specific occupational activities. Considering the average age of the patients, around 2, the source of contamination must be sought for within asymptomatic adults being contaminated during their occupational activities and in local and regional movements.



Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3

Supplementary Table 2. Characteristics of reported HFMD cases in Hai Phong city, 2011-2012

Characteristics	HFMD cases in Hai Phong city during			Children(< 14 years old) in Hai Phong city (%)	*p* value
	2011 (n=3029) n (%)	2012 (n=6592) n (%)	Total (n=9621) n (%)		
Gender					
girls	1,162(38.36)	2,711(41.13)	3,873(40.26)	186.445 (47)	
boys	1,867(61.64)	3,881(58.87)	5,748(59.74)	206.291 (53)	*p*< 0.01
Age (year)					
median (IQR)	2 (2)	2 (2)	2 (2)	NA	
< 0.5	71(2,41)	101(1.63)	172 (1.88)	NA	
0.5 - 1	726 (24.60)	2109 (34.08)	2835 (31.02)	NA	
1-2	970 (32.87)	2097 (33.88)	3067 (33.56)	NA	
2-3	629 (21.31)	1143 (18.47)	1772 (19.39)	NA	
3-4	283 (9.59)	407 (6.58)	690 (7.55)	NA	
4-5	139 (4.71)	180 (2.91)	319 (3.49)	NA	
5-10	119 (4.03)	129 (2.08)	248 (2.71)	NA	
>10	14 (0.47)	23 (0.37)	37 (0.40)	NA	NA
Severity*					
mild	2109 (69.63)	1993 (30.41)	4102 (42.81)	NA	
moderate	849 (28.03)	4413 (67.34)	5262 (54.92)		
severe	71 (2.34)	147 (2.24)	218 (2.28)	NA	NA
fatal	0	0	0		
Living area					
not urban	1474 (48.66)	4080 (61.89)	5554 (57.73)	177.518 (0.55)	
urban	1555 (51.33)	2512 (38.11)	4067 (42.27)	215.219 (0.45)	*p*<0.01
Pathogen					
Non EV-A71	13 (20)	68 (58.12)	81(44.51)	NA	
EV-A71	52 (80)	49 (41.88)	101 (55.49)	NA	*p*<0.01

p value (Chi-square test); NA: not available

*Mild cases correspond to uncomplicated disease (severity level =1).

*Moderate cases correspond to uncomplicated disease (severity level =2a).

*Severe cases correspond to more complicated forms of the disease (severity level = 2b,3 or 4).

Supplementary Table 3: Age of HFMD patients by epidemic waves in Hai Phong City (2011-2012)

Age groups of HFMD cases	Wave 1	Wave 2	Wave 3	*p* value
under 2 years	1150 (34.57)	907 (25.41)	1191 (59.11)	*p*< 0.01
2 years & more	2177 (65.43)	2663 (74.59)	824 (40.89)	
Total	3327 (100%)	3570 (100%)	2015 (100%)	

p value (Chi-square test)

Supplementary Table 4: Gender, age, living area, pathogen, delay of admission of HFMD reported cases by severity in Hai Phong city, 2011 - 2012.

Characteristics		**Mild cases**	**Moderate cases**	**Severe cases**	***p* value**
Gender	female	1680 (40.96)[b]	2094 (39.79)	86 (39.45)	*p* = 0.50
	male	2422 (59.04)	3168 (60.21)	132 (60.55)	
Age	>= 2 years	2838 (69.19)	3023 (57.45)	142 (65.14)	*p* < 0.01*
	< 2 years	1264 (30.81)	2239 (42.55)	76 (34.86)	
Living area	not urban	2431 (59.26)	2976 (56.56)	125 (57.34)	*p* < 0.05*
	urban	1671 (40.74)	2286 (43.44)	93 (42.66)	
Pathogen	Non EV-A71	54 (78.26)	15 (19.23)	12 (34.29)	*p* < 0.01*
	EV-A71	15 (21.74)	63 (80.77)	23 (65.71)	
Delay of admission	> 1 day	2451 (59.75)	4551 (86.49)	193 (88.53)	*p* < 0.01*
	= < 1 day	1651 (40.25)	711 (13.51)	25 (11.47)	

p value (Chi-square test)

Mild cases correspond to uncomplicated disease (severity level =1).
Moderate cases correspond to uncomplicated disease (severity level =2a).
Severe cases correspond to more complicated forms of the disease (severity level = 2b,3,4).

Supplementary Table 5. Age groups and severity of reported HFMD cases by epidemic outcomes in Hai Phong city between 2011 and 2012

Severity	Wave 1			Wave 2			Wave 3		
	Age of HFMD patient			Age of HFMD patient			Age of HFMD patient		
	under 2 years	2 years & more	*p* value	under 2 years	2 years & more	*p* value	under 2 years	2 years & more	*p* value
Mild	727 (63.33)	1492 (68.76)		203 (22.43)	1027 (38.98)		190 (15.95)	174 (21.12)	
Moderate	387 (33.71)	636 (29.31)	*P* < 0.05	683 (75.47)	1528 (57.99)	*p*< 0.01	981 (82.37)	635 (77.06)	*p*< 0.01
Severe	34 (2.96)	42 (1.94)		19 (2.1)	80 (3.04)		20 (1.68)	15 (1.82)	
Total	1148 (100%)	2170 (100%)		905 (100%)	2635 (100%)		1191 (100%)	824 (100%)	

p value (Chi-square test)

Mild cases correspond to uncomplicated disease (severity level =1).

Moderate cases correspond to uncomplicated disease (severity level =2a).

Severe cases correspond to more complicated forms of the disease (severity level = 2b,3,4).

$p < 0.05$, indicating that the severity distribution differs significantly between both age groups.

Supplementary Table 6. Gender, Severity, Living area and Delay of admission of reported HFMD cases by epidemic waves in Hai Phong city (2011-2012)

Epidemic waves	Gender			Severity				Living area			Delay of admission		
	Male	female	Test*	mild	moderate	severe	Test§	urban	rural	Test*	mean	SD	test#
All period	5748 (59.74)	3874 (40.26)	*p*< 0.01	3813 (42.97)	4850 (54.66)	210 (2.37)	NA	4067 (42.27)	5554 (57.73)	*p*< 0.01	2.08	1.06	NA
Wave 1	2023 (60.81)	1304 (39.19)	*p*< 0.01	2219 (66.88)	1023 (30.83)	76 (2.29)		1628 (48.93)	1699 (51.07)	*p*< 0.01	2.47	1.37	*p*< 0.01
Wave 2	2063 (57.79)	1507 (42.21)	*p*< 0.01	1230 (34.75)	2211 (62.46)	99 (2.8)	*p*< 0.01	1286 (36.02)	2284 (63.98)	*p*< 0.01	1.85	0.79	
Wave 3	1227 (60.89)	788 (39.11)	*p*< 0.01	364 (18.06)	1616 (80.2)	35 (1.74)		838 (41.59)	1177 (58.41)	*p*< 0.01	1.87	0.73	

*: Chi2 one sample, proportion comparison with whole population (male rate = 0.53 and urban rate = 0.45)

§: Chi2 test for proportion comparison of multi groups

#: one-way ANOVA test for mean comparison of multi groups

Mild cases correspond to uncomplicated disease (severity level =1).

Moderate cases correspond to uncomplicated disease (severity level =2a).

Severe cases correspond to more complicated forms of the disease (severity level = 2b,3,4).

$p < 0.001$, indicating that the severity distribution differs significantly between the three waves

Supplementary Table 7. Age groups, severity, delay duration and living area of reported HFMD cases by epidemic period in Hai Phong city between 2011 and 2012

Epidemic periods	*Age groups*			*Severity*				*Delay duration*					*Living area*		
	under 2 years	2 years & more	*p* value	mild	moderate	severe	*p* value	1 day & less	2 days	3 days	4 days & more	*p* value	*Not urban*	*urban*	*p value*
1st period	1337 (39.30)	2850 (55.93)	*P* < 0.01	2817 (80.28)	1271 (25.59)	88 (44.44)	*p*< 0.01	1265 (63.44)	1118 (27.14)	1036 (66.71)	768 (92.42)	*p*< 0.01	2168 (45.91)	2019 (53.47)	*p*< 0.01
2nd period	2065 (60.70)	2246 (47.07)		692 (19.72)	3509 (73.41)	110 (55.56)		729 (36.56)	3002 (72.86)	517 (33.29)	63 (7.85)		2554 (54.09)	1757 (46.53)	
Total	3402 (100%)	5096 (100%)		3509 (100%)	4780 (100%)	198 (100%)		1994 (100%)	4120 (100%)	1553 (100%)	831 (100%)		4722 (100%)	3776 (100%)	

p value (Chi-square test)

Mild cases correspond to uncomplicated disease (severity level =1).
Moderate cases correspond to uncomplicated disease (severity level =2a).
Severe cases correspond to more complicated forms of the disease (severity level > 2b,3,4).
1st period: first half of the epidemic (Apr 2011 – 24th Feb 2012).
2nd period: second half of the epidemic (30th Mar 2012 – 31th Dec 2012).

Supplementary Table 8. Severity of reported HFMD cases at place of admission by epidemic periods in Hai Phong city between 2011 and 2012

Epidemic periods	*Commune health station*				*District hospital*				*Hai Phong Pediatric hospital*				*At home (outpatient)*			
	mild	moderate	severe	*sum*	mild	moderate	severe	*sum*	mild	moderate	severe	sum	mild	moderate	severe	*sum*
1st period	4 (67)	-	2 (34)	6 (100)	508 (93)	34 (6.5)	3 (0.5)	545 (100)	1742 (62)	1012 (36)	59 (2)	2813 (100)	6 (60)	4 (40)	-	10 (100)
2nd period	21 (95)	-	1 (5)	22 (100)	429 (28)	1086 (71.8)	3 (0.2)	1518 (100)	93 (3.5)	2418 (93)	93 (3.5)	2604 (100)	147 (100)	0 (0)	-	147 (100)
	p value: not apply				*p value < 0.01*				*p value < 0.01*				*p value: not apply*			

p value (Chi-square test) for proportion comparison between mild group vs (moderate + severe) group

Mild cases correspond to uncomplicated disease (severity level =1).
Moderate cases correspond to uncomplicated disease (severity level =2a).
Severe cases correspond to more complicated forms of the disease (severity level > 2b,3,4).
1st period: first half of the epidemic (Apr 2011 – 24th Feb 2012).
2nd period: second half of the epidemic (30th Mar 2012 – 31th Dec 2012).

Supplementary Table 9. Age groups of reported HFMD cases at place of admission in Hai Phong city in 2 periods of the epidemic

Age group	*1st Period of epidemic*					*2nd Period of epidemic*				
	Commune health station & At home	*District hospital*	*Hai Phong Pediatric hospital*	*Sum*	*p value*	*Commune health station & At home*	*District hospital*	*Hai Phong Pediatric hospital*	*Sum*	*p value*
under 2 years	5 (0.5)	168 (16.5)	822 (83)	995 (100)	*p = 0.261*	56 (2.7)	679 (33.3)	1305 (64)	2040 (100)	*p < 0.001*
2 years & more	5 (0.2)	377 (15.8)	2002 (84)	2384 (100)		91 (4.08)	839 (37.64)	1299 (58.28)	2229 (100)	

p value (Chi2 test)

Supplementary Table 10a. Living area of reported HFMD cases at place of admission in Hai Phong city

Living area	*1st Period of epidemic*					*2nd Period of epidemic*				
	Commune health station & At home	*District hospital*	*Hai Phong Pediatric hospital*	*Sum*	*p value*	*Commune health station & At home*	*District hospital*	*Hai Phong Pediatric hospital*	*Sum*	*p value*
urban	8 (0.5)	114 (7.5)	1427 (92)	1549 (100)	*p* = 0.261*	138 (8)	235 (13.5)	1358 (78.5)	1731 (100)	*p < 0.001*
Not urban	2 (0.1)	431 (23.5)	1397 (76.4)	1830 (100)		9 (0.35)	1283 (50.55)	1246 (49.09)	2538 (100)	

p value (Chi2 test)

*p** value (Fisher exact test)

Supplementary Table 10b. Living area of reported HFMD cases at Hai Phong pediatric hospital

Living area	*Hai Phong Pediatric hospital*			
	1st period	*2nd period*	*Sum*	*p value*
urban	1427 (51.24)	1358 (48.76)	2785 (100)	*p = 0.233*
Not urban	1397 (52.86)	1246 (47.14)	2643 (100)	

p value (Chi2 test)

Supplementary Table 10c. Living area and severity of reported HFMD cases in Hai Phong pediatric hospital city

Living area	Hai Phong pediatric hospital									
	1st period					2nd period				
	mild	moderate	severe	*sum*	*p value*	mild	moderate	severe	*Sum*	*p value*
Urban	896 (63.01)	496 (34.88)	30 (2.11)	1422 (100)	*p = 0.47*	47 (3.46)	1275 (93.89)	36 (2.65)	1358 (100)	*p = 0.02*
Not urban	846 (60.82)	526 (37.10)	29 (2.08)	1391 (100)		46 (3.69)	1143 (91.73)	57 (4.57)	1246 (100)	

Supplementary Table 11: Clusters (groups) of patients obtained by hierarchical classification

Cluster	HFMD cases	Meanage	Mean gender	Mean delay	Mean severity	Urban living (%)	Wave 1	Wave 2	Wave 3	Cluster description
All	8824	2.02	0.58	2.10	1.61	3745 (42.44)	3152	3932	1740	
cluster 1	1142	2.92	0.50	0.80	1.17	415 (36.33)	455	556	121	mild cases before guidelines
cluster 2	2087	2.65	0.56	2.35	1.57	896 (42.93)	788	1061	238	homogeneous
cluster 3	440	1.01	0.60	0.77	1.57	166 (37.72)	227	117	96	young with short delay
cluster 4	3373	1.29	0.60	2.16	1.84	1447 (42.89)	652	1647	1074	after guidelines
cluster 5	423	1.4	0.60	4.14	1.34	222 (52.48)	339	40	14	W1, long delay
cluster 6	119	1.88	0.59	5.65	1.11	73 (61.34)	116	3	0	W1, long delay
cluster 7	200	3.64	0.59	4.36	1.17	123 (61.50)	180	20	0	W1, old children
cluster 8	357	4.37	0.59	2.42	1.4	173 (48.45)	166	161	30	Old children
cluster 9	69	2.69	0.62	4.36	2.13	43 (62.31)	50	15	4	sever cases W1
cluster 10	614	1.46	0.61	0.99	2.21	187 (30.45)	149	303	162	after guidelines, sever

Supplementary Table 12. Propagation of the HFMD epidemic among Hai Phong city districts according to median case. Rural and urban districts were differentiate (Type) and described according to major features (short + long description). Stratification (Group) was performed according to the relative order of median in the three waves (Suppl Fig 3). Date of the median case and relative order of the district was given for each waves.

Name	type	Short	long description	Group	Median W1	Wave1	Median W2	Wave2	Median W3	Wave3
Ngô Quyền	urban	HPC	Hai Phong city center	G1	24/11/2011	1	21/04/2012	11	14/10/2012	12
Hải An	urban	HPC	area encompassing airport and harbor	G1	25/11/2011	2	22/04/2012	13	14/10/2012	13
Hồng Bàng	urban	HPC	west suburb, industrial	G1	27/11/2011	4	18/04/2012	10	10/10/2012	10
Lê Chân	urban	HP	close to Hai Phong city center	G2	25/11/2011	3	16/04/2012	7	27/09/2012	5
Kiến An	urban	HP	new suburb, south	G2	29/11/2011	5	16/04/2012	8	15/09/2012	2
An Dương	Rural	W	rural, west, along tracks + big crossroad	G2	30/11/2011	6	21/04/2012	12	02/10/2012	7
Dương Kinh	urban	HP	new area close to highway	G3	01/12/2011	7	10/03/2012	1	19/09/2012	4
Đồ Sơn	urban	DS	city in the south, harbor, industry	G3	01/12/2011	8	14/04/2012	6	17/10/2012	14
Thủy Nguyên	Rural	N	rural, north	G3	03/12/2011	9	31/03/2012	3	13/10/2012	11
Kiến Thụy	Rural	E	rural, south, between Hai Phong and Đồ Sơn	G3	04/12/2011	10	23/03/2012	2	07/10/2012	8
Tiên Lãng	Rural	S	rural south	G3	07/12/2011	11	04/04/2012	4	09/10/2012	9
An Lão	Rural	W	rural, west, motorway junction	G4	09/12/2011	13	16/04/2012	9	02/10/2012	6
Vĩnh Bảo	Rural	S	rural, extrem south	G4	16/12/2011	14	05/04/2012	5	17/09/2012	3
Cát Hải	Rural	E	island	G5	07/12/2011	12	23/04/2012	14	29/08/2012	1

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References

1. WHO. A Guide to Clinical Management and Public Health Response for Hand, Foot and Mouth Disease (HFMD). WHO WPRO; 2011.
2. Ang LW, Koh BK, Chan KP, Chua LT, James L, Goh KT. Epidemiology and control of hand, foot and mouth disease in Singapore. Ann Acad Med Singapore. 2009; 38:106–112.
3. Chen KT, Chang HL, Wang ST, Cheng YT, Yang JY. Epidemiologic features of hand-foot-mouth disease and herpangina caused by enterovirus 71 in Taiwan, 1998–2005. Pediatrics. 2007; 120:e244–e252.
4. Bessaud M, Razafindratsimandresy R, Nougairède A, et al. Molecular Comparison and Evolutionary Analyses of VP1 Nucleotide Sequences of New African Human Enterovirus 71 Isolates Reveal a Wide Genetic Diversity. PloS One. 2014; 9:e90624.
5. Abu Bakar S, Chee HY, Al-Kobaisi MF, Xiaoshan J, Bing CK, Kit LS. Identification of enterovirus 71 isolates from an outbreak of hand, foot and mouth disease (HFMD) with fatal cases of encephalomyelitis in Malaysia. Virus Research. 1999; 61:1-9.
6. Ooi MH, Wong SC, Lewthwaite P, Cardosa MJ, Solomon T. Clinical features, diagnosis, and management of enterovirus 71. The Lancet Neurology. 2010; 9:1097–1105.
7. Zeng M, Li YF, Wang XH, et al. Epidemiology of hand, foot, and mouth disease in children in Shanghai 2007–2010. Epidemiology and infection. 2012; 140:1122-1130.
8. Tu PV, Thao NTT, Perera D, et al. 2007. Epidemiologic and virologic investigation of hand, foot, and mouth disease, southern Vietnam, 2005. Emerging Infect Dis. 2007; 13:1733-1741.
9. Nguyen NTB, Pham HV, Hoang, CQ, et al. Epidemiological and clinical characteristics of children who died from hand, foot and mouth disease in Vietnam, 2011. BMC Infectious Diseases. 2014; 14:341.
10. Huang CC, Liu CC, Chang YC, Chen CY, Wang ST, Yeh TF. Neurologic Complications in Children with Enterovirus 71 Infection. New England Journal of Medicine. 1999, 341:936-942.

11. Nix WA, Oberste MS, Pallansch MA. Sensitive, seminested PCR amplification of VP1 sequences for direct identification of all enterovirus serotypes from original clinical specimens. J Clin Microbiol. 2006; 44: 2698–2704.
12. Perera D, Podin Y, Akin W, Tan CS, Cardosa MJ. Incorrect identification of recent Asian strains of Coxsackievirus A16 as human enterovirus 71: Improved primers for the specific detection of human enterovirus 71 by RT PCR. BMC Infectious Diseases. 2004; 4:11.
13. CPHCSC (Central Population and Housing Census Steering Committee). The 2009 Vietnam population and housing census: completed results. Hanoi, Vietnam: 893 pp; 2010.
14. NGCC. 30-meter Global Land Cover Dataset (GlobeLand30). Product description. National Geomatics Center of China: 25 pp; 2014.
15. Luo ST, Chiang PS, Chao AS, et al. Enterovirus 71 Maternal Antibodies in Infants, Taiwan. Emerg Infect Dis. 2009; 15:581–584.
16. Tran CBN, Nguyen HT, Phan HTT, et al. The seroprevalence and seroincidence of enterovirus71 infection in infants and children in Ho Chi Minh City, Viet Nam. PLoS One. 2011; 6:e21116.
17. Chang LY, Tsao KC, Hsia SH, et al. Transmission and clinical features of enterovirus 71 infections in household contacts in Taiwan. Journal of the American Medical Association. 2004; 291:222-227.
18. Witso E, Palacios G, Ronningen KS, et al. Asymptomatic Circulation of HEV-A71 in Norway. Virus Research. 2007; 123: 19-29.
19. Khanh TH, Sabanathan S, Thanh TT, et al. Enterovirus 71-associated hand, foot, and mouth disease, Southern Vietnam, 2011. Emerging Infect Dis. 2012; 18:2002–2005.
20. Xiang C. Detection of human enterovirus 71 and coxsackievirus A16 in children with hand, foot and mouth disease in China. Molecular Medicine Reports; 2012.
21. Yang F, Zhang T, Hu Y, et al. Survey of enterovirus infections from hand, foot and mouth disease outbreak in China. Virol. J. 2009; 8:508.
22. Wu Y, Yeo A, Phoon MC, et al. The largest outbreak of hand; foot and mouth disease in Singapore in 2008: The role of enterovirus 71 and coxsackievirus A strains. International Journal of Infectious Diseases. 2010; 14:e1076–e1081.

23. Schmidt NJ, Lennette EH, Ho HH. An Apparently New Enterovirus Isolated from Patients with Disease of the Central Nervous System. J Infect Dis. 1974; 129:304-309.
24. Lum LC, Wong KT, Lam SK, et al. 1998. Fatal enterovirus 71 encephalomyelitis. J Pediatr. 1998; 133:795-798.
25. Chang LY, Lin TY, Huang YH, et al. Comparison of enterovirus 71 and coxsackievirus A16 clinical illnesses during the Taiwan enterovirus epidemic, 1998. Pediatr Infect Dis J. 1999; 18:1092.
26. Chiang PS, Huang ML, Luo ST, Lin TY, Tsao KC, Lee MS. Comparing Molecular Methods for Early Detection and Serotyping of Enteroviruses in Throat Swabs of Pediatric Patients. PLoS ONE. 2012; 7:e48269.
27. Umami RN, Dhenni R, Jajuli A, Nishimura Y, Shimizu H, Utama A. Detection and Identification of Human Enteroviruses among Healthy Children in Antajaya, Bogor. Journal of Biotechnology Research in Tropical Region. 2009; 2:1-7.
28. Park K, Lee B, Baek K, et al. Enteroviruses isolated from herpangina and hand-foot-and-mouth disease in Korean children. Virology Journal. 2012; 9:205.
29. He SJ, Han JF, Ding XX, Wang YD, Qin CF. Characterization of enterovirus 71 and coxsackievirus A16 isolated in hand, foot, and mouth disease patients in Guangdong, 2010. International Journal of Infectious Diseases. 2013; 17:e1025–e1030.

7. Figures legends

Figure 1. Evolution of HFMD cases and clinical parameters (age, delay of admission and severity) over the epidemic period (2011-2012)

a. Monthly HFMD cases in northern Vietnam (2011-2012)

Number of HFMD cases reported to Vietnam Ministry of Health as routine surveillance in 2011 and 2012.

b. Weekly HFMD cases and severity distribution in Hai Phong city (2011-2012)

Number of HFMD cases weekly reported to the National Institute of Hygiene and Epidemiology (NIHE), 2011-2012. Each epidemiologic week begins on Monday. Mandatory reporting of the disease began in 2011. Severity levels are based on guidelines from WHO for HFMD clinical assessment and cases management. Mild cases are cases free of complication (severity score = 1). Moderate cases are with severity score = 2a. Severe cases are characterized by febrile exanthematous symptoms affecting the central nervous system, frequently myoclonus and more severe neurological complications (severity score = 2b,3,4).

c. Monthly distribution of age groups of HFMD patients in Hai Phong city (2011-2012)

Age of the HFMD patient is divided in to 2 group; less than 2 year old and 2 year old and above.

d. Monthly distribution of delay of admission of HFMD patients in Hai Phong city (2011-2012).

The delay admission is the difference between the date of admission of the patient at the hospital and the date of onset. The distribution shows the proportion of delay of admission for the following classes: one day, two days, three days or four days and more.

Figure 2. Spatio-temporal distribution of the PCR identifications in Hai Phong

EVs represent all positive results with Enteroviruses using semi-nested PCR.

EV-A71 represent all positive results with EV-A71 using semi-nested PCR.

“Non-positive” represents all negative results with Enteroviruses using semi-nested PCR

a. Time flow of the 257 PCR identification of HFMD epidemic in Hai Phong.

b. Spatial distribution of the 257 PCR identifications in Hai Phong city.

Figure 3. Sites of emergence and expansion of the three waves of HFMD in Hai Phong city

Shades of grey represent the five goups of districts.

Supplementary Figure 1. Hierarchical classification of HFMD cases

A specific threshold (horizontal dashed line) was applied to defined ten clusters which are C1, C2, C3, C4, C5, C6, C7, C8, C9 and C10. The hierarchical classification was performed on type cases which represent all the patients displaying the same values for age, severity and time after onset to admission.

Supplementary Figure 2. Spatio-temporal distribution of HFMD case in Hai Phong city

a. Time and place of occurrence of HFMD case

Colours represent interval of occurrence of the index case in each commune. Rivers are shown in blue and roads are shown in dark grey. Line width is proportional to the importance of the system. Numbers represent the referenced commune number.

b. Land cover and human settlements

The land cover map (GLC30, 2010) shows 6 main classes of objects detected on satellite images: cultivated land, artificial surface (settlements), forest, grassland, land water bodies and wetlands. Numbers represent the referenced communes.

Supplementary Figure 3. Propagation of the HFMD epidemic among Hai Phong city districts according to median case.

Rural and urban districts were differentiated (Type) and described according to major features (supplementary table 12). Stratification (Group) was performed according to the relative order of median in the three waves. Date of the median case and relative order of the district was given for each waves.

CHAPTER 2. EVOLUTION OF EV-A71 ENTEROVIRUSES IN VIETNAM

2.1. Context of study

Hand, foot and mouth disease (HFMD) is usually mild and self-limiting, in some cases HFMD can result in severe complications such as encephalitis, aseptic meningitis, pulmonary edema, myocarditis, and death (80). HFMD is caused by members of Human Enterovirus A, a family of picornavirus which includes Coxsackievirus A (CV-A) and Human Enterovirus 71 (EV-A71) (34). Both EV-A71 and CV-A infection has been associated with severe HFMD in young children, sometimes resulting in death (35), (36), (37).

Enteroviruses are characterized by the presence of 4 structural proteins, VP4 being the internal capsid protein and VP1, VP2 and VP3 making the three external capsid proteins (30). VP1 is the most external and is the main component of the canyon on the surface of picornaviruses. VP1 is involved in viral pathogenicity, receptor binding and immune modulation of EV71 (73), (117). Differences in EV-A71 strains might contribute to the different severity of the disease (41), (44), and virulence determinants have been identified in the VP1 protein such as residues G/Q/R at position VP1-145, E at VP1-164 (118), (119), (120). VP1 is used to classified enteroviruses. Based on the VP1 gene, EV-A71 is classified into three independent genogroups: A, B, and C. The EV-A71 B and C genogroups are each further subdivided into five genotypes, B1 to B5 and C1 to C5 (39).

Although EV-A71 was isolated for the first time in Vietnam in 2003, the first outbreak of HFMD was not reported in the southern provinces until 2005. The 2005 outbreak was associated with EV-A71 C1, C4 and C5 genotypes and Coxsackievirus A16 (22), (23). Since 2011, Vietnam have experienced continuosly large outbreaks of HFMD and the disease became a notifiable one in the national communicable disease surveillance system. Besides epidemiology features, etiological agents information of this disease in Viet Nam is mostly reported the distribution and some immunology aspects but mutation, pathogenicity, vilulent ananlysis of the pathogen still limited. The 2011 - 2012 HFMD outbreak was the first one to occur in North Vietnam providing thus a ground to study the etiology, origin and dynamic of the disease. We report here the analysis of the VP1 gene of strains isolated throughout North Vietnam during the 2011-2012 outbreak.

2.2. Objective

To analyse pathogenicity-related mutations in the VP1 sequence of EV-A71 strains isolated throughout Northern Vietnam during the 2011-2012 outbreak.

2.3. Discussion and conclusions

This work provides an insight on the evolution and dynamic of the EV-A71 enterovirus during the first outbreak recorded in North Vietnam in 2011-2012. The first conclusion is that the 2011-2012 outbreak in North Vietnam was not due a single exogenous strain imported from South Vietnam where HFMD outbreaks were present (24), or from another region. All variant populations observed during the 2011-2012 outbreak were already present in North Vietnam. The only exception is the VVV population which was found only in 2011 in three different provinces. However, the phylogenetic analysis indicated that this VVV variant was the closest to the root and therefore to the mother and oldest population. The reason for the lack of VVV variants in samples older than 2011 is most likely related to the low number of samples and to the low prevalence of this population. There is no clear explanation either for the surge of variants VII and IIV in the first part of the epidemic and altogether the question remains of what triggered the outbreak in 2011 although all virus populations were already present. All I/V populations present at the beginning of the outbreak were capable of triggering it as shown by the replacement in 2012. It is not related either to the subgenogroup since the populations which emerged in 2012 belonged to two different subgenogroups, the VIV variant belonging the subgenogroup C4 and the IVI variant being a member of subgenogroup C5. A partial explanation could be a differential susceptibility of the human population which could have been slightly more susceptible to the VII and IIV groups. Another explanation might be found in the spatial distribution of the various variant groups, the socio-economic pattern and the route of dissemination. This work was not structured to address this issue and specific sampling schemes as well as transversal analyses should thus be further undertaken.

Another main outcome of this work is the observed correlation between the I/V variant groups and phylogeny, pathogenicity and ethnicity. The I/V groups, although based on the relative arrangement of only three amino-acids, overlap the different clusters

identified. These clusters correspond to genetically different populations characterized by specific polymorphism traits. This overlap between the specific combination of I/V residues at three positions and the phylogeny established over the whole sequence indicates the occurrence of a selective pressure on the I/V arrangement. The high conservation of the proteins, despite variability at the nucleotide level, indicative of a negative, or purifying, selection pressure, whereas the clustering at the protein level is driven by the I/V arrangement. The remaining question is what is the selective pressure acting on the I/V variants and what could be the role of these I/V mutations. The I/V mutations are located at positions 249, 262 and 284 of the Vp1 protein. The recurrent reports of the involvement of isoleucine and valine in the viral pathogenicity process in different viruses as well as their involvement in the selective pressure applied on the EV-A71 samples analyzed in this work suggest that the I/V pattern at positions 249, 262 and 284 on the VP1 protein might play a role in pathogenicity. The observed correlation of the I/V variant populations with severity and ethnicity strengthen this hypothesis. However, the ethnicity correlation could be a result of spatial structuration since ethnicity-2 is mostly present in the Hòa Bình province. This in turn would suggest that the various EV-A71 variants display a geographic specificity.

Altogether, these data suggest that EV-A71 strains could remain in a low level, asymptomatic state, in genomic stasis and with a geographic structuration. The cause for outbreaks should thus be sought for in the socio-economic patterns rather than in exogenous emergence. Further investigations are needed to investigate this hypothesis and to bring valuable information for the management of this pediatric diseases.

The results of this work are summarized in the article entitled "*Valine/isoleucine variants drive selective pressure in the VP1 sequence of EV-A71 enteroviruses*" is presented thereafter.

For

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Valine/isoleucine variants drive selective pressure in the VP1 sequence of EV-A71 enteroviruses

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Running title: VP1 mutations in HFMD

Key words: VP1, HFMD, Enterovirus, EV-A71, Vietnam

Abstract

Background: In 2011-2012, Northern Vietnam experienced its first large scale hand foot and mouth disease (HFMD) epidemic. In 2011, a major HFMD epidemics was also reported in South Vietnam with fatal. This 2011-2012 outbreak was the first one to occur in North Vietnam providing thus a ground to study the etiology, origin and dynamic of the disease. We report here the analysis of the VP1 gene of strains isolated throughout North Vietnam during the 2011-2012 outbreak and before.

Methods: The VP1 genes of 106 EV-A71 virus from North Vietnam and 2 from Central Vietnam were sequenced. Sequences alignments were analyzed at the nucleic acid and protein level. Gene polymorphism was also analyzed. A Factorial Correspondence Analysis was performed to correlate amino acid mutations with clinical parameters.

Results: The sequences were distributed into four phylogenetic clusters. Three clusters corresponded to the subgenogroup C4 and the last one corresponded to the subgenogroup C5. Each cluster displayed different polymorphism characteristics. Proteins were highly conserved but three site bearing only Isoleucine (I) or Valine (V) were characterized. The isoleucine / valine variability matched the clusters. Spatiotemporal analysis of the I/V variants showed that all variants which emerged in 2011 and then in 2012 were not the same but were all present in the region prior to the 2011-2012 outbreak. Some correlation was found between certain I/V variants and ethnicity and severity.

Conclusions: The 2011-2012 outbreak was not caused by an exogenous strain coming from South Vietnam or elsewhere but by strains already present and circulating at low level in North Vietnam. However, what triggered the outbreak remained unclear. A selective pressure is applied on I/V variants which matches the genetic clusters. I/V variants were shown on other viruses to correlate with pathogenicity. This should be investigated in EV-A71. I/V variants are an easy and efficient way to survey and identify circulating EV-A71 strains.

Background

Hand, foot and mouth disease (HFMD) is an acute febrile illness in children with a papulovesicular skin rash at the palms or soles of the feet, or both. Presentation can be with or without inclusion of mouth ulcers. Although the disease is usually mild and self-limiting, in some cases HFMD can result in severe complications such as encephalitis, aseptic meningitis, pulmonary edema, myocarditis, and death (WHO, 2011). HFMD is caused by members of Human Enterovirus A, a family of picornavirus which includes Coxsackievirus A (CV-A) and Human Enterovirus 71 (EV-A71) (Ang et al., 2009; Chen et al., 2007). The EV-A71 viruses are genetically related to CV-A; indeed, it has been suggested that these viruses may have diverged as recently as the 1940s (Tee et al., 2010). Both EV-A71 and CV-A infection has been associated with severe HFMD in young children, sometimes resulting in death (Abu Bakar et al., 1999; Ooi et al., 2010; Zeng et al., 2010).

Enteroviruses are characterized by the presence of 4 structural proteins, VP4 being the internal capsid protein and VP1, VP2 and VP3 making the three external capsid proteins (Carter and Saunders, 2007). VP1 is the most external and is the main component of the canyon on the surface of picornaviruses. VP1 is involved in viral pathogenicity, receptor binding and immune modulation of EV-A71 (Kataoka et al., 2015; Yang et al., 2011). Differences in EV-A71 strains might contribute to the different severity of the disease (McMinn et al., 2001; Sanders et al., 2006) and virulence determinants have been identified in the VP1 protein such as residues G/Q/R at position VP1-145, E at VP1-164 (Li et al., 2011; Huang et al., 2015; Caine et al., 2016). VP1 is used to classified enteroviruses. Based on the VP1 gene, EV-A71 is classified into three independent genogroups: A, B, and C. The EV-A71 B and C genogroups are each further subdivided into five subgenogoups, B1 to B5 and C1 to C5 (Brown et al., 1999).

Although EV-A71 was isolated for the first time in Vietnam in 2003, the first outbreak of HFMD was not reported in the southern provinces until 2005. The 2005 outbreak was associated with EV-A71 C1, C4 and C5 genotypes and Coxsackievirus

A16 (Tu et al., 2007; Khanh et al., 2012). In 2011, a major HFMD epidemics was reported in South Vietnam with fatal cases reported (Nguyen et al., 2014). This 2011-2012 outbreak was the first one to occur in North Vietnam providing thus a ground to study the etiology, origin and dynamic of the disease. We report here the analysis of the VP1 gene of strains isolated throughout North Vietnam during the 2011-2012 outbreak.

Methods

Epidemiological information and source of specimens. All HFMD cases in Hai Phong city were reported to the National Institute of Hygiene and Epidemiology (NIHE) through the national communicable disease surveillance system since 2011. HFMD patients that were present to health centers or hospitals were diagnosed and classified in 4 severity levels. The evaluation of the disease was performed according to the guidelines specifically published by the Vietnamese Ministry of Health which are based on but slightly differ from WHO and Taiwanese guidelines (Huang et al., 1999, WHO, 2011).

Sampling and culture. Ninety four samples were obtained from hospitalized patients diagnosed with EV A71 HFMD in 19 out of 28 provinces in North Vietnam in 2011 and 2012 and stored at - 80°C. They were completed by fourteen reference samples obtained from previous cases of EV A71 HFMD between 2003 and 2010 in seven provinces in North Vietnam and two provinces in Central Vietnam (Table 1). All samples were sent to the Enterovirus Laboratory of NIHE for etiological assays. Enterovirus-positive and EV-A71-positive samples were identified according to Nix et al (2006) using *SO* (SO224/SO222), *AN* (AN88/AN89) and *MAS* (MAS01S/MAS02A) (Perera et al., 2004) primer sets. EV A71 viruses were cultivated on RD cell according to WHO procedure (WHO, 2011). Viral RNA was extracted from throat swab using QIAamp® Viral RNA Mini Kit (Qiagen, Valencia, USA). The cDNA was first synthesized from the RNA for 10 min at 25^{o}C and followed by amplification at 42^{o}C for 50 min, 72^{o}C for 15 min using primers AN32, AN33, AN34 and AN35 (13).

Sequence analyses. Sequences were deposited in GenBank and accession numbers are provided in Table 1. The VP1 genetic sequences were aligned in Seaview 4.6 (Gouy et al., 2010) using Muscle algorithm (Edgar, 2004). Best-fitting evolutionary models were determined by JModelTest 2.1 (Darriba et al., 2012) or by ProtTest 2.4 (Abascal et al., 2005) using the corrected version of the Akaike Information Criterion (AICc). The phylogeny of VP1 was performed by Maximum Likelihood (ML) inference using the model GTR+G+F with Seaview 4.6 (Gouy et al., 2010). The

robustness of nodes was assessed with 500 bootstrap replicates. ML analysis of the amino acid sequences was performed using the model JTT+I+G with Seaview 4.6 (Gouy et al., 2010). The robustness of nodes was assessed with 500 bootstrap replicates. Sequence polymorphism was investigated using the DnaSP 5.10.01 package (Librado and Rozas, 2009).

Bias and Ethics. Training session HFMD cases definition and reporting were organized for the staff of the routine surveillance system to enhance quality and consistency of case report. This work was conducted following the requirements of the Vietnamese Ministry of Health and under the Law of Communicable Diseases Prevention and Control passed in 2007.

Factorial correspondence analysis. A factorial correspondence analysis (FCA) was performed using XLSTAT software (Addinsoft®). Variables considered were: amino acid profiles on positions 151, 164 and 186 (V/I), respectively in this work (249, 262 and 284 on the full length VP1 protein), severity level, ethnicity, age of patients, and patient location. The best descriptive axes were retained, explaining 34% of the data spread.

Spatio-temporal analysis. Administration data were obtained from GADM database of Global Administrative Areas (version 2.8, November 2015). Spatial analyses were conducted with Quantum GIS, version 2.8.2. All spatial data are in the WGS 84 coordinate system.

Results

Clinical and epidemiological features. Data are shown in Table 1. Patients age ranged from 2 months to 12 years old (median at 1.8 years, IQR of 1.5 years). 102/106 (96.23%) patients were under 5. The age-specific incidence highest in the 1-2 years age group (44 cases, 41.51%) and remained very low for older children. The lowest incidence was observed in infants under 6 months (2.83%) and children above 10 years old (0.94%). Patients came from all parts of the region including mountainous, rural and urban areas. Out of 83 cases, 59 (71.08 %) belonged to main Vietnamese ethnicity (Ethnicity 1) while the rest of patients belonged to the minority Hmong ethnicity (Ethnicity 2) (Table 1). All severity levels were reported for the patients. Mild forms (severity level 1) made the majority of cases (57 cases, 61.29%) while 15 patients displayed severe symptoms (16.13%). Among this group, 3 patients displayed a severity score of 3. No case with the highest level of 4 was recorded. Moderate forms of HFMD were found in 21 patients (22.58). (Table 1)

VP1 phylogeny and population structure. Phylogenetic analysis of the VP1 gene sequences indicated the presence of four clusters (Figure 1a). Cluster 1, the closest to the root, was the main one, comprising 56 sequences and structured into several subclusters characterized by low bootstrap values. Cluster 2 and cluster 3 comprised respectively 23 and 12 sequences and segregated from cluster 1 to which they were separated with a low bootstrap of 30. The last cluster, cluster 4, comprising 17 sequences was characterized by a high bootstrap value (100) and was a derivate from cluster 3. A similar tree topology was observed when using protein alignments (Figure 1b). With respect to the current genogroup classification, clusters 1, 2 and 3 belonged to the subgenogroup C4 whereas cluster 4 belonged to the subgenogroup C5 (Table 1). The VP1 protein was characterized by a high rate of conservation. However, three sites displayed a consistent Valine (V) / Isoleucine (I) variation. These sites, sites A, B and C were at position 151, 164 and 186, respectively in this work and 249, 262 and 284 on the full length protein. Six types of I/V variants were observed when considering the amino acids at sites A, B and C: VVI (1 sequence), IVI (15 sequences), IIV (28 sequences), VII (38 sequences), VIV (23 sequences) and

VVV (3 sequences) (Table 1). When attributing a color code for each I/V variant population and applying it to the phylogenetic tree a clear overlap with the previously detected clusters was observed (Figure 1). Cluster 4 overlapped with the IVI population while clusters 2 and 3 comprised the VII variants. The VVV, IVV and VIV variants all corresponded to cluster 1. However they did not mix and each one corresponded to a specific subcluster. The VVI variant derived from the cluster 4 / IVI group. Three exceptions were found, with VII variants present in clusters 1 and 4. With respect to phylogeny, the I/V variant closest to the root is VVV, the VIV population emerged from this group and gave rise in turn to the IIV group. The VII groups, derived from the VIV group with first cluster 2 from which cluster 3 evolved. The well separated cluster 4 / IVI variants evolved from cluster 3. This overlap between clusters and I/V populations was even stronger at the protein level since almost all the variability was borne by the I/V mutations, the rest of the protein being highly conserved. Groups VII, IIV, VVV and VIV belonged to the subgenogroup C4 whereas groups IVI and VVI belonged to the subgenogroup C5.

Nucleic acid polymorphism. When considering the polymorphism of the various clusters identified, very different traits were observed (Table 2). Cluster 1 was polymorphic (= 14.58) but with a 2.5 times more parsimony informative sites than singletons and 10-times more synonymous mutations than non-synonymous ones, suggesting that it is not a recent polymorphism or expanding population. The Ka/Ks ratio was also characterized by a low value. Conversely, cluster 2 displayed a very low level of polymorphism with a q of 4.06 and a low number of mutations (η = 15). The Ka/Ks was slightly higher at 0.107. This is suggesting the existence of a bottleneck at the origin of cluster 2. Cluster 3 which originated from cluster 2 was more polymorphic with a slightly increasing (= 6.92) and number of mutations η (η = 19). Only synonymous mutations were observed resulting in turn in a very low Ka/Ks ratio of 0.029. Cluster 4 was on the other hand displaying a very high polymorphism with a η of 113 for 109 and a very high level of synonymous mutations (105 out of 110) suggesting a strong negative selection acting on a mutating population. As a consequence, the Ka/Ks ratio was also very low at 0.011.

Distribution of mutations and correlation analysis. The correlation analysis indicated a partial structuration of cases (34% of dispersion explained) on different parameters (Figure 2). The VVI variant was not included in the analysis because all information was not available. The analysis was therefore conducted on only five variants, i.e. IIV, VII, IVI, VIV and VVV. Severity of HFMD seemed to correlate with the age of patient and the highest severity level was not observed above 11-month old. The VII variant segregated from the other variants on the F1 axis and was associated with both low severity and with the ethnicity-2 group, 56,5% of patients from this ethnicity-2 group were infected by the VII variant, but this represented only 46,4% of all samples harboring the VII protein. No variant was specifically associated with the highest severity whereas the IIV variant was correlated with mild severity.

Spatiotemporal distribution of the virus populations. I/V variants present in the 2011 outbreak belonged mostly the IIV and VII populations which were already present in Northern Vietnam (Figure 3a) The IIV population was previously detected in 2008 in the Ninh Binh province whereas VII variants were detected in Cao Bang and Hai Phong in 2007 and in Nam Dinh and Ninh Binh 2008. VII and IIV variants represented 46% and 33.3% of the samples collected in 2011, respectively (Figure 3b, 3d). Other mutant populations detected in 2011 were: IVI (1.7%) already detected in 2007 in Yen Bai and Han Nam, in 2008 in Ninh Binh and in 2010 in Hai Phong and Bac Kan; VIV (14.3%) previously found in 2003 in Ha Noi and in 2006 in Phu Yen; and VVV (4.8%) (Figure 3a). The VVV variants were not detected in samples collected prior to the 2011-212 outbreak. The mutant populations detected in 2012 were IIV and VII which prevalence was reduced to 16.2% and 19.3% and VIV and IVI which prevalence rose to 38.7% and 25.8%, respectively (Figure 3c, 3d). The VVV mutant was found only in 2011 in Thanh Hoa, Nam Dinh and Ha Noi (Figure 3b, 3d). With respect to spatial distribution, the rise of variants VII and IIV observed in 2011 was not located in a specific area but covered most of the sampling sites (8 out of 11). The replacement of the IIV and VII variants by the IVI and VIV variants followed a similar pattern confirming the wide-spread diffusion of the outbreak. The number of sites with more than two variants was higher in 2011 than

in 2012. The IIV variant was the most widely spread in 2011 but became the least widely spread in 2012 (Figure 3b, 3c). Conversely, the IVI variant which was the least widely spread in 2011 and found in only one province, i.e. Hoa Binh, became the most widely spread in 2012. The VVV variant was found only in 2011 in three provinces, in the South Eastern part of North Vietnam each time in association with the IIV variant (Figure 3b, 3c). The VVI variant was detected only in Quang Nam, Central Vietnam and prior to 2011.

Discussion

This work provides an insight on the evolution and dynamic of the EV-A71 enterovirus during the first outbreak recorded in North Vietnam in 2011-2012. The first conclusion is that the 2011-2012 outbreak in North Vietnam was not due to a single exogenous strain imported from South Vietnam where HFMD outbreaks were present (Nguyen et al., 2014) or from another region. All variant populations observed during the 2011-2012 outbreak were already present in North Vietnam. The only exception is the VVV population which was found only in 2011 in three different provinces. However, the phylogenetic analysis indicated that this VVV variant was the closest to the root and therefore to the mother and oldest population. The reason for the lack of VVV variants in samples older than 2011 is most likely related to the low number of samples and to the low prevalence of this population. Furthermore, this 2011-2012 outbreak was also characterized by the cocirculation of the same four variant populations with a replacement between 2011 and 2012. The VII and IIV variants which were the most prevalent in 2011 were replaced by IVI and VIV populations in 2012. There is no clear explanation for the replacement of the main variant populations between 2011 and 2012 but it could be related to immunoresistance acquired during the first half of the outbreak in 2011 The surge of variants VII and IIV in the first part of the epidemic could not be related to any measured parameters and altogether the question remains of what triggered the outbreak in 2011 although all virus populations were already present. All I/V populations present at the beginning of the outbreak were capable of triggering it as shown by the replacement in 2012. It is not related either to the subgenogroup since the populations which emerged in 2012 belonged to two different subgenogroups, the VIV variant belonging the subgenogroup C4 and the IVI variant being a member of subgenogroup C5. A partial explanation could be a differential susceptibility of the human population which could have been slightly more susceptible to the VII and IIV groups. Another explanation might be found in the spatial distribution of the various variant groups, the socio-economic pattern and the route of dissemination. This work was not structured to address this issue and specific sampling schemes as well as transversal analyses should thus be further undertaken.

Another main outcome of this work is the observed correlation between the I/V variant groups and phylogeny, pathogenicity and ethnicity. One hypothesis is that fixation of mutations in VP1 could be related to the VP1 function itself. The I/V groups, although based on the relative arrangement of only three amino-acids, overlap the different clusters identified. These clusters correspond to genetically different populations characterized by specific polymorphism traits. This overlap between the specific combination of I/V residues at three positions and the phylogeny established on the nucleotide sequences suggests the occurrence of a selective pressure on the I/V arrangement. The high conservation of the proteins, despite variability at the nucleotide level, indicative of a negative, or purifying, selection pressure, indicates that the clustering at the protein level is driven by the I/V arrangement. The remaining question is what is the selective pressure acting on the I/V variants and what could be the role of these I/V mutations. The I/V mutations are located at positions 249, 262 and 284 of the VP1 protein. The region from amino acid 132 to 297 on the EV-A71 VP1 protein was shown to be crucial for increasing the strength of protein-protein interactions in the capsid and its stability. This increased stability strongly enhances the pathogenicity and survival of the virus in the gastrointestinal track (Lal et al., 2006). Isoleucine and valine are aliphatic hydrophobic amino acid mediating the core structure of the protein and have been reported to be involved in virulence and pathogenicity in several viruses. In the related cocksackievirus B3, a conformational change occurred when leucine at position 1092 was substituted for isoleucine or valine leading to susceptibility to pleconaril (Schmidtke et al., 2005). A valine to isoleucine substitution at position 25 in $gPr80^{env}$, the envelope precursor polyprotein of the Moloney murne Leukemia Virus, was shown to mediate temperature sensitivity, inefficient processing of the protein and neurovirulence (Szurek et al., 1989). A Valine to Isoleucine substitution at position 4 in the VP1 protein of the Infectious Bursal Disease virus (IBD), causing is a highly contagious disease in chickens known as Gumboro disease, was shown to be responsible for a decrease of pathogenicity and differential growth in cell culture (Yu et al., 2013). Similarly, a valine to isoleucine at positon 3 in the NS4A protein of the Japanese Encephalitis virus was shown to increase the virulence (Yamagushi et al, 2011). An I71V mutation in the capsid protein of the Simian-Human

Immunodeficiency Virus mediates the escape from cytotoxic T-lymphocyte (Peyerl et al., 2003). The recurrent reports of the involvement of isoleucine and valine in the viral pathogenicity process in different viruses as well as their involvement in the selective pressure applied on the EV-A71 samples analyzed in this work suggest that the I/V pattern at positions 249, 262 and 284 on the VP1 protein might play a role in pathogenicity. The observed correlation of the I/V variant populations with severity and ethnicity strengthen this hypothesis. However, the ethnicity correlation could be a result of spatial structuration since ethnicity-2 is mostly present in the Hòa Bình province. This in turn would suggest that the various EV-A71 variants display a geographic specificity.

Conclusion

Altogether, these data suggest that EV-A71 strains could remain in a low level, asymptomatic state, in genomic stasis and with a geographic structuration. The cause for outbreaks should thus be sought for in the socio-economic patterns rather than in exogenous emergence. Further investigations are needed to investigate this hypothesis and to bring valuable information for the management of this major pediatric disease.

Declarations

Ethics approval and consent to participate

This work was conducted strictly following the requirements of the Vietnamese Ministry of Health and under the Law of Communicable Diseases Prevention and Control passed in 2007. This work was conducted under the control of NIHE Ethic committee.

Consent for publication

Not Applicable

Availability of data and material

All data are publicly available and sequences have been deposited in Genbank

Competing interests

The authors declare that there is no competing interests

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Authors' contributions

NDN participated to all parts of the work

OMS, YAH, RC and DJG generated all sequences

LTTH, LTSH, VDT and NTHT participated to sample collection and molecular analysis and amplification

AA designed all maps and spatiotemporal analysis

LG, CM, PR, GK, EC and RF participated to all bioinformatic, statistic and phylogenetic analyses

CD, NTH and TND have provided fruitful advises and discussions

RF supervised the work and participated to all analyses and to the writing

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References

Abascal F, Zardoya R, Posada D. ProtTest: selection of best-fit models of protein evolution. Bioinformatics. 2005; 21:2104-5.

Abu Bakar S, Chee HY, Al-Kobaisi MF, Xiaoshan J, Bing CK, Kit LS. Identification of enterovirus 71 isolates from an outbreak of hand, foot and mouth disease (HFMD) with fatal cases of encephalomyelitis in Malaysia. Virus Research. 1999; 61:1-9.

Ang LW, Koh BK, Chan KP, Chua LT, James L, Goh KT. Epidemiology and control of hand, foot and mouth disease in Singapore. Ann Acad Med Singapore. 2009; 38:106–12.

Brown BA, Oberste MS, Alexander JP Jr, Kennett ML, Pallansch MA. Molecular epidemiology and evolution of enterovirus 71 strains isolated from 1970 to 1998. Journal of virology. 1999; 73:9969-75.

Caine EA, Moncla LH, Ronderos MD, Friedrich TC, Osorio JE. A Single Mutation in the VP1 of Enterovirus 71 Is Responsible for Increased Virulence and Neurotropism in Adult Interferon-Deficient Mice. Journal of Virology. 2016; 90: 8592–604.

Carter JB, Saunders VA. Virology: Principles and Application. John Wiley & Sons; 2007..

Chen KT, Chang HL, Wang ST, Cheng YT, Yang JY. Epidemiologic features of hand-foot-mouth disease and herpangina caused by enterovirus 71 in Taiwan, 1998–2005. Pediatrics. 2007; 120:e244–e252.

Darriba D, Taboada GL, Doallo R, Posada D. jModelTest 2: more models, new heuristics and parallel computing. Nature methods. 202; 9:772.

Edgar RC. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC bioinformatics. 2004; 5:113.

Gouy M, Guindon S, Gascuel O. SeaView version 4: A multiplatform graphical user interface for sequence alignment and phylogenetic tree building. Molecular biology and evolution. 2010; 27:221-4.

Huang CC, Liu CC, Chang YC, Chen CY, Wang ST, Yeh TF. Neurologic Complications in Children with Enterovirus 71 Infection. New England Journal of Medicine. 1999; 341:936-42.

Huang S W, Tai CH, Fonville JM, Lin CH., Wang SM, Liu CC, .Su IJ, Smith DJ, Wang JR. Mapping enterovirus A71 antigenic determinants from viral evolution. Journal of Virology. 2005; 89:11500-06.

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

Khanh TH, Sabanathan S, Thanh TT, Thoa le PK, Thuong TC, Hang VT, Farrar J, Hien TT, Chau NV, van Doorn HR. Enterovirus 71-associated hand, foot, and mouth disease, Southern Vietnam, 2011. Emerging Infect Dis. 2012; 18: 2002–5.

Lal SK, Kumar P, Yeo WM, Kar- Roy A, Chow VT. The VP1 protein of human enterovirus 71 self- associates via an interaction domain spanning amino acids 66–297. Journal of Medical Virology. 2006; 78:582-90.

Li R, Zou Q, Chen L, Zhang H, Wang Y. Molecular analysis of virulent determinants of enterovirus 71. PloS One. 2011; 6(10):e26237.

Librado P, Rozas J. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics. 2009; 25:1451–1452.

McMinn P, Lindsay K, Perera D, Chan HM, Chan KP, Cardosa MJ. Phylogenetic analysis of enterovirus 71 strains isolated during linked epidemics in Malaysia, Singapore, and Western Australia. Journal of Virology. 2001; 75: 7732-8.

Nguyen NTB, Pham H, Hoang CQ, Nguyen TM, Nguyen LT, Phan HC, Phan LT, Vu LN, Minh NNT. Epidemiological and clinical characteristics of children who died from hand, foot and mouth disease in Vietnam, 2011. BMC Infectious Diseases. 2014; 14:341

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

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

Perera D, Podin Y, Akin W, Tan CS, Cardosa MJ. Incorrect identification of recent Asian strains of Coxsackievirus A16 as human enterovirus 71: Improved primers for the specific detection of human enterovirus 71 by RT PCR. BMC Infectious Diseases. 2004; 4:11.

Peyerl FW, Barouch DH, Yeh WW, Bazick HS, Kunstman J, Kunstman KJ, Letvin NL. Simian-human immunodeficiency virus escape from cytotoxic T-lymphocyte recognition at a structurally constrained epitope. Journal of virology. 2003; 77:12572-8.

Sanders S, Herrero L, McPhie K, Chow S, Craig M, Dwyer D, Rawlinson W, McMinn PC. Molecular epidemiology of enterovirus 71 over two decades in an Australian urban community. Archives of virology; 2006; 151: 1003–13.

Schmidtke M, Hammerschmidt E, Schüler S, Zell R, Birch-Hirschfeld E, Makarov VA, Wutzler P. Susceptibility of coxsackievirus B3 laboratory strains and clinical isolates to the capsid function inhibitor pleconaril: antiviral studies with virus chimeras demonstrate the crucial role of amino acid 1092 in treatment. Journal of Antimicrobial Chemotherapy. 2005; 56:648-656.

Szurek PF, Yuen, PH, Ball, JK, Wong PK. A Val-25-to-Ile substitution in the envelope precursor polyprotein, gPr80env, is responsible for the temperature sensitivity, inefficient processing of gPr80env, and neurovirulence of ts1, a mutant of Moloney murine leukemia virus TB. Journal of virology. 1990; 64; 467-75.

Tee KK, Lam TTY, Chan YF, Bible JM, Kamarulzaman A, Tong C, Takebe Y, Pybus OG. Evolutionary genetics of human enterovirus 71: origin, population dynamics, natural selection, and seasonal periodicity of the VP1 gene. Journal of virology. 2010; 84:3339-50.

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

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

Yamaguchi Y, Nukui Y, Tajima S, Nerome R, Kato F, Watanabe H, Kurane I. An amino acid substitution (V3I) in the Japanese encephalitis virus NS4A protein increases its virulence in mice, but not its growth rate in vitro. Journal of General Virology. 2011; 92:1601-6.

Yang SL, Chou YT, Wu CN, Ho MS. 2011. Annexin II binds to capsid protein VP1 of enterovirus 71 and enhances viral infectivity. J Virol. 2011; 85:11809-20.

Yu F, Ren X, Wang Y, Qi X, Song J, Gao Y, Wang X. A single amino acid V4I substitution in VP1 attenuates virulence of very virulent infectious bursal disease virus (vvIBDV) in SPF chickens and increases replication in CEF cells. Virology. 2013; 440:204-9.

Zeng M, Li YF, Wang XH, Lu GP., Shen HG, Yu H, Zhu QR. Epidemiology of hand, foot, and mouth disease in children in Shanghai 2007–2010. Epidemiology and infection. 2012; 140: 1122-30.

Figures legends

Figure 1. Phylogenetic analysis of partial VP1 sequences

a) Phylogenetic analysis of the nucleic acid sequences

b) Distribution of the protein sequences

Trees were designed using Maximum Likelihood.

Color code: Black: VVV; Light blue: VII; Yellow: IIV; Purple: VIV; Dark blue: VVI

Figure 2. Factorial correspondence analysis.

Variables analyzed were: amino acid profiles on positions 151, 164 and 186 (V/I), respectively in this work (249, 262 and 284 on the full length VP1 protein), severity level, ethnicity, age of patients, and patient location.

Figure 3. Spatiotemporal distribution of I/V variants

Color code: Black: VVV; Light blue: VII; Yellow: IIV; Purple: VIV; Dark blue: VVI

Table 1. Characteristics of the isolated EV-A71 strains

Strain	Age (month)	Gender	Province	Ethnicity	Onset	Date of collection	Severity	V/I Type	Subgenogroup	Accession number
2003019	12	NA[a]	Hà Nội	NA	13/05/2003	17/03/2003	NA	VIV	C4	KX906272
2005184	0	NA	Quảng Nam	NA	08/05/2005	10/05/2005	NA	VVI	C5	KX906332
2006023	0	NA	Phú Yên	NA	07/04/2006	10/04/2006	NA	VIV	C4	KX906278
2007015	24	NA	Hà Nam	NA	27/01/2007	30/01/2007	NA	IVI	C5	KX906365
2007037	24	NA	Yên Bái	NA	23/03/2007	24/03/2007	NA	IVI	C5	KX906264
2007041	144	NA	Cao Bằng	NA	28/03/2007	30/03/2007	NA	VII	C4	KX906262
2007053	30	NA	Hải Phòng	NA	05/04/2007	08/04/2007	NA	VII	C4	KX906261
2008014	24	1[b]	Nam Định	NA	12/05/2008	14/05/2008	NA	VII	C4	KX906263
2008017	30	1	Ninh Bình	NA	10/05/2008	13/05/2008	NA	VII	C4	KX906267
2008021	30	2[c]	Ninh Bình	NA	14/05/2008	16/05/2008	NA	IVI	C5	KX906299
2008022	24	2	Ninh Bình	NA	14/05/2008	14/05/2008	NA	IIV	C4	KX906273
2008044	30	1	Ninh Bình	NA	15/06/2008	17/06/2008	NA	IVI	C5	KX906300
2008065	30	1	Hải Phòng	1[d]	07/06/2008	10/06/2008	1	IVI	C5	KX906345
2010002	19	2	Bắc Kạn	NA	30/04/2010	02/05/2010	NA	IVI	C5	KX906358
2011011	20	2	Hòa Bình	2[e]	10/06/2011	11/06/2011	1	VII	C4	KX906301
2011020	24	2	Hòa Bình	2	15/06/2011	16/06/2011	1	VII	C4	KX906289
2011022	28	1	Hòa Bình	2	13/06/2011	14/06/2011	1	VII	C4	KX906315

2011031	72	2	Hà Nội	1	19/06/2011	22/06/2011	2a	VIV	C4	KX906338
2011033	26	1	Hòa Bình	2	31/07/2011	01/08/2011	1	VII	C4	KX906274
2011034	48	2	Hòa Bình	1	18/06/2011	19/06/2011	1	VIV	C4	KX906292
2011047	21	1	Sơn La	NA	27/06/2011	01/07/2011	1	VII	C4	KX906269
2011048	22	1	Sơn La	NA	27/06/2011	01/07/2011	2a	VIV	C4	KX906352
2011060	21	2	Thanh Hóa	1	17/06/2011	20/06/2011	2a	VVV	C4	KX906265
2011063	24	1	Hòa Bình	1	11/07/2011	13/07/2011	1	VII	C4	KX906290
2011095	19	1	Hòa Bình	2	20/07/2011	21/07/2011	1	VII	C4	KX906337
2011096	12	2	Hòa Bình	NA	21/07/2011	24/07/2011	1	VII	C4	KX906308
2011097	7	1	Hòa Bình	1	22/07/2011	28/09/2011	1	VII	C4	KX906335
2011117	42	1	Thanh Hóa	1	22/07/2011	25/07/2011	2a	IIV	C4	KX906350
2011123	12	2	Hòa Bình	2	27/07/2011	26/07/2011	1	VIV	C4	KX906281
2011124	9	1	Hòa Bình	2	25/07/2011	26/07/2011	1	VII	C4	KX906329
2011125	13	1	Hòa Bình	NA	27/07/2011	28/07/2011	1	VII	C4	KX906368
2011158	11	2	Hòa Bình	NA	30/07/2011	01/08/2011	1	IVI	C5	KX906309
2011161	21	1	Hòa Bình	2	31/07/2011	31/07/2011	1	VII	C4	KX906296
2011165	22	1	Hòa Bình	1	31/07/2011	01/08/2011	1	VII	C4	KX906349
2011278	21	1	Nam Định	1	16/08/2011	18/08/2011	1	VVV	C4	KX906310
2011282	23	1	Nam Định	1	24/08/2011	24/08/2011	1	IIV	C4	KX906343
2011340	2	1	Lào Cai	2	29/08/2011	30/08/2011	2a	VII	C4	KX906293
2011488	60	2	Hòa Bình	NA	12/09/2011	14/09/2011	1	VII	C4	KX906319

2011490	38	1	Hòa Bình	2	12/09/2011	13/09/2011	1	VII	C4	KX906276
2011521	43	2	Hà Nội	1	18/09/2011	21/09/2011	1	VVV	C4	KX906305
2011571	32	1	Hòa Bình	2	27/09/2011	28/09/2011	1	VIV	C4	KX906331
2011573	12	1	Hòa Bình	NA	26/09/2011	27/09/2011	1	VII	C4	KX906307
2011575	12	1	Hòa Bình	2	23/09/2011	27/09/2011	1	VIV	C4	KX906341
2011579	46	1	Hà Nội	1	28/09/2011	30/09/2011	1	IIV	C4	KX906277
2011586	20	2	Hà Nội	1	23/09/2011	26/09/2011	2a	IIV	C4	KX906366
2011598	26	1	Thanh Hóa	2	27/09/2011	28/09/2011	1	VII	C4	KX906339
2011600	51	1	Bắc Kạn	1	03/10/2011	11/10/2011	2a	VIV	C4	KX906355
2011647	22	2	Thanh Hóa	1	09/10/2011	17/10/2011	2a	VII	C4	KX906268
2011662	11	1	Hà Nội	1	11/10/2011	13/10/2011	1	IIV	C4	KX906297
2011664	16	2	Hà Nội	1	17/10/2011	18/10/2011	2a	IIV	C4	KX906325
2011665	11	2	Hà Nội	1	07/10/2011	10/10/2011	1	IIV	C4	KX906266
2011676	4	1	Điện Biên	2	19/10/2011	22/10/2011	1	IIV	C4	KX906271
2011677	13	1	Điện Biên	1	20/10/2011	23/10/2011	1	IIV	C4	KX906322
2011679	12	1	Thanh Hóa	NA	17/10/2011	21/10/2011	1	IIV	C4	KX906270
2011754	8	1	Hòa Bình	NA	04/10/2011	14/10/2011	1	IIV	C4	KX906334
2011799	12	1	Thanh Hóa	1	22/10/2011	24/10/2011	2b	VII	C4	KX906275
2011805	9	2	Thanh Hóa	2	25/10/2011	28/10/2011	2a	VII	C4	KX906286
2011816	22	1	Điện Biên	2	20/10/2011	28/10/2011	2a	IIV	C4	KX906357
2011823	32	2	Tuyên Quang	1	01/11/2011	11/04/2011	1	IIV	C4	KX906364

2011835	18	1	Hà Nội	1	31/10/2011	02/11/2011	1	VII	C4	KX906313
2011840	15	2	Hà Nội	1	30/10/2011	02/11/2011	2a	VII	C4	KX906340
2011866	26	2	Bắc Ninh	1	06/11/2011	08/11/2011	1	IIV	C4	KX906354
2011868	52	2	Bắc Kạn	1	06/11/2011	15/11/2011	1	VIV	C4	KX906330
2011872	13	2	Điện Biên	1	02/11/2011	04/11/2011	2a	IIV	C4	KX906347
2011881	26	2	Hải Phòng	1	18/11/2011	21/11/2011	2b	VII	C4	KX906360
2011882	25	1	Hải Phòng	1	15/11/2011	21/11/2011	2b	IIV	C4	KX906287
2011888	25	2	Hải Phòng	1	17/11/2011	21/11/2011	2b	VII	C4	KX906333
2011891	26	1	Thanh Hóa	1	20/11/2012	23/11/2011	1	VII	C4	KX906361
2011894	20	1	Hải Phòng	1	19/11/2011	24/11/2011	2a	IIV	C4	KX906312
2011896	17	2	Hải Phòng	1	19/11/2011	24/11/2011	2b	IIV	C4	KX906336
2011897	11	1	Hải Phòng	1	18/11/2011	24/11/2011	2b	VIV	C4	KX906317
2011925	13	2	Hải Phòng	1	24/11/2011	25/11/2011	2b	IIV	C4	KX906363
2011927	41	2	Hòa Bình	1	06/11/2011	09/11/2011	1	VII	C4	KX906283
2011956	64	2	Tuyên Quang	2	15/11/2011	28/11/2011	3	VII	C4	KX906304
2011958	26	1	Tuyên Quang	2	21/11/2011	28/11/2011	3	VII	C4	KX906323
2011970	32	1	Hà Nội	1	13/11/2011	14/11/2011	2a	IIV	C4	KX906298
2011984	13	1	Hải Phòng	1	30/11/2011	04/12/2011	2a	IIV	C4	KX906327
2012018	18	2	Hải Dương	1	12/02/2012	15/02/2012	1	IVI	C5	KX906295
2012019	18	1	Hải Dương	1	13/02/2012	14/02/2012	1	VIV	C4	KX906353
2012053	12	1	Lào Cai	1	25/02/2012	28/02/2012	2a	VIV	C4	KX906359

2012095	20	1	Bắc Giang	1	28/02/2012	01/03/2012	2a	IIV	C4	KX906367
2012114	20	1	Lào Cai	1	01/03/2012	02/03/2012	2a	VII	C4	KX906303
2012117	20	1	Lào Cai	2	04/03/2012	06/03/2012	1	IVI	C5	KX906280
2012126	11	1	Hải Phòng	1	01/03/2012	06/03/2012	2b	IIV	C4	KX906346
2012151	13	1	Hải Dương	1	11/03/2012	13/03/2012	1	VII	C4	KX906320
2012159	36	2	Phú Thọ	1	27/02/2012	02/03/2012	2a	VIV	C4	KX906314
2012161	16	1	Phú Thọ	1	03/03/2012	07/03/2012	2a	VIV	C4	KX906302
2012164	27	1	Phú Thọ	2	27/02/2012	29/02/2012	1	IIV	C4	KX906306
2012165	54	1	Phú Thọ	2	22/02/2012	22/02/2012	1	IIV	C4	KX906362
2012166	14	1	Phú Thọ	1	04/03/2012	09/03/2012	1	VIV	C4	KX906356
2012169	5	2	Phú Thọ	1	06/03/2012	09/03/2012	1	IVI	C5	KX906294
2012189	15	1	Hải Dương	NA	10/03/2012	15/03/2012	1	VIV	C4	KX906285
2012225	7	1	Hà Nội	1	07/03/2012	12/03/2012	3	VIV	C4	KX906348
2012233	40	1	Bắc Kạn	2	01/03/2012	16/03/2012	1	IIV	C4	KX906351
2012237	84	2	Bắc Giang	1	13/03/2012	16/03/2012	2b	IVI	C5	KX906288
2012260	25	1	Thanh Hóa	1	18/03/2012	20/03/2012	1	VIV	C4	KX906311
2012262	30	1	Thanh Hóa	NA	12/03/2012	15/03/2012	NA	VIV	C4	KX906328
2012264	9	1	Thanh Hóa	1	20/03/2012	23/03/2012	1	IVI	C5	KX906284
2012271	34	1	Ninh Bình	1	19/03/2012	20/03/2012	1	IVI	C5	KX906279
2012284	44	2	Hải Phòng	1	18/03/2012	20/03/2012	2b	IVI	C5	KX906282
2012296	24	2	Sơn La	NA	20/03/2012	22/03/2012	NA	VII	C4	KX906344

2012297	22	1	Tuyên Quang	1	14/03/2012	27/03/2012	1	VII	C4	KX906342
2012300	28	1	Tuyên Quang	1	18/03/2012	27/03/2012	1	VIV	C4	KX906326
2012319	57	2	Bắc Kạn	2	27/03/2012	27/03/2012	1	VIV	C4	KX906318
2012353	17	2	Hà Nội	1	31/03/2012	02/04/2012	1	VII	C4	KX906316
2012387	17	2	Lai Châu	1	06/04/2012	09/04/2012	2a	IVI	C5	KX906291
20111000	14	2	Hải Phòng	1	05/12/2011	08/12/2011	2b	IIV	C4	KX906324
20111003	19	1	Hải Phòng	1	04/12/2011	09/12/2011	2b	VIV	C4	KX906321

a) NA: Not Available

b) Gender 1 = Male

c) Gender 2 = Female

d) Ethnicity 1 = Main Vietnamese ethnic group

e) Ethnicity 2 = Hmong ethnic minority

Table 2. Polymorphism and divergence data

	N	Hp	Nt	S	η	Pa	Si	θ	MC	Na	Ns	Ka/Ks
Cluste	56	43	561	67	70	48	19	14.58	64	6	58	0.053
Cluste	23	15	561	15	15	9	6	4.06	15	6	9	0.107
Cluste	12	10	561	19	19	11	8	6.92	16	0	16	0.029
Cluste	17	13	561	109	113	57	52	32.24	110	5	105	0.011
Total		108	81	561	148	162	128	20	28.16	147	9	138	0.019

N: Number of sequences

Hp: Number of haplotypes

Nt: Sequence size in nucleotides

S: Number of mutated sites

η: Number of mutations

Pa: Number of parsimony informative sites

Si: Number of singletons

: Number segregating sites (per sequence from S)

MC: Number of mutated codons

Na: Number of non-synonymous mutations

Ns: Number of synonymous mutations

Ka/Ks: Ka/Ks ratio

F1 + F2 = 34,2 %

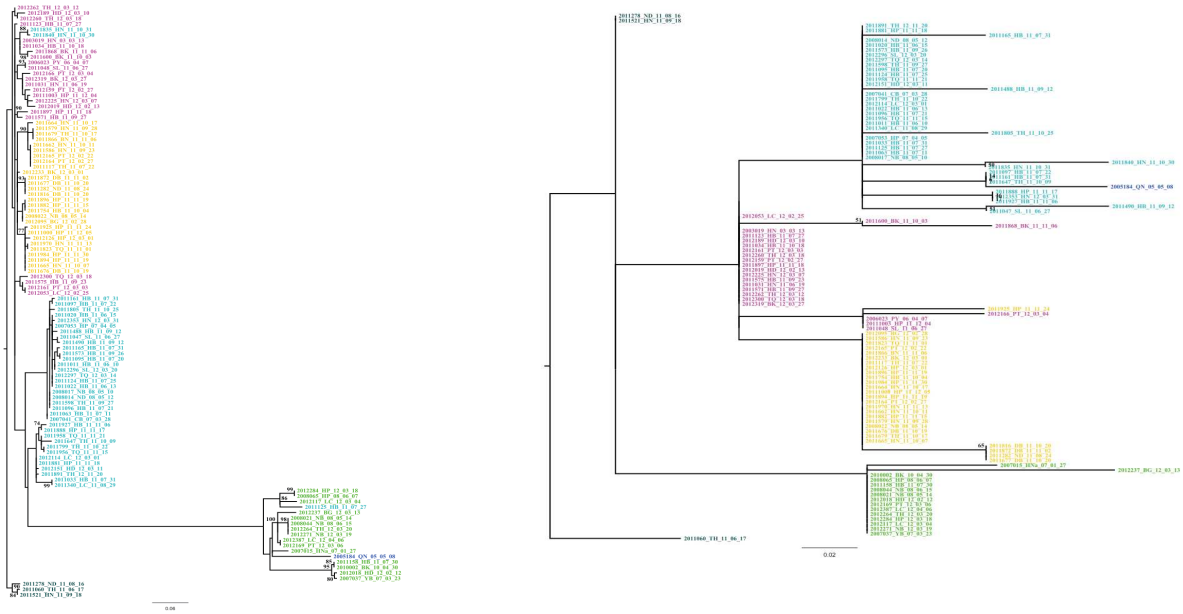
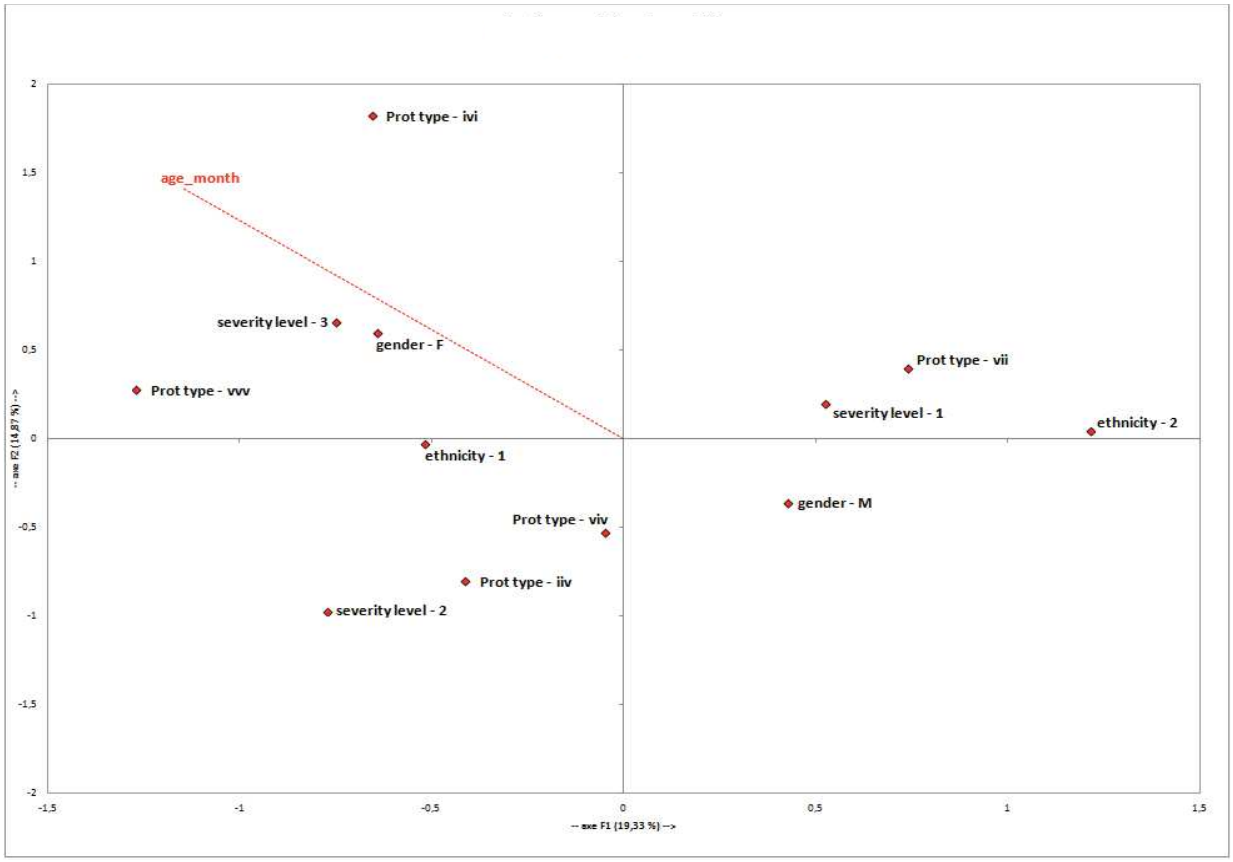
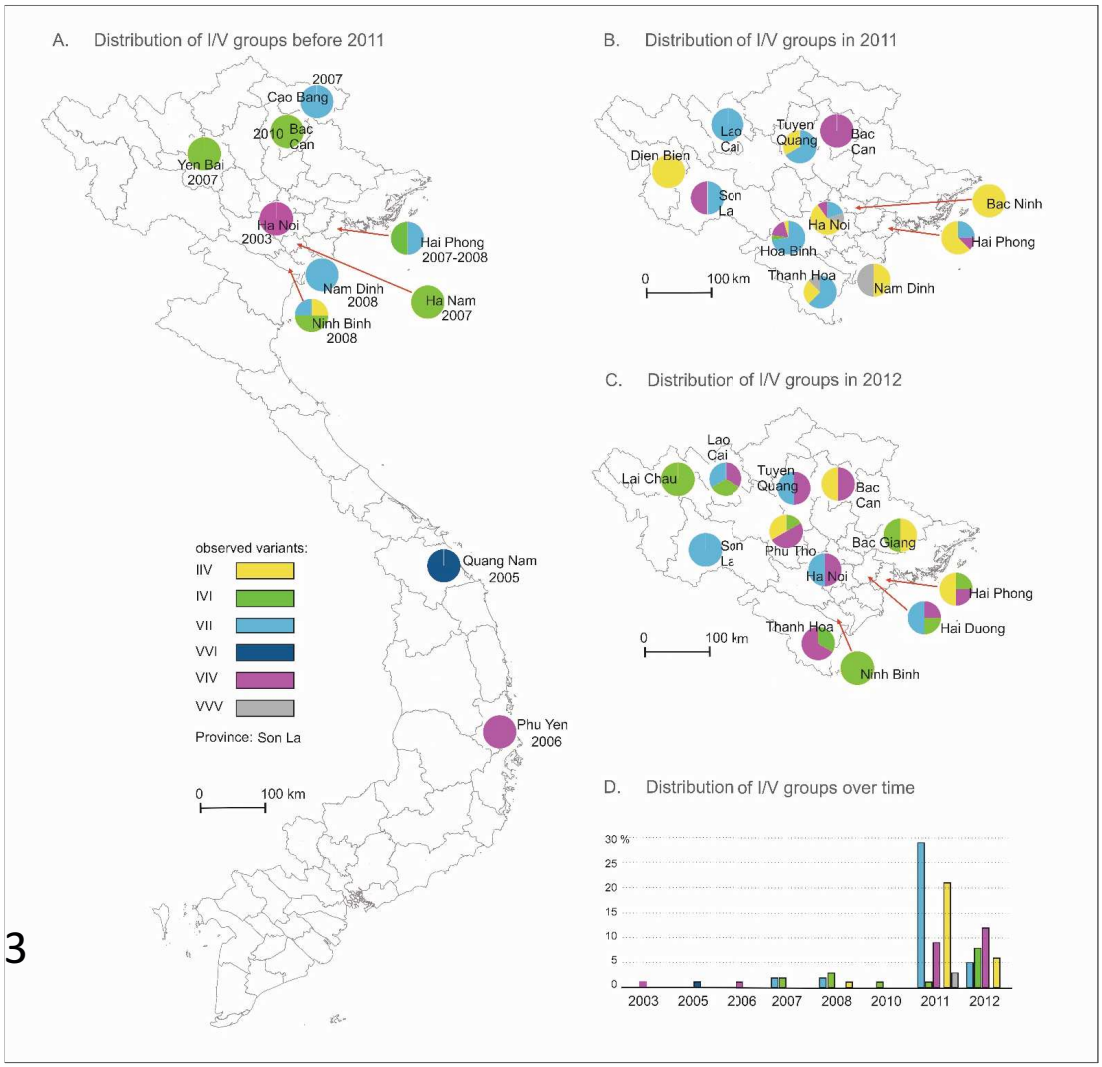


Figure 1

gure 2




A. Distribution of I/V groups before 2011
2007
Cao Bang
2010
Bac Can
Yen Bai
2007
Ha Noi
2003
Hai Phong
2007-2008
Nam Dinh
2008
Ha Nam
2007
Ninh Binh
2008
Quang Nam
2005
Phu Yen
2006
observed variants:
IIV
IVI
VII
VVI
VIV
VVV
Province: Son La
0
100 km
B. Distribution of I/V groups in 2011
Lao Cai
Tuyen Quang
Bac Can
Dien Bien
Son La
Bac Ninh
Ha Noi
Hai Phong
Hoa Binh
Thanh Hoa
Nam Dinh
0
100 km
C. Distribution of I/V groups in 2012
Lao Cai
Lai Chau
Tuyen Quang
Bac Can
Son La
Phu Tho
Bac Giang
Ha Noi
Hai Phong
Hai Duong
Thanh Hoa
Ninh Binh
0
100 km
D. Distribution of I/V groups over time
30 %
25
20
15
10
5
0
2003
2005
2006
2007
2008
2010
2011
2012

igure 3

CHAPTER 3. MODEL OF A MULTIPHASE HFMD EPIDEMIC

1.1. Context of study

Hand, foot and mouth disease (HFMD) is usually a multiphase disease involving often a succession of different viruses. HFMD is not the only disease displaying this characteristic. A major problem in epidemiology is to distinguish the different phases and therefore to understand at what time a novel virus, with differing pathogenicity and virulence charcteristics, was involved in the ongoing epidemics and has replaced the previous causative agent. Although veru often mild, HFMD can result in severe complications such as encephalitis, aseptic meningitis, pulmonary edema, myocarditis, and death (WHO, 2011). This large panel of symptoms and variation over time depends for a good parts on the succession of viruses driving the epidemics. HFMD is caused by different viruses members of Human Enterovirus A, a family of picornavirus which includes Coxsackievirus A (CV-A) and Human Enterovirus 71 (EV-A71) (Ang et al., 2009; Chen et al., 2007). This diversity of agents along with the aquired immunoresistance developed by patients generates a high dynamic of the disease which could become difficult to monitor and control in some cases. Such outbreaks occurred for instance in Cambodia in July 2012 causing the death of 52 young children out of 59 cases.

However, if properly monitoring the disease is essential to detect the presence of a potential novel agent and adapt treatments and precautionary measures, it is not really possible to determine when a novel virus has replaced a previous one. For instance, during the 2011-2012 HFMD epidemics in Hai Phong, novel guidelines were edicted but in the middle of phase two and without any ink with the etiology.

Current mathematical models are capable to represent a single phase and several phases in a separate, individual way, but not a series of several waves within a single outbreak as it is the case with the 2011-2012 HFMD outbreak in Hai Phong and more generally with many diseases. Such models cannot be developed owing the lack of

appropriate cohorts, large enough in size. In this study, we could have access to a large cohort allowing thus to develop a modelling approach.

1.2. Objective

The objective of this work was thus to take advantage of the very large number of cases collected (more than 9000) with a comprehensive and consistent clinical and epidemiological record, to develop a mathematical model capable of describing a multiphase epidemics. A second objective was to confront this model with actual data.

1.3. Discussion and conclusion

A model was developed based on Bernoulli differential equations to assess in a simple and accurate way the number of patients who present a community of symptoms as a function of time. The model is based on the following postulate: 1) If all along the epidemic the virulence is the same, then this epidemic is considered a single-wave epidemic. 2) If during the outbreak differential levels of virulence and/or pathogenicity are recorded, then this epidemic is considered a multi-wave epidemic. A single-wave outbreak can be considered as caused by a single pathogen or, but less likely, by different pathogens expressing the same phenotypic traits. A multi-wave epidemic is considered as caused by a series of successive causative agents diplaying different phenotypic traits.

The model presented in this chapter allows to show that during the 2011-2012 Hai Phong epidemic:

1) The waves were clearly present

2) The level of interaction between the virus and the host, i.e. virulence and pathogenicity, was different for each wave

3) Virulence was a discriminating factor, each wave displaying a different speed

Wave 1 was characterized by the highest speed (highest virulence)

Wave 2 was characterized by an intermediate speed (intermediate virulence).

Wave 3 was characterized by the lowest speed (lowest virulence)

4) Starting from dates clinically assessed the model is capable to assign the beginning and end of each wave, with a highly significant fit, and to show that waves overlap in part, each wave starting before the end of the previous one. The model can thus well describe the replacement of virus along the epidemic.

5) It is possible to calculate the probability for a patient to belong to a given wave and to classify the patients in specific clusters with a high level of confidence.

6) The patient clusters can be associated with a specific typology of symptoms

7) It is possible to estimate the end of an ongoing wave and to determine if a wave if starting, raising thus the possibility of a differing clinical context.

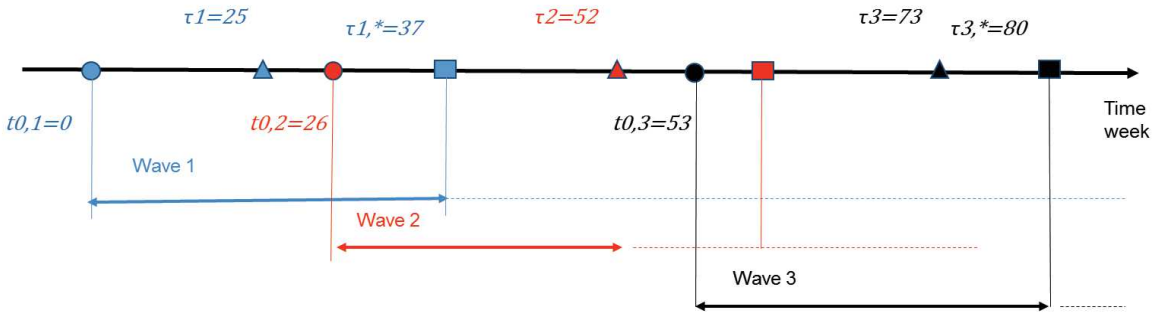


Figure 1

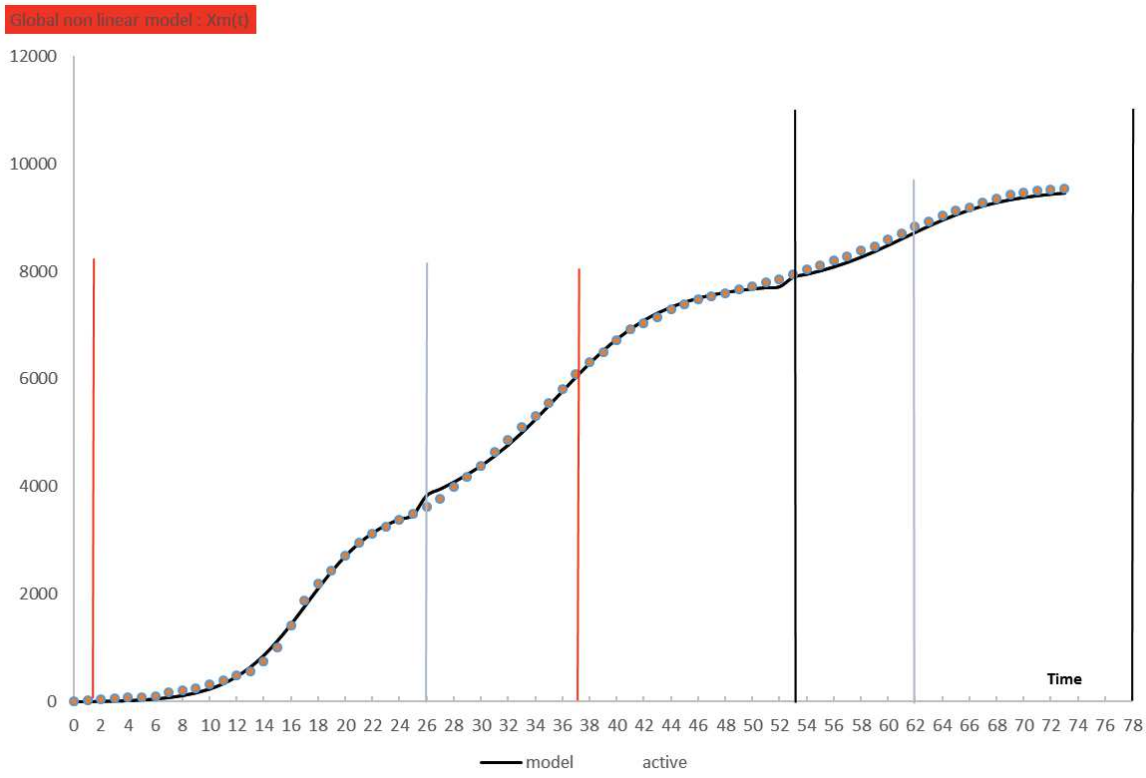


Figure 2

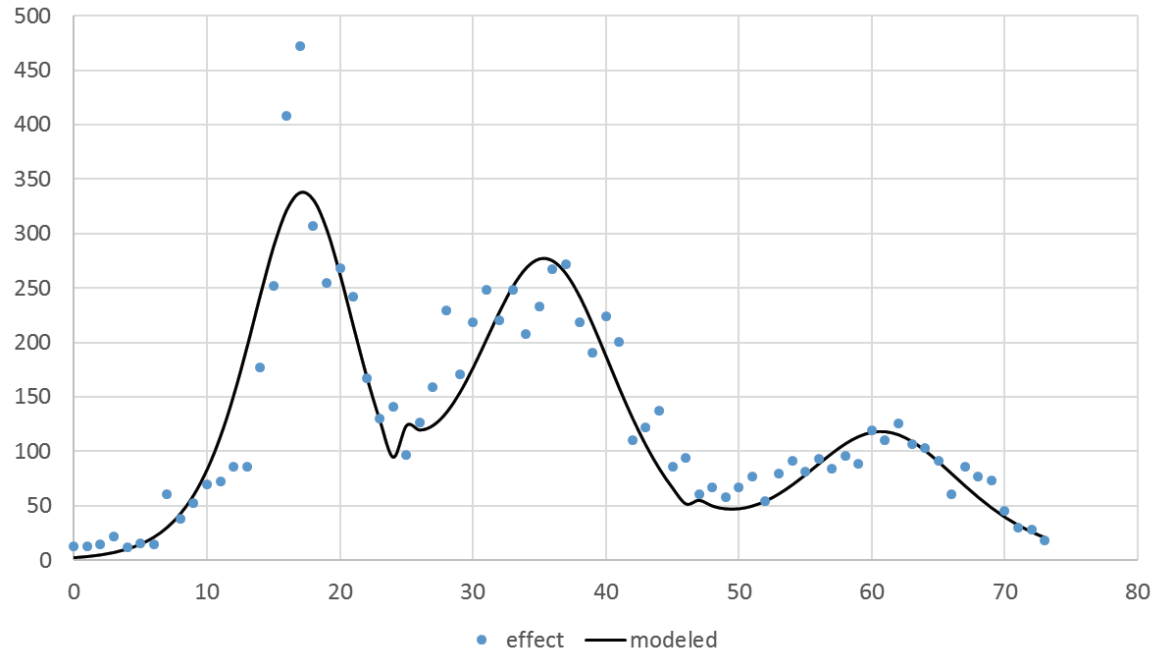


Figure 3

The perspective with this part of the work is now to assess the predictive limit of the model and to determine the minimum number of patients required to identify a new wave. This would in turn permit the identification of the viral lineage involved and take necessary actions to adapt treatments and precautionary measures.

The results of this work are summarized in the manuscript entitled article entitled “Modelling the dynamic of Hand Foot and Mouth Disease during the 2011-2012 Hai Phong outbreak”. This manuscript, currently under preparation, is presented thereafter.

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Modeling the dynamic of Hand, Foot and Mouth Disease during the 2011-2012 Hai Phong outbreak

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Running title: Mathematical modelling of HFMD

Keywords:

Abstract

The proposed approach is a general method to model over time a multi-wave epidemic. The originality of this model is to be based on the detection of differing virulence/pathogenicity. If the same rate of virulence/pathogenicity is observed over time it is a single, long wave epidemic which can analyzed with existing models. If difference of virulence/pathogenicity occurs it is a sign of a multi-wave epidemic which cannot be analyzed by currently available models ad for which the model described in this work was pecifically designed. This model was developed using the HFMD outbreak that spread over the city of Hai Phong from 08/2011 to 10/2012. This epidemic displayed 3 separate waves. A complete dataset was collected for more than 8000 patients during this period, allowing thus to develop the model with a good statistical confidence. The model showed a high accuracy at the adjustment of data for both the total number of cases and for the number of cases per week. As a consequence, the model was able to accurately determine the dates of beginning and end of each wave and to show that they overlapped. Using mathematical functions associated with this model, it was possible to calculate the probability for a patient to belong to a specific wave. This model is applicable to other diseases and can detect relatively early the occurrence of a differing wave and thus trigger appropriate clinical and preventive actions.

Introduction

Hand, foot and mouth disease (HFMD) is often a multiphase disease involving a succession of different viruses and waves. The co-circulation of different serotypes and alternation between enterovirus A71(EV-A71) and coxackievirus A (CV-A) were commonly observed during HFMD epidemics [1], [2], [3], [4]. However, co-infection is not a primary cause of severe forms [5], [6]. Epidemics could be due to both the accumulation of susceptible individuals in the community, patients are mostly young children under five and introduction of new genotypes or strains into the Asia-Pacific region [30]7. Outbreaks in Taiwan in 1998 and 2000 were caused by EV-A71 C2 and B4 strains, respectively [8]. Sentinel surveillance in Sarawak, Malaysia, demonstrated that the emergence of the novel subgenotype C1 of EV-A71 was the cause of the 2003 outbreak [9]. Although very often mild, HFMD can result in severe complications such as encephalitis, aseptic meningitis, pulmonary edema, myocarditis, and death [10] and can be devastating like in Cambodia in 2012 with a death rate of 88% ([11]. This large panel of symptoms and variation over time depends for a good parts on the succession of viruses driving the epidemics.

A major problem in epidemiology is to distinguish the different phases and therefore to understand at what time a novel virus, with differing pathogenicity and virulence characteristics, has replaced the previous causative agent. Understanding the process of emergence of disease can be related to the ability to identify type samples from outliers. Dynamic models are therefore required for analyzing epidemics. However, if current models can analyze individual waves [12], there is still a need to model and compute a mutliwave epidemic. The 2011-2012 HFMD was the largest experienced in Vietnam and the first one to occur in North Vietnam. Hai Phong displayed the highest prevalence in North Vietnam providing thus a large cohort of ca. 9000 patients to analyze. This 2011-2012 outbreak in Hai Phong was also a three-wave epidemic involving EV-A71, CV-A6, CV-A16 and a still uncharacterized virus [13]. Based on this large cohort we report here a mathematical approach for discrimination of individuals within an outbreak time frame and a mathematical model for characterizing multi-wave outbreaks displaying differing wave-associated virulence or pathogenicity.

Results and discussion

1. Model

The number of cases over the one-year period of the study displayed three different waves, each separated by a minima, i.e. a drop in the number of cases followed by an increase. Each step was analyzed separately using the same Bernoulli model.

In a first step, we assume that each wave is relatively well separated. It means when a wave is closing than the next wave is starting in a sense to be specified later. The function was therefore aiming at describing HFMD cases over time. The total number of HFMD cases for one wave is N. Over a defined period h, the number of new cases depends of the number of susceptible patients in the population Xs(t) and two parameters related to the epidemic: the probability K(t) to be exposed to the virus and K0 the probability to present clinical symptoms. These two probabilities reflect at epidemiological level two the virus associated parameters which are virulence and pathogenicity, respectively.

The cumulative number Xm(t+h) of patients, is governed by the equation for **one wave**:

$$X_m(t+h) = X_m(t) + K_0 K(t) X_S(t) * h, t \geq t_0 \quad (1)$$

with:

$$X_s(t) = N - X_m(t) \text{ and } K(t) = \frac{X_m(t)}{N},$$

The dot K(t) Xs(t) is the expected number of exposed patients. The constant K0 is called interaction factor. This is the probability of an exposed patient to become sick.

By passing to the limit as h tends to 0, we obtain a differential equation of Bernoulli (2)

$$\frac{X_m^{'}(t)}{X_m^2(t)} - K_0 \frac{1}{X_m(t)} + \frac{K_0}{N} = 0, \forall t \geq t_0 \text{ and } X_m(t) \neq 0 \quad (2)$$

with the two limits conditions:

Indiquer le changement de variable pour resoudre l'équation

The general solution of equation (2) is the well-known logistic function defined by three parameters (K0, K1, K2):

$$X_m(t) = \frac{K_2}{(1+\exp(-K_1 - K_0(t-t_0))}, \forall t \geq t_0 \quad (3)$$

With the help of the vector ***Y*** of observed values $Y(t_i)_{0 \leq i \leq k}$, the constant K0, K1, K2 are computed with a jacquard algorithm (ref) by minizing

$$Ec(K_0, K_1, K_2) = \sum_{i=0}^{\tau} \left(y(t_i) - X_m(t_i) \right)^2 \quad (4).$$

Note that τ_s is the experimental end of the wave. This corresponds to the time of the last observed case of a given wave. The time t0 is the date of the first case observed for a given wave. Nevertheless, equation (3) can be interpreted with two limits conditions:

$$\lim_{t \to +\infty} Xm(t) = N \; and \; Xm(t0) = N_0$$

N and N0 are theoretical values. They are respectively the total number of cases at the theoretical end of the waves $t = +\infty$ and the number of cases at the beginning of the wave. With equation (3), we have N=K2 and $N_0 = \frac{K_2}{(1+\exp(-K_1)}$).

Owing to the limits conditions and N>N0, it is possible to demonstrate that the interaction factor K0 is positive and less than 1. Therefore, this factor can be seen as a probability.

2. Wave specific model

We assume that the time of the beginning of each wave (t0) is known. Therefore, the beginning of the wave 2: $t_{0,2}$ occurred after the date of the last observed case of the wave 1 τ_1. The same condition is assumed for wave 2 and wave 3. This means that: $t_{0,2} > \tau_1$ and $t_{0,3} > \tau_2$. These dates are provided by physicians and clinical files. The theoretical time of the end of a wave is therefore t=+00. τ^* is an under estimation of this date. It is defined as: $\tau_* = \inf\{t/|Xm(t) - N| < \epsilon\}$, (Figure 1).

To compute values (K0, K1, K2) of equation (3), we first began with wave 1 using the Jacquard algorithm, (Table 1 and Figure 2.a). The end of wave τ_{1*} was estimated to occur at the 37th week. For wave 2, the observed frequencies were therefore corrected between the following date: beginning of the wave 2: $t_{0,2} = 26$ and $\tau_{1*} = 37$ (numbers express weeks). The correction involved subtracting the frequencies of wave 1 to wave 2 of those observed during the reporting period. The corrected frequencies allowed thus to take into account the overlapped of the two consecutive waves before applying the jacquard algorithm to wave 2, (Table 1). The end of wave 2 was estimated at week 62. As the observed frequencies were underestimated compared with the mathematical model (Figure 2.b), no correction was necessary for wave 3 (Figure 2.c). The computed parameters for wave 3 are given in Table 1. For all the waves, the quality of fit was high with a coefficient of determination R^2 higher than 0.997, (Figure 2). The computation of the total number of cases:

$$X_W(t) = X_{m,1}(t)I_{[0,+\infty[} + X_{m,2}(t)I_{[26,+\infty[} + X_{m,3}(t)\, I_{[53,+\infty[}, \forall t \geq t_0 \quad (4),$$ during the three waves is shown in Figure 3.

Parameters of interaction between disease and environment

K0 parameter represents the observed speed of spread of the outbreak (Table 1). Wave 1 was significantly ($p<0.05$) the most virulent, followed by wave 3 and wave 2 in decreasing order.

Patient classification

With the help of the definition of $X_M(t)$ (3) applied to a wave W, it is possible to build the probability for an individual to belong to a specific wave. Indeed, with a specific constant A

$$f_w(t) = \frac{1}{A} X_m(t - t_0), \forall t \geq t_0 \text{ with } A = \int_{t_0}^{\infty} X_m(t - t_0)\,dt \quad (4),$$

fw(t) is a density probability. So

$$P\left(t_0 \leq u \leq T \middle/ W\right) = \int_{t_0}^{T} f_w(t - t_0)\,dt,$$

is the probability to have an inclusion time between [t0, T] when a patient belongs to a given wave W. It is therefore possible to extract patients belonging to a specific wave with a certain probability and then to express the probability for an individual to belong a specific wave..

Mapping the occurrence of cases for each wave

HFMD cases were mapped for each wave for 14 days after the observed beginning of the wave (Figure 4). The observed beginning of the waves was selected as a starting point to avoid the overlap of waves.

Discussion

This work was motivated by the need to develop a model capable of describing at once a multiwave epidemic and by the availability of a very large cohort making possible the computation and validation of the model. The model developed in this work is a simple model based on the virulence/pathogenicity constant, in other words, on the differing dynamic of host-virus interaction. The model is based on the principle that the key factor is not the overall number of patients but indeed the differing dynamic of host-virus interaction. Compiling the number of cases has for consequence to smooth all data and to show the epidemic as monomodal. Regardless of its true nature, the epidemic is considered associated with a single causative agent. The model developed in this work allows to breakdown the epidemic in its various components when they exist and display the epidemic as a multimodal curve. In this case each mode corresponds to a specific agent displaying a specific dynamic.

The model demonstrates that three distinct waves occurred during the 2011-2012 Hai Phong HFMD epidemic, each one displaying a specific dynamic of expansion. The level of host-virus interaction, in particular the virulence, was different for each wave. Virulence was therefore a discriminating factor, each wave displaying a different speed, the highest virulence was associated to wave 1. Wave 2 displayed an intermediate speed whereas wave 3 was characterized by the slowest speed of expansion.

The model developed in this work also allowed to assign the beginning and end of each wave with a highly significant fit with observed data. It was thus possible to show that the three waves partly overlapped, each wave starting before the beginning of the previous one. This indicates that the replacement of causative agent occurs during the span a given wave and not after the wave. It is therefore difficult to only explain this phenomenon by an adaptation of the human population through immunoresistance. A hypothesis could be that each causative agent is capable of infecting only a specific, susceptible part of the human population and disappears when the available naive population falls below a given threshold. However, it is in this case difficult to explain why all viral strains do not expand at the same time providing they do not target the same populations and why there is a pattern of successive waves. The relative virulence of each viral strain might therefore play

a key role, in particular in asymptomatic patients where competition between strains might occur. Nevertheless, this also suggests that all strains circulate at the same time and that a given strain will predominate and expand depending on the ratio immunoresistance/virulence. Although we cannot clearly explain the phenomenon, the model developed in this work can thus well describe the replacement of virus along the epidemic and accurately determine the start and end of each individual component of the epidemic.

Beyond that, the model is also capable of addressing the very important issue of calculating the probability of a patient to belong to a given wave and to classify the patients into specific clusters with a high level of confidence. These clusters can be associated with a specific typology of symptoms and dynamic traits. Doing so, the model allows to estimate during the span of the epidemic if a new wave occurred, before the peak of this new wave and to identify the patients affected by this new wave comparatively with patients affected by the previous wave. This in turn open ways for clinician to determine if a new treatment strategy or a new crisis management is needed and if so provides also the means of identifying the patients to be addressed. The most important aspect being that this can be done during the span of the epidemic. The model provides thus means to facilitate real time actions.

Beyond the specific case of HFMD, the model described in this work can be applied to other diseases. If the disease considered is a single phase, monomodal disease caused by a single agent, the model will bring nothing more than existing single-wave models. Similarly, if the disease is a multiphase, multimodal, disease caused by different agents but displaying the same virulence/pathogenicity traits, it can thus be considered similar to a single wave disease and here also the model will bring nothing more than existing single-wave models. However, if the disease is a multiwave disease involving causative agents characterized by differing virulence / pathogenicity traits, this model will bring very useful applications for managing this epidemic and for identifying an emerging wave associated to a potentially new strains. This work and the model presented can therefore bring very valuable support to public health in the management of multiwave infectious diseases. Indeed, it is to our knowledge the first time such a multiwave model with a very high fit

with observed data, capable of typing patients based on clinical description and determining the emergence of a new wave has been developed. Since it is not relaying on molecular or serological test, but only on clinical parameters, the model is fast and easy to implement with no delay in response. The implementation of such model on HFMD and other multiwave diseases will therefore bring valuable support in managing these important sanitary burdens.

References

1. P. V. Tu, N. T. T. Thao, D. Perera, T. K. Huu, N. T. K. Tien, T. C. Thuong, O. M. How, M. J. Cardosa, and P. C. McMinn, “Epidemiologic and virologic investigation of hand, foot, and mouth disease, southern Vietnam, 2005,” Emerg. Infect. Dis., vol. 13, no. 11, pp. 1733–1741, Nov. 2007.
2. T. H. Khanh, S. Sabanathan, T. T. Thanh, L. P. K. Thoa, T. C. Thuong, V. thi T. Hang, J. Farrar, T. T. Hien, N. van V. Chau, and H. R. van Doorn, “Enterovirus 71-associated hand, foot, and mouth disease, Southern Vietnam, 2011,” Emerg. Infect. Dis., vol. 18, no. 12, pp. 2002–2005, Dec. 2012.
3. C. Xiang, “Detection of human enterovirus 71 and coxsackievirus A16 in children with hand, foot and mouth disease in China,” Mol. Med. Rep., Jan. 2012.
4. F. Yang, L. Ren, Z. Xiong, J. Li, Y. Xiao, R. Zhao, Y. He, G. Bu, S. Zhou, J. Wang, and others, “Enterovirus 71 outbreak in the People’s Republic of China in 2008,” J. Clin. Microbiol., vol. 47, no. 7, p. 2351, 2009.
5. K. P. Chan, K. T. Goh, C. Y. Chong, E. S. Teo, G. Lau, and A. E. Ling, “Epidemic hand, foot and mouth disease caused by human enterovirus 71, Singapore,” Emerg. Infect. Dis., vol. 9, no. 1, p. 78, 2003.
6. M. H. Ooi, S. C. Wong, Y. Podin, W. Akin, S. del Sel, A. Mohan, C. H. Chieng, D. Perera, D. Clear, D. Wong, and others, “Human enterovirus 71 disease in Sarawak, Malaysia: a prospective clinical, virological, and molecular epidemiological study,” Clin. Infect. Dis., vol. 44, no. 5, p. 646, 2007.
7. H. Shimizu, A. Utama, N. Onnimala, C. Li, Z. Li-Bi, M. Yu-Jie, Y. Pongsuwanna, and T. Miyamura, “Molecular epidemiology of enterovirus 71 infection in the Western Pacific Region,” Pediatr. Int. Off. J. Jpn. Pediatr. Soc., vol. 46, no. 2, pp. 231–235, 2004.
8. J. R. Wang, Y. C. Tuan, H. P. Tsai, J. J. Yan, C. C. Liu, and I. J. Su, “Change of major genotype of enterovirus 71 in outbreaks of hand-foot-and-mouth disease in Taiwan between 1998 and 2000,” J. Clin. Microbiol., vol. 40, no. 1, p. 10, 2002.
9. Y. Podin, E. L. M. Gias, F. Ong, Y.-W. Leong, S.-F. Yee, M. A. Yusof, D. Perera, B. Teo, T.-Y. Wee, S.-C. Yao, S.-K. Yao, A. Kiyu, M. T. Arif, and M. J. Cardosa,

"Sentinel surveillance for human enterovirus 71 in Sarawak, Malaysia: lessons from the first 7 years," BMC Public Health, vol. 6, p. 180, 2006.

10. WHO WPRO, "A Guide to Clinical Management and Public Health Response for Hand, Foot and Mouth Disease (HFMD)," WHO WPRO, 2011. [Online]. Available: http://webcache.googleusercontent.com/search?q=cache:Dtg4fDp6fqcJ:www.wpro.who.int/publications/PUB_9789290615255.htm+A+guide+to+clinical+management+and+public+health+response+for+Hand,+Foot+nad+Mouth+Disease&cd=2&hl=en&ct=clnk&gl=fr&client=firefox-a.
11. Duong, V., Mey, C., Eloit, M., Zhu, H., Danet, L., Huang, Z., ... & Laurent, D. (2016). Molecular epidemiology of human enterovirus 71 at the origin of an epidemic of fatal hand, foot and mouth disease cases in Cambodia. Emerging Microbes & Infections, 5(9), e104.
12. Patrice – REF Models
13. Nghia Ngu Duy, Le Thi Thanh Huong, Patrice Ravel, Le Thi Song Huong, Ankit Dwivedi, October Michael Sessions, Yan'An Hou, Robert Chua, Guilhem Kister, Aneta Afelt, Laurent Gavotte, Catherine Moulia, Christian Devaux, Duane J Gubler, Vu Dinh Thiem, Nguyen Thi Hien Thanh, Tran Nhu Duong, Nguyen Tran Hien, Emmanuel Cornillot, Roger Frutos. Monitoring influence of Hand, Foot and Mouth Disease new guidelines on patients care during 2011-2012 outbreak in Hai Phong City, Vietnam. Asian Pacific Journal of Public Health.

Table 1.

Wave	Parameter	Value	Standard error
1	K1	-6.33	0.209
	K0	0.368	0.014
	K2	3673.906	67.640
2	K1	-2.541	0.075
	K0	0.259	0.009
	K2	4104.45	44.29
3	K1	-2.28	0.052
	K0	0.275	0.008
	K2	1746.58	21.290

K0 is the interaction factor

For wave 2, parameters displayed are the corrected parameters

Figures legends

Figure 1. Temporal characteristics of the 2011-2012 HFMD epidemic

T0 is the beginning of a wave.

The time τ corresponds to the last known case for a given wave.

The time τ^* is an estimation of τ obtained by the model (equation 3).

Figure 2. Representation of the dynamic of the outbreak for the three different waves

2a. Representation of the dynamic for wave 1

The observed t0 is 0 for wave 1

2b. Representation of the dynamic for wave 2

The observed t0 is 26 for wave 2

2c. Representation of the dynamic for wave 3

The observed t0 is 53 for wave 3

Dots are associated to the observed values Y(t(i)).

The solid curve is the theoretical number of case.

Figure 3. Representation of the dynamic of the outbreak for the three waves together

Dots are associated to the observed values Y(t(i)).

The solid curve is the theoretical number of case.

Figure 4. Location of the initial foci of each of the three HFMD waves in Hai Phong

a) First wave

b) Second wave

c) Third wave

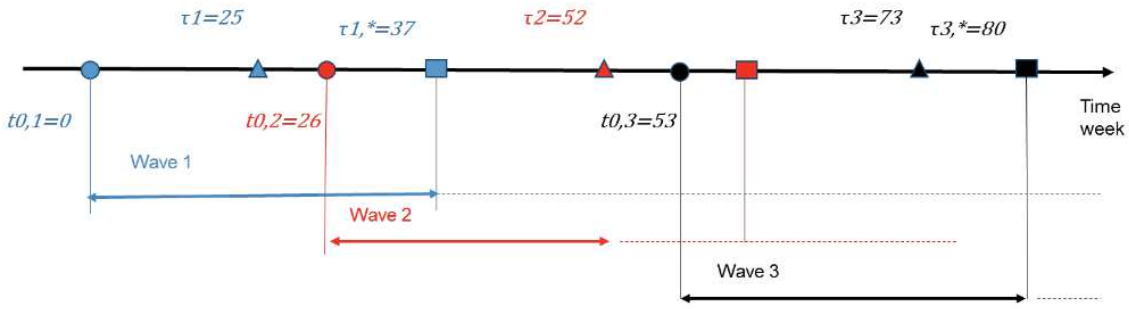

Figure 1

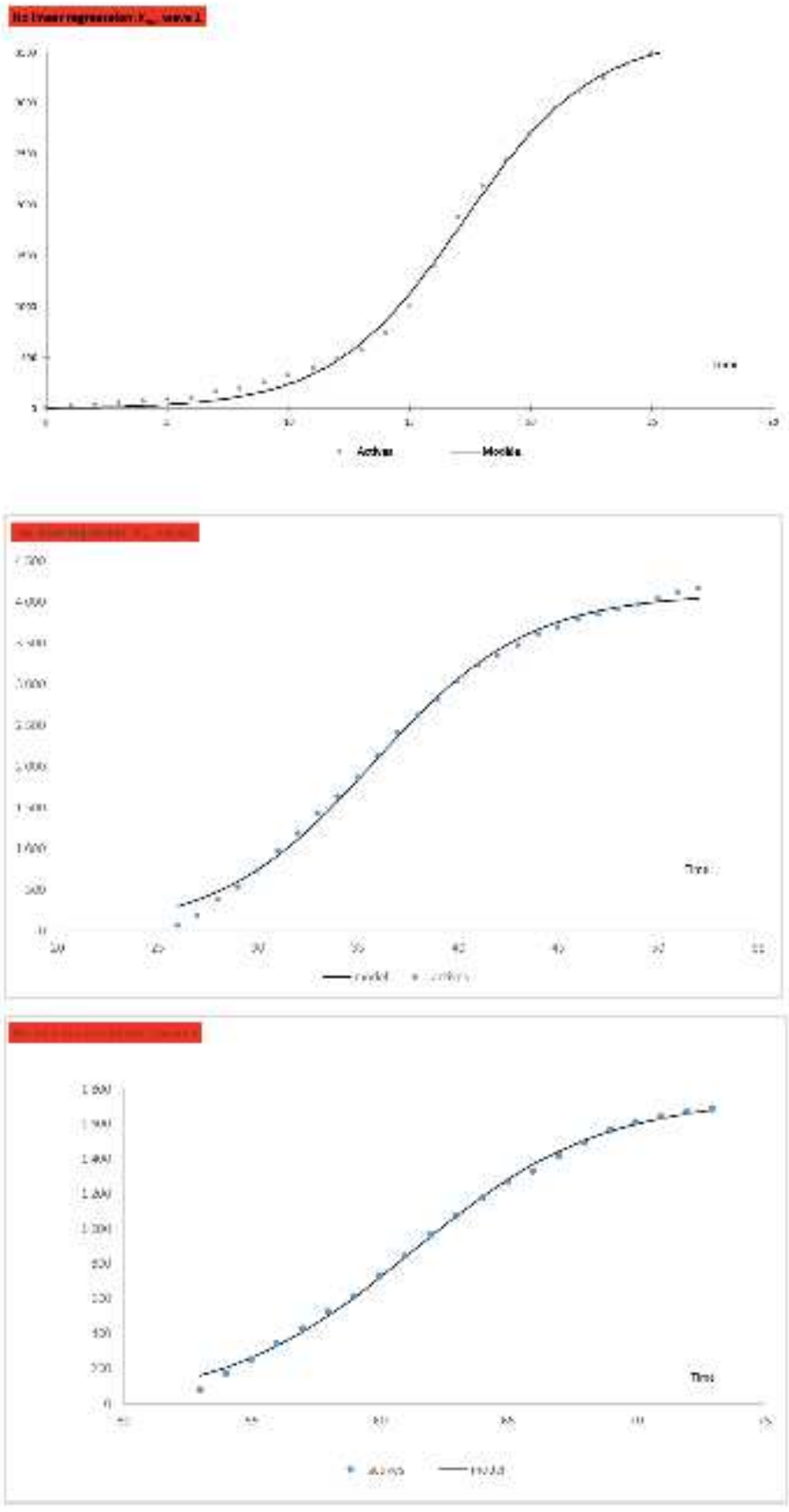

Figure 2

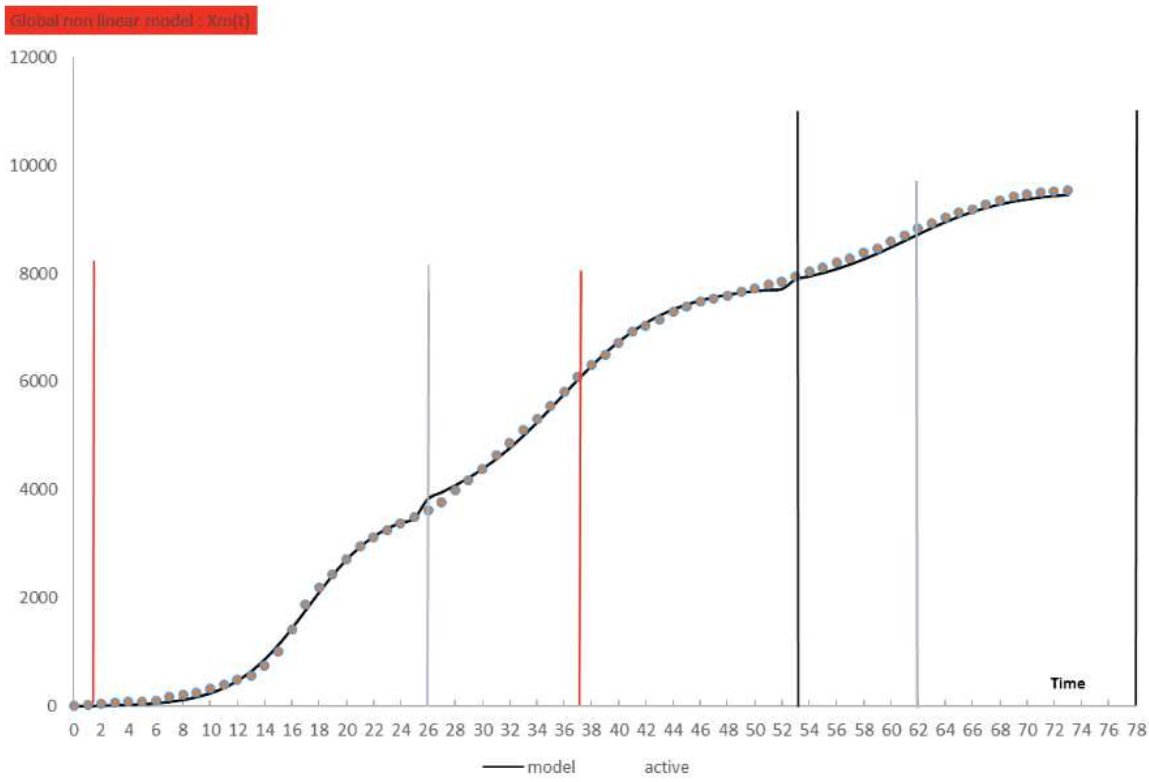
Global non linear model : Xns(t)
12000
10000
8000
6000
4000
2000
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58 60 62 64 66 68 70 72 74 76 78
Time
model
active

Figure 3

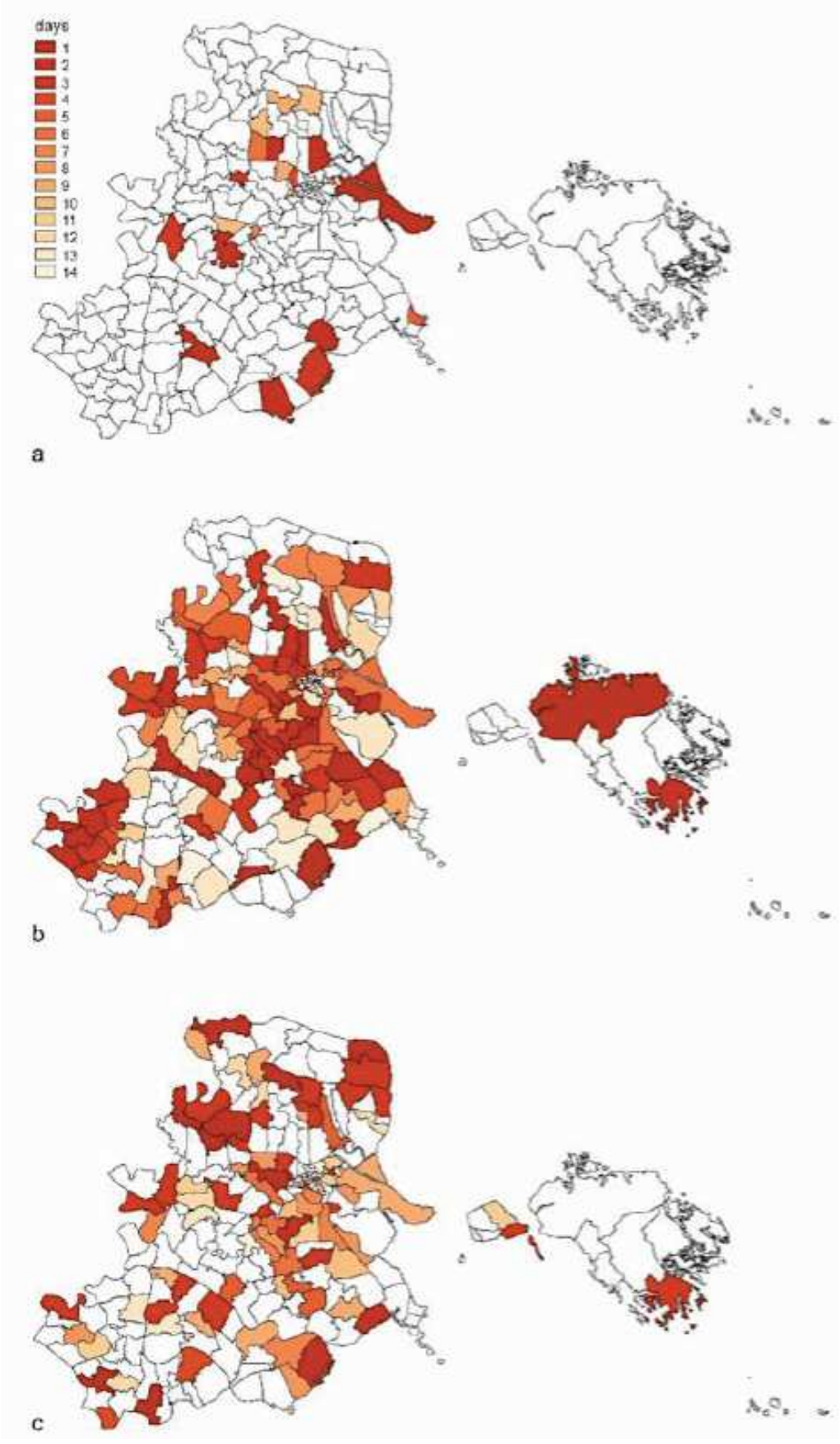

Figure 4

GENERAL CONCLUSION AND PERSPECTIVES

This work analyzed all HFMD cases reported both in the community and hospitals in 2011 and 2012 outbreak which was the largest to have ever occurred in Vietnam and the first recorded in the northern part of the country while Hai Phong city experienced the highest HFMD incidence in North Vietnam. It was a good model for investigating the dynamic of the disease without interference and potential remains from previous outbreaks or patient immunological adaptation and giving a broad view of the dynamic of the diseases.

Beside the basic epidemiological features as 3 distinct epidemic waves; median age at 2, the most < 5 yrs (highest in 1-2 yrs group); boys is higher prevalence rate; severity: mild (42.80%); moderate (54.92%), severe (2.28%) and no fatal; both urban and rural areas were affected, the study provides also findings related to influence of HFMD guidelines during the outbreak period that never described before. The first effect of the guideline release was a significant increase of the severity score. Another positive effect was the reduced delay between onset and admission after guidelines publication. The most important feature of the second guideline was the decentralization and transfer of responsibility to health care facilities. This positive effect of guidelines is also of an increased awareness and precautious approach from parents and physicians leading to patients being majoritarily declared with severe symptoms in order to ensure a better treatment and surveillance. Awareness led to the modification of guidelines but changes occurred only after publication, suggesting that the legal framework created by the guidelines is needed for implementation even though awareness is present. Public and professional awareness are not sufficient for implementing changes. Evolution of clinical patterns should not be considered only in the light of the evolution or replacement of pathogens or host-pathogen interactions but also according to the evolution of behavior and social perception.

This work is also an integrative analysis including genetic evolution and spatial analysis to describe the dynamic of HFMD in a well delimited area. Circulation of both EV-A71 and CV-A, alternatively replacement was recorded. EV-A71 detection with MAS primers should thus be systematically performed on SO primers products and SO222 primer should be redesigned to match with the 5’ part of the AN88 primer used for EVs detection. More attention should be therefore paid to the PCR negative patients. The observed correlation

between the I/V variant groups and phylogeny, pathogenicity and ethnicity. The EV-A71 strains could remain in a low level, asymptomatic state and with a geographic structuration. The 2011-2012 outbreak in the North was not due to a single exogenous strain imported from outside. The cause for outbreaks should thus be sought for in the socio-economic patterns rather than in exogenous emergence. Further investigations are needed to investigate this hypothesis and to bring valuable information for the management of this pediatric diseases.

Another main outcome of this work is the observed correlation between the I/V variant groups and phylogeny, pathogenicity and ethnicity. The recurrent reports of the involvement of isoleucine and valine in the viral pathogenicity process in different viruses as well as their involvement in the selective pressure applied on the EV-A71 samples analyzed in this work suggest that the I/V pattern at positions 249, 262 and 284 on the VP1 protein might play a role in pathogenicity. The observed correlation of the I/V variant populations with severity and ethnicity strengthen this hypothesis. However, the ethnicity correlation could be a result of spatial structuration since ethnicity-2 is mostly present in the Hòa Bình province. This in turn would suggest that the various EV-A71 variants display a geographic specificity.

This PhD work demonstrates the need of a comprehensive and coordinated approach when analyzing the dynamic of HFMD. Indeed, EV-A71 is first reported in Viet Nam in 2003. There were then several small outbreaks occurring in the southern of the country. The cause of very large outbreak with exist pathogen since 2011 is still not explained clearly. The trigger for the outbreak need to be studied further especially in aspects of climate, immunity, the host factors and also for the molecular aspects. A first recommendation and perspective from this work would be therefore to analyze correlation of the variant with severity and ethnicity by deeply analysis on molecular from full gene of pathogen.

The disease seems to have expanded following the eastbound river system to reach densely populated settlements from where it secondarily expanded through local roads. The average age of the patients, around 2, the source of contamination must be sought for within asymptomatic adults being contaminated during their occupational activities and in local and regional movements.

The PhD work also addresses the mathematical modelling of a multiphase disease such as HFMD. It is essential to detect as soon as possible the emergence of new wave, associated to a novel agent. Owing to the large size of the cohort available for this work

(ca. 9000 patients), we have been able to develop a differential equation model providing a very high fit with the observed data. The model confirmed that three waves were present in 2011-2012 with differing virulence. It also allows to characterize each wave, detect the start of a new one and associate groups of patients with specific patterns of symptoms.

All together, there are some recommendations after this work. The first for surveillance: I/V variants are an easy and efficient way to survey and identify circulating EV-A71 strains. EV-A71 detection with MAS primers should be systematically performed on SO primers products. More attention should be paid to the PCR negative patients. AN89 primer should be redesigned for better detection EVs. The seconde for disease control: Continue apply the guidelines for surveillance and disease control. Focus on high risk group (children < 5 yrs, and their carers, households members) at both urban and rural areas. Most important periods of time are in: Mar - May and Sep – Nov. Careful attention to patient age under 12 months because of severity risk regardless causative strains. Modeling should be applied for managing the epidemic of any multiwave infectious diseases in real time actions (new treatment strategy/ crisis management). The last for research: EV-A71 full gene should be investigated for correlation to the virulence/ pathogen and susceptible population. The cause for outbreaks should be sought for other aspects as socio-economic, climatology, environment patterns rather than in etiological emergence.

As a final word, this PhD work as underlined some key issues to be addressed in a coordinated way in order help developing an efficient surveillance and monitoring system for HFMD in Vietnam. Developing these systems and tools will thus be both a challenge and an exciting outcome of this work.

REFFERENCES

1. Schmidt NJ, Lennette EH, Ho HH. An apparently new enterovirus isolated from patients with disease of the central nervous system. J Infect Dis. 1974;129(3):304.

2. Solomon T, Lewthwaite P, Perera D, Cardosa MJ, McMinn P, Ooi MH. Virology, epidemiology, pathogenesis, and control of enterovirus 71. Lancet Infect Dis. 2010;10(11):778–790.

3. R D, TD F, V S. Central nervous system infections in New York State. Etiologic and epidemiologic observations, 1974. N Y State J Med. 75(13):2337.

4. Hagiwara A, Tagaya I, Yoneyama T. Epidemic of hand, foot and mouth disease associated with enterovirus 71 infection. Intervirology. 1978;9(1):60–63.

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

6. Kennett ML, Birch CJ, Lewis FA, Yung AP, Locarnini SA, Gust ID. Enterovirus type 71 infection in Melbourne. Bull World Health Organ. 1974;51(6):609–15.

7. Chumakov M, Voroshilova M, Shindarov L, Lavrova I, Gracheva L, Koroleva G, et al. Enterovirus 71 isolated from cases of epidemic poliomyelitis-like disease in Bulgaria. Arch Virol. 1979;60(3):329–340.

8. Nagy G, Takátsy S, Kukán E, Mihály I, Dömök I. Virological diagnosis of enterovirus type 71 infections: experiences gained during an epidemic of acute CNS diseases in Hungary in 1978. Arch Virol. 1982;71(3):217–227.

9. WHO weekly epidemiology report. World Health Organization: Enterovirus type 71 surveillance. Weekly Epidemiol Rec 1979;54:219. 1979.

10. SAMUDA GM, CHANG WAIK, YEUNG CY, TANG PAKS. Monoplegia caused by enterovirus 71: an outbreak in Hong Kong. Pediatr Infect Dis J. 1987;6(2):206.

11. GILBERT GL, DICKSON KE, WATERS MJO, KENNETT ML, LAND SA, SNEDDON M. Outbreak of enterovirus 71 infection in Victoria, Australia, with a high incidence of neurologic involvement. Pediatr Infect Dis J. 1988;7(7):484.

12. Hayward JC, Gillespie SM, Kaplan KM, Packer R, Pallansch M, Plotkin S, et al. Outbreak of poliomyelitis-like paralysis associated with enterovirus 71. Pediatr Infect Dis J. 1989;8(9):611.

13. Chan L, Parashar UD, Lye M, Ong F, Zaki SR, Alexander JP, et al. Deaths of children during an outbreak of hand, foot, and mouth disease in Sarawak, Malaysia: clinical and pathological characteristics of the disease. Clin Infect Dis. 2000;31(3):678.

14. Ho M, Chen ER, Hsu KH, Twu SJ, Chen KT, Tsai SF, et al. An epidemic of enterovirus 71 infection in Taiwan. N Engl J Med. 1999;341(13):929–935.

15. Podin Y, Gias ELM, Ong F, Leong Y-W, Yee S-F, Yusof MA, et al. Sentinel surveillance for human enterovirus 71 in Sarawak, Malaysia: lessons from the first 7 years. BMC Public Health. 2006;6:180.

16. Mizuta K, Abiko C, Murata T, Matsuzaki Y, Itagaki T, Sanjoh K, et al. Frequent importation of enterovirus 71 from surrounding countries into the local community of Yamagata, Japan, between 1998 and 2003. J Clin Microbiol. 2005;43(12):6171.

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

18. Yang F, Ren L, Xiong Z, Li J, Xiao Y, Zhao R, et al. Enterovirus 71 outbreak in the People's Republic of China in 2008. J Clin Microbiol. 2009;47(7):2351.

19. Zhang Y, Zhu Z, Yang W, Ren J, Tan X, Wang Y, et al. An emerging recombinant human enterovirus 71 responsible for the 2008 outbreak of Hand Foot and Mouth Disease in Fuyang city of China. Virol J. 2010;7(1):94.

20. Huang CC, Liu CC, Chang YC, Chen CY, Wang ST, Yeh TF. Neurologic complications in children with enterovirus 71 infection. N Engl J Med. 1999;341(13):936–942.

21. Ooi MH, Wong SC, Podin Y, Akin W, del Sel S, Mohan A, et al. Human enterovirus 71 disease in Sarawak, Malaysia: a prospective clinical, virological, and molecular epidemiological study. Clin Infect Dis. 2007;44(5):646.

22. Tu PV, Thao NTT, Perera D, Huu TK, Tien NTK, Thuong TC, et al. Epidemiologic and virologic investigation of hand, foot, and mouth disease, southern Vietnam, 2005. Emerg Infect Dis. 2007 Nov;13(11):1733–41.

23. Khanh TH, Sabanathan S, Thanh TT, Thoa LPK, Thuong TC, Hang V thi T, et al. Enterovirus 71-associated hand, foot, and mouth disease, Southern Vietnam, 2011. Emerg Infect Dis. 2012 Dec;18(12):2002–5.

24. Nguyen NT, Pham HV, Hoang CQ, Nguyen TM, Nguyen LT, Phan HC, et al. Epidemiological and clinical characteristics of children who died from hand, foot and mouth disease in Vietnam, 2011. BMC Infect Dis. 2014;14:341.

25. Tran CBN, Nguyen HT, Phan HTT, Van Tran N, Wills B, Farrar J, et al. The seroprevalence and seroincidence of enterovirus71 infection in infants and children in Ho Chi Minh City, Viet Nam. PLoS One. 2011;6(7):e21116.

26. Mahy BWJ, Regenmortel MHV van. Desk Encyclopedia of General Virology. Academic Press; 2009. 663 p.

27. King A, Lefkowitz E, Adams MJ. Virus Taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses. Elsevier; 2011. 1463 p.

28. Norder H, Palma AMD, Selisko B, Costenaro L, Papageorgiou N, Arnan C, et al. Picornavirus non-structural proteins as targets for new anti-virals with broad activity. Antiviral Res. 2011;

29. Maclachlan NJ, Dubovi EJ. Fenner's Veterinary Virology. Academic Press; 2010. 531 p.

30. Venetia Saunders JC. Virology: Principles and Application. WILEY; 2007.

31. VR RV. Picornaviridae : the viruses and their replication. Fields Virol. 2007;1:795–838.

32. Ehrenfeld E, Domingo E, Roos RP, Microbiology AS for. The Picornaviruses. ASM Press; 2010.

33. Agol VI, Gmyl AP. Viral security proteins: counteracting host defences. Nat Rev Microbiol. 2010;8(12):867–878.

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

35. AbuBakar S, Chee H-Y, Al-Kobaisi MF, Xiaoshan J, Bing Chua K, Kit Lam S. Identification of enterovirus 71 isolates from an outbreak of hand, foot and mouth disease (HFMD) with fatal cases of encephalomyelitis in Malaysia. Virus Res. 1999 May;61(1):1–9.

36. Ooi MH, Wong SC, Lewthwaite P, Cardosa MJ, Solomon T. Clinical features, diagnosis, and management of enterovirus 71. Lancet Neurol. 2010;9(11):1097–1105.

37. Zeng M, Li YF, Wang XH, Lu GP, Shen HG, Yu H, et al. Epidemiology of hand, foot, and mouth disease in children in Shanghai 2007–2010. Epidemiol Infect. 2012;140(6):1122.

38. Yu H, Chen W, Chang H, Tang R, Zhao J, Gan L, et al. Genetic analysis of the VP1 region of enterovirus 71 reveals the emergence of genotype A in central China in 2008. Virus Genes. 2010;41(1):1–4.

39. Brown BA, Oberste MS, Alexander Jr JP, Kennett ML, Pallansch MA. Molecular epidemiology and evolution of enterovirus 71 strains isolated from 1970 to 1998. J Virol. 1999;73(12):9969.

40. Cardosa MJ, Perera D, Brown BA, Cheon D, Chan HM, Chan KP, et al. Molecular epidemiology of human enterovirus 71 strains and recent outbreaks in the Asia-Pacific region: comparative analysis of the VP1 and VP4 genes. Emerg Infect Dis. 2003 Apr;9(4):461–8.

41. McMinn P, Lindsay K, Perera D, Chan HM, Chan KP, Cardosa MJ. Phylogenetic analysis of enterovirus 71 strains isolated during linked epidemics in Malaysia, Singapore, and Western Australia. J Virol. 2001 Aug;75(16):7732–8.

42. Shimizu H, Utama A, Onnimala N, Li C, Li-Bi Z, Yu-Jie M, et al. Molecular epidemiology of enterovirus 71 infection in the Western Pacific Region. Pediatr Int Off J Jpn Pediatr Soc. 2004 Apr;46(2):231–5.

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

44. Sanders S, Herrero L, McPhie K, Chow S, Craig M, Dwyer D, et al. Molecular epidemiology of enterovirus 71 over two decades in an Australian urban community. Arch Virol. 2006;151(5):1003–1013.

45. Tee KK, Lam TTY, Chan YF, Bible JM, Kamarulzaman A, Tong C, et al. Evolutionary genetics of human enterovirus 71: origin, population dynamics, natural selection, and seasonal periodicity of the VP1 gene. J Virol. 2010;84(7):3339.

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

47. Outbreak news. Enterovirus, China. Relevé Épidémiologique Hebd Sect Hygiène Secrétariat Société Nations Wkly Epidemiol Rec Health Sect Secr Leag Nations. 2008 May 9;83(19):169–70.

48. Jee Y, Cheon DS, Kim K, Cho J, Chung YS, Lee J, et al. Genetic analysis of the VP1 region of human enterovirus 71 strains isolated in Korea during 2000. Arch Virol. 2003;148(9):1735–1746.

49. Iwai M, Masaki A, Hasegawa S, Obara M, Horimoto E, Nakamura K, et al. Genetic changes of coxsackievirus A16 and enterovirus 71 isolated from hand, foot, and mouth disease patients in Toyama, Japan between 1981 and 2007. Jpn J Infect Dis. 2009;62(4):254–259.

50. Chen X, Zhang Q, Li J, Cao W, Zhang J-X, Zhang L, et al. Analysis of recombination and natural selection in human enterovirus 71. Virology. 2010 Mar 15;398(2):251–61.

51. Huang S-C, Hsu Y-W, Wang H-C, Huang S-W, Kiang D, Tsai H-P, et al. Appearance of intratypic recombination of enterovirus 71 in Taiwan from 2002 to 2005. Virus Res. 2008 Feb;131(2):250–9.

52. Yoke-Fun C, AbuBakar S. Phylogenetic evidence for inter-typic recombination in the emergence of human enterovirus 71 subgenotypes. BMC Microbiol. 2006;6(1):74.

53. Phuektes P. Development of a reverse genetic system for Human enterovirus 71 (HEV71) and the molecular basis of its growth phenotype and adaptation to mice. 2009;

54. Ooi E-E, Phoon M-C, Ishak B, Chan S-H. Seroepidemiology of human enterovirus 71, Singapore. Emerg Infect Dis. 2002 Sep;8(9):995–7.

55. Pallansch M, Roos R. Enteroviruses: polioviruses, coxsackieviruses, echoviruses, and newer enteroviruses. Fields Virol. 2001;1:723–775.

56. Samuel Baron. Medical Microbiology, 4th edition [Internet]. 1996 [cited 2011 Dec 10]. Available from: http://www.ncbi.nlm.nih.gov/books/NBK7627/

57. Chung PW, Huang YC, Chang LY, Lin TY, Ning HC, others. Duration of enterovirus shedding in stool. J Microbiol Immunol Infect. 2001;34(3):167–170.

58. Li-mei S, Huan-ying Z, Hui-zhen Z, Xue G, Jian-feng H, Da-wei G, et al. An enterovirus 71 epidemic in Guangdong Province of China, 2008: epidemiological, clinical, and virogenic manifestations. Jpn J Infect Dis. 2011;64(1):13–18.

59. Chen YC, Yu CK, Wang YF, Liu CC, Su IJ, Lei HY. A murine oral enterovirus 71 infection model with central nervous system involvement. J Gen Virol. 2004;85(1):69.

60. Wong KT, Munisamy B, Ong KC, Kojima H, Noriyo N, Chua KB, et al. The distribution of inflammation and virus in human enterovirus 71 encephalomyelitis suggests possible viral spread by neural pathways. J Neuropathol Exp Neurol. 2008 Feb;67(2):162–9.

61. Wang YF, Chou CT, Lei HY, Liu CC, Wang SM, Yan JJ, et al. A mouse-adapted enterovirus 71 strain causes neurological disease in mice after oral infection. J Virol. 2004;78(15):7916.

62. Arita M, Shimizu H, Nagata N, Ami Y, Suzaki Y, Sata T, et al. Temperature-sensitive mutants of enterovirus 71 show attenuation in cynomolgus monkeys. J Gen Virol. 2005;86(5):1391.

63. Lin T, Chang L, Huang Y, Hsu K, Chiu C, Yang K, et al. Different proinflammatory reactions in fatal and non-fatal enterovirus 71 infections: implications for early recognition and therapy. Acta Paediatr. 2002;91(6):632–635.

64. Wang SM, Lei HY, Su LY, Wu JM, Yu CK, Wang JR, et al. Cerebrospinal fluid cytokines in enterovirus 71 brain stem encephalitis and echovirus meningitis infections of varying severity. Clin Microbiol Infect. 2007;13(7):677–682.

65. Wang SM, Lei HY, Yu CK, Wang JR, Su IJ, Liu CC. Acute chemokine response in the blood and cerebrospinal fluid of children with enterovirus 71-associated brainstem encephalitis. J Infect Dis. 2008;198(7):1002.

66. Wang SM, Lei HY, Huang KJ, Wu JM, Wang JR, Yu CK, et al. Pathogenesis of enterovirus 71 brainstem encephalitis in pediatric patients: roles of cytokines and cellular immune activation in patients with pulmonary edema. J Infect Dis. 2003;188(4):564.

67. Chang LY, Chang I, Chen WJ, Huang YC, Chen GW, Shih SR, et al. HLA-A33 is associated with susceptibility to enterovirus 71 infection. Pediatrics. 2008;122(6):1271.

68. Yamayoshi S, Yamashita Y, Li J, Hanagata N, Minowa T, Takemura T, et al. Scavenger receptor B2 is a cellular receptor for enterovirus 71. Nat Med. 2009;15(7):798–801.

69. Nishimura Y, Shimojima M, Tano Y, Miyamura T, Wakita T, Shimizu H. Human P-selectin glycoprotein ligand-1 is a functional receptor for enterovirus 71. Nat Med. 2009;15(7):794–797.

70. Chang LY, King CC, Hsu KH, Ning HC, Tsao KC, Li CC, et al. Risk factors of enterovirus 71 infection and associated hand, foot, and mouth disease/herpangina in children during an epidemic in Taiwan. Pediatrics. 2002;109(6):e88.

71. Lu CY, Lee CY, Kao CL, Shao WY, Lee PI, Twu SJ, et al. Incidence and case-fatality rates resulting from the 1998 enterovirus 71 outbreak in Taiwan. J Med Virol. 2002;67(2):217–223.

72. Lin Y-W, Chang K-C, Kao C-M, Chang S-P, Tung Y-Y, Chen S-H. Lymphocyte and Antibody Responses Reduce Enterovirus 71 Lethality in Mice by Decreasing Tissue Viral Loads. J Virol. 2009 Jul 1;83(13):6477–83.

73. Yang KD, Yang M-Y, Li C-C, Lin S-F, Chong M-C, Wang C-L, et al. Altered Cellular but Not Humoral Reactions in Children with Complicated Enterovirus 71 Infections in Taiwan. J Infect Dis. 2001 Mar 15;183(6):850–6.

74. Chang L-Y, Hsiung CA, Lu C-Y, Lin T-Y, Huang F-Y, Lai Y-H, et al. Status of Cellular Rather Than Humoral Immunity is Correlated with Clinical Outcome of Enterovirus 71. Pediatr Res. 2006 Oct;60(4):466–71.

75. Yu C-K, Chen C-C, Chen C-L, Wang J-R, Liu C-C, Yan J-J, et al. Neutralizing antibody provided protection against enterovirus type 71 lethal challenge in neonatal mice. J Biomed Sci. 2000 Nov;7(6):523–8.

76. Foo DGW, Alonso S, Chow VTK, Poh CL. Passive protection against lethal enterovirus 71 infection in newborn mice by neutralizing antibodies elicited by a synthetic peptide. Microbes Infect. 2007;9(11):1299–1306.

77. Wu TC, Wang YF, Lee YP, Wang JR, Liu CC, Wang SM, et al. Immunity to avirulent enterovirus 71 and coxsackie A16 virus protects against enterovirus 71 infection in mice. J Virol. 2007;81(19):10310.

78. Luo S-T, Chiang P-S, Chao A-S, Liou G-Y, Lin R, Lin T-Y, et al. Enterovirus 71 Maternal Antibodies in Infants, Taiwan. Emerg Infect Dis. 2009 Apr;15(4):581–4.

79. Chiu C-H, Chu C, He C-C, Lin T-Y. Protection of neonatal mice from lethal enterovirus 71 infection by maternal immunization with attenuated Salmonella enterica serovar Typhimurium expressing VP1 of enterovirus 71. Microbes Infect. 2006 Jun;8(7):1671–8.

80. WHO WPRO. A Guide to Clinical Management and Public Health Response for Hand, Foot and Mouth Disease (HFMD) [Internet]. WHO WPRO. 2011 [cited 2012 Jan 27]. Available from: http://webcache.googleusercontent.com/search?q=cache:Dtg4fDp6fqcJ:www.wpro.who.int/publications/PUB_9789290615255.htm+A+guide+to+clinical+management+and+public+health+response+for+Hand,+Foot+nad+Mouth+Disease&cd=2&hl=en&ct=clnk&gl=fr&client=firefox-a

81. Ooi MH, Solomon T, Podin Y, Mohan A, Akin W, Yusuf MA, et al. Evaluation of different clinical sample types in the diagnosis of human enterovirus 71 associated hand-foot-and-mouth disease. J Clin Microbiol. 2007;JCM–01394.

82. Wang JR, Tsai HP, Chen PF, Lai YJ, Yan JJ, Kiang D, et al. An outbreak of enterovirus 71 infection in Taiwan, 1998. II. Laboratory diagnosis and genetic analysis. J Clin Virol. 2000;17(2):91–99.

83. Arita Minetaro, Ling Hua, Yan Dongmei, Nishimura Yorihiro, Yoshida Hiromu, Wakita Takaji, et al. Development of a reverse transcription-loop-mediated isothermal amplification (RT-LAMP) system for a highly sensitive detection of enterovirus in the stool samples of acute flaccid paralysis cases [Internet]. 2009 [cited 2012 Jan 30].

Available from: http://www.biomedcentral.com/1471-2334/9/208, http://www.doaj.org/doaj?func=openurl&genre=article&issn=14712334&date=2009&volume=9&issue=1&spage=208

84. Shima T, Saito T, Kondo M, Watanabe S, Mizuno K, Sato Y, et al. Enterovirus Detection Status from Patients with Herpangina and Hand, Foot and Mouth Disease in Kanagawa Prefecture, Japan. Virus. 2001;2002(2003):2004–2005.

85. Oberste MS, Maher K, Kilpatrick DR, Flemister MR, Brown BA, Pallansch MA. Typing of Human Enteroviruses by Partial Sequencing of VP1. J Clin Microbiol. 1999 May 1;37(5):1288–93.

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

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

88. Tan EL, Chow VTK, Quak SH, Yeo WCA, Poh CL. Development of multiplex real-time hybridization probe reverse transcriptase polymerase chain reaction for specific detection and differentiation of Enterovirus 71 and Coxsackievirus A16. Diagn Microbiol Infect Dis. 2008 Jul;61(3):294–301.

89. Perera D, Podin Y, Akin W, Tan C-S, Cardosa MJ. Incorrect identification of recent Asian strains of Coxsackievirus A16 as human enterovirus 71: improved primers for the specific detection of human enterovirus 71 by RT PCR. BMC Infect Dis. 2004 May 4;4:11.

90. Chiueh TS, Huang CP, Yen CT, Liao SJ, Lu JJ, Wang CC. Multiplex Reverse Transcription-semi-nested PCR for Differentiating Enterovirus 71, Coxsackievirus A16, and Polioviruses from Other Enteroviruses. J Med Sci-TAIPEI. 2001;21(6):277–282.

91. Chen TC, Chen GW, Hsiung CA, Yang JY, Shih SR, Lai YK, et al. Combining multiplex reverse transcription-PCR and a diagnostic microarray to detect and differentiate enterovirus 71 and coxsackievirus A16. J Clin Microbiol. 2006;44(6):2212.

92. Mizuta K, Aoki Y, Suto A, Ootani K, Katsushima N, Itagaki T, et al. Cross-antigenicity among EV71 strains from different genogroups isolated in Yamagata, Japan, between 1990 and 2007. Vaccine. 2009;27(24):3153–3158.

93. Wang SY, Lin TL, Chen HY, Lin TS. Early and rapid detection of enterovirus 71 infection by an IgM-capture ELISA. J Virol Methods. 2004;119(1):37–43.

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

95. Wang JR, Tuan YC, Tsai HP, Yan JJ, Liu CC, Su IJ. Change of major genotype of enterovirus 71 in outbreaks of hand-foot-and-mouth disease in Taiwan between 1998 and 2000. J Clin Microbiol. 2002;40(1):10.

96. Van Der Sanden S, Koopmans M, Uslu G, Van Der Avoort H, others. Epidemiology of enterovirus 71 in the Netherlands, 1963 to 2008. J Clin Microbiol. 2009;47(9):2826.

97. Bible JM, Iturriza-Gomara M, Megson B, Brown D, Pantelidis P, Earl P, et al. Molecular epidemiology of human enterovirus 71 in the United Kingdom from 1998 to 2006. J Clin Microbiol. 2008;46(10):3192.

98. Merovitz L, Demers AM, Newby D, McDonald J. Enterovirus 71 infections at a Canadian center. Pediatr Infect Dis J. 2000;19(8):755.

99. Pérez-Vélez CM, Anderson MS, Robinson CC, McFarland EJ, Nix WA, Pallansch MA, et al. Outbreak of neurologic enterovirus type 71 disease: a diagnostic challenge. Clin Infect Dis. 2007;45(8):950.

100. Witso E, Palacios G, Ronningen KS, Cinek O, Janowitz D, Rewers M, et al. Asymptomatic circulation of HEV71 in Norway. Virus Res. 2007;123(1):19–29.

101. Chakraborty R, Iturriza-Gómara M, Musoke R, Palakudy T, D'Agostino A, Gray J. An epidemic of enterovirus 71 infection among HIV-1-infected orphans in Nairobi. Aids. 2004;18(14):1968.

102. Wong S, Yip C, Lau S, Yuen K, others. Human enterovirus 71 and hand, foot and mouth disease. Epidemiol Infect. 2010;138(8):1071–1089.

103. Chang LY, Tsao KC, Hsia SH, Shih SR, Huang CG, Chan WK, et al. Transmission and clinical features of enterovirus 71 infections in household contacts in Taiwan. JAMA J Am Med Assoc. 2004;291(2):222.

104. Kuramitsu M, Kuroiwa C, Yoshida H, Miyoshi M, Okumura J, Shimizu H, et al. Non-polio enterovirus isolation among families in Ulaanbaatar and Tov province, Mongolia: prevalence, intrafamilial spread, and risk factors for infection. Epidemiol Infect. 2005;133(6):1131–1142.

105. Shao Q, Lou G, Yan J. [The clinical epidemiology characteristics of HFMD in 2010 of Hangzhou]. Zhonghua Shi Yan He Lin Chuang Bing Xue Za Zhi Zhonghua Shiyan He Linchuang Bingduxue Zazhi Chin J Exp Clin Virol. 2011 Aug;25(4):289–91.

106. Ooi EE, Phoon MC, Ishak B, Chan SH. Seroepidemiology of human enterovirus 71, Singapore. Emerg Infect Dis. 2002;8(9):995.

107. Wu C-N, Lin Y-C, Fann C, Liao N-S, Shih S-R, Ho M-S. Protection against lethal enterovirus 71 infection in newborn mice by passive immunization with subunit VP1 vaccines and inactivated virus. Vaccine. 2001 Dec 12;20(5–6):895–904.

108. Lin Y-C, Wu C-N, Shih S-R, Ho M-S. Characterization of a vero cell-adapted virulent strain of enterovirus 71 suitable for use as a vaccine candidate. Vaccine. 2002 Jun 7;20(19–20):2485–93.

109. Arita M, Shimizu H, Nagata N, Ami Y, Suzaki Y, Sata T, et al. Temperature-sensitive mutants of enterovirus 71 show attenuation in cynomolgus monkeys. J Gen Virol. 2005;86(5):1391.

110. Arita M, Ami Y, Wakita T, Shimizu H. Cooperative effect of the attenuation determinants derived from poliovirus Sabin 1 strain is essential for attenuation of enterovirus 71 in the NOD/SCID mouse infection model. J Virol. 2008;82(4):1787.

111. Arita M, Nagata N, Iwata N, Ami Y, Suzaki Y, Mizuta K, et al. An attenuated strain of enterovirus 71 belonging to genotype a showed a broad spectrum of antigenicity with attenuated neurovirulence in cynomolgus monkeys. J Virol. 2007;81(17):9386–9395.

112. Ho M. Enterovirus 71: the virus, its infections and outbreaks. J Microbiol Immunol Infect. 2000;33(4):205–216.

113. Tung WS, Bakar SA, Sekawi Z, Rosli R. DNA vaccine constructs against enterovirus 71 elicit immune response in mice. Genet Vaccines Ther. 2007;5(6):1–13.

114. Chung Y-C, Huang J-H, Lai C-W, Sheng H-C, Shih S-R, Ho M-S, et al. Expression, purification and characterization of enterovirus-71 virus-like particles. World J Gastroenterol WJG. 2006 Feb 14;12(6):921–7.

115. Chung YC, Ho MS, Wu JC, Chen WJ, Huang JH, Chou ST, et al. Immunization with virus-like particles of enterovirus 71 elicits potent immune responses and protects mice against lethal challenge. Vaccine. 2008;26(15):1855–1862.

116. Chung C-Y, Chen C-Y, Lin S-Y, Chung Y-C, Chiu H-Y, Chi W-K, et al. Enterovirus 71 virus-like particle vaccine: Improved production conditions for enhanced yield. Vaccine. 2010 Oct 8;28(43):6951–7.

117. Kataoka C, Suzuki T, Kotani O, Iwata-Yoshikawa N, Nagata N, Ami Y, et al. The Role of VP1 Amino Acid Residue 145 of Enterovirus 71 in Viral Fitness and Pathogenesis in a Cynomolgus Monkey Model. PLOS Pathog. 2015 Jul 16;11(7):e1005033.

118. Li R, Zou Q, Chen L, Zhang H, Wang Y. Molecular Analysis of Virulent Determinants of Enterovirus 71. PLOS ONE. 2011 Oct 19;6(10):e26237.

119. Huang S-W, Tai C-H, Fonville JM, Lin C-H, Wang S-M, Liu C-C, et al. Mapping Enterovirus A71 Antigenic Determinants from Viral Evolution. J Virol. 2015 Nov;89(22):11500–6.

120. Caine EA, Moncla LH, Ronderos MD, Friedrich TC, Osorio JE. A Single Mutation in the VP1 of Enterovirus 71 is Responsible for Increased Virulence and Neurotropism in Adult Interferon Deficient Mice. J Virol. 2016 Jul 20;JVI.01370-16.

Résumé

Ce travail a analysé tous les cas de HFMD déclarés à Hai Phong pendant l'épidémie de 2011 et 2012 qui a été la plus importante au Vietnam et la première enregistrée dans le nord du pays. Hai Phong a connu la plus forte incidence au Nord Vietnam. C'était donc un bon modèle pour étudier la dynamique de cette maladie sans interférence et reliquats de précédentes épidémies ou de patients immunoadaptés.

La première section est consacrée à une revue de la littérature sur EV-A71 et les entérovirus. La seconde section est divisée en trois chapitres, chacun abordant un aspect spécifique du projet.

Le premier chapitre aborde la dynamique de la maladie et le rôle des directives officielles pour la gestion de l'épidémie de 2011-2012. Outre les éléments de base, cette étude apporte des résultats sur l'influence des directives HFMD durant l'épidémie, ce qui n'avait pas encore été fait. La publication des directives a conduit à un accroissement du score de sévérité et d'une réduction du délai entre le premier pic de fièvre et l'admission. Cet effet est associé à un accroissement de la sensibilisation qui a conduit à déclarer la plupart des patients avec des symptômes sévères pour assurer de meilleurs traitements et suivis. The travail décrit dans ce chapitre a aussi démontré que trois vagues avec des caractéristiques différentes et causées par trois virus différents s'étaient succédées. La vague 1 et la vague 3 ont été causées respectivement par EV-A71 et par une combinaison de CV-A6 et CV-A16 alors que la vague 2 a été causée par un virus inconnu. Ce travail est aussi une analyse intégrative incluant une analyse spatiotemporelle. La maladie semble s'être étendue vers l'est en suivant les rivières pour atteindre les des zones plus peuplées à partir desquelles elle s'est répandue par les routes secondaires locales. Etant donné l'âge moyen des patients, environ 2 ans, la source de contamination doit être cherchée chez les adultes asymptomatiques contaminés lors de leurs activités professionnelles et des mobilités locales.

Le deuxième chapitre aborde la phylogénie et la distribution spatiotemporelle de EV-A71 dans le nord du Vietnam et apporte un éclairage sur l'évolution et la dynamique de cet entérovirus. La protéine de capside VP1 a été ciblée. La première conclusion de ce chapitre est que l'épidémie de 2011 et 2012 n'a pas été causée par une souche exogène mais par des souches d'EV-A71 déjà présentes au Nord Vietnam. Ceci indique qu'elles

peuvent se maintenir à faible niveau, asymptomatique, en stase génomique et avec une structuration géographique. La cause de l'épidémie devrait donc être recherchée dans le tissu socio-économique plutôt que dans une émergence extérieure. Une autre conclusion de ce chapitre est la corrélation observée ente les groupes de variants I/V et phylogénie, pathogénicité et groupe ethnique. Les profils des mutations I/V aux positions 249, 262 et 284 sur la protéine VP1 pourraient jouer un rôle dans la pathogénicité, ce qui est appuyé par la corrélation entre variants I/V et sévérité/ethnicité.

Le dernier chapitre aborde la modélisation mathématique d'une maladie multiphases telle que HFMD. Il est essentiel de détecter aussi tôt que possible une nouvelle vague associée à un nouvel agent. Grace à la grande taille de la cohorte disponible pour ce travail (environ 9000 patients), nous avons pu développer un système d'équations différentielles apportant une forte correspondance avec les données observées. Le modèle a confirmé l'existence de trois vagues en 2011-2012, ayant des niveaux de virulence différents. Il permet aussi de caractériser chaque vague, de détecter l'apparition d'une nouvelle vague et d'associer des groupes patients à un tableau clinique.

En conclusion, ce travail de thèse a permis de souligner plusieurs éléments clés à aborder de façon coordonnée afin de faciliter une surveillance efficace de l'HFMD au Vietnam.

Summary

This work analyzed all HFMD cases reported in Hai Phong in 2011 and 2012 outbreak which was the largest to have ever occurred in Vietnam and the first recorded in the northern part of the country. Hai Phong city experienced the highest HFMD incidence in North Vietnam. It was thus a good model for investigating the dynamic of the disease without interference and potential remains from previous outbreaks or patient immunological adaptation.

The first section is dedicated to a review of the literature on EV-A71 and enteroviruses. The second section is divided in three chapters, each one addressing a specific issue of the project.

The first chapter addresses the dynamic of the disease and the role of official guidelines in the handling of the 2011-2012 epidemic. Beside basic epidemiological features, the study also provides findings relating to the influence of HFMD guidelines during the outbreak period that has never been described before. The guideline release led to a significant increase of the severity score and reduced delay between onset and admission. This effect is linked to an increased awareness leading to patients being mostly declared with severe symptoms in order to ensure a better treatment and surveillance. The work presented in this chapter also demonstrated that three waves occurred with different characteristics and caused by three different viruses. Wave 1 and wave 3 were caused by EV-A71 and a combination of CV-A6 and CV-A16, respectively while Wave 2 was caused by an unknown virus. This work is also an integrative analysis including a spatiotemporal analysis. The disease seems to have expanded following the eastbound river system to reach densely populated settlements from where it secondarily expanded through local roads. Owing to the average age of the patients, around 2, the source of contamination must be sought for within asymptomatic adults being contaminated during their occupational activities and in local movements.

The second chapter addresses the phylogeny and spatiotemporal distribution of EV-A71 in North Vietnam and provides an insight on the evolution and dynamic of the EV-A71 enterovirus. The VP1 capsid protein was used as target. The first conclusion of this chapter is that the 2011-2012 outbreak was not caused by an incoming strain but by EV-A71 strains which were already present in North Vietnam. This indicates that they can

remain in a low level, asymptomatic state, in genomic stasis and with a geographic structuration. The cause for outbreaks should thus be sought for in the socio-economic patterns rather than in exogenous emergence. Another outcome of this chapter is the observed correlation between I/V variant groups and phylogeny, pathogenicity and ethnicity. The I/V pattern at positions 249, 262 and 284 on the VP1 protein might play a role in pathogenicity. The observed correlation of the I/V variant populations with severity and ethnicity strengthen this hypothesis.

The last chapter addresses the mathematical modelling of a multiphase disease such as HFMD. It is essential to detect as soon as possible the emergence of new wave, associated to a novel agent. Owing to the large size of the cohort available for this work (ca. 9000 patients), we have been able to develop a differential equation model providing a very high fit with the observed data. The model confirmed that three waves were present in 2011-2012 with differing virulence. It also allows to characterize each wave, detect the start of a new one and associate groups of patients with specific patterns of symptoms.

As a conclusion, this PhD work as underlined some key issues to be addressed in a coordinated way in order help developing an efficient surveillance and monitoring system for HFMD in Vietnam.