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A prospective, comparative study of severe neurological and uncomplicated hand, foot and mouth forms of paediatric enterovirus 71 infections

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ABSTRACT

Objectives: In this study, we document the clinical characteristics and investigated risk factors for uncomplicated and severe forms of EV-A71 disease in Cambodian children.
Methods: From March to July 2014 inclusive, all patients with suspicion of EV-A71 infection presenting to Kantha Bopha Hospitals in Phnom Penh and Siem Reap and confirmed by the Virology Unit at the Institut Pasteur du Cambodge were prospectively enrolled in this study. Throat swabs, rectal swabs and serum samples were collected from all consecutive patients with suspected EV-A71 infection. In addition, CSF was also collected from patients with suspected EV-A71 associated encephalitis. A total of 122 patients (29 with uncomplicated disease and 93 with severe disease) with confirmed EV-A71 infection with all available demographic and clinical data for clinical classification and further analysis were included in the study.
Results: In this prospective EV-A71 study in Cambodia, we confirmed the previously reported association of male gender and absence of mouth or skin lesions with severe disease. We also highlighted the strong association of neutrophils in blood, but also in CSF in patients with pulmonary oedema. More importantly, we identified new putative nutrition-related risk factors for severe disease.
Conclusions: EV-A71 is an important cause of encephalitis in the Asia-Pacific region. Further studies to determine the risk factors associated with severe EV-A71 disease are needed.

Introduction

Enterovirus 71 (EV-A71) is a genotype within the species Enterovirus A, genus Enterovirus, family picornaviridae. The virus is one of the most common aetiologies of hand, foot and mouth disease (HFMD) in children. EV-A71 associated disease is usually mild with children typically recovering within 4-6 days. However, the virus has been associated with fulminant disease during large outbreaks in many parts of the World, including Bulgaria (Shindarov et al., 1979), Hungary (Nagy et al., 1982), Malaysia (Chan et al., 2000), Taiwan (Huang et al., 1999), Singapore (Chong et al., 2003), and China (Li et al., 2012; Liu et al., 2014; Huang et al., 2014). A meta-analysis has estimated case-fatality rates for hospitalized cases of HFMD associated with EV-A71 at 1.7% (Zhao et al., 2015).

In 2012, an outbreak of EV-A71 in Cambodia associated with cardiovascular collapse and pulmonary oedema gained international attention following the deaths of nearly 100 young children

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over a short period (Duong et al., 2016). Although this was the first EV-A71 epidemic reported in Cambodia, retrospective seroepidemiological testing showed that there was widespread circulation of the virus for at least a decade prior to the outbreak (Horwood et al., 2016). The pathogenetic and epidemiological mechanisms of how this virus intermittently causes large deadly outbreaks remains unclear.

We report factors associated with clinical characteristics of 122 Cambodian pediatric patients during an EV-A71 outbreak in 2014. We focus on environmental, clinical and biological risk factors for severe neurological and/or pulmonary disease associated with confirmed EV-A71 infection.

Methods

Clinical methods

During an EV-A71 disease outbreak in Cambodia from March to July 2014 inclusive, all patients with confirmed EV-A71 infection presenting at either of two Kantha Bopha Hospitals (one in Cambodia's southeastern capital Phnom Penh and the other in Siem Reap in the north-west of the country) were enrolled in the study. The clinical definitions used throughout the study are listed in Table 1. Patients with uncomplicated EV-A71 HFMD were compared to patients with severe forms of infection, including isolated encephalitis without pulmonary oedema (ECP) and pulmonary oedema with or without neurological involvement (PO). Demographic and clinical data were recorded for further analysis on a case report form (CRF) that was specifically designed for the study. Clinical outcome was not available for the study and patients were not followed-up.

Virological methods

Throat swabs, rectal swabs and serum samples were collected from all patients presenting to Kantha Bopha Hospitals with suspected EV-A71 infection. In addition, cerebrospinal fluid (CSF) was also collected from patients with suspected EV-A71 associated encephalitis. Nucleic acids were extracted from all samples using the QIAamp Viral RNA Minikit (Qiagen, Hilden, Germany), as outlined in the manufacturer's instructions. EV-A71 infections were detected by testing all clinical samples using an EV-A71 specific qRT-PCR assay (Khanh et al., 2012) or culture isolation of EV-A71 from original clinical material in Vero E6 cells. EV-A71 infection was confirmed if PCR or culture was positive in any of the tested samples. Severe EV-A71 cases with neurological symptoms were confirmed by the detection of EV-A71 by qRT-PCR or culture from CSF or blood; and/or detection of EV-A71 in both throat and rectal swabs, as previously recommended (Jain et al., 2014). Laboratory test results were integrated in the light of clinical findings to provide diagnosis (Figure 1).

EV-A71 phylogenetic analysis

EV-A71 isolates were randomly selected from throughout the outbreak period for sequence analysis of the VP-1 region. Amplicons (~500 bp) of the VP-1 region were produced using RT-PCR (Nix et al., 2006) for EV-A71 isolates associated with uncomplicated HFMD (n = 6) and severe EV-A71 infection (n = 5). The amplicons were sequenced at a commercial facility (Macrogen, South Korea) by Sanger method. Contiguous sequences were assembled using CLC Workbench (CLC bio) and compared to representative EV-A71 sequences downloaded from the NCBI GenBank database. Neighbour-joining trees were constructed with MEGA5 (Tamura et al., 2011) and bootstrap values were calculated and expressed as a percentage from 1,000 replicates.

Data collection and analysis

Data was collected at the bedside using a specifically designed CRF that was focused on socio-demographic variables as well as general and neurological clinical findings. Demographic and clinical data were collected for age, sex, hospital of inclusion, province, fever, peak body temperature, and neurological and extra neurological symptoms and signs at presentation. While in hospital, bodyweight, heart rate, blood pressure, body temperature, mental status, general and neurological examination and treatment were assessed. Weight-for-age z scores were computed for all patients and compared with the World Health Organization child growth standards (www.who.int/childgrowth).

Laboratory data including blood-cell count and differentiated white-blood-cell count, haemoglobin, platelet count, creatinin, liver enzymes, and blood glucose were collected from all patients. Cell count, differentiated cell count, glucose, and protein were also collected from the cerebrospinal fluid of patients with neurological symptoms. Chest radiography, brain computed tomography, and brain magnetic resonance imaging scans were reviewed by radiologists and recorded. Data was entered using standardized paper forms and then exported into an Excel® spreadsheet.

Statistical analysis

Data was analysed with the SPSS statistical package (Version 22; IBM Corp. Armonk, NY: USA). The Student's t-test for

Table 1
Clinical definitions used in this study.

- Suspected EV-A71 infection was defined as an acute illness with either systemic (e.g., fever), respiratory, neurological or skin signs and symptoms suggestive of viral infection.
- Confirmed EV-A71 infection was defined as a suspected EV-A71 infection plus the isolation of EV-A71 virus or molecular detection of EV-A71 RNA in a rectal swab, throat swab, serum or cerebrospinal fluid sample.
- Uncomplicated Hand Foot Mouth Disease patients (HFMD) were defined as patients with a confirmed viral infection presenting with the classical signs and symptoms of HFMD and without neurological symptoms.
- Skin lesions were defined as vesiculo-papular or maculo-papular rash (mostly present over the hands, soles and/or buttocks).
- Mouth lesions were defined as oral ulceration usually observed on anterior tonsillar pillars, soft palate, buccal mucosa, or uvula.
- Neurological symptoms were defined as Glasgow Coma Scale (GCS) score (or modified GCS in less than 2 years old) less than 14 or clinical rhombencephalitis or limb weakness.
- Neurological involvement was defined as neurological symptoms and/or CSF white cell count >5/mm3 and/or abnormal brain imaging (computed tomography scan or magnetic resonance imaging) suggestive of encephalitis.
- Pulmonary oedema was defined as respiratory symptoms and bilateral alveolar congestion on chest radiography.
- Encephalitis patients (ECP) were defined as patients with neurological involvement without pulmonary oedema.
- Pulmonary oedema patients (PO) were patients with pulmonary oedema with or without neurological involvement.
- ECP and PO were termed severe patients.

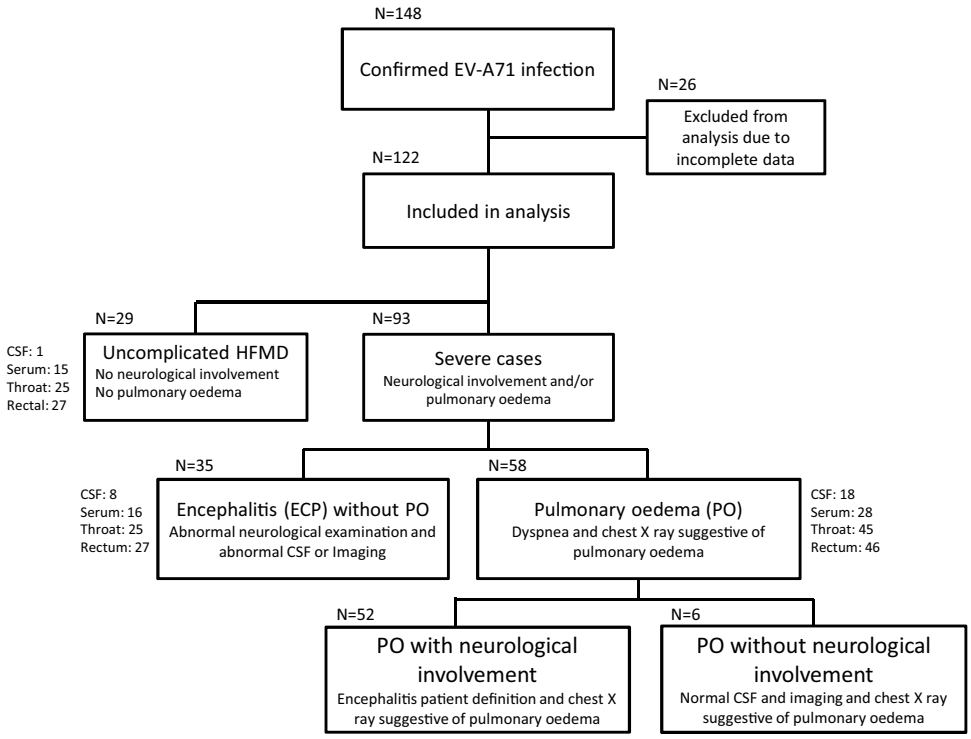

Figure 1. Flow chart showing enrolment into the study.

continuous variables and Mann-Whitney test and Chi 2 test were used for categorical data. Fisher's exact test was used adjunctively if the expected values were less than 5. Bonferroni correction was used as a correction for multiple comparison analysis. Binary logistic regression analysis was used to examine the multivariate-adjusted odds ratios for risk factors that were significant (p value of less than 0.05) in the univariate analysis.

Factors significantly associated with ECP or PO patients in the univariate analysis were included in the multivariate analysis. Forward stepwise regression was used for identifying the optimum variable for the model. A p value of less than 0.05 was considered statistically significant.

Ethics

Ethical clearance was obtained from the Cambodian National Ethics Committee for Human Research before testing commenced. All parents/guardians of sick children who participated in this study provided written informed consent; and clinical CRFs were anonymized to protect the identity of patients.

Results

Patient characteristics

During the five-month period, 148 patients with laboratory confirmed EV-A71 infection were enrolled. Of these 117 (79.1%) patients were recruited in Phnom Penh and 31 (20.9%) were recruited in Siem Reap hospital sites. The recruited patients originated from 17 out of 25 of Cambodia's provinces, mainly from the three most populated provinces in Cambodia: Phnom Penh (25.9%), Kandal (14.2%) and Kampong Cham (12.2%).

The mean age at onset of disease was 28.6 months (range 5 months to 15 years; median 20 months). The majority (n = 133; 90%) of the patients included in the study were less than five years old. The male:female ratio was 1.5:1. Patients had a low median weight for age adjusted z score of −1.11 (range −4.05 to 2.40; mean −0.94; IQR 2.39) (Table 2), compared with the World Health Organization child growth standards.

A total of 122 (82%) patients were enrolled in the study with all available data for clinical classification and further analysis. The median time to hospitalization for these children was two days (range from 0 to 31 days; mean 3.2 days). Twenty-nine (19.6%) patients were classified as uncomplicated HFMD patients and 93 patients were classified with severe EV-A71 disease, including 35 (23.6%) who had ECP and 58 (39.2%) with PO (with or without neurological involvement in 52 and 6 cases respectively).

Overall, 84 (56.8%) patients had either mouth or skin lesions on admission examination. Typical clinical course began with cough and fever 3.2 ± 1.4 days on average before admission. Skin and mouth lesions then appeared 2.4 ± 0.82 days before admission, and in severe cases dyspnea and vomiting would begin 2.2 ± 1.23 days before admission, followed by coma and convulsions 1.6 ± 0.99 days before admission. Lumbar puncture was performed in all ECP patients, in 48 (82.8%) PO patients and in 1 (3.4%) HFMD patient. Chest X-ray was available for 117 (79.1%) patients. Seven (4.7%) patients underwent a brain CT scan and 36 (24.4%) patients had a brain MRI.

Overall, rectal swabs had the highest detection rate of EV-A71 by PCR or culture (100/122; 82.0%), followed by throat swabs (95/122; 77.9%), serum samples (59/122; 48.4%) and CSF samples (26/93; 28.0%) (Table 3).

Comparison of HFMD and ECP patients

By definition, ECP patients had a higher rate of neurological signs and symptoms (headache, limb weakness and convulsion), abnormal neurological examination (lower mean GCS and higher rate of brainstem clinical involvement) and MRI brainstem involvement. In univariate analysis, compared to HFMD cases, ECP patients were significantly more often male, and more often

Table 2
Statistical analyses of the demographic and clinical characteristics associated with uncomplicated and severe cases of enterovirus 71 infections.

Characteristics	HFMD n = 29	N[a]	Encephalitis (ECP) n = 35	N[a]	Pulmonary oedema (PO) n = 58	N[a]	p value HFMD vs ECP	p value ECP vs PO
Demographic								
Male gender	17 (58.6)		10 (28.6)		18 (31)		0.015	0.802
Age, months	21 [12–72]	29	20 [5–168]	35	19 [5–96]	58	0.962	0.221
Province	11 (37.9)		5 (14.3)		10 (17.2)		0.003	0.052
Phnom Penh	7 (24.1)		2 (5.7)		9 (15.5)			
Kandal	0 (0)		6 (17.1)		1 (1.7)			
Battambang	11 (37.9)		22 (62.9)		37 (63.8)			
Other	78.6 [5.8–231]	29	108 [2.90–302]	35	86 [2.9–315]	57	0.022	0.734
Mean distance to hospital, Km	2 [0–3]	18	3 [1–7]	28	3 [0–31]	49	0.006	0.690
History of disease								
Fever	23 (79.3)		34 (97.1)		58 (100)		0.023	0.196
Duration of fever	2 [2–4]	22	3 [1–16]	33	3 [1–10]	53	0.010	0.329
Cough	4 (13.8)		15 (42.9)		32 (55.2)		0.011	0.25
Sputum	0 (0)		2 (5.7)		14 (24.1)		0.191	0.023
Dyspnea	2 (6.9)		8 (22.9)		46 (79.3)		0.080	<0.001
Vomiting	3 (10.3)		21 (60)		44 (75.9)		<0.001	0.106
Abdominal pain	0 (0)		4 (11.4)		10 (17.2)		0.060	0.448
Diarrhea	0 (0)		6 (17.1)		10 (17.2)		0.019	0.99
Mouth ulcer	26 (89.7)		11 (31.4)		15 (25.9)		<0.001	0.562
Duration of mouth lesions	2 [1–3]	26	3 [2–5]	11	2 [1–4]	11	0.043	0.401
Skin lesion	28 (96.6)		16 (45.7)		13 (22.4)		<0.001	0.019
Duration of skin lesion	2 [1–3]	28	3 [1–6]	16	2.5 [2–4]	10	0.028	0.623
Headache	1 (3.4)		10 (28.6)		13 (22.4)		0.008	0.505
Neck stiffness	0 (0)		3 (8.6)		3 (5.2)		0.106	0.518
Limb weakness	0 (0)		8 (22.9)		9 (15.5)		0.006	0.375
Seizure	0 (0)		7 (20)		34 (58.6)		0.011	<0.001
Coma	0 (0)		4 (11.4)		16 (27.6)		0.060	0.066
Examination findings								
Weight, Kg	11 [7.5–21]	29	9.6 [7–30]	34	10 [6–20]	58	0.284	0.390
Weight Age adjusted z score	−0.5 [−2.87–1.58]	28	−1.51 [−3.25–0.81]	29	−1.26 [−4.05–1.96]	53	0.004	0.597
Temperature, °C	37 [36.5–40]	29	38.3 [36.5–40]	34	38.3 [35–40]	58	0.007	0.828
Pulse, bpm	120 [108–180]	29	124 [100–160]	32	140 [60–190]	44	0.154	<0.001
Respiratory rate per min	40 [32–70]	29	40 [30–60]	31	60 [24–120]	43	0.400	<0.001
Glasgow Coma Scale	15 [14–15]	29	15 [3–15]	32	11 [3–15]	55	<0.001	0.015
Oral lesions	27 (93.1)		10 (28.6)		14 (24.1)		<0.001	0.636
Skin lesions	28 (96.6)		14 (40)		14 (24.1)		<0.001	0.106
Abnormal chest examination	27 (93.1)		30 (90.9)		16 (28.6)		0.752	<0.001
Enlarged liver	0 (0)		5 (14.3)		15 (25.9)		0.058	0.188
Brainstem clinical involvement	0 (0)		6 (19.4)		8 (14.8)		0.013	0.587
Paraplegia	0 (0)		1 (2.9)		7 (12.1)		0.359	0.125
Investigation findings								
Haemoglobin, g/dL	11.8 [9.8–13.2]	16	10.5 [7.9–12.9]	35	10.6 [6.4–19.1]	55	0.004	0.497
Mean corpuscular volume, fl	71 [51–80]	17	66 [55–83]	35	66.5 [42–83]	54	0.036	0.762
White cells count, $\times 10^9$ cells/L	11.7 [7.1–22.1]	18	17.4 [6.7–46.8]	35	19.6 [7.1–54.3]	56	0.001	0.029
Neutrophils count, $\times 10^9$ cells/L	5.36 [3.08–13.48]	17	9.51 [0.47–28.22]	35	12.22 [0–35.84]	55	0.003	0.028
Lymphocytes count, $\times 10^9$ cells/L	4.75 [2.62–8.0]	17	4.18 [1.51–16.38]	35	5.06 [0.5–23.80]	55	0.689	0.211
Platelets count, $\times 10^9$ cells/L	336 [162–575]	18	482 [67–743]	35	468 [86–874]	55	0.012	0.970
Creatinine, µmol/L	38.5 [13–97]	18	35 [3.5–75]	35	38.5 [1–105]	52	0.104	0.129
AST, IU/L	37 [25–93]	18	43 [11.4–175]	34	52 [11–365]	53	0.923	0.095
ALT, IU/L	18.5 [15–74]	18	26.5 [11–119]	34	22 [10–549]	54	0.046	0.574
Blood glucose, mmol/L	4.9 [1.3–16]	18	5 [2–14.5]	32	9.1 [1.1–88]	53	0.262	0.000
CSF white cells count, $\times 10^9$ cells/L	4 [4–4]	1	33 [2–549]	35	61 [2–927]	42	0.333	0.539
CSF lymphocytes, %	NA	0	56.5 [32–71]	20	39 [30–68]	22	NA	0.023
CSF neutrophils, %	NA	0	44 [29–68]	20	60.5 [32–70]	22	NA	0.037
CSF proteins, g/L	NA	0	0.31 [0.12–0.75]	35	0.32 [0.1–14]	40	NA	0.778
CSF glucose, mmol/L	4.4 [4.4–4.4]	1	4 [2–35.2]	35	5.3 [1.75–98]	42	0.722	<0.001
Abnormal chest X Ray	1 (5.6)		2 (5.9)		58 (100)		0.962	<0.001
Abnormal brain CT scan	0 (0)		1 (100)		5 (83.3)		NA	0.659
Abnormal brain MRI	0 (0)		20 (100)		14 (93.3)		NA	0.241
MRI brainstem involvement	0 (0)		8 (22.9)		5 (8.6)		0.006	0.069

The round brackets contain percentage; the square brackets contain vales range.
NA: not available.
[a] N: the number of patients with available data.

Table 3
Virological detection rates of enterovirus 71 from different clinical samples.

Detection	Mild disease		Severe disease	
	HFMD (n = 29)	ECP (n = 35)	PO (n = 58)	Total (n = 93)
CSF PCR	NA	7 (20.0%)	17 (29.3%)	24 (25.8%)
CSF culture	NA	5 (14.3%)	3 (5.2%)	8 (8.6%)
CSF total[a]	0/1 (0%)	8 (22.9%)	18 (31.0%)	26 (28.0%)
Serum PCR	15 (51.7%)	14 (40.0%)	28 (48.3%)	42 (45.2%)
Serum culture	1 (3.4%)	3 (8.6%)	1 (1.7%)	4 (4.3%)
Serum total[a]	15 (51.7%)	16 (45.7%)	28 (48.3%)	44 (47.3%)
Throat PCR	25 (86.2%)	25 (71.4%)	45 (77.6%)	70 (75.3%)
Throat culture	10 (34.5%)	7 (20.0%)	20 (34.5%)	27 (29.0%)
Throat total[a]	25 (86.2%)	25 (71.4%)	45 (77.6%)	70 (75.3%)
Rectal PCR	27 (93.1%)	27 (77.1%)	45 (77.6%)	72 (77.4%)
Rectal culture	11 (37.9%)	7 (20.0%)	15 (25.9%)	22 (23.7%)
Rectal total[a]	27 (93.1%)	27 (77.1%)	46 (79.3%)	73 (78.5%)

[a] total values are the number of samples positive by PCR OR culture.

resided outside Phnom Penh and had a longer median distance from home to the hospital. ECP patients also had significantly lower weight-for-age adjusted z score (−1.51 [−3.25–0.81] vs −0.5 [−2.87–1.58]; p = 0.004) (Table 2).

ECP patients had a longer prodromal phase before admission, including a higher percentage of fever, cough and digestive prodromes (diarrhea or vomiting). They also had higher median fever on examination. They had significantly lower rate of mouth or skin lesions and, when they appeared, a longer duration of skin or mouth lesions. ECP patients had lower haemoglobin titers, lower mean corpuscular volume, higher neutrophil and platelets counts, and ALT value.

Comparison of ECP and PO patients

By definition, PO patients had higher rate of sputum, dyspnea, abnormal chest examination or chest X-Ray and higher respiratory and pulse rates compared to ECP patients (Table 2). Blood pressure at admission, however, did not differ within groups. Compared to ECP patients, PO patients were less likely to have skin lesions and had a higher frequency of neurological signs (lower GCS score and higher frequency of convulsion). They had higher white cells count, especially neutrophil count and higher median blood glucose level (9.1 [1.1–88] mmol/L vs 5 [2–14.5] mmol/L, p = 0.000). PO patients were also more likely to have elevated PNN counts in CSF. There was no significant difference in clinical or MRI brainstem involvement between PO patients with neurological involvement and ECP patients.

Multivariate analysis

In logistic regression, PNN count (OR = 2.57 CI95[1.25–5.31], p = 0.011), and haemoglobin titers (OR = 0.181, CI95[0.037–0.881], p = 0.034) were significantly associated with ECP. Time to hospitalization didn't reach statistical significance (OR = 3.065, CI95[0.846–11.11], p = 0.088). Blood PNN count (OR = 2.57 CI95[1.25–5.31], p = 0.011) and CSF neutrophil differential count (OR = 1.079, CI95 [1.011–1.151], p = 0.021) were significantly associated with PO patients.

Phylogenetic analysis

Sequence comparison and phylogenetic analysis of representative EV-A71 strains associated with uncomplicated HFMD and severe cases found no differences in the VP-1 region (Figure 2). There was no discernible clustering of EV-A71 strains according to severity of illness. All of the viruses analyzed were EV-A71 C4a strains, which were previously associated with the 2012 outbreak in Cambodia. The 2014 viruses clustered closest to EV-A71 strains associated with the Cambodian EV-A71 epidemic in 2012; and Vietnamese strains from 2011-2013.

Discussion

In this prospective study, comparative analysis showed uncomplicated HFMD and ECP patients differed significantly regarding gender, province of residence, weight-for-age adjusted z score, time to admission, fever, mouth and skin lesions, haemoglobin, mean corpuscular volume, neutrophil and platelets counts. ECP and PO patients also differed regarding skin lesions, neurological signs, neutrophil count in blood or CSF and median blood glucose level. Provinces outside Phnom Penh, distance to hospital reference center, weight-for-age adjusted z score, haemoglobin, mean corpuscular volume and thrombocytosis were associated with severe forms of EV-A71 infection.

Patients experiencing severe EV-A71 infection had a significantly lower percentage of mouth or skin lesions. In 29 fatal cases of EV-A71 infections, one of the largest case series previously reported, oral ulcers and extremity rash were reported in 66% and 62% of patients respectively (Chan et al., 2000), contrasting with the lower rate of skin or mucosal lesions among PO patients (24%) in our study. The absence of history or clinical findings of mouth or skin lesion have already been associated with severe disease (Chong et al., 2003; Ooi et al., 2007). This association could be simply related to a bias in patient recruitment. Indeed, by an implicit definition, non-severe symptomatic patients included in our study went to hospital because they had skin or mucosal lesions, whereas patients with neurological or respiratory symptoms were hospitalized regardless of their dermatological status. Furthermore, EV-A71 related rash has been reported as often subtle and scant, especially in younger patients with natural tanned skin, and could also have been missed because of delayed hospitalization (Chan et al., 2000; Zhang et al., 2014). No known mutations associated with skin or neurotropism (Ishimaru et al., 1980; Hsueh et al., 2000) were found in the VP1 region, other genes were not sequenced. A final hypothesis could be that, in a particular population, skin or dermatological lesions reflect the host's ability to mount an efficient immune response against EV-A71, as reported in other viral infections, such as parvovirus B19 infection (Bültmann et al., 2004).

Our findings confirm previously reported risk factors for severe forms of EV-A71 infection, including male gender (Zhang et al., 2014), delayed time to hospitalization (Fang et al., 2014), high or prolonged fever (Ooi et al., 2007; Zhang et al., 2014; Ooi et al., 2009; Chang et al., 1999) and vomiting (Chong et al., 2003; Ooi et al., 2007; Chang et al., 1999). A clear and well-documented overrepresentation of males exists in many infectious diseases, with an apparent influence of testosterone and X chromosome on immune responses and disease progression (Libert et al., 2010; Bernin and Lotter, 2014). However, a recent meta-analysis of 19 studies investigating the risk factors for severe EV-A71 disease found no association with gender (Fang et al., 2014).

Our study did not confirm other risk factors for CNS disease identified following large previous EV-A71 outbreaks. Young age (Ooi et al., 2007; Zhang et al., 2014; Chang et al., 1999), home care (Fang et al., 2014) and neurological signs (headache, history of lethargy, limb weakness) (Zhang et al., 2014; Ooi et al., 2009; Chang et al., 1999) have been reported. Viral factors such as genogroup (Ooi

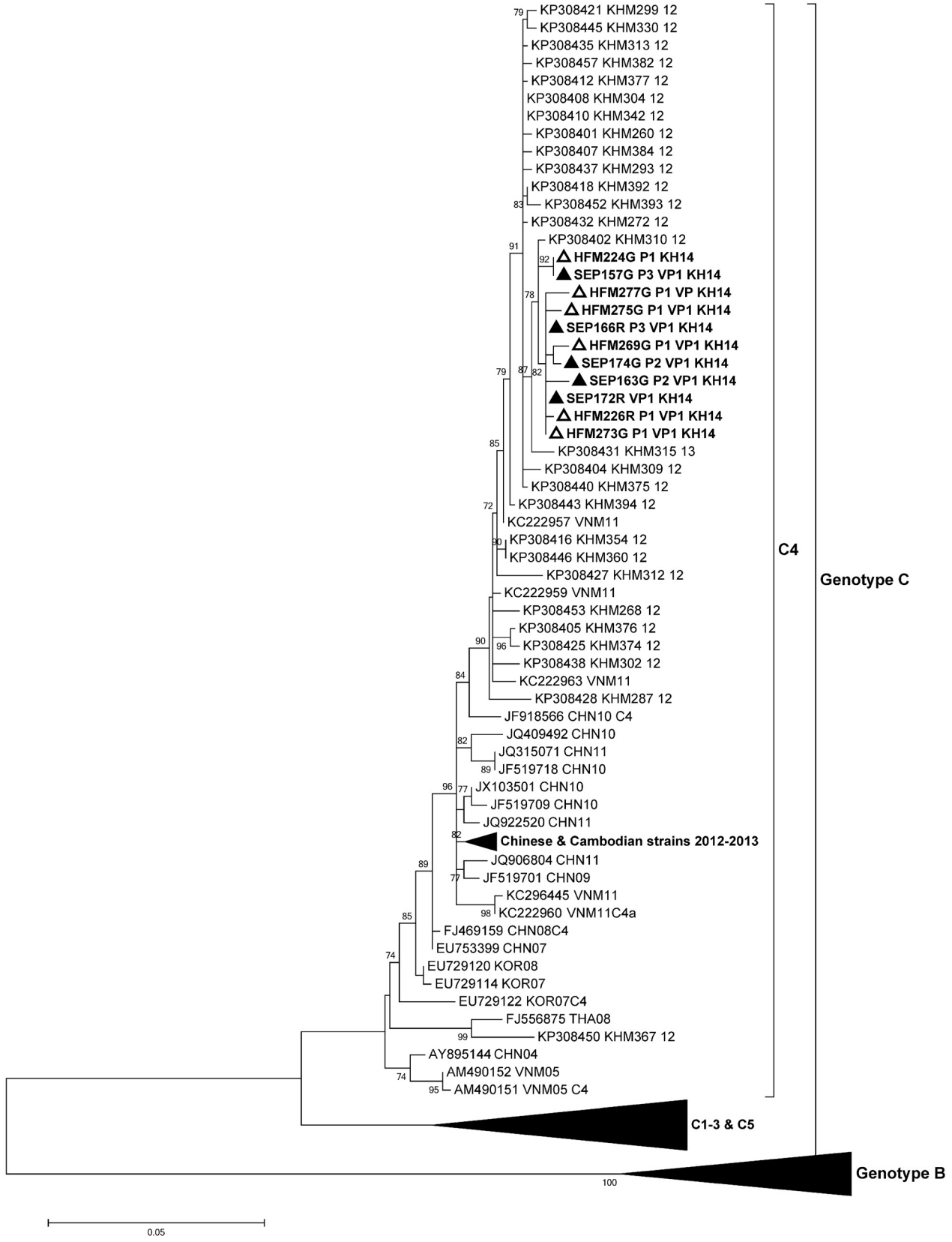

Figure 2. Phylogenetic tree based on VP-1 sequences of enterovirus 71 strains, generated in MEGA5 (Ooi et al., 2007). The numbers next to the branches indicate the percentage of 1000 bootstrap replicates that support each phylogenetic branch. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

▲ EV-A71 strains sequenced in the present study associated with severe illness.

△ EV-A71 strains sequenced in the present study associated with uncomplicated illness.

et al., 2007) or VP1 amino acid substitution (Liu et al., 2014) have also been studied. Co-infection and antibody responses have not been shown to be related to severe forms (Ooi et al., 2007; Yang et al., 2011a). In multivariate meta-analysis, male gender, residing in rural areas and absence of mouth ulcers at examination were not significantly associated with severity (Fang et al., 2014).

Our results are conflicting regarding typical CSF in EV-A71 CNS infection. In one study in children with EV-A71 meningitis, CSF showed a neutrophil predominance in 64% of cases and >200 WBC/mm^3 in 25% (Goldberg and Weiner, 1981) In a case series of 29 children with EV-A71 fatal infections, lymphocytic predominance was reported in 11/12 patients with pleiocytosis on lumbar puncture (Chan et al., 2000). Our results confirm previous findings on the association between EV-A71 related pulmonary oedema and high neutrophil count or hyperglycemia (Chang et al., 1999; Lin et al., 2002) but also highlights the higher rate of neutrophilic CSF in PO patients and lymphocytic CSF in encephalitis patients .

Interestingly, PO patients in our study did not differ significantly in terms of clinical or MRI brainstem lesions or dysautonomic symptoms, compared to ECP patients. Our findings suggest that PO pathophysiology in Cambodian children may be more closely related to cytokinic inflammatory cardiac dysfunction rather than to neurogenic pulmonary oedema.

Our study has important limitations, including the lack of outcome data for patients. Although many surrogate markers of malnutrition were identified as putative risk factors for severe disease, only low haemoglobin titres were independently associated with encephalitis in multivariate analysis. Lack of power and some degree of variable colinearity could explain the lack of differences across groups in multivariate analysis. This study contained potential biases in the recruitment of patients due to the fact that parents of rural origin are less likely to travel long distances to bring their children to the hospital for mild disease such as uncomplicated HFMD, hence the overrepresentation and late presentation of severe illness among children of rural origin. Indeed, there was a negative correlation between distance to hospital and GCS on admission (Pearson −2.56, p = 0.004), suggesting people from remote areas tend to come to hospital for more severe symptoms. However, there was no correlation between distance to hospital and weight adjusted z score (Pearson = −0.06, p = 0.43), suggesting malnutrition may be an independent key factor for severe disease. In addition, the significant association between rash and mouth ulcerations with mild illness may be an artefact due to these factors being included in the definition for uncomplicated HFMD. Finally, the possibility that co-infection with other viral pathogens contributed to the clinical characteristics of children infected with EV-A71 was not explored in this study. However, previous studies suggest that co-infections with other viruses rarely occur in EV-A71 associated encephalitis or HFMD cases (Le et al., 2010; Sapkal et al., 2009; Yang et al., 2011b).

In this prospective EV-A71 outbreak study in Cambodia, we confirmed the previously reported association of male gender and absence of mouth or skin lesions with severe disease. We also highlighted the strong association of neutrophils in blood but also in the CSF of patients with pulmonary oedema. More importantly, we identified new putative malnutrition-related risk factors of severe disease that warrant further investigations, including a more detailed analysis of nutritional status.

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Ethical approval

Ethical clearance was obtained from the Cambodian National Ethics Committee for Human Research before testing commenced. All parents/guardians of sick children who participated in this study provided written informed consent; and clinical case report forms were anonymized to protect the identity of patients.

Conflict of interest

Philippe Buchy is currently an employee of GSK Vaccines but this position has no link with the work presented here. The other authors declare no conflict of interest.

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References

Bültmann BD, Sotlar K, Klingel K. Parvovirus B19. N Engl J Med 2004;350:2006–7.
Bernin H, Lotter H. Sex bias in the outcome of human tropical infectious diseases: influence of steroid hormones. J Infect Dis 2014;209:S107–13.
Chan LG, Parashar UD, Lye MS, Ong FG, Zaki SR, Alexander JP, et al. Deaths of children during an outbreak of hand, foot, and mouth disease in Sarawak, Malaysia: clinical and pathological characteristics of the disease. Clin Infect Dis 2000;31:678–83.
Chang LY, Lin TY, Hsu KH, Huang YC, Lin KL, Hsueh C, et al. Clinical features and risk factors of pulmonary oedema after enterovirus-71-related hand, foot, and mouth disease. Lancet 1999;354:1682–6.
Chong C-Y, Chan K-P, Shah VA, Ng WYM, Lau G, Teo TE-S, et al. Hand, foot and mouth disease in Singapore: a comparison of fatal and non-fatal cases. Acta Paediatr 2003;92:1163–9.
Duong V, Mey C, Eloit M, Zhu H, Danet L, Huang Z, et al. Molecular epidemiology of human enterovirus 71 at the origin of an epidemic of fatal hand, foot and mouth disease cases in Cambodia. Emerg Microbes Infect 2016;5:e104.
Fang Y, Wang S, Zhang L, Guo Z, Huang Z, Tu C, et al. Risk factors of severe hand, foot and mouth disease: A meta-analysis. Scand J Infect Dis 2014;46:515–22.
Goldberg F, Weiner LB. Cerebrospinal fluid white blood cell counts and lactic acid dehydrogenase in enterovirus type 71 meningitis. Clin Pediatr (Phila) 1981;20:327–30.
Horwood PF, Andronico A, Tarantola A, Salje H, Duong V, Mey C, et al. Seroepidemiology of human enterovirus 71 infection among Cambodian children. Emerg Infect Dis 2016;22:92–5.
Hsueh C, Jung SM, Shih SR, Kuo TT, Shieh WJ, Zaki S, et al. Acute encephalomyelitis during an outbreak of enterovirus type 71 infection in Taiwan: report of an autopsy case with pathologic, immunofluorescence, and molecular studies. Mod Pathol Off J U S Can Acad Pathol Inc 2000;13:1200–5.
Huang C-C, Liu C-C, Chang Y-C, Chen CY, Wang ST, Yeh TF. Neurologic complications in children with enterovirus 71 infection. N Engl J Med 1999;341:936–42.
Huang W-C, Shih W-L, Yang S-C, Yen TY, Lee JT, Huang YC, et al. Predicting severe enterovirus 71 infection: Age, comorbidity, and parental behavior matter. J Microbiol Immunol Infect 2014;, doi:http://dx.doi.org/10.1016/j.jmii.2014.11.013 pii: S1684-1182(14)00243-6. [Epub ahead of print].
Ishimaru Y, Nakano S, Yamaoka K, Takami S. Outbreaks of hand, foot, and mouth disease by enterovirus 71. High incidence of complication disorders of central nervous system. Arch Dis Child 1980;55:583–8.
Jain S, Patel B, Bhatt GC. Enteroviral encephalitis in children: clinical features, pathophysiology, and treatment advances. Pathog Glob Health. 2014;108:216–22.
Khanh TH, Sabanathan S, Thanh TT, Thoa le PK, Thuong TC, Hang Vt, et al. Enterovirus 71-associated hand, foot, and mouth disease, Southern Vietnam, 2011. Emerg Infect Dis 2012;18:2002–5.
Le VT, Phan TQ, Do QH, Nguyen BH, Lam QB, Bach V, et al. Viral etiology of encephalitis in children in southern Vietnam: results of a one-year prospective descriptive study. PLoS Negl Trop Dis 2010;4:e854.
Li J, Chen F, Liu T, Wang L. MRI findings of neurological complications in hand-foot-mouth disease by enterovirus 71 infection. Int J Neurosci 2012;122:338–44.
Libert C, Dejager L, Pinheiro I. The X chromosome in immune functions: when a chromosome makes the difference. Nat Rev Immunol 2010;10:594–604.
Lin T-Y, Chang L-Y, Hsia S-H, Huang YC, Chiu CH, Hsueh C, et al. The 1998 enterovirus 71 outbreak in Taiwan: pathogenesis and management. Clin Infect Dis Off Publ Infect Dis Soc Am 2002;34:S52–7.
Liu N, Xie J, Qiu X, Jia L, Wu Z, Ma Y, et al. An atypical winter outbreak of hand, foot, and mouth disease associated with human enterovirus 71, 2010. BMC Infect Dis 2014;14:123.
Nagy G, Takátsy S, Kukán E, Mihály I, Dömök I. Virological diagnosis of enterovirus type 71 infections: experiences gained during an epidemic of acute CNS diseases in Hungary in 1978. Arch Virol 1982;71:217–27.

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

Ooi MH, Wong SC, Podin Y, Akin W, del Sel S, Mohan A, et al. Human enterovirus 71 disease in Sarawak, Malaysia: a prospective clinical, virological, and molecular epidemiological study. Clin Infect Dis Off Publ Infect Dis Soc Am 2007;44:646–56.

Ooi MH, Wong SC, Mohan A, Podin Y, Perera D, Clear D, et al. Identification and validation of clinical predictors for the risk of neurological involvement in children with hand, foot, and mouth disease in Sarawak. BMC Infect Dis 2009;9:3.

Sapkal GN, Bondre VP, Fulmali PV, Patil P, Gopalkrishna V, Dadhania V, et al. Enteroviruses in patients with acute encephalitis, uttar pradesh, India. E merg Infect Dis 2009;15:295–8.

Shindarov LM, Chumakov MP, Voroshilova MK, Bojinov S, Vasilenko SM, Iordanov I, et al. Epidemiological, clinical, and pathomorphological characteristics of epidemic poliomyelitis-like disease caused by enterovirus 71. J Hyg Epidemiol Microbiol Immunol 1979;23:284–95.

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

Yang C, Deng C, Wan J, Zhu L, Leng Q. Neutralizing antibody response in the patients with hand, foot and mouth disease to enterovirus 71 and its clinical implications. Virol J 2011a;8:306.

Yang F, Du J, Hu Y, Wang X, Xue Y, Dong J, et al. Enterovirus coinfection during an outbreak of hand, foot, and mouth disease in Shandong, China. Clin Infect Dis Off Publ Infect Dis Soc Am 2011b;53:400–1.

Zhang Q, MacDonald NE, Smith JC, Cai K, Yu H, Li H, et al. Severe enterovirus type 71 nervous system infections in children in the Shanghai region of China: clinical manifestations and implications for prevention and care. Pediatr Infect Dis J 2014;33:482–7.

Zhao YY, Jin H, Zhang XF, Wang B. Case-fatality of hand, foot and mouth disease associated with EV-A71: a systematic review and meta-analysis. Epidemiol Infect 2015;143:3094–102.