HUMAN ENTEROVIRUS 71 INFECTION

IN SARAWAK, MALAYSIA

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by

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Abstract

Human Enterovirus 71 Infection in Sarawak, Malaysia Mong How Ooi

Introduction: Hand, foot and mouth disease (HFMD) is a childhood exanthema caused by enteroviruses, such as coxsackie virus A (CVA) 16. However since 1997 large epidemics of HFMD caused by human enterovirus (EV) 71 and associated with severe and sometimes fatal neurological complications have occurred across Asia.

Aims: To examine: (i) the diagnostic approach for detection of EV71, (ii) the clinical and molecular epidemiology of the virus in Sarawak, (iii) the clinical predictors for neurological involvement, and (iv) the viral determinants for clinical phenotype of EV71 infection.

Methods: A prospective study was set up to examine children with HFMD presenting to Sibu Hospital, Sarawak, Malaysia between January 2000 and December 2006. Detailed history and clinical examination was performed and recorded on standardised forms. Throat and rectal swabs, and swabs from skin vesicles and mouth ulcers, if present, were taken from every patient. Lumbar puncture was performed in patients with suspected neurological involvement. Virus isolation and RT-PCR for enteroviruses were performed on all specimens. Isolated enteroviruses were typed by nucleotide sequencing of VP1 and VP4 genes and genogrouped by phylogenetic analysis.

Results: Throat and vesicle swabs were the most useful samples for detection of EV71. Using virus culture results as the reference, an EV71-specific assay originally developed for molecular typing of EV71 clinical isolates had a sensitivity of 76.9% (258/337), specificity of 82.6% (133/161), positive predictive value of 90.2% (259/287) and negative predictive value of 63.0% (133/211) when evaluated with 337 EV71-positive, 161 non-EV71 culture-positive clinical specimens. Epidemics of EV71-associated HFMD occurred every 3 years in Sarawak, and were caused by genogroups B4 and B5, and C1. The genogroups of EV71 differ in their risk of causing neurological disease and family clusters. Total duration of fever $\geq$3 days, peak temperature $\geq$38.5°C and history of lethargy were identified and validated as independent risk factors for neurological involvement. EV71-positive children were more likely to have neurological disease when compared to CVA16-positive children.

Discussion: EV71 has become a major public health problem in Asia and may continue to spread globally. The transmission dynamic of the virus is poorly understood. The public health intervention measure to date has been empirical and generic, but they have considerable socioeconomic implication. There is neither specific antiviral nor vaccine for EV71. Intravenous immunoglobulin is now used presumptively for severe EV71 infection in many Asian countries, although there are little data on its efficacy. Early diagnosis of neurological involvement may help reduce the mortality. A better understanding on diagnosis and management of this neurological infectious disease can help public health doctors and clinicians manage the epidemic caused by the virus when it spread to a new territory.

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Declaration

Except for the assistance as outlined in the acknowledgements above, the work described is my own work and has not been submitted for a degree or other qualification to this or any other university.

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Publications and presentations arising from this work

Publications

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

2. **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, C. H. Chieng, E. Blake, D. Perera, S. C. Wong and J. Cardosa (2007)**. Evaluation of different clinical sample types in diagnosis of human enterovirus 71-associated hand-foot-and-mouth disease. *J Clin Microbiol* **45**(6): 1858-66.

3. **M.H. Ooi, S. C. Wong, A. Mohan, Y. Podin, D. Perera, D. Clear, S. del Sel, C. Chieng, P. Tio, M. Cardosa and T. Solomon (2009)**. Identification and validation of clinical predictors for the risk of neurological involvement in children with hand, foot, and mouth disease in Sarawak. *BMC Infectious Diseases* **9**(1): 3.

4. **M. H. Ooi, S. C. Wong, P. Lewthwaite, M. J. Cardosa and T. Solomon (2010)**. Clinical features, diagnosis, and management of enterovirus 71. *Lancet Neurol* **9**(11): 1097-105.

5. **T. Solomon, P. Lewthwaite, D. Perera, M. J. Cardosa, P. McMinn and M. H. Ooi (2010)**. Virology, epidemiology, pathogenesis, and control of enterovirus 71. *Lancet Infect Dis* **10**(11): 778-90.

Invited Presentations

1. **M. H. Ooi** Hand, Foot, and Mouth Disease *Malaysian Medical Association-The Ministry of Health Infectious Disease Conference: Issues and Challenges* 21 November 2010, Kuala Lumpur, Malaysia.

2. **M. H. Ooi** Clinical Management of Severe HFMD Caused by EV71: A WHO WPRO Clinical Guidance *2nd Meeting of WHO WPRO Regional Clinical Network on Emerging Infectious Disease* 17-19 Nov 2010 Manila, Philippines.

3. **M. H. Ooi** Management of Complicated Hand, Foot, and Mouth Disease in Sarawak, Malaysia *The 6th Congress of Asian Society for*

Paediatric Research & 51st Annual Meeting of Taiwan Paediatric Association, 15-18 April, 2010, Taipei, Taiwan.

4. **M. H. Ooi** Clinical Features and Management of Hand, Foot, and Mouth Disease *Informal WHO Consultation on Hand, Foot and Mouth Disease* 10-12 March 2010, Kuala Lumpur, Malaysia.

5. **M. H. Ooi** Early Recognition and Management of Complicated Hand, Foot, and Mouth Disease: The Sarawak Experience *APEC Conference for the Surveillance, Treatment, Laboratory Diagnosis and Vaccine Development of Enteroviruses* 13-14 August 2009, Taipei, Chinese Taipei.

6. **M. H. Ooi** Clinical Management of Enterovirus 71 associated Hand, Foot, and Mouth Disease in Sarawak *Forum on Hand, Foot and Mouth Disease in Asian Pacific Region,* 21-22 August 2008, Singapore.

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List of Abbreviations

293	human embryonal kidney
Ad	adenovirus
AFP	Acute flaccid paralysis
ASM	aseptic meningitis
cDNA	complementary deoxyribonucleic acid
CI	confidence interval
CNS	central nervous system
CSF	cerebrospinal fluid
CT	computer tomography
CTL	cytotoxic T lymphocytes
CV	coxsackie virus
DENV	dengue virus
DHF	dengue haemorrhagic fever
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
EMG	electromyography
EV	human enterovirus
GCS	Glasgow coma score
HFMD	hand, foot and mouth disease
HFMD-CNS	HFMD with CNS complications
HFMD-Non-CNS	HFMD without CNS involvement

HLA	human leukocyte antigen system
IgG	immunoglobulin G
IgM	immunoglobulin M
IL	interleukin
IFN- γ	interferon gamma
IP-10	interferon-gamma-induced protein
IVIG	intravenous immunoglobulin
JEV	Japanese encephalitis virus
MAC-ELISA	IgM capture enzyme-linked immunosorbent assay
MCP	monocyte chemoattractant protein
MIG	monokine induced by interferon-gamma
MRC5	human fetal lung fibroblast
MRI	magnetic resonance imaging
NPV	negative predictive value
OD	optical density
OR	odds ratio
PCR	polymerase chain reaction
PPV	positive predictive value
RD	rhabdomyosarcoma
RNA	ribonucleic acid
RT-PCR	reverse-transcriptase polymerase chain reaction
TNF-α	tumour necrosis factor alpha

UTR	untranslated region
Vero	African green monkey kidney epithelium
VP	viral protein
VPg	viral protein genome-linked
WBC	white blood cells
WHO	World Health Organisation

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Chapter 1: Introduction

1.1 Overview

Enteroviruses are small single stranded positive sense RNA viruses from the genus *Enterovirus* in the family *Picornaviridae* (Pallansch et al. 2001). They are responsible for a very wide range of clinical manifestations, including cutaneous, visceral and neurological disease. For many years polioviruses were the most important enteroviruses, on account of the large outbreaks of paralytic disease that they caused; however in the last few decades polio has all but disappeared from Europe, the Americas, and much of Africa and Asia, secondary to the global polio eradication campaign. However, over the last 10 years, a related virus, human enterovirus (EV) 71 has emerged across Asia, where it threatens to become the "new polio" (Prager et al. 2003). The virus is a member of the enterovirus genus, which includes coxsackie viruses, echoviruses. Being a more recently identified enterovirus serotype, it is assigned a type number. EV71 first appeared in California in the 1960s, subsequently causing sporadic cases, or small outbreaks of hand foot and mouth disease (HFMD), neurological disease, or both (Pallansch et al. 2001). However, in 1997 the virus caused an unexpected large and severe outbreak in Sarawak, Malaysia, with many fatalities (Cardosa et al. 1999;

Chan et al. 2000). Since then countries of the Asia Pacific Region have been hit by regular epidemics of EV71, including an epidemic in Taiwan, in which millions of people were thought to be infected (Ho et al. 1999) (Table 1.1). Whilst the virus circulates across most of the globe, the largest outbreaks of disease have so far mostly been confined to the Asia-Pacific region, for reasons that are incompletely understood (Cardosa et al. 1999; Ho et al. 1999; Komatsu et al. 1999; Chan et al. 2000; McMinn et al. 2001; Cardosa et al. 2003; Chan et al. 2003; Jee et al. 2003; Shimizu 2004; Chen et al. 2007; Tu et al. 2007; Zhang et al. 2009; Zhang et al. 2010). During outbreaks, tens of thousands of children can develop HFMD, and whilst most will have a self-limiting illness, a small proportion can rapidly develop neurological and systemic complications which may be fatal. The neurological manifestations range from aseptic meningitis, to acute flaccid paralysis and a brainstem encephalitis, which is often associated with systemic features such as severe pulmonary oedema and shock, which are thought to be neurogenic in origin (Chang et al. 1998; Huang et al. 1999).

1.1 Virology

1.1.1 Classification of human enterovirus

As well as the Enterovirus genus, the large Picornaviridae family, includes Hepatovirus (e.g. human hepatitis A virus), Parechovirus (e.g. human parechovirus 1-14), and nine important animal virus genera Cardiovirus (e.g. encephalomyocarditis virus), Aphthovirus (foot and mouth disease virus), Avihepatovirus (e.g. Duck hepatitis A virus), Erbovirus (e.g. Equine rhinitis B virus), Kobuvirus (e.g. Bovine kobuvirus), Sapelovirus (e.g. Avian sapelovirus), Senecavirus (e.g. Seneca Valley virus), Teschovirus (e.g. Porcine teschovirus), Tremovirus (e.g. Avian encephalomyelitis virus) (Virus Taxonomy: 2009 Release, http://www.ictvonline.org/virusTaxonomy.asp?version=2009&bhcp=1). The human enteroviruses were traditionally classified into four subgroups according to their pathogenicity in humans and experimental animals, and their cytopathic effects in tissue culture; these subgroups were polioviruses (3 serotypes), coxsackie virus A (23 serotypes) and B (6 serotypes), and echoviruses (28 serotypes) (Pallansch et al. 2001). However, because of the limitations of this system newer serologically distinct human enteroviruses, isolated since 1970, have been designated by serotype numbers, beginning with EV68. More recently the original classification of human enteroviruses has been substituted by a new taxonomical scheme based on molecular and biological properties of the

viruses (King et al. 2000; Nasri et al. 2007). This revised classification recognises at least 90 types, and divides them into 4 species, which tend to be associated with particular clinical presentations, though there is considerable overlap (Table 1.2) (http://www.picornaviridae.com/enterovirus/enterovirus.htm). Of note, polioviruses have been designated as members of human enterovirus species C because they are genetically closely related to other members of human enterovirus species C (Brown et al. 2003).

1.1.2 Physicochemical properties

The human enteroviruses are small non-enveloped icosahedral spherical particles (approximately 30nm) containing a single-stranded, positive-sense polyadenylated virus RNA of approximately 7.4 kilo base pairs. The virus capsid is made of 60 identical subunits (protomers); each of which comprises a copy of the four structural viral proteins (VP1 to VP4) (Figure 1.1) (Brown et al. 1995). The VP1, VP2, and VP3 form the mature virion surface, while VP4 is completely internalised within the capsid, and is not exposed to the immune pressure from the host antibody response (Pallansch et al. 2001). The property of the lack of a lipid envelope confers the human enterovirus' considerable stability in the environment, and hence the viruses remain stable on exposure to human gastric acid, and can survive at room temperature for several days

(Pallansch et al. 2001). EV71 and other enteroviruses have also been detected in surface and ground water and hot spas (Chen et al. 2008; Hsu et al. 2008). Enteroviruses are resistant to organic solvents (such as ether and chloroform) and alcohol, and freezing, but may be inactivated by temperatures above 56° C, chlorination, formaldehyde and ultraviolet irradiation (Pallansch et al. 2001).

1.1.3 Virus life cycle

Humans are the only known natural hosts of human enteroviruses. The viruses are ubiquitous and transmitted primarily through the faeco-oral mode. In addition, they may spread from person to person through contact of virus-contaminated oral secretion or vesicular fluid, as well as via respiratory droplets (Pallansch et al. 2001). Like most other enteroviruses the replication cycle of EV71 is generally considered to be similar to that of polioviruses which have been studied extensively. A comprehensive review on replication cycle on polioviruses is available (Jesus 2007). Viral entry into susceptible host cells is dependent on the presence of specific receptors. Seven distinct receptors for different enteroviruses have been identified in humans to date (Nasri et al. 2007). The specific receptors include the poliovirus receptor (PVR; CD155), three integrins ($\alpha 2\beta 1$, $\alpha v\beta 3$, and $\alpha v\beta 6$), decay-accelerating factor (DAF; CD55), the coxsackievirus-adenovirus receptor (CAR), and intracellular adhesion

molecule 1 (ICAM-1). Some enteroviruses are able to use more than one receptor to infect a host cell, but the specific receptor for EV71 has yet to be identified.

After the binding of enteroviruses with a specific receptor on the cell surface, a series of structural changes occur at the virus capsid, yet to be defined in EV71, and pores are formed in the cell membrane through which the virion RNA is released into the host cell cytoplasm (Pallansch et al. 2001; Jesus 2007). Being a positive-sensed RNA virus, the parent virus RNA acts directly as a messenger RNA and is translated into a large polypeptide, which is promptly cleaved by the viral proteases into a total of 11 mature structural and non-structural proteins. The replication of the virus genome takes place in a vesicle membrane structure (viral replication complex) in the host cell cytoplasm, and is carried out by the error-prone RNA-dependent RNA polymerase (3Dpol). It is estimated that the polymerase mis-incorporates 1 to 2 bases in every genome copying event, which explains why this RNA virus mutates and evolves rapidly when compared to the DNA viruses (Pallansch et al. 2001). Mutation occurs during the process of viral replication when there is a faulty deletion, insertion or an exchange of nucleotide in a gene encoding a specific protein. Such changes to the nucleotide sequence of the gene may lead to a change in the structure and function of the protein product. Substitution is a type of point mutation involving replacement of a single

nucleotide base with a different nucleotide. It is considered a synonymous substitution when such replacement causes no change in the resultant protein because the altered codon continues to encode the same amino acid. On the other hand a non-synonymous substitution occurs when the replacement result in a codon that encodes a different amino acid. The newly synthesized protein with altered amino acid sequence may be ineffective in its structure and function. Within the VP1 gene there are estimated to be 4.2-4.6 $x10^{-3}$ nucleotide substitutions per site per year, which is similar to that of poliovirus and greater than that of influenza viruses (0.5-2.6 $x10^{-3}$ nucleotide substitutions per site per year) (Kew et al. 1998; Nobusawa et al. 2006; Tee et al. 2010). While the machinery of the host cellular protein synthesis is shut down by the viral protease (2A), the viral protein synthesis remains unaffected. An infectious virus particle is formed following the packaging of a progeny viral RNA into a virus capsid in the cytoplasm of the infected cells. Mature infectious virus particles are released to infect new susceptible cells when an infected cell is lysed.

1.2 Clinical features

EV71 infection may present with a board spectrum of clinical manifestations, although central nervous system (CNS) infection and

HFMD are the two most commonly recognised disease associations (Pallansch et al. 2001).

1.2.1 Mucocutaneous and respiratory manifestations

HFMD is a common childhood exanthema characterised by a brief, usually mild, febrile illness with papulovesicular rashes over the palms and soles and multiple oral ulcers (Figure 1.2). Herpangina, a closely related childhood enanthema, is characterised by a febrile illness and the presence of multiple oral ulcers predominantly affecting the posterior aspect of the oral cavity including the anterior pharyngeal folds, uvula, tonsils and soft palate. Although the classical HFMD picture is typically seen in older children with EV71, in children aged 2 years and below more widespread and atypical rashes occur. Of the many enteroviruses that can cause HFMD or herpangina, EV71 and coxsakie virus (CV) A16 are the most common, both causing epidemic disease. CVA16 is not normally associated with neurological disease (Pallansch et al. 2001), but the rash it causes is indistinguishable from that caused by EV71. Other respiratory manifestations in young children include exacerbation of bronchial asthma, bronchiolitis, and pneumonia (Merovitz et al. 2000).

1.2.2 Neurological and systemic manifestations

Like other enteroviruses, EV71 can cause aseptic meningitis, acute flaccid paralysis, encephalitis and other rarer manifestations (Table 1.3) (McMinn 2002); in EV71 encephalitis is typically a brainstem encephalitis (or rhombencephalitis) and unlike most other enteroviruses, this is often accompanied by marked cardiorespiratory problems; these are also seen in poliomyelitis, and have been attributed to neurogenic pulmonary oedema.

Myoclonic jerks are seen more often in EV71 than other enteroviruses, and may be an early indicator of neurological involvement, particularly in the brainstem (Huang et al. 1999; Lu et al. 2004); however myoclonus has also been reported in other virus CNS infection including Japanese encephalitis, Nipah virus, subacute sclerosing panencephalitis, herpes simplex virus, HIV and varicella-zoster virus. In addition, myoclonic jerks are not uncommonly seen in otherwise healthy young infants particularly when they are asleep and may occur spontaneously or be provoked by a loud noise.

Seizures, if they occur at all in EV71 infection, tend to be seen in younger children, and are short lived with good recovery of consciousness, suggesting that they are febrile seizures, rather than due to CNS infection itself; Unlike other viral encephalitides, recurrent and prolonged seizures

are very rare, probably reflecting the fact that this is a brainstem, rather than a cortical encephalitis (Huang et al. 1999).

Brainstem encephalitis with associated pulmonary oedema has been the hallmark of EV71 CNS infection in Asia since the late 1990s (Chang et al. 1998; Chang et al. 1999; Huang et al. 1999). This distinctive clinical syndrome is characterised by a prodromal illness of HFMD in followed by a sudden deterioration that typically occurs after 3 to 5 days of fever. Children then develop acute rapidly progressing acute cardiorespiratory failure which presents as shock and pulmonary oedema or haemorrhages. Without intensive care support, the vast majority of such children die within 24 hours of hospital admission, and some may die before reaching hospital.

Magnetic resonance (MR) imaging findings of children with brainstem encephalitis correlated well with those of postmortem examination; both of which shows frequent involvement of the medulla oblongata, reticular formation, pons and midbrain in several studies (Figure 1.3) (Shen et al. 1999; Chen et al. 2000; Wong et al. 2000). Acute flaccid paralysis is the primary presenting feature of a number of neurological syndromes caused by EV71 including poliomyelitis-like anterior horn cell destruction (anterior myelitis), Guillian-Barré syndrome and transverse myelitis. Anterior horn cell destruction is probably the most common of these,

though it may be less severe that that caused by polioviruses, with a higher recovery rate (McMinn 2002).

During the 1998 Taiwan epidemic the severity of brainstem encephalitis was categorised into Grade I disease with myoclonic jerks and tremor or ataxia, or both; Grade II disease with cranial nerve palsies evident by eye movement disorders (nystagmus, strabismus, or gaze paresis), facial weakness and bulbar palsy (dysphagia, dysarthria and dysphonia); Grade III disease, the acute onset of intractable cardiorespiratory failure, which is often fatal as described above (Huang et al. 1999). A separate clinical staging system of stage 1 through to stage 4 has been used by other clinicians to help monitor progress through the clinical course of EV71 infection, and management, from febrile illness through CNS involvement to cardiorespiratory failure and development of sequelae (Lin et al. 2002; Chang et al. 2004).

1.2.3 Outcome

Published reports on acute outcome showed that many children developed neurological sequelae (Huang 2001). Long term outcome is still unknown involving those with mild CNS infection. It is not known if children with mild EV71 CNS infection will have complete recovery like those affected by other enteroviruses such as echoviruses.

1.3 Clinical management and diagnosis

During outbreaks of EV71 tens of thousands of children develop symptoms, and whilst most of them will have mild self limiting illness, a small proportion of apparently well children can rapidly deteriorate to severe and fatal neurological and systemic complications over days or even hours. Whereas in the past children with mild HFMD tended to be looked after at home, with increasing public awareness about the potentially fatal complications, many are now brought directly to hospital, and health services can easily become swamped. The challenge for the front-line clinician is to recognise which patients are likely to deteriorate, to know which investigations give the best diagnostic yield, and to understand which treatments might be appropriate, given the lack of controlled clinical trials.

1.3.1 Differential diagnosis

The rash of HFMD can be caused a range of enteroviruses in addition to EV71, the most common being CVA16 (Table 1.4). There are many other childhood exanthamata that can be confused with HFMD, particularly measles, rubella, and chicken pox. Two particularly important causes to consider are meningococcus, because of the need for antimicrobial

treatments, and dengue, because of the risk of developing dengue haemorrhagic fever, which in severe forms requires careful fluid resuscitation. Herpangina, on the other hand, can be confused with apthous ulcer and herpetic gingivostomatitis. For aseptic meningitis, which is a common neurological manifestation of EV71, the differential is broad, and includes other viruses, especially echoviruses and other enteroviruses, adenoviruses, mumps and occasionally Japanese encephalitis virus; partially treated bacterial meningitis, and tuberculous meningitis should also be considered. Most patients with severe CNS disease due to EV71, also have features of shock and collapse, and septicaemia is an important differential; however other causes of encephalopathy need to be excluded, particularly malaria. Where acute flaccid paralysis is the predominant feature, the differential includes poliomyelitis due to wild type polioviruses or vaccine associated disease, other enteroviruses, flaviviruses, rabies, Guillain-Barré syndrome, and bacterial toxins, such as diphtheria.

1.3.2 Virological diagnosis

Laboratory diagnosis of EV71 is established primarily through virus isolation or molecular detection of the virus nucleic acid in appropriate clinical specimens.

Choice of sample

A wide range of samples may be available, depending on the disease manifestations; these include throat and rectal swabs, serum, urine, and when taken, cerebrospinal fluid (CSF), as well as fluid from vesicles and swabs from ulcers, if they are present. Prolonged viral shedding from the gastrointestinal tract (throat, rectum or stool) may occur after complete resolution of EV71 infection, as it does for other enteroviruses.

Virus isolation, serotyping and nucleic acid detection

Traditionally the gold standard for diagnosis of enterovirus infection is virus isolation; several human and non-human primate cell lines may be used including rhabdomyosarcoma (RD), which is most efficient, human lung fibroblast cells (MRC5), and African green monkey kidney cells (Vero) (Pallansch et al. 2001). In RD cells a characteristic cytopathic effect is observed typically 7 to 10 days after inoculation. However, to improve the yield, blind passage may necessary before cytopathic effects become apparent. Once a cytopathic effect is observed, the virus is identified by neutralization tests using intersecting pools of type-specific anti-sera, EV71 type-specific anti-sera, or by an indirect immunofluorescence assay using EV71 type-specific monoclonal antibodies (Pallansch et al. 2001). More recently a molecular "serotyping" approach has been developed by amplifying part of the VP1 gene of the cultured virus, using the polymerase chain reaction (PCR) and pan-

enterovirus specific primers and then sequencing the product (Nasri et al. 2007). To this end, several sets of primers directed at different regions of the VP1 gene of human enterovirus have been developed (Brown et al. 2000; Perera et al. 2004).

Serology

Serological diagnosis of an acute virus infection is classically established by demonstrating a four-fold rise in specific neutralizing antibody between the acute and convalescent samples (Pallansch et al. 2001). However, in the case EV71, very high levels of neutralizing antibodies are often detectable within the first few days of illness, and thus a four-fold rise cannot be demonstrated (Chumakov et al. 1979; Nagy et al. 1982). Furthermore, although homologous antibody is produced when young children encounter their first enterovirus infection, heterologous cross-reacting IgG and IgM antibodies are produced by older children and adults following repeated infection with different enterovirus serotypes; this reduces their usefulness in diagnosis. A rapid IgM ELISA test for EV71 has recently been developed to try to overcome some of these limitations (Wang et al. 2004); however the possibility of cross reactivity still remains an issue (Xu et al. 2010), and the duration of detectable EV71-specific IgM following an infection is also uncertain.

1.3.3 Other laboratory diagnosis

In mild disease the full blood count is usually normal, but in severe disease the white cell count is often elevated with a neutrophilia (Chang et al. 1999). The urea and electrolytes are typically unchanged, but there may be hyperglycaemia in severe disease (Chang et al. 1999). Among the cardiac enzymes, creatine kinase is sometimes elevated in patients with cardiac involvement (Fu et al. 2004), and elevated cardiac tropinin I has been reported as a predictor of imminent cardiopulmonary failure in children with brainstem encephalitis (Huang et al. 2003). If there is pulmonary oedema it will usually be obvious on a chest X-ray, and the normal heart size will suggest this is not cause by an acute viral myocarditis, or congenital heart disease. The ECG often shows non-specific changes (Fu et al. 2004), and continuous monitoring can demonstrate abnormal beat to beat variability, which may predict imminent cardiovascular collapse (Lin et al. 2006). In children that are haemodynamically unstable with tachycardia, hypotension or pulmonary oedema, an echocardiogram shows generalised left ventricular hypokinesia which may be accompanied by mitral regurgitation (Fu et al. 2004); pericardial effusion is not usually seen. The lumbar puncture is essential in children who are unwell with suspected CNS involvement. In some patients the clinical features, such as meningism or myoclonic jerks, may clearly point to the CNS. However in other children particularly those less than two years old, there may just be high fever, vomiting or

lethargy, but a lumbar puncture reveals CNS disease. There is typically a mild CSF lymphocytic pleocytosis of 10 to 100 cells per mm3, but occasionally there may be none (Perez-Velez et al. 2007). The CSF to plasma glucose ratio is usually normal, but can be low.

1.3.4 Imaging

Computer tomography scans can be helpful to exclude other pathologies, but in EV71 encephalitis, where the pathology is mostly in the brainstem, CT scans are almost always normal. MR imaging in these patients shows characteristic high signal intensities on T2 weighted images in the dorsal pons and medulla, most of the midbrain, and the dentate nuclei of the cerebellum. Similar high signal lesions may also be found in the anterior horn cells of cervical spinal cord (Figure 1.3) (Huang et al. 1999; Shen et al. 1999). However, the usefulness of these changes, in terms of sensitivity, specificity, positive and negative predictive value has yet to be demonstrated. In children with acute flaccid paralysis magnetic resonance imaging typically shows unilateral high signal lesions in the anterior horn cells of spinal cord on T2-wieghted images and contrast-enhancing ventral root on T1 weighted images (Huang et al. 1999; Shen et al. 2000; Chen et al. 2001).

1.3.5 Predictors of severe disease

Several clinical features and laboratory abnormalities have been associated with neurological and fatal EV71 disease, but few have been prospectively validated (Chang et al. 1999; Huang et al. 2003; Hsia et al. 2005). Young age at disease onset is associated with increased risk of severe disease (Chang et al. 2007). Hyperglycemia and leucocytosis were also associated fatal EV71 disease in a retrospective evaluation of the fatal cases (Chang et al. 1999).

1.4 Epidemiology

1.4.1 Clinical Epidemiology

EV71 was first isolated from the stool of a 9-month-old infant with encephalitis in California, USA in 1969 (Schmidt et al. 1974). Before long small outbreaks of neurological infection including encephalitis and aseptic meningitis attributed to the newly identified neurotrophic virus were reported in New York, Melbourne and Sweden in the early 1970s (Blomberg et al. 1974; Kennett et al. 1975; Deibel et al. 1979).

The dermotrophic properties of EV71 were first recognised when the virus caused epidemics of HFMD in Japan in 1973 (Hagiwara et al. 1978; Ishimaru et al. 1980). In the 1970s, two large and severe EV71 epidemics

occurred in Europe. The first was in Bulgaria, and was initially attributed to polioviruses because the epidemiological, clinical and pathological characteristics mimicked that disease (Chumakov et al. 1979; Shindarov et al. 1979). Indeed country-wide administration of Sabin's live-attenuated poliovirus vaccine was one of the public health measures instituted at the height of the epidemic. EV71 infection, confirmed by either virus isolation or neutralization test, was later determined to be the causative agent in 347 (77%) of 451 children who presented with non-specific febrile illness, or neurological disease; forty-four children died. Three years later, the second major European EV71 epidemic took place in Hungary, with 1550 cases (826 aseptic meningitis, 724 encephalitis), with 47 deaths; unlike the Bulgarian epidemic, the Hungarian one also had a small number of patients with HFMD (Nagy et al. 1982).

Recent EV71 activity in the Asia-Pacific region

After the Australian and Japanese EV71 epidemics of the 1970s, further small epidemics and sporadic clusters occurred in Hong Kong (1985) and Australia (1986) (Samuda et al. 1987; Gilbert et al. 1988). Then in 1997 a large outbreak of EV71 in Sarawak, Malaysia heralded the start of a new series of large outbreaks across the Asia-Pacific region, which is still ongoing; these epidemics have confirmed EV71 as a major public health problem across the region, and have provided setting for the work in this thesis (Table 1.1) (Figure 1.4) (Brown et al. 1999; McMinn et al. 2001;

Cardosa et al. 2003; Shimizu 2004; Li et al. 2005; Lin et al. 2006; Chua et al. 2007; Tu et al. 2007; Huang et al. 2008; Huang et al. 2009; Zhang et al. 2009; Chatproedprai et al. 2010; Jeong et al. 2010; Zhang et al. 2010).

During the 1997 Sarawak outbreak, a total of 2618 HFMD cases and 34 deaths were recorded between May and July; around the same time EV71 caused four deaths in peninsular Malaysia and a number of severe neurological diseases in Japan (Lum et al. 1998; Cardosa et al. 1999; Komatsu et al. 1999). In 1998, the largest ever EV71 epidemic to date occurred in Taiwan (Ho et al. 1999). Four hundred and five children were hospitalised for serious neurological complications, 78 of whom died; but epidemiological studies estimated that almost 1.5 million people were infected with the virus.

The clinico-epidemiological features of the EV71 epidemics in Asia in recent years differ from earlier epidemics in that in addition to HFMD, aseptic meningitis, and flaccid paralysis, a brainstem encephalitis associated with cardiopulmonary dysfunction also occurs, which has been the primary cause of death in most fatal cases in Asia (Cardosa et al. 1999; Huang et al. 1999; Prager et al. 2003; Tu et al. 2007; Zhang et al. 2010). Children so affected typically present with a brief febrile illness with subtle neurological signs, following which they develop signs of tachycardia, poor perfusion and tachypnoea, which rapidly develops into

acute intractable cardiac dysfunction and fulminant often fatal pulmonary oedema or haemorrhage (Chang et al. 1998; Chang et al. 1999). Imaging and autopsy findings indicate this is associated with encephalitis in the brainstem, especially the medulla, and neurogenic pulmonary oedema is thought to be the main pathogenic process (Chang et al. 1998; Lum et al. 1998; Lum et al. 1998; Huang et al. 1999). Such rapidly fatal HFMD was not observed in epidemics caused by EV71 in the 1970's and 1980's, where aseptic meningitis was the most commonly observed neurological involvement (Blomberg et al. 1974; Kennett et al. 1975).

EV71 circulation outside the Asia-Pacific region

Outside the Asia-Pacific region, EV71 has continued to circulate in America, Europe, and Africa at a low level causing sporadic cases or small outbreaks. During a one-year prospective study in Canada in 1998, 20 children with EV71 were admitted to a tertiary hospital, most during the summer or fall; half had aseptic meningitis, and a third had respiratory symptoms, but none was severe and all improved rapidly (Merovitz et al. 2000).

Clearly EV71 circulates across most of the globe. Why the large outbreaks have been confined to the Asia-Pacific region, and whether they might be seen elsewhere is not completely understood, though recent molecular epidemiological work on of the evolution of the different

genotypes of virus has given some key insights into this important question.

1.4.2 Molecular epidemiology of enterovirus 71

Virus genogroup, evolution and geographical distribution

Before 1999 there was relatively little information about the molecular epidemiology of EV71. However, systematic laboratory surveillance established in several Asian countries following the first epidemics in the late 1990's has provided invaluable information on the geographical distribution, spread and evolution of the virus.

The first complete phylogenetic analysis of EV71 based on the structural VP1 gene identified three independent lineages of EV71, and designated them genogroup A, B and C (Brown et al. 1999). A sequence diversity of at least 15% in the VP1 gene was used to distinguish genogroups. Genogroup A consists of a single member, the prototype BrCr strain, which was first identified in California in 1970 and until very recently never been reported outside the USA (see Final discussion and overall conclusion chapter). The genogroup B viruses, subdivided into subgenogroups B1 and B2 with divergence of 12% at the nucleotide level, were the predominant circulating strains in the 1970's and 1980's. The

genogroup C viruses, also similarly subdivided into C1 and C2, were only identified later in the mid-1980's (Figure 1.5).

Recombination

Recombination events occur frequently within an enterovirus species (Simmonds et al. 2006). Recombination between EV71 viruses, and occasionally between EV71 and other enteroviruses, such as CVA16 or CVA8 has been reported (Chan et al. 2004; Huang et al. 2009); most often this is in the non-structural gene regions or untranslated region, rather than structural genes, and so diagnostics PCR based on VP1 gene is thought to be robust.

1.5 Pathogenesis

1.5.1 Viral determinants of virulence

The factors that determine whether EV71 infection will be asymptomatic or result in HFMD or severe neurological disease are not known. For poliovirus, the 5' untranslated region (UTR) and VP1 genes are known to contain virulence determinants (Jesus 2007). Several studies have therefore examined the nucleotide sequence of these genes, or the whole genome, comparing isolates from fatal and non-fatal cases, but in most cases the isolates have been identical, or near identical, and significant

changes have not been found (Shih et al. 2000; Singh et al. 2002). The incidence of CNS disease and other severe complications of EV71 infection appear to have varied among the recent outbreaks in Asia, leading to the suggestion that differences in the virulence of the various subgenogroups may have a role. However, comparisons between outbreaks have been hampered by the retrospective nature of many of the studies, and differences in inclusion criteria, definitions of severity, and viral diagnostic capabilities. Perhaps the strongest data supporting the hypothesis that strain virulence determinants play an important role in the pathogenesis of severe neurological disease come from a study in Perth. During the EV71 epidemic in Perth in 1999, two subgenogroups, B3 and C2, co-circulated, thus providing a unique opportunity to examine the role of virulence determinants in a single epidemic setting (McMinn et al. 2001; McMinn 2002). In this outbreak, subgenogroup C2 viruses linked to the Taiwan epidemic of 1998 were almost exclusively isolated from children with severe neurological disease, and only a single isolate came from a case of uncomplicated HFMD. In contrast, subgenogroup B3 viruses, similar to those from the Sarawak 1997 epidemic, were isolated mainly from children with uncomplicated HFMD. aseptic meningitis, or post-infectious neurological disease, none of whom died (McMinn et al. 2001).

1.5.2 Dual infection

During the first of the recent EV71 outbreaks in Sarawak in 1997, which was due to subgenogroup B3, an adenovirus type 21 was also implicated in the fatal cases as well as in some cases with acute flaccid paralysis (Cardosa et al. 1999; Ooi et al. 2003). The virus was isolated from sterile sites such as CSF, brain and heart in fatal cases, and indeed was more frequently detected than EV71 itself; this led to the suggestion that in this early outbreak the fatalities were due to dual infection, rather than EV71 alone (Cardosa et al. 1999).

1.5.3 Host susceptibility

A range of host factors may affect pathogenesis, particularly partial cross protective immunity from prior outbreaks; this may partially explain why young age is a risk factor for severe disease (Chang et al. 2002; Lu et al. 2002; Chang et al. 2004).

1.6 Pathophysiology of severe disease

1.6.1 Viral entry and spread

EV71 is transmitted predominantly via the faeco-oral route, with respiratory spread also implicated (Pallansch et al. 2001). As for other enteroviruses, initial viral replication is presumed to occur in the

lymphoid tissues of the oropharyngeal cavity (tonsils) and small bowel (Peyer's patches), with further multiplication in the regional lymph nodes (deep cervical nodes, mesenteric nodes), giving rise to a mild viraemia. The majority of infections are controlled at this point, and are asymptomatic. However in vivo studies show that if enteroviruses disseminate further they reach target organs, particularly the reticuloendothelial system (liver, spleen, bone marrow and lymph nodes), heart, lung, pancreas, skin, mucous membranes and central nervous system, coinciding with the onset of clinical features.

1.6.2 Pathological findings

CNS inflammation is observed predominantly in the spinal cord gray matter, and the whole medulla oblongata including the dorsal nucleus of the vagus, tractus solitarius, and nucleus, and reticular formation; in addition the hypothalamus and subthalamic and dentate nuclei, and to a lesser degree motor cortex of the cerebrum are involved (Figure 1.6) (Lum et al. 1998; Huang et al. 1999; Shen et al. 1999; Hsueh et al. 2000; Shieh et al. 2001; Wong et al. 2008). Inflammatory changes were absent in cerebellar cortex, thalamus, basal ganglia, peripheral nerve and autonomic ganglia. The histopathological changes, characterised by perivascular cuffs, variable edema, neuronophagia and microglia nodules, are similar to encephalitis caused by other viruses (German et al. 2006).

However, virus inclusion has not been observed, and viral antigens and RNA can only be seen in a small number of neuronal processes and phagocytic cells (Wong et al. 2008).

1.7 Prevention and control

1.7.1 Surveillance and prevention

The only measures available currently for disease control are public health approaches. Outbreak control measures are primarily targeted at interrupting the virus transmission and spread from person-to-person through contact with throat and nose secretions, saliva, stool and vesicular fluid; in addition contact with virus contaminated surfaces, toys or fomites is important. Hence health education focuses on personal hygiene and good sanitation including frequent hand washing, proper disposal of soiled diapers and disinfection of soiled surfaces with chlorinated (bleach) disinfectants.

1.7.2 Vaccine development

There are currently no vaccines against EV71, but by analogy with poliomyelitis, vaccines probably offer the strong best for future disease control.

1.8 Treatment

There are no established antiviral treatments for EV71, and there have been no clinical trials of antiviral or ancillary treatments.

1.8.1 Antiviral agents

Pleconaril is an antiviral drug that inhibits entry into cells for a number of enteroviruses by blocking viral attachment and uncoating, which has been used in clinical trials of aseptic meningitis (Rotbart et al. 1998; Rotbart 2000; Rotbart et al. 2001); however it is not active against EV71 (McMinn 2002; Chen et al. 2008).

1.8.2 Intravenous Immunoglobulin

During the initial large outbreaks of EV71 in Asia, clinicians in Sarawak and Taiwan used intravenous immunoglobulin on the presumptive basis that it might prove beneficial because of anti-EV71 neutralizing antibodies and non-specific anti-inflammatory properties (Wang et al. 1999; Ooi et al. 2007).

1.8.3 Fluid balance and ionotrope support

In routine paediatric practice the most common cause for shock and peripheral shut down is hypovolaemia and dehydration, for example

following gastrointestinal infections, which are treated with rapid fluid resuscitation. However when similar approaches were used in the early EV71 outbreaks in Asia, this often precipitated pulmonary oedema. Once it became clear that impaired cardiac function is an important contributor to shock, clinicians were more judicious in their use of intravenous fluids, and used additional inotrope support. Where possible fluid management should be guided by central venous pressure monitoring. In Taiwan, management algorithms based on this approach appear to have improved outcome (Lin et al. 2002; Chang et al. 2004).

1.9 Scope of this thesis

EV71 has become a major emerging virus across Asia, and has become responsible for frequent explosive epidemics of HFMD with severe neurological complications. A better understanding of which clinical samples are best for the detection of the neurotrophic virus will help in the laboratory diagnosis. Rapid laboratory identification of the virus is critical in guiding public health doctors to institute appropriate intervention and outbreak response measures because EV71 can spread very rapidly in a susceptible population. Clearly a rapid and reliable detection assay of EV71 in primary clinical samples such as an RT-PCR approach would be an ideal alternative to the classical time-consuming tissue culture technique. EV71 infection has emerged as a growing public

health concern in Asia with new clinical manifestations. In the absence of a rapid diagnostic test clinicians will have to depend on their clinical skills to identify children at risk of severe disease. Early recognition of children at risk of severe disease may be the key to reducing acute mortality and morbidity. However which clinical features predict severe disease is not known. Molecular epidemiological studies of the virus indicate that the virus is undergoing rapid evolution as it spreads across Asia in recent years. However the role of the virus genogroup in the viral transmission dynamics and neurovirulence is still poorly understood.

This thesis therefore focuses on the clinical and laboratory diagnosis, case management and pathogenesis of EV71 infection that has emerged in Sarawak, Malaysia. In particular the thesis sets out to answer the following questions:

1. Which type of clinical samples are best for detection of EV71?
2. Can RT-PCR techniques be a rapid diagnostic approach to detect enterovirus 71 in primary clinical samples?
3. What are the clinical features, virological characteristics and molecular epidemiology of enterovirus 71 infection in Sarawak? Is viral genogroup an important determinant of the disease phenotype?
4. How to recognise and manage children at risk of neurological disease and death?

5. What are the differences between cases of hand, foot and disease caused by CVA16 and that by enterovirus 71?

Table 1.1 Worldwide reports of enterovirus 71 outbreaks between 1969 and 1998

Location	Year of the outbreak	No. of cases reported	No. of fatal cases reported	Main clinical presentations	References
California, USA	1969 to 1972	20	1	encephalitis, meningitis, "coxsackie virus"	(Schmidt et al. 1974)
Sweden	1973	195	None	aseptic meningitis, HFMD (some cases)	(Blomberg et al. 1974)
New York, USA	1972	11	None	aseptic meningitis, encephalitis, HFMD (only 1 case)	(Deibel et al. 1979)
Bulgaria	1975	705	44	aseptic meningitis,	(Chumakov et al.

				paralytic disease including bulbar encephalitis	1979)
Japan (included 3 outbreaks)		1031	some deaths, exact number not reported	HFMD (in most patients), cerebellar encephalitis, meningitis, acute flaccid paralysis	(Ishimaru et al. 1980)
New York, USA	1977	12	none	CNS disease, HFMD, acute respiratory illness, acute gastroenteritis	(Chonmaitree et al. 1981)
Hungary	1978	323 laboratory-	47 (not clear if all due to EV71)	meningitis, encephalitis, poliomyelitis, HFMD	(Nagy et al. 1982)

		Confirmed EV71 cases		(only 4 cases)	
Australia	1986	114	none	HFMD (in most cases), meningitis, encephalitis, encephalomyelopathy	(Gilbert et al. 1988)
Philadelphia	1987	5	none	acute flaccid paralysis	(Hayward et al. 1989)
USA	1977-1991	193 lab confirmed EV71 cases	not known	1985-1989: paralysis, meningitis, encephalitis, rash, other	(Alexander et al. 1994)
Sarawak, Malaysia	1997	2628	34	HFMD, aseptic meningitis, acute flaccid paralysis,	(Chan et al. 2000)

				cardiorespiratory dysfunction	
Otsu, Japan	1997	12	none	HFMD, herpangina, meningoencephalitis, encephalitis, meningitis	(Komatsu et al. 1999)
Taiwan	1998	129,106	78	encephalitis, aseptic meningitis, pulmonary oedema or haemorrhage, acute flaccid paralysis, and myocarditis.	(Ho et al. 1999)

Table 1.2 Human enterovirus species and serotypes

Species	Serotype
A	CV-A2-8, CV-A10, CV-A12, CV-A14, CV-A16, EV-71, EV-76, EV-89-92
B	CV-A9, CV-B1-6, E-1-7, E-9, E-11-21, E-24-27, E-29-33, EV-69, EV-73, EV-74-75, EV-77-88, EV-93, EV-97, EV-98, EV-100, EV-101, EV-106, EV-107
C	CV-A1, CV-A11, CV-A13, CV-A17, CV-A19-A22, CV-A24, EV-95, EV-96, EV-99, EV-102, EV-104, EV-105, EV-109, PV-1-3
D	EV-68, EV-70, EV-94

Table 1.3 Neurological syndromes association with enterovirus 71 infection

Purely neurological manifestations:	
Encephalitis, especially brainstem	Common
Acute flaccid paralysis (anterior myelitis)	Common
Encephalomyelitis	Common
Aseptic meningitis	Very common
Cerebellar ataxia	Uncommon
Transverse myelitis	Rare
Neurological plus systemic manifestations:	
Brainstem encephalitis with cardiorespiratory failure	Common
Manifestations indicative of immune-mediated (para-infectious) mechanisms	
Guillain-Barreé syndrome	Uncommon
Opsoclonus-myoclonus syndrome	Rare
Benign intracranial hypertension	Rare

. Modified from (McMinn 2002)

Table 1.4 Differential Diagnosis of enterovirus 71 infection

Rash	
	Viral Infections
	*Coxsackie virus A16, and other enteroviruses
	*Measles
	*Rubella
	*Chicken pox (varicella zoster virus)
	Roseola infantum (human herpes viruses 6 & 7)
	Erythema infectiosum ("slapped cheek disease", parvovirus B19)
	Dengue Hemorrhagic fever (and other flavivirus infections, e.g. West Nile virus)
	Chikungunya (and other alphavirus infections, e.g. O'nyong nyong, Western equine encephalitis)
	Epstein Barr virus
	Primary HIV infection
	Non-specific viral rashes
	Bacterial Infections
	Meningococcemia (*Neisseria meningitides*)
	Scarlet fever *(Streptococcus pyogenes)*
	Leptospirosis (Weil's disease;*Leptospira* Spp)
	Relapsing fever (*Borrelia recurrentis*)
	Lyme Disease (*Borrelia burgdorferi*)
	Syphilis (*Treponema pallidum*)
	Typhus and other Rickettsial infections
	Other diseases
	*Scabies (*Sarcoptes scabei*)
	Drug reaction
	Allergy
	Paraneoplastic syndrome

Aseptic Meningitis

Viral Infections

*Echoviruses, Coxsackie viruses and other enteroviruses,

Herpes simplex virus type 2

HIV

Mumps

Flaviviruses (Japanese encephalitis virus, West Nile virus, Tick-borne encephalitis virus)

Alphaviruses (Western Eastern and Venezuelan encephalitis virus)

Bunyaviruses (eg La Cross virus)

Lymphocytic choriomeningitis

Bacterial Infections

*Bacterial meningitis (partially treated)

Tuberculous meningitis

Parameningeal bacterial infections

Listeria (*Listeria monocytogenes*)

Syphilis

Lyme disease (*Borrelia burgdorferi*)

Weil's disease (Leptospira spp)

Mycoplasma pneumoniae

Other

Drug reactions (antibiotics, non-steroidal anti-inflammatory drugs)

Fungi (e.g. *Cryptococcus* spp, *Candida* spp, *Aspergillus* spp)

Flaccid paralysis

Viral Infections

*Poliomyelitis and other enteroviruses

Vaccine-associated paralytic poliomyelitis

Flaviviruses (Japanese encephalitis virus, West Nile virus, Tick-borne encephalitis virus)

Rabies

Adenoviruses

Bacterial Infections

Botulism

Diphtheria

Other

Intramuscular injection into buttock causing sciatic nerve damage

Guillain-Barre syndrome (esp acute motor axonal neuropathy)

Brainstem encephalitis

Viral Infections

*Poliomyelitis and other enteroviruses

*Flaviviruses (Japanese encephalitis virus, West Nile virus, Tick-borne encephalitis virus)

Nipah virus

Bacterial Infections

Listeria (*Listeria monocytogenes)*

Tuberculosis (*Mycobacterium tuberculosis*)

Brucellosis (*Brucella abortens*)

Lyme disease (*Borrelia burgdorferi*)

Other

Paraneoplastic syndromes

NB the relative importance of the pathogens listed in the differential varies greatly according to the age of the patient, and the geographical location

*Those which are especially likely to be confused with enterovirus 71 infection are marked with an asterix. Modified from (Solomon 2009)

Figure 1.1 Enterovirus 71: three dimensional structure and genome structure

VP1, VP2, VP3 and VP4 are viral structural proteins, coded for by the P1 region of the genome; P2 and P3 regions encode seven non-structural proteins 2A-2C and 3A-3D, UTR is untranslated region. VPg is virus encoded protein. Modified from http://www.expasy.ch/viralzone/all_by_species/97.html and (Brown et al. 1995).

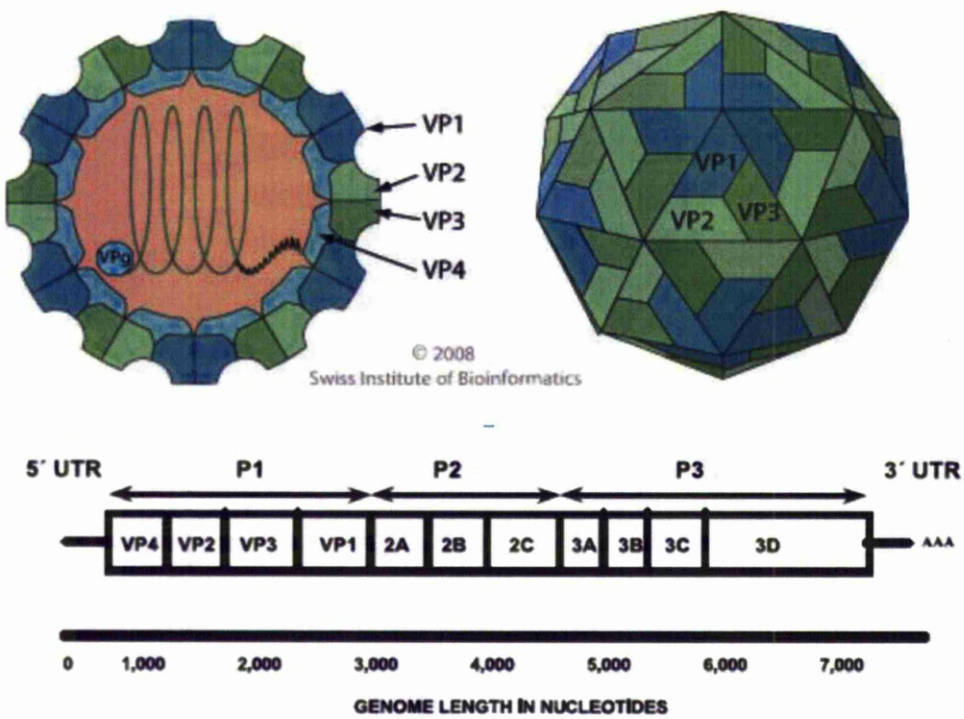


Figure 1.2 Mucocutaneous lesions in hand, foot and mouth disease

This Malaysian child has ulcers are seen inside the upper lip (top), and vesicular and macular lesions on the wrists (middle) and soles (bottom).Photo credit: T Solomon

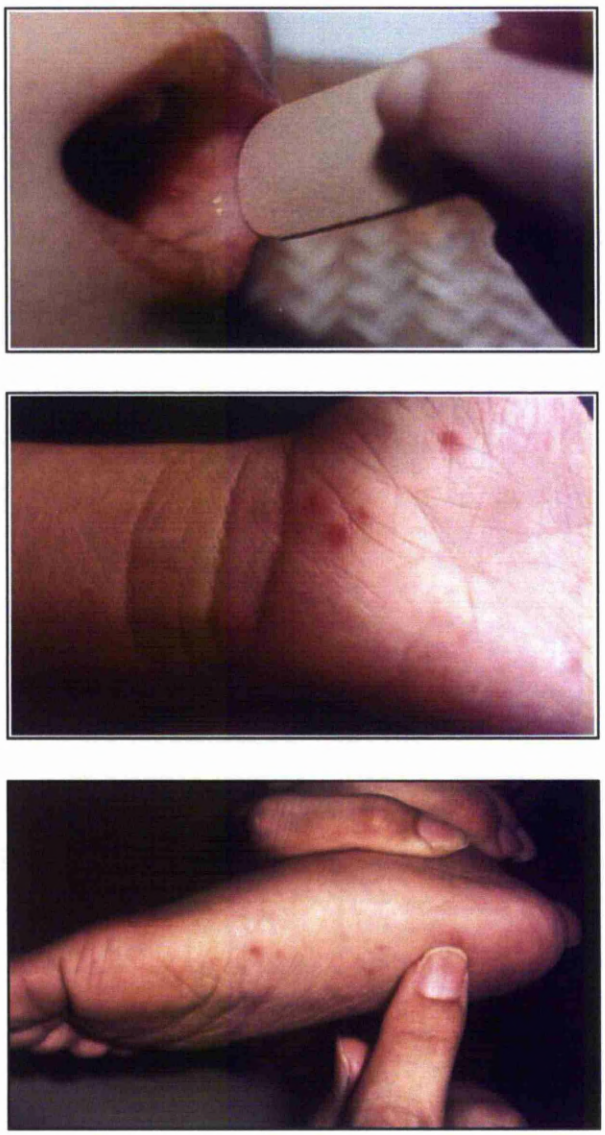


Figure 1.3 MRI changes in enterovirus 71 encephalomyelitis

T-2 weighted images of a 10 month old female who presented three months earlier with somnolence tachycardia, tachypnea and coma, and who recovered consciousness but remained ventilator dependent. A. sagital section showing high signal intensity in the posterior portion of the pons and medulla (black arrows) and anterior cervical cord (white arrows). B. Axial section showing the high signal intensity in the two anterior horns of the cervical cords (black arrows) , modified from (Shen et al. 1999).

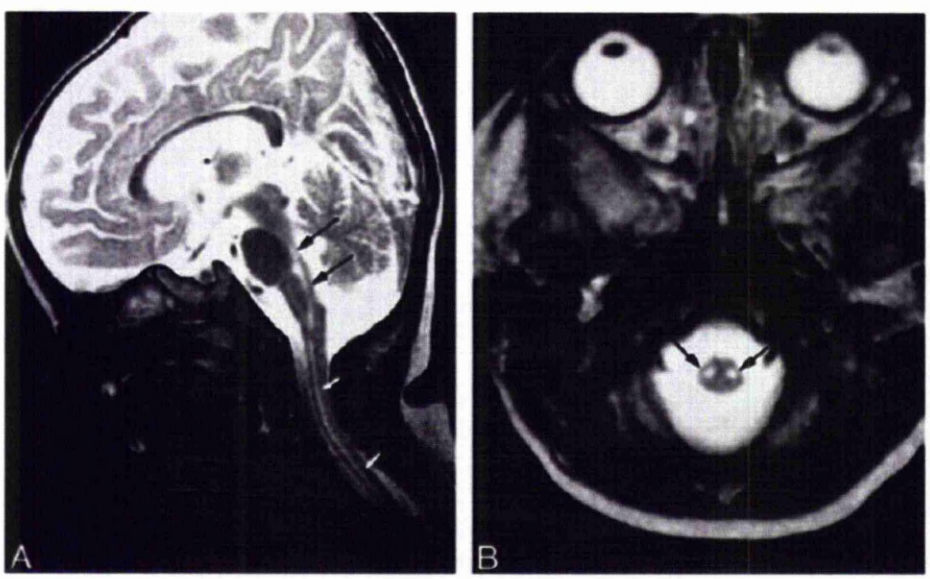
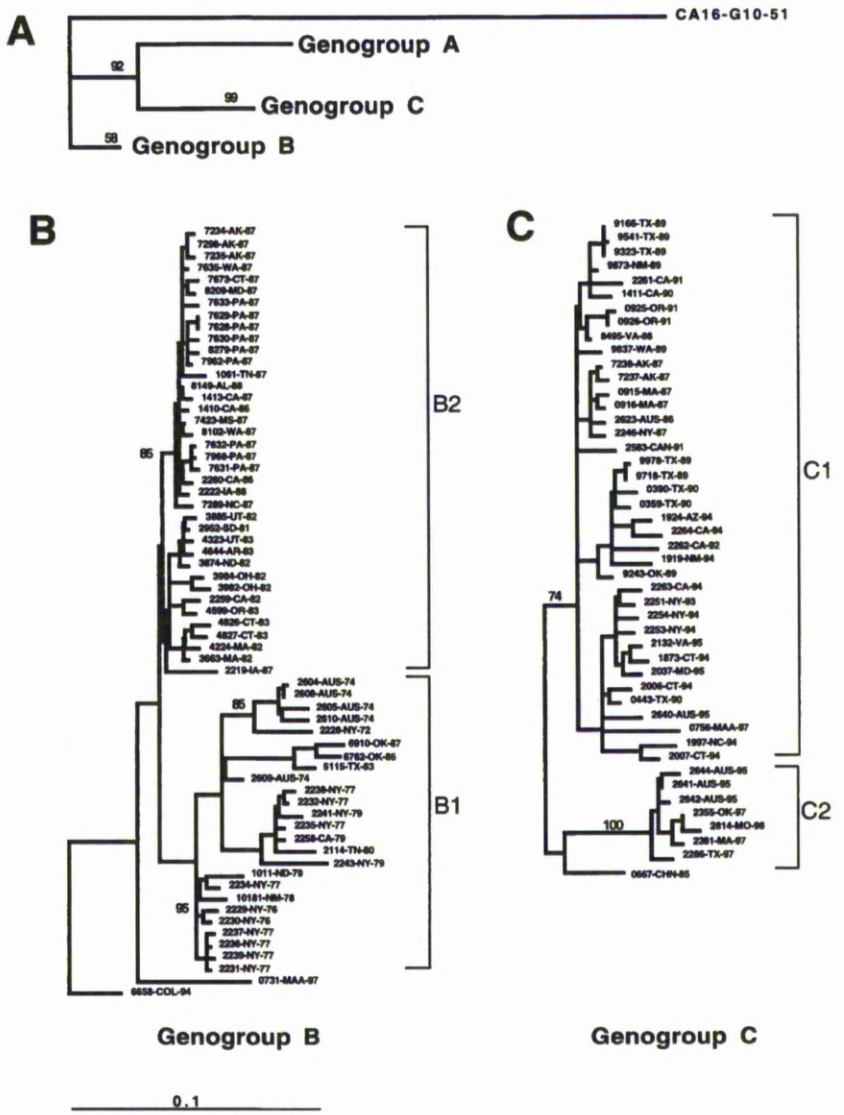


Figure 1.4 Map of Southeast Asia

The map shows the geographical location of the state of Sarawak, Malaysia. The country consists of two regions, West and East Malaysia, which are separated by the South China Sea. Sarawak is situated at the Northern region of the Borneo Island, where it shares its borders with Brunei and Kalimantan, Indonesia. Sibu Hospital (the study site) is situated at Sibu, which was the epicentre of the Sarawak 1997 epidemic. All the virological investigations performed in the thesis were carried out at Institute of Health and Community Medicine, Universiti Malaysia Sarawak at Kuching (the capital city of Sarawak).

Figure 1.5 Phylogenetic tree of enterovirus 71 isolated between 1970 and 1998

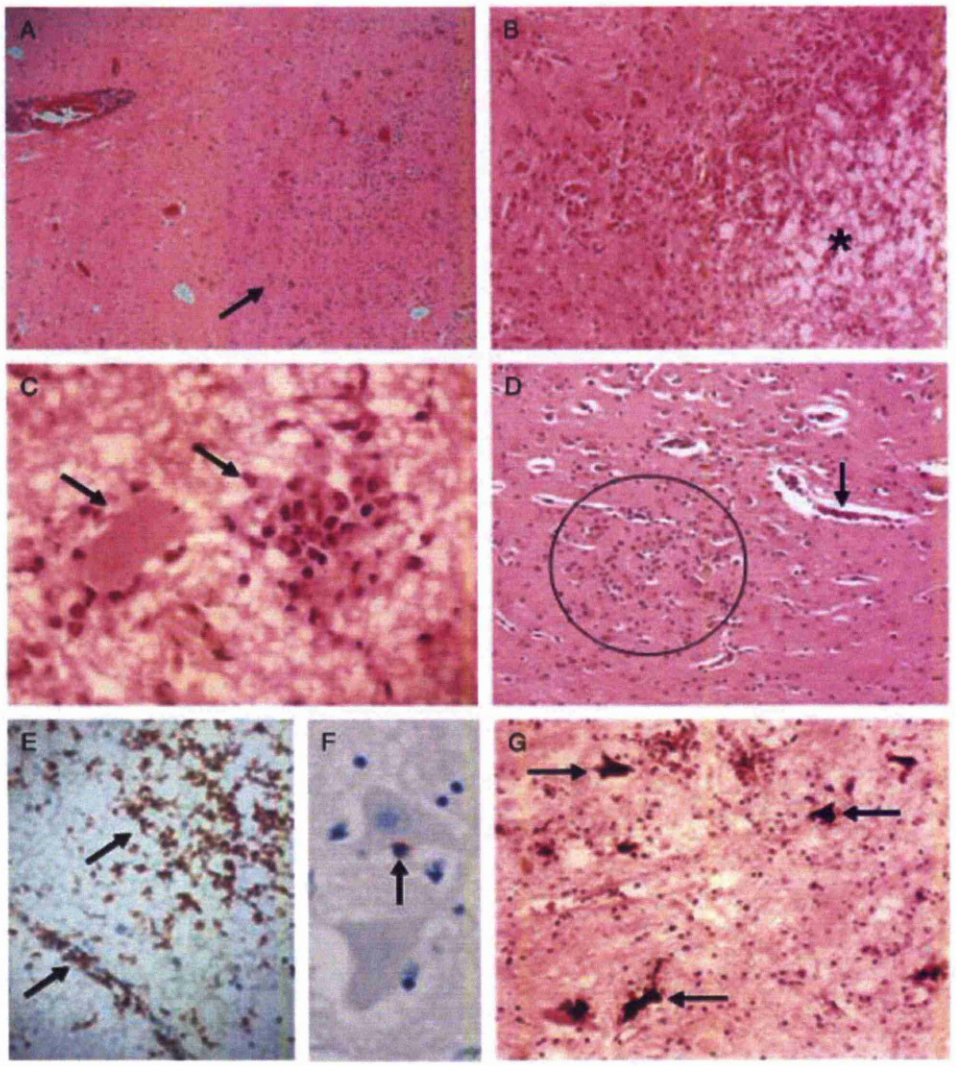
The dendrogram was generated by the neighbor-joining method with the DNADIST distance measure program (PHYLIP, version 3.5). The phylogram was calculated based on the nucleotide divergence of the VP1 gene (position 2442 to 3332). The last four or five characters of each strain name indicate the state or country and year of isolation. Branch lengths are proportional to the number of nucleotide differences; the frequencies with which the branches for genotypes A, B, and C appeared in 1,000 bootstrap replications were 898, 543, and 999, respectively. Clades with bootstrap numbers are expressed in percentile. The marker denotes a measurement of the relative phylogenetic distance. (A) The branch length for the outgroup, CA16-G10-51, was reduced by 0.75 to save space.



Adapted from (Brown et al. 1999)

Figure 1.6 Pathological findings in enterovirus 71 encephalitis

Parenchymal inflammation (arrows) and perivascular cuffing in the the medulla **(A);** more severely inflamed areas **(B),** with edema (*) and neuronophagia (**C,** arrows). More subtle inflammation in the motor cortex with mild perivascular cuffing (arrow) and parenchymal inflammatory cells (circle) (**D**). Numerous CD68-positive macrophages/microglial (**E**), CD8-positive lymphocyte adjacent to a neuron (**F**). Viral RNA in the anterior horn cells of the spinal cord (**G**). (**A–D**: hematoxylin and eosin stains; **E, F**: immunohistochemistry / peroxidise / DAB; **G**: in situ hybridization / nitroblue tetrazolium/ 5-bromo-4-chloro-3-indolyl phosphate stains. Original magnification: (**A**) 4x; (**B, D**) 10x; (**C, F**) 40x; (**G**) 20x.


A
B
C
D
E
F
G

Chapter 2: The usefulness of different clinical samples in laboratory diagnosis of enterovirus 71

Abstract

Human enterovirus 71 and coxsackie virus A16 are important causes of hand, foot and mouth disease (HFMD). Like other enteroviruses they can be isolated from a range of sterile and non-sterile sites, but which clinical sample, or combination of samples is the most useful is not clear. Virus culture was attempted on 2916 samples from 628 of 725 children with HFMD studied over a 3½ year period (between January 2000 and July 2003), which included 2 large outbreaks. Overall, throat swabs were the single most useful specimen, being positive for any enterovirus for 288 (49%) of 592 patients with a full set of samples. Vesicle swabs were positive for 169 (48%) of 333 patients with vesicles, the yield being greater if two or more vesicles were swabbed. The combination of throat plus vesicle swabs identified virus for 224 (67%) of the 333 patients with vesicles; for this patient group, the addition of rectal and ulcer swabs diagnosed just 27 (8%) extra patients. Of 259 patients without vesicles, the combination of throat plus rectal swab identified virus for 138 (53%).

60 patients had virus isolated from both vesicle and rectal swabs, but for 12 (20%) of these, the isolate differed. Such discordance occurred for just 11 (10%) of 112 patients with virus isolated from vesicle and throat swabs. During large HFMD outbreaks it is recommended to collect swabs from the throat plus one other site: vesicles, if these are present (at least two should be swabbed), or the rectum if there are no vesicles. Vesicle swabs give a high diagnostic yield, with the added advantage they are from a sterile site.

2.1 Introduction

Hand, foot and mouth disease (HFMD) is a common febrile illness in young children characterised by lesions on the skin, and oral mucosa. The skin rash, which may be maculopapular or vesicular, typically occurs on the palms and soles, but can also involve the buttocks, elbows and knees. Mouth ulcers are the most common enanthema, but some patients have herpangina (multiple oral ulcers predominantly affecting the posterior part of the oral cavity), and others have no oral lesions (Pallansch et al. 2001; McMinn 2002).

Many human enteroviruses (family *Picornoviridae*, genus *enterovirus*) can cause HFMD, but human enterovirus (EV) 71 and the closely related coxsackie virus (CV) A16 are the most important (Pallansch et al. 2001;

McMinn 2002). Since the late 1990's, EV71 has caused a series of large HFMD epidemics in the Asia-Pacific region, associated with a rapid fulminant course, severe neurological complications and a large number of fatalities (Cardosa et al. 1999; Ho et al. 1999; Komatsu et al. 1999; McMinn et al. 2001). CVA16 causes a similar clinical illness initially, but neurological and other severe complications are extremely rare (Chang et al. 1999). In much of Asia there is now epidemiological and virological surveillance for HFMD so that effective public health measures, such as closing nurseries and schools, can be instituted early. However, because of the similar clinical presentation, establishing the actual cause of HFMD cases relies on laboratory identification of the virus. Diagnostic techniques include isolating the virus in susceptible continuous cell lines or detecting viral RNA using the polymerase chain reaction (PCR) (Lina et al. 1996; Yerly et al. 1996). Though laborious and time consuming, virus isolation remains the gold standard for enterovirus diagnosis; it is a relatively cheaper than PCR, and is the most widely used method, particularly in developing countries such as in Sarawak, Malaysia.

There is a wide range of samples from which virus isolation can be attempted, including rectal and throat swabs, serum, and CSF (when taken) and vesicles and ulcers when they are present. However for EV71-associated HFMD outbreaks there has been relatively little work examining which samples or combination of samples are the most useful.

This question becomes especially important in the context of large outbreaks with many thousands of patients. Rectal and throat swabs are available for all patients, and do not require the presence of mucocutaneous stigmata. However, they have the disadvantage that, because these are not sterile sites, isolation of virus here may represent coincidental asymptomatic carriage, rather than the causative agent (Rotbart et al. 1995): many enterovirus infections are asymptomatic, and viral shedding may persist for up to 2 weeks from the throat and up to 11 weeks from the rectum (Chung et al. 2001; Pallansch et al. 2001). In the absence of virus isolation from a sterile site, isolates from non-sterile sites are usually accepted as surrogate markers for enterovirus infections (Rotbart 1995; Pallansch et al. 2001). There is little data available on the validity of this approach for EV71-associated HFMD. I therefore set out to answer three important clinical microbiological questions during a 3½ year prospective clinical and diagnostic study of HFMD, which included two large outbreaks: firstly which single specimen is most often positive for the different HFMD patient groups; secondly, which combination of samples is the most efficient in terms of diagnostic yield; and thirdly how reliable are samples from non-sterile sites, compared with sterile sites.

2.2 Materials and methods

2.2.1 Clinical and laboratory methods

Between January 2000 and July 2003 all children with HFMD in the paediatric ward and intensive care unit at Sibu Hospital, Sarawak, Malaysia were included into the study.

Case definitions

a. Hand, foot and mouth disease

A child was defined as having HFMD if they had new onset of at least one of the following: maculopapular and/or vesicular rash on the palms and/or soles; vesicles or ulcers in the oral cavity; herpangina (defined as multiple oral ulcers predominantly affecting the posterior parts of the oral cavity).

b. Suspected central nervous system involvement

A child with HFMD was suspected of having central nervous system (CNS) involvement if he or she had a history of fever, or fever on examination ($\geq 38^{\circ}C$), and at least one of the following: toxic and ill in appearance, recurrent vomiting (at least twice), tachycardia (heart rate >150/min) breathlessness, poor perfusion (cold clammy skin), reduced consciousness (irritability, lethargy, drowsiness, coma), limb weakness, meningism (neck stiffness or positive Kernig's sign), seizures.

Collection of clinical samples

All children with HFMD admitted into the hospital were assessed by the paediatricians of the study team. A detailed history and clinical examination, including for mucocutaneous lesions, was recorded on standardised forms. A rectal and throat swab was taken for each child. The skin was examined carefully for vesicles, and the oral cavity for ulcers, and if they were present swabs were taken from at least one of each (usually the largest and most accessible lesions). Rectal swabs were taken with a gentle circular motion on the rectal wall. Throat swabs were taken with the aid of a tongue depressor, by carefully swabbing the lateral and posterior pharynx without touching the tongue or buccal mucosa. For vesicle swabs the skin was cleaned gently with 0.9% sterile normal saline, but not alcohol which kills viruses. A sterile 24G needle was used to rupture the vesicle, and the fluid absorbed onto a swab. Alternatively the swab was gently rolled over the vesicle to squeeze out fluid. Mouth ulcers were sampled by rolling the swab over the floor of the ulcer. When more than one vesicle or ulcer was swabbed, a fresh swab was used for each lesion and put into a separate tube of viral transport material, because I was interested in the yield from each swab. Swab samples were collected by study team members or nursing staff, after training. Cerebrospinal fluid (CSF) and serum were collected from children with suspected central nervous system (CNS) involvement. The clinical samples were stored immediately in a -70°C freezer until further testing. Out of hours,

when immediate storage at -70°C was not possible, clinical samples were stored at 4°C overnight, and were transferred to a -70°C freezer the following morning.

Virological method

Virus isolation was attempted on all swabs, CSF and any serum which had adequate volume. Specimens were inoculated into rhabdomyosarcoma (RD) and 293 (human embryonal kidney) cells, and observed daily for cytopathic effects. Cell cultures were harvested when the monolayer showed extensive cytopathic effect. A blind passage was done in the same cell line with all cultures showing no cytopathic effect after 10 to 14 days. Enteroviruses isolated were typed by nucleotide sequencing of the VP1 or the VP4 genes (McMinn et al. 2001; Cardosa et al. 2003).

For the purposes of analysis, swabs from herpangina lesions were grouped with other mouth ulcers. Vesicles, serum and CSF were considered to be sterile sites, and swabs from the throat, mouth ulcers and rectum to be from non-sterile sites. All samples were investigated, irrespective of the results of other samples from the same patient.

2.2.2 Analytical approach

The patients were divided into 4 groups according to their presenting mucocutaneous lesions, and thus the availability of samples: those with a papulovesicular rash and mouth ulcers (referred to hereafter as HFMD with vesicles and ulcers); those with a papulovesicular rash only (HFMD with vesicles); those with a maculopapular rash and mouth ulcers only (HFMD with ulcers), and patients with maculopapular rash only (HFMD with maculopapular rash). To determine which combination of samples gives the best diagnostic yield, a stepwise approach was adopted to the analysis for each patient group. First I determined which sample type gave the most positives. Then I looked at the remaining undiagnosed patients, and determined which of the remaining samples gave the most positives. I continued like this until all sample types had been assessed. I decided to use data from the first outbreak to determine the usefulness of different samples, and combinations of samples. I then applied these findings to the second outbreak to see if the predicted samples remained useful. However, the sample analysis was not begun until the end of the study, to avoid any bias in sample collection.

2.2.3 Statistical analysis

Statistical analysis was performed by using statistical software Statview 4.02 Abacus Concepts Inc. Sensitivity, specificity, positive predictive value and negative predictive value were calculated from a 2 X 2 table.

2.2.4 Ethical approval

The study was approved by the Director of Health for Sarawak (Malaysia) and the Ethics Committee of the Liverpool School of Tropical Medicine (Liverpool, United Kingdom). Informed consent was obtained from each child's accompanying parent or guardian.

2.3 Results

Seven-hundred-and-twenty-five patients were entered into the study: 471 (299 [63%] males, median [range] age 28 [4-120] months) were enrolled in the first half (the majority in an outbreak between January 2000 and March 2001), and 254 (158 [62%] males, median [range] age 28 [2-153] months) were enrolled in the second half (mostly during an outbreak between January and July 2003). The 471 patients in the first half of the study included 110 with vesicles and ulcers, 112 patients with vesicles only, 78 patients with ulcers only, and 171 patients with a maculopapular rash only. Of the 254 patients in the second half of the study 98 had

vesicles and ulcers, 29 had vesicles only, 87 had ulcers only, and 40 had a maculopapular rash only. The median (range) duration of illness before admission was 2 (0-8) days, and did not differ significantly between patient groups.

2.3.1 Virology results

Viral isolation was attempted on a total of 2916 samples: 1666 from 389 (83%) of the 471 patients in the first half of the study, and 1250 from 239 (94%) of the 254 patients in the second half of the study. Most patients had a single throat swab and single rectal swab cultured. In addition, 127 patients with vesicles had at least one (median 2; range 1-10) vesicle investigated, and 185 patients with ulcers had at least one (median 2; range 1-6) sample investigated. For a single swab, 35% of vesicles and 17% of ulcers were positive (Table 2.1), but the percentages increased as more swabs were taken.

The number of patients tested for each sample type, and the number that were positive for any enterovirus, for EV71, and for CVA16 are shown in Table 2.2. During the first half of the study a throat swab was most likely to be positive (being positive for any enterovirus for 191 [52%] of 367 patients), followed by vesicle, ulcer and then rectal swabs. During the second half, vesicle swabs were most likely to be positive (positive for

any enterovirus for 63 [50%] of 127 patients), followed by throat, rectal and then ulcer swabs. Most (>95%) viruses were isolated following a single passage.

In the first half of the study, 167 (65%) of 255 patients with positive viral isolation were EV71 positive (11 of whom were also infected with CVA16, 2 with CVA4, 2 with CVA24 and 1 with adenoviruses [Ad]-7); CVA16 was isolated from a further 69 patients (2 of whom were also infected with another virus). In addition 19 patients were infected with other enteroviruses, adenoviruses or unidentified viruses (6 of whom had multiple viruses isolated). In the 2nd half of the study, 106 (44%) of 239 patients had EV71 isolated, 10 of whom had a second virus isolated: 3 with CVA16, 2 with CVA5, and 1 each with CVA10, Poliovirus 1 Sabin vaccine strain, Ad1, an untypeable enterovirus, and an unidentified virus. CVA16 was isolated from 16 further patients (2 of whom were also infected with a second virus, Ad2 and Ad4). In addition 31 patients were infected with other enteroviruses, adenoviruses or unidentified viruses (4 of whom had multiple viruses isolated). For most patients with multiple isolates the viruses came from different clinical samples, however in 20 cases two viruses were isolated from the same clinical sample. These comprised 9 rectal swabs, 5 throat swabs, and 2 ulcer swabs, which gave different isolates in different cell lines; in addition four patients with mild HFMD had two different viruses grown from different vesicles (three

with EV71 and CVA16, and one with EV71 and poliovirus Sabin strain type 1). The serotyping of enterovirus isolates was repeated and verified.

Across the whole study 79 (51%) of 156 patients who required CSF examination had elevated CSF cell counts (>5/mm^3), but only 3 had virus cultured (two EV71 and one other). Enteroviruses were isolated from the serum of 7(9%) of 81 patients, 2 were EV71, 2 were CVA16, and there was a single CVA6, CVA9, and CVA10.

2.3.2 Analysis of sample combinations during first outbreak

Figure 2.1a shows for each patient group, the possible incremental increases in number of patients diagnosed virologically, by different combinations of samples assessed stepwise according to the analytical plan. For this part of the analysis, only patients with full sets of samples were studied. For vesicles and ulcer swabs, the results for multiple swabs of a single type were treated as a single result, so that if at least one swab was positive, this was taken as a positive result for that sample type.

For the 105 patients with vesicles and ulcers in the first half of the study, the throat swab alone diagnosed 63 (60%) patients, whereas the vesicle swabs alone would have diagnosed 54 patients, the rectal swab alone 29 patients, and the ulcer swab alone 26 patients. Throat swabs were

therefore taken as the first sample (Figure 2.1a). The number of patients with a virological diagnosis (the diagnostic yield) would increase to 73 patients if the results of vesicle swabs were added next, 70 if rectal swabs were added, or 68 if ulcer swabs were added. The vesicle swab results were therefore added as a next step. From here the addition of the rectal swab results would increase the yield to 79 patients, whereas ulcer swab results would increase it to 75 patients; so the rectal swab was therefore added next, and the ulcer swab was added last, increasing the yield to 82 (78%) patients.

For patients with HFMD and a vesicular rash, a throat swab was again the most useful single sample, diagnosing 57 (52%) of 109 patients. The addition of the rectal swab result would increase the diagnostic yield to 70 (64%), whereas the addition of the vesicle swab result would increase it to 72 (66%). The vesicle swab result was therefore used as the second investigation; finally the addition of the rectal swab result increased the number of patients diagnosed to 79 (72%). For patients with ulcers only, a throat swab diagnosed virologically 33 (48%) of 69 patients; addition of either the rectal or vesicle swab increased the diagnostic yield to 38 (55%) patients, and combining all three samples diagnosed 42 (61%) patients. Finally, for patients with a maculopapular rash only, a throat swab alone diagnosed 35 (43%) of 82 patients, whereas a rectal swab alone diagnosed 22 patients. A throat swab was therefore used as the first

sample, followed by the rectal swab, which increased the number of patients diagnosed to 44 (54%) of 82 patients.

In figure 2.2a, the proportion of patients diagnosed at each step, using the best combination of samples as determined above, is compared with the total number of samples analyzed at each step. It is clear that although the detection rate increased as more clinical sample types were included, the number of samples analyzed increased to a much greater extent. For example for the patients with vesicles and ulcers, 63 patients were diagnosed by throat swab samples alone (105 samples, 1.7 samples per patient diagnosed); adding the vesicle swab results diagnosed a further 10 patients, but required the processing of a further 264 samples (26.4 samples per patient); adding the rectal swab results diagnosed 6 more patients for a further 105 samples (17.5 per patient), and adding ulcer swab diagnosed 3 more patients for 213 samples (71 per patient). Thus the total the number of samples needed to be analyzed to diagnose each additional patient increased dramatically for each additional sample type included.

2.3.3 Recommendation based on the first outbreak

Based on these observations, it was decided that during a large outbreak, if one wanted to limit the number of samples, the following could be

recommended. For patients with both vesicles and ulcers, investigation of throat and vesicle swabs diagnosed most patients (70%), and the addition of rectal or ulcer swabs added little value, for the extra work and cost involved. Similarly for patients with vesicles only, the combination of throat swabs and vesicles gave a good diagnostic yield, and the addition of rectal swabs only increased the yield marginally (6%). For patients with ulcers only, throat and ulcer swabs, or throat and rectal swabs were equally useful, but combining all three swabs only increased the diagnostic yield by 6%. For patients with a maculopapular rash only, both throat and rectal swabs should be tested.

2.3.4 Application of findings to the second outbreak

The validity of these recommendations was tested on data from the second outbreak. The same analytic process was applied to determine which combination of samples gave the best diagnostic yield and to see if the recommended combinations would prove to be the most useful. Figures 2.1b and 2.2b show that, for the most part, the approach remained valid. For patients with vesicles plus ulcers, and for patients with vesicles only, the combination of throat and vesicle swabs gave a good diagnostic yield (66 and 67% respectively), with further samples not improving the yield greatly. Interestingly, though, for the first patient group vesicles, rather than throat swabs, were the single most useful sample. For patients

with a maculopapular rash only, throat swabs and then rectal swabs were most useful. However, for those with ulcers only, the addition of rectal swabs to throat swabs proved more useful than the addition of ulcer swabs, increasing the yield to 41 (48%) of 85 patients, as compared to 36 (42%).

Thus, summarising the data from both outbreaks together, the combination of throat swabs plus vesicle swabs was the most useful approach for patients with vesicles (whether or not they also had ulcers), identifying virus for 134 (64%) of 208 patients with vesicles and ulcers, and 90 (66%) of 136 patients with vesicles only; the combination of throat swab and rectal swab was most useful for patients without vesicles (whether or not they had ulcers), identifying virus for 79 (51%) of 154 patients with ulcers only, and 59 (56%) of 105 patients with maculopapular rash only.

2.3.5 Concordance of viral diagnosis between samples

To examine the concordance of virus isolates from non-sterile sites (rectal, throat and ulcer swabs), with those from a sterile site (vesicle swabs), all HFMD patients with swabs taken from vesicles plus another site were studied (Table 2.3). The isolation results from 212 (63%) of 337 patients with throat swabs, 187(55%) of 342 with rectal swabs and 112

(54%) of 208 with ulcer swabs were in agreement with the results from vesicle swabs of the same patients (either the same virus isolated or no virus isolated). However, a different virus was isolated for 11 (10%) of 112 patients with positive throat and vesicle swabs, 4 (12%) of 33 patients with positive ulcer and vesicle swabs, and 12 (20%) of 60 patients with positive rectal and vesicle swabs. Overall taking the vesicle swab as a reference, the sensitivity of the throat swabs for isolating the same virus was 67%, the specificity 63%, and the positive and negative predictive values were 61% and 69%. Equivalent values for the rectal swabs were 31%, 79%, 56%, and 57%, and for ulcer swabs were 28%, 81%, 60% and 53%. The detailed viral isolation results of these patients with different viruses isolated from vesicle and non-sterile sites are shown in Table 2.4.

2.4 Discussion

The outbreaks of EV71-associated hand foot and mouth disease which have swept across the Asia Pacific region since 1997 have posed a great economic and social burden, not least on the health care facilities and laboratories that have to diagnose and manage such patients. The three large outbreaks in Sarawak (1997, 2000 and 2003) are estimated to have affected several thousand children, whilst the 1998 Taiwanese outbreak was estimated to have affected 1.5 million children (Ho et al. 1999; Chan et al. 2000; Cardosa et al. 2003). To diagnose EV71 infection, clinicians

are faced with a wide range of samples to choose from, but there has been relatively little work comparing them. Whilst for the occasional patient, taking every sample might be possible, faced with a large outbreak with hundreds to thousands of patients, a more rational approach is needed.

Most of the recent studies of EV71 infection have relied on stool culture and throat swabs, and have found the latter to have greater sensitivity, with throat swabs being positive in 90 to 95% compared to stool culture being positive in 40-65% of patients tested (Chang et al. 1999; Wang et al. 1999; Wang et al. 2000). Few studies have looked systematically at all samples from a large patient group. One report of 175 patients with HFMD during the 2000 outbreak in Singapore found that rectal swabs most often yielded virus, followed by stool samples, vesicle swabs, and then throat swabs (Chan et al. 2003). However, throat swab was the single specimen most likely to be positive in most patient groups in this study, being positive for 288 (49%) of the 592 patients with a full set of samples, and 292 (49%) of all 598 patients studied. Approximately half of the patients had skin vesicles, and the result showed that vesicle swabs were also very useful. In patients with vesicles, they gave the second highest yield, being positive for 169 (48%) of 333 patients studied and in one patient group (during the second outbreak) they were the single most sensitive specimen (positive for 50%). Vesicle swabs have not been widely used to diagnose HFMD previously. One study from the EV71

outbreak in Taiwan in 1998 reported virus isolation from four children who had vesicle swabs investigated, all of which were positive (Chang et al. 1999). But for most outbreaks vesicle fluid was not investigated (Komatsu et al. 1999; Wang et al. 1999; Fujimoto et al. 2002; Nolan et al. 2003; Prager et al. 2003). However, in the report from the Singapore 2000 outbreak, vesicles were positive for 50% of 62 HFMD patients with vesicles (Chan et al. 2003).

To examine the optimal number of lesions to sample, separate swabs were taken from each vesicle. Swabs from single vesicle were positive 35% of the time, but increased to 49% with two vesicles and 60% with 3 vesicles were sampled. Based on this observation, applying a single swab to two or more vesicles is recommended. This maximises the chance of isolating virus, without doubling the number of samples to be processed. In the study approximately half the patients had vesicles. They usually appeared early in the illness, and resolved after a few days, so that their presence may depend on the time of presentation. Like previous reports for EV71 (Chang et al. 1999; Chang et al. 1999; Wang et al. 1999; McMinn et al. 2001; Fujimoto et al. 2002; Chan et al. 2003; Nolan et al. 2003) and other human enteroviruses (Rotbart 1995; Pallansch et al. 2001), the isolation rate of CSF was low in this study. Less than 3% (3 of 102) of HFMD patients with aseptic meningitis had a positive CSF culture. The yield would likely have been higher if PCR had been used (Rotbart et al. 1997)

but this investigation is not available in most developing countries. The isolation rate for rectal swabs was not as high as that of throat and vesicle swabs. This could be a reflection of the timing of the samples: the median time of presentation was just 2 days, which may be before viral shedding in the stool has become fully established.

In addition to looking at individual samples, I examined which combinations of samples were the most useful. This was achieved by determining the extent to which the addition of a sample type increased the number of patients diagnosed; this is different to asking how frequently a sample is positive. Thus, for example in the first half of the study, although ulcer swabs were positive more often than rectal swabs, they were less useful diagnostically, because most of the patients in whom they were positive had already been diagnosed by a throat swab. Determining the "added diagnostic value" of a sample type allowed us to produce predictions about which combinations of samples should prove useful, that I were then able to test in the second outbreak. Although the two outbreaks differed in size, and the proportion of patients in the different groups, they were similar in terms of demographics and causative agents, with EV71 being the predominant virus followed by CVA16. The predictions were basically sound. The combination of throat swabs plus vesicle swabs was the most useful for patients with vesicles, whether or not they also had ulcers, identifying virus for 224 (67%) of

333 such patients across the whole study. For these patients, the addition of rectal and ulcer swabs diagnosed just 27 (8%) extra patients. For patients without vesicles (whether or not they had mouth ulcers), the combination of throat swab and rectal swab was most useful, identifying virus for 138 (53%) of 259 such patients. Thus during large outbreaks it is recommended that a throat swab plus one other sample type should be taken for each patient: if no vesicles are present, this should be a rectal swab; but if there are vesicles a swab should be applied to as many of these as possible (but at least 2). Although a limited sampling particularly for community surveillance and during large outbreaks is advocated, for individual patients, especially those that are critically ill, physicians will want to maximise the chance of obtaining a diagnosis. One approach might be to collect all sample types, but to investigate them in a stepwise manner, starting with the most useful samples, until a diagnosis has been made.

The study also examined the concordance of virus isolates from non-sterile sites (rectal, throat and ulcer swabs), with those from a sterile site (vesicle swabs). Twenty percent of rectal isolates differed from vesicle isolates (in individual patients with isolates from both sites), where-as such discordance with vesicle isolates occurred for only 10% of throat isolates. While most would accept the virus isolated from a vesicle as the causative pathogen, the significance of other viruses concomitantly

detected from throat and/or rectum is not always clear. The data show that in the majority of patients with HFMD throat and rectal isolates do reflect viruses isolated from sterile sites, but in a minority they may be co-incidental infections. However, it may not be correct to assume that virus isolated from vesicle is always the most important pathogen. For example one patient (HFM-178, Table 2.4) with severe HFMD and CNS disease had CVA16 isolated from a vesicle swab, but CVB1 from the throat. CVA16, a common cause of HFMD, is not known to cause CNS disease; in contrast CVB1, is not known to cause HFMD, and being a species B human enterovirus is a more likely neuropathogen (Thivierge et al. 1982). So in this patient with dual infection, the two virus isolates may have been responsible for two clinical syndromes: the throat isolate CVB1 causing CNS disease and the vesicle isolate CVA16 causing HFMD. For four patients I also isolated different viruses from different vesicles: three with EV71 and CVA16, and one (with mild HFMD) with EV71 and Poliovirus 1 Sabin strain. These patients clearly demonstrate that systemic infection with two viruses can occur simultaneously. In addition, the latter case suggests the possibility that occasionally during dual infection, viruses may cross tissue barriers that they would not normally be able to (Poliovirus Sabin strain type 1 does not itself cause HFMD, and thus is not normally found in vesicles (Pallansch et al. 2001). The findings on dual infection underscore the need to look for a possible second pathogen,

before attributing pathogenesis to a virus rarely associated with a severe disease phenotype.

EV71 looks likely to continue causing large outbreaks across the Asia pacific region. In summary I have shown that the throat swab is the single most useful sample from patients with HFMD during an EV71 outbreak. Vesicle swabs, which have been relatively neglected until now, can also be extremely useful. Although they are not as easy to obtain as throat and rectal swabs, and are not available for approximately half the patients, the viral yield is almost as good as that of throat swabs, with the added advantage that they come from sterile sites. Whilst virological culture is the standard method of diagnosis of enterovirus infection, rapid detection with PCR may offer new approach to laboratory detection of the virus.

Table 2.1 The relationship between the numbers of swabs collected and the number positive, for vesicles and ulcers.

The relationship between the numbers of swabs collected and the number positive, for vesicles and ulcers.

Sample type	No. of swabs per patient	No. of patients with at least one swab positive
Vesicle	1	62/177 (35)
	2	40/81 (49)
	3	18/30 (60)
	≥4	46/61 (75)
Ulcer	1	26/152 (17)
	2	21/103 (20)
	3	37/77 (48)
	≥4	13/40 (33)

Table 2.2 Detection rate of different clinical sample types

Positive isolation rates of different clinical sample types according to virus [No. (%)] for different viruses according to sample type. The number of patients positive for each sample type is shown as a proportion of the EV71 positive patients, the CVA16 positive patients, all enterovirus positive patients, and all patients (whether positive or negative).

Note:

* 11 patients in the 1st half of the study, and 3 in the 2nd half of the study had co-isolation of EV71 and CVA16.

EV=human enterovirus, CVA=Coxsackie virus A, CSF=cerebrospinal fluid.

	First half of the study** (n=471)				Second half of the study (n=254)			
	EV71 (n=167)	CVA16 (n=80)	Any EV (n=255)	All patients (389)	EV71 (n=106)	CVA16 (n=19)	Any EV (n=153)	All patients (239)
Sample type	No. of patients tested positive/ Total no. of EV71 patients tested	No. of patients tested positive/ Total no. of CVA16 patients tested	No. of patients tested positive/ Total no. of EV positive patients tested	No. of patients tested positive All patients tested	No. of patients tested positive/ Total no. of EV71 patients tested	No. of patients tested positive/ Total no. of CVA16 patients tested	No. of patients tested positive/ Total no. of EV positive patients tested	No. of patients tested positive/ All patients tested
Rectal	65/166 (39)	22/78 (28)	100/252 (40)	100/378 (27)	38/103 (37)	7/16 (44)	68/147 (46)	68/229 (30)
Throat	127/164 (77)	50/76 (66)	191/248 (77)	191/367 (52)	66/104 (64)	9/15 (60)	101/148 (68)	101/231 (44)
Vesicle	66/111 (60)	36/57 (63)	106/167 (63)	106/222 (47)	54/81 (67)	10/13 (77)	63/97 (65)	63/127 (50)
Ulcer	36/85 (42)	11/42 (26)	53/129 (41)	53/188 (28)	28/87 (32)	7/15 (47)	44/117 (38)	44/185 (24)
Serum	0/9 (0)	1/3 (33)	1/10 (10)	1/20 (5)	2/26 (8)	1/11 (9)	6/43 (14)	6/61 (10)
CSF	2/43 (5)	0/11(0)	2/58 (3)	2/96 (2)	0/29 (0)	0/0 (0)	1/42 (2)	1/60 (2)

Table 2.3 Comparison of virus isolates grown from sterile sites with those from non-sterile sites

Pair-wise comparison [No.(%)] of virus isolates grown from vesicles (sterile site) with isolates grown from throat, rectum, or ulcers (non-sterile sites) in the same patient*

Concordance findings with that of vesicle swab (sterile site)	Throat swab	Rectal swab	Ulcer swab
Same virus was isolated from vesicle swab	101 (30)	48 (14)	29 (14)
No virus was isolated from either swab	111 (33)	139 (41)	83 (40)
Total	212 (63)	187 (55)	112 (54)

Discordance findings with that of vesicle swab (sterile site)	Throat swab	Rectal swab	Ulcer swab
Different virus was isolated from vesicle swab	11 (3)	12 (4)	4 (2)
Vesicle swab negative but sample positive	65 (19)	37 (11)	19 (9)
Vesicle swab positive but sample negative	49 (15)	106 (31)	73 (35)
Total	125 (37)	155 (45)	96 (46)
Grand total	337	342	208

* Many patients had swabs from multiple sites. The total number of patients represented here is 349.

Table 2.4 Virus isolation results of patients with different viruses grown from sterile and non-sterile sites.

Study No.	Age (months)	Gender	Clinical severity	Sterile site			Non-sterile site		
				Vesicle	Serum	CSF	Rectum	Throat	Ulcer
HFM-4	46	Male	Severe HFMD with CNS	EV71	-	NEG	EV71; AD7 [1]	EV71	EV71
HFM-9	52	Female	Mild HFMD	EV71	CVA16	-	NEG	NEG	NEG
HFM-90	37	Female	Mild HFMD	CVA16	-	-	NEG	CVA16	EV71
HFM-112	16	Male	Mild HFMD	EV71	-	-	CVA16	EV71	NEG

HFM-152	45	Male	Mild HFMD	EV71; CVA16 [2]	-	-	NEG	EV71	NEG
HFM-174	73	Male	Mild HFMD	CVA16	-	-	EV71	EV71	NEG
HFM-178	14	Male	Severe HFMD with CNS	CVA16	-	NEG	CVA16	CVB1	-
HFM-193	36	Female	Severe HFMD, non CNS	EV71	-	NEG	CVA17	EV71	-
HFM-198	16	Female	Mild HFMD	EV71	-	-	EV71; CVA17 [3]	EV71	-

HFM-228	11	Female	Mild HFMD	CVA10	-	-	CVA6	CVA6; CVB5 [4]	NEG
HFM-298	22	Female	Mild HFMD	CVA10	-	-	NEG	CVB1; CVA10 [5]	NEG
HFM-302	15	Female	Mild HFMD	EV71	-	-	CVA16	EV71	EV71
HFM-328	19	Male	Severe HFMD, non CNS	EV71	-	NEG	EV71	EV71; CVA24 [6]	EV71
HFM-337	36	Male	Mild HFMD	CVA16	-	-	EV71	EV71	NEG

HFM-338	27	Male	Mild HFMD	Unidentified virus	-	-	EV71	CVA16	NEG
HFM-435	11	Male	Mild HFMD	CVA16	-	-	NEG	NEG	EV71
HFM-489	13	Male	Mild HFMD	CVA16	-	-	AD4	CVA16	NEG
HFM3-7	38	Male	Mild HFMD	EV71; P1S [7]	NEG	-	NEG	EV71	NEG
HFM3-35	20	Female	Mild HFMD	EV71	NEG	-	CVA5	CVA5	-
HFM3-63	17	Female	Mild HFMD	EV71; CVA16 [8]	-	-	CVA16	NEG	CVA16

HFM3-97	61	Male	Severe HFMD, non CNS	EV71	-	-	NEG	NEG	EV71; Untyped EV[9]
HFM3-161	32	Male	Mild HFMD	EV71	-	-	NEG	CVA16	NEG
HFM3-164	5	Male	Severe HFMD, non CNS	NEG	-	Unidentified virus	NEG	EV71	EV71

AD = Adenovirus, CSF = cerebrospinal fluid, CVA = Coxsackie virus A, CVB = Coxsackie virus B, EV = enterovirus, HFMD = Hand foot and mouth disease, NEG = negative, P1S = Poliovirus 1 Sabin strain, Severe HFMD with CNS = severe hand foot and mouth disease with central nervous system involvement, Severe HFMD, non-CNS = severe hand foot and mouth disease with no central nervous system involvement.

*Details of dual viruses isolated from a single site, using different cell lines are given below.

1=EV71 isolated using RD; Adenovirus 7 using 293 cells

2=EV71 isolated from right palm vesicle using RD and CVA16 from left sole vesicle using RD

3=EV71 isolated using RD and CVA17 using 293

4=CVA6 isolated using RD and CVB5 using 293

5=CVB1 isolated using RD and CVA10 using 293

6=EV71 isolated using RD and CVA24 using 293

7=EV71 isolated from right and left palm vesicles using RD and P1S from right and left sole vesicles using RD

8=EV71 isolated from right sole vesicles using RD and CVA16 from right knee and left sole vesicles using RD

9=EV71 isolated from lip and tongue ulcers using RD and untyped EV isolated from right buccal ulcer using using RD

Figure 2.1 Diagnostic yields of different clinical sample combinations

Analysis of which combination of samples gave the greatest diagnostic yield for the four groups of HFMD patients, assessed stepwise according to the analytical plan during the first half of the study (2.1a) and 2nd half of the study (2.1b). The number (%) of positive patients for each sample type at each step is shown; the sample boxed gave the greatest diagnostic yield, and thus was the one used for the next step. Only patients with complete sets of samples were analyzed. For the 1st half of the study this comprised 105 (95%) of 110 HFMD patients with vesicles and ulcers, 109 (97%) of 112 with vesicles only, 69 (88%) of 78 with ulcers only, and 82 (48%) of 171 HFMD patients with maculopapular rash. For the 2nd half this comprised 92 (94%) of 98 HFMD patients with vesicles and ulcers, 27 (93%) of 29 with vesicles, 85 (98%) of 87 with ulcers, and 23 (58%) of 40 HFMD patients with maculopapular rash. RS=rectal swab, TS=throat swab, US=ulcer swab, VS=vesicle swab.

Figure 2.1a

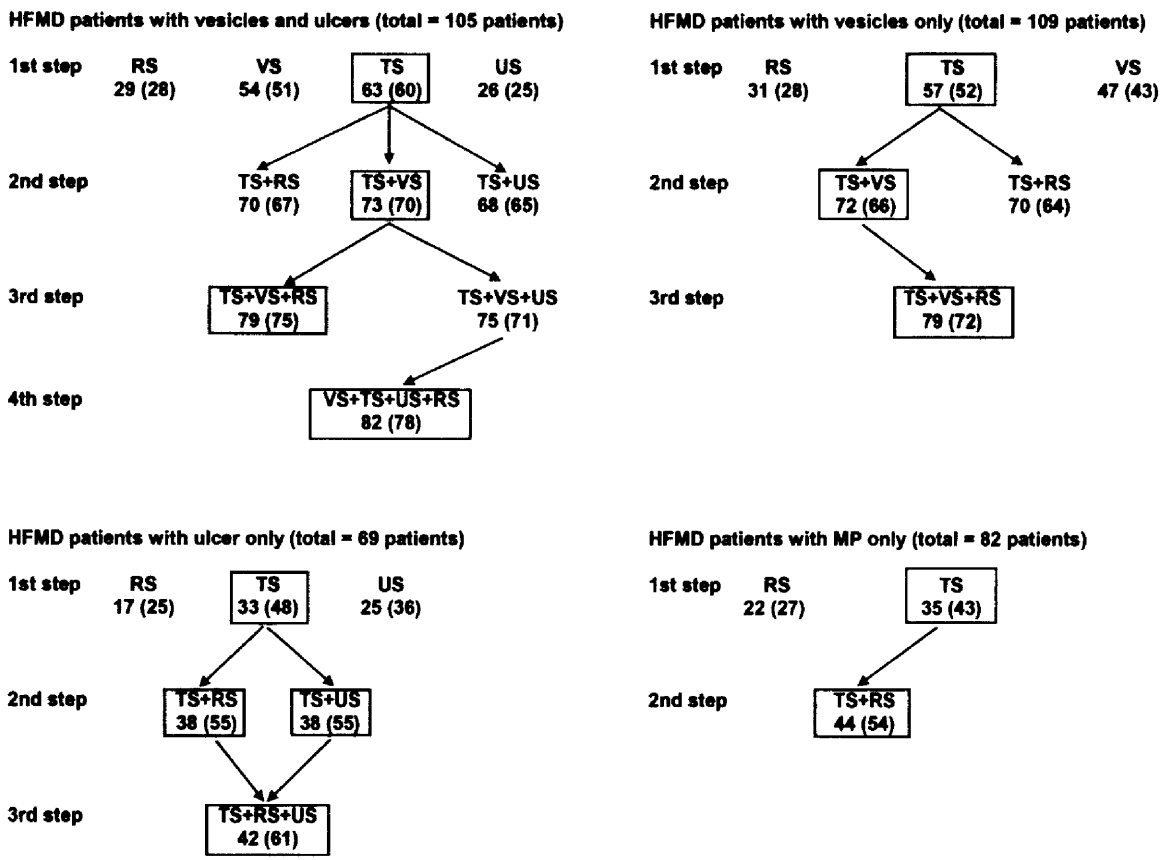
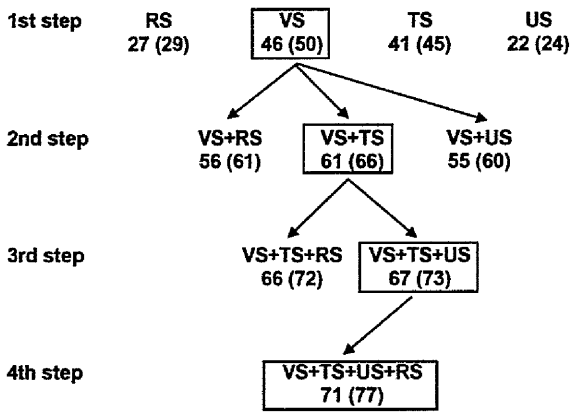
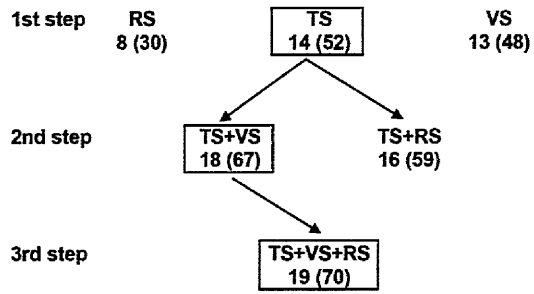

HFMD patients with vesicles and ulcers (total = 105 patients)
1st step
RS 29 (28)
VS 54 (51)
TS 63 (60)
US 26 (25)
2nd step
TS+RS 70 (67)
TS+VS 73 (70)
TS+US 68 (65)
3rd step
TS+VS+RS 79 (75)
TS+VS+US 75 (71)
4th step
VS+TS+US+RS 82 (78)
HFMD patients with vesicles only (total = 109 patients)
1st step
RS 31 (28)
TS 57 (52)
VS 47 (43)
2nd step
TS+VS 72 (66)
TS+RS 70 (64)
3rd step
TS+VS+RS 79 (72)
HFMD patients with ulcer only (total = 69 patients)
1st step
RS 17 (25)
TS 33 (48)
US 25 (36)
2nd step
TS+RS 38 (55)
TS+US 38 (55)
3rd step
TS+RS+US 42 (61)
HFMD patients with MP only (total = 82 patients)
1st step
RS 22 (27)
TS 35 (43)
2nd step
TS+RS 44 (54)

Figure 2.1b

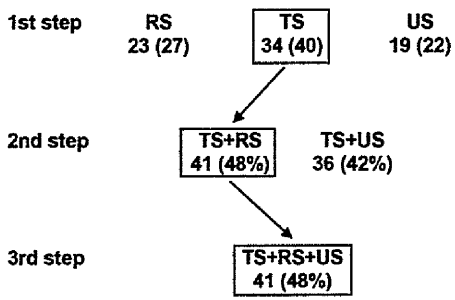
HFMD patients with vesicles and ulcers (total = 92 patients)



HFMD patients with vesicles only (total = 27 patients)



HFMD patients with ulcer only (total = 85 patients)



HFMD patients with MP only (total = 23 patients)

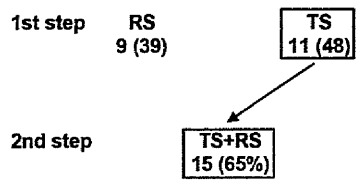
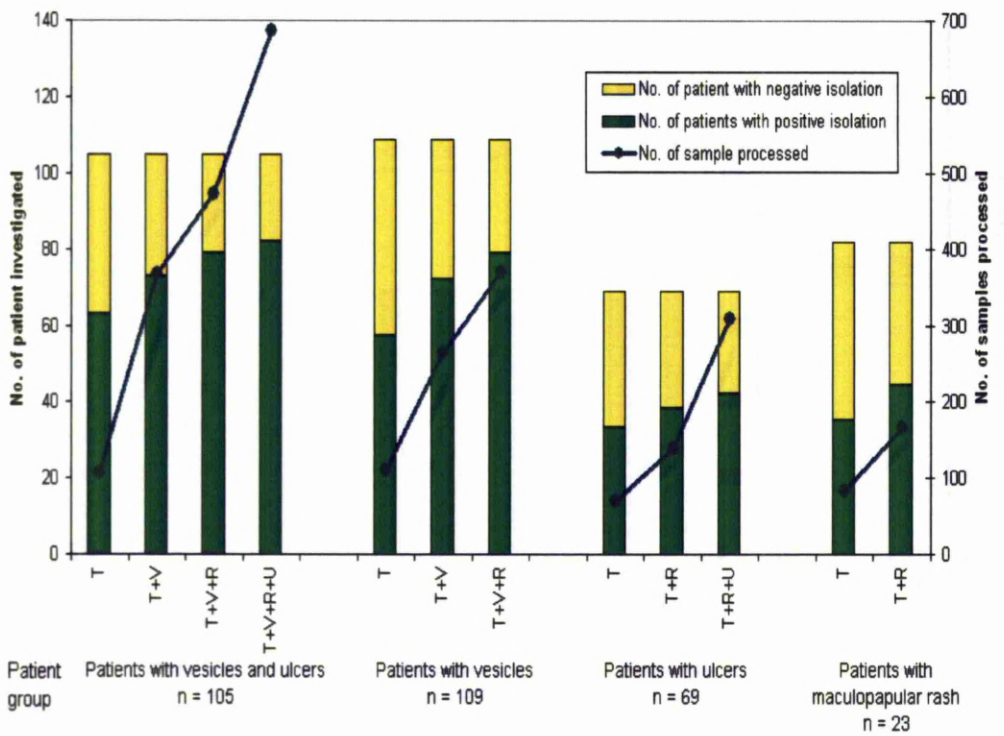


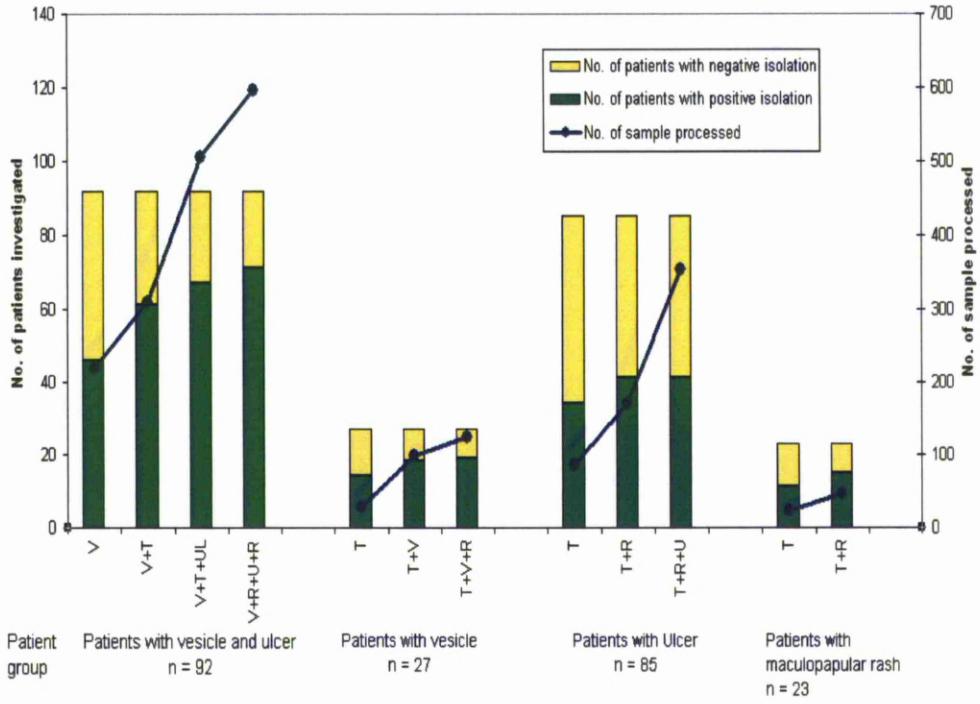
Figure 2.2 Diagnostic yields of different combination of clinical sample types

Histograms showing the proportion of patients diagnosed at each step, for the different patient groups, using the optimum combination of samples as determined in figure 2.1, and the total number of samples analysed at each step. Figure 2.2a shows the first half of the study and figure 2.2b shows the 2nd half. T-throat swab, V-vesicle swab, R-rectal swab, U-ulcer swab

2.2a



2.2b


No. of patients with negative isolation
No. of patients with positive isolation
No. of sample processed
No. of patients investigated
No. of sample processed
140
120
100
80
60
40
20
0
700
600
500
400
300
200
100
0
V
V+T
V+T+UL
V+R+U+R
T
T+V
T+V+R
T
T+R
T+R+U
T
T+R
Patient group
Patients with vesicle and ulcer n = 92
Patients with vesicle n = 27
Patients with Ulcer n = 85
Patients with maculopapular rash n = 23

Chapter 3: Rapid detection of enterovirus 71 in primary clinical specimens

Abstract

A human enterovirus 71 (EV) 71 type-specific RT-PCR was developed in Institute of Health and Community Medicine, University Malaysia Sarawak, Malaysia for molecular typing of EV71 clinical isolates. In this chapter, I evaluated the diagnostic performance of the EV71 type-specific RT-PCR when tested with clinical specimens collected from children with hand, foot and mouth disease (HFMD) admitted at hospitals and at sentinel surveillance clinics during an outbreak of HFMD in 2006. Without altering PCR conditions originally optimised for typing of clinical isolates, and using virus culture results as the reference standard, the EV71 type-specific assay had a sensitivity of 76.9% (258/337), specificity of 82.6% (133/161), positive predictive value of 90.2% (259/287) and negative predictive value of 63.0% (133/211) when evaluated with a total of 498 (337 EV71 positive, 161 non-EV71 virus) culture positive clinical specimens. The sensitivity of the assay in detection of EV71 from EV71 culture-positive clinical specimens was lower than that of pan-enterovirus RT-PCR [73.4% (152/207) versus 97.6% (202/207)]. By lowering the PCR stringency, the sensitivity of the

assay improved to 94.9% (297/313) and 95.1% (176/185) when compared to virus culture method and pan-enterovirus RT-PCR, respectively. The evaluation indicated that the application of the EV71 type-specific RT-PCR can be extended to be used as a rapid diagnostic assay for direct detection of EV71 from primary clinical specimens. As a single step PCR assay with no additional step of amplicon sequencing, the EV71 type-specific assay may be a useful alternative to clinical laboratories that have limited access to the conventional virus culture and neutralization test method, and nucleotide sequencing facilities.

3.1 Introduction

Hand, foot, and mouth disease (HFMD) is a common febrile illness in young children, and is characterised by maculopapular or vesicular skin lesions on the palms and soles, and mouth ulcers. Many species A human enteroviruses (EV-A, family *Picornaviridae*, genus *Enterovirus*) can cause HFMD, but human enterovirus 71 (EV) 71 and coxsackievirus (CV) A16 are the most common (Pallansch et al. 2001). A series of EV71-associeted HFMD outbreaks have occurred in Asia in the past decade, and some of these were associated with severe neurological manifestations and many deaths (Cardosa et al. 1999; Ho et al. 1999; McMinn et al. 2001; Chan et al. 2003; Ooi et al. 2007). In contrast, CVA16 infection is a benign disease (Pallansch et al. 2001). In many

parts of Asia, there is now epidemiological and virological surveillance for HFMD so that effective public health measures, such as closing nurseries and schools, and broadcasting messages about personal hygiene can be instituted early to control outbreaks (Podin et al. 2006; Chen et al. 2007). However, because the clinical presentations of these virus infections are indistinguishable clinically, establishing the aetiological diagnosis of HFMD cases relies on laboratory identification of the infecting virus. Hence, it is important to develop an accurate diagnostic method that can rapidly discriminate these two viruses particularly at the start of an outbreak in order to guide public health interventions.

The gold standard method for the identification of human enterovirus infection is to isolate the virus from the patient using susceptible cell lines followed by typing using the neutralization test (Pallansch et al. 2001). However, the method is slow and labour-intensive. In addition, it is dependent on the availability of the Lim-Benyesh-Melnick antiserum pools that are not available to every clinical virology laboratory. Indirect immunofluorescence employing commercially available monoclonal antibodies is an often used alternative, but some commercially available monoclonal antibodies do not have the specificity claimed. Therefore, a molecular diagnostic assay is more useful to allow rapid and sensitive detection of EV71. A universal enterovirus (pan-enterovirus) primer sets targeting the highly conserved 5' untranslated region (5'UTR) of the

enterovirus genome were developed in the late 1980's and the early 1990's to detect enterovirus from primary clinical specimens (Rotbart et al. 1995). The pan-enterovirus primer sets have substantially higher sensitivity than virus culture in the detection of enterovirus, and are used widely on primary clinical specimens for rapid virological diagnosis (Romero 1999). However, the major drawback of the pan-enterovirus primers is that the method does not distinguish enterovirus serotypes (Rotbart et al. 1995). More recently, a molecular typing method based on sequencing a segment of the VP1 gene, which codes for the most immunodominant region of viral capsid protein, has been shown to be a useful and rapid approach to typing of human enterovirus (Oberste et al. 1999). This method, however, requires access to a DNA sequencing facility and is therefore more expensive and less accessible in resource limited situations.

Several EV71 type-specific RT-PCR assays directed at different regions of the VP1 gene of human enterovirus have been developed following the large outbreaks of HFMD in Malaysia and Taiwan (Brown et al. 2000; Yan et al. 2001; Singh et al. 2002; Tsao et al. 2002). Although all the published primer sets have been shown to be highly sensitive and specific when tested with virus isolates, only one of these sets has been evaluated for its utility in the detection of EV71 from primary clinical specimens (Singh et al. 2002). Furthermore, misidentification of CVA16 as EV71

has been observed with some primer sets (Singh et al. 2002; Perera et al. 2004). A set of EV71 type-specific primer (MAS01A and MAS02S) was developed in Institute of Heath and Community Medicine, Universiti Malaysia Sarawak, Sarawak, Malaysia. The EV71 type-specific primers were designed to only amplify a part of the VP1 gene of EV71 but not other enteroviruses, including CVA16. The utility of these primers on identification of clinical isolates has been reported (Perera et al. 2004). The diagnostic performance of the MAS01S/MAS02A primer set when its application was extended to direct detection of EV71 from primary clinical specimens was evaluated during an outbreak of HFMD in Sarawak in 2006. The performance of this type specific primer set was compared with a widely used pan-enterovirus primer set in detection of EV71 from primary clinical specimens.

3.2 Materials and methods

3.2.1 Setting and Clinical specimens

Clinical specimens including throat swabs, rectal swabs, ulcer swabs, vesicular fluid, urine, serum and cerebrospinal fluid (CSF) were collected from the hospitalised patients with HFMD as well as patients treated in the sentinel surveillance clinics during an outbreak of HFMD in 2006. A sentinel surveillance clinic programme for HFMD was established in

Sarawak in 1998 (Podin et al. 2006). The doctors of the sentinel clinics in this study have actively participated in the surveillance programme since 1998. They were provided with a standard reporting and specimen collection form, sterile swabs and virus transport medium, a telephone number for obtaining assistance for transport of specimens to the laboratory and a facsimile number to report cases to the Sarawak Health Department. The clinical specimens from the hospitalised patients were stored immediately in a -70°C freezer until transported to the laboratory. When immediate storage at -70°C was not possible, the specimens were stored at 4°C overnight and were transferred to a -70°C freezer the following morning. Specimens from the sentinel clinics were stored at 4 °C after collection and transported on ice to the laboratory. Swabs in virus transport medium were vortexed and aliquoted before testing.

3.2.2 Virus isolation

Specimens were inoculated into human rhabdomyosarcoma (RD) and human embryonal kidney (293) cell lines as described in the section of materials and methods of Chapter 2.

3.2.3 Nucleic acid extraction

In this chapter RNA was extracted from infected tissue culture supernatants and from primary clinical specimens using Chemagic Viral

RNA/DNA kits (Chemagen, Baesweiler, Germany) according to the manufacturer's instructions. An automated magnetic particle separator (Kingfisher mL, Thermo Electron Corporation) was used to separate the magnetic beads from eluted viral RNA. The extracted RNA was stored at -80°C until use.

3.2.4 Pan-enterovirus RT-PCR for identification of enterovirus

The presence of any enterovirus in primary clinical specimens was determined by the pan-enterovirus RT-PCR using the primer MD90 and MD91 as described previously (Romero et al. 1993). The expected PCR product for pan-enterovirus PCR was 154 base pairs.

3.2.5 Enterovirus 71 type specific RT-PCR

The presence of EV71 RNA in primary clinical specimens was determined by using the MAS01S (5'-ATAATAGCA(C/T)T(A/G)GCGGCAGCCCA -3') and MAS02A (5' – AGAGGGAG(A/G)TCTATCTC(C/T)CC -3') primer set that flank a region within the VP1 gene unique to EV71 and amplify an expected product size of 376 base pairs (Perera et al. 2004). Briefly, first strand of cDNA was prepared in a 10 µl reaction mixture containing 5.5 µl RNA

template, 0.5 mM dNTP, 200 U Moloney murine leukemia virus reverse transcriptase (M-MuLV RT) (Fermentas), 1X M-MuLV RT buffer (Fermentas) and the antisense primer MAS02A. cDNA synthesis was performed for 1 hour at 37°C. In the PCR step, the VP1 gene was amplified using 10 µl of cDNA in a 20 µl reaction volume containing 1.5 mM $MgCl_2$, 1 µM each of primer MAS01S and MAS02A, 0.3 mM dNTP (Fermentas), 2.5 U Taq DNA polymerase (Fermentas) and 1X Taq polymerase buffer with $(NH_4)_2SO_4$ (Fermentas). DNA amplification was performed by initial denaturation at 94°C for 5 minutes and followed by 35 cycles of denaturation at 95°C, annealing at 55°C and extension at 72°C for 30 seconds each. A final extension at 72°C for 5 minutes was performed after the last cycle. The PCR products were analyzed by electrophoresis on 2% agarose gel containing 0.5 µg/ml ethidium bromide.

3.2.6 Nucleotide sequencing of enterovirus isolated

Enterovirus VP4 gene nucleotide sequencing was used to screen, and to identify clinical isolates according to enterovirus species (Ishiko et al. 2002; Cardosa et al. 2003). Further typing of EV-A isolates was provided by VP4 gene sequencing as it has been previously shown that the results of the VP4 gene were 100% in concordance with that provided by the VP1 gene for EV-A (Tu et al. 2007). Sequencing reactions were

performed using the Big Dye Terminator Cycle Sequencing Kit version 3.1 (Applied Biosystems, Foster City, CA, USA).

3.2.7 Statistical analysis

Differences between proportions were tested using the Chi-Square test with Yates' correction or Fisher's exact test (Epi Info version 6 CDC, Atlanta, USA). The diagnostic accuracy of the EV71 specific assay was compared with two reference standards - virus isolation and Pan-enterovirus RT-PCR. Using the results of virus culture (the conventional gold standard) as the reference standard, the sensitivity of EV71 specific assay was defined as the percent of EV71 PCR positive specimens that also yielded an EV71 isolate by virus culture, and the specificity was defined by the percent of EV71 PCR negative specimens that yielded a non-EV71 virus by virus culture. Likewise, using the results of pan-enterovirus RT-PCR using MD90/MD91 primer set (a widely used RT-PCR method for enteroviruses) as the reference standard, the sensitivity of EV71 specific assay in the detection of EV71 from EV71 isolate positive specimens was defined as the percent of EV71 PCR positive specimens that also were positive by pan-enterovirus RT-PCR, and the specificity was defined by the percent of EV71 PCR negative specimens that were also negative by pan-enterovirus RT-PCR. The sensitivity, specificity, positive predictive value (PPV), negative predictive value

(NPV) of the EV71 specific assay were calculated from a 2 X 2 table, and reported according to the guideline of The STARD statement (Bossuyt et al. 2003).

3.3 Results

A total of 1515 clinical specimens (1256 throat swabs, 100 vesicle swabs, 56 rectal swabs, 30 CSF, 30 ulcer swabs, 23 serum, 9 endotracheal aspirate, 6 urine, 2 brain tissue and 3 swabs from unknown site) from hospitals, and 311 samples (180 throat swabs, 86 ulcer swabs and 45 vesicle swabs) from 2 sentinel clinics were tested (Figure 3.1). All the clinical specimens were subjected to virus isolation. EV71 type-specific RT-PCR and Pan-enterovirus RT-PCR on the primary clinical specimens were performed before the results of the virus isolation became available.

3.3.1 Virus isolation

Virus was isolated from 498 (27.3%, 449 from the hospitals, 49 from sentinel clinics) of 1826 specimen tested. Three hundred and twelve (69.5%) of 449 hospital specimens had EV71, and 3 of these specimens also had a second non-EV71 virus. The remaining 137 (30.5%) hospital isolates were not EV71 and included EV-A and EV-B with some adenoviruses, herpes simplex virus and human rhinovirus. At least 3 of

these specimens also yielded more than one virus isolate. Of the 49 culture positive specimens from the sentinel clinics, only 25 (51%) yielded EV71 and the remaining yielded other EV-A. No specimen from the sentinel clinics yielded more than one virus isolate. Table 3.1 shows the distribution of the viruses according to specimen types. EV71 was more likely to be isolated from specimens collected from hospitalised children than that from children treated at sentinel clinics [312 (69.5%) of 449 versus 25 (51%) of 49, Odds ratio (95% confidence interval) 2.19 (1.16-4.13), p=0.0138].

3.3.2 The diagnostic accuracy of enterovirus 71 type specific RT-PCR compared to virus isolation followed by serotype identification

Table 3.2 shows the results of the EV71 type-specific assay when tested with a total of 498 culture positive clinical specimens. The MAS01A/MAS02S primers correctly identified 259 (76.9%) of the 337 specimens that subsequently cultured EV71 isolates (true positive), but failed to amplify the remaining 78 specimens (false negative). The MAS01S/MAS02A primers also generated a PCR product of the expected size in 28 (17.4%) of the 161 primary specimens from which a non-EV71 isolate was obtained (false positive). Using the results from virus culture as the reference standard, the type specific RT-PCR had a sensitivity of

76.9% (259/337), specificity of 82.6% (113/161), PPV of 90.2% (259/287) and NPV of 63.2% (113/211). When the performance of the primers was analyzed according to the samples collected from 2 different clinical settings, the primer set had a sensitivity of 76.9% (240/312), specificity of 82.5% (113/137)), PPV of 89.6% (240/264) and NPV of 61.1% (113/185) when tested with the hospital samples, and a sensitivity of 76% (19/25), specificity of 83.3% (20/24), PPV of 82.6% (19/23) and NPV of 76.9% (20/26) when tested with the sentinel clinic samples.

In addition, nearly a third (415/1328) of the culture negative clinical specimens was also positive with the EV71 type-specific assay (Table 3.2). To exclude the likelihood of non-specific amplification in the primary clinical specimens, the PCR products obtained from 16 of the 415 culture negative specimens were sequenced, and confirmed as EV71.

3.3.3 Reason for the non-detection of enterovirus 71 by enterovirus 71 type specific RT-PCR

The possible reasons for the failure of EV71 PCR product amplification in a total of 78 EV71 culture-positive clinical specimens (72 from hospitals, 6 from sentinel clinics) may be due to multiple reasons including (a) degradation of viral RNA because of prolonged storage, (b)

primer-template sequence mismatch, (c) low viral load or low RT-PCR assay sensitivity.

The information on the duration of template storage before RT PCR was available in 314 of 337 EV71 isolate positive specimens. There was no difference in the median duration of template storage (days, range) before the assay was performed between the true positive group (24, 0-193) and the false negative group (18, 3-86) (Mann-Whitney U test, p=0.1729).

I attempted to exclude the likelihood of primer-template mismatch in the false negative group by investigating 68 (87.2%) of 78 EV71-infected tissue culture supernatants that were available for testing with the MAS01A/MAS02S primers. A PCR product of expected size was obtained in all the 68 (100%) infected tissue culture supernatants tested indicating that the failure of PCR amplification was unlikely due to primer-template mismatch in the primary clinical specimens, although the availability of large amount of templates that would normally be present in tissue culture supernatants may have overcome the inefficiency of the PCR reaction with mismatched target.

Fifty five (70.5%) of the 78 primary clinical specimens in the false negative group were tested with pan-enterovirus PCR to determine if the universal enterovirus primer set could detect the presence of EV71 that

were missed by the EV71 type specific primers (Figure 3.1). Apart from 5 (4 throat, 1 urine) samples that were negative by both pan-enterovirus RT-PCR and EV71 type specific RT-PCR, 90.9% (50/55) of the samples were positive using the pan-enterovirus RT-PCR suggesting that the non-detection of EV71 was not due to low viral load but lower sensitivity of the EV71 type-specific assay compared to the pan-enterovirus primer set.

3.3.4 Comparison of enterovirus 71 type-specific RT-PCR results with pan-enterovirus RT-PCR results in enterovirus 71 culture-positive clinical specimens

Table 3.3 compares the performance of EV71 type-specific RT-PCR with pan-enterovirus RT-PCR in the detection of EV71 from a total of 207 (195 from hospitals, 12 from sentinel clinics) EV71 culture-positive clinical specimens. Of the 202 EV71 culture-positive specimens that were positive by pan-enterovirus RT-PCR, the MAS01A/MAS02S primers correctly identified 152 (75.2%) but missed the remaining 50 (24.8%) specimens. The primer set failed to identify EV71 in 5 culture positive specimens that were also missed by pan-enterovirus RT-PCR.

3.3.5 Further improvement in the sensitivity of EV71 specific assay

Compared with virus isolation followed by serotype identification

The stringency of the PCR conditions was reduced to improve the sensitivity of EV71 specific assay - the annealing temperature was lowered from 55°C to 50°C. Fifty four (69.2%) of the 78 false negative specimens were available for re-testing using the modified RT-PCR protocol (Figure 3.1), and PCR product was obtained in 38 (70%) of the 54 specimens tested (Figure 3.2). Repeating the analysis based on the repeat assay results of the 54 specimens, with the assumption that all the PCR positive samples would remain positive when they were assayed with the modified RT-PCR protocol, the sensitivity of the primer set when compared to virus culture improved from 76.9% (259/337) to 94.9% (297/313).

Compared with Pan-enterovirus RT-PCR

Thirty four of 50 samples that were negative by the EV71 type-specific RT-PCR but positive by the pan-enterovirus RT-PCR, as well as 3 of 5 samples that were negative by both PCR methods were also retested using the modified protocol. By lowering the annealing temperature to 50° C, the primer set detected EV71 in 25 (73.5%) of the 34 samples that were

previously EV71 RT-PCR negative, but continued to fail to amplify EV71 in all 3 samples that were negative earlier by both PCR methods. Using a similar analytical approach and assumption, the sensitivity of the primer set, when compared to pan-enterovirus PCR improved from 75.2 (152/202) to 95.1% (176/185).

3.4 Discussion

Two consecutive epidemics of EV71 associated HFMD in Sarawak and Taiwan in the late 1990's heralded the emergence of EV71 as the most important non-polio enterovirus in children in the Asia Pacific region (Cardosa et al. 1999; Ho et al. 1999). The memory of the previous massive outbreaks in Sarawak and Taiwan has led the general public in Asia, including in Sarawak, to perceive HFMD as a potentially fatal infection. It often causes panic among the parents of the affected children, as well as the doctors, and attracts widespread media publicity. Previous experience in Sarawak, Malaysia showed the epidemic activity of EV71 can rise exponentially and reach its height within 4 weeks after its first appearance (Podin et al. 2006). However, enterovirus typically takes 7 to 10 days to grow and produce cytopathic effect on the RD cell culture, which was developed for the detection of enteroviruses belonging to species A including EV71 (Schmidt et al. 1975). Therefore to meet the primary objectives of providing an early alert for an impending outbreak,

and to guide public health intervention, an effective virological surveillance programme for HFMD requires not only virus culture, but also a rapid and accurate molecular diagnostic tool for the identification of EV71. Although preventive measures such as dissemination of health information including the importance of personal hygiene and avoidance of crowded public areas, disinfection and cleaning of areas are useful and inexpensive, more aggressive approaches such as closure of childcare centres and schools or extension of school vacation may be necessary to effectively prevent further transmission of EV71 in a community. Due to the socio-economic impact of some of these measures, the identification of the infecting virus is critical in guiding the public health doctors to institute appropriate public health interventions. In addition, timely clinical intervention including use of intravenous immunoglobulin in children with severe EV71 infection may reduce acute mortality (Chang et al. 2004; Wang et al. 2006). Nonetheless, intravenous immunoglobulin treatment is expensive, and not completely free of serious side effects. As a result, clinical laboratories are often under tremendous pressure to provide a timely diagnosis to public health doctors, clinicians and the community when there is a cluster of HFMD in the community.

Several nested and semi-nested PCR assays had been developed for direct identification of enterovirus serotypes from primary clinical specimens. The assays, however, require a panel of primer sets and involve the

additional step of PCR product sequencing (Miren et al. 2006; Nix et al. 2006; Oberste et al. 2006). At least four sets of EV71 type specific primers have been published previously, but only one set developed by Singh et al had been evaluated on clinical specimens (Brown et al. 2000; Yan et al. 2001; Singh et al. 2002; Tsao et al. 2002). The reported sensitivity of the primers was less than 5% (1/21) when used as a single-round RT-PCR and 53% (17/32) as a semi-nested PCR. A two-step duplex real-time RT-PCR assay for rapid detection of enteroviruses and parechoviruses has been shown to have superior diagnostic sensitivity over virus isolation when tested on CSF and throat swab samples (Corless et al. 2002). In this chapter, a set of EV71 type-specific primers originally developed for molecular typing of EV71 clinical isolates, for rapid detection of EV71 from clinical specimens was evaluated. Without altering the thermal cycling conditions that were originally optimised for typing of enterovirus isolates, the MAS01A/MAS02S primer set had a sensitivity of 76.9% and a specificity of 82.6% when tested with 498 culture positive clinical specimens. The assay performed equally well when tested with clinical specimens collected from hospitals and primary care clinics.

The false negative rate of theEV71 specific assay was 23.1%. The reason for non-detection in EV71 culture positive specimens is likely related to lower assay sensitivity when compared to the pan-enterovirus RT-PCR.

The study showed that the sensitivity of the assay can be improved significantly by lowering the PCR stringency. Nonetheless, the overall negative predictive value (NPV) is only 63% (133/211) and it is important to exercise caution in the interpretation of negative results (Table 3.2).

In this study, there were 28 specimens that were EV71 PCR-positive but subsequently obtained a non-EV71 isolate. It is notable that there were at least 6 specimens that yielded more than one virus. Three of these had both EV71 as well as another enterovirus or adenovirus isolate. The most plausible explanations for the non-cultivation of EV71 may be related to low viral load, non-viable virus, virus interference or other reasons. EV71 was detected in a significant proportion (415/1328) of culture negative specimens. The ability of the primer set in detecting EV71 from such clinical specimen types indicates that the primer set can be a valuable back-up diagnostic method to virus culture in clinical settings where a definitive aetiological diagnosis is crucial. Although there is no doubt that real time RT-PCR methods may also provide similar information rapidly, a higher cost associated with this technology render it less useful in Sarawak, Malaysia, which is that of a developing country setting.

A number of EV71-associated HFMD outbreaks have been reported in the past decade in Asia, and some of these were linked epidemiologically (McMinn et al. 2001; McMinn et al. 2001; Fujimoto et al. 2002; Cardosa

et al. 2003; Chan et al. 2003; Jee et al. 2003; Shimizu et al. 2004; Lin et al. 2006; Hosoya et al. 2007). The virus can cause cyclical outbreaks and may have established an endemic circulation in some countries (Podin et al. 2006; Chen et al. 2007). There is also concern that it may spread globally. The MAS01A/MAS02S primer set may be used to provide rapid genogroup information about EV71 (Perera et al. 2004). Molecular epidemiological linkage of outbreaks may be established through the sequenced PCR product obtained directly from clinical specimens before clinical isolates become available through virus culture. Indeed, the detection of partial sequence of VP1 of EV71 through this approach has helped the laboratory in Universiti Malaysia Sarawak to issue early warning of an impending outbreak, and provide preliminary information about the molecular epidemiology of the outbreak virus strains in Sarawak in early 2006 (http://www2.ichr.uwa.edu.au/apnet/news/index.lasso?q=/apnet/news/index.lasso&news=current_ev71 and http://www2.ichr.uwa.edu.au/apnet/news/docs/Sarawak_EV71_2006.pdf). As WHO is close to eradicating wild poliovirus from the world, there is great concern that EV71 may emerge as the most important neurotrophic enterovirus in children (Prager et al. 2003; Modlin 2007; Perez-Velez et al. 2007), and thence underscores the need for a reliable diagnostic tool for EV71 infection. Although all recent major EV71 epidemics in Asia were closely associated with HFMD (McMinn 2002), a valuable clinical

cue for the clinicians who manage children with suspected neurological infection, the cutaneous stigmata of the virus may be absent in some cases, as reported in a recent outbreaks of EV71 in Colorado, USA in 2003 and 2005 (Perez-Velez et al. 2007). To heighten the index of suspicion in the laboratory and to improve the detection rate of EV71, the authors proposed an algorithm that considers the results of pan-enterovirus RT-PCR on CSF, throat swab and rectal swab, followed by further tests such as nucleotide sequencing and virus isolation. A single step type-specific EV71 assay is therefore a useful and practical alternative for most clinical laboratories that have limited access to nucleotide sequencing and virus isolation facilities, and may indeed even be a useful screening tool for the most well-resourced laboratories.

There were some limitations in the study. The molecular diagnostic approaches in this study were developed to detect enteroviruses in the clinical samples. Children with CNS involvement were not tested for human parechoviruses. Human parechoviruses are increasingly recognized as important cause of viral CNS infection in children. Apart from gastrointestinal and respiratory infections, human parechovirus type 1 has been associated with encephalitis and paralysis, while human parechovirus type 3 has been found to be an important cause of aseptic meningitis in young infants (Harvala et al. 2009).

In summary, the study evaluated a single step EV71 type-specific RT-PCR method for an extended application of direct detection of EV71 from primary clinical specimens. The primers performed satisfactorily when tested with a large set of clinical specimens from 2 different clinical settings, and has compared very favourably to a previously published type specific semi-nested PCR method. The performance of the assay improved further with a lower annealing temperature, and was comparable to that of pan-enterovirus RT-PCR. A lower stringency may be employed when the primer set is used to analyze primary clinical specimens, but the higher stringency conditions should be maintained when used to type isolates.

Until RT-PCR become part of routine diagnostic practice, virus isolation will remain the mainstay of laboratory diagnosis of enterovirus infection. Both virus isolation and PCR allow determination of the genogroup of the infecting virus, and thus examination of possible clinical difference between different subgenogroups, as addressed in the next chapter.

Table 3.1 Distribution of virus isolates according to specimen types and clinical settings

Specimen type	Hospital			Sentinel clinic			Total
	EV71 isolated	Non-EV71 virus isolated	Negative	EV71 isolated	Non-EV71 virus isolated	Negative	
Throat Swabs	277*	116**	863	10	7	163	410
Vesicle	16	9	75	1	1	43	27
Mouth ulcer	3	1	26	14	16	56	34
Rectal swabs	13	8	35	-	-	-	21
Swab (site unknown)	1	0	2	-	-	-	1
CSF	1	2	27	-	-	-	3
Urine	1	1	4	-	-	-	2
Total	312	137	1032	25	24	262	498

Notes:

* 3 specimens yielded both EV71 and a second virus (1 each with EV species A, B and C)

** 3 specimens yielded 2 different non-EV71 isolates

\- no sample tested

Table 3.2 Enterovirus 71 type-specific RT PCR results on clinical specimens from which virus isolates were obtained

	EV71 isolated	Non-EV71 virus isolated	No virus isolated	Total number of specimens tested
EV71 type-specific RT-PCR positive	259	28	415	702
EV71 type-specific RT-PCR negative	78	133	913	1124
Total	337	161	1328	1826

Table 3.3 Comparison of the performance of enterovirus 71 type-specific RT-PCR with pan-enterovirus RT-PCR in the detection of enterovirus 71 from enterovirus 71-culture positive clinical samples

	Pan-enterovirus RT-PCR positive	Pan-enterovirus RT-PCR negative	Total
EV71 type-specific RT-PCR positive	152	0	152
EV71 type-specific RT PCR negative	50	5	55
Total	202	5	207

Figure 3.1 Evaluation of a total of 1826 clinical specimens collected from the patients with hand, foot and mouth disease.

A flowchart showing the evaluation of a total of 1826 clinical specimens collected from the patients with hand, foot and mouth disease.

HFMD: hand, foot and mouth disease; EV71 spec RT-PCR: enterovirus 71 type-specific RT-PCR; EV71 spec RT-PCR ΔT50: enterovirus 71 type-specific RT-PCR with an annealing temperature of 50°C; Pan-enterovirus RT-PCR: universal enterovirus RT-PCR.

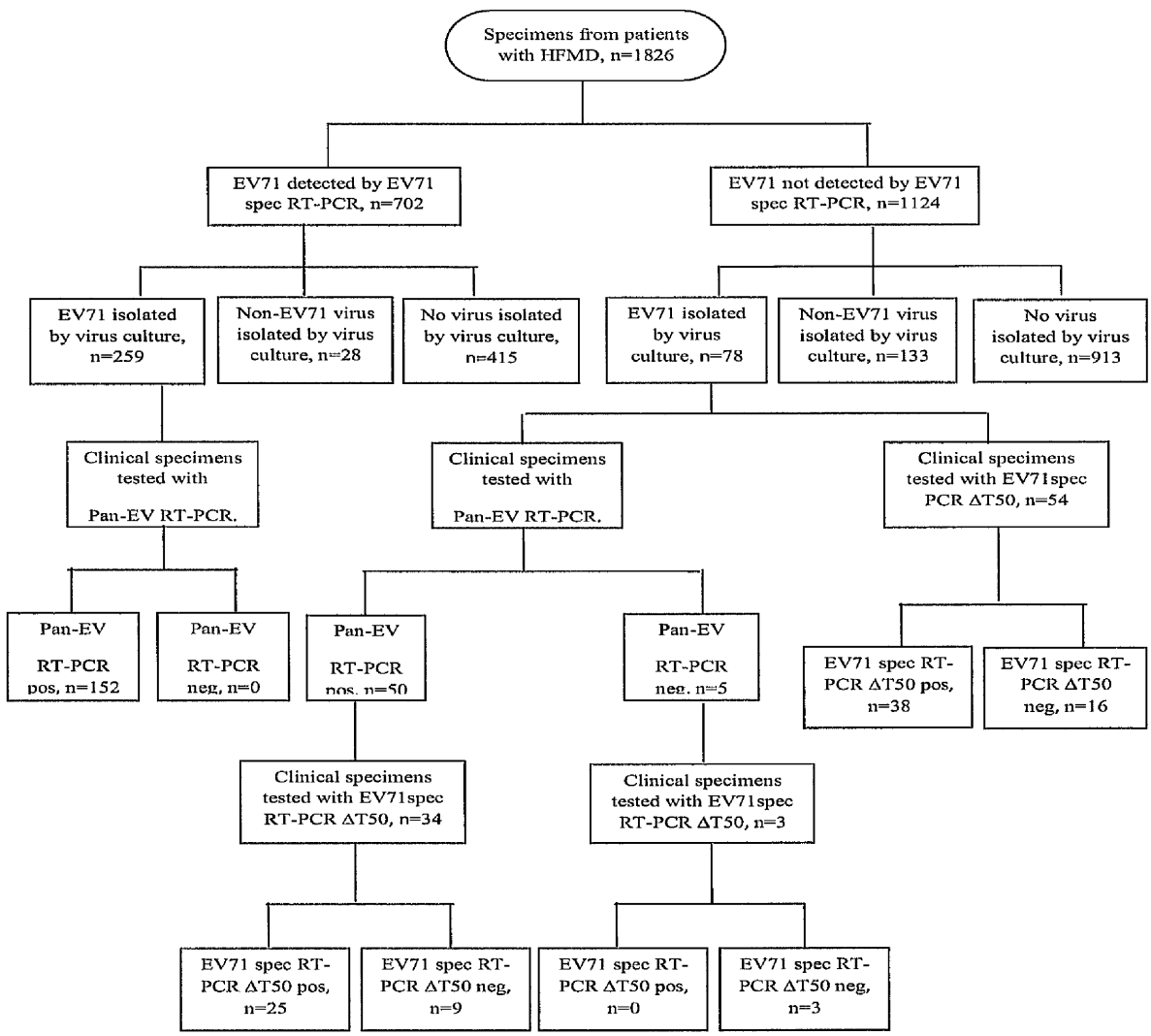
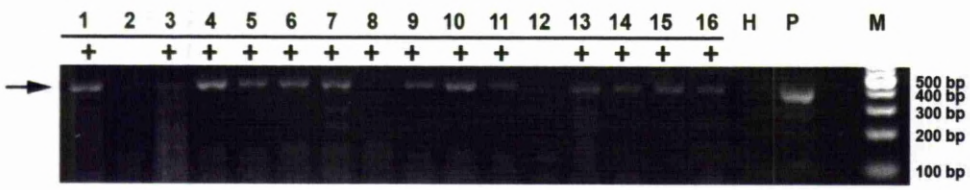


Figure 3.2 Amplification of enterovirus 71 from primary clinical material using re-optimised PCR cycling conditions with primer pair MAS01S/MAS02A.

Lanes, 1-16, primary clinical material previously not positive for EV71 using published PCR conditions (Perera et al., 2004); H, water control; P, EV71 positive control; M, 100 bp DNA ladder (Fermentas). '+' indicates samples that were tested positive for EV71. The expected position of the amplicon is indicated by the arrow.



Chapter 4: Clinical features, virological characteristics and molecular epidemiology of enterovirus 71 disease in Sarawak, Malaysia

Abstract

Human enterovirus (EV) 71 causes large outbreaks of hand, foot and mouth disease with neurological complications, but the role of EV71 genogroups or dual infection with other viruses, in causing severe disease is unclear. Children with suspected EV71 infection (hand, foot and mouth disease, central nervous system disease, or both) were studied over 3½ years, with detailed virological investigation and genogrouping of all isolates. A total of 773 children were recruited, 277 of whom were EV71 positive, including 28 co-infected with other viruses. Risk factors for central nervous system disease in EV71 included young age, fever, vomiting, mouth ulcers, breathlessness, cold limbs, and poor urine output. Genogroup analysis for the EV71-infected patients showed 168 were infected with genogroup B4, 68 with C1, and 41 with a newly emerged genogroup B5. Children with EV71 genogroup B4 were less likely to have central nervous system complications than those with other genogroups [26 (15%) of 168 versus 30 (28%) of 109, odds ratio (OR) [95% CI] 0.48 [0.26 – 0.91], p=0.0223] and less likely to be part of a

family cluster [12 (7%) of 168 versus 29 (27%) of 109, OR 0.21 [0.10-0.46], p<0.0001]; family clusters were more likely with genogroup B5 (OR 6.26 [2.77-14.18], p<0.0001). Children with EV71 and co-infection with another enterovirus or adenovirus were no more likely to have CNS disease. In conclusion genogroups of EV71 may differ in their risk of causing CNS disease, and family clusters. Dual infections are common, and all possible causes should be excluded before accepting that the first virus identified is the causal agent.

4.1 Introduction

Since 1997 countries of the Asia-Pacific rim have been affected by large outbreaks of human enterovirus (EV) 71 associated hand foot and mouth disease (HFMD), with hundreds of thousands of cases, and many deaths (Cardosa et al. 1999; Ho et al. 1999; Komatsu et al. 1999; Chan et al. 2000; McMinn 2002; Chan et al. 2003; Nolan et al. 2003). HFMD is a common exanthema of young children, characterised by fever, rash on the palms and soles, and ulcers in the oral cavity. Most patients have mild disease, but some patients develop severe neurological complications (aseptic meningitis, acute flaccid paralysis and encephalitis) or systemic disease (shock, cardiac dysfunction). HFMD is caused by enteroviruses (genus *enterovirus*, family *Picornoviridae*), particularly Coxsackie virus (CV) A10, CAV16 and EV71, which are mostly transmitted by the faeco-

oral route. Phylogenetic studies have divided EV71 strains into genogroups A, B and C, which have been further sub-divided (Brown et al. 1999; McMinn et al. 2001; McMinn 2002; Cardosa et al. 2003). The incidence of CNS disease, and of other severe complications, appears to have varied between outbreaks. The reason for this is unclear, but differences between genogroups (Dolin 1999; McMinn et al. 2001; Wang et al. 2002), and co-infection with other viruses, such as a newly characterised adenovirus (Cardosa et al. 1999; Ooi et al. 2003), have been postulated. However making comparisons between outbreaks has been hampered by the retrospective nature of many studies, and differences in inclusion criteria, in definitions of severe disease, and in viral diagnostic capabilities. A prospective clinical, diagnostic and molecular virological study of EV71-associated HFMD in one setting was conducted over 3½ years focusing on risk factors for neurological disease, and the putative role of different EV71 genogroups and dual infections in pathogenesis.

4.2 Materials and methods

Setting

The study was conducted on the paediatric wards and intensive care unit at Sibu Hospital, Sarawak, Malaysia, from January 2000 for 3 ½ years. This 550 bed hospital serves Sibu town (population 200,000) and receives referrals from district hospitals in the Rejang basin (total population

approximately 650,000). The study was approved by the Director of Health for Sarawak (Malaysia) and the Ethics Committee of the Liverpool School of Tropical Medicine (Liverpool, UK). Informed consent was obtained from each child's accompanying parent or guardian.

Case definitions

Because enteroviruses can cause HFMD, CNS infection, or both, children (age 1 month to <12y) were screened for both these conditions. Children with HFMD were classified according to an algorithm as having Mild HFMD, Severe non-CNS HFMD, or HFMD with CNS complications (Figure 4.1). Children with suspected CNS infections, but no HFMD, were classified as having aseptic meningitis (ASM) or viral encephalitis, based on the clinical and CSF findings; however there were no children with enterovirus infection in this last group, so they are not discussed further.

Clinical methods

A detailed history was taken, and a clinical examination performed by a paediatrician in the study team, looking especially for mucocutaneous lesions, cardiovascular and neurological signs; all details were recorded on standardised forms. For viral culture, swabs were taken from the throat, and rectum of every patient, as well as at least one swab from vesicles on the skin and oral ulcers (if present). Blood was taken for viral

studies. In patients with severe disease full blood count, urea, electrolytes, glucose, electrocardiogram and echocardiogram was performed. CSF was examined for cell count and differential, protein, glucose, Gram stain, bacterial culture and viral studies; lumbar puncture was repeated if there was a strong clinical suspicion of viral CNS infection, but the initial examination was not confirmatory. Lumbar punctures were delayed in those severely unwell. Patients were examined daily or more frequently as indicated, by a member of the study team. Children with HFMD and CNS complications were treated with intravenous immunoglobulin (IVIG, Intragam P, CSL which is derived from local Malaysian blood donors) on a presumptive basis (Cardosa et al. 1999; Lin et al. 2002), at the discretion of the treating physician.

Virological methods

Virus isolation was attempted on all swabs, CSF and any serum remaining after other investigations had been completed, by inoculating into rhabdomyosarcoma (Rd) and 293 cells (Ooi et al. 2003). Enteroviruses isolated were typed by nucleotide sequencing of VP1 and VP4 genes (Brown et al. 1999; Ishiko et al. 2002), and genogrouped by phylogenetic analysis (Brown et al. 1999; McMinn et al. 2001; Cardosa et al. 2003). Adenoviruses were cultured and typed as described previously (Ooi et al. 2003). Paired sera (taken on admission and day 7 or discharge) and CSF were also tested for IgM against dengue and Japanese

encephalitis virus (JEV) in parallel, using an IgM capture ELISA (Cardosa et al. 2002). Seroconversion from a negative sample to a positive 2nd sample or a rising IgM optic density (OD) was considered evidence of acute infection; antibody in the CSF was diagnostic of flavivirus CNS infection (Solomon et al. 1998; Solomon et al. 2000). A falling IgM OD was considered evidence of a recent infection, and low IgM OD in serum of children recently vaccinated against JEV was ascribed to vaccination.

Statistical analysis

Normally distributed data were compared using Student's t-test; data that were not normally distributed were compared by the Mann-Whitney U test (Staview 4.02 Abacus Concepts Inc). Differences between proportions were tested using the chi-square test with Yates' correction or Fisher's exact test (Epi-info Version 6). Variables that might relate to disease pathogenesis that were associated with CNS disease in univariate analyses were also examined in a stepwise logistic regression (SPSS version 9).

4.3 Results

4.3.1 Epidemiology

During the study 773 children (485, 63% males) were recruited. Five-hundred-and-forty (70%) had mild HFMD, 83 (11%) had severe HFMD with no CNS complications, 102 (13%) had HFMD with CNS disease, three of whom died, and 48 (6%) had ASM, one of whom died. Most children were recruited during two large outbreaks of EV71 associated HFMD, during 2000/1 and 2003 (Figure 4.2). Both outbreaks began in the months of January/February and peaked around the months of May and June, which coincided with the dry season of Sarawak. Genogroup analysis showed that in the 2000/1 outbreak most EV71 isolates belonged to genogroup B4 (McMinn et al. 2001; Cardosa et al. 2003), with some in C1. In 2003 as well as genogroup C1 strains, a previously un-described genogroup emerged during the outbreak which has been named B5 (see below).

Most patients entered the study just once, but four boys were admitted to the study twice. For the purposes of analysis, each hospitalization was considered as a separate entity. Thirteen other children had also been hospitalised in the past history with HFMD (outside the study). For 211 (27%) children, there was a history of contact with another HFMD case. 103 children (13%) children had at least one other child in the family

(sibling or cousin; maximum 4) admitted into the study, and were considered to be part of a family cluster. Seventy-seven of the children in these 44 family clusters had mild disease, 9 had severe non-CNS HFMD, and 17 had HFMD with CNS complications. Where the index case in a family cluster could be identified clearly, they were no more or less likely to have CNS disease than subsequent cases. Forty-one of the children in clusters had EV71 isolated (with only one genogroup isolated from each family), 3 of these children also had CVA16 isolated, so that in two families both CVA16 and EV71 were isolated. Twelve other children had CVA16, and three had other viruses.

4.3.2 Virology

Dual infections

Virus isolation was attempted on 3006 samples (622 throat swabs, 631 rectal swabs, 768 mouth ulcer swabs, 706 vesicle swabs, 91 serum and 188 CSF samples) from 672 (87%) of the 773 patients seen over the study period. There were no important demographic differences between those investigated and those not investigated. Two-hundred-and-seventy-seven (41%) patients had EV71 isolated. Twenty eight of these EV71 patients had a second virus isolated, including 14 patients coinfected with CVA16, and 9 co-infected with other enteroviruses (including CVA4, CVA5, CVA16, CVA10, Echo 25, CVB1, CVA24) and one each co-infected

with an oral polio vaccine virus, an untyped enterovirus, adenovirus (Ad) 1 (isolated from the rectum), Ad7 (rectum), and an unidentified virus (isolated from the CSF). CVA16 was isolated from a further 85 patients, four of whom had co-infection: two with CVB1 (isolated from the throat) and two with adenoviruses isolated from the rectum (one Ad2 and one Ad4). Fifty-eight patients were infected with other enteroviruses, adenoviruses or unidentified viruses (12 of whom had multiple viruses isolated). The EV71 positive and virus isolation-negative patients were similar in most of the presenting clinical features, except that 26 (10%) of the 252 patients with no virus isolated had seizures, compared with only 6 (2%) of the 263 EV71 positive patients [OR 0.20 [0.07-0.53], $p<0.001$].

Seven-hundred-and-sixteen patients had serum, and when available CSF (233 patients) tested for antibodies to JEV and dengue. Three had serological evidence of acute JEV infection: a girl with mild HFMD and EV71 isolated from vesicles and the throat, and a boy with mild HFMD and fever who had EV71 isolated from vesicles, and CVA16 isolated from the throat had acute peripheral JEV infection (seroconversion in the serum); in addition An 18m old boy with HFMD and CNS disease (manifested by a generalised seizure and a CSF pleocytosis of 10 cells/μL) had CVA16 isolated from a vesicle, but seroconverted to JEV in both the serum and CSF; he made a full recovery. Three patients had acute dengue. All had HFMD and CNS disease (lethargy, irritability, CSF

pleocytosis), and EV71 isolated: one from the CSF, one from the throat and vesicles, and one from vesicles and the rectum. Dengue was diagnosed by IgM seroconversion, but there was no dengue antibody in the CSF. Two HFMD patients had serological evidence of a recent JEV infection or recent vaccination, and 6 patients had serological evidence of recent dengue. Only two of the CVA16 children with HFMD had CNS disease: one of these was the child co-infected with JEV, described above; for the other child, CVA16 was isolated from rectal and vesicle swabs, and CVB1 was isolated from the throat.

4.3.3. Enterovirus 71 genogroups

Phylogenetic analysis of all 277 EV71 isolates showed that 168 (61%) of the isolates belonged to genogroup B4 (165 from 2000, 1 from 2002, and 2 from 2003); 68 (25%) of the isolates belonged to C1 (4 of these were isolated in 2000 and 64 in the 2003 outbreak); in addition, 41 (15%) EV71 strains isolated in 2003 were from a new genogroup (B5) that emerged during the course of the study (Cardosa et al. 2003; Mizuta et al. 2005; Podin et al. 2006). Neighbour-joining trees of the VP1 [Figure 4.3a] genes showed that representative Sibu EV71 strains belonging to B4, B5 and C1 genogroups clustered tightly with their respective genogroup reference strains with a bootstrap value 93.6%, 100% and 100%, respectively.

4.3.4 Clinical features of enterovirus 71 infection

Nearly two thirds of the EV71 patients had mild HFMD; 13% had severe non-CNS disease, 20% had HFMD with CNS disease, and only 1% had ASM (Table 4.1). For the purposes of analysis, those with CNS infection, defined by a CSF pleocytosis (with or without HFMD) were compared with the patients with no CNS disease (including mild and severe non-CNS HFMD). Patients with CNS disease were younger, and were more likely to give a history of fever, vomiting, mouth ulcers, breathlessness, cold limbs, poor urine output, and neurological features including altered sensorium, and seizures (Table 4.1). On examination patients with CNS disease were more likely to look toxic, be dehydrated and febrile, have altered consciousness, and neck stiffness, but they were less likely to have mouth ulcers (Table 4.2). Excluding the four patients with ASM and no rash did not significantly alter these findings. The CSF was examined in 90 (34%) children infected with EV71, of whom 56 (62%) had a CSF white cell count > 5/μL. For these patients the median (range) white cell count was 75 (6-1090) cells/μL. Typically this was a lymphocytic CSF with a normal glucose ratio, but 12 (21%) had neutrophil predominance, and 10 (18%) had a CSF to plasma glucose ratio < 50%.

Three children had HFMD complicated by acute flaccid paralysis: one had EV71 B4 genogroup isolated from his throat; the two others were culture negative. Sixty-two children with severe HFMD had cardiac enzymes measured (27 EV71 positive). Seventy-eight of the 233 more severe HFMD cases were treated with IVIG on a presumptive basis, including 42 that were EV71 positive. Five EV71 positive patients required ionotropic support, and three required ventilation. Four children died, including two that had EV71 isolated (one genogroup B4, one C1).

4.3.5 Pathogenesis of neurological enterovirus 71 disease

Children from whom EV71 plus another virus was isolated were no more likely than those with EV71 alone, to have neurological involvement [2 (7%) of 28, versus 54 (22%) of 249, OR 0.28 (0.04 to 1.26), p=0.1167]. Repeating this analysis excluding children with only rectal isolates (which could represent continued carriage rather than acute pathogenic infection) did not alter these findings [2 (7%) of 28, versus 47 (21%) of 226, OR 0.29 (0.05 to 1.34) p=0.1407]. All three children with EV71 and serological evidence of acute dengue infection, had HFMD with CNS disease, compared with 52 (20%) of 264 EV71-infected children with no dengue (p =0.008). In contrast the two EV71-infected children with serological evidence of JEV infection (as shown by seroconversion in the

serum) had HFMD with no CNS disease, compared with 55 (21%) of 266 with no JEV infection (p=1).

To examine the possible role of different EV71 genogroups in the clinical phenotype of EV71 infection, the likelihood of CNS disease for each genogroup was determined. Children with B4 genogroup were less likely to have CNS infection that those with other genogroups: 26 (15%) of 168 children infected with genogroup B4 had CNS infection, compared with 30 (28%) of 109 with other genotypes (OR [95% CI] 0.48 [0.26 – 0.91], p = 0.0223) (Table 4.2). Pair wise comparisons of the genogroups showed that genogroup B4 was associated with less risk of CNS disease than C1 [26 (15%) of 168, versus 19 (28%) of 68, OR 0.47 (0.23-0.98) p=0.0429]. There was also a trend showing that genogroup B4 was associated reduced incidence of CNS disease when compared to B5 [26 (15%) of 168, versus 11 (27%) of 41, OR 0.50 (0.21-1.21) p=0.139]. The observed difference did not reach the statistical significance likely because of the small sample size. There was no altered risk for B5 compared with C1 [11 (27%) of 41, versus 19 (28%) of 68 (28%) OR 0.95 (0.36-2.45) p=0.9240]. Children with EV71 genogroup B4 were also less likely to be part of a family cluster: 12 (7%) of 168 B4 positive were in clusters, versus 29 (27%) of 109 with other genogroups (OR 0.21 [0.10-0.46], p <0.0001). In contrast those with B5 genogroup were more likely to be part of a cluster: 17 (41%) of 41 B5 positive children were in a cluster,

versus 24 (10%) of 236 with other genogroups (OR 6.26 [2.77-14.18], p<0.0001). Genogroup, age, and being part of a family cluster were initially entered into a multiple logistic regression model: increasing age and genogroup B4 versus C1 were associated with a reduced risk of CNS infection (Table 4.3), whereas being in a family cluster did not affect risk.

4.4 Discussion

Since 1997, EV71 has become a major public health problem in both developed and developing countries in the Asia-Pacific region, and has the potential to spread further. Molecular epidemiological studies have documented remarkable changes in the circulating EV71 genogroups in the Asia-Pacific region during this time (McMinn et al. 2001; Cardosa et al. 2003; Jee et al. 2003; Shimizu 2004; Lin et al. 2006). However, whether such genetic differences explain the differing clinical presentations is not clear (McMinn et al. 2001; McMinn 2002). Other postulated factors include differences in pre-existing immunity of the paediatric population (Lu et al. 2002), in host genetic susceptibility (Dolin 1999; Yang et al. 2001; Lin et al. 2003), and co-infection with other viruses (Cardosa et al. 1999). The detailed investigation of 773 patients, including 277 with EV71 infection has produced intriguing clinic-epidemiological evidence suggesting that genogroups of EV71 may differ in their behaviour in humans. Children infected with genogroup B4 were

less likely to present with CNS infection than those infected with C1 or B5, and were also less likely to be part of a family cluster. In contrast children infected with B5 were more likely to be part of a family cluster, and there was a trend towards a greater incidence of CNS disease in these patients.

However, these findings must be interpreted with caution. Although I attempted to minimise confounding variables, there were factors beyond my control. In particular the likelihood of children presenting to the hospital with HFMD may have varied during the study, although altered referral rates would be unlikely to explain the different risk of family clustering for the different genogroups. There may also have been other inherent features that differed between the cohort studied in 2000 and 2003, through the basic demographic features were similar (data not shown).

This study has also confirmed those of earlier reports that young age, fever, vomiting and hyperglycaemia are associated with severe EV71 infection (Chang et al. 1999; Wang et al. 1999); in addition, tachycardia, breathlessness, and absence of mouth ulcers were noted to have associated with CNS disease. Overall, the incidence of severe disease was milder in this study than previous series, with just four fatal cases, all of

whom had pulmonary oedema. In addition, the study was unusual in having just four patients with pure aseptic meningitis.

This study has also made an important contribution to the debate about the role of dual infections in viral CNS disease. As others have shown previously (Hooi et al. 2002), several enteroviruses were co-circulating during the HFMD outbreaks in Sarawak. Instances of co-infection of EV71 with other enteroviruses in individual patients were documented. There was no evidence for an increased likelihood of CNS disease in such patients, though there was an intriguing association of co-infection with dengue viruses and neurological presentation. Nevertheless the findings must be interpreted with cautions in view of small number of cases with proven co-infection. Previously a new adenovirus type-21 was postulated to have made an important contribution to fatal disease during an EV71-associated HFMD outbreak (Cardosa et al. 1999; Ooi et al. 2003), however adenoviruses was isolated from only 4 patients in this study, and none was adenovirus type-21. Rigorous investigations for multiple viral agents in the study showed the importance of pursuing all possible avenues before attributing causation to the first pathogen encountered. For example in two children, CNS disease would have been attributed to CVA16, which is an unusual cause of CNS disease, had the thorough diagnostic work-up not revealed alternative more likely viral causes (JEV in one child, CBV1 in the other). Whether unsuspected dual infections lie

behind other "unusual manifestations" of common relatively benign enteroviruses, such as acute flaccid paralysis recently attributed to CVA24 is not known (Chaves et al. 2001).

In summary the study provides clinical epidemiological evidence for different biological behaviour of EV71 genogroups, in terms of transmissibility within families, and risk of CNS disease. It also highlights the importance of detailed investigations for multiple pathogens during HFMD outbreaks. Only with such thorough investigations will the pathophysiology of EV71 be fully determined.

Clearly neurological disease is one of the most important manifestations of EV71 infection. Clinical predictors of such disease, as described in the next chapter can help clinicians focus their attention on patients who most need it.

Table 4.1 Features in the history for 277 patients with enterovirus 71 isolated

	Non-CNS disease			CNS disease			Odds Ratio*	95% CI	p
Disease Classification^	Mild HFMD	Severe Non-CNS HFMD	Total	HFMD with CNS complications	ASM	Total			
No.(%) of patients	187 (68)	34 (12)	221 (80)	52 (19)	4 (1)	56 (20)			
Age (range, months)	32 (6-112)	24 (5-97)	31 (5-112)	23 (5-87)	25.5 (6-58)	23 (5-87)	..	..	0.003
Male	120	18	138	38	3	41	1.64	0.82-3.32	0.1321
Known HFMD contact	60	15	75	21	0	21	1.17	0.61-2.24	0.9632
Fever at home	141	29	170	51	4	55	16.5	2.37-328.65	0.0003
Coryza	61	13	74	19	3	22	1.29	0.67-2.45	0.4151
Cough	48	12	60	19	1	20	1.49	0.76-2.90	0.1364
Vomiting	19	12	31	22	1	23	4.27	2.11-8.65	<0.0001
Diarrhoea	3	2	5	3	1	4	3.32	0.63-15.95	0.0851

Mouth ulcers	175	31	206	46	0	46	0.33	0.13-0.86	0.0098
Breathlessness	1	0	1	5	0	5	21.57	2.31-1024.52	0.0015
Cold limbs	1	0	1	4	0	4	16.92	1.61-837.01	0.0065
Reduced urine output	15	7	22	12	1	13	2.37	1.19-6.22	0.0076
Headache	2	3	5	4	0	4	3.32	0.63-15.95	0.08
Irritability	14	12	26	21	1	22	4.85	2.34-10.06	<0.0001
Lethargy/drowsiness	21	16	37	30	3	33	7.14	3.60-14.24	<0.0001
Seizures	0 (0)	1 (2.9)	1 (0.5)	3 (5.8)	2 (50.0)	5 (8.9)	21.57	2.31-1024.52	0.0015

All values are number (%), or mean (SD) except age

*HFMD = hand foot and mouth disease, CNS = central nervous system, ASM = aseptic meningitis

Odds ratio (95%CI) and p values are for the comparison of all patients with CNS disease versus all those with non-CNS disease

Repeating the analysis for 254 children (using only the first child from each family cluster), did not significantly alter any of the associations.

Table 4.2 Examination and Investigation findings for 277 patients with enterovirus 71 isolated.

	Non-CNS disease			CNS disease			Odds Ratio*	95% CI	p
Disease Classification^	Mild HFMD	Severe Non-CNS HFMD	Total	HFMD with CNS complications	ASM	Total			
No.(%) of patients	187 (68)	34 (12)	221 (80)	52 (19)	4 (1)	56 (20)			
Examination									..
Toxic	4	24	28	33	2	35	11.49	5.59-23.83	<0.0001
Dehydration	9	3	12	11	2	13	5.27	2.08-13.37	<0.0001
Admission Temperature (range, °C)	37 (36.4-38.20	37.7 (36.5-39)	37.1 (36.4-39)	38 (36.5-39.5)	38.9 (37.1-39.9)	38 (36.5-39.9)	..	..	<0.0001
Pulse >150/min	2	0	2	7	2	9	20.97	4.09-202.37	<0.0001
Respiratory abnormalities	0	0	0	4	0	4	..	..	0.0015
Hepatomegaly	12	4	16	12	0	12	3.49	1.43-8.49	0.0017
Rash	184	34	218	52	2	54	0.37	0.04-4.57	0.2663

Mouth ulcer	180	32	212	47	0	47	0.22	0.08-0.65	0.0011
Herpangina	3	0	3	0	0	0	0	0.00-9.62	>0.9999
Irritable	0	7	7	11	0	11	7.47	2.51-22.79	<0.0001
Lethargic/drowsy	1	13	14	27	1	28	14.79	6.56-33.80	<0.0001
Neck stiffness	0	0	0	3	1	4	..	..	0.0015
Investigation									..
Plasma glucose (mmol/L)	5 (2.9-11.4)	5.3 (2.4-7.7)	5.1 (2.4-11.4)	6.1 (2.8-24.1)	6.3 (4.8-8.8)	6.1 (2.8-24.1)	..	..	0.0388
[no tested]	[31]	[24]	[55]	[44]	[4]	[48]			
EV71 Genogroup									
Genogroup B4	120	22	142	24	2	26	0.48	0.26-0.91	0.0223
Genogroup B5	24	6	30	10	1	11	1.56	0.68-3.53	0.3515
Genogroup C1	43	6	49	18	1	19	1.8	0.91-3.57	0.0985

All values are number (%), or mean (SD) except age

*HFMD = hand foot and mouth disease, CNS = central nervous system, ASM = aseptic meningitis

Odds ratio (95%CI) and p values are for the comparison of all patients with CNS disease versus all those with non-CNS disease

Repeating the analysis for 254 children (using only the first child from each family cluster), did not significantly alter any of the associations.

Table 4.3 Multivariate logistic regression analysis of variables associated with neurological disease in enterovirus 71 infection

Variable	B-coefficient	Odds Ratio (95% CI)	P
Age	-0.022	0.978 (0.962-0.996)	0.015
Genogroup C1 versus B4	0.751	2.12 (1.070-4.202)	0.031
Genogroup B5 versus B4	0.795	2.215 (0.972-5.048)	0.058

Genogroup, age, and being part of a family cluster were initially entered into a multiple logistic regression model. Terms were entered into the model and remained in only if they were statistically associated with CNS disease ($p<0.05$). Both forward selection and backward elimination methods were used. Increasing age and genogroup B4 versus C1 were associated with a reduced risk of CNS infection where-as being in a family cluster did not affect risk. Forward selection and backward elimination procedures generated the same model, indicating its robustness. The Hosmer-Lemeshow statistic indicated a non-significant lack of fit (Chi2 8.338, $p=0.401$).

Figure 4.1 Algorithm showing the classification of patients in the study.

Because EV71 can cause HFMD, CNS infection or both, children with HFMD or suspected CNS infections were studied. Children with HFMD were classified into mild HFMD, Severe non-CNS HFMD, and HFMD complicated by CNS disease; those with viral CNS infection were classified as aseptic meningitis (ASM), or viral encephalitis, but there were no children in the latter group infected with enteroviruses during this study. The final number of EV71 positive patients in each diagnostic group is shown in the figure. * Herpangina was defined as multiple oral ulcers predominantly affecting the posterior parts of the oral cavity.

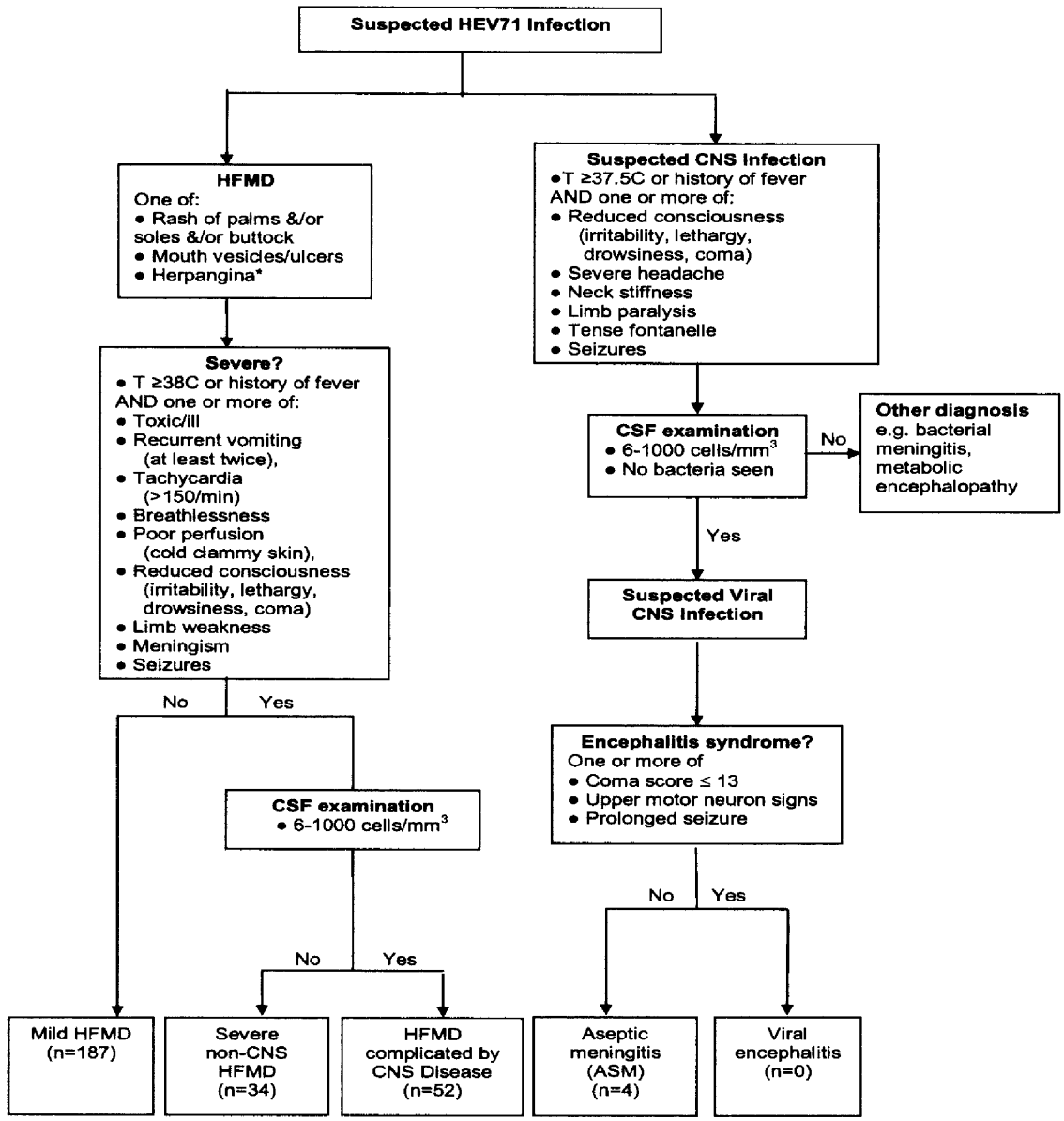

Suspected HEV71 Infection
HFMD
One of:
• Rash of palms &/or soles &/or buttock
• Mouth vesicles/ulcers
• Herpangina*
Severe?
• T ≥38C or history of fever AND one or more of:
• Toxic/ill
• Recurrent vomiting (at least twice),
• Tachycardia (>150/min)
• Breathlessness
• Poor perfusion (cold clammy skin),
• Reduced consciousness (irritability, lethargy, drowsiness, coma)
• Limb weakness
• Meningism
• Seizures
No
Yes
CSF examination
• 6-1000 cells/mm3
No
Yes
Mild HFMD (n=187)
Severe non-CNS HFMD (n=34)
HFMD complicated by CNS Disease (n=52)
Suspected CNS Infection
•T ≥37.5C or history of fever AND one or more of:
• Reduced consciousness (irritability, lethargy, drowsiness, coma)
• Severe headache
• Neck stiffness
• Limb paralysis
• Tense fontanelle
• Seizures
CSF examination
• 6-1000 cells/mm3
• No bacteria seen
No
Other diagnosis e.g. bacterial meningitis, metabolic encephalopathy
Yes
Suspected Viral CNS Infection
Encephalitis syndrome?
One or more of
• Coma score ≤ 13
• Upper motor neuron signs
• Prolonged seizure
No
Yes
Aseptic meningitis (ASM) (n=4)
Viral encephalitis (n=0)

Figure 4.2 Epidemic curve of 773 children with hand, foot and mouth disease and/or aseptic meningitis between 1st Jan 2000 and 31st July 2003.

The continuous line describes the total number of patients recruited into the study each month; the histogram below shows the number of patients with a positive virus isolate; grouped as EV71 – EV71 isolated, EV71/CVA16 – EV71 and CVA16 isolated, CVA16 – CVA16 isolated, CVA – other CVAs isolated, but not CVA16 or EV71; CVB – other CVBs isolated but not CVA16 or EV71; Echo – echoviruses isolated, but not CVA16 or EV71. The inset boxes show the number of patients with different genogroups of EV71 isolates during the 2000 and 2003 outbreaks.

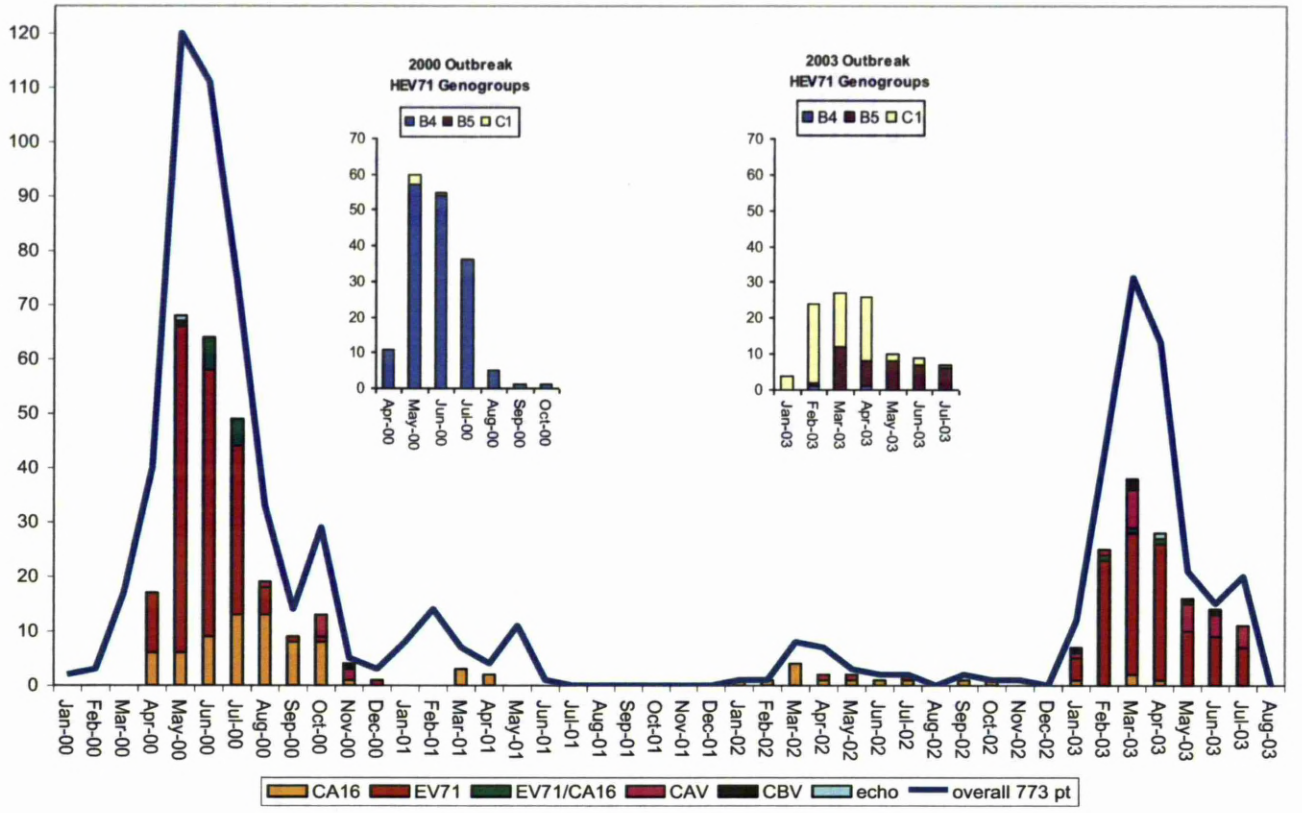

2000 Outbreak
HEV71 Genogroups
B4 B5 C1
2003 Outbreak
HEV71 Genogroups
B4 B5 C1
CA16 EV71 EV71/CA16 CAV CBV echo overall 773 pt

Figure 4.3 Neighbor-joining phylogenetic trees of human enterovirus 71 strains isolated in this study

(a) Tree based on the alignment of a partial VP1 (nucleotide positions 2442–3281) or complete VP1 (nucleotide positions 2442–3332) gene sequences. The tree show 14 representative strains isolated in this study (boxed), and representative EV71 strains for other genogroups.

(b) Tree based on the alignment of a partial VP4 (nucleotide positions 744-950). The trees shows 7 representative strains isolated in this study (boxed), and representative EV71 strains for other genogroups.

For both trees sequence from the prototype CVA16-G10 was used as an outgroup. The year of isolates is given as a suffix, and Genbank accession numbers are in parentheses. Horizontal branch lengths are proportional to the number of nucleotide changes between viruses, and a scale shown, and the bootstrap values in 1,000 pseudoreplicates for major lineages within the dendrogram are shown as percentages. PCR products were sequenced using Big Dye ver3.0 and run on an ABI377 automated sequencer (Applied Biosciences, Foster City, CAV, USA), and nucleotide sequences were aligned (DNASTAR Inc., Madison WI, USA).

Figure 4.3a

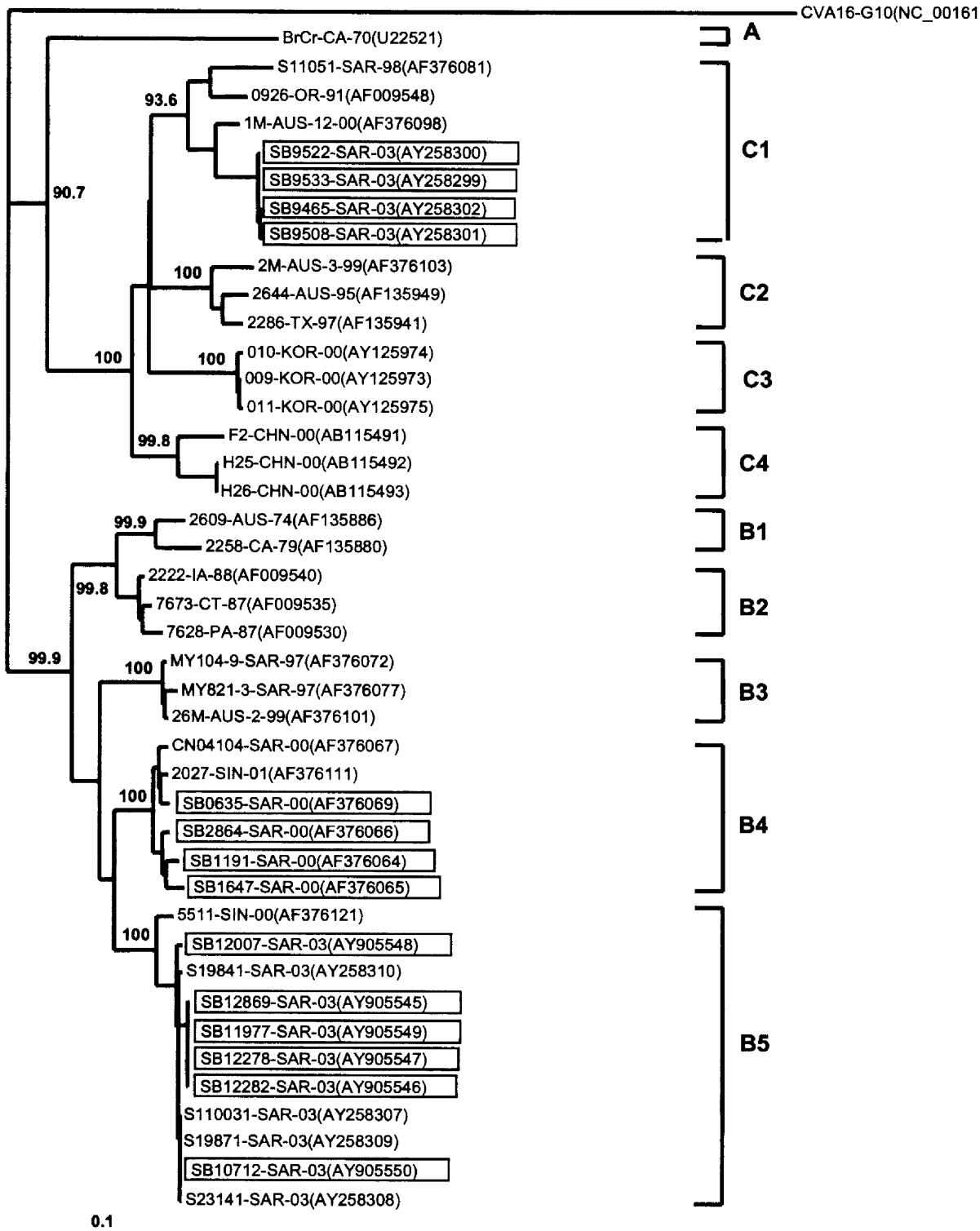
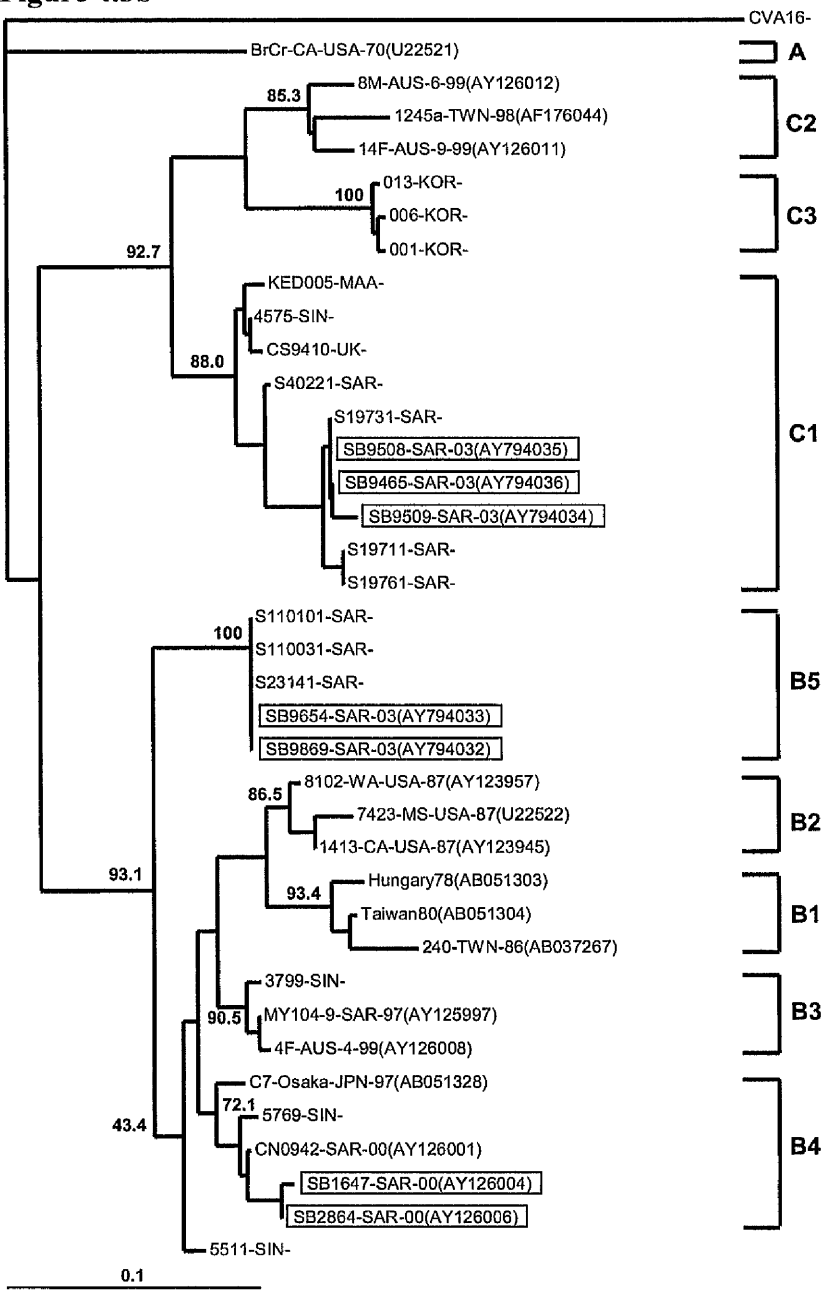

CVA16-G10(NC_00161
BrCr-CA-70(U22521)
S11051-SAR-98(AF376081)
0926-OR-91(AF009548)
1M-AUS-12-00(AF376098)
SB9522-SAR-03(AY258300)
SB9533-SAR-03(AY258299)
SB9465-SAR-03(AY258302)
SB9508-SAR-03(AY258301)
2M-AUS-3-99(AF376103)
2644-AUS-95(AF135949)
2286-TX-97(AF135941)
010-KOR-00(AY125974)
009-KOR-00(AY125973)
011-KOR-00(AY125975)
F2-CHN-00(AB115491)
H25-CHN-00(AB115492)
H26-CHN-00(AB115493)
2609-AUS-74(AF135886)
2258-CA-79(AF135880)
2222-IA-88(AF009540)
7673-CT-87(AF009535)
7628-PA-87(AF009530)
MY104-9-SAR-97(AF376072)
MY821-3-SAR-97(AF376077)
26M-AUS-2-99(AF376101)
CN04104-SAR-00(AF376067)
2027-SIN-01(AF376111)
SB0635-SAR-00(AF376069)
SB2864-SAR-00(AF376066)
SB1191-SAR-00(AF376064)
SB1647-SAR-00(AF376065)
5511-SIN-00(AF376121)
SB12007-SAR-03(AY905548)
S19841-SAR-03(AY258310)
SB12869-SAR-03(AY905545)
SB11977-SAR-03(AY905549)
SB12278-SAR-03(AY905547)
SB12282-SAR-03(AY905546)
S110031-SAR-03(AY258307)
S19871-SAR-03(AY258309)
SB10712-SAR-03(AY905550)
S23141-SAR-03(AY258308)
93.6
90.7
100
100
100
99.8
99.9
99.8
99.9
100
100
100
A
C1
C2
C3
C4
B1
B2
B3
B4
B5
0.1

Figure 4.3b


CVA16-
BrCr-CA-USA-70(U22521)
A
8M-AUS-6-99(AY126012)
85.3
1245a-TWN-98(AF176044)
14F-AUS-9-99(AY126011)
C2
100
013-KOR-
006-KOR-
001-KOR-
C3
92.7
KED005-MAA-
4575-SIN-
CS9410-UK-
88.0
S40221-SAR-
S19731-SAR-
SB9508-SAR-03(AY794035)
SB9465-SAR-03(AY794036)
SB9509-SAR-03(AY794034)
S19711-SAR-
S19761-SAR-
C1
100
S110101-SAR-
S110031-SAR-
S23141-SAR-
SB9654-SAR-03(AY794033)
SB9869-SAR-03(AY794032)
B5
8102-WA-USA-87(AY123957)
86.5
7423-MS-USA-87(U22522)
1413-CA-USA-87(AY123945)
B2
93.1
Hungary78(AB051303)
93.4
Taiwan80(AB051304)
240-TWN-86(AB037267)
B1
3799-SIN-
90.5
MY104-9-SAR-97(AY125997)
4F-AUS-4-99(AY126008)
B3
C7-Osaka-JPN-97(AB051328)
72.1
5769-SIN-
43.4
CN0942-SAR-00(AY126001)
SB1647-SAR-00(AY126004)
SB2864-SAR-00(AY126006)
B4
5511-SIN-
0.1

Chapter 5: Clinical predictors for the risk of neurological involvement in hand, foot and mouth disease

Abstract

Human enterovirus (EV) 71 can cause Hand, foot, and mouth disease (HFMD) with neurological complications, which may rapidly progress to fulminant cardiorespiratory failure, and death. Early recognition of children at risk is the key to reduce acute mortality and morbidity. Data collected through a prospective clinical study of HFMD conducted between 2000 and 2006 that included 3 distinct outbreaks of EV71 were examined to identify risk factors associated with neurological involvement in children with HFMD. Total duration of fever ≥3 days, peak temperature ≥38.5°C and history of lethargy were identified as independent risk factors for neurological involvement (evident by CSF pleocytosis) in the analysis of 725 children admitted during the first phase of the study. When they were validated in the second phase of the study, two or more (≥2) risk factors were present in 162 (65%) of 250 children with CSF pleocytosis compared with 56

(30%) of 186 children with no CSF pleocytosis (OR 4.27, 95% CI 2.79-6.56, p<0.0001). The usefulness of the three risk factors in identifying children with CSF pleocytosis on hospital admission during the second phase of the study was also tested. Peak temperature ≥38.5°C and history of lethargy had the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of 28%(48/174), 89%(125/140), 76%(48/63) and 50%(125/251), respectively in predicting CSF pleocytosis in children that were seen within the first 2 days of febrile illness. For those presented on the 3rd or later day of febrile illness, the sensitivity, specificity, PPV and NPV of ≥2 risk factors predictive of CSF pleocytosis were 75% (57/76), 59% (27/46), 75% (57/76) and 59% (27/46), respectively. In summary three readily elicited clinical risk factors were identified to help detect children at risk of neurological involvement. These risk factors may serve as a guide to clinicians to decide the need for hospitalization and further investigation, including cerebrospinal fluid examination, and close monitoring for disease progression in children with HFMD.

5.1 Introduction

Hand, foot and mouth disease (HFMD) is a common childhood exanthema caused by species A human enteroviruses (EV-A), particularly

coxsackievirus (CV) A16 (Pallansch et al. 2001). In most instances, this is a mild self-limiting illness. The affected children are often given out-patient care with symptomatic treatment. However over the last decade HFMD has emerged as a growing public health problem in Asia following frequent outbreaks of death-associated HFMD caused by a more virulence member of EVA, human enterovirus (EV) 71, in a number of countries in the region (Cardosa et al. 1999; Komatsu et al. 1999; Chan et al. 2003). This was first recognised with large outbreaks of HFMD associated with neurological disease and alarming fatalities in Sarawak, Malaysia in 1997 and in Taiwan in 1998 (Cardosa et al. 1999; Ho et al. 1999). Fatal cases typically presented with a brief duration of febrile illness, subtle neurological signs and died dramatically of acute refractory cardiac dysfunction and fulminant pulmonary oedema within hours of developing signs of tachycardia, poor peripheral perfusion and tachypnea. Indeed, most of them died shortly after hospital admission, and some even before or on arrival at hospital (Cardosa et al. 1999; Chang et al. 1999; Wang et al. 1999; Huang et al. 2002). Although severe neurological complications and death only occur in a small minority of children with HFMD, the fulminant disease course of the fatal cases has caused great public alarm in Asia. Experience from recent outbreaks of EV71 associated HFMD in Asia showed that primary care doctors are often overwhelmed with large number of children with HFMD seeking medical

attention for the fear of neurological complications and death. Because of the risk of sudden death, coupled with tremendous parental pressure to admit children with HFMD into hospital for observation, children with HFMD are often routinely admitted into hospital for observation in Sarawak, which has imposed a huge burden on the healthcare system. Cerebrospinal fluid (CSF) pleocytosis has so far been the universal finding in fatal cases even though many have no obvious neurological signs prior to sudden onset of cardiorespiratory failure and death (Cardosa et al. 1999; Chang et al. 1999; Wang et al. 1999). In the absence of clear neurological sign, CSF pleocytosis (indicative of neurological involvement) has thus been considered an objective marker of complicated disease, allowing clinicians to focus their attention and provide timely intervention in these patients before they develop fatal cardiorespiratory failure. Data collected through a prospective study of HFMD were examined to identify and validate risk factors associated with neurological involvement in children with HFMD that may be used by clinicians managing children with HFMD.

5.2 Materials and methods

5.2.1 Setting and study period

A prospective clinical study from January 2000 through December 2006, which included 3 distinct outbreaks that occurred in 2000/1, 2003 and 2006, was conducted at the paediatric wards and intensive care unit at Sibu Hospital (Sarawak, Malaysia). The study was approved by the Director of Health for Sarawak and the Ethics Committee of the Liverpool School of Tropical Medicine (UK). Informed consent was obtained verbally from each child's accompanying parent or guardian.

5.2.2 Case definitions

Figure 5.1 shows the algorithm of the investigation and the classification of the disease severity of children with HFMD in the study, as described in Chapter 4. Briefly a child was defined as having HFMD if they had new onset of at least one (≥1) of the following: maculopapular or vesicular rash on the palms and/or soles; vesicles or ulcers in the mouth or herpangina (defined as multiple oral ulcers predominantly affecting the posterior parts of the oral cavity). Children with HFMD were considered to have more serious illness if they have the following features: a history of fever, or fever on

examination (≥38°C), and ≥1 of the following features indicative of more serious illness: toxic and ill in appearance, recurrent vomiting (at least twice), tachycardia (heart rate ≥150/min) breathlessness, poor perfusion (cold clammy skin), reduced consciousness (irritability, lethargy, drowsiness, coma), limb weakness, meningism (neck stiffness or positive Kernig's sign), seizures. They were subjected to CSF examination after written consent to exclude central nervous system (CNS) involvement. Children with >5 cells/μL (i.e. CSF pleocytosis) and negative microscopy and culture for bacteria were classified as "HFMD with CNS complications" (HFMD-CNS), while those with normal CSF examination were considered to have "severe HFMD without CNS involvement" (HFMD-Non-CNS). Children with HFMD-CNS were diagnosed to have aseptic meningitis (ASM) if they were fully conscious, had headache, meningism, and no focal neurological signs. Encephalitis was defined by the presence of impaired consciousness including lethargy, drowsiness or coma, seizures or myoclonus. Acute flaccid paralysis (AFP) was characterised by the acute onset of areflexic limb weakness. Cardiorespiratory failure was 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. Children without features of more serious illness were classified as "mild HFMD", and were

observed in hospital until they became afebrile for at least 12-24 hours. A child was considered to be positive for EV71 if EV71 was isolated by tissue culture or EV71 RNA was detected by EV71 type-specific RT-PCR from ≥1 clinical sample, as described in Chapter 3.

5.2.3 Clinical methods

All children with HFMD admitted into the hospital were assessed by paediatricians of the study team. A detailed history and clinical examination was performed with special attention to mucocutaneous lesions, cardiovascular and neurological signs. All details were recorded on standardised forms. Swabs were taken from the throat and rectum of every patient, as well as ≥1 swab from vesicles on the skin and oral ulcers (if present). The clinical samples were stored immediately in a -70°C freezer until further testing. Blood was taken for flavivirus serology, and in patients with suspected CNS involvement for full blood count, urea, electrolytes, and glucose. Electrocardiogram and echocardiogram was also performed on children with suspected CNS involvement. CSF was examined for cell count and differential, protein, glucose, Gram stain, bacterial culture and processed for viral studies. If there was a strong clinical suspicion of viral CNS infection, but the initial CSF examination was acellular, a second lumbar

puncture was performed. Lumbar punctures were delayed in those with unstable vital signs. Patients were examined daily or more frequently as indicated, by a member of the study team. Children with HFMD-CNS complications (particularly those with encephalitis and acute flaccid paralysis) were treated with intravenous immunoglobulin (IVIG) at the discretion of the treating physician (Lin et al. 2002).

5.2.4 Virological methods

Virus isolation was attempted on all swab specimens, CSF specimens, and any serum samples remaining after other investigations had been completed through the inoculation of human rhabdomyosarcoma and human embryonic kidney cells. Isolated enteroviruses were typed by nucleotide sequencing of VP1 and VP4 genes and genogrouped by phylogenetic analysis (McMinn et al. 2001; Cardosa et al. 2003). During the 2006 outbreak, in addition to virus isolation, all swab specimens were also tested for presence of EV71 RNA using a EV71 type-specific RT-PCR (Perera et al. 2004). Paired serum samples (obtained on the day of admission and on day 7, or on the day of discharge or after death) and CSF specimens were also tested for IgM against dengue and Japanese encephalitis virus (JEV) in parallel, using an IgM-

capture ELISA that distinguishes responses to these two viruses (Cardosa et al. 2002).

5.2.5 Statistical analysis

Data from HFMD patients recruited in the first phase of the study (mostly during 2 outbreaks that occurred between January 2000 and July 2003) were used to identify risk factors for neurological involvement (evident by CSF pleocytosis). The primary analysis was for variables associated with neurological involvement by comparing children with HFMD-CNS (i.e. with CSF pleocytosis) to those with HFMD-Non-CNS (i.e. no CSF pleocytosis). Variables that were considered potentially useful to primary care doctors in identifying children with neurological involvement were included in a multiple logistic regression analysis to look for independent risk factors for neurological involvement (i.e. CSF pleocytosis). Variables were selected backward and remained in the model only if they were statistically associated with neurological involvement ($p<0.05$). (SPSS software, Version 13.0; SPSS). The association between the independent risk factors identified and neurological involvement were validated in the second phase of the study, where most patients were admitted during the 2006 outbreak. The utility of the identified risk factors as clinical predictors for neurological involvement

at the point of first contact for care was also examined. Normally distributed data were compared using Student's t test; data that were not normally distributed were compared by the Mann-Whitney U test (Statview 4.02; Abacus Concepts). Differences between proportions were tested using the Chi-square test with Yates's correction or Fisher's exact test as appropriate (Epi Info, version 6; Centers for Disease Control and Prevention). A p value <0.05 was considered statistically significant.

5.3 Results

A total of 725 children (457, 63% males) were recruited between 1st January 2000 and 31st July 2003 (Figure 5.1) Most children were recruited during 2 large outbreaks of EV71-HFMD that occurred during 2000/2001 and 2003. Five hundred and forty (74%) children had mild HFMD. One hundred and eighty five (26%) children had suspected CNS involvement and required CSF examination; 102 (55%) of them had CSF pleocytosis (HFMD-CNS) and the remaining 83 (45%) had normal CSF findings (HFMD-Non-CNS). Of the 102 children with HFMD-CNS, 63 (62%) had ASM, 33 (32%) had encephalitis, 3 (3%) had AFP, and 3 (3%) had encephalitis associated with cardiorespiratory failure (all of the 3 died). Of the 273 EV71 culture positive children, 187 (69%) had mild HFMD, 34 (13%) had HFMD-Non-CNS, and

52 (19%) had HFMD-CNS (30 had ASM, 19 had encephalitis, 1 had AFP and 2 had encephalitis with cardiorespiratory failure). Detailed results of the epidemiology, diagnostic virology and molecular epidemiology of this phase of the study have been described in the earlier chapters.

5.3.1 Clinical features

Comparison of patients with HFMD with CNS complications with those had more serious HFMD without CNS involvement (January 2000 to July 2003)

The clinical features of the children with HFMD-CNS (i.e. with CSF pleocytosis) are compared to those with HFMD-Non-CNS (i.e. no CSF pleocytosis) (Table 5.1). Children with HFMD-CNS were more likely to be male and of Chinese ethnic group. Children of Iban ethnic group, however, were less likely to have HFMD with CNS complications. Children with HFMD-CNS complications were more likely to have higher mean peak temperature, peak temperature ≥38.5°C, longer mean total duration of fever and total duration of fever ≥3 days. Findings of lethargy (from the parent's history or physical examination), faster mean heart rate, mean heart rate ≥150/min and limb weakness on examination were more frequently present in children with HFMD-CNS. There was no difference in the proportion of

children with positive EV71 isolation between children with HFMD-CNS and those with HFMD-Non-CNS.

To look for independent risk factors that could be used to predict neurological involvement evident by CSF pleocytosis, total duration of fever ≥3 days, peak temperature ≥38.5°C, being lethargic (from the parent's history or physical findings), history of breathlessness, history of vomiting, history of or witnessed myoclonus, neck stiffness were included in a multiple logistic regression analysis. Total duration of fever ≥3 days, peak temperature ≥38.5°C and history of lethargy were found to be independent risk factors of neurological involvement after multivariate analysis (Table 5.2). Table 5.3 shows the number and type of the risk factors that were present in the 725 children with HFMD seen during the first phase of the study according to the disease severity. Two or more (≥2) risk factors were present in 83% (85/102) of patients that had HFMD-CNS when compared to 43% (36/83) of patients with HFMD-Non-CNS (OR 6.53, 95% CI 3.15-13.66, $p<0.0001$). Further analysis on the EV71-positive subset showed that ≥2 risk factors were present in 82% (43/52) of children with HFMD-CNS when compared to 32% (11/34) patients with HFMD-Non-CNS (OR 9.99, 95%CI 3.26-31.82). A separate analysis on children with mild HFMD showed that ≥2 risk factors

were present in 6% of cases with mild HFMD (32/540)(Table 2), and EV71-postive mild HFMD (11/187), respectively.

5.3.2 Validation of the association between the risk factors and neurological involvement in children with HFMD in 2006 outbreak

The association between the identified risk factors (total duration of fever ≥3 days, peak temperature ≥38.5°C and history of lethargy) and neurological involvement were validated in the 2006 outbreak. A total of 730 children with HFMD were admitted between January and December 2006. Two hundred and ninety four (40%) children had mild HFMD. Four hundred and thirty six (60%) children had features of more serious illness and warranted CSF examination; 250 (34%) of them had HFMD-CNS and the remaining 186 (26%) had HFMD-Non-CNS. Of the 250 children with HFMD-CNS, 65 (26%) had ASM, 172 (69%) had encephalitis, 2 (0.8%) had encephalitis associated with AFP, and 11 (4.4%) had encephalitis associated with cardiorespiratory failure (6 of them died). EV71 was isolated from 157 (27%) of 586 children who had virus isolation done. A further 44 (7%) children had other species A enteroviruses (n=29) and species B EV (n=15). No patient had CVA16 isolated. EV71 RNA was detected in 239 (50%) of 477 children

that were tested with EV71 type-specific RT-PCR. In short, 291 (45%) of 653 children were positive for EV71. Of the 291 EV71-positive children, 104 (36%) had mild HFMD, 73 (25%) had HFMD-Non-CNS, 114 (39%) had HFMD-CNS (22 had ASM, 83 had encephalitis, 2 had encephalitis associated with AFP, 7 had encephalitis associated with cardiorespiratory failure). EV71 was detected in 4 (67%) of the 6 fatal case children that had encephalitis associated with cardiorespiratory failure. The table 5.4 shows the clinical features of the 730 children that were admitted during the 2006 outbreak according to the disease severity. Total duration of fever ≥3 days, peak temperature ≥38.5°C and history of lethargy were similarly more frequently present in children with HFMD-CNS than those with HFMD-Non-CNS. Two or more risk factors were present in 65% (162/250) of children that had HFMD-CNS when compared with 30% (56/186) of children with HFMD-Non-CNS (OR 4.27, 95%CI 2.79-6.56, p<0.0001) (Table 5.3). Among children with EV71-positive HFMD, ≥2 risk factors were present in 61% (69/114) of children with HFMD-CNS when compared with 26% (19/73) of children with HFMD-Non-CNS (OR 4.36, 95%CI 2.19-8.75, p<0.0001). A separate analysis on children with mild HFMD showed that history of lethargy, total duration of fever ≥3 days and peak temperature ≥38.5°C was present in 6.4% (19/294), 11% (33/294) and 14% (42/294) of the children with mild HFMD, respectively (table 5.4). Two or more 2 risk

factors were found in only 5 (2%) of 294 with mild HFMD (Table 5.3) and in 1 (1%) of 104 of children with EV71-positive mild HFMD.

5.3.3 The usefulness of the risk factors in predicting neurological involvement in children with HFMD in the 2006 outbreak

The utility of the three clinical risk factors in predicting neurological involvement in children with HFMD at the point of first contact for care was assessed. While a febrile illness ≥3 day was an important risk factor for severity, primary care physicians often see many children on the first 2 days of HFMD illness. To determine if peak temperature ≥38.5°C and history of lethargy are useful in identifying children who sought treatment within the first 2 days of the febrile illness a separate analysis for children who presented within the first 2 days of the illness during the 2006 outbreak was performed. Figure 5.2 shows the distribution and classification of disease severity of 730 children with HFMD in the 2006 outbreak according to the duration of febrile illness and the risk factors that were present when they first presented to hospital. Five hundred and seventy nine (79%) of 730 children were admitted within the first 2 days of febrile illness. Sixty five (11%) of the 579 children had history of lethargy plus peak temperature

≥38.5C. All but two (97%) of the 65 children had features of more serious illness and warranted CSF examination. About three quarter of them had CSF pleocytosis and was classified as HFMD-CNS. Only 2 (3%) of the 65 children were labelled as mild HFMD. Two hundred and twenty (38%) children had only either history of lethargy or peak temperature ≥38.5C. Of the 167 (76%) children who warranted a CSF examination, 102 (61%) of them had CSF pleocytosis, and were classified as HFMD-CNS. The remaining 53 (24%) children without feature of more serious illness were considered as mild HFMD. Two hundred and ninety four (51%) children had neither of the two risk factors. Eighty four (29%) of the 294 children, however, had other features of more serious illness, and hence underwent CSF examination. CSF pleocytosis was found in 24 (29%) of the 84 children, and were classified as HFMD-CNS. Two hundred and ten (71%) of the 294 children without features of more serious illness were labelled as mild HFMD. In summary CSF pleocytosis was found in 48(74%) of 65 children with 2 risk factors (temperature ≥38.5°C and history of lethargy) on hospital admission compared with that in 126 (25%) of 514 children with ≤1 risk factors (OR 8.69; 95%CI 4.66-16.37, $p<0.0001$).

One hundred and fifty one (21%) of the 730 children were seen on the 3rd or later days of their febrile illness. Twenty two (15%) of the 151 children had

all the 3 risk factors associated with neurological involvement. All the 22 children warranted CSF examination. Seventeen (77%), including 4 fatal cases, of the 22 children had CSF pleocytosis and were classified as HFMD-CNS. Of the 55 (36%) children that had 2 risk factors, all except one child required CSF examination to exclude CNS involvement. Forty (74%) of the 54 children had CSF pleocytosis and were classified as HFMD-CNS. Being febrile for ≥3 days was the sole risk factor in 74 (49%) of the 151 children. Forty six (62%) children had features of more serious illness, and underwent CSF examination – 19 (41%) had CSF pleocytosis and were classified as HFMD-CNS. The remaining 28 (38%) children were labelled as mild HFMD. In short CSF pleocytosis was found in 57(74%) of 77 children that had ≥2 risk factors on hospital admission compared with in 19 (26%) of 74 children with isolated risk factor of being febrile ≥3 days (OR 8.25; 95%CI 3.75-18.38, p<0.0001). Further analysis on the EV71-positive subset showed that 24% (21/86) of children with HFMD-CNS presented within the first 2 days of febrile illness had ≥2 risk factors compared with 10% (6/60) of children with HFMD-Non-CNS (OR 2.91; 95% CI 1.03-9.38, p=0.0464). For the EV71-positive children presented on the 3rd or later days of febrile illness , 71% (20/28) of children with HFMD-CNS had ≥2 risk factors compared with 31% (4/13) of children with HFMD-Non-CNS (OR 5.63; 95% CI 1.11-31.35, p=0.0341). The sensitivity, specificity, positive predictive value and

negative predictive value of the risk factors in predicting CSF pleocytosis in children with HFMD at presentation in the 2006 outbreak is shown in Table 5.5.

Between 2000 and 2006, a total of 352 children with CNS involvement were admitted into the study. One hundred and twenty eight (36%) children had ASM (a mild and benign CNS involvement) and 224 (64%) had severe and potentially fatal CNS complications (205 had encephalitis, 14 had encephalitis associated with cardiorespiratory failure, 2 had encephalitis associated with AFP, 3 had AFP). Among the 224 children that had severe CNS complications, 204 (95%) of 215 children that survived had timely hospital admission and IVIG treatment compared to one (11%) of 9 children that died (OR 148.36, 95% CI 16.34-6609.04, $p<0.0001$) (Figure 5.3). Table 5.6 shows the clinical details and the risk factors that were present in the 9 fatal case children on hospital admission. Two or more risk factors associated with neurological involvement were present in all the 9 fatal case children, and were noted for ≥24-48 hours before hospital admission. Three of the fatal case children had repeated white cell counts and serum glucose examination, which showed leucocytosis and hyperglycaemia were observed on the day or the day after the onset of cardiorespiratory failure (Figure 5.4)

5.4 Discussion

Early recognition of children at risk of neurological involvement and death (particularly those with encephalitis and encephalomyelitis) is critical as the disease progression from the onset of neurological involvement to fulminant cardiorespiratory failure may be remarkably rapid (Chang et al.). However the clinical manifestations of neurological involvement may be very subtle, particularly in young children with early CNS disease (Chang et al. 1999; Lin et al. 2006). While the signs of cardiorespiratory distress such as breathlessness, tachypnea, tachycardia, poor perfusion are easy to recognise, they invariably appear late and only shortly before most fatal case children collapsed. The results of this work and other published studies showed that timely diagnosis and intervention, including the use of IVIG infusion, may reduce acute mortality (Lin et al. 2002; Chang et al. 2004; Wang et al. 2006; Wang et al. 2006). Hence the primary care doctors are confronted with a clinical challenge of identifying a small fraction of children who are at risk of neurological complication from an overwhelmingly large number of children who would have uncomplicated course of HFMD. For this reason it is important to find clinical predictors for neurological involvement that can guide primary care doctors perform a proper patient triage, which should be aimed to admit high risk children into hospital early for close observation and

further management, while those at low risk of neurological complication may be given out-patient care after parental education and advice. Few studies have systemically examined how to identify children at risk early before they develop cardiorespiratory failure, particularly at the primary care setting where the majority of children with HFMD would first seek treatment during a community outbreak of HFMD. A history of lethargy, mean peak temperature ≥38.5°C and total duration of fever ≥3 days were identified and validated as important risk factors for neurological involvement in this study. The study also showed that neurological involvement occurs at early course of complicated HFMD, and may be detectable within the first 2 days of the febrile illness because CSF pleocytosis was present in 174 (30%) of 579 children seen within the first 2 days of febrile illness, where they also formed 70% (174/250) of children with HFMD-CNS in the 2006 outbreak (Figure 5.2). Since CSF pleocytosis may be detectable within the first 2 days of the febrile illness and fulminant cardiorespiratory failure seen in the fatal case children typically occurred on the 3rd or later day of febrile illness, it is imperative to attempt to identify children at risk of neurological involvement before the 3rd day of febrile illness so that they can be admitted into hospital early for close monitoring and investigation, and intervention may be instituted when necessary.

Examination of body temperature and careful enquiry into history of lethargy, duration of fever and home record of body temperature should form an integral part of HFMD patient triage at the primary care level. The three risk factors are readily elicited, and can also be used after minimal training by paramedics, who are the key primary care providers in many developing countries including in Sarawak (Malaysia) in Asia. The parents of children with HFMD can also play an important role in early diagnosis of neurological complication in children with HFMD. They should be educated about the 3 risk factors, and be encouraged to monitor the children's body temperature regularly and observe the children's physical activity closely. Body temperature ≥38.5°C and history of lethargy may be particularly useful clinical clues for neurological involvement during the first 2 days of febrile illness since at this time the presentation of complicated HFMD is typically undifferentiated and subtle, even to the experienced clinicians (Chang et al. 1999). Indeed both history of lethargy and temperature ≥38.5°C were observed for 24-48 hours in all the 9 fatal case children before they succumbed to unexpected fulminant cardiorespiratory failure (Table 5.6). Primary care doctors should have high index of suspicions of neurological complication when they are presented with children with HFMD who have been febrile ≥3 days. The children should be admitted into hospital for close observation and investigated for CNS involvement, if necessary. This study

showed that 92 (31%) of 293 children with total duration of fever ≥3 days in the 2000/3 outbreak, and 183 (61%) of 300 children in the 2006 outbreak had neurological involvement (Table 5.3). CSF pleocytosis was present in 25% (19/74) of children with a single risk factor of being febrile ≥3 days on hospital admission (Figure 5.2). The risk of CNS complication is increased significantly when there are added risk factors of having history of lethargy and temperature ≥38.5°C. In contrast children who have a brief duration of low grade fever (≤38.5°C) and no history of lethargy are of low risk of neurological disease, and may be provided with out-patient care and parental reassurance.

The results of this study are in keeping with findings reported by Chang and co-authors where fever ≥39°C, fever duration ≥3 days and lethargy were more frequently observed in children with CNS involvement and in children with EV71-HFMD than in those with CVA16-HFMD (Chang et al. 1999). Although several other clinical features and laboratory abnormalities have been associated with fatal EV71-HFMD, they have yet been validated, and been shown useful in detecting neurological disease or disease progression (Huang et al. 1999; Huang et al. 2003) (Chong et al. 2003). For example, Chong and co-authors reported that absence of mouth ulcers predicted a more complicated or fatal HFMD, and have recommended that children without

mouth ulcers should be monitored closely (Chong et al. 2003). However, children without mouth ulcers were more likely than children with mouth ulcers to have features of more severe HFMD or develop neurological complication in this study. Not all the risk factors identified in these published studies can readily be translated into clinical practice, particularly at primary care settings. Hyperglycaemia and leucocytosis have been shown as risk factors for fatal EV71 disease (Chang et al. 1999). However, in this study hyperglycaemia and leucocytosis were late laboratory changes in children with fulminant cardiorespiratory failure, and thus are not helpful clinically in identifying children at high risk of complication and death. Elevated cardiac troponin I, a sensitive cardiac-specific biomarker for myocardial injury, has been noted in children who subsequently developed left ventricular failure, and may be useful in identifying patients at risk of left ventricular failure and pulmonary oedema (Huang et al. 2003). Although cardiac troponin I has been used widely in developed countries for early diagnosis of acute coronary syndrome, it is expensive and not widely available in many developing countries, including in Sarawak. Screening for heart rate variability abnormalities, an index of autonomic nervous system, through non-invasive continuous electrocardiography monitoring may provide early warning of impending cardiorespiratory failure about 7 hours before its onset (Lin et al. 2006). The labour-intensive approach is most

suited in a critical care setting for children already diagnosed of CNS involvement because it requires the use of expensive and sophisticated device, and close monitoring for the heart rate abnormalities.

Children of Chinese ethnic group were more likely to develop CNS complication while children of Iban ethnic group were less likely to have CNS disease. Host genetic susceptibility may be a possible explanation for the observed difference in the risk of developing HFMD-CNS among children of different ethnic group. HLA-A33 has been reported to have significant association with increased susceptibility to EV71 infection in a genetic study in Taiwan (Chang et al. 2008). The authors also reported that HLA-A2 was closely associated with the risk of cardiorespiratory failure often observed in fatal cases (Chang et al. 2008).

A limitation of this study is that the clinical predictors developed for use at primary care setting were identified and tested using data collected through a hospital-based study. Clinical characteristics of children treated at primary care settings may differ from hospitalised children. However, as a large number of children with mild HFMD were admitted into the study, it provided an opportunity to systemically examine the clinical feature of HFMD of varying severity, including children with mild disease that would

normally be treated at primary care clinics, where history of lethargy, peak temperature 38.5C and total duration of fever 3 days were reported infrequently in children with mild HFMD.

Currently there is no vaccine against EV71 infection. Early recognition of children at risk of fulminant pulmonary oedema and cardiac dysfunction may be the key to reduce acute mortality and morbidity. The three clinical risk factors identified in this study may help detect children at risk of neurological involvement and death at primary care settings, which may guide primary care doctors decide if hospital admission is warranted when they see with children with HFMD. These risk factors are readily elicited through history taking and measurement of body temperature. They may also provide useful guide to help clinicians to decide the need for CSF examination, as well as to monitor disease progression in children with HFMD.

Whilst EV71 remains the most important cause of HFMD with severe manifestations, the related enterovirus, coxsackie virus A16 also causes HFMD. Whether it too can cause neurological disease has been a subject of some controversies.

Figure 5.1 The algorithm of the investigation and the classification of the disease severity of children with hand, foot and mouth disease

The flow chart shows the algorithm of the investigation and the classification of the disease severity of children with HFMD used in the study

HFMD: Hand, foot, and mouth disease, CSF: Cerebrospinal fluid, CNS: Central nervous system

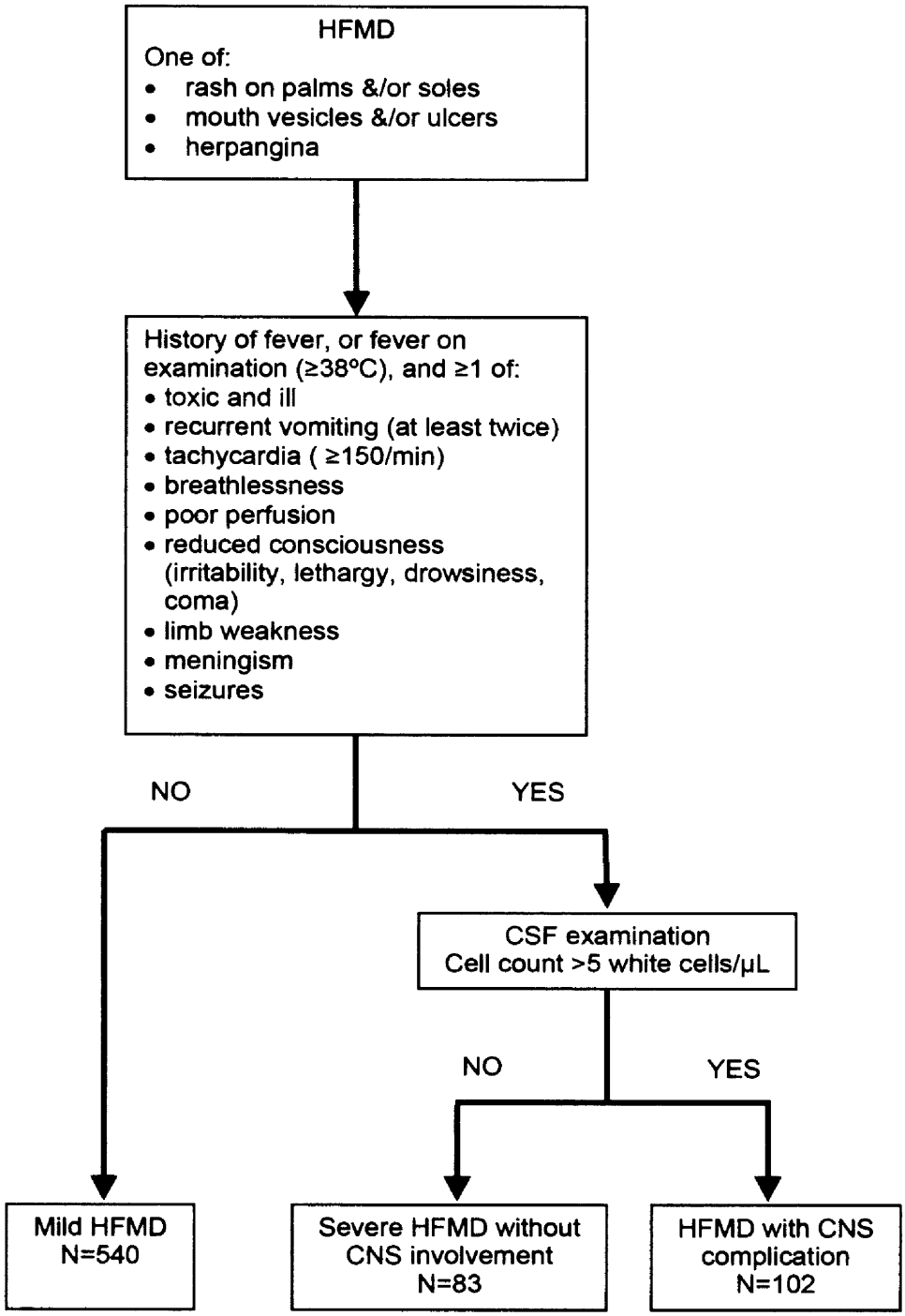

HFMD
One of:
• rash on palms &/or soles
• mouth vesicles &/or ulcers
• herpangina
History of fever, or fever on examination (≥38°C), and ≥1 of:
• toxic and ill
• recurrent vomiting (at least twice)
• tachycardia (≥150/min)
• breathlessness
• poor perfusion
• reduced consciousness (irritability, lethargy, drowsiness, coma)
• limb weakness
• meningism
• seizures
NO
YES
CSF examination
Cell count >5 white cells/μL
NO
YES
Mild HFMD
N=540
Severe HFMD without CNS involvement
N=83
HFMD with CNS complication
N=102

Figure 5.2 Classification of 730 Children with hand, foot and mouth disease

The flow chart shows the distribution and classification of disease severity of 730 children with HFMD in the 2006 outbreak according to the duration of fever and the risk factors that were present when they first presented to the hospital. CSF examination is indicated if the children have features indicative of more serious illness of HFMD (see case definition in main text)

Hx lethargy: History of lethargy, Temp ≥38.5°C: body temperature ≥38.5°C, CSF exam: cerebrospinal fluid examination, HFMD: Hand, foot, and mouth disease, HFMD-CNS: Hand, foot, and mouth disease with central nervous system complication, HFMD-Non-CNS: Severe HFMD without central nervous system involvement, BENC: brainstem encephalitis, ASM: aseptic meningitis.

a. Of the 48 children with HFMD-CNS, 40 had BENC, 6 had ASM and 2 had BENC associated with cardiorespiratory failure (1 of whom died).
b. Of the 102 children with HFMD-CNS, 74 had BENC, 26 had ASM, 1 had encephalitis and 1 had fatal BENC associated with cardiorespiratory failure.
c. Of the 24 children with HFMD-CNS, 13 had BENC, 8 had ASM, 1 each had BENC associated with cardiorespiratory failure, encephalitis, and encephalitis associated with acute flaccid paralysis.

d. Of the 17 children with HFMD-CNS, 11 had BENC, 5 had BENC associated with cardiorespiratory failure (4 of whom died) and 1 had ASM

e. Of the 40 children with HFMD-CNS, 22 had BENC, 17 had ASM and 1 had fatal BENC associated with cardiorespiratory failure.

f. Of the 19 children with HFMD-CNS, 10 had BENC, 7 had ASM and 1 each had encephalitis with acute flaccid paralysis, and BENC associated with cardiorespiratory failure.

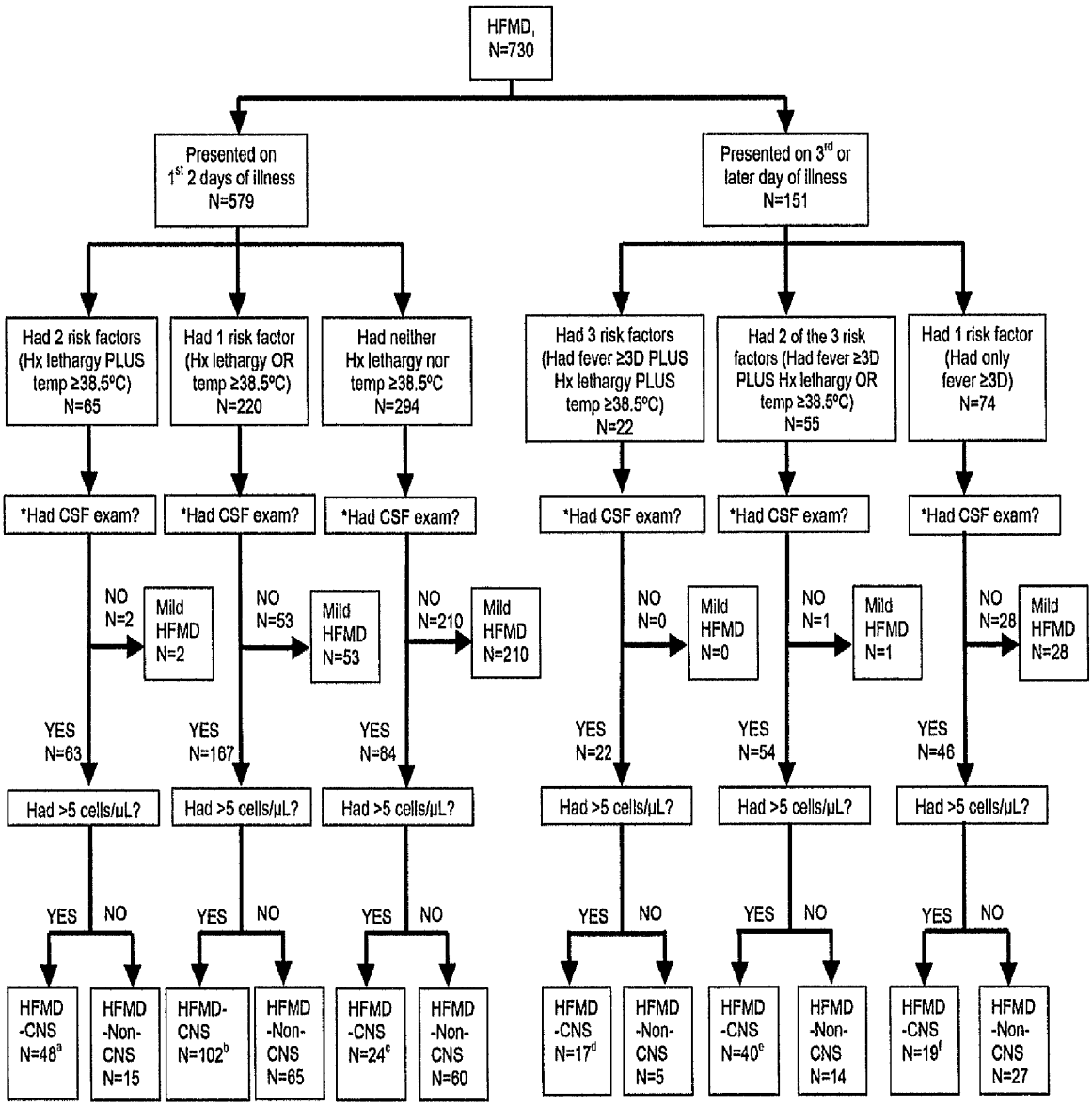

HFMD, N=730
Presented on 1st 2 days of illness N=579
Presented on 3rd or later day of illness N=151
Had 2 risk factors (Hx lethargy PLUS temp ≥38.5°C) N=65
Had 1 risk factor (Hx lethargy OR temp ≥38.5°C) N=220
Had neither Hx lethargy nor temp ≥38.5°C N=294
Had 3 risk factors (Had fever ≥3D PLUS Hx lethargy PLUS temp ≥38.5°C) N=22
Had 2 of the 3 risk factors (Had fever ≥3D PLUS Hx lethargy OR temp ≥38.5°C) N=55
Had 1 risk factor (Had only fever ≥3D) N=74
*Had CSF exam?
NO N=2
Mild HFMD N=2
NO N=53
Mild HFMD N=53
NO N=210
Mild HFMD N=210
NO N=0
Mild HFMD N=0
NO N=1
Mild HFMD N=1
NO N=28
Mild HFMD N=28
YES N=63
YES N=167
YES N=84
YES N=22
YES N=54
YES N=46
Had >5 cells/μL?
YES
NO
HFMD -CNS N=48a
HFMD -Non-CNS N=15
HFMD-CNS N=102b
HFMD -Non-CNS N=65
HFMD -CNS N=24c
HFMD -Non-CNS N=60
HFMD -CNS N=17d
HFMD -Non-CNS N=5
HFMD -CNS N=40e
HFMD -Non-CNS N=14
HFMD -CNS N=19f
HFMD -Non-CNS N=27

Figure 5.3 Use of intravenous immunoglobulin treatment and the clinical outcome

Between 2000 and 2006 there were 1455 children with hand, foot and mouth disease were admitted; 352 of those had central nervous system (CNS) involvement. One hundred and twenty eight (36%) children had aseptic meningitis, a mild and benign CNS disease, and were not treated with intravenous immunoglobulin. The other 224 (64%) children had severe and potentially fatal CNS complications, including 205 had encephalitis, 14 had encephalitis associated with cardiorespiratory failure, 2 had encephalitis associated with acute flaccid paralysis, 3 had isolated acute flaccid paralysis, and were treated with intravenous immunoglobulin. There were a total of 20 deaths occurred during the Sarawak 1997 (April - June) epidemic (Cardosa et al. 1999).

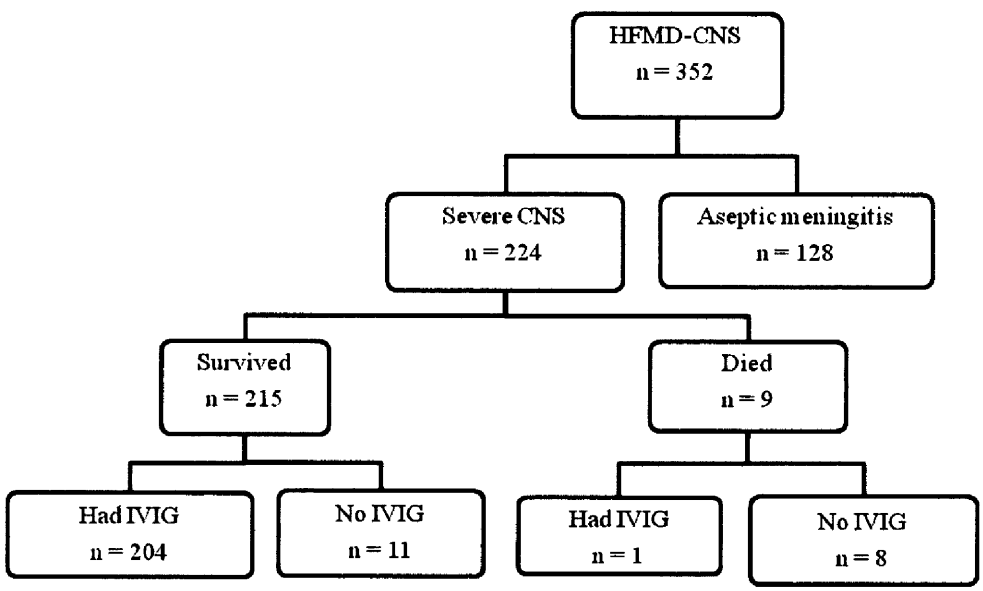


Figure 5.4 White cell counts and serum glucose of three children with fatal hand, foot and mouth disease caused by enterovirus 71

The line graphs show the white cell counts (top panel, Y axis indicates the number of white cells per uL) and serum glucose (bottom panel, Y axis indicates the measurement unit in mmol/L) of three patients (Pt) with fatal hand, foot and mouth disease the day before (day -1), on the day (day 0) and the day after the onset of cardiorespiratory failure.

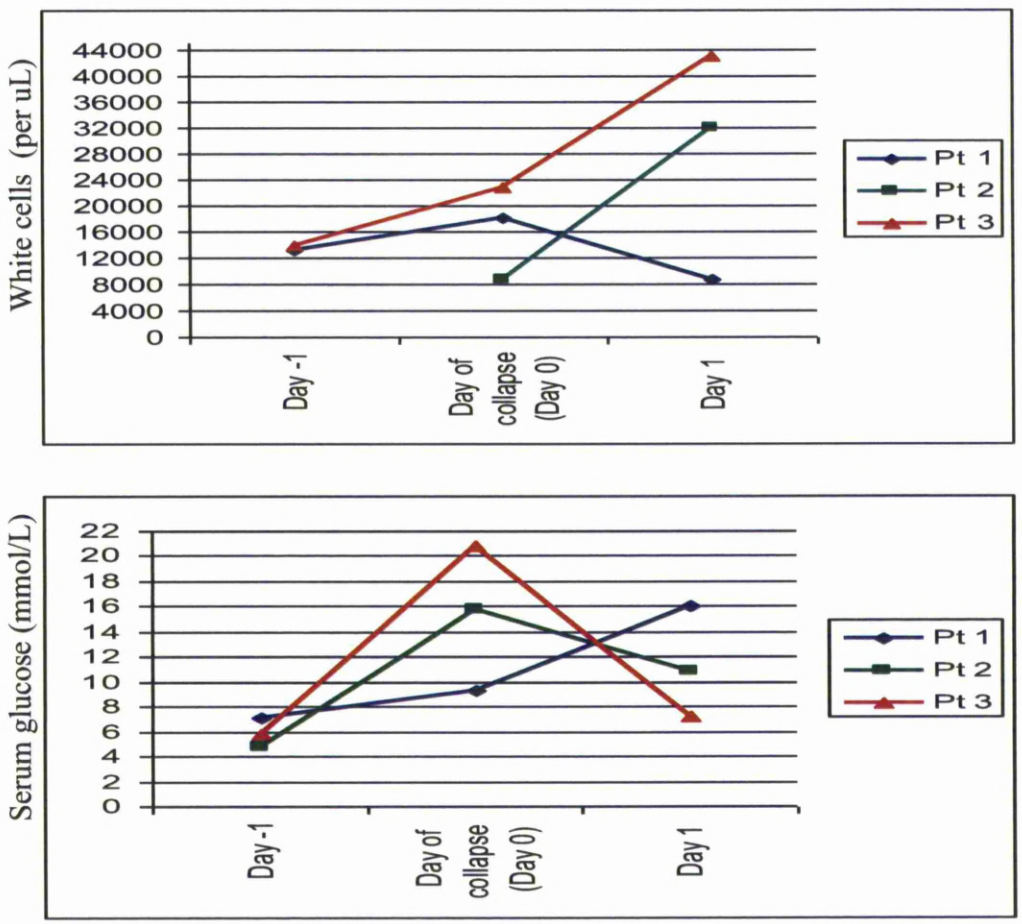


Table 5.1 Clinical features of 725 children with hand, foot, and mouth disease that were admitted between January 2000 and July 2003 according to the clinical severity

Severity	HFMD-CNS (with CSF pleocytosis)	HFMD-Non-CNS (no CSF pleocytosis)	p value*	Mild HFMD
Number of children	102	83	--	540
History				
Median age in months (range)	25 [5-120]	25 [5-104]	0.7582	29 [2-153]
Male, no (%)	73 [71.6%]	47 [56.6%]	0.0497	337 [62.4%]
Chinese	44 [43.1%]	22 [26.5%]	0.0282	209 [38.7%]
Iban	28 [27.5%]	46 [55.4%]	0.0002	188 [34.8%]
Malay/Melanau	30 [29.4%]	15 [18.1%]	0.1062	125 [23.1%]
Fever at home	100 [98%]	77 [92.8%]	0.1425	402 [74.4%]
Mean duration of fever at home (days)	2.6 [0-8]	2.0 [0-7]	0.0078	1.6 [0-7]
Mean total duration of fever (days)	4.5 [1-12]	2.9 [0-8]	<0.0001	1.8 [0-10]

Severity	HFMD-CNS (with CSF pleocytosis)	HFMD-Non-CNS (no CSF pleocytosis)	p value*	Mild HFMD
Total duration of fever ≥ 3 days	92 [90.2%]	48 [57.8%]	<0.0001	153 [28.3%]
Past history of HFMD	3 [2.9%]	3 [3.6%]	>0.9999	12 [2.2%]
Had history of contact with children with HFMD	34 [33.3%]	21 [25.3%]	0.3044	156 [28.9%]
Rash	92 [90.2%]	70 [84.3%]	0.3279	486 [90%]
Mouth ulcers	85 [83.3%]	76 [91.6%]	0.1502	491 [90.9%]
Coryza	33 [32.4%]	33 [39.8%]	0.3722	165 [30.6%]
Cough	35 [34.3%]	32 [38.6%]	0.6571	146 [27%]
Breathlessness	7 [6.9%]	1 [1.2%]	0.0759	2 [0.4%]
Cold peripheries / poor perfusion	9 [8.8%]	3 [3.6%]	0.2306	2 [0.4%]
Vomiting	49 [48%]	29 [34.9%]	0.0996	59 [10.9%]
Poor feeding	79 [77.5%]	70 [84.3%]	0.3217	303 [56.1%]
Diarrhea	7 [6.9%]	6 [7.2%]	>0.9999	17 [3.1%]
Constipation	8 [7.8%]	2 [2.4%]	0.1890	20 [3.7%]

Severity	HFMD-CNS (with CSF pleocytosis)	HFMD-Non-CNS (no CSF pleocytosis)	p value*	Mild HFMD
Reduced urine output	24 [23.5%]	20 [24.1%]	>0.9999	48 [8.9%]
Irritability	36 [35.3%]	29 [34.9%]	>0.9999	36 [6.7%]
Lethargy	65 [63.7%]	36 [43.4%]	0.0089	51 [9.4%]
Seizures	7 [6.9%]	8 [9.6%]	0.6755	0
Reduced limb movement	6 [5.9%]	1 [1.2%]	0.1318	0
Headache	14 [13.7%]	8 [9.6%]	0.5315	7 [1.3%]
Examination				
Toxic looking	69 (67.6)	53 (63.9)	0.6994	7 (1.3)
Dehydration	18 [17.6%]	10 [12%]	0.3944	22 [4.1%]
Mean peak body temperature (°C, range)	38.6 [36.8-40.9]	38.1 [36.8-40.5]	<0.0001	37.2 [36.4-39.8]
Mean peak body temperature ≥ 38.5°C	60 [58.8%]	32 [38.6%]	0.0094	20 [3.7%]

Severity	HFMD-CNS (with CSF pleocytosis)	HFMD-Non-CNS (no CSF pleocytosis)	p value*	Mild HFMD
Mean heart rate (per min, range)	132 [72-219]	124 [80-160]	0.0038	123 [80-238]
Mean heart rate >150/min	12 [11.8%]	1 [1.2%]	0.0069	6 [1.1%]
Rash	97 [95.1%]	78 [94%]	0.9915	516 [95.6%]
Mouth ulcers	90 [88.2%]	78 [94%]	0.2785	496 [91.9%]
Lethargy/drowsy	35	15	0.0210	1
Irritability	21 [20.6%]	13 [15.7%]	0.5025	0
Limb weakness	9 [8.8%]	0	0.0046	0
Neck stiffness	7 [6.9%]	1 [1.2%]	0.0759	0
History of or witnessed myoclonus	21 [20.6%]	9 [10.8%]	0.1121	4 [0.7%]
Cerebellar signs	2 [2%]	0	0.5026	0
Abnormal lung examination	7 [6.9%]	3 [3.6%]	0.5157	0
Abnormal cardiovascular examination	4 [3.9%]	2 [2.4%]	0.6925	9 [1.7%]

Severity	HFMD-CNS (with CSF pleocytosis)	HFMD-Non-CNS (no CSF pleocytosis)	p value*	Mild HFMD
Hepatomegaly	16 [15.7%]	10 [12%]	0.6195	34 [6.3%]
Splenomegaly	1 [1.8%]	0	>0.9999	4 [0.7%]
Vesicle present	49 [48%]	39 [47%]	0.9953	367 [68%]
Viral isolation				
EV71 isolated	52 [51%]	34 [41%]	0.2258	173 [32%]
CVA16 isolated	2 [2%]	9 [10.8%]	0.0102	74 [13.7%]
Both EV71 and CVA16 isolated	0	0		14 [2.6%]
Other virus isolated	8 [7.8%]	11 [13.3%]	0.3360	31 [5.7%]
Negative isolation	35 [34.3%]	24 [28.9%]	0.5320	161 [29.8%]
No virus isolation done	5 [4.9%]	5 [6%]	0.7551	87 [16.1%]
Laboratory results				

Severity	HFMD-CNS (with CSF pleocytosis)	HFMD-Non-CNS (no CSF pleocytosis)	p value*	Mild HFMD
Mean haemoglobin (g/dL, range)	11.5 [8.9-15.1]	11.3 [8.5-13.4]	0.4866	11.6 [7.3-17.3]
Mean white cell count (cells/μL, range)	13247 [5200-264000]	13300 [5700-283000]	0.9543	12010 [5100-31100]
Mean platelet count (platelelt/μL, range)	387537 [152000-994000]	353220 [180000-504000]	0.1473	356336 [125000-775000]
Mean serum sodium (mmol/L, range)	138 [130-146]	137 [131-145]	0.6392	138 [131-148]
Mean blood glucose (mmol/L, range)	5.9 [2.8-24.1]	5.4 [1.9-13.4]	0.1618	
Median CSF cell count (cells/μL, range)	41[0-1090]	2 [0-5]		

Severity	HFMD-CNS (with CSF pleocytosis)	HFMD-Non-CNS (no CSF pleocytosis)	p value*	Mild HFMD
CSF neutrophilia	17	0		
Median CSF protein concentration (g/dL, range)	0.28 [0.06-1.26]	0.18 [0.06-0.57]	<0.0001	
CSF protein concentration > 0.45	22	2		
Mean CSF: plasma glucose ratio	0.66 [0.2-1.1]	0.67 [0.14-1.1]	0.7374	

Note:

* Comparison between children with HFMD-CNS and those with HFMD-Non-CNS

HFMD: Hand, foot and mouth disease

CSF: Cerebrospinal fluid

HFMD-CNS: HFMD with central nervous system complication

HFMD-Non-CNS: Severe HFMD without central nervous system involvement

EV71: human enterovirus 71

CVA16: Coxsackie virus A16

Table 5.2 Risk factors that were significantly associated with cerebrospinal fluid pleoytosis in children with hand, foot and mouth in the first phase of the study (2000 to 2003).

Risk factors	p value	Odds ratio	95% CI
Total duration of fever ≥3 days	<0.0001	6.52	2.83 – 14.99
Peak temperature ≥38.5°C	0.0192	2.27	1.14 - 4.51
History of lethargy	0.001	3.18	1.60 - 6.35

Note: The Hosmer-Lemeshow statistics indicated a non-significance of lack of fit ($\chi^2 = 2.163$, p=0.904).

CSF: Cerebrospinal fluid

HFMD: Hand, foot, and mouth disease

Table 5.3 The number and type of the risk factors that were present in the children with hand, foot and mouth disease seen in the 2000/3 and 2006 outbreaks

Risk factors that were present	First phase of study (2000/3)			Second phase of study (2006)		
	HFMD-CNS (N=102)	HFMD-Non-CNS (N=83)	Mild HFMD (N=540)	HFMD-CNS (N=250)	HFMD-Non-CNS (N=186)	Mild HFMD (N=294)
Number (%) of patients with none of the 3 risk factor	**2 (2)**	**9 (11)**	**352 (65)**	**11 (4)**	**52 (28)**	**208 (71)**
Peak temperature ≥38.5°C only	1	8	7	29	32	34
History of lethargy only	5	15	27	16	11	17
Total duration of fever ≥3 only	9	15	122	32	35	30
Number (%) of patients with 1 risk factor	**15 (15)**	**38 (46)**	**156 (29)**	**77 (31)**	**78 (42)**	**81 (28)**
Peak temperature ≥38.5°C	2	3	1	11	7	2

plus history of lethargy						
Peak temperature ≥38.5°C	25	15	8	76	31	3
plus total duration of fever ≥3 days						
Total duration of fever ≥3 days	26	12	19	21	5	0
plus history of lethargy						
Number (%) of patients with 2 risk factors	**53 (52)**	**30 (36)**	**28 (5)**	**108 (43)**	**43 (23)**	**5 (2)**
Number (%) of patients with 3 risk factors (Peak temperature ≥38.5°C plus total duration of fever ≥3 days plus history of lethargy)	**32 (31)**	**6 (7)**	**4 (<1)**	**54 (22)**	**13 (7)**	**0**

Note: HFMD: Hand, foot, and mouth disease; HFMD-CNS: Hand, foot, and mouth disease with central nervous system complication; HFMD-Non-CNS: Severe HFMD without central nervous system involvement

Table 5.4 Clinical features of 730 children with hand, foot, and mouth disease that were admitted during the 2006 outbreak according to clinical severity

Severity	HFMD-CNS (with CSF pleocytosis)	HFMD-Non-CNS (no CSF pleocytosis)	p value*	Mild HFMD
Number of children	250	186		294
History				
Median age in months (months, range)	23.9 (0.3-126.0)	27.8 (2.2-134.2)	0.1456	31.2 (1.6-128.0)
Male, no (%)	152 (60.8)	97 (52.2)	0.0878	163 (54.9)
Chinese	97 (38.8)	52 (28.0)	0.0239	64 (21.5)
Iban	106 (42.4)	88 (47.3)	0.3559	166 (55.9)
Malay/Melanau	40 (16)	42 (22.6)	0.1063	63 (21.2)
Fever at home	247 (98.8)	178 (95.7)	0.0610	253 (85.2)
Mean duration of fever at home (days)	2 (0-6)	1.9 (0-7)	0.2095	1.3 (0-10)
Duration of fever at home $\geq$ 3 days	76 (30.4)	46 (24.7)	0.2316	29 (9.8)

Severity	HFMD-CNS (with CSF pleocytosis)	HFMD-Non-CNS (no CSF pleocytosis)	p value*	Mild HFMD
Mean total duration of fever (days)	3.6 (0.5-9.5)	2.7 (0-7.5)	<0.0001	1.5 (0-10)
Total duration of fever ≥ 3 days	183 (73.2)	84 (45.2)	<0.0001	33 (11.1)
Past history of HFMD	2 (0.8)	0	0.5096	4 (1.3)
Had history of contact with children with HFMD	103 (41.2)	50 (26.9)	0.0027	96 (32.3)
Rash	191 (76.4)	114 (61.3)	0.0010	213 (71.7)
Mouth ulcers	218 (87.2)	158 (84.9)	0.5926	272 (91.6)
Coryza	42 (16.8)	28 (15.1)	0.7194	41 (13.8)
Cough	48 (19.2)	37 (19.9)	0.9535	36 (12.1)
Breathlessness	5 (2.0)	3 (1.6)	>0.9999	1 (0.3)
Cold peripheries / poor perfusion	5 (2.0)	2 (1.1)	0.7039	2 (0.7)
Vomiting	62 (24.8)	35 (18.8)	0.1710	14 (4.7)
Poor feeding	203 (81.2)	154 (82.8)	0.7625	185 (62.3)

Severity	HFMD-CNS (with CSF pleocytosis)	HFMD-Non-CNS (no CSF pleocytosis)	p value*	Mild HFMD
Diarrhoea	11 (4.4)	10 (5.4)	0.8066	8 (2.7)
Constipation	3 (1.2)	0	0.2645	0
Reduced urine output	92 (36.8)	68 (36.6)	0.9610	72 (24.2)
Irritability	54 (21.6)	41 (22.0)	0.9948	15 (5.1)
Lethargy	90 (36.0)	36 (19.4)	0.0002	19 (6.4)
Seizures	13 (5.3)	9 (4.8)	0.9595	0
Reduced limb movement	1 (0.4)	0	>0.9999	0
Headache	17 (6.8)	8 (4.3)	0.3672	16 (5.4)
Examination				
Toxic looking	52 (20.8)	14 (7.5)	0.0002	1 (0.3)
Dehydration	85 (34.0)	50 (26.9)	0.1375	38 (12.8)
Mean peak body temperature (°C, range)	38.7 (36.8-40.3)	38.3 (36.8-40.4)	<0.0001	37.6 (36.4-39.9)
Mean peak body temperature ≥38.5°C	170 (68)	83 (44.6)	<0.0001	42 (14.1)

Severity	HFMD-CNS (with CSF pleocytosis)	HFMD-Non-CNS (no CSF pleocytosis)	p value*	Mild HFMD
Mean heart rate (bears per min, range)	145 (86-204)	141 (82-209)	0.0607	130 (80-199)
Mean heart rate >150/min	105 (42)	69 (37.1)	0.3497	49 (16.5)
Rash	222 (88.8)	147 (79.0)	0.0077	250 (84.2)
Mouth ulcers	235 (94.0)	181 (97.3)	0.1605	287 (96.6)
Lethargy	74 (29.6)	31 (16.7)	0.0026	5 (1.7)
Irritability	37 (14.8)	34 (18.3)	0.3997	6 (2.0)
Limb weakness	2 (0.8)	0	0.5096	0
Neck stiffness	17 (6.8)	4 (2.2)	0.0438	0
History of or witnessed myoclonus	176 (70.4)	91 (48.9)	<0.0001	31 (10.4)
Abnormal lung findings	19	6	0.0828	1
Abnormal cardiovascular findings	1 (0.4)	1 (0.5)	>0.9999	0

Severity	HFMD-CNS (with CSF pleocytosis)	HFMD-Non-CNS (no CSF pleocytosis)	p value*	Mild HFMD
Hepatomegaly	20 (8)	7 (3.8)	0.1064	4 (1.3)
Splenomegaly	3 (1.3)	1 (0.5)	0.6394	0
Vesicle present	41 (16.4)	22 (11.8)	0.2281	61 (20.5)
Viral isolation				
EV71 isolated	53	46		58
CVA16 isolated	0	0		0
Other EV isolated	12	19		13
Negative isolation	123	114		148
Pan-enterovirus PCR				
positive	163 (65.2)	133 (71.5)		188
negative	57 (22.8)	18 (9.7)		35
EV71 specific PCR				
positive	96 (38.4)	60 (32.3)		83
negative	63 (25.2)	88		87

Severity	HFMD-CNS (with CSF pleocytosis)	HFMD-Non-CNS (no CSF pleocytosis)	p value*	Mild HFMD
Number of EV71 positive children	114	73		104
Number of EV71 negative children	109	109		144

Note:

* Comparison between children with HFMD-CNS and those with HFMD-Non-CNS

HFMD: Hand, foot, and mouth disease

CSF: Cerebrospinal fluid

HFMD-CNS: HFMD with central nervous system complication

HFMD-Non-CNS: Severe HFMD without central nervous system involvement

EV71: human enterovirus 71

CVA16: Coxsackie virus A16

Table 5.5 The diagnostic value of the risk factors in predicting CSF pleocytosis

Risk factors that were present	Presented within the first 2 days of febrile illness				Presented on the 3rd or later day of febrile illness			
	Sensitivity	Specificity	PPV	NPV	Sensitivity	Specificity	PPV	NPV
Both peak temperature ≥38.5°C and history of lethargy	28% (48/174) [23-33%]	89% (125/140) [86-92%]	76% (48/63) [71-81%]	50% (125/251) [44-56%]	22% (17/76) [15-29%]	89% (41/46) [83-95%]	77% (17/22) [70-84%]	41% (41/100) [32-50%]
Peak temperature ≥38.5°C &/or history of lethargy	86% (150/174) [82-90%]	43% (60/140) [38-48%]	65% (150/230) [60-70%]	71% (60/84) [66-76%]	75% (57/76) [67-83%]	59% (27/46) [50-68%]	75% (57/76) [67-83%]	59% (27/46) [50-68%]

Note:

CSF: Cerebrospinal fluid

CNS: Central nervous system

Sensitivity = The proportion of children with CSF pleocytosis that are correctly identified by the presence of the risk factors

Specificity = The proportion of children without CSF pleocytosis that are correctly identified by the absence of the risk factors

Positive predictive value (PPV) = The proportion of individuals with the risk factors that have CSF pleocytosis

Negative predictive value (NPV) = The proportion of individuals without the risk factors that do not have CSF pleocytosis

Table 5.6 The clinical details and risk factors for neurological involvement of the nine fatal case children with hand, foot and mouth disease seen in the study.

Patient	Year of the outbreak	Age (months)	Day of illness at presentation	Risk factors that were present at presentation	Disease severity	EV71 detected?	IVIG treatment	Note
1	2000	11	Day 3	fever ≥3D, history of lethargy, temperature ≥38.5°C	HFMD-CNS	Yes	No	a.
2	2003	34	Day 5	fever ≥3D, history of lethargy	HFMD-CNS	Yes	No	b.
3	2003	32	Day 3	fever ≥3D, history of lethargy	HFMD-CNS	No	No	a.
4	2006	9	Day 1	history of lethargy, temperature ≥38.5°C	HFMD-CNS	Yes	Yes	c.
5	2006	8	Day 3	fever ≥3D, history of lethargy, temperature ≥38.5°C	HFMD-CNS	Yes	No	a.
6	2006	14	Day 3	fever ≥3D, history of lethargy, temperature ≥38.5°C	HFMD-CNS	Yes	No	a.
7	2006	34	Day 4	fever ≥3D, history of lethargy,	HFMD-CNS	Yes	No	a.

				temperature ≥38.5°C				
8	2006	25	Day 4	fever ≥3D, history of lethargy, temperature ≥38.5°C	HFMD-CNS	No	No	a.
9	2006	47	Day 4	fever ≥3D, history of lethargy	HFMD-CNS	No	No	a.

Note:

a. Presented in the moribund state with fulminant cardiorespiratory failure and pulmonary oedema. The patient died within 24 hours of the hospitalization. The risk factors were present for ≥48 hours before hospital admission.

b. Developed acute cardiorespiratory collapse and died 12 hours after hospitalization. Had peak temperature ≥38.5°C in the hospital. The patient was lethargic for ≥48 hours before hospital admission.

c. Deteriorated progressively because of cardiorespiratory failure despite intensive care support. Died on day 4 of the hospitalization. The patient was lethargic for 24 hours before hospital admission

HFMD-CNS: Hand, foot and mouth disease with central nervous system complication

IVIG: Intravenous immunoglobulin

Chapter 6: Neurological disease in children infected with coxsackie virus A16 in Sarawak: cause or coincidence?

Abstract

Coxsackievirus (CV) A16 infection commonly causes a mild self-limiting illness, and neurological complications are rarely reported. Recent reports of neurological disease and deaths associated with CVA16 infection have raised concerns about the possible emergence of a more virulent strain of CVA16. Data collected through a prospective study of hand, foot, and mouth disease (HFMD) conducted between 2000 and 2003 were examined to investigate for possible severe CVA16 infection in Sarawak, Malaysia, and review whether any CVA16 infection was likely to be co-incidental or causal. The clinical features and disease severity of the CVA16-positive children were compared to the human enterovirus (EV) 71-positive children. CVA16-positive children with neurological disease were identified, and the results of laboratory investigation for the aetiology of the children's neurological disease were reviewed. CVA16-positive children were more likely to have

mild HFMD than HFMD with central nervous system complications when compared to EV71-positive children. There were two CVA16-positive children who had encephalitis in the study. While Japanese encephalitis, confirmed by the intrathecal Japanese encephalitis virus-specific IgM production, was determined to be the cause of encephalitis in the first child, the co-isolation of coxsackie virus B1 and an adenovirus from the second child has raised doubts about CVA16 being the aetiology of the second child's encephalitis.

6.1 Introduction

Hand, foot, and mouth disease (HFMD) is a common childhood illness characterised by a short febrile illness and the development of oral ulcers and typical skin rashes over the palms and soles of the affected children. The disease is most commonly caused by human enterovirus (EV) species A, particularly coxsackie virus (CV) A16 and EV71 (Pallansch et al. 2001). While CVA16 and EV71 are genetically very closely related and often co-circulate during outbreaks of HFMD and result in clinically indistinguishable skin lesions, the outcome and disease severity associated with the two viruses may be remarkably different. In most instances, CVA16 infection causes a mild self-limiting illness, and neurological complications are rarely

reported. EV71 infection, however, has been associated with severe neurological complications and fulminant cardiorespiratory failure (Huang et al. 1999). There have been two case reports of fatal CVA16 infections involving young infants in the literature between 1960 and 1997 (Wright et al. 1963) (Goldberg et al. 1963). Further cases of severe CVA16 infection associated with neurological complication and fatal myocarditis in older children during an outbreak of HFMD, as well as fatal pneumonitis in an adult have been reported more recently (Chang et al. 1999) (Wang et al. 2004) (Legay et al. 2007). This has raised some concerns about the possible emergence of a new, more virulent strain of CVA16 (Legay et al. 2007). However, in many parts of Asia where these outbreaks have occurred, other neurotrophic viruses, such as JEV co-circulate (Solomon 2004). In addition an adenovirus has been associated with unexpected neurological disease during a large HFMD outbreak in Sarawak in 1997 (Cardosa et al. 1999). In this chapter data collected through a prospective clinical study of HFMD conducted between 2000 and 2003, a period which included 2 large epidemics of HFMD caused by EV71 and CVA16, were examined for possible severe CVA16 infection in Sarawak, Malaysia, and review whether any CVA16 infection was likely to be co-incidental or causal.

6.2 Materials and methods

6.2.1 Setting and study period

A prospective clinical study of HFMD was conducted between 2000 and 2003 at Sibu Hospital (Sarawak, Malaysia). The study was approved by the Director of Health for Sarawak and the Ethics Committee of the Liverpool School of Tropical Medicine (UK). Informed consent was obtained from each child's accompanying parent or guardian.

6.2.2 Case definitions and clinical methods

The case definitions and clinical methods of the study have been described in earlier chapters. Briefly clinical data obtained through history taking and clinical examination were recorded on standardised forms. Swabs were taken from the throat and rectum of every patient, as well as skin vesicles and oral ulcers (if present). The clinical samples were stored immediately in a -70°C freezer until further testing. Blood was taken for flavivirus serology, and in patients with suspected CNS involvement for full blood count, urea, electrolytes, and glucose. Cerebrospinal fluid (CSF) was examined for cell count and differential, protein, glucose, Gram stain, bacterial culture. If there was a strong clinical suspicion of viral CNS infection, but the initial CSF

examination was acellular, a second lumbar puncture was performed. Lumbar punctures were delayed in those with unstable vital signs. Patients were examined daily or more frequently as indicated, by a member of the study team.

6.2.3 Virological methods

Virus isolation was attempted on all swab specimens and CSF specimens and any available serum samples through the inoculation of human rhabdomyosarcoma (RD) and human embryonic kidney cells (293). Isolated enteroviruses were typed by nucleotide sequencing of VP1 and VP4 genes as described in earlier chapters. Adenoviruses were isolated in 293 cells.

6.2.4 Serological diagnosis of flaviviruses

All children with suspected CNS infection were routinely investigated for Japanese encephalitis (JE) in the hospital (Ooi et al. 2008; Wong et al. 2008). JEV shares cross reactive epitopes with dengue virus (DENV). Both CSF and serum samples were tested by an IgM-capture ELISA that distinguishes between responses to these 2 viruses (Cardosa et al. 2002). Paired serum samples (obtained at hospital admission and at day 7, at discharge from the hospital, or after death) and either one CSF specimen or paired CSF

specimens were tested for IgM against DENV and JEV in parallel. As JEV-specific IgM may be undetectable in the CSF at the early stage of JE, a second CSF examination is routinely performed in the hospital to provide laboratory confirmation of JEV by demonstrating JEV-specific IgM seroconversion in the CSF (WHO 2006). A sample was considered to be IgM positive for JEV if the optical density against JEV was higher than that against DENV in the same test run (Cardosa et al. 2002; Ooi et al. 2008).

6.2.5 Statistical analysis

Normally distributed data were compared using Student's t test; data that were not normally distributed were compared by the Mann-Whitney U test (Statview 4.02; Abacus Concepts). Differences between proportions were tested using the Chi-square test with Yates's correction or Fisher's exact test as appropriate (Epi Info, version 6; Centers for Disease Control and Prevention). A p value <0.05 was considered statistically significant.

6.3 Results

A total of 725 patients were recruited into the study. Five hundred and forty (74%) patients had mild HFMD, 83 (11%) had severe HFMD without CNS

involvement and 102 (14%) patients had HFMD with CNS complication. EV71 and CVA16 were the two most frequently isolated enteroviruses during the study. The detailed results of epidemiological, diagnostic virology and molecular epidemiology of EV71 isolated in the study have been reported in Chapter 4.

Of 628 (87%) of the 725 patients tested in the study, 259 (41%) patients had EV71 isolated (14 of these patients also had other non-CVA16 viruses isolated), 85 (14%) had CVA16 isolated (4 of whom also had another non-EV71 virus isolated), 14 (2%) had both EV71 and CVA16 co-isolated and a further 50 (8%) had other viruses isolated. The remaining 220 (35%) patients had no virus isolated. For the purpose of the analysis, children with CVA16 isolation were compared to those with EV71 isolation. The 14 children that had co-isolation of EV71 and CVA16 (all of whom had mild HFMD) were excluded in the analysis. Compared to EV71-positive children, CVA16-positive children were more likely to have mild HFMD. They were less likely to have a total duration of fever ≥3 days, history or examination findings lethargy and myoclonus, and hepatomegaly (Table 6.1). There was also a trend to suggest that recurrent vomiting was less frequently seen in children with CVA16. Interestingly, two (2%) of the 85 children with CVA16-positive HFMD developed neurological disease. The detailed

clinical presentation and investigation results of the 2 children with CVA16-positive HFMD complicated by neurological disease are presented here.

Case 1

A previously healthy 18-month old boy was admitted into hospital in September 2000 with a 5-day history of fever, painful mouth ulcers and skin rash. He fed poorly and became increasingly irritable and lethargic during the illness. On the day of admission, he developed a generalised seizure. There was no history of limb weakness, vomiting, diarrhoea or respiratory symptoms. His parents reported that his elder brother had just recovered from a febrile illness associated with similar skin lesions a few days before he was admitted. On examination at hospital admission, he looked ill, drowsy and was febrile (39°C). There were multiple papulovesicular skin lesions over the palms, soles, buttocks and knees, and multiple oral ulcers. Apart from the altered state of consciousness there was no sign of meningism or focal neurological deficit. The rest of the systemic examination, including heart, lung and abdomen, was unremarkable. His first CSF examination on admission showed an elevated cell count of 10 lymphocytes/μL, a protein level of 0.1 g/dL and a CSF-to-plasma glucose ratio of 0.57. A second CSF examination performed 4 days after the first showed that the CSF cell counts raised further to 39 cells/μL (90% were lymphocytes); an elevated protein

level of 0.69 g/dL. Bacterial cultures for blood and CSF were sterile. CVA16 was isolated from a vesicle in the RD cell culture. JEV-specific IgM was absent in the first CSF specimen but the JEV-specific IgM seroconversion was demonstrated in the second CSF specimen taken 4 days after the first (Figure 6.1). This child was thus considered infected with both CVA16, and JEV.

Case 2

A previously healthy 14-month old boy presented to the hospital in October 2000 with a 2-day history of fever, painful mouth ulcers and skin rash. He fed poorly, became less active and cried inconsolably. There was no history of seizures, limb weakness, vomiting, diarrhoea, respiratory symptoms or contact with cases of HFMD. On examination at hospital admission, he appeared ill and lethargic, and was febrile (38°C). There were multiple papulovesicular skin lesions over the palms and soles, as well as multiple oral ulcers. There was no sign of meningism or focal neurological deficit apart from increased irritability. The other systemic examinations, including heart, lung and abdomen, were unremarkable. His CSF examination on admission showed a marginally elevated cell count of 6 cells/μL (all were lymphocytes); a protein level of 0.22g/dL and a CSF glucose level of 3.1. CVA16 was isolated from a vesicle and rectum in the RD cell cultures while

coxsackie virus B1 and an adenovirus was cultured from the throat in the RD and 293 cell cultures, respectively. JEV-specific IgM was negative in both serum and CSF samples. These results confirmed that this child was infected with CVA16, coxsackie virus B1 and an adenovirus.

6.4 Discussion

The study confirms that CVA16-associated HFMD is a brief uncomplicated virus infection. Although there were two children with CVA16-associated HFMD who developed encephalitis in the study, the concurrent detection of other neurotrophic viruses (JEV in the first, coxsackie virus B1 and an adenovirus in the second case) has raised doubts if CVA16, which is not normally known to cause neurological disease, was the plausible cause of neurological disease in the two children.

In Case 1 the intrathecal production of JEV-specific IgM, demonstrated by JEV-specific IgM seroconversion in the paired CSF samples, provided strong evidence to support JEV infection as the underlying cause of the 18-month-old boy's acute neurological symptoms. Fatal JEV infection during an epidemic of EV71-associated HFMD has been reported previously. Shieh and co-workers documented a case of fatal JEV infection in a child presented

with HFMD and fulminant neurogenic pulmonary oedema during the largest ever EV71-associated HFMD epidemic in Taiwan in 1998 (Shieh et al. 2001). JEV, the causative agent of JE, is the principal cause of virus encephalitis in children in Asia, including in Sarawak, Malaysia (Ooi et al. 2008; Wong et al. 2008). The neurotrophic flavivirus is transmitted naturally among birds, pigs and other vertebrae hosts by *Culex spp* mosquitoes that breed in rice fields and stagnant water. In Sarawak the flavivirus encephalitis occurs sporadically throughout the year with a seasonal peak during the rainy season that lasts between the months of September and December, which coincide with the seasonal increase in *Culex tritaeniorynchus* population (Wong et al. 2008). The first case is a good example to show that during a virus epidemic a patient may be simultaneously infected by the outbreak virus and the endemically circulating virus. Therefore it is important to always consider endemic encephalitis viruses as possible causes of CNS infection in the diagnostic work-up of patients with acute neurological syndrome even during an epidemic of an unrelated virus. The 18-month-old boy could have been misdiagnosed as having an unusually severe CVA16-associated HFMD had I failed to consider JEV, the most important endemic neurotrophic virus in Sarawak, as a cause of the encephalitis.

Apart from CVA16, coxsackie virus B1 and an adenovirus were also isolated from the 14-month-old boy described in Case 2. The serotyping of coxsackie virus B1 isolated in Case 2 was performed by nucleotide sequencing of VP4 gene. Coxsackie virus B1 is a member of enterovirus species B. A recent study compared the VP4 with VP1 gene regions for its usefulness in typing human enterovirus species B (Perera et al. 2010). The authors showed that a VP4-based approach often did not provide the same serotype identification as that obtained through VP1-based nucleotide sequencing, although the species identity was correct. Therefore it is possible that the coxsackie virus identified as B1 during the study, using VP4 nucleotide sequencing, might actually be a different species B enterovirus. Although the three viruses were isolated from the throat, which may not necessarily represent acute infection but only related to asymptomatic carriage state or continuing shedding from a recent virus infection, the pathogenic role of these viruses isolated in the second case could not be discounted. The isolation finding is particularly relevant because both enterovirus species B and adenovirus are well-recognised possible CNS pathogens while neurological CVA16 infection is rarely reported. Pre-existing immunodeficiency may predispose a susceptible individual to multiple concurrent virus infection, which in turn lead to unusually severe infection caused by viruses that normally only cause mild illness. However this is less likely in this case because the child's subsequent

follow-up examination showed that he recovered fully from the neurological infection and has since remained healthy. Alternatively transient immunosuppression due to multiple concurrent virus infections may have predisposed the child to neurological CVA16 infection.

Cases of serious and fatal CVA16 infection have been published by several authors previously; though they were varied in the diagnostic rigour in investigating for co-infecting viruses. The first two reports of fatal CVA16 infection in the literature were published in 1963, and involved two infants who died of encephalitis and myocarditis, respectively (Goldberg et al. 1963; Wright et al. 1963). CVA16 was isolated from the intestinal content of both fatal cases, and also from the blood and heart tissues of the first infant. More recently CVA16 has been implicated as the pathogen responsible for a fatal case of HFMD complicated by CNS (i.e. seizures and CSF pleocytosis) and cardiac involvement, and for a further 4 cases of HFMD complicated by aseptic meningitis (Chang et al. 1999), during the same HFMD outbreak in 1998 where Shieh and co-workers showed JEV infection was the cause of death in a child presented with HFMD and fulminant neurogenic pulmonary oedema (Shieh et al. 2001). It was not clear, however, from the two reports that JEV infection has been considered in the investigation of the patients. Legay and co-workers reported a fatal case of CVA16 pneumonitis in a 76-

year-old man who had history of contact with HFMD. CVA16, isolated from bronchoalveolar lavage, was determined to be the cause of death following extensive investigation for other common respiratory pathogens (Legay et al. 2007). Apart from the advanced age, an important risk factor for mortality in community-acquired pneumonia, it is not clear from the report if there were underlying co-morbid conditions that may have contributed to the fatal outcome of this normally benign virus infection (Janssens 2005; Chong et al. 2008).

Conclusions

In conclusion, CVA16-associated HFMD is a brief uncomplicated virus infection and rarely associated with neurological disease. During a virus epidemic concurrent infection caused by the outbreak virus and other endemically circulating virus can occur in an individual. It is particularly important for clinicians to consider common and endemically circulating encephalitis viruses being the possible aetiological agents before ascribing the neurological disease observed in children with CVA16-associated HFMD to CVA16 infection.

Table 6.1 Comparisions between children with coxsackie virus A16-positive hand, foot and mouth and those with enterovirus 71-positive hand, foot and mouth disease that were admitted between 2000 and 2003

Clinical features	CVA16-positive children	EV71-positive children	p value
Number of patients	85	259	
History			
Median age in months (range)	25 (8-100)	28 (5-112)	0.2582
Male, no (%)	55 (64.7)	166 (64.1)	>0.9999
Fever at home	64 [75.3]	210 [81.1]	0.3199
Median total duration of fever (days)	2 [0-4.5]	2 [0-12]	0.0044
Total duration of fever ≥3 days	19 [22.4]	112 [43.2]	0.0009

Past history of HFMD	3 [3.5]	5 [1.9]	0.4137
Had history of contact with children with HFMD	25 [29.4]	92 [35.5]	0.3683
Rash	80 [94.1]	249 [96.1]	0.6271
Mouth ulcers	83 [97.6]	239 [92.3]	0.1218
Coryza	24 [28.2]	86 [33.2]	0.4725
Cough	20 [23.5]	77 [29.7]	0.3353
Breathlessness	2 [2.4]	6 [2.3]	>0.9999
Cold peripheries / poor perfusion	0	5 [1.9]	0.3386
Vomiting	9 [10.6]	50 [19.3]	0.0922
Poor feeding	56 [65.9]	177 [68.3]	0.7743
Reduced urine output	6 [7.1]	33 [12.7]	0.2162
Irritability	14 [16.5]	47 [18.1]	0.8513

Lethargy	12 [14.1]	66 [25.5]	0.0432
Seizures	0	4 [1.5]	0.5757
Reduced limb movement	1 [1.2]	1 [0.4]	0.4337
Headache	2 [2.4]	9 [3.5]	>0.9999
Examination			
Dehydration	3 [3.5]	22 [8.5]	0.1526
Mean peak temperature (°C, range)	37.2 [36.6-39]	37.6 [36.4-40.9]	<0.0001
Mean Peak temperature ≥38.5°C	5 [5.9]	45 [17.4]	0.0151
Mean heart rate (beats per minute, range)	124 [90-142]	125 [72-238]	0.8339
Mean heart rate >150 beats per minute	0	9 [3.5]	0.1195
Rash	85 [100]	256 [98.8]	>0.9999
Mouth ulcer	81 [95.3]	245 [94.6]	>0.9999

Lethargy/drowsy	1 [1.2]	25 [9.7]	0.0080
Irritability	4 [4.7]	18 [7]	0.6123
Limb weakness	0	2 [0.8]	>0.9999
Neck stiffness	1 [1.2]	3 [1.2]	>0.9999
History or observed myoclonus	0	21 [8.1]	0.0031
Cerebellar signs including nystagmus	0	2	>0.9999
Abnormal lung examination	2 [2.4]	4 [1.5]	0.6395
Abnormal cardiovascular examination	2 [2.4]	5 [1.9]	0.6840
Hepatomegaly	2 [2.4]	28 [10.8]	0.0142
Vesicle present	60 [70.6]	180 [69.5]	0.8125
Disease severity			
Mild HFMD	74 [87.1]	173 [66.8]	0.0005

Severe HFMD without CNS involvement	9 [10.6]	34 [13.1]	0.6707
HFMD with CNS complication	2 [2.4]	52 [20.1]	0.0002

Note:

HFMD: Hand, foot, and mouth disease

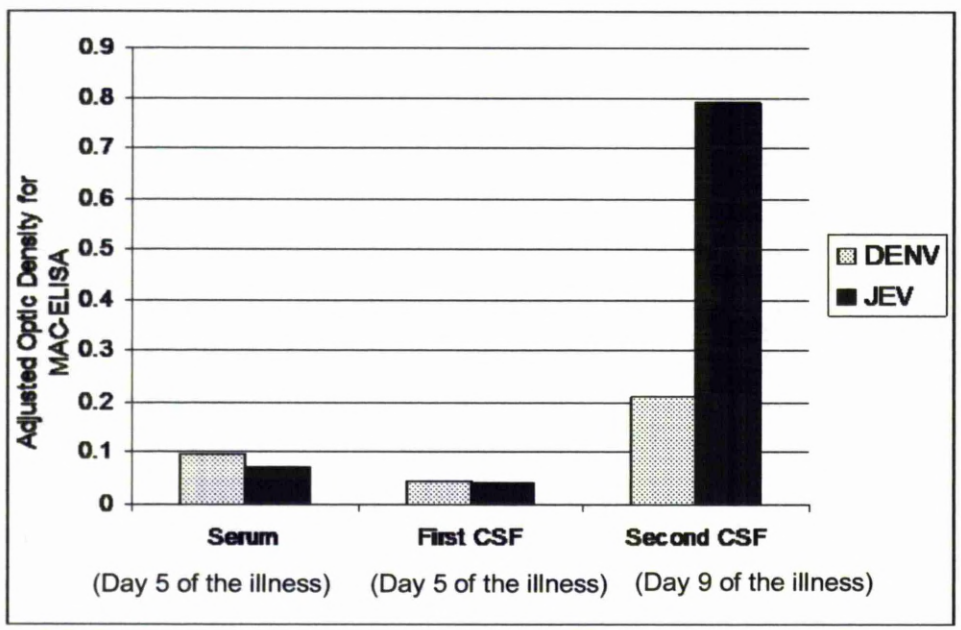
CVA16: Coxsackie virus A16

EV71: human enterovirus 71

CNS: Central nervous system

Figure 6.1 Serological diagnosis of Japanese encephalitis in a child with CVA16-associated hand, foot and mouth disease

The bar chart shows the adjusted optic density (adj. OD) values against Japanese encephalitis virus (JEV) and dengue virus (DENV) of serum and cerebrospinal fluid samples of an 18-month old boy (Case 1) that were tested on the same IgM-capture ELISA run. The cutoff adj. OD values for JEV and DENV were 0.210 and 0.395, respectively. Coxsackie virus A16 was isolated from a vesicle in the human rhabdomyosarcoma cell culture.



Chapter 7: Final discussion and overall conclusion

Human enterovirus 71 continues to cause large cyclical epidemics of hand, foot and mouth disease over the last 14 years, and is now considered the most important non-polio enterovirus in children in Asia. Of note the virus often sweeps across the affected countries very rapidly causing an alarming number of deaths or patients with neurological sequelae, not to mention the marked socioeconomic disruption. The neurotrophic virus, which first emerged in Sarawak, Malaysia in 1997 with new clinical manifestations of hand, foot and mouth disease complicated by brainstem encephalitis and potentially fatal cardiorespiratory failure, has become a major public health problem in Asia, and had posed many clinical and public health challenges to clinicians, public health doctors and virologists in the region.

7.1 The usefulness of different clinical samples in laboratory diagnosis of enterovirus 71

A wide range of samples may be used to detect EV71 infection, depending on the disease manifestations; these include throat and rectal swabs, serum,

urine, and when taken, cerebrospinal fluid (CSF), as well as fluid from vesicles and swabs from ulcers, if they are present. The results in Chapter 2 have shown that the sensitivity, specificity and usefulness vary according to the sample. In particular virus detection in sterile sites such as vesicular fluid, CSF, serum, urine, serum, or autopsy material more reliably indicates a causative organism, than detection from non-sterile sites such as throat or rectum, which may indicate coincidental carriage. However many of the sterile sites only occasionally yield virus. For example virus was isolated from less than 3% of the CSF of patients with neurological disease, and the low yield from CSF in this work is consistent with other published reports (Kennett et al. 1975; Chumakov et al. 1979; Ishimaru et al. 1980; Nagy et al. 1982; Gilbert et al. 1988; Chang et al. 1999; Ooi et al. 2007; Ooi et al. 2009), because, as for poliomyelitis, the viral load in the CSF is very low (Fujimoto et al. 2008). The yield for serum is similarly low. Vesicular fluid, when present, is more useful, though care is needed to collect it (Figure 7.1). Although throat and rectal swabs are more likely to have an enterovirus detected, the virus isolated from the non-sterile sites was not always the same enterovirus as that isolated from a sterile site: using the isolate from vesicle swabs as a reference, 10% of positive throat swabs gave a different isolate, and for rectal swabs the figure was 20%. Presumably the isolate from

the non-sterile site represented coincidental carriage, where-as that from the vesicles was actually pathogenic.

Prolonged viral shedding from the gastrointestinal tract (throat, rectum or stool) may occur after complete resolution of EV71 infection, as it does for other enteroviruses; a study in Taiwan showed that EV71 may be detected in the throat up to 2 weeks after recovery from HFMD or herpangina; in the stool it can be detected up to 11 weeks (Chung et al. 2001; Han et al. 2010). Given that there are so many potential samples which could be positive, during an outbreak, laboratories can soon become overwhelmed. The results Chapter 2 have shown that the most efficient approach was to examine throat swabs for all patients, plus swabs from at least two vesicles, if they were present, or from the rectum for patients with no vesicles.

7.2 Rapid diagnostic assay for direct detection of enterovirus 71 from primary clinical specimens

The gold standard for diagnosis of enterovirus infection is virus isolation, but PCR has increasingly become diagnostic method of choice in routine viral diagnostics. The advantage of PCR is that it provides quicker results and less labour intensive than virus culture. The evaluation of the application of

an EV71 type-specific RT-PCR (MAS01S/MAS02A) as a rapid diagnostic assay for direct detection of EV71 from primary clinical specimens was shown in Chapter 3. The EV71 type-specific RT-PCR was originally developed for rapid molecular typing of EV71 clinical isolates. The evaluation indicated that the EV71 type-specific RT-PCR can be extended to be used as a rapid diagnostic assay for direct detection of EV71 from primary clinical specimens. As a single step PCR assay with no additional step of amplicon sequencing, the EV71 type-specific RT-PCR may be a useful alternative to clinical laboratories that have limited access to the conventional virus culture and neutralization test method, and nucleotide sequencing facilities. This can be especially useful in providing rapid diagnostics given the explosive nature of some EV71 outbreaks, and the need for urgent public health interventions. The disadvantage is that such an approach will only detect the virus looked for, and thus will miss the chance to identify other unexpected causes (Perera et al. 2004; Chen et al. 2006; Tan et al. 2008; Xiao et al. 2009). Other diagnostic platforms have been explored for the need for rapid diagnosis of EV71 in recent years. DNA microarray is a powerful, though expensive, new tool designed to detect multiple pathogen targets by hybridization of pathogen-specific probes. Two groups have recently reported such an approach to distinguish EV71 and CVA16 infection in primary clinical specimens (Shih et al. 2003; Chen et al. 2006).

7.3 Clinical predictors for neurological disease

While there are advances in the rapid laboratory detection method for EV71 as alternative to the laborious time-consuming tissue culture technique there is still lack of a point-of-care diagnostic kit for EV71 to help clinicians identify children with EV71 infection who may at risk of neurological disease and death. Hence clinicians still need to rely on good clinical history and examination skill to identify children at risk of neurological disease and death. In Chapter 5 nearly 1500 children recruited through three EV71 outbreaks over 7 years were studied. Peak temperature of 38·5°C or more, duration of fever for 3 or more days, and a history of lethargy were found to be useful clinical predictors for neurological involvement. The presence of at least two of these factors was strongly associated with the subsequent development of neurological disease. The results of Chapter 5 confirmed the findings from early retrospective studies. However other findings from earlier studies, such as an association between the absence of mouth ulcers and development of complicated or fatal disease were not confirmed (Chong et al. 2003). Hyperglycaemia and leucocytosis were also associated fatal EV71 disease in a retrospective evaluation (Chang et al. 1999), and although these findings were confirmed in the prospective study, they were late

changes occurring at approximately the same time as the fulminant disease as have been shown in Chapter 5. Therefore they are not helpful clinically in identifying children at high risk of complications and death.

Not all the children with CNS involvement in EV71 infection will progress to cardiorespiratory collapse. A small study involving 46 patients showed that children developed abnormal heart rate variability, an index of autonomic nervous system disease, about 7 hours before the clinical onset of cardiorespiratory instability (Lin et al. 2006). The authors proposed that screening children with CNS involvement for abnormalities in heart rate variability may provide early warning of impending cardiorespiratory failure and allow timely institution of appropriate interventions. Cardiac troponin I is a cardiac-specific biomarker for myocardial injury, used for early diagnosis of acute coronary syndrome in adults. Elevated cardiac troponin I has been observed in children with EV71 infection, brainstem encephalitis cardiac dysfunction and pulmonary oedema (Huang et al. 2003); in some cases it was elevated prior to the development of cardiopulmonary failure, suggesting that serial measurement of cardiac troponin may be helpful in identifying children at risk of left ventricular failure. However neither measurement of heart rate variability nor troponin I level evaluations have become routine clinical practice in the management of EV71, probably

because the former requires relatively sophisticated equipment which is not widely available, and the latter, though more widely available, is relatively expensive in developing countries. Even in wealthier Asian countries such as Taiwan these measures have not entered routine clinical practice. Overall simple clinical parameters such as length of illness, height of fever, and lethargy are probably more useful indicators of potentially severe disease.

7.4 Clinical feature of EV71 infection

Similar to many enteroviruses most of the infection caused by EV71 are either mild or asymptomatic. More than 20% of adult contacts during one Taiwanese outbreak had symptoms of an upper respiratory tract infection, whilst asymptomatic infection occurred in more than 50% (Chang et al. 2004). EV71 and CVA16 are the most important causative agents for HFMD. While EV71 has caused massive outbreaks with alarming number of deaths in Asia, CVA16 has rarely been associated with neurological disease and deaths. Although both viruses cause indistinguishable skin lesion there may be other clues that may suggest a child's HFMD is due to EV71 rather than CVA16. In Chapter 6, children with EV71 were shown to be more likely to have a fever for longer than 3 days, a peak temperature greater than 38·5oC, lethargy and myoclonus. The study confirmed the findings observed

in a retrospective study by Chang et al. In Chapter 6, two cases of HFMD caused by CA16 who also had neurological disease were investigated in detail. The investigation showed that there were other plausible explanations for the neurological disease in these two children, underscoring the importance of thorough diagnostic investigation for common aetiological agents before attributing severe disease manifestations to CVA16 infection.

Although brainstem encephalitis severity grading and clinical staging has been proposed shortly after the 1998 Taiwan epidemic, the grading and staging systems have not come into widespread usage in clinical practice during the ongoing Asian EV71 epidemics, possibly because they are not always easy to remember, and imply sequential progression through the various disease grades or stages, which does not always occur. A WHO Informal Consultation on Hand Foot and Mouth disease which met in Kuala Lumpur in March 2010 discussed the use of simple clinical descriptions of disease manifestations (Ooi et al. 2010).

7.5 Epidemiology

7.5.1 Clinical Epidemiology of EV71

EV71 continues to cause large epidemics of HFMD with deaths across Asia. The most recent large epidemic in Asia occurred in China in 2008. Approximately 490,000 children including 126 deaths were reported nationwide; at the epicentre in Anhui Province, more than 6,000 HFMD cases and 22 deaths were reported (Zhang et al. 2010). In addition to these very large outbreaks, many areas, including Sarawak in Malaysia, Taiwan, Singapore, Vietnam and Japan have experienced cyclical epidemics that occur at 2 to 3 year intervals (Figure 7.2) (Mizuta et al. 2005; Hosoya et al. 2006; Podin et al. 2006).

Outside the Asia-Pacific region two small community outbreaks of neurological EV71 disease, with no HFMD, occurred in 2003 and 2005 in Denver, USA, and affected 16 children, aged 4 weeks to 9 years, with 1 fatality (Perez-Velez et al. 2007). A retrospective analysis of stool samples collected from children with aseptic meningitis in Austria showed that EV71 was detected in 16 (9%) of 181 patients admitted between 2001 and 2004 (Ortner et al. 2009). A similar study identified 32 sporadic cases of EV71 infection in the UK between 1998 and 2006, presenting primarily as

neurological disease and/or HFMD (Bible et al. 2008). A cluster of 58 hospitalised cases with predominant clinical manifestations of fever, gastrointestinal symptoms and CNS infections was observed in 2007 following a period of 21 years of low endemicity (Van der Sanden et al. 2009). Widespread asymptomatic circulation of EV71 was also noted between October 2002 and October 2003 in Norway where the virus was isolated from 19 (17%) of 113 children who were completely well (Witso et al. 2007). EV71 was among a range of enteroviruses detected by screening blood donations in Scotland over 22 months; the pick-up rate for any enterovirus was 1 in 4000 donations, and the significance remains uncertain (Welch et al. 2003). In Nairobi Kenya, there were two small institutional outbreaks of EV71 infection in an HIV-orphanage in 1999 and 2000 (Chakraborty et al. 2004). Although EV71 was first isolated in 1969 in California, a retrospective analysis of samples from the Netherlands shows that EV71 was circulating as early as 1963 (Van der Sanden et al. 2009).

7.5.2 Molecular epidemiology

Chapter 4 describes the emergence of two new subgenogroups B4 and B5 in Sarawak during the two consecutive epidemics in 2000 and 2003, respectively. A number of new subgenogroups within genogroup B and C

have emerged in the Asia-Pacific region over the last 12 years (Table 7.1) Phylogenetic studies of the virus isolated from the ongoing epidemics in Asia showed that subgenogroup B3 and B4 viruses are likely to have co-circulated in the region since 1997 (McMinn et al. 2001; Cardosa et al. 2003; Shimizu 2004). Subgenogroup B5, was first isolated in Sarawak and Japan in 2003, and was responsible for epidemics in Sarawak, Taiwan and Brunei in 2006 (Mizuta et al. 2005; Podin et al. 2006; Huang et al. 2008; AbuBakar et al. 2009). Except for the major community outbreak in Sydney in 1986, subgenogroup C1 viruses have mostly been isolated from sporadic cases since the mid-1980, suggesting a low level of endemic circulation across the globe (Brown et al. 1999; Sanders et al. 2006). Subgenogroup C2 viruses were responsible for the large EV71 epidemic in Taiwan in 1998, and the Perth outbreak in 1999 (McMinn et al. 2001; McMinn 2002; Cardosa et al. 2003; Lin et al. 2006). Subgenogroup C3 was isolated in Japan in 1994 and Korea in 2000 (Cardosa et al. 2003; Jee et al. 2003; Iwai et al. 2009). Subgenogroup C4 has been the predominant circulating genogroup in mainland China since 2000, including the most recent epidemic in 2008, as well as occurring in Japan, Vietnam and Taiwan (Shimizu 2004; Lin et al. 2006; Tu et al. 2007; Zhang et al. 2010). Subgenogroup C5 has been reported from Southern Vietnam and Taiwan (Tu et al. 2007; Huang et al. 2008). A genetically distinct EV71 strain (R13223, Genbank accession no. AY179600

to AY179602), when compared to other EV71 strains isolated in the Asia-Pacific region in recent years, was isolated from a single child who with acute flaccid paralysis in India in 2001 (Deshpande et al. 2003). Thus, two major EV71 genogroups (B and C) have co-circulated and co-evolved into subgenogroups in the Asia-Pacific region over the past 14 years. In Malaysia and Singapore, the genogroup B viruses have dominated, where-as in East Asia, particularly in mainland China and Vietnam, genogroup C viruses have dominated (Figure 7.3).

Recently isolates of genogroup A have been reported from 5 of 22 children presenting with HFMD in Anhui province of Central China during the 2008 outbreak - the first ever report of genogroup A viruses, since the original prototype was isolated in America (Yu et al. 2010). Sequencing of the complete VP1 gene shows the isolates cluster with the prototype genogroup A virus, with very little divergence. This highly unexpected occurrence might indicate that the virus has been circulating undetected in central China, with very little evolutionary change for 40 years, or raises queries about the source of the virus templates that were sequenced, which may be from laboratory contamination or perhaps be related to laboratory-acquired infection. Surveillance from the same outbreak by the Chinese Centre for Disease Control does not appear to have identified any genogroup A viruses

(Zhang et al. 2010). Clearly, good surveillance programs need to be implemented in many different geographical regions to provide accurate and relevant information about EV71 transmission and evolution and it would be prudent to await confirmation by other laboratories before concluding that genogroup A viruses have re-emerged. A recent phylogenetic analysis suggests that EV71 may have emerged from the genetically closely related coxsackie virus A16 as recently as 1940 (Tee et al. 2010).

7.6 Transmission and epidemic potential of genogroups

Surveillance systems for EV71 have been established in a number of countries in the Asia-Pacific region; whilst primarily aimed at monitoring transmission and spread, they have also provided invaluable insights into how the virus evolves in the community during outbreaks. For example, data from a virological surveillance system in Sarawak, Malaysia has shown increased viral activity every 3 years (1997, 2000, 2003, 2006 and 2008/09) which is closely associated with an increased incidence of HFMD in the community (Podin et al. 2006) (Solomon et al. 2010). The phenomenon of cyclical epidemics that take place at regular intervals has also been observed in Fukushima Prefecture, Japan (Hosoya et al. 2006). Such cyclical activity

is presumed to relate to the availability of new birth cohorts of children who have not been exposed to the virus during the earlier epidemics (Chang et al. 2002; Lu et al. 2002). Trying to make predictions about the epidemic potential of particular subgenogroups has proved difficult, beyond the observation that some subgenogroups, such as C1, appear to be less virulent, causing endemic disease, rather than the large epidemics, whereas other subgenogroups, particularly those that appear to have emerged in Asia in recent years, are associated with large epidemics.

More than one subgenogroup may co-circulate, and during outbreaks in Sarawak and Vietnam shift from one dominant genogroup to another has been described (Cardosa et al. 2003; Podin et al. 2006; Tu et al. 2007). In Japan and Taiwan subgenogroup B and C viruses have caused epidemics at different times (Table 7.1) (Wang et al. 2002; Lin et al. 2006; Huang et al. 2008). In contrast in the Netherlands a permanent shift appears to have occurred, from genogroup B viruses before 1986 to genogroup C viruses since 1987; cross-neutralization among the genogroup B viruses, but not with genogroup C viruses is postulated as one explanation for this, though other experimental cross neutralization data would not appear to support it (Kung et al. 2007; Mizuta et al. 2009; Van der Sanden et al. 2009; Van der Sanden et al. 2010). Whilst the older subgenogroups of virus have been

circulating and causing low levels of disease for many years, some of the more recently evolved subgenogroups such as genogroup B5, which possess distinctive antigencity from other viruses may have the potential to cause very large outbreaks (Huang et al. 2009; Van der Sanden et al. 2010). Although these have been confined to the Asia-Pacific region, the fact these viruses are carried by human, and that there is increasing global travel means that every region should consider itself potentially at risk.

7.7 Pathogenesis

7.7.1 Viral determinants of virulence

Subgenogroups may indeed vary in their biological behaviour with regard to risk of neurological disease and transmissibility within families, as depicted in Chapter 4. Children infected with subgenogroup B4 viruses were less likely to present with CNS infection than those infected with C1 or B5 viruses, and that they were also less likely to be part of a family cluster. In contrast, children infected with B5 viruses were more likely to be part of a family cluster, and there was a trend towards a greater incidence of CNS disease in these patients.

7.7.2 Dual infection

Co-infection with adenovrus 21 has been postulated as an important factor for the development of systemic complications and death during HFMD epidemic in Sarawak in 1997 (Cardosa et al. 1999). Nevertheless I did not find in this longitudinal study evidence of adenovirus 21 infection in other HFMD or neurological cases, though dual infection of EV71 with other viruses, including dengue and Japanese encephalitis, has been found but with no increase in disease severity or mortality.

7.7.3 Host susceptibility

The potential for host genetic variants to explain the variability in clinical epidemiological patterns of EV71 has also been studied. One genetic study in Taiwan reported that HLA-A33 is associated with increased susceptibility to EV71 although the role of the major histocompatibility complex in the virus infection is still unknown (Chang et al. 2008). The authors noted that HLA-A33 is more frequently found in the Asian population than that in the Caucasian population, and this may help explain frequent occurrence of EV71 epidemics in Asia. The authors also reported that HLA-A2, in a mechanism yet to be defined may be linked to the risk of cardiopulmonary failure often observed in fatal cases (Chang et al. 2008). Cytotoxic T lymphocyte antigen–4 (CTLA-4) gene is an important regulator of T cell

cytotoxicity, and it has a role in the regulation of an immune response. In a study of 78 children with EV71 infection in Taiwan, those with meningoencephalitis were found to have a higher frequency of G/G genotype at position 49 of exon 1 in the CTLA-4 gene, than those without meningoencephalitis and control subjects (Yang et al. 2001). However a subsequent study found no such association (Chang et al. 2008).

7.4 Pathophysiology of severe disease

7.4.1 Viral entry and spread

The mechanism by which enteroviruses enter the CNS is not completely understood. A number of epidemiological and experimental animal studies on polioviruses indicate that the virus can invade the CNS system by permeation through a disrupted blood-brain barrier, or retrograde axonal spread along cranial or peripheral nerves. For EV71, this latter route has been implicated both from mouse models, and by examining the distribution of virus and inflammation in fatal human cases (Chen et al. 2007; Ong et al. 2008; Wong et al. 2008).

7.4.2 Pathogenesis of severe pulmonary oedema and heart failure

While it is clear that fulminant pulmonary oedema is closely associated with, and preceded by CNS involvement, there is no consensus on its cause, especially the relative contributions of neurogenic pulmonary oedema, cardiac dysfunction, increased vascular permeability and a cytokine storm (Chang et al. 1998; Chang et al. 1999; Lin et al. 2002; Fu et al. 2003; Lin et al. 2003; Wang et al. 2003; Fu et al. 2004; Wang et al. 2007; Wang et al. 2008)(Figure 7.4).

Neurogenic pulmonary oedema is classically seen following a head injury, where the associated raised intracranial pressure is thought to be important. Although the pathogenesis is not completely understood, studies from animal models suggest that the hypothalamus, and vasomotor centres of the medulla, and nuclei in the cervical spinal cord are important; lesions to various nuclei in these regions can increase activity along the sympathetic trunk, resulting in profound systemic and pulmonary hypertension and consequent pulmonary oedema (Hoff et al. 1981). Pulmonary oedema was also seen in poliomyelitis, and because it was associated with damage to brainstem nuclei, was thought to be neurogenic in origin (Baker 1957). Thus when severe pulmonary oedema was first seen in EV71 encephalitis, and brainstem

inflammatory changes were observed too, the oedema was thought to be due to neurogenic causes. Post-mortem examination and magnetic resonance imaging studies of children with EV71 brainstem encephalitis showed that there was extensive inflammation of gray matter of the spinal cord and the whole medulla oblongata, as described above (Lum et al. 1998; Huang et al. 1999; Shen et al. 1999; Hsueh et al. 2000; Wong et al. 2008). The observations of hyperglycaemia and leucocytosis were also postulated to be due to increased sympathetic discharges (Chang et al. 1999). However, detailed hemodynamic observations of children with EV71 and pulmonary oedema have not always shown the profound systemic and pulmonary hypertension that would be expected (Lin et al. 2002; Wu et al. 2002; Fu et al. 2004). This may be because the changes in vascular pressures in neurogenic pulmonary oedema are only transient, as was shown for one child with EV71 (Wu et al. 2002). Others have argued that cardiac dysfunction is a major contributor to the pulmonary oedema. Although there is no histological or virological evidence of a viral myocarditis, elevated levels of the cardiac specific troponin I suggest a degree of cardiac damage (Chang et al. 1998; Lum et al. 1998; Huang et al. 2003; Fu et al. 2004). One detailed echocardiographic study of cardiac function in 11 children with EV71 brainstem encephalitis show that it was impaired, with significantly reduced left ventricular ejection fraction (Fu et al. 2004). Two children whose cardiac

output was supported with a left ventricular assist device survived, whereas all the others died (Fu et al. 2003). Interestingly, in a separate report the same authors described very high levels of norepinephrine and epinephrine in 3 of the 17 children with left ventricular dysfunction (Fu et al. 2004). Although there is no myocardial inflammation, histological examination of cardiac ventricular tissue from six fatal cases and one survivor, obtained though a biopsy, revealed significant coagulative myocytolysis, myofibrillar degeneration, and cardiomyocyte apoptosis which are the characteristic features of catecholamine cardiotoxicity (Fu et al. 2004; Fu et al. 2006). Thus it is argued by some that the high catecholamine levels caused by brainstem encephalitis may have a direct effect on cardiac function, as well as causing pulmonary oedema via raised pulmonary pressures.

The other potential contributor to pulmonary oedema is increased vascular permeability which is postulated to occur secondary to the systemic inflammatory response. A series of studies have examined cytokine and chemokine profiles in EV71: interleukin (IL)-6, IL-1B, IL-10, IL-13, tumour necrosis factor (TNF)-α and interferon (IFN)-γ are all significantly higher in EV71 patients with pulmonary oedema, than those with encephalitis uncomplicated by oedema, and several of these, including IL-1, IL-6, IL-13 and IFN-γ are known mediators of increased vascular permeability (Lin et al.

2002; Lin et al. 2003; Wang et al. 2003). In addition increased plasma levels of several chemokine including interferon-gamma-induced protein (IP-10), monocyte chemoattractant protein (MCP)-1, monokine induced by interferon-gamma (MIG), and IL-8 have been found in children with pulmonary oedema when compared to those with uncomplicated brainstem encephalitis (Wang et al. 2008). Children with oedema also had depleted a lymphocyte population particularly in CD4 and 8 and natural killer cells (Yang et al. 2001; Wang et al. 2003). Thrombocytosis, neutrophilia and hyperglycaemia, are all thought to be indicative of a systemic inflammatory response (Lin et al. 2003; Wang et al. 2003). Fewer studies have looked at cytokines in the CSF, but one showed elevated CSF IL-1b in patients with encephalitis complicated by oedema, compared to those with encephalitis alone (Lin et al. 2003).

Because the development of pulmonary oedema in patients with EV71 encephalitis appears to be strongly associated with a dysregulated systemic and CNS inflammatory response; this has formed at least part of the basis for treatment with the anti-inflammatory drug intravenous immunoglobulin, which appears to be effective as I have shown in Chapter 5 and in other studies (Lin et al. 2002; Chang et al. 2004; Wang et al. 2006; Ooi et al. 2007; Ooi et al. 2009).

In summary, the exact mechanism for pulmonary oedema in EV71 encephalitis is still unclear. Neurogenic mechanisms secondary to brainstem inflammation appear to be important, but pathologically similar changes are seen in other encephalitides, for example Japanese encephalitis, without pulmonary oedema being such a prominent feature. Cardiac dysfunction and the effects of the systemic inflammatory response on the vascular endothelium may also make an important contribution. In vivo models including those in mouse and non-human primates have replicated some of the features of severe EV71 disease, such as neuroinvasiveness with marked inflammatory changes; however none has yet been able to reproduce the severe systemic features such as pulmonary oedema (Chen et al. 2004; Wang et al. 2004; Arita et al. 2005; Chen et al. 2007; Ong et al. 2008; Wong et al. 2008).

7.5 Treatment of enterovirus 71 infection

7.5.1 Antiviral agents

Several other newer capsid-function inhibitors have been investigated and some have demonstrated promising anti-EV71 activities in pre-clinical studies (Chen et al. 2008). In vitro and in vivo studies show that both

ribavirin and interferons, may be useful (Liu et al. 2005; Li et al. 2008), and RNA interference approaches are being explored (Sim et al. 2005; Tan et al. 2007; Wu et al. 2009). Several receptors for EV71 have been identified recently, and may be an important step towards the development of EV71-specific antiviral. A ubiquitously expressed cellular receptor, scavenger receptor B2, and a functional receptor, human P-selectin glycoprotein ligand-1, found on white blood cells have recently been identified by two groups of Japanese investigators as specific receptors for EV71(Nishimura et al. 2009; Yamayoshi et al. 2009). In addition sialic acid-link glycan, expressed in abundance in respiratory and gastrointestinal tracts, and dendritic cell-specific iIntercellular adhesion molecule-3-Grabbing non-integrin (DC-SIGN, CD209), found exclusively in dendritic cells that are present in lymphoid tissues, have also been identified as receptors for EV71 by two groups of Taiwanese investigators (Zhou et al. 2006; Lin et al. 2009; Yang et al. 2009).

7.5.2 Intravenous Immunoglobulin

In Chapter 5 the effectiveness of IVIG in children with neurological disease who are at risk of cardiorespiratory failure was examined. Retrospective comparisons of patients that received IVIG with those that did not suggest

that there may be benefit from IVIG if given early, and this is consistent with a study in Taiwan (Chang et al. 2004; Ooi et al. 2009). Analysis of cytokine profiles before and after IVIG treatment showed significant reductions in some pro-inflammatory cytokines in EV71 patients if they had encephalitis with autonomic dysfunction, but not if they had less severe disease (Lin et al. 2002; Lin et al. 2003; Wang et al. 2003; Wang et al. 2006). IVIG has therefore becoming routine treatment for severe EV71 disease (Chang et al. 2004; Wang et al. 2006; Ooi et al. 2007; Ooi et al. 2009), and in Taiwan has been introduced into the national treatment guidelines (Lin et al. 2002). However uncertainty exists over whether this treatment is really effective, and most accept that randomised placebo controlled trials are needed. Such trials would be logistically and ethically challenging to establish, given the extent to which the treatment is used and the strong beliefs held by some; in the first instance carefully designed phase II trials with surrogate end points of disease progression, such as failure of resolution of tachycardia would be needed.

7.5.3 Milrinone

Milrinone is a cyclic nucleotide phosphodiesterase inhibitor currently used in the treatment of congestive heart failure. Inhibition of cyclic nucleotide

phosphodiesterase subtype III by this cardiotrophic agent results in an increase in intracellular cyclic adenosine monophosphate (cAMP), which in turn leads to increased cardiac output and decreased peripheral vascular resistance. A small non-randomised retrospective comparison involving 24 children with EV71-induced pulmonary oedema showed that those treated with milrinone had reduced tachycardia, and a lower mortality (Wang et al. 2005; Wang et al. 2006). Intriguingly the peripheral white cell count, platelet count, and plasma IL-13 were also lower (Wang et al. 2005), possibly suggesting an immunomodulatory as well as a cardiovascular effect of the drug. A clinical study examining efficacy of milrinone is said to be ongoing(Wang et al. 2008).

7.6 Prospects for control

7.6.1 Surveillance and prevention

The only measures available currently for disease control are public health approaches. Since public health intervention at an early phase of an outbreak may help mitigate the spread of the virus in the communities, many countries in the region including Malaysia, Singapore, Taiwan, Japan and Vietnam have implemented heightened surveillance for EV71 (Mizuta et al. 2005; Podin et al. 2006; Chen et al. 2007; Ang et al. 2009; 2009). HFMD has now

become a notifiable disease in many countries, including Malaysia, Singapore, Thailand, Taiwan, Vietnam and China (Anonymous 2009). However as HFMD may be caused by a number of other EV-A viruses including CVA8, 10 and 16, concurrent virological surveillance is necessary. Virological surveillance also provides invaluable molecular epidemiological data about the circulating EV71 genotype and may thus help to track the spread of the virus across the region.

Outbreak control measures are primarily targeted at interrupting the virus transmission and spread from person-to-person through contact with throat and nose secretions, saliva, stool and vesicular fluid; in addition contact with virus contaminated surfaces, toys or fomites is important. Hence health education focuses on personal hygiene and good sanitation including frequent hand washing, proper disposal of soiled diapers and disinfection of soiled surfaces with chlorinated (bleach) disinfectants. A good hand hygiene among children and their caregivers has been shown to be effective in reducing risk of HFMD and herpangina caused by EV71 (Ruan et al. 2011). Since the transmission of enteroviruses including EV71 is most efficient in overcrowded settings, most countries in the region including Malaysia, Singapore, Taiwan, Hong Kong and China have adopted "social distancing" measures during epidemics, such as closures of childcare facilities and

schools, and cancellation of public functions involving children (Ang et al. 2009; 2009). It is unclear though whether they should be instituted as soon as there is a HFMD outbreak or should await proof that it is EV71-related. However, it is not known if the distancing measures, which carry significant socioeconomic implications, are effective. If EV71 is like other directly transmissible viruses, then controls such as school closure will decrease the peak incidence of disease, but this may be associated with a prolonged outbreak, and no reduction in the overall number of cases (Figure 7.5) (Solomon et al. 2010). Paradoxically, if this means that more children catch the infection from their siblings, rather than from the peer-group at school, then this could theoretically lead to an increased incidence of severe cases; because family transmission of EV71 between siblings may be associated with more severe disease (presumably from a higher inoculum) (Chang et al. 2004). Clearly more clinical and epidemiological studies are necessary to guide public health specialists into instituting evidence-based interventions to control the spread of EV71. Although there has been little systematic research to examine the effectiveness of such measures, studies from Singapore and Hong Kong appeared to show some benefit (Ma et al. ; Ang et al. 2009).

7.6 2 Vaccine development

There are currently no vaccines against EV71, but by analogy with poliomyelitis, vaccines probably offer the best strategy for future disease control. One limitation in EV71 vaccine development is the lack of a good mouse model of human disease. Adult mice are resistant to infection. Although suckling mice are susceptible, by the time potential vaccines inoculated into them have led to the development of immunity, the animals have matured and become resistant to infection anyway. One way around this is to vaccinate female adult mice, allow them to become pregnant, and then determine the level of protective maternal antibody transferred to their offspring, as judged by protection against lethal infection (Wu et al. 2001). In humans the target population should be younger children, especially those less than three years, because they are susceptible to severe disease. One important issue is whether vaccines derived against one genogroup will provide cross protection against all genotypes: data so far are contradictory (Kung et al. 2007; Mizuta et al. 2009; Van der Sanden et al. 2010). Several comprehensive reviews on the development of EV71 vaccine candidates have been published (Xu et al. 2010; Lee et al. 2010). A range of approaches to developing vaccines have been adopted including inactivated whole virus vaccines, live attenuated vaccines, production of subviral particles and DNA vaccines. They are all in relatively early stages of development, with the

most advanced undergoing preclinical trials in murine and non-human primates (Lee et al. 2010).

Candidate inactivated vaccines include those derived in Taiwan from subgenogroup B4 viruses, EV71-075 and EV71-0117, which are highly immunogenic, and EV71-1207, which is a C2 virus, and is less immunogenic; in one study they were found to be more immunogenic in mice than recombinant VP1 protein, or DNA vectored vaccines (Liu et al. 2007). Virus like particles for EV71, that resemble the virus in appearance, capsid structure, and protein have been produced and purified as potential vaccines (Chung et al. 2008). After immunization of BALB/c mice, the particles induced potent and long-lasting humoral immune responses as evidenced by high total IgG titer and neutralization titer. Splenocytes collected from the immunised mice exhibited significant cell proliferation and produced high levels of IFN-gamma, IL-2 and IL-4 after stimulation, indicating the induction of Th1 and Th2-type immune responses. Immunization of female mice conferred protection (survival rate up to 89%) to neonatal mice against virus challenge with a dose 1000 times that normally required to kill 50% of animals (Chung et al. 2008).

A potential DNA vaccine has been developed by inserting the VP1 gene into the pVAX1 vector, and transforming the constructs into E coli cells, and expressing in a mammalian cell line (Tung et al. 2007). Immunization of mice with the DNA vaccine constructs resulted in the production of anti-VP1 IgG and neutralizing antibody against EV71. An alternative approach, immunizing mice orally with an attenuated Salmonella enteric serovar Typhimurium expressing the VP1 gene, also proved protective of newborn mice, following maternal immunization (Chiu et al. 2006). Transgenic tomatoes expressing the VP1 protein have also been developed, and used as an oral vaccine, again resulting in the development of VP1-specific antibodies in BALB/c mice, as well as evidence of cell mediated immunity, and providing protection to their offspring in the neonatal challenge models (Chen et al. 2006).

Linear neutralizing epitopes from the VP1 capsid protein were identified by raising anti-sera in mice against overlapping peptides from this protein, and two peptides were found to elicit neutralizing antibody responses (Foo et al. 2007). One of them, SP70 was particularly potent, and comparison with sequences from other strains showed it was conserved among the different subgenotypes of EV71, suggesting it is a promising candidate to take forward.

A live attenuated strain of EV71, EV71(S1-3'), was derived from the genotype A prototype strain BrCr using genetic manipulations (Arita et al. 2007). These were based on knowledge of the temperature sensitive determinants of poliovirus type 1 vaccine strain, some of which are located in the 5' and 3' untranslated regions and the 3Dpol gene. Cynomolgus monkeys inoculated intravenously with the EV71 vaccine strain develop antibodies with cross reactivity against a broad spectrum of EV71 genotypes, and survived challenge with intravenous virulent EV71 (BrCr-TR strain) which is lethal in non-immunised monkeys. However the vaccine strain itself causes mild neurological symptoms (tremor), and was found to be neurotropic, entering the spinal cord, indicating that further work on attenuation is needed (Arita et al. 2007).

Among the various vaccine candidates, inactivated whole virus vaccines are in some ways the most ready to take forward, because the principles of vaccines based on inactive whole virus are well established. However, although the technology may be the most readily adaptable, and quickest to develop, those developing enterovirus vaccines would do well to remember the lessons learnt from vaccines against Japanese encephalitis virus, another major neurological infection in Asia. An effective formalin inactivated

mouse brain derived vaccine has been available since 1950s, but the vaccine has not been widely used in many Japanese encephalitis virus endemic countries until more recently because of its high cost. Therefore; ultimately, if a vaccine is to be used across the whole of Asia, it needs to be cheap, easily produced, and readily available (Solomon 2004).

7.7 Final concluding remarks

The emergence of EV71 in the Asia Pacific Region over the last 14 years has had a major public health impact. The virus has emerged from being a relatively rare and sporadic cause of HFMD to a cause of major and regular epidemic across the Asia-Pacific region, which is often associated with severe and sometimes fatal neurological complications. Of the many thousands who become infected with the virus there are still no good ways of predicting who will develop HFMD, and who will have neurological complications. Nor are there clear indicators of which patients, with CNS involvement, are at risk of disease progression. Molecular epidemiological studies suggest that some subgenogroups appear to have massive potential for explosive epidemics, whilst others have more indolent low level circulation. However the biological determinants of these differences are poorly understood. The epidemiological differences observed between EV71

in Asia-Pacific region and that in Europe and USA is also a mystery. Similarly the virological and host determinants of the wide ranging clinical phenotypes in those infected are unclear. There is still no specific antiviral drug available for EV71 infection although the identification of several EV71 receptors may help in drug discovery. IVIG is now used presumptively for severe EV71 infection in many Asian countries, even though there are almost no data on its efficacy. Several vaccine candidates are under development but how to make sure they ultimately reach the paediatric populations that really need them remains an important issue. Public health intervention and control measures of EV71 epidemics so far have been empirical and generic in nature; but they have a significant socioeconomic impact.

7.8 Future Direction

Across Asia a range of approaches to diagnosis is used, with different techniques and different controls; standardisation via some form of laboratory network with proficiency panels to allow comparability between laboratories and ensure quality control may prove useful. There are relatively good animal models of neurological disease caused by EV71, but there is an urgent need for an animal model of cardiorespiratory dysfunction to help

understand pathogenesis better. IVIG is an expensive and potentially dangerous treatment, but is being widely in Asia. A clinical trial aimed at examining the efficacy of IVIG is urgently required. Evidence-based clinical practise guidelines on best approaches to diagnosis and treatment would considerably help the management of this fascinating emerging neurological infectious disease. Further research on the transmission dynamic of the virus is needed to guide the best public health intervention strategies to limit the havoc wreaked by future EV71 outbreaks.

Table 7.1 Enterovirus 71 genogroups circulating in Asia-Pacific region between 1973 and 2008

Countries	1973	1980	1986	1990	1993	1994	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009
Singapore	-	-	-	-	-	-	B3, B4	B3, C1	B3	B4	B4	C1, B4	B4	-	-	B5	-	B5	-
Peninsular Malaysia	-	-	-	-	-	-	B3, B4, C1[#], C2[#]	C1	B4, C1	B4, C1	-	-	-	-	B5, C1	-	-	-	-
Sarawak, Malaysia	-	-	-	-	-	-	B3	C1	None	B4, C1	None	C1	B5, C1	None	B5	B5			-
Perth	-	-	-	-	-	-	-	-	B3, C2	C1	None	None	-	-	-	-	-	-	-

Japan	B1	-	-	B2, C1	B2	C3	B3, B4, C2	C2	C2	B4	C2	B4, C2	C4, B5	C4	-	C4	C4	-	-
Taiwan	-	B1	B1	-	-	-	-	C2, B4#	B4	B4	B4	B4, C4#	B4, B5#	C4	C4, C5#	C5	C5, B5	B5	-
Korea	-	-	-	-	-	-	-	-	-	C3	None	None	C4	-	-	-	-	-	C4
Brunei	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	B5	-	-	-
Vietnam	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C1, C4, C5	-	-	-	
Thailand	-	-	-	-	-	-	-	-	-	-	-	C1	C1	C1	-	B5, C1, C2,	B5, C1, C2,	B5#, C1, C2#,	-

																C4	C4, C5	C4		
China	-	-	-	-	-	-	C3	C4	-	C4	C4	C4	C4	C4	-	-	C4	C4	-	
Hong Kong	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C4	

Footnotes:

*Subgenogroups underlined are those which caused large outbreaks.

Subgenogrous that were isolated in a small number

None: No EV71 was detected despite active surveillance

- No data

References: (Brown et al. 1999; McMinn et al. 2001; Cardosa et al. 2003; Shimizu 2004; Li et al. 2005; Lin et al. 2006; Chua et al. 2007; Tu et 2009; Zhang et al. 2009; Chatproedprai et al. 2010; Jeong et al. 2010; Zhang et al. 2010)

Figure 7.1 Collection of vesicular fluid from palmar lesions for virological diagnosis of hand foot and mouth disease

The hand is gripped firmly to prevent movement, the skin stretched slight and a small needle is used to puncture the vesicle (left); a clean swab is then applied to collect the fluid released, before placing into viral transport medium (right). (Photos T Solomon)

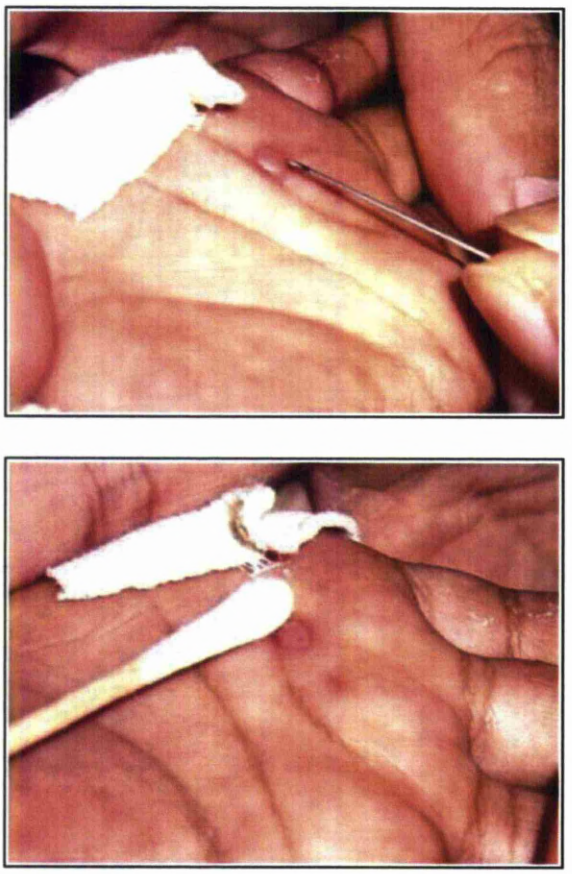


Figure 7.2 Distribution of HFMD cases, and enteroviruses isolated from sentinel clinics in Sarawak, Malaysia from March 1998 through December 2008

The top panel shows the distribution of cases of HFMD seen in sentinel clinics. The bottom panel shows the distribution of EV71 isolates. Adapted from (Podin et al. 2006; Solomon et al. 2010)

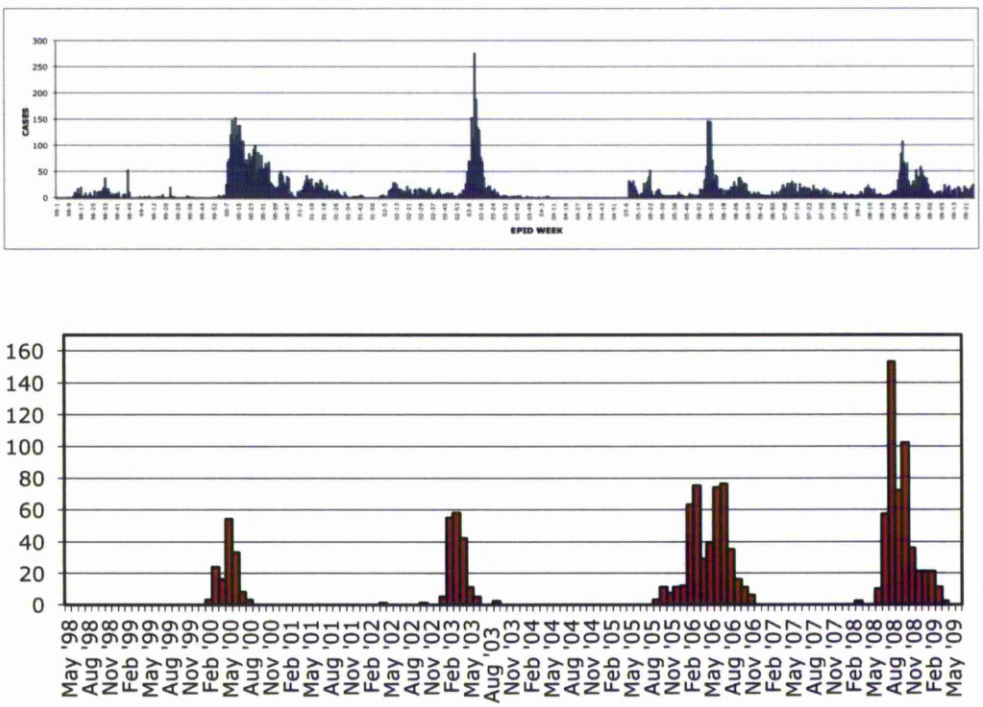


Figure 7.3 Phylogenetic analysis of enterovirus 71 VP1 gene sequences. A neighbor-joining tree constructed using the Kimura-2 parameter as a model for nucleotide substitution. The robustness of the tree was determined by bootstrapping using 1000 pseudoreplicates.. Sequences are labeled according to the following convention: "GenBank accession number" – "Country of origin" – "Year of isolation". The scale bar represents nucleotide changes per site per year.

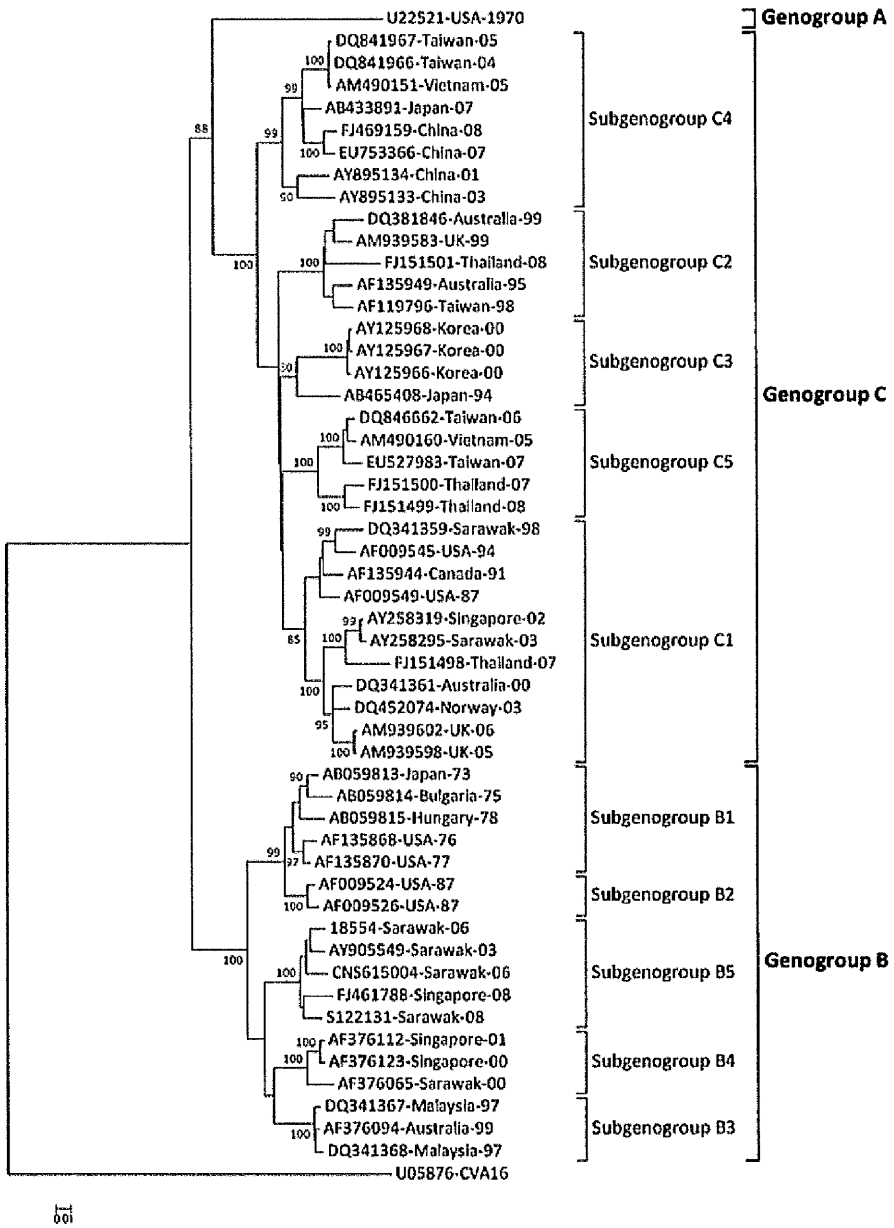

U22521-USA-1970
DQ841967-Taiwan-05
DQ841966-Taiwan-04
AM490151-Vietnam-05
AB433891-Japan-07
FJ469159-China-08
EU753366-China-07
AY895134-China-01
AY895133-China-03
DQ381846-Australia-99
AM939583-UK-99
FJ151501-Thailand-08
AF135949-Australia-95
AF119796-Taiwan-98
AY125968-Korea-00
AY125967-Korea-00
AY125966-Korea-00
AB465408-Japan-94
DQ846662-Taiwan-06
AM490160-Vietnam-05
EU527983-Taiwan-07
FJ151500-Thailand-07
FJ151499-Thailand-08
DQ341359-Sarawak-98
AF009545-USA-94
AF135944-Canada-91
AF009549-USA-87
AY258319-Singapore-02
AY258295-Sarawak-03
FJ151498-Thailand-07
DQ341361-Australia-00
DQ452074-Norway-03
AM939602-UK-06
AM939598-UK-05
AB059813-Japan-73
AB059814-Bulgaria-75
AB059815-Hungary-78
AF135868-USA-76
AF135870-USA-77
AF009524-USA-87
AF009526-USA-87
18554-Sarawak-06
AY905549-Sarawak-03
CNS615004-Sarawak-06
FJ461788-Singapore-08
S122131-Sarawak-08
AF376112-Singapore-01
AF376123-Singapore-00
AF376065-Sarawak-00
DQ341367-Malaysia-97
AF376094-Australia-99
DQ341368-Malaysia-97
U05876-CVA16
Subgenogroup C4
Subgenogroup C2
Subgenogroup C3
Subgenogroup C5
Subgenogroup C1
Subgenogroup B1
Subgenogroup B2
Subgenogroup B5
Subgenogroup B4
Subgenogroup B3
Genogroup A
Genogroup C
Genogroup B
0.01

Figure 7.4 The postulated pathogenesis of enterovirus 71 associated acute pulmonary oedema

Major postulated pathogenic pathways are shown with thick lines; lesser contributory pathways are shown with thinner lines. The solid boxes indicate strong supporting evidence from human clinical or pathological studies, whilst the dotted boxes indicate hypothetical but unproven steps or evidence from animal models only.

EV71: human enterovirus 71

BBB: Blood brain barrier

CNS: central nervous system

↑↑: markedly high; SVR: systemic vascular resistance

SBP: systemic blood pressure

HR: heart rate

LV: left ventricular

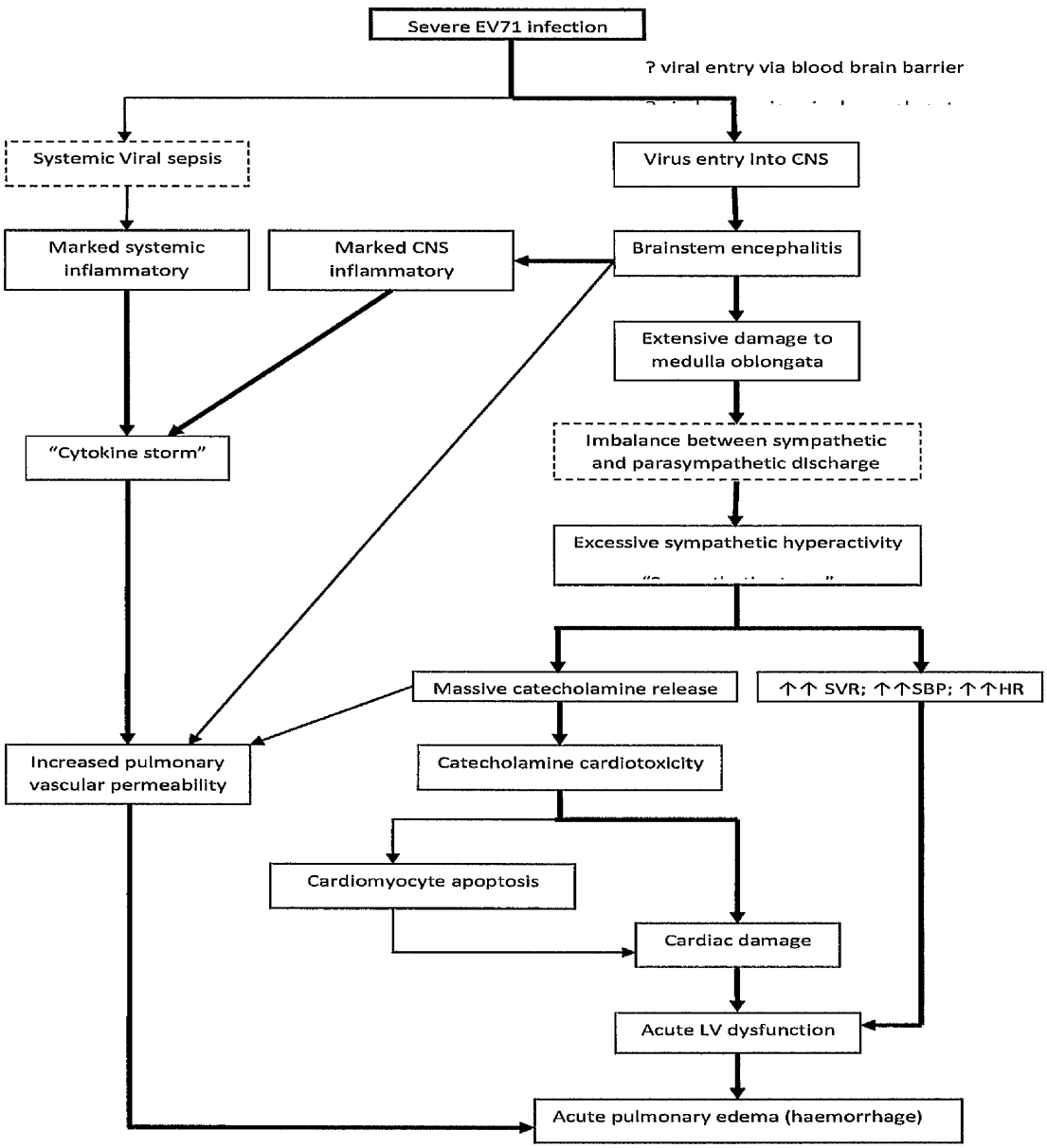
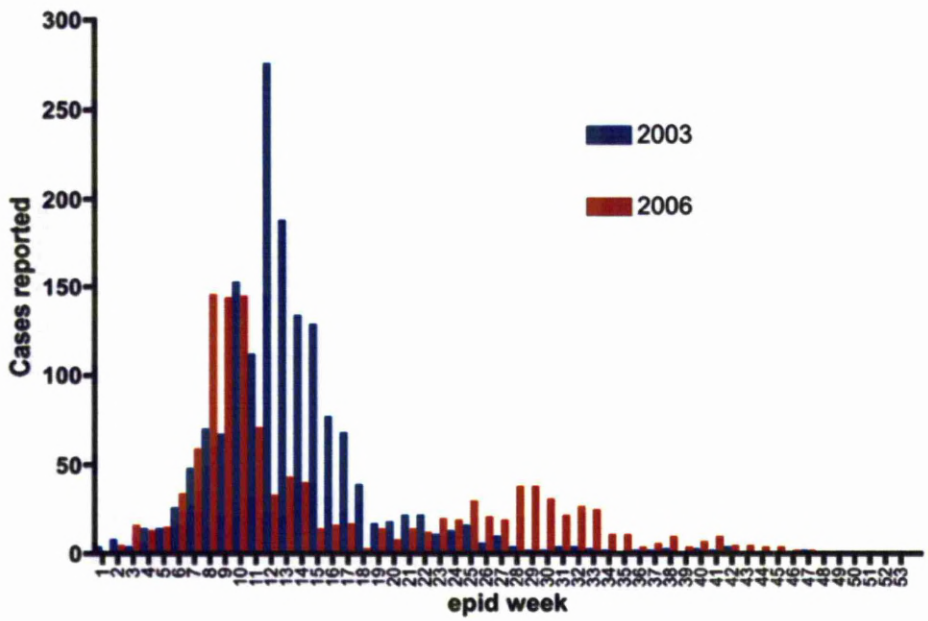

Severe EV71 infection
? viral entry via blood brain barrier
Systemic Viral sepsis
Virus entry into CNS
Marked systemic inflammatory
Marked CNS inflammatory
Brainstem encephalitis
Extensive damage to medulla oblongata
"Cytokine storm"
Imbalance between sympathetic and parasympathetic discharge
Excessive sympathetic hyperactivity
Massive catecholamine release
↑↑ SVR; ↑↑SBP; ↑↑HR
Increased pulmonary vascular permeability
Catecholamine cardiotoxicity
Cardiomyocyte apoptosis
Cardiac damage
Acute LV dysfunction
Acute pulmonary edema (haemorrhage)

Figure 7.5 The effect of public health interventions on hand foot and mouth disease outbreaks

The effect of public health interventions on hand foot and mouth disease outbreaks, comparing sentinel surveillance data from the 2003 outbreak in Sarawak Malaysia when the public health response was limited, and the 2006 outbreak, when more rigorous social distancing measures were encouraged.



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Appendix 1: Publications arising from this work

MAJOR ARTICLE

Human Enterovirus 71 Disease in Sarawak, Malaysia: A Prospective Clinical, Virological, and Molecular Epidemiological Study

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Background. Human enterovirus (HEV)–71 causes large outbreaks of hand-foot-and-mouth disease with central nervous system (CNS) complications, but the role of HEV-71 genogroups or dual infection with other viruses in causing severe disease is unclear.

Methods. We prospectively studied children with suspected HEV-71 (i.e., hand-foot-and-mouth disease, CNS disease, or both) over 3.5 years, using detailed virological investigation and genogroup analysis of all isolates.

Results. Seven hundred seventy-three children were recruited, 277 of whom were infected with HEV-71, including 28 who were coinfected with other viruses. Risk factors for CNS disease in HEV-71 included young age, fever, vomiting, mouth ulcers, breathlessness, cold limbs, and poor urine output. Genogroup analysis for the HEV-71–infected patients revealed that 168 were infected with genogroup B4, 68 with C1, and 41 with a newly emerged genogroup, B5. Children with HEV-71 genogroup B4 were less likely to have CNS complications than those with other genogroups (26 [15%] of 168 vs. 30 [28%] of 109; odds ratio [OR], 0.48; 95% confidence interval [CI], 0.26–0.91; $P = .0223$) and less likely to be part of a family cluster (12 [7%] of 168 vs. 29 [27%] of 109; OR, 0.21; 95% CI, 0.10–0.46; $P < .0001$); children with HEV-71 genogroup B5 were more likely to be part of a family cluster (OR, 6.26; 95% CI, 2.77–14.18; $P < .0001$). Children with HEV-71 and coinfected with another enterovirus or adenovirus were no more likely to have CNS disease.

Conclusions. Genogroups of HEV-71 may differ with regard to the risk of causing CNS disease and the association with family clusters. Dual infections are common, and all possible causes should be excluded before accepting that the first virus identified is the causal agent.

Since 1997, countries of the Asia-Pacific rim have been affected by large outbreaks of human enterovirus (HEV)–71-associated hand-foot-and-mouth disease (HFMD), which have resulted in hundreds of thousands of cases and many deaths [1–7]. HFMD is a common exanthema of young children, characterized by fever, rash on the palms and bottoms of the feet, and ulcers in the oral cavity. Most patients have mild cases of disease, but some patients develop severe neurological complications (i.e., aseptic meningitis, acute flaccid paralysis, and encephalitis) or systemic disease (i.e., shock and cardiac dysfunction). HFMD is caused by enteroviruses (genus *Enterovirus*, family Picornoviridae), particularly Coxsackie A virus (CAV)–10, CAV-16, and HEV-71, which are mostly transmitted by the fecal-oral route. Phylogenetic studies have divided HEV–71 strains into genogroups A, B, and C, which have been further subdivided [4, 8–10]. The incidence of CNS disease and other severe complications appears to have varied among outbreaks of HEV-71 infection. The reason for this is unclear, but differences between genogroups [10–12] and coinfection with other viruses, such as a newly characterized adenovirus [1, 13], have been postulated. However, comparisons between outbreaks have been hampered by the retrospective nature

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of many studies and differences in inclusion criteria, definitions of severe disease, and viral diagnostic capabilities. We conducted a prospective, clinical, diagnostic, and molecular virological study of HEV-71–associated HFMD in 1 context for 3.5 years. We focused on risk factors for neurological disease and the putative role of different HEV-71 genogroups and dual infections in pathogenesis.

MATERIALS AND METHODS

Setting. The study was conducted at the pediatric wards and intensive care unit at Sibu Hospital (Sarawak, Malaysia); it commenced in January 2000 and continued for 3.5 years. This 550-bed hospital serves Sibu (population, 200,000) and receives referrals from district hospitals in the Rejang basin (total population, ~650,000). The study was approved by the Director of Health for Sarawak (Malaysia) and the Ethics Committee of the Liverpool School of Tropical Medicine (Liverpool, UK). Informed consent was obtained from each child's accompanying parent or guardian.

Case definitions. Because enteroviruses can cause HFMD, CNS infection, or both, children (age 1 month to <12 years) were screened for both of these conditions. Children with HFMD were classified according to an algorithm as having mild HFMD, severe non–CNS-HFMD, or HFMD with CNS complications (figure 1). Children with suspected CNS infection, but no HFMD, were classified as having aseptic meningitis (ASM) or viral encephalitis on the basis of clinical and CSF findings; however, there were no children with enterovirus infection in this last group; thus, they are not discussed further.

Clinical methods. A detailed history was recorded, and a clinical examination was performed by a pediatrician of the study team who looked especially for mucocutaneous lesions and cardiovascular and neurological signs; all details were recorded on standardized forms. For viral culture, specimens were swabbed from the throat and rectum of every patient, as well as from at least 1 of the vesicles on the skin and oral ulcers (if present). Blood samples were obtained for viral studies. In patients with severe disease, complete blood cell count, urea level, electrolyte level, and glucose level were determined, and electrocardiography and echocardiography were performed. CSF specimens were examined for cell count and differential, protein level, and glucose level, and Gram stain, bacterial culture, and viral studies were performed; lumbar puncture was repeated if there was a strong clinical suspicion of viral CNS infection and if the initial examination was not confirmatory. Lumbar puncture was delayed in patients who were severely unhealthy. Patients were examined daily, or more frequently (as indicated), by a member of the study team. Children with HFMD and CNS complications were treated with intravenous immunoglobulin (IVIG; Intragam P-CSL, which is derived from local Malaysian blood donors), on a presumptive basis [1, 14], at the discretion of the treating physician.

Virological methods. Viral isolation was attempted on all swab specimens, CSF specimens, and any serum samples remaining after other investigations had been completed through the inoculation of rhabdomyosarcoma (Rd) and 293 cells [13]. Isolated enteroviruses were typed by nucleotide sequencing of VP1 and VP4 genes [8, 15] and genogrouped by phylogenetic analysis [8–10]. Adenoviruses were cultured and typed in accordance with the methods described previously [13]. Paired serum samples (obtained on the day of admission, on day 7, or on the day of discharge) and CSF specimens were also tested for IgM against dengue and Japanese encephalitis virus (JEV) in parallel, using an IgM capture ELISA [16]. Seroconversion from a negative result to a positive result for a second sample or an increasing IgM optic density was considered evidence of acute infection; antibody in the CSF was diagnostic of flavivirus CNS infection [17, 18]. A decreasing IgM optic density was considered evidence of a recent infection, and low IgM optic density in the serum of children recently vaccinated against JEV was ascribed to vaccination.

Statistical analysis. Normally distributed data were compared using Student's t test; data that were not normally distributed were compared by the Mann-Whitney U test (Staview 4.02; Abacus Concepts). Differences between proportions were tested using the χ^2 test with Yates's correction or Fisher's exact test (EpiInfo, version 6; Centers for Disease Control and Prevention). Variables that might relate to disease pathogenesis that were associated with CNS disease in univariate analyses were also examined in a stepwise logistic regression (SPSS software, version 9; SPSS).

RESULTS

Epidemiology

During the study, 773 children (485 boys [63%]) were recruited. Five hundred forty children (70%) had mild HFMD; 83 (11%) had severe HFMD with no CNS complications; 102 (13%) had HFMD with CNS disease, 3 of whom died; and 48 (6%) had ASM, 1 of whom died. Most of the children were recruited during 2 large outbreaks of HEV-71–associated HFMD during 2000, 2001, and 2003 (figure 2). Genogroup analysis showed that in the outbreak that occurred during 2000–2001, most HEV-71 isolates belonged to genogroup B4 [9, 10], and some belonged to C1. In 2003, in addition to genogroup C1 strains, a previously undescribed genogroup, which has been named B5, emerged during the outbreak.

Most of the patients entered the study once, but 4 boys were admitted to the study twice. For the purposes of analysis, each hospitalization was considered to be a separate entity. Thirteen other children had also been hospitalized in the past with

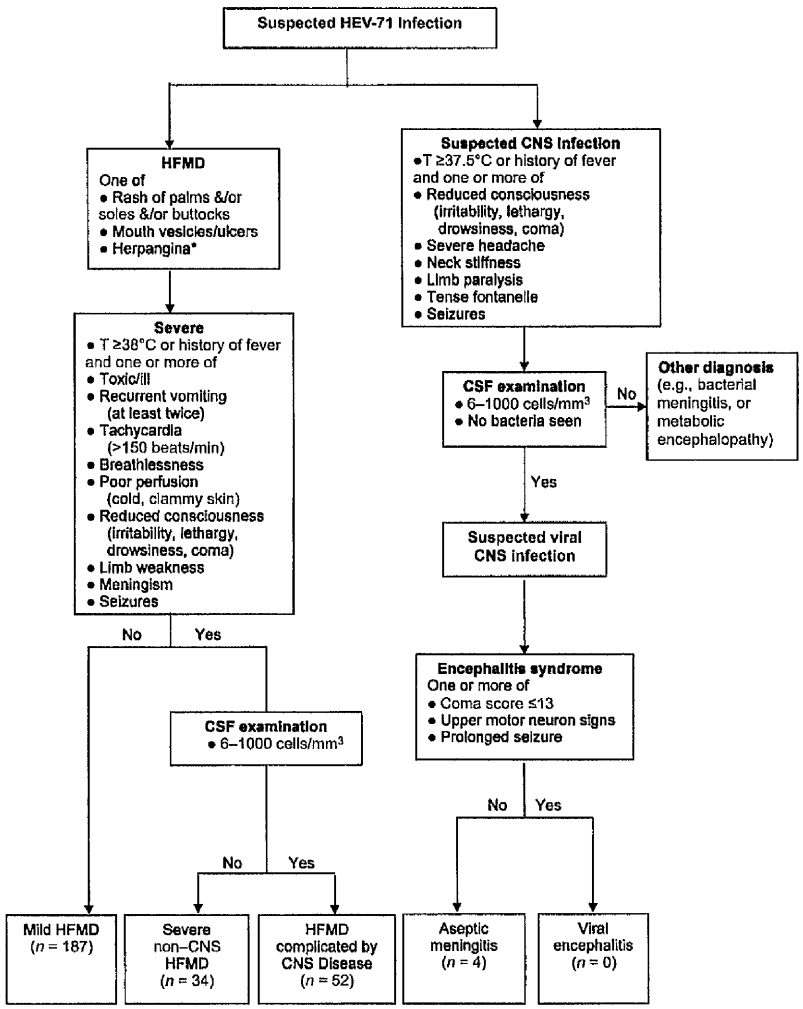


Figure 1. Algorithm showing the classification of patients in the study. Because human enterovirus (HEV)–71 can cause hand-foot-and-mouth disease (HFMD), CNS infection, or both, children with HFMD or suspected CNS infection were studied. Cases of HFMD were classified as mild HFMD, severe non-CNS HFMD, and HFMD complicated by CNS disease; children with viral CNS infection were classified as having aseptic meningitis (ASM) or viral encephalitis, but there were no children in the latter group infected with enteroviruses during this study. The final number of patients infected with HEV-71 in each diagnostic group is shown in the figure. T, temperature. *Herpangina was defined as multiple oral ulcers predominantly affecting the posterior parts of the oral cavity.

HFMD (outside the study). For 211 children (27%), there was a history of contact with another person infected with HFMD. One hundred three children (13%) had at least 1 other child in the family (sibling or cousin; maximum, 4) admitted into the study and were considered to be part of a family cluster. Seventy-seven of the children of these 44 family clusters had mild disease, 9 had severe non-CNS HFMD, and 17 had HFMD with CNS complications. When the index case of a family cluster could be identified clearly, they were no more or less likely to have CNS disease than subsequent cases. Forty-one of the children in family clusters had HEV-71 isolated (with only 1 genogroup isolated from each family); 3 of these children also had CAV-16 isolated; thus, in 2 families, both CAV-16 and HEV-71 were isolated. Twelve other children had CAV-16, and 3 had other viruses.

Virology

Dual infections. We attempted virus isolation on 3006 samples (622 throat swab specimens, 631 rectal swab specimens, 768 mouth ulcer swab specimens, 706 vesicle swab specimens, 91 serum samples, and 188 CSF samples) from 672 (87%) of the 773 patients seen during the study period. There were no important demographic differences between those who were investigated and those who were not investigated. Two hundred seventy-seven patients (41%) had HEV-71 isolated. Twenty-

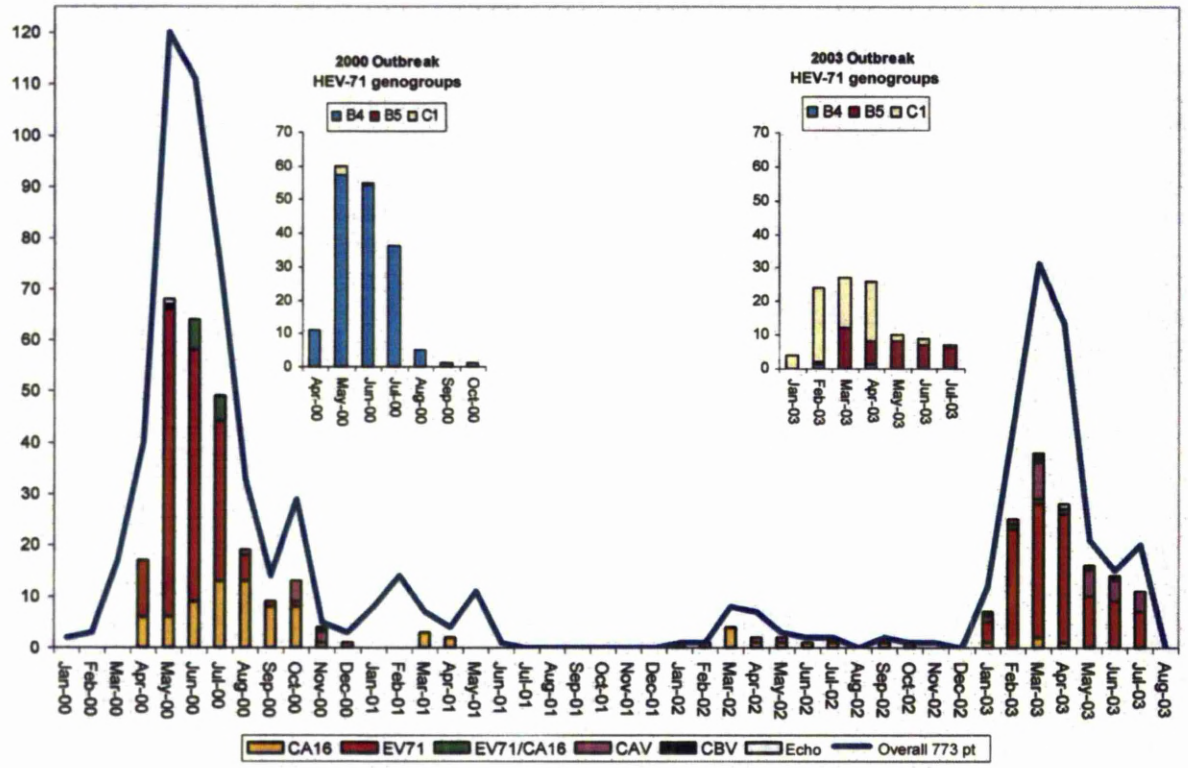


Figure 2. Epidemic curve of 773 children with hand-foot-and-mouth disease (HFMD) and/or aseptic meningitis (ASM) during the period from 1 January 2000 through 31 July 2003. The continuous line describes the total number of patients recruited into the study each month; the histogram below shows the number of patients with a positive virus isolate, who are grouped as follows: human enterovirus (HEV)–71, HEV-71 isolated; HEV-71/Coxsackie A virus (CAV)–16, HEV-71 and CAV-16 isolated; CAV-16, CAV-16 isolated; CAV, other CAVs isolated, but not CAV-16 or HEV-71; CBV, other CBVs isolated but not CAV-16 or HEV-71; and Echo, echoviruses isolated, but not CAV-16 or HEV-71. The inset boxes show the number of patients with different genogroups of HEV-71 isolates during the 2000 and 2003 outbreaks.

eight of these patients with HEV-71 had a second virus isolated, including 14 patients coinfected with CAV-16 and 9 coinfected with other enteroviruses (CAV-4, CAV-5, CA-16, CAV-10, Echo 25, CBV-1, and CAV-24); 1 of each was coinfected with an oral polio vaccine virus, an untyped enterovirus, adenovirus (Ad)–1 (isolated from the rectum), Ad7 (rectum), or an unidentified virus (isolated from the CSF). CAV-16 was isolated from an additional 85 patients, 4 of whom had coinfection; 2 had CBV-1 isolated from the throat, and 2 had adenoviruses isolated from the rectum (Ad2 in one and Ad4 in the other). Fifty-eight patients were infected with other enteroviruses, adenoviruses, or unidentified viruses (12 of whom had multiple viruses isolated). Most of the presenting clinical features of the HEV-71–infected and virus-isolation–negative patients were similar, with the exception of 26 (10%) of the 252 patients who had no virus isolated and had seizures, compared with only 6 (2%) of the 263 patients infected with HEV-71 (OR, 0.20; 95% CI, 0.07–0.53; $P < .001$).

Seven hundred sixteen patients had serum samples, and, when available, CSF specimens (233 patients) tested for antibodies to JEV and dengue. Three patients had serological evidence of acute JEV infection, including a girl with mild HFMD and HEV-71 isolated from vesicles and the throat and a boy with mild HFMD and fever who had HEV-71 isolated from vesicles and CAV-16 isolated from the throat that had acute peripheral JEV infection (seroconversion in the serum). In addition, an 18-month-old boy with HFMD and CNS disease (manifested by a generalized seizure and a CSF pleocyte level of 10 cells/μL) had CAV-16 isolated from a vesicle, which seroconverted to JEV in both the serum and CSF; he made a full recovery. Three patients had acute dengue. All had HFMD and CNS disease (defined as lethargy, irritability, and CSF pleocytosis) and HEV-71 isolated (1 from the CSF, 1 from the throat and vesicles, and 1 from vesicles and the rectum). Dengue was diagnosed by IgM seroconversion, but there was no dengue antibody in the CSF. Two patients with HFMD had serological evidence of a recent JEV infection or recent vaccination, and 6 patients had serological evidence of recent dengue. Only 2 of the children with CAV-16 and HFMD had CNS disease; 1 of these was the child coinfected with JEV described above and

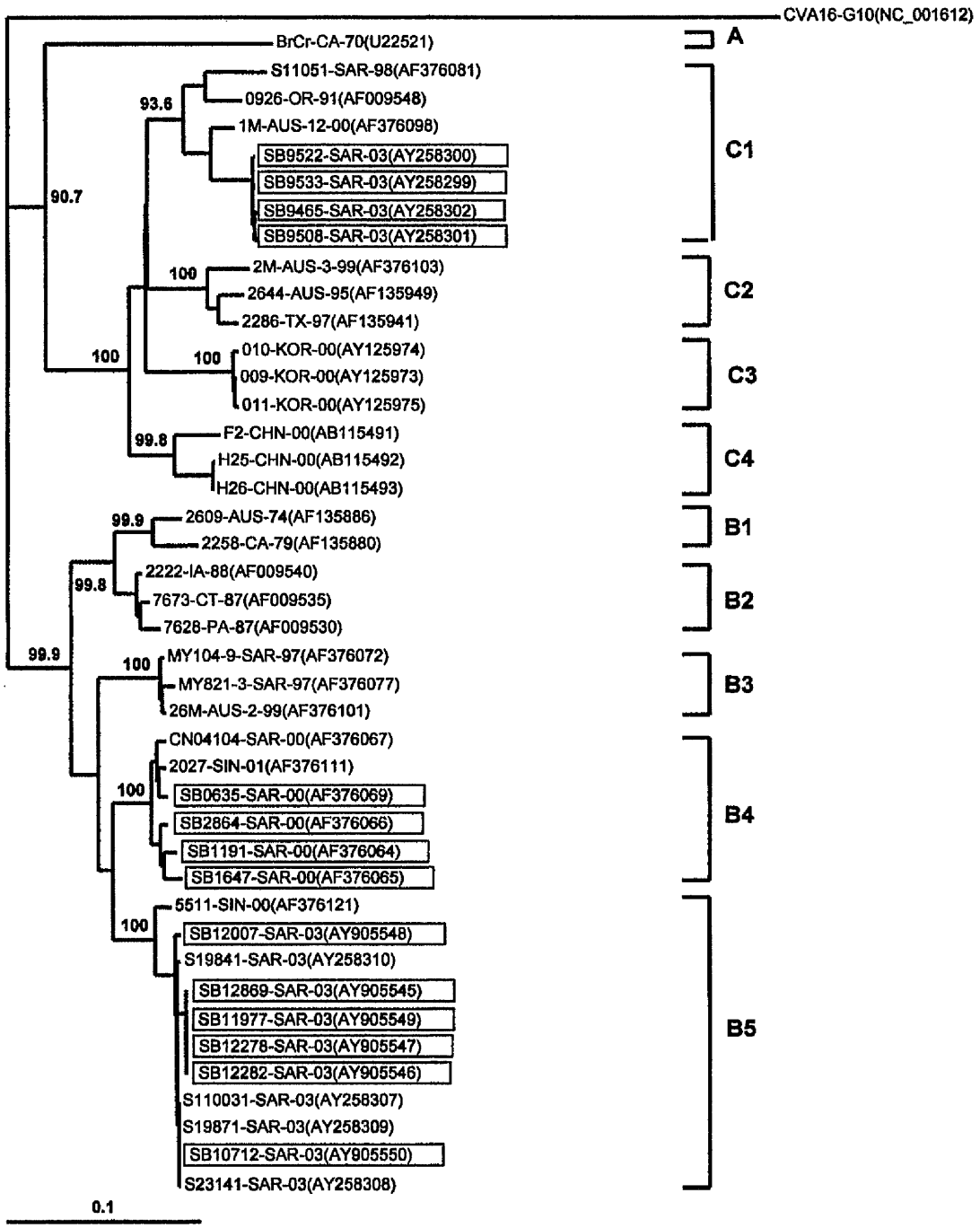
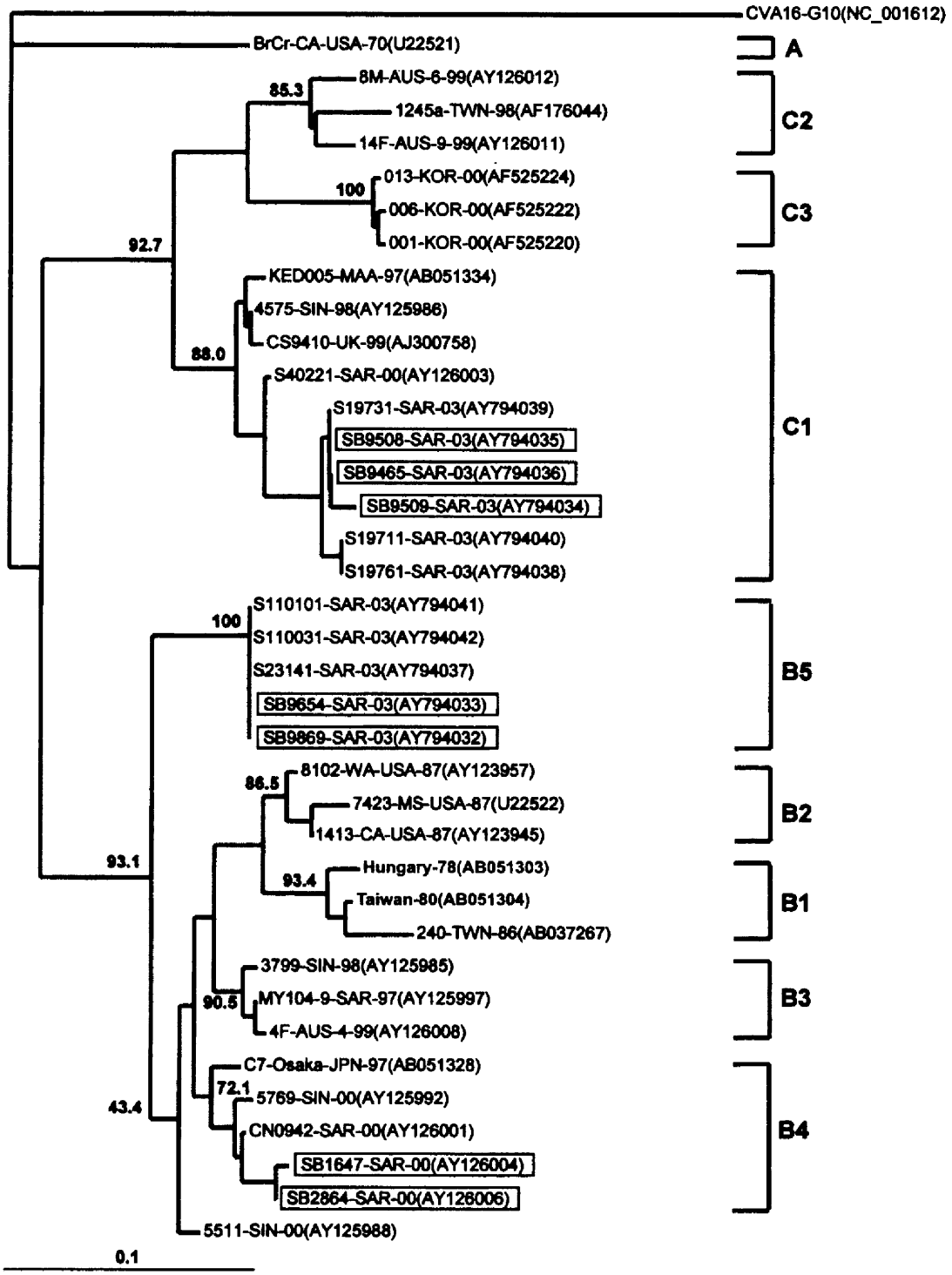


Figure 3. Neighbor-joining phylogenetic trees of human enterovirus (HEV)–71 strains isolated in this study. *A*, Tree based on the alignment of a partial VP1 (nucleotide positions 2442–3281) or complete VP1 (nucleotide positions 2442–3332) gene sequences. The tree shows 14 representative strains isolated in this study (boxed) and representative HEV-71 strains for other genogroups. *B*, Tree based on the alignment of a partial VP4 (nucleotide positions 744–950). The tree shows 7 representative strains isolated in this study (boxed) and representative HEV-71 strains for other genogroups. For both trees, sequence from the prototype Coxsackie A virus (CVA)–16-G10 was used as an outgroup. The year of isolates is given as a suffix, and Genbank accession numbers are in parentheses. Horizontal branch lengths are proportional to the number of nucleotide changes between viruses, and the scale and the bootstrap values in 1000 pseudoreplicates for major lineages within the dendrogram are shown as percentages. PCR products were sequenced using Big Dye, version 3.0, and run on an ABI377 automated sequencer (Applied Biosciences), and nucleotide sequences were aligned (DNASTAR).



(Figure 3 continued)

for the other child, CAV-16 was isolated from rectal and vesicle swab specimens, and CBV-1 was isolated from the throat.

HEV-71 genogroups. Phylogenetic analysis of all 277 HEV-71 isolates showed that 168 (61%) of the isolates belonged to genogroup B4 (165 of these were isolated in 2000, 1 was isolated in 2002, and 2 were isolated in 2003), and 68 (25%) of the isolates belonged to C1 (4 of these were isolated in 2000, and 64 were isolated in the 2003 outbreak); in addition, 41 HEV-71 strains (15%) isolated in 2003 were from a new genogroup (B5) that emerged during the course of the study [9, 19, 20]. Neighbor-joining trees of the VP1 (figure 3*A*) and VP4 (figure 3*B*) genes revealed that representative HEV-71 strains from all 3 genogroups (B4, B5, and C1), described at Sibu, clustered tightly with bootstrap support values >90% and >70%, respectively.

Clinical Features of HEV-71 Infection

Nearly two-thirds of the patients with HEV-71 infection had mild HFMD; 12% had severe non-CNS disease, 19% had HFMD with CNS disease, and only 1% had ASM (table 1). For the purposes of analysis, patients with CNS infection, defined by a CSF pleocytosis (with or without HFMD), were compared with patients with no CNS disease (including mild and severe non-CNS HFMD). Patients with CNS disease were younger and were more likely to give a history of fever, vomiting, mouth ulcers, breathlessness, cold limbs, poor urine output, and neurological features, including altered sensorium and seizures (table 1). During examination, patients with CNS disease were more likely to look toxic, be dehydrated and febrile, have altered consciousness, and experience neck stiffness, but they were less likely to have mouth ulcers (table 2). Excluding the 4 patients with ASM and no rash did not significantly alter these findings. The CSF was examined in 90 children (34%) infected with HEV-71, of whom 56 (62%) had a CSF WBC count >5 cells/μL. For these patients, the median WBC count was 75 cells/μL (range, 6–1090 cells/μL). Typically, this was a lymphocytic CSF with a normal glucose ratio, but 12 (21%) had neutrophil predominance, and 10 (18%) had a CSF to plasma glucose ratio <50%.

Three children had HFMD complicated by acute flaccid paralysis; 1 had HEV-71–B4 genogroup isolated from his throat, and the 2 others had negative culture results. Sixty-two children with severe HFMD had cardiac enzyme levels measured (27 children with HEV-71). Seventy-eight of the 233 patients with more severe HFMD were treated with IVIG on a presumptive basis, including 42 patients who were infected with HEV-71. Five patients with HEV-71 required ionotropic support, and 3 required ventilation. Four children died, including 2 who had HEV-71 isolated (1 genogroup B4 and 1 genogroup C1).

Pathogenesis of Neurological HEV-71 Disease

Children from whom HEV-71 and another virus were isolated were no more likely than those with HEV-71 alone to have neurological involvement (2 [7%] of 28 vs. 54 [22%] of 249; OR, 0.28; 95% CI, 0.04–1.26; $P = .1167$). Repeating this analysis excluding children with only rectal isolates (which could represent continued carriage rather than acute pathogenic infection) did not alter these findings (2 [7%] of 28 vs. 47 [21%] of 226; OR, 0.29; 95% CI, 0.05–1.34; $P = .1407$). All 3 of the children with HEV-71 and serological evidence of acute dengue infection had HFMD with CNS disease, compared with 52 (20%) of 264 HEV-71–infected children with no dengue infection ($P = .008$). In contrast, the 2 HEV-71–infected children with serological evidence of JEV infection (as shown by seroconversion in the serum) had HFMD with no CNS disease, compared with 55 (21%) of 266 children with no JEV infection ($P = 1$).

To examine the possible role of different HEV-71 genogroups in the clinical phenotype of HEV-71 infection, we determined the likelihood of CNS disease for each genogroup. Children with B4 genogroup were less likely to have CNS infection than were those with other genogroups; 26 (15%) of 168 children infected with genogroup B4 had CNS infection, compared with 30 (28%) of 109 with other genotypes (OR, 0.48; 95% CI, 0.26–0.91; $P = .0223$) (table 2). Pair-wise comparisons of the genogroups showed that this occurred because genogroup B4 was associated with less risk of CNS disease than genogroup C1 (26 [15%] of 168 vs. 19 [28%] of 68; OR, 0.47; 95% CI, 0.23–0.98; $P = .0429$), whereas there was no altered risk for genogroup B4 versus B5 (26 [15%] of 168 vs. 11 [27%] of 41; OR, 0.50; 95% CI, 0.21–1.21; $P = .139$) or for B5 versus C1 (11 [27%] of 41 vs. 19 [28%] of 68 [28%]; OR, 0.95; 95% CI, 0.36–2.45; $P = .9240$). Children with HEV-71 genogroup B4 were also less likely to be part of a family cluster; 12 (7%) of 168 children with genogroup B4 positive were in family clusters versus 29 (27%) of 109 children with other genogroups (OR, 0.21; 95% CI, 0.10–0.46; $P < .0001$). In contrast, those with B5 genogroup were more likely to be part of a family cluster; 17 (41%) of 41 children with genogroup B5 were in a cluster versus 24 (10%) of 236 children with other genogroups (OR, 6.26; 95% CI, 2.77–14.18; $P < .0001$). Genogroup, age, and being part of a family cluster were initially entered into a multiple logistic regression model; increasing age and genogroup B4 versus C1 were associated with a reduced risk of CNS infection (table 3), whereas being in a family cluster did not affect risk.

DISCUSSION

Since 1997, HEV-71 infection has become a major public health problem in developed and developing countries in the Asia-Pacific region, and it has the potential to spread further. Mo-

Table 1. Features in the history for 277 patients who had human enterovirus (HEV)–71 isolated.

Characteristic	Patients with non-CNS disease			Patients with CNS disease			OR (95% CI)	*P*
	Mild HFMD	Severe Non–CNS HFMD	Total	HFMD with CNS complications	ASM	Total		
No. (%) of patients	187 (68)	34 (12)	221 (80)	52 (19)	4 (1)	56 (20)	...	...
Age, median months (range)	32 (6–112)	24 (5–97)	31 (5–112)	23 (5–87)	25.5 (6–58)	23 (5–87)	...	.003
Male sex	120	18	138	38	3	41	1.64 (0.82–3.32)	.1321
Known contact with a person infected with HFMD	60	15	75	21	0	21	1.17 (0.61–2.24)	.9632
Fever before admission	141	29	170	51	4	55	16.5 (2.37–328.65)	.0003
Coryza	61	13	74	19	3	22	1.29 (0.67–2.45)	.4151
Cough	48	12	60	19	1	20	1.49 (0.76–2.90)	.1364
Vomiting	19	12	31	22	1	23	4.27 (2.11–8.65)	<.0001
Diarrhea	3	2	5	3	1	4	3.32 (0.63–15.95)	.0851
Mouth ulcers	175	31	206	46	0	46	0.33 (0.13–0.86)	.0098
Breathlessness	1	0	1	5	0	5	21.57 (2.31–1024.52)	.0015
Cold limbs	1	0	1	4	0	4	16.92 (1.61–837.01)	.0065
Reduced urine output	15	7	22	12	1	13	2.37 (1.19–6.22)	.0076
Headache	2	3	5	4	0	4	3.32 (0.63–15.95)	.08
Irritability	14	12	26	21	1	22	4.85 (2.34–10.06)	<.0001
Lethargy/drowsiness	21	16	37	30	3	33	7.14 (3.60–14.24)	<.0001
Seizures	0 (0)	1 (2.9)	1 (0.5)	3 (5.8)	2 (50.0)	5 (8.9)	21.57 (2.31–1024.52)	.0015

NOTE. Data are no. (%) of patients, unless otherwise indicated. OR (95% CI) and *P* values are for the comparison of all patients with CNS disease versus all those with non-CNS disease. ASM, aseptic meningitis; HFMD, hand-foot-and-mouth disease.

Table 2. Examination and investigation findings for 277 patients who had human enterovirus (HEV)–71 isolated.

Characteristic	Patients with non–CNS disease			Patients with CNS disease			OR (95% CI)	*P*
	Mild HFMD	Severe Non–CNS HFMD	Total	HFMD with CNS complications	ASM	Total		
No. (%) of patients	187 (68)	34 (12)	221 (80)	52 (19)	4 (1)	56 (20)	...	...
Examination findings								
Toxic	4	24	28	33	2	35	11.49 (5.59–23.83)	<.0001
Dehydration	9	3	12	11	2	13	5.27 (2.08–13.37)	<.0001
Admission temperature, median °C (range)	37 (36.4–38.20)	37.7 (36.5–39)	37.1 (36.4–39)	38 (36.5–39.5)	38.9 (37.1–39.9)	38 (36.5–39.9)	...	<.0001
Pulse of >150 beats/min	2	0	2	7	2	9	20.97 (4.09–202.37)	<.0001
Respiratory abnormalities	0	0	0	4	0	4	...	.0015
Hepatomegaly	12	4	16	12	0	12	3.49 (1.43–8.49)	.0017
Rash	184	34	218	52	2	54	0.37 (0.04–4.57)	.2663
Mouth ulcer	180	32	212	47	0	47	0.22 (0.08–0.65)	.0011
Herpangina	3	0	3	0	0	0	0 (0.00–9.62)	>.9999
Irritable	0	7	7	11	0	11	7.47 (2.51–22.79)	<.0001
Lethargic/drowsy	1	13	14	27	1	28	14.79 (6.56–33.80)	<.0001
Neck stiffness	0	0	0	3	1	4	...	.0015
Investigation findings								
Plasma glucose level								
Median mmol/L (range)	5 (2.9–11.4)	5.3 (2.4–7.7)	5.1 (2.4–11.4)	6.1 (2.8–24.1)	6.3 (4.8–8.8)	6.1 (2.8–24.1)	...	.0388
Patients with plasma glucose tested	31	24	55	44	4	48	...	...
HEV-71 genogroup								
Genogroup B4	120	22	142	24	2	26	0.48 (0.26–0.91)	.0223
Genogroup B5	24	6	30	10	1	11	1.56 (0.68–3.53)	.3515
Genogroup C1	43	6	49	18	1	19	1.8 (0.91–3.57)	.0985

NOTE Data are no. (%) of patients, unless otherwise indicated. ASM, aseptic meningitis; HFMD, hand-foot-and-mouth disease. Repeating the analysis for 254 children (using only the first child from each family cluster) did not significantly alter any of the associations.

Table 3. Multivariate logistic regression analysis of variables associated with CNS disease in human enterovirus (HEV)–71 infection.

Characteristic	β Coefficient	OR (95% CI)	P
Age	−0.022	0.978 (0.962–0.996)	.015
Genogroup C1 vs. B4	0.751	2.12 (1.070–4.202)	.031
Genogroup B5 vs. B4	0.795	2.215 (0.972–5.048)	.058

NOTE. Genogroup, age, and being part of a family cluster were initially entered into a multiple logistic regression model. Terms were entered into the model and remained only if they were statistically associated with CNS disease ($P < .05$). Both forward selection and backward elimination methods were used. Increasing age and genogroup B4 versus C1 were associated with a reduced risk of CNS infection, whereas being in a family cluster did not affect risk. Forward selection and backward elimination procedures generated the same model, indicating its robustness. The Hosmer-Lemeshow statistic indicated a nonsignificant lack of fit (χ^2, 8.338; $P = .401$).

lecular epidemiological studies have documented remarkable changes in the circulating HEV-71 genogroups in the Asia-Pacific region during this time [9, 10, 21–23]. However, whether such genetic differences explain the differing clinical presentations is not clear [4, 24]. Other postulated factors include differences in pre-existing immunity of the pediatric population [25], differences in host genetic susceptibility [12, 26, 27], and coinfection with other viruses [1]. Our detailed investigation of 773 patients, which included 277 patients with HEV-71 infection, has produced intriguing clinical epidemiological evidence and suggests that the behavior of genogroups of HEV-71 may differ. Children infected with genogroup B4 were less likely to present with CNS infection than those infected with C1 or B5 and were also less likely to be part of a family cluster. In contrast, children infected with B5 were more likely to be part of a family cluster, and there was a trend towards a greater incidence of CNS disease in these patients.

However, our findings must be interpreted with caution. Although we attempted to minimize confounding variables, there were factors beyond our control. In particular, the likelihood of children presenting to the hospital with HFMD may have varied during the study, although altered referral rates would be unlikely to explain the different risk of family clustering for the different genogroups. There may also have been other inherent features that differed between the cohort studied in 2000 and 2003, although the basic demographic features were similar (data not shown).

Our study has also confirmed findings from earlier reports that young age, fever, vomiting, and hyperglycemia are associated with severe HEV-71 infection [28, 29]; in addition, we found tachycardia, breathlessness, and the absence of mouth ulcers were associated with CNS disease. Overall, the incidence of severe disease was milder in our study than in previous studies, because only 4 fatalities occurred in our study, all of which were a result of pulmonary edema. In addition, our series was unusual in having only 4 patients with pure aseptic meningitis.

Our study has also made an important contribution to the debate about the role of dual infections in viral CNS disease. As others have shown previously [30], we found several enteroviruses were cocirculating during the HFMD outbreaks. We also documented instances of coinfection of HEV-71 with other enteroviruses in individual patients. We found no evidence of an increased likelihood of CNS disease in such patients, although we did find an intriguing association of coinfection with dengue viruses and neurological presentation. Previously, we postulated that a new adenovirus type 21 might make an important contribution to fatal disease during an HEV-71–associated HFMD outbreak [1, 13]; in this new study, we isolated adenoviruses from only 4 patients, and none was adenovirus type 21. Our rigorous investigations for multiple viral agents showed the importance of pursuing all possible avenues before attributing causation to the first pathogen encountered. For example in 2 children, CNS disease would have been attributed to CAV-16, which is an unusual cause of CNS disease, had our thorough diagnostic examination not revealed alternative, more likely viral causes (JEV in 1 child and CBV-1 in the other). Whether unsuspected dual infections lie behind other unusual manifestations of common, relatively benign enteroviruses, such as acute flaccid paralysis recently attributed to CAV-24 [31], is not known .

In summary, our detailed study provides clinical epidemiological evidence for different biological behavior of HEV-71 genogroups, with regard to transmissibility within families and risk of CNS disease. It also highlights the importance of detailed investigations for multiple pathogens during HFMD outbreaks. Only with such thorough investigations will the pathophysiology of HEV-71 be fully determined.

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Evaluation of Different Clinical Sample Types in Diagnosis of Human Enterovirus 71-Associated Hand-Foot-and-Mouth Disease[▽]

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Human enterovirus 71 and coxsackievirus A16 are important causes of hand-foot-and-mouth disease (HFMD). Like other enteroviruses, they can be isolated from a range of sterile and nonsterile sites, but which clinical sample, or combination of samples, is the most useful for laboratory diagnosis of HFMD is not clear. We attempted virus culture for 2,916 samples from 628 of 725 children with HFMD studied over a 3 1/2-year period, which included two large outbreaks. Overall, throat swabs were the single most useful specimen, being positive for any enterovirus for 288 (49%) of 592 patients with a full set of samples. Vesicle swabs were positive for 169 (48%) of 333 patients with vesicles, the yield being greater if two or more vesicles were swabbed. The combination of throat plus vesicle swabs enabled the identification of virus for 224 (67%) of the 333 patients with vesicles; for this patient group, just 27 (8%) extra patients were diagnosed when rectal and ulcer swabs were added. Of 259 patients without vesicles, use of the combination of throat plus rectal swab identified virus for 138 (53%). For 60 patients, virus was isolated from both vesicle and rectal swabs, but for 12 (20%) of these, the isolates differed. Such discordance occurred for just 11 (10%) of 112 patients with virus isolated from vesicle and throat swabs. During large HFMD outbreaks, we suggest collecting swabs from the throat plus one other site: vesicles, if these are present (at least two should be swabbed), or the rectum if there are no vesicles. Vesicle swabs give a high diagnostic yield, with the added advantage of being from a sterile site.

Hand-foot-and-mouth disease (HFMD) is a common febrile illness in young children and is characterized by lesions on the skin and oral mucosa. The skin rash, which may be maculopapular or vesicular, typically occurs on the palms and soles but can also involve the buttocks, elbows, and knees. Mouth ulcers are the most common enanthema, but some patients have herpangina (multiple oral ulcers affecting predominantly the posterior part of the oral cavity), and others have no oral lesions (16, 20).

Many human enteroviruses (family *Picornaviridae*, genus *Enterovirus*) can cause HFMD, but human enterovirus 71 (HEV71) and the closely related coxsackievirus A16 (CVA16) are the most important (16, 20). Since the late 1990s, HEV71 has caused a series of large HFMD epidemics in the Asia-Pacific region, associated with a rapid fulminant course, severe neurological complications, and a large number of fatalities (1–4, 8–11, 14, 17, 18, 21). CVA16 causes a similar clinical illness initially, but neurological and other severe complications are extremely rare (5). In much of Asia, there is now epidemiological and virological surveillance for HFMD so that effective public health measures, such as closing nurseries and schools, can be instituted early. However, because of the similar clinical presentations of the viruses, establishing the actual cause of HFMD cases relies on laboratory identification of the virus. Diagnostic techniques include isolating the virus in susceptible continuous cell lines or detecting viral RNA by PCR (12, 28). Though laborious and time consuming, virus isolation remains the gold standard for enterovirus diagnosis; it is cheaper than PCR and is the most widely used method, particularly in developing countries.

There is a wide range of samples from which virus isolation can be attempted, including rectal and throat swabs, serum, and cerebrospinal fluid (CSF) (when taken) and vesicles and ulcers when they are present. However, for HEV71-associated HFMD outbreaks, there has been relatively little work examining which sample, or combination of samples, is the most useful. This question becomes especially important in the context of large outbreaks with many thousands of patients. Rectal and throat swabs are available for all patients and do not require the presence of mucocutaneous stigmata. However, they have the disadvantage that, because they are not sterile sites, isolation of virus there may represent coincidental asymptomatic carriage rather than the causative agent (24): many enterovirus infections are asymptomatic, and viral shedding may persist for up to 2 weeks from the throat and up to 11 weeks from the rectum (7, 20, 24). In the absence of virus isolation from a sterile site, isolates from nonsterile sites are usually accepted as surrogate markers for enterovirus infec-

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tions (20, 22, 24), but there is little data available on the validity of this approach for HEV71-associated HFMD. We therefore set out to answer three important clinical microbiological questions during a 3 1/2-year prospective clinical and diagnostic study of HFMD, which included two large outbreaks: first, which single specimen is most often positive for the different HFMD patient groups; second, which combination of samples is the most efficient in terms of diagnostic yield; and third, how reliable samples from nonsterile sites are compared with those from sterile sites.

MATERIALS AND METHODS

Clinical and laboratory methods. Between January 2000 and July 2003, we studied all children with HFMD in the pediatric ward and intensive care unit at Sibu Hospital, Sarawak, Malaysia. Children were defined as having HFMD if they had new onset of at least one of the following: maculopapular and/or vesicular rash on the palms and/or soles, vesicles or ulcers in the oral cavity, or herpangina (defined as multiple oral ulcers affecting predominantly the posterior parts of the oral cavity). All children with HFMD admitted into the hospital were assessed by the pediatricians of the study team. A detailed history and results of a clinical examination, including an examination for mucocutaneous lesions, were recorded on standardized forms. A rectal swab and a throat swab were taken for each child. The skin was examined carefully for vesicles, and the oral cavity for ulcers; if they were present, swabs were taken from at least one of each (usually the largest and most accessible lesions). Rectal swabs were taken with a gentle circular motion on the rectal wall. Throat swabs were taken with the aid of a tongue depressor, by carefully swabbing the lateral and posterior pharynx without touching the tongue or buccal mucosa. For vesicle swabs, the skin was cleaned gently with 0.9% sterile normal saline, but not with alcohol, which kills viruses. A sterile 24-gauge needle was used to rupture the vesicle, and a swab was used to absorb the fluid. Alternatively, the swab was gently rolled over the vesicle to squeeze out fluid. Mouth ulcers were sampled by rolling the swab over the floor of the ulcer. When more than one vesicle or ulcer was swabbed, a fresh swab was used for each lesion and put into a separate tube of viral transport material, because we were interested in the yield from each swab. Swab samples were collected by study team members or by nursing staff, after training.

CSF and serum were collected from children with suspected central nervous system (CNS) involvement if they had a history of fever, or fever on examination (≥38°C), and at least one of the following: toxic and ill in appearance, recurrent vomiting (at least twice), tachycardia (heart rate, >150/min), breathlessness, poor perfusion (cold, clammy skin), reduced consciousness (irritability, lethargy, drowsiness, coma), limb weakness, meningism (neck stiffness or positive Kernig's sign), or seizures.

The clinical samples were stored immediately in a −70°C freezer until further testing. Out of hours, when immediate storage at −70°C was not possible, clinical samples were stored at 4°C overnight and were transferred to a −70°C freezer the following morning. Five percent of samples were handled in this way, but their isolation rates did not differ significantly from those of other samples. Virus isolation was attempted for all swabs and for CSF and any serum which had adequate volume. Specimens were inoculated into rhabdomyosarcoma (RD) and 293 cells as described previously (1, 19). Enteroviruses isolated were typed by nucleotide sequencing of the VP1 or the VP4 genes (2, 13).

For the purposes of analysis, swabs from herpangina lesions were grouped with those from other mouth ulcers. Vesicles, serum, and CSF were considered sterile sites, and the throat, mouth ulcers, and the rectum were considered nonsterile sites. All samples were investigated, irrespective of the results for other samples from the same patient.

Analytical approach. The patients were divided into four groups according to their presenting mucocutaneous lesions and, thus, the availability of samples: those with a papulovesicular rash and mouth ulcers (referred to hereafter as HFMD with vesicles and ulcers), those with a papulovesicular rash only (HFMD with vesicles), those with a maculopapular rash and mouth ulcers only (HFMD with ulcers), and those with maculopapular rash only (HFMD with maculopapular rash). As we were interested in which combination of samples gives the best diagnostic yield, we adopted a stepwise approach to the analysis for each patient group. First, we determined which sample type gave the most positive results. Then we looked at the remaining undiagnosed patients and determined which of the remaining samples gave the most positive results. We continued in this manner until all sample types had been assessed. We decided to use data from the first outbreak to determine the usefulness of different samples and combinations of samples. We then applied these findings to the second outbreak to see if the predicted samples remained useful. However, the sample analysis was not begun until the end of the study, to avoid any bias in sample collection.

TABLE 1. Relationship between the number of vesicle and ulcer swabs collected and the number positive

Sample type	No. of swabs per patient	No. of patients with at least one swab positive/no. of patients tested (%)
Vesicle	1	62/177 (35)
	2	40/81 (49)
	3	18/30 (60)
	≥4	46/61 (75)
Ulcer	1	26/152 (17)
	2	21/103 (20)
	3	37/77 (48)
	≥4	13/40 (33)

Statistical analysis. Statistical analysis was performed by using the statistical software Statview 4.02 (Abacus Concepts, Inc.). Sensitivity, specificity, positive predictive value, and negative predictive value were calculated from a 2 × 2 table.

Ethical approval. The study was approved by the Director of Health for Sarawak (Malaysia) and the Ethics Committee of the Liverpool School of Tropical Medicine (Liverpool, United Kingdom). Informed consent was obtained from each child's accompanying parent or guardian.

RESULTS

Seven hundred twenty-five patients were entered into the study: 471 (299 [63%] males, with a median age of 28 months [range, 4 to 120 months]) were enrolled in the first half (the majority from an outbreak between January 2000 and March 2001) and 254 (158 [62%] males, with a median age of 28 months [range, 2 to 153 months]) were enrolled in the second half (mostly during an outbreak between January and July 2003). The 471 patients in the first half of the study included 110 patients with vesicles and ulcers, 112 patients with vesicles only, 78 patients with ulcers only, and 171 patients with a maculopapular rash only. Of the 254 patients in the second half of the study, 98 had vesicles and ulcers, 29 had vesicles only, 87 had ulcers only, and 40 had a maculopapular rash only. The median duration of illness before admission was 2 days (range, 0 to 8 days) and did not differ significantly between patient groups.

Virology results. We attempted viral isolation for 2,916 samples: 1,666 samples from 389 (83%) of the 471 patients in the first half of the study and 1,250 samples from 239 (94%) of the 254 patients in the second half of the study. For most patients, a single throat swab and single rectal swab were cultured. In addition, for 127 patients with vesicles, at least one (median, 2; range, 1 to 10) vesicle was investigated, and for 185 patients with ulcers, at least one (median, 2; range, 1 to 6) ulcer sample was investigated. For a single swab, 35% of vesicle and 17% of ulcer samples were positive (Table 1), but the percentages increased as more swabs were taken.

The number of patients tested for each sample type and the number that tested positive for any enterovirus, for HEV71, and for CVA16 are shown in Table 2. During the first half of the study, a throat swab was most likely to be positive (being positive for any enterovirus for 191 [52%] of 367 patients), followed by vesicle, ulcer, and then rectal swabs. During the

TABLE 2. Positive isolation rates for different viruses according to sample type[a]

Sample type	No. of patients with positive results/total no. of patients tested (%)							
	First half of study (n = 471)				Second half of study (n = 254)			
	HEV71 (n = 167)	CVA16 (n = 80)	Any HEV (n = 255)	All (n = 389)	HEV71 (n = 106)	CVA16 (n = 19)	Any HEV (n = 153)	All (n = 239)
Rectal	65/166 (39)	22/78 (28)	100/252 (40)	100/378 (27)	38/103 (37)	7/16 (44)	68/147 (46)	68/229 (30)
Throat	127/164 (77)	50/76 (66)	191/248 (77)	191/367 (52)	66/104 (64)	9/15 (60)	101/148 (68)	101/231 (44)
Vesicle	66/111 (60)	36/57 (63)	106/167 (63)	106/222 (47)	54/81 (67)	10/13 (77)	63/97 (65)	63/127 (50)
Ulcer	36/85 (42)	11/42 (26)	53/129 (41)	53/188 (28)	28/87 (32)	7/15 (47)	44/117 (38)	44/185 (24)
Serum	0/9 (0)	1/3 (33)	1/10 (10)	1/20 (5)	2/26 (8)	1/11 (9)	6/43 (14)	6/61 (10)
CSF	2/43 (5)	0/11 (0)	2/58 (3)	2/96 (2)	0/29 (0)	0/0 (0)	1/42 (2)	1/60 (2)

[a] The number of patients with positive results for each sample type is shown as a proportion of the HEV71-positive patients, the CVA16-positive patients, all HEV-positive patients, and all patients (whether their samples tested positive or negative). Eleven patients in the first half of the study and three in the second half of the study had co-isolation of HEV71 and CVA16.

second half of the study, vesicle swabs were most likely to be positive (positive for any enterovirus for 63 [50%] of 127 patients), followed by throat, rectal, and then ulcer swabs. Most viruses (>95%) were isolated following a single passage.

In the first half of the study, 167 (65%) of 255 patients with positive viral isolation were HEV71 positive (11 of whom were also infected with CVA16, 2 with CVA4, 2 with CVA24, and 1 with adenovirus 7 [Ad-7]); CVA16 was isolated from a further 69 patients (2 of whom were also infected with another virus). In addition, 19 patients were infected with other enteroviruses, Ads, or unidentified viruses (6 of whom had multiple viruses isolated). In the second half of the study, 106 (44%) of 239 patients had HEV71 isolated, 10 of whom had a second virus isolated: there were 3 with CVA16, 2 with CVA5, and 1 each with CVA10, poliovirus 1 Sabin vaccine strain, Ad-1, an untypeable enterovirus, and an unidentified virus. CVA16 was isolated from 16 further patients (2 of whom were also infected with a second virus, Ad2 or Ad4). In addition, 31 patients were infected with other enteroviruses, Ads, or unidentified viruses (4 of whom had multiple viruses isolated). For most patients with multiple isolates, the viruses came from different clinical samples; however, in 20 cases, two viruses were isolated from the same clinical sample. These comprised nine rectal swabs, five throat swabs, and two ulcer swabs, which gave different isolates in different cell lines; in addition, four patients with mild HFMD had two different viruses grown from different vesicles (three with HEV71 and CVA16 and one with HEV71 and P1S). The serotyping of enterovirus isolates was repeated and verified.

Across the whole study, 79 (51%) of 156 patients who required CSF examination had elevated CSF cell counts (>5/mm^3), but only 3 had virus cultured (two HEV71 and one other). Enteroviruses were isolated from the serum of 7 (9%) of 81 patients: two of these were identified as HEV71, two as CVA16, and one (each) as CVA6, CVA9, and CVA10.

Analysis of sample combinations during the first outbreak. Figure 1A shows, for each patient group, the possible incremental increases in the numbers of patients diagnosed virologically, by different combinations of samples assessed stepwise according to the analytical plan. For this part of the analysis, only patients with full sets of samples were studied. For vesicles and ulcer swabs, the results for multiple swabs of a single type were treated as a single result, so that at least one swab testing positive was taken as a positive result for that sample type. Figure 1B shows similar data for a later outbreak.

For the 105 patients with vesicles and ulcers in the first half of the study, use of the throat swab alone diagnosed 63 patients (60%), whereas use of the vesicle swabs alone would have diagnosed 54 patients, the rectal swab alone 29 patients, and the ulcer swab alone 26 patients. Throat swabs were therefore taken as the first sample (Fig. 1A). The number of patients with a virological diagnosis (the diagnostic yield) would increase to 73 patients if the results for vesicle swabs were added next, 70 if results for rectal swabs were added, or 68 if results for ulcer swabs were added. The vesicle swab results were therefore added as a next step. From here, the addition of the rectal swab results would increase the yield to 79 patients, whereas ulcer swab results would increase it to 75 patients; the rectal swab was therefore added next, and the ulcer swab was added last, increasing the yield to 82 patients (78%).

For patients with HFMD and a vesicular rash, a throat swab was again the most useful single sample, allowing for the diagnosis of 57 (52%) of 109 patients. The addition of the rectal swab result would increase the diagnostic yield to 70 (64%), whereas the addition of the vesicle swab result would increase it to 72 (66%). The vesicle swab result was therefore used as the second investigation; finally, the addition of the rectal swab result increased the number of patients diagnosed to 79 (72%). For patients with ulcers only, a throat swab result diagnosed virologically 33 (48%) of 69 patients; the addition of either the rectal or vesicle swab result increased the diagnostic yield to 38 patients (55%), and results from the combination of all three samples diagnosed 42 patients (61%). Finally, for patients with a maculopapular rash only, results from a throat swab alone diagnosed 35 (43%) of 82 patients, whereas results from a rectal swab alone diagnosed 22 patients. A throat swab was therefore used as the first sample, followed by the rectal swab, which increased the number of patients diagnosed to 44 (54%) of 82 patients.

In Fig. 2A, the proportion of patients diagnosed at each step,by use of the best combination of samples as determined above, is compared with the total number of samples analyzed at each step. It is clear that although the detection rate increased as more clinical sample types were included, the number of samples analyzed increased to a much greater extent. For example, for the patients with vesicles and ulcers, 63 patients were diagnosed by

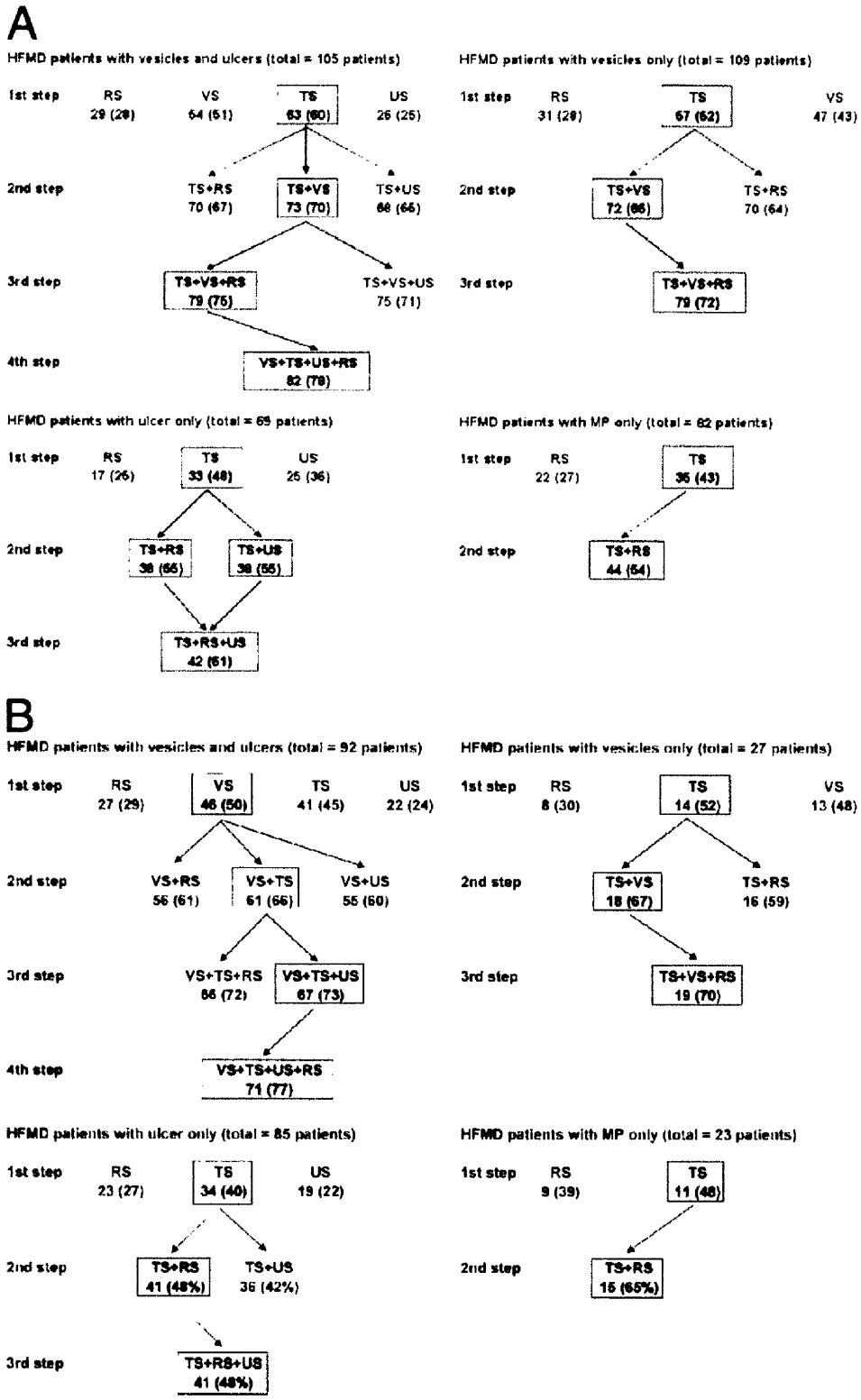

FIG. 1. Analysis of which combination of samples gave the greatest diagnostic yield for the four groups of HFMD patients, assessed stepwise according to the analytical plan during the first half of the study (A) and second half of the study (B). The number (%) of positive patients for each sample type at each step is shown; the boxed sample gave the greatest diagnostic yield and thus was the one used for the next step. Only patients with complete sets of samples were analyzed. For the first half of the study, this comprised 105 (95%) of 110 HFMD patients with vesicles and ulcers, 109 (97%) of 112 with vesicles only, 69 (88%) of 78 with ulcers only, and 82 (48%) of 171 with maculopapular rash. For the second half, this comprised 92 (94%) of 98 HFMD patients with vesicles and ulcers, 27 (93%) of 29 with vesicles, 85 (98%) of 87 with ulcers, and 23 (58%) of 40 with maculopapular rash. RS, rectal swab; TS, throat swab; US, ulcer swab; VS, vesicle swab.

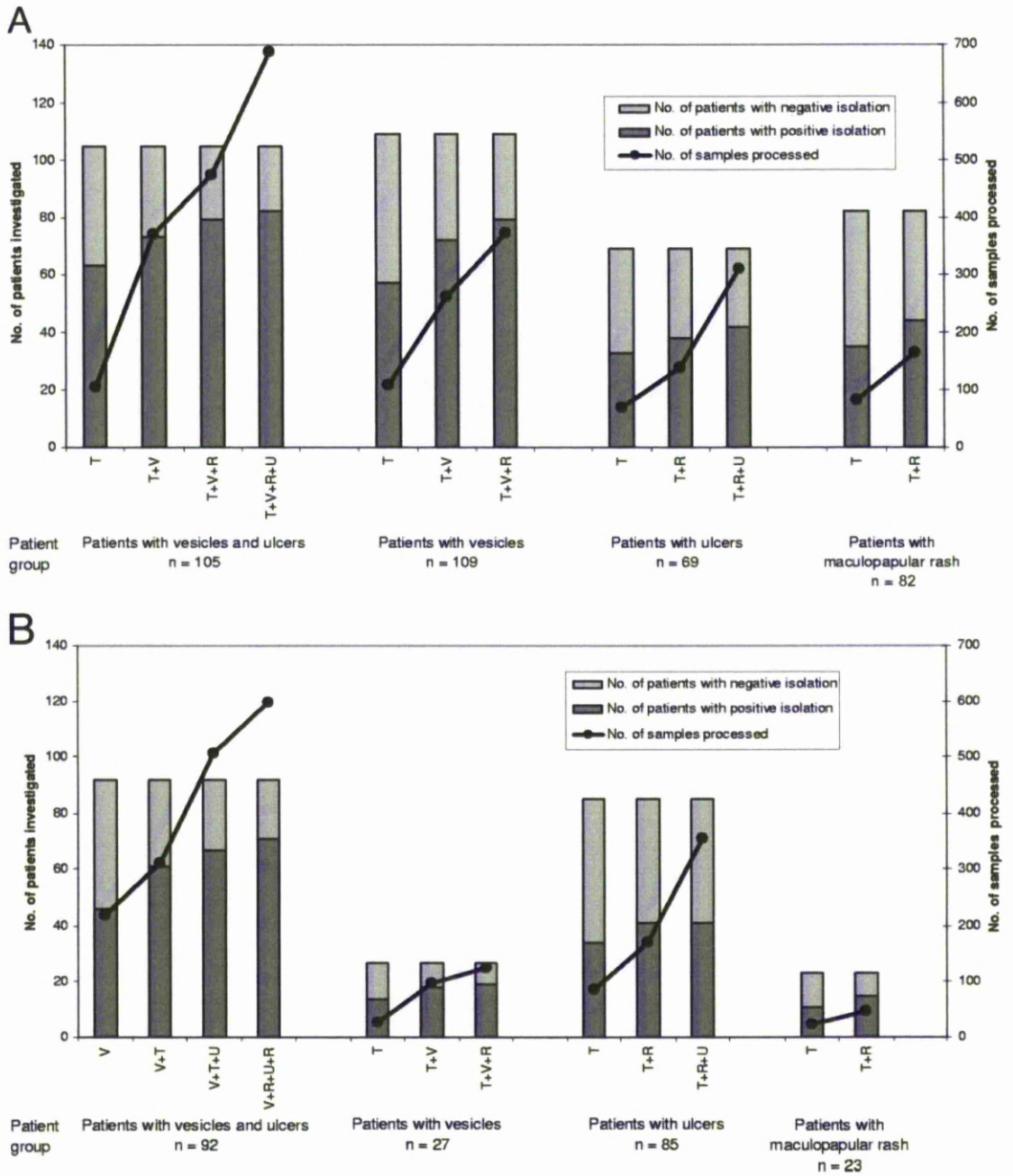

FIG. 2. Histograms showing the proportion of patients diagnosed at each step for the different patient groups, using the optimum combination of samples as determined in Fig. 1, and the total number of samples analyzed at each step. Panel A shows the first half of the study, and panel B shows the second half. T, throat swab; V, vesicle swab; R, rectal swab; U, ulcer swab.

throat swab samples alone (105 samples; 1.7 samples per patient diagnosed). The addition of the vesicle swab results enabled the diagnosis of a further 10 patients but required the processing of a further 264 samples (26.4 samples per patient), the addition of the rectal swab results allowed the diagnosis of 6 more patients with a further 105 samples (17.5 per patient) processed, and the addition of ulcer swab allowed the diagnosis of 3 more patients with 213 additional samples (71 per patient) analyzed. Thus, the total number of samples needed to be analyzed to diagnose each additional patient increased dramatically for each additional sample type included.

Recommendation based on the first outbreak. Based on these observations, it was decided that during a large outbreak, if one wanted to limit the number of samples, the following could be recommended. For patients with both vesicles and ulcers, most patients (70%) could be diagnosed by investigat-

TABLE 3. Pairwise comparison of virus isolates grown from a sterile site with isolates grown from nonsterile sites in the same patient[a]

Result	Finding	No. (%) of swabs testing positive from:		
		Throat (n = 337)	Rectum (n = 342)	Ulcer (n = 208)
Concordance	Same virus isolated as vesicle swab	101 (30)	48 (14)	29 (14)
	No virus isolated from either swab	111 (33)	139 (41)	83 (40)
Total		212 (63)	187 (55)	112 (54)
Discordance	Different virus isolated compared with vesicle swab	11 (3)	12 (4)	4 (2)
	Vesicle swab negative but sample positive	65 (19)	37 (11)	19 (9)
	Vesicle swab positive but sample negative	49 (15)	106 (31)	73 (35)
Total		125 (37)	155 (45)	96 (46)

[a] Vesicles were the sterile site; throat, rectum, and ulcers were the nonsterile sites. Many patients had swabs from multiple sites. The total number of patients represented here is 349.

ing throat and vesicle swabs, and the addition of rectal or ulcer swabs added little value for the extra work and cost involved. Similarly, for patients with vesicles only, the combination of throat swabs and vesicles gave a good diagnostic yield, and the addition of rectal swabs only increased the yield marginally (6%). For patients with ulcers only, throat and ulcer swabs or throat and rectal swabs were equally useful, but combining all three swabs increased the diagnostic yield by only 6%. For patients with a maculopapular rash only, both throat and rectal swabs should be tested.

Application of findings to the second outbreak. The validity of these recommendations was tested with data from the second outbreak. The same analytic process was applied to determine which combination of samples gave the best diagnostic yield and to see if the recommended combinations would prove to be the most useful. Figures 1B and 2B show that, for the most part, the approach remained valid. For patients with vesicles plus ulcers, and for patients with vesicles only, the combination of throat and vesicle swabs gave a good diagnostic yield (66 and 67%, respectively), with further samples not improving the yield greatly. Interestingly, though, for the first patient group, vesicles, rather than throat swabs, were the single most useful sample. For patients with a maculopapular rash only, throat swabs and then rectal swabs were most useful. However, for those with ulcers only, the addition of rectal swabs to throat swabs proved more useful than the addition of ulcer swabs, increasing the yield to 41 (48%) of 85 patients compared to 36 (42%).

Thus, to summarize the data from both outbreaks together, the combination of throat swabs plus vesicle swabs was the most useful approach for patients with vesicles (whether or not they also had ulcers), identifying virus for 134 (64%) of 208 patients with vesicles and ulcers and 90 (66%) of 136 patients with vesicles only; the combination of throat swab and rectal swab was most useful for patients without vesicles (whether or not they had ulcers), identifying virus for 79 (51%) of 154 patients with ulcers only and 59 (56%) of 105 patients with maculopapular rash only.

Concordance of viral diagnosis between samples. To examine the concordance of virus isolates from nonsterile sites (rectal, throat, and ulcer swabs) with those from a sterile site (vesicle swabs), all HFMD patients with swabs taken from vesicles plus another site were studied (Table 3). The isolation results from 212 (63%) of 337 patients with throat swabs, 187 (55%) of 342 with rectal swabs, and 112 (54%) of 208 with ulcer swabs were in agreement with the results for vesicle swabs from the same patients (either the same virus was isolated or no virus was isolated). However, a different virus was isolated for 11 (10%) of 112 patients with positive throat and vesicle swabs, 4 (12%) of 33 patients with positive ulcer and vesicle swabs, and 12 (20%) of 60 patients with positive rectal and vesicle swabs. Overall, by taking the vesicle swab as a reference, the sensitivity of the throat swabs for isolating the same virus was 67%, the specificity was 63%, and the positive and negative predictive values were 61% and 69%, respectively. Equivalent values were 31%, 79%, 56%, and 57% for the rectal swabs and 28%, 81%, 60%, and 53% for the ulcer swabs. The detailed viral isolation results of these patients with different viruses isolated from vesicle and nonsterile sites are shown in Table 4.

DISCUSSION

The outbreaks of HEV71-associated HFMD which have swept across the Asia-Pacific region since 1997 have posed a great economic and social burden, not least on the health-care facilities and laboratories that have to diagnose and manage such patients. To diagnose HEV71 infection, clinicians are faced with a wide range of samples to choose from, but there has been relatively little work comparing them.

Most of the recent studies of HEV71 infection have relied on stool culture and throat swabs and have found the latter to have greater sensitivity, with throat swabs being positive for 90 to 95% and stool culture being positive for 40 to 65% of patients tested (6, 26, 27). Few studies have looked systematically at all samples from a large patient group. One report of 175 patients with HFMD during the 2000 outbreak in Singapore found that rectal swabs most often yielded virus, followed by stool samples, vesicle swabs, and then throat swabs (3). However, our study found that in most patient groups, a throat swab was the single specimen most likely to be positive, being positive for 288 (49%) of the 592 patients with a full set of samples and 292 (49%) of all 598 patients studied. Approximately half of our patients had skin vesicles, and we showed that vesicle swabs were also very useful. In patients with vesicles, they gave the second highest yield, being positive for 169 (48%) of 333 patients studied; in one patient group (during the second outbreak), they were the single most sensitive specimen (positive for 50%). Vesicle swabs have not been widely used to diagnose HFMD previously. One study reported virus isolation

TABLE 4. Virus isolation results for patients with different viruses grown from sterile and nonsterile sites[a]

Study no.	Age (mo)	Gender	Clinical severity	Virus isolated from:					
				Sterile site			Nonsterile site		
				Vesicle	Serum	CSF	Rectum	Throat	Ulcer
HFM-4	46	Male	Severe HFMD with CNS	HEV71	—	NEG	HEV71, Ad-7[b]	HEV71	HEV71
HFM-9	52	Female	Mild HFMD	HEV71	CA16	—	NEG	NEG	NEG
HFM-90	37	Female	Mild HFMD	CVA16	—	—	NEG	CVA16	HEV71
HFM-112	16	Male	Mild HFMD	HEV71	—	—	CVA16	HEV71	NEG
HFM-152	45	Male	Mild HFMD	HEV71, CVA16[c]	—	—	NEG	HEV71	NEG
HFM-174	73	Male	Mild HFMD	CVA16	—	—	HEV71	HEV71	NEG
HFM-178	14	Male	Severe HFMD with CNS	CVA16	—	NEG	CVA16	CVB1	—
HFM-193	36	Female	Severe HFMD, no CNS	HEV71	—	NEG	CVA17	HEV71	—
HFM-198	16	Female	Mild HFMD	HEV71	—	—	HEV71, CVA17[d]	HEV71	—
HFM-228	11	Female	Mild HFMD	CVA10	—	—	CVA6	CVA6, CVB5[e]	NEG
HFM-298	22	Female	Mild HFMD	CVA10	—	—	NEG	CVB1, CVA10[f]	NEG
HFM-302	15	Female	Mild HFMD	HEV71	—	—	CVA16	HEV71	HEV71
HFM-328	19	Male	Severe HFMD, no CNS	HEV71		NEG	HEV71	HEV71, CVA24[g]	HEV71
HFM-337	36	Male	Mild HFMD	CVA16	—	—	HEV71	HEV71	NEG
HFM-338	27	Male	Mild HFMD	Unidentified virus	—	—	HEV71	CVA16	NEG
HFM-435	11	Male	Mild HFMD	CVA16	—	—	NEG	NEG	HEV71
HFM-489	13	Male	Mild HFMD	CVA16	—	—	Ad-4	CVA16	NEG
HFM3-7	38	Male	Mild HFMD	HEV71, P1S[h]	NEG	—	NEG	HEV71	NEG
HFM3-35	20	Female	Mild HFMD	HEV71	NEG	—	CVA5	CVA5	
HFM3-63	17	Female	Mild HFMD	HEV71, CVA16[i]	—	—	CVA16	NEG	CVA16
HFM3-97	61	Male	Severe HFMD, no CNS	HEV71	—	—	NEG	NEG	HEV71, Untyped EV[j]
HFM3-161	32	Male	Mild HFMD	HEV71	—	—	NEG	CVA16	NEG
HFM3-164	5	Male	Severe HFMD, no CNS	NEG	—	Unidentified virus	NEG	HEV71	HEV71

[a] Details of dual viruses isolated from a single site by using different cell lines are given below. NEG, negative; P1S, poliovirus 1 Sabin strain; with CNS, with CNS involvement; no CNS, no CNS involvement; —, no sample or insufficient sample for testing.
[b] HEV71 isolated using RD cells; Ad-7 isolated using 293 cells.
[c] HEV71 isolated from right palm vesicle using RD cells; CVA16 isolated from left sole vesicle using RD cells.
[d] HEV71 isolated using RD cells; CVA17 isolated using 293 cells.
[e] CVA6 isolated using RD cells; CVB5 isolated using 293 cells.
[f] CVB1 isolated using RD cells; CVA10 isolated using 293 cells.
[g] HEV71 isolated using RD cells; CVA24 isolated using 293 cells.
[h] HEV71 isolated from right and left palm vesicles using RD cells; P1S isolated from right and left sole vesicles using RD cells.
[i] HEV71 isolated from right sole vesicles using RD cells; CVA16 isolated from right knee and left sole vesicles using RD cells.
[j] HEV71 isolated from lip and tongue ulcers using RD cells; untyped EV isolated from right buccal ulcer using using RD cells.

from four children for whom vesicle swabs were investigated (5), and another reported 50% positive vesicles for 62 HFMD patients with vesicles (3). But for most outbreaks, vesicle fluid was not investigated (8, 11, 18, 21, 27).

Because we wanted to examine the optimal number of lesions to sample, in our study we took separate swabs from each vesicle. We found a single vesicle was positive 35% of the time, but this increased to 49% with two vesicles and 60% with three vesicles. Our recommended practice now is to apply a single swab to two or more vesicles. This maximizes the chance of isolating virus without doubling the number of samples to be processed. In our study, approximately half the patients had vesicles. They usually appeared early in the illness and resolved after a few days, so that their presence may depend on the time of presentation. Like previous reports for HEV71 (3, 6, 8, 10, 15, 18, 27) and other HEVs (20, 24), our study reports a low isolation rate of CSF (3 of 102 HFMD patients with aseptic meningitis). The yield would likely have been higher if PCR had been used (23), but this investigation is not available in most developing countries. We found that the isolation rate for rectal swabs was not as high as that for throat and vesicle swabs; the median time of presentation (and thus sampling) was just 2 days, which may be before viral shedding in the stool has become fully established.

In addition to looking at individual samples, we examined which combinations of samples were the most useful. This was achieved by determining the extent to which the addition of a sample type increased the number of patients diagnosed; this is different from asking how frequently a sample is positive. Thus, for example, in the first half of the study, although ulcer swabs were positive more often than rectal swabs, they were less useful diagnostically, because most of the patients in whom they were positive had already been diagnosed by a throat swab. Determining the "added diagnostic value" of a sample

type allowed us to produce predictions about which combinations of samples should prove useful, which we were then able to test in the second outbreak. We found that our predictions were basically sound. Results from the combination of throat swabs plus vesicle swabs were the most useful for patients with vesicles whether or not they also had ulcers, identifying virus for 224 (67%) of 333 such patients across the whole study. For these patients, the addition of rectal and ulcer swabs enabled the diagnosis of just 27 extra patients (8%). For patients without vesicles (whether or not they had mouth ulcers), the combination of throat swab and rectal swab was most useful, identifying virus for 138 of 259 such patients (53%). Thus, during large outbreaks, we suggest that a throat swab plus one other sample type be taken for each patient. If no vesicles are present, the second sample type should be a rectal swab, but if there are vesicles, a swab should be applied to as many of these as possible (but at least two). Although we advocate limited sampling, particularly for community surveillance and during large outbreaks, for individual patients, especially those that are critically ill, physicians will want to maximize the chance of obtaining a diagnosis. One approach might be to collect all sample types but to investigate them in a stepwise manner, starting with the most useful samples, until a diagnosis has been made.

During our study, we were also able to examine the concordance of virus isolates from nonsterile sites (rectal, throat, and ulcer swabs) with those from a sterile site (vesicle swabs). We found that 20% of rectal isolates differed from vesicle isolates (in individual patients with isolates from both sites), whereas such discordance with vesicle isolates occurred for only 10% of throat isolates. While most would accept the virus isolated from a vesicle as the causative pathogen, the significance of other viruses concomitantly detected from throat and/or rectum is not always clear. One plausible explanation is that the isolation of a virus from throat or rectum may be only a confounding factor related to asymptomatic carriage or ongoing shedding from recent enterovirus infection. Our data show that in the majority of patients with HFMD, throat and rectal isolates do reflect viruses isolated from sterile sites, but in a minority, they may be coincidental infections. However, it may not be correct to assume that virus isolated from vesicle is always the most important pathogen. For example, one patient (HFM-178) (Table 4) with severe HFMD and CNS disease had CVA16 isolated from a vesicle swab but CVB1 isolated from a throat swab. CVA16, a common cause of HFMD, is not known to cause CNS disease; in contrast, CVB1 is not known to cause HFMD and, being a species B HEV, is a more likely neuropathogen (25). So in this patient with dual infection, the two virus isolates may have been responsible for two clinical syndromes: the throat isolate CVB1 causing CNS disease and the vesicle isolate CA16 causing HFMD. For four patients, we also isolated different viruses from different vesicles: three with HEV71 and CVA16 and one (with mild HFMD) with HEV71 and poliovirus 1 Sabin strain. These patients clearly demonstrate that systemic infection with two viruses can occur simultaneously. In addition, the latter case suggests the possibility that occasionally during dual infection, viruses may cross tissue barriers that they would not normally be able to (poliovirus Sabin strain type 1 does not itself cause HFMD and thus is not normally found in vesicles) (20). Our findings on dual infection underscore the need to look for a possible second pathogen before attributing pathogenesis to a virus rarely associated with a severe disease phenotype.

In summary, we have shown that the throat swab is the single most useful sample from patients with HFMD during an HEV71 outbreak. Vesicle swabs, which have been relatively neglected until now, can also be extremely useful. Although they are not as easy to obtain as throat and rectal swabs and are not available for approximately half the patients, the viral yield is almost as good as that of throat swabs, with the added advantage that they come from sterile sites.

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Identification and validation of clinical predictors for the risk of neurological involvement in children with hand, foot, and mouth disease in Sarawak

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Abstract

Background: Human enterovirus 71 (HEV71) can cause Hand, foot, and mouth disease (HFMD) with neurological complications, which may rapidly progress to fulminant cardiorespiratory failure, and death. Early recognition of children at risk is the key to reduce acute mortality and morbidity.

Methods: We examined data collected through a prospective clinical study of HFMD conducted between 2000 and 2006 that included 3 distinct outbreaks of HEV71 to identify risk factors associated with neurological involvement in children with HFMD.

Results: Total duration of fever ≥ 3 days, peak temperature ≥ 38.5°C and history of lethargy were identified as independent risk factors for neurological involvement (evident by CSF pleocytosis) in the analysis of 725 children admitted during the first phase of the study. When they were validated in the second phase of the study, two or more (≥ 2) risk factors were present in 162 (65%) of 250 children with CSF pleocytosis compared with 56 (30%) of 186 children with no CSF pleocytosis (OR 4.27, 95% CI2.79–6.56, $p < 0.0001$). The usefulness of the three risk factors in identifying children with CSF pleocytosis on hospital admission during the second phase of the study was also tested. Peak temperature ≥ 38.5°C and history of lethargy had the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of 28%(48/174), 89%(125/140), 76%(48/63) and 50%(125/251), respectively in predicting CSF pleocytosis in children that were seen within the first 2 days of febrile illness. For those presented on the 3rd or later day of febrile illness, the sensitivity, specificity, PPV and NPV of ≥ 2 risk factors predictive of CSF pleocytosis were 75%(57/76), 59%(27/46), 75%(57/76) and 59%(27/46), respectively.

Conclusion: Three readily elicited clinical risk factors were identified to help detect children at risk of neurological involvement. These risk factors may serve as a guide to clinicians to decide the need for hospitalization and further investigation, including cerebrospinal fluid examination, and close monitoring for disease progression in children with HFMD.

Background

Hand, foot, and mouth disease (HFMD) is a common childhood exanthema caused by species A human enteroviruses (HEVA), particularly Coxsackievirus A16 (CVA16)[1]. In most instances, this is a mild self-limiting illness. The affected children are often given out-patient care with symptomatic treatment. However over the last decade HFMD has emerged as a growing public health problem in Asia following frequent outbreaks of death-associated HFMD caused by a more virulence member of HEVA, human enterovirus 71 (HEV71), in a number of countries in the region [2-5]. This was first recognized with large outbreaks of HFMD associated with neurological disease and alarming fatalities in Sarawak, Malaysia in 1997 and in Taiwan in 1998 [2,3]. Fatal cases typically presented with a brief duration of febrile illness, subtle neurological signs and died dramatically of acute refractory cardiac dysfunction and fulminant pulmonary oedema within hours of developing signs of tachycardia, poor peripheral perfusion and tachypnea. Indeed, most of them died shortly after hospital admission, and some even before or on arrival at hospital [2,6-8]. Although severe neurological complications and death only occur in a small minority of children with HFMD, the fulminant disease course of the fatal cases has caused great public alarm in Asia. Experience from recent outbreaks of HEV71 associated HFMD (HEV71-HFMD) in Asia showed that primary care doctors are often overwhelmed with large number of children with HFMD seeking medical attention for the fear of neurological complications and death. Because of the risk of sudden death, coupled with tremendous parental pressure to admit children with HFMD into hospital for observation, children with HFMD are often routinely admitted into hospital for observation in Sarawak, which has imposed a huge burden on the health-care system. Cerebrospinal fluid (CSF) pleocytosis has so far been the universal finding in fatal cases even though many have no obvious neurological signs prior to sudden onset of cardiorespiratory failure and death [2,6,8]. In the absence of clear neurological sign, CSF pleocytosis (indicative of neurological involvement) has thus been considered an objective marker of complicated disease, allowing clinicians to focus their attention and provide timely intervention in these patients before they develop fatal cardiorespiratory failure. We therefore examined data collected through a prospective study of HFMD to identify and validate risk factors associated with neurological involvement in children with HFMD that may be used by clinicians managing children with HFMD.

Methods

Setting and study period

A prospective clinical study was conducted from January 2000 through December 2006, which included 3 distinct outbreaks that occurred in 2000/1, 2003 and 2006, at the paediatric wards and intensive care unit at Sibu Hospital (Sarawak, Malaysia). The study was approved by the Director of Health for Sarawak and the Ethics Committee of the Liverpool School of Tropical Medicine (UK). Informed consent was obtained verbally from each child's accompanying parent or guardian.

Case definitions

Figure 1 shows the algorithm of the investigation and the classification of the disease severity of children with HFMD in the study [9]. A child was defined as having HFMD if they had new onset of at least one (≥ 1) of the following: maculopapular or vesicular rash on the palms and/or soles; vesicles or ulcers in the mouth or herpangina (defined as multiple oral ulcers predominantly affecting the posterior parts of the oral cavity). Children with HFMD were considered to have more serious illness if they have the following features: a history of fever, or fever on examination ($\geq 38°C$), and ≥ 1 of the following features indicative of more serious illness: toxic and ill in appearance, recurrent vomiting (at least twice), tachycardia (heart rate ≥ 150/min) breathlessness, poor perfusion (cold clammy skin), reduced consciousness (irritability, lethargy, drowsiness, coma), limb weakness, meningism (neck stiffness or positive Kernig's sign), seizures. They were subjected to CSF examination after written consent to exclude central nervous system (CNS) involvement. Children with > 5 cells/μL (i.e. CSF pleocytosis) and negative microscopy and culture for bacteria were classified as "HFMD with CNS complications" (HFMD-CNS), while those with normal CSF examination were considered to have "severe HFMD without CNS involvement" (HFMD-Non-CNS). Children with HFMD-CNS were diagnosed to have aseptic meningitis (ASM) if they were fully conscious, had headache, meningism, and no focal neurological signs. Encephalitis was defined by the presence of impaired consciousness including lethargy, drowsiness or coma, seizures or myoclonus. Acute flaccid paralysis (AFP) was characterized by the acute onset of areflexic limb weakness. Cardiorespiratory failure was 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. Children without features of more serious illness were classified as "mild HFMD", and were observed in hospital until they became afebrile for at least 12–24 hours. A child was considered to be positive for HEV71 if HEV71 was isolated by tissue culture or HEV71 RNA was detected by HEV71 specific RT-PCR from ≥ 1 clinical sample.

Clinical methods

All children with HFMD admitted into the hospital were assessed by pediatricians of the study team. A detailed history and clinical examination was performed with special

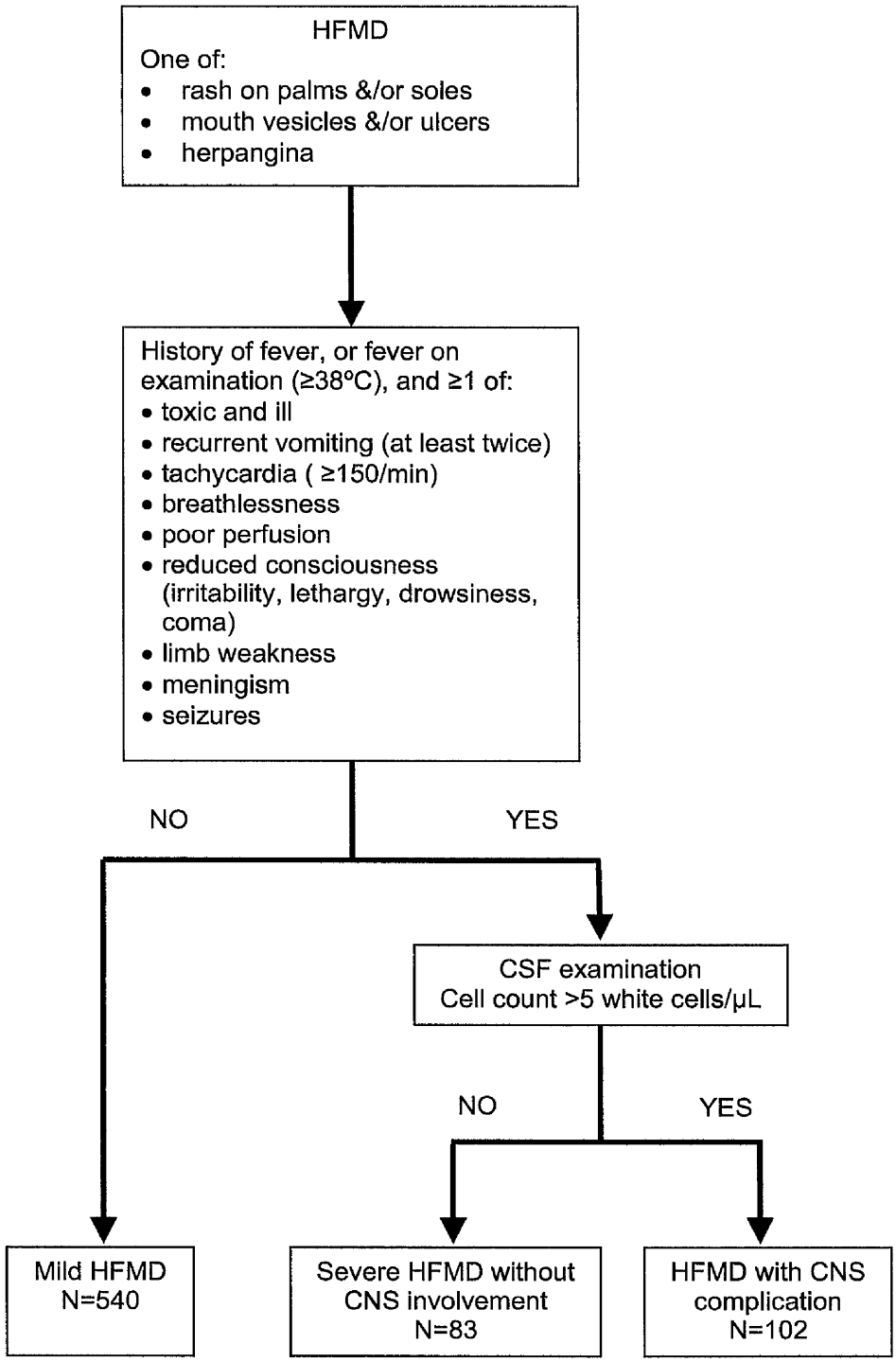

Figure 1
Case definitions. The flow chart shows the algorithm of the investigation and the classification of the disease severity of children with HFMD used in the study. HFMD: Hand, foot, and mouth disease, CSF: Cerebrospinal fluid, CNS: Central nervous system.

attention to mucocutaneous lesions, cardiovascular and neurological signs. All details were recorded on standardized forms. Swabs were taken from the throat and rectum of every patient, as well as ≥ 1 swab from vesicles on the skin and oral ulcers (if present). The clinical samples were stored immediately in a -70 °C freezer until further testing. Blood was taken for flavivirus serology, and in patients with suspected CNS involvement for full blood count, urea, electrolytes, and glucose. Electrocardiogram and echocardiogram was also performed on children with suspected CNS involvement. CSF was examined for cell count and differential, protein, glucose, Gram stain, bacterial culture and processed for viral studies. If there was a strong clinical suspicion of viral CNS infection, but the initial CSF examination was acellular, a second lumbar puncture was performed. Lumbar punctures were delayed in those with unstable vital signs. Patients were examined daily or more frequently as indicated, by a member of the study team. Children with HFMD-CNS complications (particularly those with encephalitis and acute flaccid paralysis) were treated with intravenous immunoglobulin (IVIG) at the discretion of the treating physician [10].

Virological methods

Virus isolation was attempted on all swab specimens, CSF specimens, and any serum samples remaining after other investigations had been completed through the inoculation of human rhabdomyosarcoma and human embryonic kidney cells. Isolated enteroviruses were typed by nucleotide sequencing of VP1 and VP4 genes and genogrouped by phylogenetic analysis [11,12]. During the 2006 outbreak, in addition to virus isolation, all swab specimens were also tested for presence of HEV71 RNA using a HEV71 specific RT-PCR [13]. Paired serum samples (obtained on the day of admission and on day 7, or on the day of discharge or after death) and CSF specimens were also tested for IgM against dengue and Japanese encephalitis virus (JEV) in parallel, using an IgM-capture ELISA that distinguishes responses to these two viruses [14].

Statistical analysis

Data from HFMD patients recruited in the first phase of the study (mostly during 2 outbreaks that occurred between January 2000 and July 2003) were used to identify risk factors for neurological involvement (evident by CSF pleocytosis). The primary analysis was for variables associated with neurological involvement by comparing children with HFMD-CNS (i.e. with CSF pleocytosis) to those with HFMD-Non-CNS (i.e. no CSF pleocytosis). Variables that were considered potentially useful to primary care doctors in identifying children with neurological involvement were included in a multiple logistic regression analysis to look for independent risk factors for neurological involvement (i.e. CSF pleocytosis). Variables were selected backward and remained in the model only if they were statistically associated with neurological involvement ($p < 0.05$). (SPSS software, Version 13.0; SPSS). The association between the independent risk factors identified and neurological involvement were validated in the second phase of the study, where most patients were admitted during the 2006 outbreak. The utility of the identified risk factors as clinical predictors for neurological involvement at the point of first contact for care was also examined. Normally distributed data were compared using Student's *t* test; data that were not normally distributed were compared by the Mann-Whitney *U* test (Statview 4.02; Abacus Concepts). Differences between proportions were tested using the Chi-square test with Yates's correction or Fisher's exact test as appropriate (Epi Info, version 6; Centers for Disease Control and Prevention). A p value < 0.05 was considered statistically significant.

Results

A total of 725 children (457, 63% males) were recruited between 1st January 2000 and 31st July 2003. Most children were recruited during 2 large outbreaks of HEV71-HFMD that occurred during 2000/2001 and 2003. Five hundred and forty (74%) children had mild HFMD. One hundred and eighty five (26%) children had suspected CNS involvement and required CSF examination; 102 (55%) of them had CSF pleocytosis (HFMD-CNS) and the remaining 83 (45%) had normal CSF findings (HFMD-Non-CNS). Of the 102 children with HFMD-CNS, 63 (62%) had ASM, 33 (32%) had encephalitis, 3 (3%) had AFP, and 3 (3%) had encephalitis associated with cardiorespiratory failure (all of the 3 died). Of the 273 HEV71 culture positive children, 187 (69%) had mild HFMD, 34 (13%) had HFMD-Non-CNS, and 52 (19%) had HFMD-CNS (30 had ASM, 19 had encephalitis, 1 had AFP and 2 had encephalitis with cardiorespiratory failure). Detailed results of the epidemiology, diagnostic virology and molecular epidemiology of this phase of the study have been reported previously [9,15].

Clinical features

Comparison of patients with HFMD with CNS complications with those had more serious HFMD without CNS involvement (January 2000 to July 2003)

The clinical features of the children with HFMD-CNS (i.e. with CSF pleocytosis) are compared to those with HFMD-Non-CNS (i.e. no CSF pleocytosis) (see Additional file 1). Children with HFMD-CNS were more likely to be male and of Chinese ethnic group. Children of Iban ethnic group, however, were less likely to have HFMD with CNS complications. Children with HFMD-CNS complications were more likely to have higher mean peak temperature, peak temperature ≥ 38.5 °C, longer mean total duration of fever and total duration of fever ≥ 3 days. Findings of leth-

argy (from the parent's history or physical examination), faster mean heart rate, mean heart rate ≥ 150/min and limb weakness on examination were more frequently present in children with HFMD-CNS. There was no difference in the proportion of children with positive HEV71 isolation between children with HFMD-CNS and those with HFMD-Non-CNS.

To look for independent risk factors that could be used to predict neurological involvement evident by CSF pleocytosis, total duration of fever ≥ 3 days, peak temperature ≥ 38.5°C, being lethargic (from the parent's history or physical findings), history of breathlessness, history of vomiting, history of or witnessed myoclonus, neck stiffness were included in a multiple logistic regression analysis. Total duration of fever ≥ 3 days, peak temperature ≥ 38.5°C and history of lethargy were found to be independent risk factors of neurological involvement after multivariate analysis (Table 1). Table 2 shows the number and type of the risk factors that were present in the 725 children with HFMD seen during the first phase of the study according to the disease severity. Two or more (≥ 2) risk factors were present in 83% (85/102) of patients that had HFMD-CNS when compared to 43% (36/83) of patients with HFMD-Non-CNS (OR 6.53, 95% CI 3.15–13.66, $p < 0.0001$). Further analysis on the HEV71-positive subset showed that ≥ 2 risk factors were present in 82% (43/52) of children with HFMD-CNS when compared to 32% (11/34) patients with HFMD-Non-CNS (OR 9.99, 95%CI 3.26–31.82). A separate analysis on children with mild HFMD showed that ≥ 2 risk factors were present in 6% of cases with mild HFMD (32/540)(Table 2), and HEV71-postive mild HFMD (11/187), respectively.

Validation of the association between the risk factors and neurological involvement in children with HFMD in 2006 outbreak

The association between the identified risk factors (total duration of fever ≥ 3 days, peak temperature ≥ 38.5°C and history of lethargy) and neurological involvement were validated in the 2006 outbreak. A total of 730 children with HFMD were admitted between January and December 2006. Two hundred and ninety four (40%) children had mild HFMD. Four hundred and thirty six (60%) children had features of more serious illness and warranted CSF examination; 250 (34%) of them had HFMD-CNS and the remaining 186 (26%) had HFMD-Non-CNS. Of the 250 children with HFMD-CNS, 65 (26%) had ASM, 172 (69%) had encephalitis, 2 (0.8%) had encephalitis associated with AFP, and 11 (4.4%) had encephalitis associated with cardiorespiratory failure (6 of them died). HEV71 was isolated from 157 (27%) of 586 children who had virus isolation done. A further 44 (7%) children had other HEVA (n = 29) and species B HEV (n = 15). No patient had CVA16 isolated. HEV71RNA was detected in 239 (50%) of 477 children that were tested with HEV71 specific RT-PCR. In short, 291 (45%) of 653 children were positive for HEV71. Of the 291 HEV71-positive children, 104 (36%) had mild HFMD, 73 (25%) had HFMD-Non-CNS, 114 (39%) had HFMD-CNS (22 had ASM, 83 had encephalitis, 2 had encephalitis associated with AFP, 7 had encephalitis associated with cardiorespiratory failure). HEV71 was detected in 4 (67%) of the 6 fatal case children that had encephalitis associated with cardiorespiratory failure. The Additional file 2 shows the clinical features of the 730 children that were admitted during the 2006 outbreak according to the disease severity. Total duration of fever ≥ 3 days, peak temperature ≥ 38.5°C and history of lethargy were similarly more frequently present in children with HFMD-CNS than those with HFMD-Non-CNS. Two or more risk factors were present in 65% (162/250) of children that had HFMD-CNS when compared with 30% (56/186) of children with HFMD-Non-CNS (OR 4.27, 95%CI 2.79–6.56, $p < 0.0001$) (Table 2). Among children with HEV71-positive HFMD, ≥ 2 risk factors were present in 61% (69/114) of children with HFMD-CNS when compared with 26% (19/73) of children with HFMD-Non-CNS (OR 4.36, 95%CI 2.19–8.75, $p < 0.0001$). A separate analysis on children with mild HFMD showed that history of lethargy, total duration of fever ≥ 3 days and peak temperature ≥ 38.5°C was present in 6.4% (19/294), 11% (33/294) and 14% (42/294) of the children with mild HFMD, respectively (Additional file 2). Two or more 2 risk factors were found in only 5 (2%) of 294 with mild HFMD (Table 2) and in 1 (1%) of 104 of children with HEV71-positive mild HFMD.

Table 1: Risk factors that were significantly associated with CSF pleocytosis in children with HFMD in the first phase of the study (2000 to 2003).

Risk factors	p value	Odds ratio	95% CI
Total duration of fever ≥ 3 days	< 0.0001	6.52	2.83 – 14.99
Peak temperature ≥ 38.5°C	0.0192	2.27	1.14 – 4.51
History of lethargy	0.001	3.18	1.60 – 6.35

Note: The Hosmer-Lemeshow statistics indicated a non-significance of lack of fit ($\chi^2 = 2.163$, $p = 0.904$).
CSF: Cerebrospinal fluid
HFMD: Hand, foot, and mouth disease

Table 2: The number and type of the risk factors that were present in the children with HFMD seen in the 2000/3 and 2006 outbreaks

Risk factors that were present	First phase of study (2000/3)			Second phase of study (2006)		
	HFMD-CNS (N = 102)	HFMD-Non-CNS (N = 83)	Mild HFMD (N = 540)	HFMD-CNS (N = 250)	HFMD-Non-CNS (N = 186)	Mild HFMD (N = 294)
No. of patients with none of the 3 risk factor	**2**	**9**	**352**	**11**	**52**	**208**
Peak temperature ≥ 38.5°C only	1	8	7	29	32	34
History of lethargy only	5	15	27	16	11	17
Total duration of fever ≥ 3 only	9	15	122	32	35	30
No. of patients with 1 risk factor	**15**	**38**	**156**	**77**	**78**	**81**
Peak temperature ≥ 38.5°C plus history of lethargy	2	3	1	11	7	2
Peak temperature ≥ 38.5°C plus total duration of fever ≥ 3 days	25	15	8	76	31	3
Total duration of fever ≥ 3 days plus history of lethargy	26	12	19	21	5	0
No. of patients with 2 risk factors	**53**	**30**	**28**	**108**	**43**	**5**
No. of patients with 3 risk factors (Peak temperature ≥ 38.5°C plus total duration of fever ≥ 3 days plus history of lethargy)	**32**	**6**	**4**	**54**	**13**	**0**

Note:
HFMD: Hand, foot, and mouth disease
HFMD-CNS: Hand, foot, and mouth disease with central nervous system complication
HFMD-Non-CNS: Severe HFMD without central nervous system involvement

The usefulness of the risk factors in predicting neurological involvement in children with HFMD in the 2006 outbreak

We were particularly interested to assess the utility of the three clinical risk factors in predicting neurological involvement in children with HFMD at the point of first contact for care. While a febrile illness ≥ 3 day was an important risk factor for severity, primary care physicians often see many children on the first 2 days of HFMD illness. To determine if peak temperature ≥ 38.5°C and history of lethargy are useful in identifying children who sought treatment within the first 2 days of the febrile illness we performed a separate analysis for children who presented within the first 2 days of the illness during the 2006 outbreak. Figure 2 shows the distribution and classification of disease severity of 730 children with HFMD in the 2006 outbreak according to the duration of febrile illness and the risk factors that were present when they first presented to hospital. Five hundred and seventy nine (79%) of 730 children were admitted within the first 2 days of febrile illness. Sixty five (11%) of the 579 children had history of lethargy plus peak temperature ≥ 38.5C. All but two (97%) of the 65 children had features of more serious illness and warranted CSF examination. About three quarter of them had CSF pleocytosis and was classified as HFMD-CNS. Only 2 (3%) of the 65 children were labeled as mild HFMD. Two hundred and twenty (38%) children had only either history of lethargy or peak temperature ≥ 38.5C. Of the 167 (76%) children who warranted a CSF examination, 102 (61%) of them had CSF pleocytosis, and were classified as HFMD-CNS. The remaining 53 (24%) children without feature of more serious illness were considered as mild HFMD. Two hundred and ninety four (51%) children had neither of the two risk factors. Eighty four (29%) of the 294 children, however, had other features of more serious illness, and hence underwent CSF examination. CSF pleocytosis was

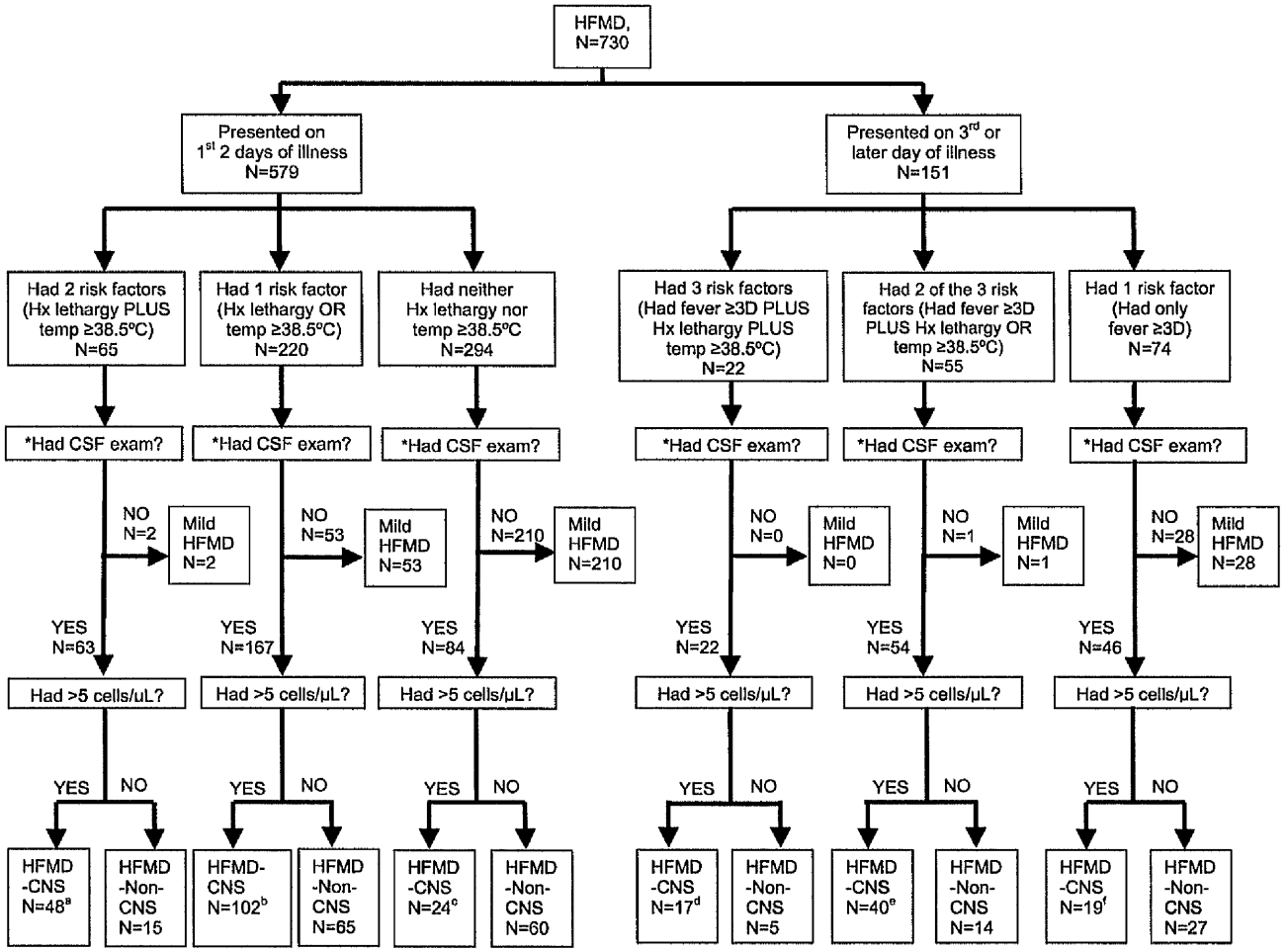

Figure 2

Classification of 730 Children with HFMD. The flow chart shows the distribution and classification of disease severity of 730 children with HFMD in the 2006 outbreak according to the duration of fever and the risk factors that were present when they first presented to the hospital. CSF examination is indicated if the children have features indicative of more serious illness of HFMD (see case definition in main text). Hx lethargy: History of lethargy, Temp ≥ 38.5°C: body temperature ≥ 38.5°C, CSF exam: cerebrospinal fluid examination, HFMD: Hand, foot, and mouth disease, HFMD-CNS: Hand, foot, and mouth disease with central nervous system complication, HFMD-Non-CNS: Severe HFMD without central nervous system involvement, BENC: brainstem encephalitis, ASM: aseptic meningitis. a. Of the 48 children with HFMD-CNS, 40 had BENC, 6 had ASM and 2 had BENC associated with cardiorespiratory failure (1 of whom died). b. Of the 102 children with HFMD-CNS, 74 had BENC, 26 had ASM, 1 had encephalitis and 1 had fatal BENC associated with cardiorespiratory failure. c. Of the 24 children with HFMD-CNS, 13 had BENC, 8 had ASM, 1 each had BENC associated with cardiorespiratory failure, encephalitis, and encephalitis associated with acute flaccid paralysis. d. Of the 17 children with HFMD-CNS, 11 had BENC, 5 had BENC associated with cardiorespiratory failure (4 of whom died) and 1 had ASM. e. Of the 40 children with HFMD-CNS, 22 had BENC, 17 had ASM and 1 had fatal BENC associated with cardiorespiratory failure. f. Of the 19 children with HFMD-CNS, 10 had BENC, 7 had ASM and 1 each had encephalitis with acute flaccid paralysis, and BENC associated with cardiorespiratory failure.

found in 24 (29%) of the 84 children, and were classified as HFMD-CNS. Two hundred and ten (71%) of the 294 children without features of more serious illness were labeled as mild HFMD. In summary CSF pleocytosis was found in 48(74%) of 65 children with 2 risk factors (temperature ≥ 38.5°C and history of lethargy) on hospital admission compared with that in 126 (25%) of 514 children with ≥ 1 risk factors (OR 8.69; 95%CI 4.66–16.37, p < 0.0001).

One hundred and fifty one (21%) of the 730 children were seen on the 3rd or later days of their febrile illness.

Twenty two (15%) of the 151 children had all the 3 risk factors associated with neurological involvement. All the 22 children warranted CSF examination. Seventeen (77%), including 4 fatal cases, of the 22 children had CSF pleocytosis and were classified as HFMD-CNS. Of the 55 (36%) children that had 2 risk factors, all except one child required CSF examination to exclude CNS involvement. Forty (74%) of the 54 children had CSF pleocytosis and were classified as HFMD-CNS. Being febrile for ≥ 3 days was the sole risk factor in 74 (49%) of the 151 children. Forty six (62%) children had features of more serious illness, and underwent CSF examination - 19 (41%) had CSF pleocytosis and were classified as HFMD-CNS. The remaining 28 (38%) children were labeled as mild HFMD. In short CSF pleocytosis was found in 57(74%) of 77 children that had ≥ 2 risk factors on hospital admission compared with in 19 (26%) of 74 children with isolated risk factor of being febrile ≥ 3 days (OR 8.25; 95%CI 3.75–18.38, p < 0.0001). Further analysis on the HEV71-positive subset showed that 24% (21/86) of children with HFMD-CNS presented within the first 2 days of febrile illness had ≥ 2 risk factors compared with 10% (6/60) of children with HFMD-Non-CNS (OR 2.91; 95% CI 1.03–9.38, p = 0.0464). For the HEV71-positive children presented on the 3rd or later days of febrile illness, 71% (20/28) of children with HFMD-CNS had ≥ 2 risk factors compared with 31% (4/13) of children with HFMD-Non-CNS (OR 5.63; 95% CI 1.11–31.35, p = 0.0341). The sensitivity, specificity, positive predictive value and negative predictive value of the risk factors in predicting CSF pleocytosis in children with HFMD at presentation in the 2006 outbreak is shown in Table 3.

Between 2000 and 2006, a total of 352 children with CNS involvement were admitted into the study. One hundred and twenty eight (36%) children had ASM (a mild and benign CNS involvement) and 224 (64%) had severe and potentially fatal CNS complications (205 had encephalitis, 14 had encephalitis associated with cardiorespiratory failure, 2 had encephalitis associated with AFP, 3 had AFP). Among the 224 children that had severe CNS complications, 204 (95%) of 215 children that survived had timely hospital admission and IVIG treatment compared to one (11%) of 9 children that died (OR 148.36, 95%CI 16.34–6609.04, p < 0.0001). Table 4 shows the clinical details and the risk factors that were present in the 9 fatal case children on hospital admission. Two or more risk factors associated with neurological involvement were present in all the 9 fatal children, and were noted for ≥ 24–48 hours before hospital admission.

Discussion

Early recognition of children at risk of neurological involvement and death (particularly those with encephalitis and encephalomyelitis) is critical as the disease progression from the onset of neurological involvement to fulminant cardiorespiratory failure may be remarkably rapid [8]. However the clinical manifestations of neurological involvement may be very subtle, particularly in young children with early CNS disease [8,16]. While the signs of cardiorespiratory distress such as breathlessness, tachypnea, tachycardia, poor perfusion are easy to recognize, they invariably appear very late shortly before most fatal case collapsed. Our results and other published studies showed that timely diagnosis and intervention, including the use of IVIG infusion, may reduce acute mortality [10,17-19]. Hence the primary care doctors are confronted with a clinical challenge of identifying a small fraction of children who are at risk of neurological complication from an overwhelmingly large number of chil-

Table 3: The sensitivity, specificity, positive predictive value and negative predictive value of the risk factors.

Risk factors that were present	Presented within the first 2 days of febrile illness				Presented on the 3rd or later day of febrile illness			
	Sensitivity	Specificity	PPV	NPV	Sensitivity	Specificity	PPV	NPV
Both peak temperature ≥ 38.5°C and history of lethargy	28% (48/174) [23–33%]	89% (125/140) [86–92%]	76% (48/63) [71–81%]	50% (125/251) [44–56%]	22% (17/76) [15–29%]	89% (41/46) [83–95%]	77% (17/22) [70–84%]	41% (41/100) [32–50%]
Peak temperature ≥ 38.5°C &/ or history of lethargy	86% (150/174) [82–90%]	43% (60/140) [38–48%]	65% (150/230) [60–70%]	71% (60/84) [66–76%]	75% (57/76) [67–83%]	59% (27/46) [50–68%]	75% (57/76) [67–83%]	59% (27/46) [50–68%]

(%, proportion, [95% confidence interval]) in predicting CSF pleocytosis in the 436 children with suspected CNS involvement seen in the 2006 outbreak

Note:

CSF: Cerebrospinal fluid

CNS: Central nervous system

Sensitivity = The proportion of children with CSF pleocytosis that are correctly identified by the presence of the risk factors

Specificity = The proportion of children without CSF pleocytosis that are correctly identified by the absence of the risk factors

Positive predictive value (PPV) = The proportion of individuals with the risk factors that have CSF pleocytosis

Negative predictive value (NPV) = The proportion of individuals without the risk factors that do not have CSF pleocytosis

Table 4: The clinical details and risk factors for neurological involvement of the nine fatal case children with HFMD seen in the study.

Patient	Year of the outbreak	Age (months)	Day of illness at presentation	Risk factors that were present at presentation	Disease severity	HEV71 detected?	IVIG treatment	Note
1	2000	11	Day 3	fever ≥ 3D, history of lethargy, temperature ≥ 38.5°C	HFMD-CNS	Yes	No	a.
2	2003	34	Day 5	fever ≥ 3D, history of lethargy	HFMD-CNS	Yes	No	b.
3	2003	32	Day 3	fever ≥ 3D, history of lethargy	HFMD-CNS	No	No	a.
4	2006	9	Day 1	history of lethargy, temperature ≥ 38.5°C	HFMD-CNS	Yes	Yes	c.
5	2006	8	Day 3	fever ≥ 3D, history of lethargy, temperature ≥ 38.5°C	HFMD-CNS	Yes	No	a.
6	2006	14	Day 3	fever ≥ 3D, history of lethargy, temperature ≥ 38.5°C	HFMD-CNS	Yes	No	a.
7	2006	34	Day 4	fever ≥ 3D, history of lethargy, temperature ≥ 38.5°C	HFMD-CNS	Yes	No	a.
8	2006	25	Day 4	fever ≥ 3D, history of lethargy, temperature ≥ 38.5°C	HFMD-CNS	No	No	a.
9	2006	47	Day 4	fever ≥ 3D, history of lethargy	HFMD-CNS	No	No	a.

Note:
a. Presented in the moribund state with fulminant cardiorespiratory failure and pulmonary oedema. The patient died within 24 hours of the hospitalization. The risk factors were present for ≥ 48 hours before hospital admission.
b. Developed acute cardiorespiratory collapse and died 12 hours after hospitalization. Had peak temperature ≥ 38.5°C in the hospital. The patient was lethargic for ≥ 48 hours before hospital admission.
c. Deteriorated progressively because of cardiorespiratory failure despite intensive care support. Died on day 4 of the hospitalization. The patient was lethargic for 24 hours before hospital admission
HFMD-CNS: Hand, foot and mouth disease with central nervous system complication
IVIG: Intravenous immunoglobulin

dren who would have uncomplicated course of HFMD. For this reason it is important to find clinical predictors for neurological involvement that can guide primary care doctors perform a proper patient triage, which should be aimed to admit high risk children into hospital early for close observation and further management, while those at low risk of neurological complication may be given outpatient care after parental education and advice. Few studies have systemically examined how to identify children at risk early before they develop cardiorespiratory failure, particularly at the primary care setting where the majority of children with HFMD would first seek treatment during a community outbreak of HFMD.

In this study we identified and validated that history of lethargy, mean peak temperature ≥ 38.5°C and total duration of fever ≥ 3 days were important risk factors for neurological involvement. Our study also shows that neurological involvement occurs at early course of complicated HFMD, and may be detectable within the first 2 days of the febrile illness because CSF pleocytosis was present in 174 (30%) of 579 children seen within the first 2 days of febrile illness, where they also formed 70% (174/250) of children with HFMD-CNS in the 2006 outbreak (Figure 2). Since CSF pleocytosis may be detectable within the first 2 days of the febrile illness and fulminant cardiorespiratory failure seen in the fatal case children typically occurred on the 3rd or later day of febrile illness, it is imperative to attempt to identify children at risk of neurological involvement before the 3rd day of febrile illness so that they can be admitted into hospital early for close monitoring and investigation, and intervention may be instituted when necessary.

Examination of body temperature and careful enquiry into history of lethargy, duration of fever and home record of body temperature should form an integral part of HFMD patient triage at the primary care level. The three risk factors are readily elicited, and can also be used after minimal training by paramedics, who are the key primary care providers in many developing countries including in Sarawak (Malaysia) in Asia. The parents of children with HFMD can also play an important role in early diagnosis of neurological complication in children with HFMD. They should be educated about the 3 risk factors, and be encouraged to monitor the children's body temperature regularly and observe the children's physical activity closely. Body temperature ≥ 38.5°C and history of lethargy may be particularly useful clinical clues for neurological involvement during the first 2 days of febrile illness since at this time the presentation of complicated HFMD is typically undifferentiated and subtle, even to the experienced clinicians [8]. Indeed both history of lethargy and temperature ≥ 38.5°C were observed for 24–48 hours in all the 9 fatal case children before they succumbed to unexpected fulminant cardiorespiratory failure (Table 4). Primary care doctors should have high index of suspicions of neurological complication when they are presented with children with HFMD who have been febrile ≥ 3 days. The children should be admitted into hospital for close observation and investigated for CNS involvement, if necessary. Our study showed that 92 (31%) of 293 children with total duration of fever ≥ 3 days in the 2000/3 outbreak, and 183 (61%) of 300 children in the 2006 outbreak had neurological involvement (Table 2). CSF pleocytosis was present in 25% (19/74) of children with a single risk factor of being febrile ≥ 3 days on hospital admission (Figure 2). The risk of CNS complication is increased significantly when there are added risk factors of having history of lethargy and temperature ≥ 38.5°C. In contrast children who have a brief duration of low grade fever (≥ 38.5°C) and no history of lethargy are of low risk of neurological disease, and may be provided with outpatient care and parental reassurance.

Our results are in keeping with findings reported by Chang and co-authors where fever ≥ 39°C, fever duration ≥ 3 days and lethargy were more frequently observed in children with CNS involvement and in children with HEV71-HFMD than in those with CVA16-HFMD [8,20]. Although several other clinical features and laboratory abnormalities have been associated with fatal HEV71-HFMD, they have yet been validated, and been shown useful in detecting neurological disease or disease progression [8,21,22]. For example, Chong and co-authors reported that absence of mouth ulcers predicted a more complicated or fatal HFMD, and have recommended that children without mouth ulcers should be monitored closely [22]. However, in our study we did not find that children without mouth ulcers were more likely than children with mouth ulcers to have features of more severe HFMD or develop neurological complication. Not all the risk factors identified in these published studies can readily be translated into clinical practice, particularly at primary care settings. Hyperglycemia and leucocytosis have been shown as risk factors for fatal HEV71 disease [8]. However, in our experience hyperglycemia and leucocytosis are late laboratory changes in children with fulminant cardiorespiratory failure (unpublished observations), and thus are not helpful clinically in identifying children at high risk of complication and death. Elevated cardiac Troponin I, a sensitive cardiac-specific biomarker for myocardial injury, has been noted in children who subsequently developed left ventricular failure, and may be useful in identifying patients at risk of left ventricular failure and pulmonary oedema [21]. Although cardiac Troponin I has been used widely in developed countries for early diagnosis of acute coronary syndrome, it is expensive and not widely available in many developing countries, including in Sarawak. Screening for heart rate variability abnormalities, an index of autonomic nervous system, through non-invasive continuous ECG monitoring may provide early warning of impending cardiorespiratory failure about 7 hours before its onset [16]. The labour-intensive approach is most suited in a critical care setting for children already diagnosed of CNS involvement because it requires the use of expensive and sophisticated device, and close monitoring for the heart rate abnormalities.

A limitation of our study is that the clinical predictors developed for use at primary care setting were identified and tested using data collected through a hospital-based study. Clinical characteristics of children treated at primary care settings may differ from hospitalized children. However, as a large number of children with mild HFMD were admitted into our study, we had the opportunity to systemically examine the clinical feature of HFMD of varying severity, including children with mild disease that would normally be treated at primary care clinics, where we have shown history of lethargy, peak temperature 38.5C and total duration of fever 3 days were reported infrequently in children with mild HFMD.

Conclusion

Currently there is no vaccine against HEV71 infection. Early recognition of children at risk of fulminant pulmonary oedema and cardiac dysfunction is the key to reduce acute mortality and morbidity. We have identified three clinical risk factors that may help detect children at risk of neurological involvement and death at primary care settings, which can guide primary care doctors decide if hospital admission is warranted when they see with children with HFMD. These risk factors are readily elicited through history taking and measurement of body temperature.

They may also provide useful guide to help clinicians to decide the need for CSF examination, as well as to monitor disease progression in children with HFMD.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MHO, SCW, MJC and TS conceived of the study; they were assisted by AM, CHC, DC and SS in the planning, design, and execution of the clinical aspects and by PHT, YP and DP in the analysis and interpretation of the clinical samples; all authors contributed to writing the manuscript.

Additional material

Additional file 1

Clinical features of the 725 children with Hand, foot and mouth Disease that were admitted between January 2000 and July 2003 according to the clinical severity. The clinical features of the children with HFMD-CNS (i.e. with CSF pleocytosis) are compared to those with HFMD-Non-CNS (i.e. no CSF pleocytosis). The clinical features of children with mild HFMD is also included here.

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Additional file 2

Clinical features of the 730 children with Hand, foot and mouth Disease that were admitted during the 2006 outbreak according to clinical severity. The clinical features of the children with HFMD-CNS (i.e. with CSF pleocytosis) are compared to those with HFMD-Non-CNS (i.e. no CSF pleocytosis). The clinical features of children with mild HFMD is also included here.

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Virology, epidemiology, pathogenesis, and control of enterovirus 71

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For the **revised classification** see http://www.picornaviridae.com/enterovirus/enterovirus.htm

First isolated in California, USA, in 1969, enterovirus 71 (EV71) is a major public health issue across the Asia-Pacific region and beyond. The virus, which is closely related to polioviruses, mostly affects children and causes hand, foot, and mouth disease with neurological and systemic complications. Specific receptors for this virus are found on white blood cells, cells in the respiratory and gastrointestinal tract, and dendritic cells. Being an RNA virus, EV71 lacks a proofreading mechanism and is evolving rapidly, with new outbreaks occurring across Asia in regular cycles, and virus gene subgroups seem to differ in clinical epidemiological properties. The pathogenesis of the severe cardiopulmonary manifestations and the relative contributions of neurogenic pulmonary oedema, cardiac dysfunction, increased vascular permeability, and cytokine storm are controversial. Public health interventions to control outbreaks involve social distancing measures, but their effectiveness has not been fully assessed. Vaccines being developed include inactivated whole-virus, live attenuated, subviral particle, and DNA vaccines.

Introduction

Enteroviruses are small, single-stranded, positive-sense RNA viruses from the enterovirus genus in the family Picornaviridae.[1] They cause disorders with a wide range of clinical manifestations, including cutaneous, visceral, and neurological diseases. For many years polioviruses were the most important enteroviruses, since they led to large outbreaks of paralytic disease. A global campaign has, however, almost eradicated poliomyelitis from many regions worldwide. In its place, enterovirus 71 (EV71) causes major outbreaks of hand, foot, and mouth disease (HFMD), most frequently affecting children. This virus was first described in 1969,[2] although an analysis shows that EV71 was circulating in the Netherlands as early as 1963.[3] Although present in most countries, the largest outbreaks of disease have been seen in the Asia-Pacific region, for reasons that are incompletely understood.[4–16] The neurological manifestations range from aseptic meningitis to acute flaccid paralysis and brainstem encephalitis, which is associated with systemic features, such as severe pulmonary oedema and shock, in many cases.[17,18] The clinical features, investigations, and management of severe EV71 disease are discussed in a companion article in *The Lancet Neurology*.[19] In this Review we consider the virology, clinical and molecular epidemiology, pathogenesis, and prospects for control.

Virology

Classification

As well as the enterovirus genus, the large Picornaviridae family includes Rhinovirus spp (eg, the common cold), Hepatovirus spp (eg, human hepatitis A virus), Parechovirus spp (eg, human parechovirus 1 and 2), and two important animal virus genera, Cardiovirus spp (eg, encephalomyocarditis virus) and Aphthovirus spp (foot and mouth disease virus).[1] Human enteroviruses were traditionally separated into four classifications, according to their pathogenicity in human beings and experimental animals and their cytopathic effects in tissue culture; these subgroups were polioviruses (three serotypes), coxsackievirus A (23 serotypes), coxsackievirus B (six serotypes), and echoviruses (28 serotypes).[1] However, because of the limitations of this system, serologically distinct human enteroviruses isolated since 1970 have been designated by serotype numbers, beginning with HEV68. The original classification of human enteroviruses has been substituted by a taxonomic scheme based on molecular and biological properties of the viruses.[20] This revised classification recognises at least 90 subtypes and separates them into four species (table 1). Polioviruses have been designated as members of the human enterovirus C species because they are genetically closely related.[21]

Physicochemical properties

The virus capsid comprises 60 identical subunits (protomers), each of which contains a copy of the four structural viral proteins (figure 1).[22] The lack of a lipid envelope confers human enteroviruses stability in the host environment, including on exposure to human gastric acid, and they can survive at room temperature for several days. EV71 and other enteroviruses have also been detected in surface and ground water and in hot spas.[23,24] Enteroviruses are resistant to organic solvents (eg, ether and chloroform), alcohol, and freezing, but can be inactivated by temperatures higher than 56°C, chlorination, formaldehyde, and ultraviolet irradiation. In one study EV71 was destroyed by virucidal disinfectants.[25]

Life cycle and replication

Human beings are the only known natural hosts of human enteroviruses. Like most other enteroviruses the replication cycle of EV71 is similar to that of polioviruses.[26] Viral entry into susceptible host cells is dependent on specific receptors. Seven receptors for different enteroviruses have been identified in human beings.[20] The specific receptors include the poliovirus receptor (CD155), three integrins (α2β1, αvβ3, and αvβ6), decay-accelerating factor (CD55), the coxsackievirus-adenovirus

	Serotype
A	CV-A2–8, CV-A10, CV-A12, CV-A14, CV-A16, EV71, EV76, EV89–92
B	CV-A9, CV-B1–6, E1–7, E9, E11–21, E24–27, E29–33, EV69, EV73, EV74–75, EV77–88, EV93, EV97, EV98, EV100, EV101, EV106, EV107
C	CV-A1, CV-A11, CV-A13, CV-A17, CV-A19–A22, CV-A24, EV95, EV96, EV99, EV102, EV104, EV105, EV109, PV1–3
D	EV68, EV70, EV94

The Picornaviridae Study Group and the International Committee on Taxonomy of Viruses classified the Enterovirus genus into ten species, which include four human enterovirus species (A–D), three human rhinovirus species (A–C), bovine enterovirus, simian enterovirus A, and porcine enteroviruses (http://www.ncbi.nlm.nih.gov/ICTVdb/ICTVdB/). CV-A=coxsackievirus A. CV-B=coxsackievirus B. EV=enterovirus. E=echovirus. PV=poliovirus.

Table 1: **Human enterovirus serotypes, by species**

receptor, and intracellular adhesion molecule 1. Some enteroviruses use more than one receptor to infect a host cell. Several receptors for EV71 have been identified, but a ubiquitously expressed cellular receptor, scavenger receptor B2, and a functional receptor, human P-selectin glycoprotein ligand-1, found on white blood cells, are specific for EV71.[27,28] Sialic-acid-linked glycan, which is expressed in abundance in the respiratory and gastrointestinal tracts, and dendritic-cell-specific intercellular adhesion-molecule-3-grabbing non-integrin (CD209), which is found exclusively in dendritic cells in lymphoid tissues, have also been identified.[29–31]

After an enterovirus binds with a specific receptor on the cell surface, a series of structural changes occur in the virus capsid (yet to be defined in EV71) and pores are formed in the cell membrane through which the virion RNA is released into the host cell cytoplasm. Being positive-sensed, the parent virus RNA acts directly as a messenger RNA and is translated into a large polypeptide that is promptly cleaved by the viral proteases into 11 mature structural and non-structural proteins. The replication of the virus genome by the error-prone RNA-dependent RNA polymerase 3Dpol takes place in a vesicle membrane structure (viral replication complex). The polymerase is estimated to misincorporate one or two bases in every genome copying event, which explains why the virus mutates and evolves rapidly. Within the *VP1* gene $4{\cdot}2–4{\cdot}6\times10^{-3}$ nucleotide substitutions occur per site per year, which is similar to the number in poliovirus and greater than that of influenza viruses.[32–34]

While the machinery of the host cellular protein synthesis is shut down by viral protease 2A, viral protein synthesis remains unaffected. An infectious virus particle is formed after the packaging of a progeny viral RNA into a virus capsid in the cytoplasm of the infected cells. Mature infectious virus particles are released when an infected cell is lysed.

Clinical epidemiology

Initial identification

EV71 was isolated from the stool of a child aged 9 months with encephalitis, in California, USA, in 1969,[2] although an earlier isolate has since been identified.[3] Within 5 years small outbreaks of neurological infections, including encephalitis and aseptic meningitis, attributed to EV71 were reported in Australia, Japan, Sweden, and the USA.[35–39]

The dermotrophic properties of EV71 were first recognised when the virus caused epidemics of HFMD in Japan in 1973.[38,39] In the 1970s, two large EV71 epidemics occurred in Europe. The first, in Bulgaria, was initially attributed to polioviruses because of the epidemiological, clinical, and pathological characteristics.[40,41] EV71, confirmed by virus isolation or neutralisation test, was later identified as the causative agent in 347 (77%) of 451 children who presented with non-specific febrile illness or neurological disease; 44 children died. The second major epidemic was 3 years later in Hungary, with 1550 cases (826 aseptic meningitis, 724 encephalitis) and 47 deaths reported; unlike the Bulgarian epidemic, few patients had HFMD.[42]

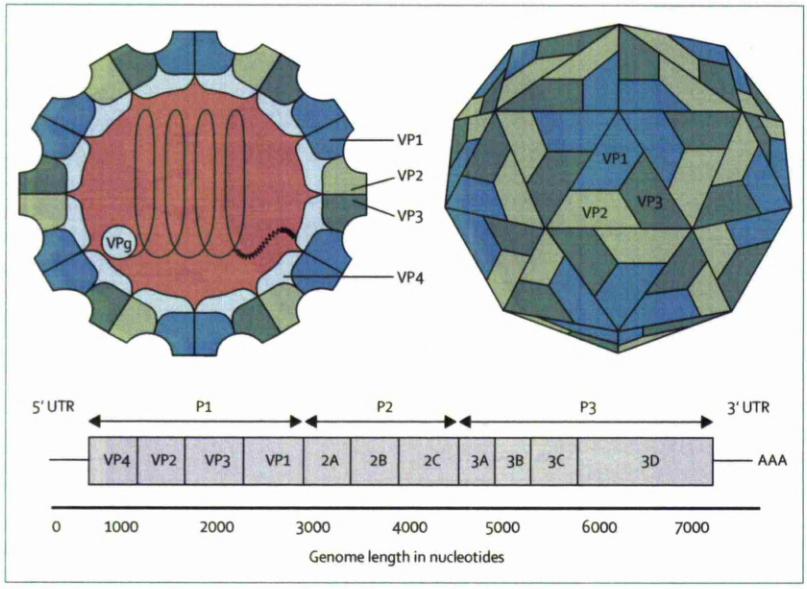

Figure 1: **Enterovirus 71 structure and genome structure of the virion**
Human enteroviruses are small (circumference around 30 nm), non-enveloped, icosahedral particles that contain a single-stranded, positive-sense, polyadenylated virus RNA of approximately 7·4 kb. Each protomer in the virus capsid contains a copy of the four structural viral proteins (VP1–VP4), of which VP1, VP2, and VP3 are external, whereas VP4 is completely internalised and is not, therefore, exposed to the host antibody response. All the structural proteins are encoded by the P1 region of the genome. The P2 and P3 regions encode seven non-structural proteins—2A–2C and 3A–3D. Reproduced from ViralZone, with permission of Swiss Institute of Bioinformatics, and from reference 70, with permission of Springer. UTR=untranslated region. VPg=virus encoded protein.

	1973	1980	1986	1990	1993	1994	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008
Singapore	..	..	..	..	..	..	B3, B4	B3, C1	B3	B4*	B4	C1, B4	B4	-	-	B5	-	B5
Peninsular Malaysia	..	..	..	..	..	..	B3*, B4*, C1†, C2†	C1	B4, C1	B4*, C1*	-	-	-	-	B5*, C1	-	-	-
Sarawak, Malaysia	..	..	..	..	..	..	B3*	C1	None‡	B4*, C1	None‡	C1	B5, C1	None‡	B5	B5		
Perth, Australia	..	..	..	..	..	..	..	..	B3, C2	C1	None‡	None‡	-	-	-	-	-	-
Japan	B1	..	..	B2, C1	B2	C3	B3, B4, C2	C2	C2	B4	C2	B4, C2	C4, B5	C4	-	C4	C4	-
Taiwan	..	B1	B1	..	..	..	..	C2*, B4†	B4	B4*	B4	B4, C4†	B4, B5†	C4*	C4*, C5†	C5	C5, B5	B5*
Korea	..	..	..	..	..	..	..	..	..	C3*	None‡	None‡	C4	-	-	-	-	-
Brunei	..	..	..	..	..	..	..	..	..	-	-	-	-	-	-	B5	-	-
Vietnam	..	..	..	..	..	..	..	..	..	-	-	-	-	-	C1, C4, C5	-	-	-
Thailand	..	..	..	..	..	..	..	..	..	-	-	C1	C1	C1	-	B5, C1, C2, C4	B5, C1, C2, C4, C5	B5†, C1, C2†, C4*
China	..	..	..	..	..	..	C3	C4	..	C4	C4	C4	C4	C4	-	-	C4	C4

*Genotypic subgroups caused large outbreaks. †Genotypic subgroup isolated in a small number of patients. ‡No enterovirus 71 detected, despite active surveillance.

Table 2: **Enterovirus 71 genotypic subgroups reported to be circulating in the Asia-Pacific region between 1973 and 2008, by year**[9-14,45-53]

Asia-Pacific region

After the Australian and Japanese EV71 epidemics of the 1970s, further small epidemics and sporadic clusters occurred in Hong Kong in 1985,[43] and in Australia in 1986.[44] In 1997, a large outbreak of EV71 in Sarawak, Malaysia, heralded the start of a new series of outbreaks across the Asia-Pacific region (table 2).[9-14,45-53]

In Sarawak, 2618 HFMD cases and 34 deaths were recorded between May and July, 1997; around the same time EV71 caused four deaths in peninsular Malaysia and several cases of severe neurological disease in Japan.[4,11,54] In 1998, the largest EV71 epidemic so far occurred in Taiwan:[7] an estimated 1·5 million people were infected and 405 children were admitted to hospital for serious neurological complications, of whom 78 died. The latest large Asian-Pacific epidemic was in China in 2008, when around 490 000 infections and 126 deaths in children were reported; at the epicentre in Anhui Province, more than 6000 HFMD cases and 22 deaths in children were reported.[12] In addition to these very large outbreaks, many areas, including Japan, Sarawak, Singapore, Taiwan, and Vietnam, have experienced cyclical epidemics that occur every 2–3 years (figure 2).[55-57]

Brainstem encephalitis, especially affecting the medulla, associated with cardiopulmonary dysfunction has become a notable feature in EV71 epidemics in Asia, and is the primary cause of death.[4,9,12,17,58] This presentation is in contrast to that in the 1980s, when aseptic meningitis was the most frequent neurological involvement.[36,37] Children typically present with a brief febrile illness and mild neurological signs, after which they develop signs of tachycardia, poor perfusion, and tachypnoea that rapidly develop into acute, intractable cardiac dysfunction and fulminant—in many cases fatal—pulmonary oedema or haemorrhage.[19] Neurogenic pulmonary oedema is thought to be the main pathogenic process.[17,18,54,59]

Other regions

Outside the Asia-Pacific region, EV71 has continued to circulate at a low level in Africa, Europe, and the USA and causes sporadic cases or small outbreaks. During a 1-year prospective study in Canada in 1998, 20 children with EV71 were admitted to a tertiary hospital, mostly in the summer or autumn months; half had aseptic meningitis, and a third had respiratory symptoms, but no symptoms were severe and all improved rapidly.[60] Two small community outbreaks of neurological EV71 disease, without HFMD, occurred in 2003 and 2005 in Denver, CO, USA, affecting 16 children aged 4 weeks to 9 years; one child died.[61] A retrospective analysis of stool samples collected from children admitted to hospital with aseptic meningitis in Austria between 2001 and 2004 showed that EV71 was detected in 16 (9%) of 181.[62] A similar study identified 32 sporadic cases of EV71 infection in the UK between 1998 and 2006, presenting primarily as neurological disease, HFMD, or both.[63] In the Netherlands, 58 people were admitted to hospital with EV71-associated fever, gastrointestinal symptoms, and CNS infections in 2007, after 21 years of low endemicity.[3] Widespread asymptomatic circulation of EV71 was also noted between October 2002 and October 2003 in Norway, where the virus was isolated from 19 (17%) of 113 well children.[64] EV71 was among a range of enteroviruses detected by the screening of blood donations in Scotland over 22 months; the detection rate for any enterovirus was one per 4000 donations,[65] although the importance of this finding remains uncertain. In Nairobi, Kenya, two small institutional outbreaks of EV71 infection were reported in an HIV orphanage in 1999 and 2000.[66]

Molecular epidemiology

Gene groups, evolution, and geographical distribution

Phylogenetic analysis suggests that EV71 emerged from the coxsackievirus type A 16, as recently as 1940.[32] The

first complete phylogenetic analysis of EV71 based on the structural *VP1* gene identified three independent lineages of EV71, designated A, B, and C;[53] each group has at least 15% divergence from the others. Group A consists of one member, the prototype BrCr strain, which was first identified in California, USA, in 1970, and was not reported outside the USA until 2008, when isolates were reported from five of 22 children presenting with HFMD in Anhui province of central China.[67] Sequencing of the complete *VP1* gene showed very little divergence between isolates. The virus might, therefore, have been circulating undetected in central China, with very little evolutionary change for 40 years, although the source of the virus templates that were sequenced could

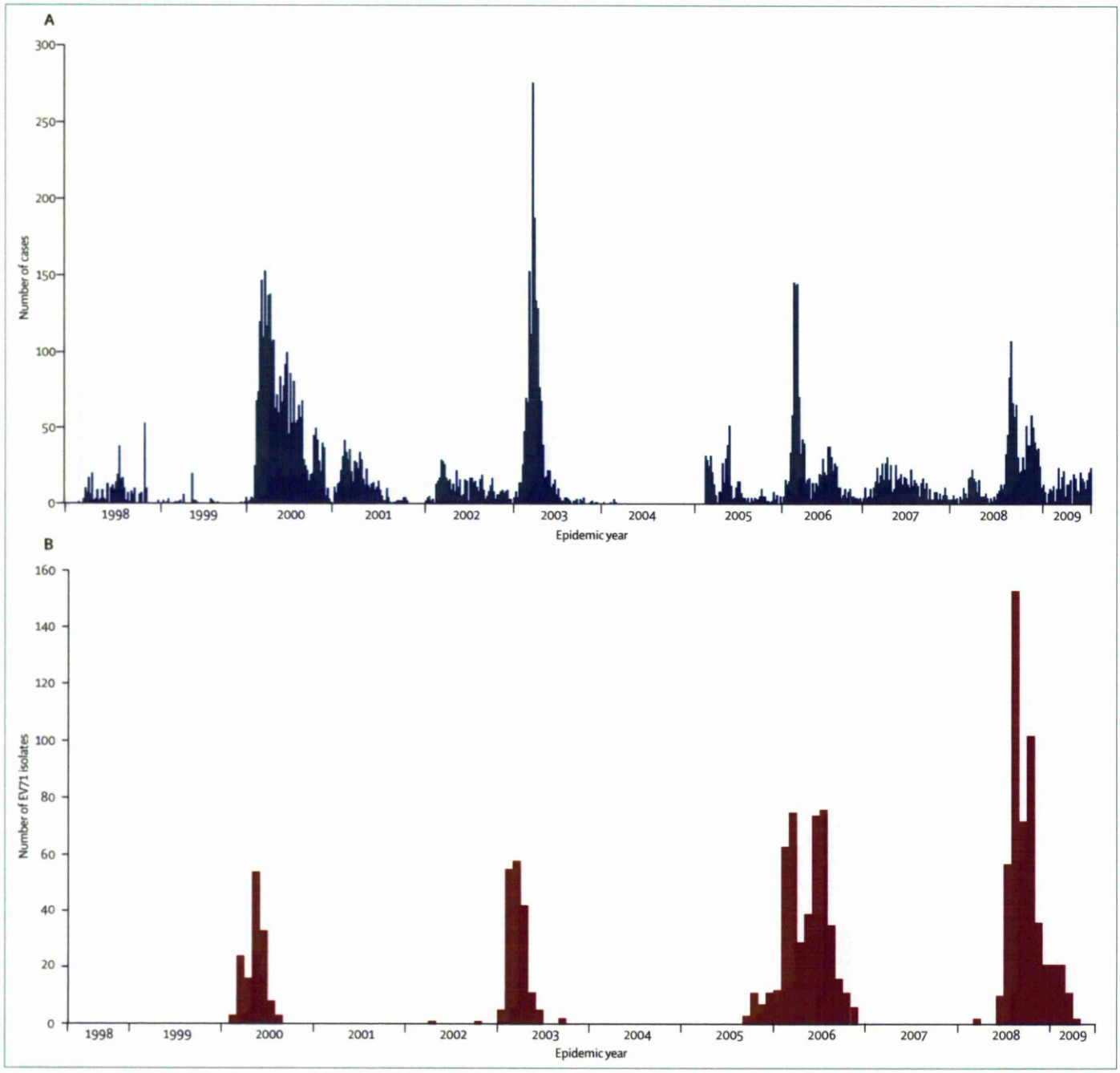

Figure 2: **Distribution of (A) hand, foot, and mouth disease and (B) enteroviruse 71 isolates identified in sentinel clinics in Sarawak, Malaysia, from March, 1998, to mid-2009**

have affected the results. Surveillance data from the same outbreak by the Chinese Center for Disease Control and Prevention do not seem to indicate any group A viruses.[12] Good surveillance programmes are needed in many different geographical regions to provide accurate and relevant information about EV71 transmission and evolution, and to confirm whether group A viruses have re-emerged.

The B group has been predominant in Malaysia and Singapore, whereas the C group has been so in east Asia, especially in mainland China and Vietnam (figure 3). Group B viruses, which were initially separated into subgroups, B1 and B2, owing to 12% divergence at the nucleotide level, were the predominant circulating strains in the 1970s and 1980s.[53] Group C viruses, which were initially separated into the C1 and C2 subgroups, were identified in the mid-1980s (figure 3). Several subgroups have been added to groups B and C in the past 12 years, according to findings in the Asia-Pacific region (figure 3, table 2). Viruses in subgroups B3 and B4 are thought to have both circulated in the region since 1997.[13,14,45] Subgroup B5, was first isolated in Japan and Sarawak in 2003, caused epidemics in Brunei, Sarawak, and Taiwan in 2006.[46,55,56,68] Except for the major community outbreak in Sydney in 1986, subgroup C1 viruses have been isolated mainly from sporadic cases since the mid-1980s, which suggests low-level circulation worldwide.[53,69] Subgroup C2 viruses caused the outbreak in 1998, and an outbreak in Perth, Australia, in 1999.[8,13,47,70] Subgroup C3 was isolated in Japan in 1994, and in Korea in 2000.[13,16,71] Subgroup C4 has been the predominant circulating subtype in mainland China since 2000, and has been reported in Japan, Vietnam, and Taiwan.[9,12,14,47] Subgroup C5 has been reported in southern Vietnam and Taiwan.[9,46] A genetically distinct EV71 strain (R13223, Genbank accession number AY179600 to AY179602), with no genetic relationship to other EV71 strains, was isolated in India in 2001 from one child with acute flaccid paralysis.[72]

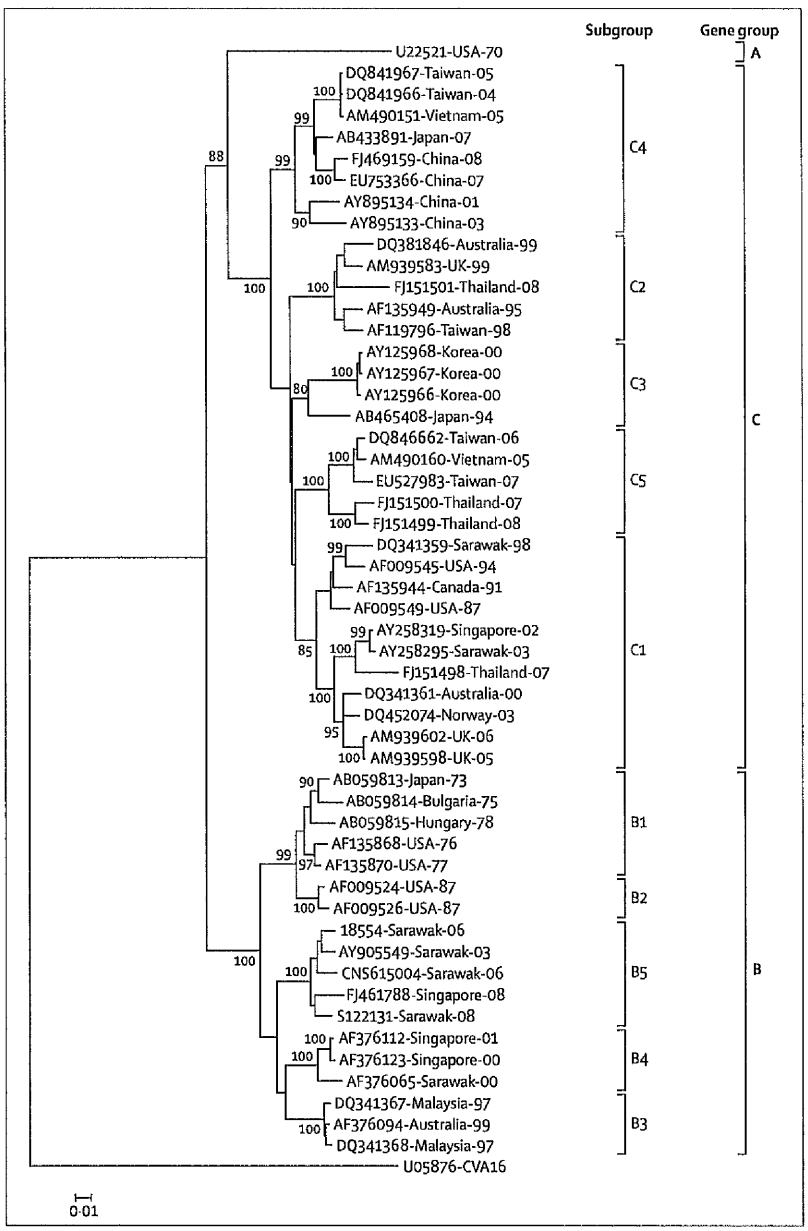

Figure 3: **Phylogenetic analysis of enterovirus 71 VP1 gene sequences**
A neighbour-joining tree constructed with the Kimura-2 parameter as a model for nucleotide substitution. The robustness of the tree was determined by bootstrapping, with use of 1000 pseudoreplicates..

Transmission and epidemic potential

Surveillance systems for EV71 established in several countries in the Asia-Pacific region, mainly to monitor transmission and spread, have provided information on virus evolution during outbreaks. In Sarawak, viral activity has increased every 3 years since 1997. This pattern is closely associated with increases in community incidence of HFMD.[55] Regular cyclical epidemics have also been seen in Fukushima Prefecture, Japan.[57] Such cyclical activity is assumed to relate to the availability of new birth cohorts of children who have not been exposed to the virus.[73,74] Prediction of the epidemic potential of particular genotypic subgroups has proved difficult, although some differences in virulence, judged by size of associated epidemics, exist.

Shifts in subgroup dominance have been reported in Sarawak and Vietnam.[9,13,55] In Japan and Taiwan subgroups of the B and C viruses have caused epidemics at different times (table 2).[46,47,75] By contrast, in the Netherlands group B viruses were predominant before 1986, but since 1987 dominance has shifted to group C viruses; cross-neutralisation among the group B but not group C viruses is a possible explanation, although experimental data seem not to support this theory.[3,76–78] Older subgroups of EV71 have been circulating and causing low levels of disease for many years, whereas some of those in newly described subgroups, such as B5, possess antigenicity distinct from other viruses and might, therefore, have the potential to

cause very large outbreaks.[48,76] Although outbreaks have so far been confined to the Asia-Pacific region, increasing rates of travel mean that every region could be at risk.

Recombination

Recombination events occur frequently within enterovirus species,[79] and recombination between EV71 viruses, and occasionally between EV71 and other enteroviruses, such as coxsackievirus types A 16 and A 8, has also been reported.[48,80] Since recombination most often involves non-structural gene regions or untranslated regions, the use of PCR that amplifies the *VP1* gene region is thought to be robust for diagnosis.

Pathogenesis

Viral determinants of virulence

The factors that determine whether EV71 infection will be asymptomatic or lead to HFMD or severe neurological disease are unknown. For polioviruses, the 5′ untranslated region and *VP1* genes contain virulence determinants.[26] Several studies have, therefore, examined the relevant nucleotide sequences or the whole genome to compare isolates from fatal and non-fatal cases, but most isolates have been identical or nearly identical.[81,82] The frequency of CNS disease and other severe complications of EV71 infection has varied between Asian outbreaks, which suggests differences in virulence of subtypes. However, comparisons of outbreak data have been hampered by differences in study designs and viral diagnostic capabilities.

Perhaps the strongest data that determinants of strain virulence have key roles in the pathogenesis of severe neurological disease come from outbreaks in Perth, Australia, and Sarawak. In Perth, in 1999, subgroups B3 and C2 were both circulating.[45,70] C2 viruses linked to the Taiwan epidemic of 1998 were almost exclusively isolated from children with severe neurological disease, and only one isolate came from a case of uncomplicated HFMD.[45,70] By contrast, B3 viruses, which were similar to those from the Sarawak 1997 epidemic, were isolated mainly from children with uncomplicated HFMD, aseptic meningitis, or those with neurological complications, none of whom died.[8] In two discrete epidemics in Sarawak in which either B4 or B5 viruses were predominant, a study of 277 children with EV71-associated HFMD showed that B4 viruses were less likely than B5 viruses to cause CNS infection or be part of a family cluster.[83]

Dual infection

During the 1997 EV71 B3 virus outbreak in Sarawak, an adenovirus type 21 was isolated in the patients who died and in some with acute flaccid paralysis.[4,84] The virus was detected at autopsy in sterile sites, such as cerebrospinal fluid and brain and heart tissue, in more patients than EV71. This finding led to the suggestion that death was related to dual infection,[4] but subsequent detailed studies, including longitudinal studies from Sarawak, have found no evidence of adenovirus 21 infection in other HFMD or neurological cases. Dual infection with EV71 and other viruses, including dengue and Japanese encephalitis, has been reported.[83] Furthermore, adenovirus 21 has not been isolated in Sarawak since 1997.

Host susceptibility

Various factors could affect pathogenesis, especially partial cross-protective immunity from previous outbreaks, which might partly explain why young age is a risk factor for severe disease.[73,74,85] One genetic study in Taiwan reported that HLA-A33 is associated with increased susceptibility to EV71 infection, although the role of MHC remains unknown.[86] The researchers noted that HLA-A33 is more frequent in Asian populations than in white populations, which might explain the high number of EV71 epidemics in Asia. They suggested also that HLA-A2, in a mechanism yet to be defined, could be linked to the risk of cardiopulmonary failure in patients with EV71.[86] The *CTLA4* gene is an important regulator of T-cell cytotoxicity, and it has a role in the regulation of an immune response. In a study of 78 children with EV71 infection in Taiwan, those with meningoencephalitis had a higher frequency of G/G genotype at position 49 of exon 1 in this gene, than those without meningoencephalitis and controls.[87] However, a subsequent study found no such association.[86]

Pathophysiology of severe disease

Virus entry and spread

EV71 is transmitted predominantly via the faeco-oral route, but can also spread through contact with virus-contaminated oral secretions, vesicular fluid, surfaces or fomites, and in respiratory droplets.[1] As with other enteroviruses, initial viral replication is presumed to occur in the lymphoid tissues of the oropharyngeal cavity (tonsils) and small bowel (Peyer's patches), with further multiplication in the regional lymph nodes (deep cervical and mesenteric nodes), giving rise to a mild viraemia. Most infections are controlled at this point and remain asymptomatic. Further dissemination of enteroviruses to the reticuloendothelial system (liver, spleen, bone marrow, and lymph nodes), heart, lung, pancreas, skin, mucous membranes, and CNS coincides with the onset of clinical features. For EV71, viral shedding from the throat can occur up to 2 weeks after an acute EV71 infection, and virus can be isolated from stool for up to 11 weeks.[88]

Epidemiological and experimental studies suggest that polioviruses can invade the CNS system through a disrupted blood–brain barrier or retrograde axonal spread along cranial or peripheral nerves. For EV71, studies in mice and assessment of the distribution of virus and inflammation in fatal human cases implicate the latter route.[88–91]

Pathological findings

CNS inflammation predominantly affects grey matter of the spinal cord and the whole medulla oblongata, including the dorsal nucleus of the vagus, tractus solitarius, the nucleus, and reticular formation. The hypothalamus and subthalamic and dentate nuclei, and to a lesser degree motor cortex of the cerebrum, are also involved (figure 4).[17,54,90,92–94] Inflammatory changes were absent in cerebellar cortex, thalamus, basal ganglia, peripheral nerve, and autonomic ganglia. Histopathological changes, characterised by perivascular cuffs, variable oedema, neuronophagia, and microglia nodules, are similar to those in encephalitis caused by other viruses.[95] However, virus inclusion has not been observed, and viral antigens and RNA can be seen in only a few neuronal processes and phagocytic cells.[90]

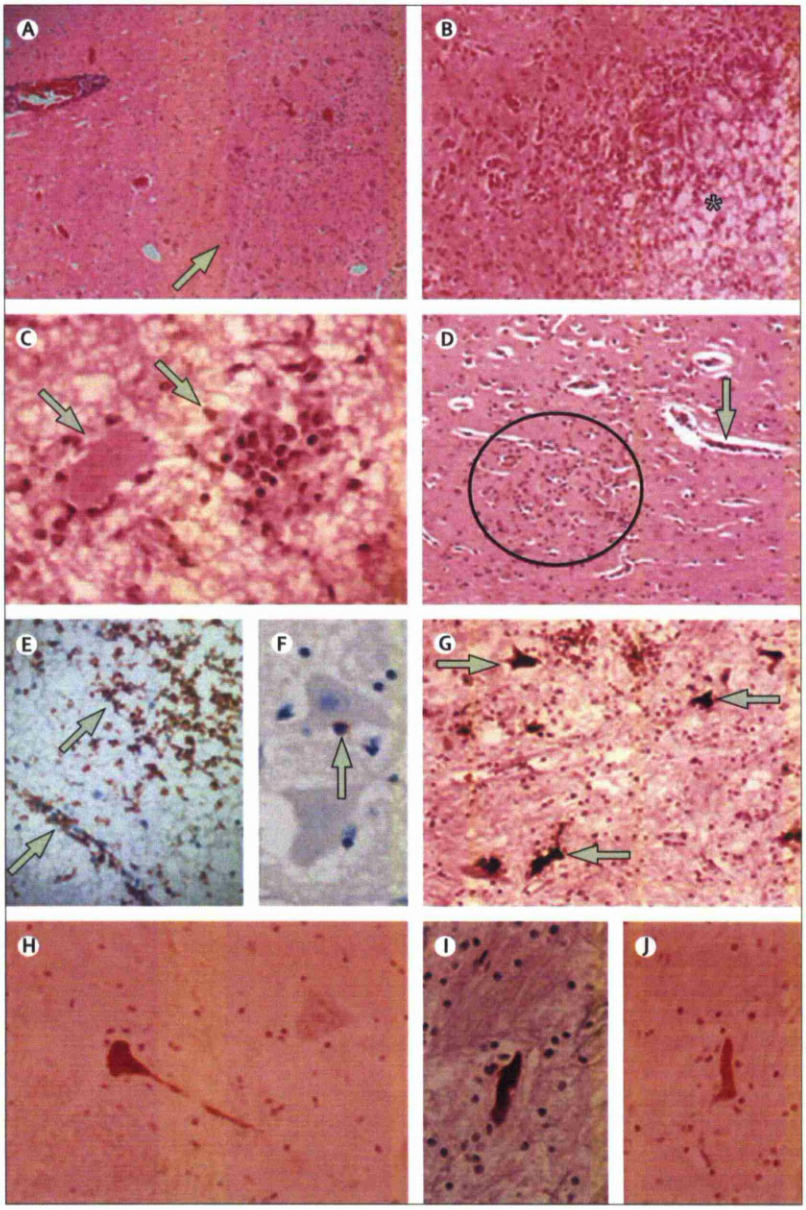

Figure 4: **Pathological findings in enterovirus 71 encephalitis**
(A) Parenchymal inflammation (arrow) and perivascular cuffing in the the medulla. Severely inflamed areas show (B) oedema (asterisk) and (C) neuronophagia (arrows). (D) More-subtle inflammation can be seen in the motor cortex, with mild perivascular cuffing (arrow) and parenchymal inflammatory cells (circle). (E) Numerous CD68-positive macrophages/microglia (arrows). (F) A CD8-positive lymphocyte adjacent to a neuron (arrow). (G) Viral RNA in the anterior horn cells of the spinal cord (arrows). (H) Viral antigens in the neuronal body and process in the hypothalamus. (I) Adjacent section of the same neuron that was positive for viral RNA, and (J) adjacent section that was positive for antigen. Stains: haematoxylin and eosin (A–D), immunohistochemistry/peroxidase/DAB (E, F), and ISH/nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (G). Original magnifications: ×4 (A), ×10 (B and D), ×40 (C and F), and ×20 (G). Modified from the *Journal of Neuropathololgy and Experimental Neurology*,[90] with permission of Wolters Kluwer.

Severe pulmonary oedema and heart failure

Although fulminant pulmonary oedema is preceded by and closely associated with CNS involvement, its cause is unclear, especially whether neurogenic pulmonary oedema, cardiac dysfunction, increased vascular permeability, and cytokine storm contribute (figure 5).

Neurogenic pulmonary oedema classically follows head injury. In these cases, raised intracranial pressure is thought to be important, but the pathogenesis is not completely understood. Experimental studies suggest that the hypothalamus, vasomotor centres of the medulla, and nuclei in the cervical spinal cord are important; lesions to various nuclei in these regions can increase activity along the sympathetic trunk, resulting in severe systemic and pulmonary hypertension and pulmonary oedema.[96] Damage to brainstem nuclei in poliomyelitis is thought to lead to pulmonary oedema of neurogenic origin.[97] Thus, when severe pulmonary oedema was first seen in EV71 encephalitis along with brainstem inflammatory changes, oedema was thought to be neurogenic. Post-mortem examination and MRI studies of children with EV71 brainstem encephalitis showed extensive inflammation of grey matter of the spinal cord and the whole medulla oblongata.[17,54,90,93,94] The observations of hyperglycaemia and leucocytosis were also postulated to be due to increased sympathetic discharges.[98]

Severe systemic and pulmonary hypertension is not always seen in children with EV71-associated pulmonary oedema.[99–101] This disparity might arise because the changes in vascular pressures in neurogenic pulmonary oedema are only transient.[96] Some commentators have argued that cardiac dysfunction is a major contributor to the pulmonary oedema. Although no histological or virological evidence of viral myocarditis is seen in patients with EV71 infection, raised concentrations of cardiac-specific troponin I suggest a degree of cardiac damage.[18,54,101,102] An echocardiographic study in 11 children with EV71 brainstem encephalitis showed that cardiac function was impaired, indicated by substantially lowered left-ventricular ejection fractions.[101] Two children whose cardiac output was supported with a left-ventricular assist device survived, whereas all the others died.[103] In a separate report the same researchers described very high concentrations of norepinephrine

and epinephrine in three of the 17 children with left-ventricular dysfunction.[101]

Although patients with EV71 infection do not have myocardial inflammation, histological examination of cardiac ventricular tissue biopsy samples from six fatal cases and one survivor revealed notable coagulative myocytolysis, myofibrillar degeneration, and cardiomyocyte apoptosis, which are the characteristic features of catecholamine-associated cardiotoxic effects.[101,104] Thus, high catecholamine concentrations caused by brainstem encephalitis are purported to have a direct effect on cardiac function, as well as to cause pulmonary oedema via raised pulmonary pressures.

The other potential contributor to pulmonary oedema, increased vascular permeability, might arise secondary to the systemic inflammatory response. Several studies have examined cytokine and chemokine profiles in EV71 patients with brainstem encephalitis: concentrations of interleukins 1B, 6, 10, and 13, tumour necrosis factor α, and interferon γ are all significantly higher in patients with EV71 with pulmonary oedema than in those without. Several of these cytokines are mediators of increased

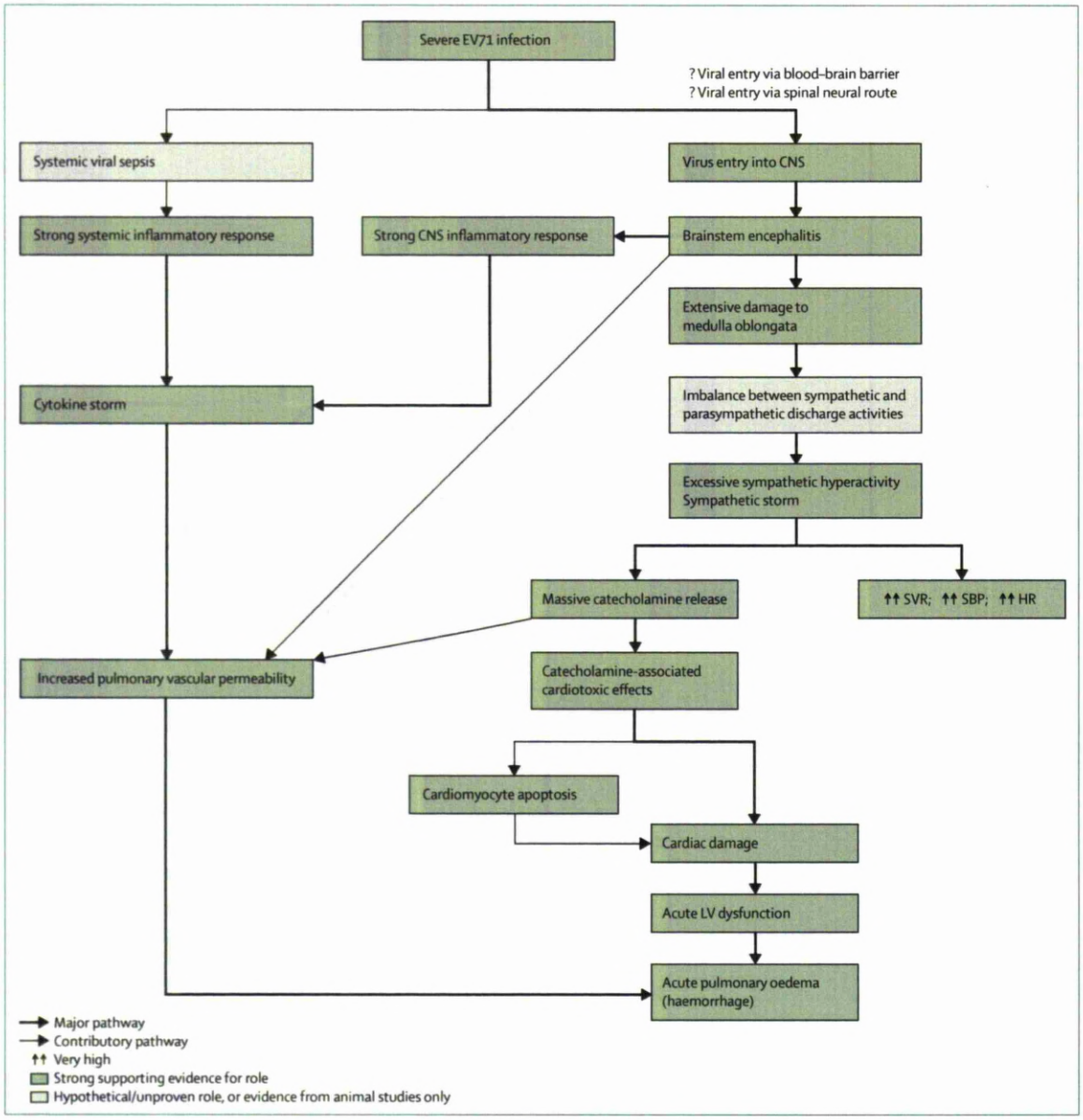

Figure 5: **The postulated pathogenesis of enterovirus-71-associated acute pulmonary oedema**
EV71=enterovirus 71. CNS=central nervous system. SVR=systemic vascular resistance. SBP=systemic blood pressure. HR=heart rate. LV=left ventricular.

vascular permeability.[105–107] High concentrations of several chemokines in plasma, including 10 kDa-interferon-γ-induced protein, monocyte chemoattractant protein, monokine induced by interferon γ, and interleukin 8, have been reported in children with brainstem encephalitis and pulmonary oedema.[108] Children with oedema also had depleted lymphocyte populations, especially CD4, CD8, and natural killer cells.[87,107] Thrombocytosis, neutrophilia, and hyperglycaemia are all thought to reflect a systemic inflammatory response.[105,107] Cytokines in the cerebrospinal fluid are less studied than those in blood, but in one study patients with encephalitis complicated by oedema had high concentrations of interleukin 1b, compared with those who had encephalitis alone.[105]

The development of pulmonary oedema in patients with EV71 encephalitis seems to be strongly associated with dysregulation of systemic and CNS inflammatory responses. This relation has formed at least part of the basis for anti-inflammatory therapy with intravenous immunoglobulin, and the approach does seem to be effective.[83,99,109–111]

The exact mechanism for pulmonary oedema in EV71 encephalitis is unclear. Neurogenic mechanisms secondary to brainstem inflammation seem to be important, but pathologically similar changes are seen in other encephalitides, such as Japanese encephalitis, without pulmonary oedema being such a prominent feature. Cardiac dysfunction and the effects of the systemic inflammatory response on the vascular endothelium may also make important contributions. In-vivo models, including those in mice and non-human primates, have replicated some of the features of severe EV71 disease, such as neuroinvasion with inflammatory changes, but none has yet been able to reproduce the severe systemic features, such as pulmonary oedema.[89–91,112–114]

Prospects for control

Surveillance and social distancing

The only measures available for disease control are public health approaches. Since early intervention can lessen the spread of the virus, many countries in the Asia-Pacific region, including Japan, Malaysia, Singapore, Taiwan, and Vietnam, have implemented heightened surveillance for EV71.[15,55,56,115,116] HFMD has now become a notifiable disease in many countries in the region.[116] However, since other enteroviruses, such as coxsackievirus types A 8, A 10, and A 16, can cause HFMD, concurrent virological surveillance is necessary. This approach can also provide invaluable molecular epidemiological data that might help to track the spread of the virus across the region.

Outbreak control measures are primarily targeted at interrupting virus transmission person to person and through contact with contaminated surfaces, toys, or fomites. Health education, therefore, focuses on personal hygiene and good sanitation, including frequent hand washing, proper disposal of soiled nappies, and disinfection of soiled surfaces with chlorinated (bleach) disinfectants.

The transmission of enteroviruses, including EV71, is most efficient in crowded settings and, therefore, most countries in the region, including Malaysia, Singapore, Taiwan, Hong Kong, and China, have adopted social distancing measures, such as closures of childcare facilities and schools, and cancellation of public functions involving children.[115,116] Little systematic research has been done to assess the effectiveness of such measures, but one study from Singapore seemed to show some benefit.[115] However, the optimum timing for implementation—as soon as an HFMD outbreak is reported or after it is confirmed to be caused by EV71—is unclear. In addition, the effectiveness of distancing measures, which have substantial socioeconomic implications, is uncertain. If EV71 is like other directly transmissible viruses, such controls will decrease the peak incidence of disease, but the outbreak could be prolonged and, therefore, the overall number of cases might not be lowered (Cardosa MJ, unpublished; figure 6). Transmission of the virus within families rather than the peer-group at school could lead to increased incidence of severe cases, as the inoculum concentration is thought to be higher.[85] Data from clinical and epidemiological studies are needed to guide public health decisions.

Vaccine development

No vaccines against EV71 exist, but by analogy with poliomyelitis, vaccination probably offers the best option for disease control. One limitation in EV71 vaccine development is the lack of a good mouse model of human disease. Adult mice are resistant to infection. Although suckling mice are susceptible, by the time immunity develops after inoculation, the animals have matured and become resistant to infection. One way around this issue is to vaccinate female adult mice, allow them to become pregnant, and then measure titres of protective maternal antibodies transferred to offspring, as judged by protection against lethal infection.[117]

In human beings the target population should be young children, especially those younger than 3 years, because they are the most susceptible to severe disease. One important issue is whether vaccines derived against one EV71 genetic subgroup will provide cross-protection against all others; available data are contradictory.[76–78] Several comprehensive reviews on the development of EV71 vaccine candidates have been published.[118,119] Various types of vaccines are being investigated, including inactivated whole-virus, live attenuated, subviral particle, and DNA vaccines. All types are in early stages of development, with the most advanced undergoing preclinical trials in mice and non-human primates.

Candidate inactivated vaccines include those derived in Taiwan from the B4 viruses EV71-075 and EV71-0117, which are highly immunogenic, and from EV71-1207, which is a C2 virus and is less immunogenic. In one

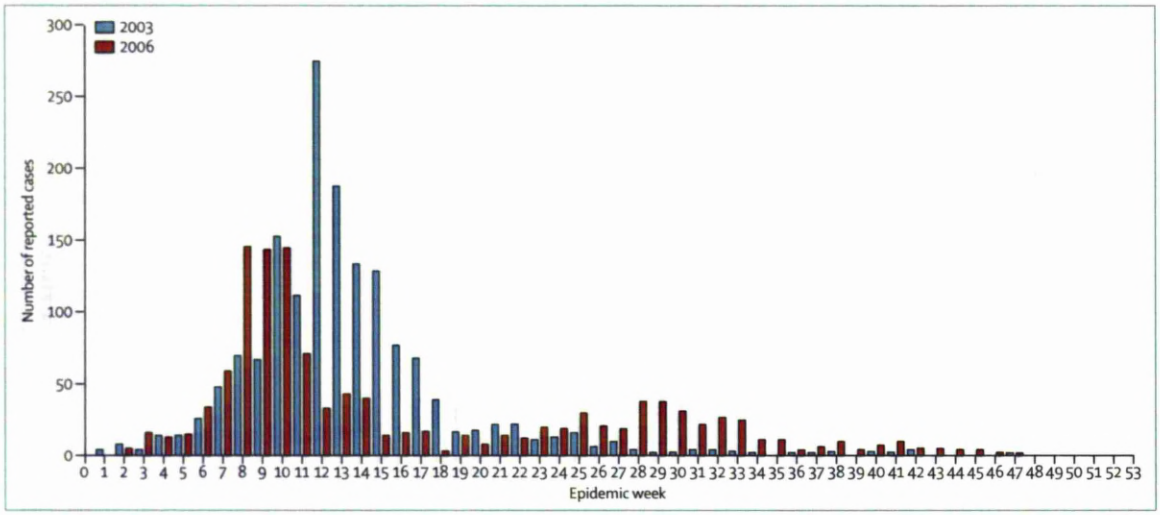

Figure 6: **Comparison of data from sentinel surveillance centres on effects of public health interventions on hand, foot, and mouth disease in Sarawak, Malaysia, in 2003 and 2006**
The public health response was limited in 2003, but more-rigorous social distancing measures were encouraged in 2006.

study all these vaccines were more immunogenic in mice than recombinant VP1 protein or DNA vectored vaccines.[120] Virus-like particles for EV71, which resemble the virus in appearance and capsid and protein structure, have been produced and purified as potential vaccines.[121] After immunisation of BALB/c mice, the particles induced potent and long-lasting humoral immune responses, reflected by high total IgG and neutralisation titres. Splenocytes collected from the immunised mice exhibited substantial cell proliferation and stimulated production of interferon γ and interleukins 2 and 4, indicating the induction of T-helper-1 and T-helper-2 immune responses. Immunisation of female mice conferred protection (survival up to 89%) to neonatal mice against virus challenge with a dose 1000 times that normally required to kill 50% of animals.[121]

A potential DNA vaccine has been developed by inserting the *VP1* gene into the pVAX1 vector, and transforming the constructs into *Escherichia coli* cells, followed by expression in a mammalian cell line.[122] Immunisation of mice with the DNA vaccine constructs resulted in the production of antibodies to VP1 IgG and neutralising antibody against EV71. Oral immunisation of female mice with an attenuated *Salmonella enterica* serovar Typhimurium expressing the *VP1* gene, also proved protective in newborn offspring.[123] Transgenic tomatoes expressing the VP1 protein have been developed. Incorporation of this protein in an oral vaccine led to the development of VP1-specific antibodies and evidence of cell-mediated immunity in BALB/c mice, and provided protection to offspring in neonatal challenge models.[124]

Linear neutralising epitopes from the VP1 capsid protein were identified in mice by raised concentrations of antisera against overlapping peptides from this protein, two of which elicited neutralising antibody responses.[125] One of these peptides, SP70, was especially potent, and comparison with sequences from other strains showed it was conserved among the different genotypic subgroups of EV71, which suggests it is a promising vaccine candidate.

A live attenuated strain of EV71, EV71(S1-3), was derived from the genotype A prototype strain BrCr by genetic manipulation,[126] on the basis of temperature-sensitive determinants of poliovirus type 1 vaccine strain, some of which are located in the 5′ and 3′ untranslated regions and the *3Dpol* gene. Intravenous inoculation of cynomolgus monkeys led to the production of antibodies with cross-reactivity against a broad spectrum of EV71 genotypes that survived challenge with intravenous virulent EV71 (BrCr-TR strain), which is lethal to non-immunised monkeys. However, the vaccine strain itself caused mild neurological symptoms (tremor) and entered the spinal cord, which indicated that further work on attenuation is needed.[126]

Among the various vaccine candidates, inactivated whole virus vaccines are in some ways the most ready to develop further, because the principles of vaccines based on inactive whole virus are well established. However, experience with vaccination against Japanese encephalitis, another major neurological infection in Asia, has shown that issues over cost and availability can limit the widespread uptake of vaccines in poor Asian countries. If a vaccine is to be used across the whole of Asia, it needs to be cheap, easily produced, and readily available.[127]

Conclusions

The increased size and frequency of EV71 outbreaks in the Asia-Pacific region over the past 12 years has been an important public health issue. Molecular epidemiological studies suggest that some viral genotypic subgroups seem

to have massive potential for explosive epidemics, whereas others have more-indolent, low-level circulation. However, the biological determinants of these differences are poorly understood. The reasons for epidemiological differences between EV71 in the Asia-Pacific region and that in Europe and the USA are also unclear, as are the virological and host determinants of the wide-ranging clinical phenotypes in infected individuals. Although some animal models of neurological disease caused by EV71 are reasonable, a good model of cardiorespiratory dysfunction is urgently required to help understand pathogenesis better.

The public health measures currently used during EV71 epidemics are empirical and generic, have high socioeconomic impact and are not clearly effective. Further research is needed on virus transmission. The identification of several EV71 receptors might help in drug discovery. Several vaccine candidates are under development, but the logistical issues of how to reach their target paediatric populations remain important.

Contributors

TS and MHO conceived and designed this Review. TS, PL, DP, MJC, PM, and MHO drafted the paper and critically revised it.

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Conflicts of Interest

MJC joined Sentinext Therapeutics, a Malaysian biotechnology start-up company involved in the development of a vaccine against enterovirus 71, as Chief Scientific Officer in September, 2010, but was not employed by the company during the design or preparation of this Review. TS, MJC, and MHO have acted as informal advisers to WHO on hand, foot, and mouth disease and enterovirus 71 infection.
PL, DP, and PM declare that they have no conflicts of interest.

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Clinical features, diagnosis, and management of enterovirus 71

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Although poliomyelitis has been mostly eradicated worldwide, large outbreaks of the related enterovirus 71 have been seen in Asia-Pacific countries in the past 10 years. This virus mostly affects children, manifesting as hand, foot, and mouth disease, aseptic meningitis, poliomyelitis-like acute flaccid paralysis, brainstem encephalitis, and other severe systemic disorders, including especially pulmonary oedema and cardiorespiratory collapse. Clinical predictors of severe disease include high temperature and lethargy, and lumbar puncture might reveal pleocytosis. Many diagnostic tests are available, but PCR of throat swabs and vesicle fluid, if available, is among the most efficient. Features of inflammation, particularly in the anterior horns of the spinal cord, the dorsal pons, and the medulla can be clearly seen on MRI. No established antiviral treatment is available. Intravenous immunoglobulin seems to be beneficial in severe disease, perhaps through non-specific anti-inflammatory mechanisms, but has not been tested in any formal trials. Milrinone might be helpful in patients with cardiac dysfunction.

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Introduction

A global campaign has all but eradicated poliomyelitis from Europe, the Americas, and much of Africa and Asia. Over the past 10 years, however, the related enterovirus 71 (EV71) has emerged across Asia, where it threatens to become what has been coined the new polio.[1] The virus is a member of the enterovirus genus, which includes coxsackieviruses and echoviruses. EV71 first appeared in California, USA, in the 1960s, and caused sporadic cases or small outbreaks of hand, foot, and mouth disease (HFMD), neurological disease, or both (table 1). In 1997, the virus caused an unexpectedly large and severe outbreak in Sarawak, Malaysia, with high mortality. Regular epidemics have since been seen in countries across the Asia-Pacific region, including an epidemic in Taiwan in 1998 that was thought to involve millions of people, and an outbreak of HFMD in China, during which nearly 500 000 cases were reported.[13,14,17,18,22,25–30]

During outbreaks, thousands of children can develop HFMD, and although most will have self-limiting illness, a small proportion can rapidly develop neurological and systemic complications that can be fatal. EV71 is, therefore, of major interest to neurologists, paediatricians, and specialists in infectious diseases, virology, and public health. In this Review we discuss clinical management, diagnosis, and treatment of EV71 disease. In a companion article in *The Lancet Infectious Diseases*,[31] we examine the virology, clinical and molecular epidemiology, pathogenesis, and public health implications of this important emerging virus.

Clinical features

EV71 infection has a wide variety of clinical manifestations, although CNS infection and HFMD are the two features most frequently seen.[14]

Mucocutaneous and respiratory manifestations

HFMD is a common childhood exanthema that is characterised by a brief, generally mild, febrile illness with papulovesicular rash on the palms and soles, and multiple oral ulcers (figure 1). Herpangina, a closely related childhood enanthema, is characterised by febrile illness and the presence of multiple oral ulcers that predominantly affect the posterior of the oral cavity, including the anterior pharyngeal folds, uvula, tonsils, and soft palate. A classic course of HFMD generally occurs in older children with EV71, but in those aged 2 years and younger more-widespread and atypical rashes are frequently seen. Other features include upper respiratory tract infection, gastroenteritis, and non-specific viral rashes,[32] and, especially in young children, exacerbation of bronchial asthma, bronchiolitis, and pneumonia.[33] More than 20% of adult contacts in one Taiwanese outbreak had symptoms of an upper respiratory tract infection, but more than 50% of infected adults remained symptom free.[32]

Neurological and systemic manifestations

As for other enteroviruses, EV71 can cause aseptic meningitis, acute flaccid paralysis, encephalitis, and other rarer manifestations (table 2).[34] EV71 encephalitis is typically a brainstem encephalitis and, unlike most other enteroviruses, is frequently accompanied by severe cardiorespiratory symptoms, similar to those associated with poliomyelitis. These symptoms have been attributed to neurogenic pulmonary oedema, although the cause remains controversial.[31]

In a large prospective clinical study of several epidemics occurring over 7 years in Sarawak, 10–30% of children admitted to hospital with EV71-related HFMD also developed CNS complications.[17,23] Brainstem encephalitis was the most frequent presentation, accounting for 58% of neurological manifestations, followed by aseptic meningitis (36%), and brainstem encephalitis with cardiorespiratory dysfunction (4%). Most children with CNS involvement also had features of HFMD, but a small proportion presented only with neurological features.[17]

Myoclonic jerks are seen more often in EV71 than in other enteroviruses, and could be an early indicator of neurological involvement, particularly in the brainstem.[35] This symptom has, however, also been reported in other

	Number of EV71 cases reported	Number of fatal cases reported	Main clinical presentations
California, USA,1969–72[2]	20	1	Encephalitis, meningitis, coxsackievirus-like illness
New York, USA, 1972[3]	11	0	Aseptic meningitis, encephalitis, HFMD (only one case)
Sweden, 1973[4]	195	0	Aseptic meningitis, HFMD (some cases)
Bulgaria, 1975[5]	705	44	Aseptic meningitis, paralytic disease, including bulbar encephalitis
Japan, 1973, 1977, 1978[6]	1031	Some deaths, exact number unknown	HFMD (in most patients), cerebellar encephalitis, meningitis, acute flaccid paralysis
New York, USA, 1977[7]	12	0	CNS disease, HFMD, acute respiratory illness, acute gastroenteritis
Hungary, 1978[8]	323 laboratory confirmed	47 (unclear whether all due to EV71)	Meningitis, encephalitis, poliomyelitis, HFMD (only four cases)
Australia, 1986[9]	114	0	HFMD (in most cases), meningitis, encephalitis, encephalomyelopathy
Philadelphia, 1987[10]	5	0	Acute flaccid paralysis
USA, 1977–91[11]	193 laboratory confirmed	Unknown	1985–89: paralysis, meningitis, encephalitis, rash, Guillain-Barré syndrome
Sarawak, Malaysia, 1997[12]	2628	34	HFMD, aseptic meningitis, acute flaccid paralysis, cardiorespiratory dysfunction
Otsu, Japan, 1997[13]	12	0	HFMD, herpangina, meningoencephalitis, encephalitis, meningitis
Taiwan, 1998[14]	129 106	78	Encephalitis, aseptic meningitis, pulmonary oedema or haemorrhage, acute flaccid paralysis, myocarditis
Kenya, 1999[15]	8	0	Dermatitis, mucositis, myositis
Hyogo, Japan, 2000[16]	60	1	HFMD, aseptic meningitis, cerebellar ataxia, acute flaccid paralysis, brainstem encephalitis
Sarawak, Malaysia, 2000[17]	169 laboratory confirmed	2	HFMD, aseptic meningitis, acute flaccid paralysis, brainstem encephalitis
Singapore, 2000[18]	3790	5	HFMD, neurological disease
Korea, 2000[19]	Unknown	0	Aseptic meningitis, HFMD, herpangina, acute flaccid paralysis
Sarawak, Malaysia, 2003[17]	107 laboratory confirmed	1	HFMD, aseptic meningitis, acute flaccid paralysis, brainstem encephalitis
Fukushima, Japan, 1983–2003[20]	Unknown	Unknown	Unknown
Denver, USA, 2003[21]	8	1	Meningitis, acute flaccid paralysis, fever, cardiopulmonary dysfunction
Southern Vietnam, 2005[22]	173 laboratory confirmed	3	HFMD, aseptic meningitis, acute flaccid paralysis, brainstem encephalitis
Sarawak, Malaysia, 2006[23]	291 laboratory confirmed	6	HFMD, aseptic meningitis, brainstem encephalitis
Denver, USA, 2005[21]	8	0	Meningitis, acute flaccid paralysis, fever, encephalitis
Brunei, 2006[24]	1681	3	HFMD, neurological disease
Shandong, China, 2007[25]	1149	3	HFMD, brainstem encephalitis, aseptic meningitis
Anhui, China, 2008[26]	488 955	128	HFMD, neurogenic pulmonary oedema

HFMD=hand, foot, and mouth disease.

Table 1: **Enterovirus 71 outbreaks worldwide, by country and year**

viral CNS infections, including Japanese encephalitis, Nipah virus, subacute sclerosing panencephalitis, herpes simplex virus, HIV, and varicella-zoster virus. In addition, myoclonic jerks seen in many otherwise healthy young children, particularly when they are asleep, can occur spontaneously or be provoked by loud noises.

Seizures, if they occur at all in EV71 infection, are seen generally in children aged younger than 2 years and are short lived with good recovery of consciousness. Thus, they are likely to be febrile seizures rather than being caused by CNS involvement. Unlike other viral encephalitides, recurrent and sustained seizures are very rare, which probably reflects the fact that EV71 is associated with a brainstem rather than a cortical encephalitis.

Brainstem encephalitis with associated pulmonary oedema has been the hallmark of EV71 CNS infection in Asia since the late 1990s.[12,18,27,36] This distinctive clinical syndrome is characterised by prodromal HFMD followed by a sudden deterioration that typically occurs after 3–5 days of fever. Children then develop acute and rapidly progressing cardiorespiratory failure, which presents as shock and pulmonary oedema or haemorrhages. Without intensive care most children affected in this way will die before reaching hospital or within 24 h of admission.[12,18,27,36]

In the few studies where it has been possible to assess children with brainstem encephalitis, MRI and post-mortem findings have correlated well, with both showing frequent involvement of the medulla oblongata, reticular formation, pons, and midbrain (figure 2).[37–39] Acute flaccid paralysis is the primary presenting feature in several neurological syndromes caused by EV71, including poliomyelitis-like anterior horn cell destruction (anterior myelitis), Guillain-Barré syndrome, and transverse myelitis. Anterior horn cell destruction is probably the most frequently seen of these syndromes, although it is generally less severe than that caused by polioviruses and has a higher recovery rate.[34]

During the 1998 Taiwan epidemic the severity of brainstem encephalitis was categorised into three grades: grade I, myoclonic jerks, tremor or ataxia, or both; grade II, cranial nerve palsies evident from eye-

movement disorders (nystagmus, strabismus, or gaze paresis), facial weakness, and bulbar palsy (dysphagia, dysarthria and dysphonia); and grade III, acute onset of intractable, frequently fatal, cardiorespiratory failure.[36] A separate clinical staging system (stages 1–4) has been used to help monitor the clinical course of EV71 infection from febrile illness, to CNS involvement, to cardiorespiratory failure, and development of sequelae, and to aid management.[40,41] These systems are not, however, widely used, possibly because they are not always easy to remember and imply sequential progression, which does not always occur. The WHO Informal Consultation on Hand, Foot, and Mouth Disease, in Kuala Lumpur, Malaysia, in March, 2010, has proposed a simple clinical description of disease manifestation to describe the natural history of the disease, which will be published next year.

Diagnosis

During outbreaks of EV71, thousands of children develop symptoms (table 1). Most of them will have mild, self-limiting illness, but a small proportion of infected children rapidly develop severe and sometimes fatal neurological and systemic complications over days or even hours. In the past, many children with mild HFMD were originally cared for at home, but an increase in public awareness about the swift development of potentially fatal complications has led to many now being taken to hospital, and health services can easily become overwhelmed. A challenge for front-line clinicians is to recognise which patients are likely to deteriorate and to know which investigations give the best diagnostic yield.

Differential diagnosis

Childhood exanthema from a wide variety of causes can be confused with HFMD, particularly those seen with measles, rubella, and chicken pox (panel).[42] Two particularly important causes to consider are meningococcus, because of the need for antimicrobial treatments, and dengue, because of the risk of developing dengue haemorrhagic fever, as severe forms require careful fluid resuscitation. Herpangina can be confused with aphthous ulcer and herpetic gingivostomatitis.

Of the many enteroviruses that can cause HFMD or herpangina, EV71 and coxsackievirus type A 16 are the most frequent, and both can cause epidemic disease. Coxsackievirus type A 16 is not generally associated with neurological disease,[43] but the rash it causes is indistinguishable from that caused by EV71. However, other features can distinguish the two causes of HFMD. For example, studies from Sarawak and Taiwan show that children with EV71 are more likely to have a fever for longer than 3 days, with a peak temperature higher than 38·5°C, lethargy, and myoclonus.[23,44]

Aseptic meningitis, which is a frequent neurological manifestation of EV71, must be distinguished from a broad range of other viruses, especially echoviruses and other enteroviruses, adenoviruses, mumps, and occasionally Japanese encephalitis virus; partly treated bacterial meningitis and tuberculous meningitis should also be considered. Most patients with severe CNS disease due to EV71 also have features of shock and collapse. Septicaemia is, therefore, an important differential diagnosis. Other causes of encephalopathy might need to be excluded, particularly malaria. When acute flaccid paralysis is the predominant feature, the differential diagnosis includes poliomyelitis caused by wild-type polioviruses or vaccination, other enteroviruses, flaviviruses, rabies, Guillain-Barré syndrome, and bacterial toxins, such as diphtheria.

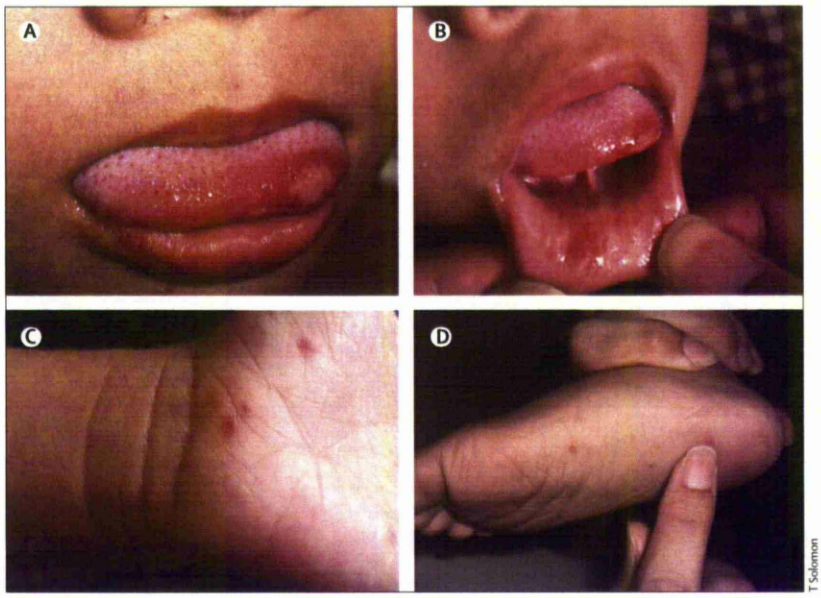

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Figure 1: **Mucocutaneous lesions in hand, foot, and mouth disease**
Ulcers on (A) the tongue and (B) inside the lip, and vesicular and macular lesions on (C) the wrists and (D) the soles of children with enterovirus 71.

	Frequency
Purely neurological manifestations	
Encephalitis, especially brainstem	Frequent
Acute flaccid paralysis (anterior myelitis)	Frequent
Encephalomyelitis	Frequent
Aseptic meningitis	Very frequent
Cerebellar ataxia	Infrequent
Transverse myelitis	Rare
Neurological and systemic manifestations	
Brainstem encephalitis with cardiorespiratory failure	Frequent
Manifestations indicative of immune-mediated mechanisms	
Guillain-Barré syndrome	Infrequent
Opsoclonus-myoclonus syndrome	Rare
Benign intracranial hypertension	Rare

Modified from McMinn,[34] with permission of John Wiley and Sons.

Table 2: **Neurological syndromes associated with enterovirus 71 infection**

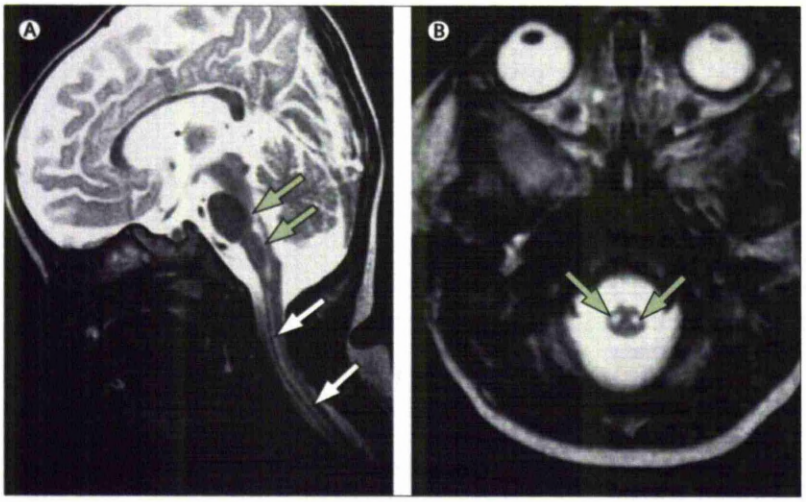

Figure 2: **MRI changes in enterovirus-71-associated encephalomyelitis**
T2-weighted images of a child aged 10 months who had presented 3 months earlier with somnolence, tachycardia, tachypnoea, and coma, and who recovered consciousness but remained dependent on a ventilator. High signal intensity can be seen in (A) the posterior portion of the pons and medulla (green arrows) and anterior cervical cord (white arrows) on a sagittal section, and (B) in the two anterior horns of the cervical cords (green arrows) on an axial section. Modified from Shen and colleagues,[39] with permission of the American Society of Neuroradiology.

Virological diagnosis

Laboratory diagnosis of EV71 is established primarily through virus isolation or molecular detection of the virus nucleic acid in appropriate clinical specimens. This approach is important to distinguish EV71 from other enteroviruses, such as coxsackievirus type A 16.

Choice of sample

Samples for laboratory investigation should be selected according to the disease manifestations. Possibilities include throat, rectal, and ulcer swabs, and samples of serum, urine, CSF, and fluid from vesicles. The sensitivity, specificity, and usefulness of findings vary according to the sample.[45] In particular, virus detection in samples from sterile sites, such as vesicular fluid, CSF, serum, urine, or those gathered at autopsy, is more reliable than that in samples from non-sterile sites, such as the throat or rectum, where the presence of the virus might merely indicate coincidental carriage.

Viral shedding from the gastrointestinal tract (via the throat or rectum or in stools) might continue after complete resolution of the symptoms of EV71 infection, as it does for other enteroviruses. A study in Taiwan showed that EV71 can be detected in the throat up to 2 weeks after recovery from HFMD or herpangina, and in stools it can be detected up to 11 weeks after recovery.[46] In addition, when an enterovirus is detected in non-sterile sites, it differs from that isolated in samples from sterile sites in 10% of throat swabs and 20% of rectal swabs.[45] In many of the sterile sites, however, the viral load is frequently very low, as for poliomyelitis; for example, virus is detected in 0–5% of CSF samples from patients with neurological disease.[6,8,17,23,47–51] The yield for serum is similarly low.[17] Vesicular fluid, when present, is more useful, although care must be taken during collection (figure 3). Given the potential number of samples, laboratories can become overwhelmed. One study showed the most efficient approach was to examine throat swabs for all patients, plus swabs from at least two vesicles or from the rectum for patients with no vesicles.[45]

Virus isolation, serotyping, and nucleic acid detection

The gold standard for diagnosis of enterovirus infection is virus isolation. Several human and non-human primate cell lines can be used, including rhabdomyosarcoma, which is most efficient, human lung fibroblast cells, and African green monkey kidney cells.[43] In rhabdomyosarcoma cells, a characteristic cytopathic effect is observed typically 7–10 days after inoculation. To improve the yield, blind passage might be necessary before cytopathic effects become apparent. Once a cytopathic effect is observed, the virus is identified by neutralisation tests in intersecting pools of type-specific antisera, EV71-specific antisera, or by an indirect immunofluorescence assay with EV71-specific monoclonal antibodies.[43] A molecular serotyping approach has been developed by amplification of part of the *VP1* gene of the cultured virus, use of PCR and pan-enterovirus EV71-specific primers, and sequencing of the product.[9] Several sets of primers directed at different regions of the *VP1* gene of EV71 have been developed.[5,9,52]

EV71-specific primers are used to perform PCR directly on clinical samples. The advantage of this approach is that it is quicker than virus culture. Speed can be especially important given the explosive nature of some EV71 outbreaks and the need for urgent public health interventions. The disadvantage is that only the virus looked for can be detected and, therefore, other unexpected causes will not be identified.[52–55] DNA microarray is a powerful, although expensive, tool designed to detect multiple pathogen targets by hybridisation of pathogen-specific probes. Two groups have reported its use to distinguish EV71 and coxsackievirus type A 16 infection in primary clinical specimens, with diagnostic accuracy of about 90%.[53,56]

Serology

Serological diagnosis of an acute virus infection classically relies on a fourfold increase being shown in the concentrations of a specific neutralising antibody between the acute and convalescent phases.[43] In the case of EV71, however, very high concentrations of neutralising antibodies are frequently detectable within the first few days of illness, and thus such a difference will not be seen.[49,51] Furthermore, although homologous antibodies are produced when young children encounter their first enterovirus infection, heterologous cross-reacting IgG and IgM antibodies are produced by older children and adults following repeated infection with different enterovirus serotypes. The usefulness of this test, therefore, decreases with increasing age.

A rapid IgM ELISA test for EV71 has been developed to try to overcome some of the current limitations of serological testing.[57] The possibility of cross reactivity still remains an issue,[58] and the duration of detectable EV71-specific IgM after an infection is also uncertain.

Other laboratory investigations

In mild EV71 disease, the full blood count and urea and electrolyte concentrations are generally normal, but in severe disease a raised white cell count with neutrophilia is frequently seen and hyperglycaemia might be present.[47] Creatine kinase concentration is sometimes raised in patients with cardiac involvement,[59] and an increased cardiac troponin I level has been reported as a predictor of imminent cardiopulmonary failure in children with brainstem encephalitis.[60] Pulmonary oedema, if present, will generally be obvious on chest radiographs; normal heart size indicates that the cause is not acute viral myocarditis or congenital heart disease. Electrocardiography frequently shows non-specific changes,[59] and continuous monitoring can demonstrate abnormal beat-to-beat variability, which can be predictive of imminent cardiovascular collapse.[61] In children who are haemodynamically unstable and have tachycardia, hypotension, or pulmonary oedema, echocardiograms show generalised left ventricular hypokinesia, which might be accompanied by mitral regurgitation;[57] pericardial effusion is seldom seen.

The lumbar puncture is essential in children who are unwell and who have suspected CNS involvement. In some patients the clinical features, such as meningism or myoclonic jerks, clearly point to the CNS. In other children, however, especially those younger than 2 years, there may just be high fever, vomiting, or lethargy, but a lumbar puncture shows CNS disease. Mild lymphocytic pleocytosis of 10–100 cells per μL is typical, but occasionally there might be none.[21] The ratio of glucose concentration in CSF to that in plasma is generally normal, but can be low.

Imaging

Although CT scanning can be helpful to exclude certain pathologies, it does not clearly identify EV71 encephalitis, where the pathology is mostly in the brainstem, as scans are almost always normal. Conversely, MRI shows characteristic high signal intensities on T2-weighted images in the dorsal pons and medulla, most of the midbrain, and the dentate nuclei of the cerebellum. Similar high-signal lesions can also be found in the anterior horn cells of cervical spinal cord (figure 2).[36,39] The usefulness of these changes in terms of sensitivity, specificity, and positive and negative predictive value, however, has yet to be demonstrated. In children with acute flaccid paralysis, MRI typically shows unilateral high-signal lesions in the anterior horn cells of the spinal cord on T2-weighted images, and contrast-enhancing ventral root on T1-weighted images.[36,62,63]

***Panel:* Characteristics to consider in differential diagnosis of enterovirus 71 infection**

Rash

- Viral infections: coxsackievirus type A 16, and other enteroviruses,* measles,* rubella,* varicella-zoster virus,* human herpes viruses 6 and 7, parvovirus B19, dengue, flavivirus infections (especially haemorrhagic fever), alphavirus infections, Epstein-Barr virus, primary HIV infection, and non-specific viral rashes
- Bacterial infections: meningococcaemia (*Neisseria meningitides*), scarlet fever (*Streptococcus pyogenes*), leptospirosis, relapsing fever (*Borrelia recurrentis*), Lyme disease (*Borrelia burgdorferi*), syphilis (*Treponema pallidum*), and typhus and other rickettsial infections
- Other disorders: scabies (*Sarcoptes scabei*),* drug reactions, allergies, and paraneoplastic syndrome

Aseptic meningitis

- Viral infections: echoviruses,* coxsackieviruses and other enteroviruses, herpes simplex virus type 2, HIV, mumps, flaviviruses, alphaviruses, and bunyaviruses, lymphocytic choriomeningitis
- Bacterial infections: partly treated bacterial meningitis, tuberculous meningitis, parameningeal bacterial infections, listeria (*Listeria monocytogenes*), syphilis, Lyme disease (*Borrelia burgdorferi*), Weil's disease (*Leptospira* spp), *Mycoplasma pneumoniae*
- Other disorders: drug reactions, fungal infections (eg, *Cryptococcus* spp, *Candida* spp, *Aspergillus* spp)

Flaccid paralysis

- Viral infections: poliomyelitis and other enteroviruses,* vaccine-associated paralytic poliomyelitis, flaviviruses (Japanese encephalitis virus, West Nile virus, tick-borne encephalitis virus), rabies, adenoviruses
- Bacterial infections: botulism, diphtheria
- Other disorders: intramuscular injection into buttock causing sciatic nerve damage, Guillain-Barré syndrome (especially acute motor axonal neuropathy)

Brainstem encephalitis

- Viral infections: poliomyelitis and other enteroviruses,* flaviviruses,* Nipah virus
- Bacterial infections: listeria (*Listeria monocytogenes*), tuberculosis (*Mycobacterium tuberculosis*), brucellosis (*Brucella abortens*), Lyme disease (*Borrelia burgdorferi*)
- Other disorders: paraneoplastic syndromes

The importance of the infections listed varies greatly according to the age and geographic location of the patient. *Especially likely to be confused with symptoms of entervirus 71. Modified from Solomon,[42] with permission of Oxford University Press.

Predictors of severe disease

Several clinical features and laboratory abnormalities have been associated with neurological and fatal EV71 disease, but few have been prospectively validated.[47,60,64] Young age at disease onset is associated with increased risk of severe disease.[65] The results of a prospective clinical study of nearly 1500 children presenting to one hospital during three EV71 outbreaks in Sarawak over 7 years showed that neurological involvement was strongly predicted by the presence of at least two of the following: peak temperature of 38·5°C or more, fever for 3 days or longer, and a history of lethargy.[23] This study confirmed the findings from early retrospective studies. However, other early findings, such as an association between the absence of mouth ulcers and development of complicated or fatal disease,[17,66] were not confirmed. In a retrospective study, hyperglycaemia and leucocytosis

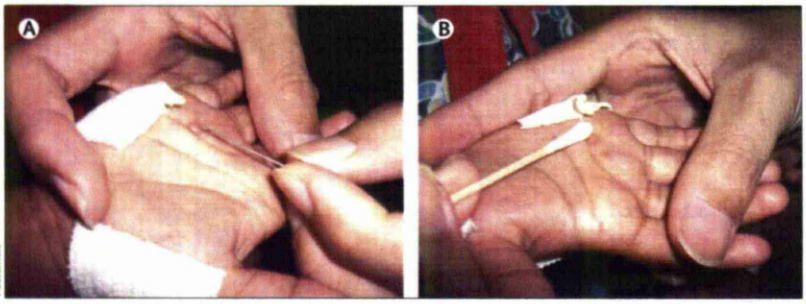

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Figure 3: **Collection of vesicular fluid from palmar lesions for virological diagnosis of hand, foot, and mouth disease**

(A) The hand should be gripped firmly to prevent movement, the skin stretched tight, and a small needle used to puncture the vesicle. (B) A clean swab is applied to collect the fluid released. The swab is placed into viral transport medium.

had been associated with fatal EV71 disease[47] and were confirmed prospectively,[23] but these are late symptoms that generally occur with fulminant disease and are not helpful clinical predictors of complications and death.[15]

Not all children with CNS involvement in EV71 infection will progress to cardiorespiratory collapse. The results of a small study involving 46 patients showed that children developed abnormal heart rate variability (an index of autonomic nervous system disease) about 7 h before the clinical onset of cardiorespiratory instability.[61] The researchers proposed that screening of children with CNS involvement for this feature should be done to predict impending cardiorespiratory failure and allow timely initiation of appropriate interventions. Cardiac troponin I is a cardiac-specific biomarker for myocardial injury and is measured in patients with suspected acute coronary syndrome in adults. Raised concentrations of this protein have been observed in children with EV71 infection, brainstem encephalitis, cardiac dysfunction, and pulmonary oedema,[60] in some cases before the development of cardiopulmonary failure.[60] Serial measurement of cardiac troponin I concentrations might, therefore, be helpful in identifying children at risk of left-ventricular failure. Measurement of heart rate variability and cardiac troponin I concentrations have not, however, become routine clinical practice in the management of EV71, probably because of the poor availability of technologically advanced equipment needed and the high cost, respectively. Even in wealthier Asian countries, such as Taiwan, these measures have not entered routine clinical practice. Overall, simple clinical parameters, such as length of illness, height of fever, and lethargy, are probably more useful indicators of potentially severe disease.

Treatment

No established antiviral treatments are available for EV71, and there are no specific clinical data on antiviral or ancillary treatments. Thus, recognition of which treatments might be appropriate remains a challenge for front-line clinicians.

Antiviral agents

Pleconaril is an antiviral drug that inhibits the entry of several enteroviruses into cells by blocking viral attachment and uncoating, and has been used in clinical trials of aseptic meningitis.[67–69] This drug it is not, however, active against EV71.[70] Several other capsid-function inhibitors have been investigated, and some have shown promising activities against EV71 in preclinical studies.[70] In-vitro and in-vivo studies show that both ribavirin and interferons might be useful,[71,72] and RNA interference approaches are being explored.[73–75]

Intravenous Immunoglobulin

During the initial large outbreaks of EV71 in Asia, clinicians in Sarawak and Taiwan used intravenous immunoglobulin on the presumptive basis that it would neutralise the virus and have non-specific anti-inflammatory properties.[17,44] Retrospective comparisons of patients who did and did not receive immunoglobulin suggest a benefit from this treatment if given early.[23,76] For example, among children with EV71 assessed in Sarawak over three seasons, 204 (95%) of 215 survivors who had severe CNS complications had received intravenous immunoglobulin treatment, typically once there was evidence of severe disease, such as tachycardia or poor perfusion or altered consciousness, compared with only one (11%) of nine fatal cases (odds ratio 148·36, 95% CI 16·34–6609·04, p<0·0001).[23] Analysis of cytokine profiles before and after immunoglobulin treatment showed substantial reductions in concentrations of some proinflammatory cytokines in patients with EV71 if they had encephalitis with autonomic dysfunction, but not if they had less severe disease.[76–80] Intravenous immunoglobulin has, therefore, become more routinely used for the treatment of severe EV71 disease, and in Taiwan has been introduced into the national treatment guidelines.[17,23,40,41,81] Uncertainty remains, however, over whether this expensive human blood product treatment is really effective, and randomised, placebo-controlled, phase 2 trials are needed. Such trials would be logistically and ethically challenging to establish because the treatment is so widely used, and would require careful design with surrogate endpoints of disease progression, such as failure of resolution of tachycardia.

Milrinone

Milrinone is a cyclic nucleotide phosphodiesterase inhibitor currently used in the treatment of congestive heart failure. Inhibition of phosphodiesterase subtype III by this cardiotrophic agent results in an increase in intracellular concentrations of cyclic AMP, which in turn leads to increased cardiac output and decreased peripheral vascular resistance. The results of a small, non-randomised, retrospective assessment of 24 children with EV71-induced pulmonary oedema showed that those treated with milrinone had reduced tachycardia and lower mortality than those who did not receive this

drug.[81,82] Peripheral white cell and platelet counts and plasma interleukin 13 concentrations were also lower,[82] which might indicate an immunomodulatory effect of the drug. A clinical study examining efficacy of milrinone is said to be ongoing.[83]

Fluid balance and inotrope support

In routine paediatric practice, the most common cause of shock and peripheral shut down is hypovolaemia and dehydration, for example after gastrointestinal infection. These disorders are treated with rapid fluid resuscitation. When similar approaches were used in the early EV71 outbreaks in Asia, however, they frequently precipitated pulmonary oedema. After it became clear that impaired cardiac function is an important contributor to shock, clinicians were more judicious in their use of intravenous fluids and used inotrope support. Fluid management should, whenever possible, be guided by measurement of central venous pressure. In Taiwan, management algorithms based on this approach seem to have improved outcome.[76]

Outcomes

Studies up to 7 years after infection show that children who present with aseptic meningitis generally have good outcomes, although parent and teacher reports indicate that the incidence of symptoms similar to those in attention deficit-hyperactivity disorder is 20% among children who have recovered from EV71 CNS infection, compared with 3% for matched controls.[84] Approximately a fifth of children with severe neurological disease, including encephalitis, poliomyelitis-like paralysis, and encephalomyelitis, have sequelae, particularly focal limb weakness and atrophy.[65,85,86] Cerebellar disorders are observed in about 10% of patients after moderately severe brainstem encephalitis, including cranial neuropathies, myoclonus, tremor, and ataxia. Of those, however, only a quarter with severe brainstem encephalitis associated with fulminant cardiorespiratory failure make a full neurological recovery.[65,86] Common sequelae include focal limb weakness and atrophy, swallowing difficulties requiring nasogastric feeding, central hypoventilation, facial nerve palsies, seizures, and psychomotor retardation.

Conclusions

Over the past 12 years, EV71 has evolved from being a rare and sporadic cause of HFMD to a cause of major and regular epidemics across the Asia-Pacific region and a disease frequently associated with severe and sometimes fatal neurological complications. Across Asia a range of diagnostic techniques is used. Standardisation via some form of laboratory network with proficiency panels to allow comparison of laboratory findings and ensure quality control might prove useful. Reliable ways of predicting who will develop HFMD and who will have neurological complications and which patients with CNS involvement are at risk of disease progression are still lacking. Intravenous immunoglobulin is now used presumptively for severe EV71 infection in many Asian countries, even though there are almost no data on its efficacy and this treatment is expensive. Evidence-based clinical practice guidelines for diagnosis and treatment would considerably help the management of this emerging neurological infectious disease.

Contributors

The Review was conceived by MHO and TS; all authors were involved in the design and drafting of the Review, and in revising it critically for important intellectual content.

Conflicts of interest

MJC joined Sentinext Therapeutics, a Malaysian biotechnology start-up company involved in the development of a vaccine against EV71, as Chief Scientific Officer in September, 2010, but was not employed by the company during the design or preparation of this Review. MHO, MJC, and TS have acted as informal advisers to WHO on hand, foot, and mouth disease and enterovirus 71 infection. SCW and PL declare that they have no conflicts of interest.

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