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SUMMARY: Hand-foot-and-mouth disease (HFMD) is caused by a group of enteroviruses, most commonly coxsackievirus A 16 (CA16) and enterovirus 71 (EV71). In general, the disease is mild and self-limited except in the case of EV71 infections, which may incur serious complications. This research focused on virus characterization of HFMD cases in Thailand from 2008–2009, related clinical findings and complications of specific enterovirus subtypes. Specimens (stool, vesicle fluid, throat swab/sputum) from 48 cases were collected during 2008–2009. Reverse transcriptase-polymerase chain reaction (PCR) followed by direct sequencing and phylogenetic analysis served to detect enterovirus and determine subtype. Enterovirus was found in 58.3% (28/48) of cases, specifically EV71 (n = 23), CA16 (n = 4), and CA10 (n = 1). Two patients infected by EV71 had brainstem encephalitis (one death). Eight patients required hospital admission due to dehydration. Of these, 3 were PCR positive for EV71, 1 for CA16, and the reminder negative. This study demonstrated EV71 as the most prevalent present cause of HFMD in Thailand in 2008–2009. Potentially fatal complications of HFMD should be taken into consideration. Surveillance of epidemiology and monitoring of disease severity should be continued, and as a prevention measure, sanitation and hygiene should be improved.

INTRODUCTION

Enteroviruses are members of the family Picornaviridae and cause various diseases in humans. Two types of them, enterovirus 71 (EV71) and coxsackievirus A16 (CA16) are the cause of hand-foot-and-mouth disease (HFMD), with CA16 the most common etiologic agent. However, it has been reported that coxsackievirus A10 (CA10) can also be the etiologic agent of HFMD and clustered without CA16 or EV71 in the outbreak from July 1981 through January 1982 in Matsue City and Gotsu City, Japan (1). Regarding the current genotyping of EV71 isolates, it can generally be classified into nine sub-genogroups of A, B1–B5, and C1–C5 (2–5).

HFMD usually affects children below the age of 10 and especially those younger than 5 years and is generally mild and self-limited. EV71 has recently been reported as the causative agent in several outbreaks of the disease, for example in Malaysia in 1997 (6), Taiwan in 1998 and 2000 (7), Japan (8), and Singapore (9,10) in 2000. In 2008, there was an outbreak in China, with more than 25,000 people infected and more than 30 deaths (11,12). EV71 is related to more serious complications such as neurological involvement, myocarditis, and pulmonary edema (6,8,9). Risk factors caused by the infection contributing to fatal HFMD were atypical physical findings (tachycardia, tachypnea, hypotension, hypertension, bleeding in the gastrointestinal tract, and neurological deficits), raised total leukocyte count, vomiting, and absence of mouth ulcers (10). Over the last 10 years the Bureau of Epidemiology, Department of Disease Control in Thailand, where all diagnosed patients have to be reported, has reported an increase in HFMD cases (Fig. 1). EV71 was the most common etiologic agent, present in 12.9% of cases, while the other enteroviruses were found in 15.1% of cases (13). The aim of this project was to elucidate viral characteristics of HFMD in Thai children as part of an epidemiology study and to ascribe clinical findings and disease severity to specific enterovirus subtypes.

SUBJECTS AND METHODS

The study protocol has been approved by the Ethics Committee, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. Prior to specimen collec-
tion, the parents of all patients were informed as to the purpose of the study and written consent was obtained.

**Study population:** The subjects were recruited for the study on being diagnosed with HFMD upon presenting to the hospital either as in- or out-patients during 2008–2009. Patient specific information such as demographic data, underlying diseases, history of contact with index case, clinical signs, and symptoms was collected. Specimens were collected from stool/rectal swab, vesicle fluid, and/or throat swab/sputum. All specimens were kept at −70°C until tested for enterovirus by reverse transcriptase-polymerase chain reaction (RT-PCR) and further subtype determination by direct sequencing.

**Laboratory tests:** (i) **Viral RNA extraction and RT-PCR:** Viral RNA was extracted from the clinical specimens by Viral Nucleic Acid Extraction Kit (RBC Bioscience, Taipei, Taiwan) according to the manufacturer’s specifications and subsequently reverse transcribed into cDNA using random hexamers. The resulting cDNA was amplified by nested PCR using primer CU-EVF2760, 5′-ATGGKTATGYYWATGTCG-3′ (nt 2760–2779) as the outer sense primer, and CU-EV3206, 5′-CTGACRTGTYTMTACTCTC-3′ (nt 3206–3226) as the outer antisense primer. CU-EVF3029, 5′-TTCTATGTRCCWCGSATGTC-3′ (nt 3029–3048) was used as the inner sense primer. Both amplification reactions were performed under the following conditions: initial denaturation at 95°C for 3 min, followed by 35 cycles comprising denaturation at 95°C for 1 min, primer annealing at 55°C for 1 min, and extension at 72°C for 1 min. The amplification reactions were concluded by a final extension step at 72°C for 10 min. Upon electrophoresis in a 2%-agarose gel stained with ethidium bromide, the expected 234 bp band was visualized on a UV transilluminator (Gel Doc 1000; Bio-Rad, Hercules, Calif., USA).

(ii) **EV71 sequencing and genotype characterization:** The PCR products were purified for sequencing using the Perfectprep Gel Cleanup Kit (Eppendorf, Westbury, N.Y., USA) and subsequently subjected to 2%-agarose gel electrophoresis in order to ascertain their purity. The concentration of the amplified DNA was determined by measuring each sample’s absorption at 260 nm in a Bio-Photometer (Eppendorf, Hamburg, Germany). The DNA concentration was calculated based on the conversion of 1 OD 260 being equivalent to 50 µg of double-stranded DNA. Between 10 and 30 ng/µl (3–6 µl) of each DNA sample were subjected to direct sequencing using the primers CU-EVF2760 and CU-EV3206 (to confirm the sequence). Direct sequencing was performed by First BASE Laboratories Sdn Bhd (Selangor Darul Ehsan, Malaysia).

(iii) **Phylogenetic analysis:** To further characterize the enterovirus strains from this study, the BLAST/Fasta program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and phylogenetic analysis were used. The sequences were edited using the programs CHROMASLITE v.2.0 (http://www.technelysium.com.au/chromas_lite.html) and SeqMan (DNASTAR, Madison, Wis., USA). To investigate the relationship between enterovirus strains, the unrooted tree topology based on multiple alignments of the VP1 nucleotide sequences with those of known genotype from Genbank (EV71, CA10, and CA16) was obtained by the neighbor-joining method calculated bootstrap values with MEGA 3.1 (http://www.megasoftware.net). Consistency of branching was tested with a bootstrap analysis with 1,000 resamplings of the data using MEGA 3.1. Multiple protein translations and sequence alignments were generated with BioEdit version 7.0.1 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). All EV71, CA10, and CA16 strains from this study were compared with the reference strains (accession nos. FJ556873, FJ556874, FJ151502, FJ151494, AF322906, AF895136, and AF286531 for C4b; EU753402, FJ556875, FJ711317, GU196833, EU753409, FJ469153, and EU753369 for C4q; AF905548, AF905546, and AF119795 for B5; AM396587, AB059818, AJ56873, AF352027, and AF376111 for B4; AM396588, AF376105, AB059819, and AM396586 for B3; AF135888, AF009540, U22522, and EU22522 for B2; AF135901, AB059814, AB059813, and AB059815 for B1; AJ258301, AF135935, AB115495, and AF135945 for C1; AE125968 and AB115494 for C3; AF304458, AF135947, AM396585, AM396584, AF376109, DQ006149, and DQ381846 for C2; AB204852, AB204853, and U22521 for A; AB167986 and ABI26614 for CA10; and CAU05876 for CA16).

**Data analysis:** Data analysis was descriptive as demographic data. Clinical signs and symptoms were analyzed (average age, standard deviation, frequency) using SPSS 13.0 for Windows.

**RESULTS**

From September 2008 to November 2009, there were 48 cases, aged 4 months to 26 years (median 3.0 years) with a male/female ratio of 1.3:1. None of the patients had any underlying disease. The associated symptoms were fever (50.0%) and vomiting (40.0%). The 28 cases with positive viral results, aged 7 months to 26 years (median 3.0 years), had a higher male/female ratio (2.1:1) (Table 1). Fever was found in 46.4% without any vomiting.

**Prevalence and subtype of enteroviruses:** Specimens were collected from stool/rectal swab in all subjects, vesicle fluid in 5 subjects, and throat swab/sputum in 3 subjects. Among the 48 subjects, the prevalence of enteroviruses was 58.3% (28/48). EV71 was the most commonly identified virus (47.9%, 23/48), whereas CA16 and the rather unusual CA10 amounted to only 8.3% (4/48) and 2.1% (1/48), respectively. Enterovirus from vesicle fluid and throat swab/sputum was detected among 60.0% (3/5) and 66.7% (2/3), respectively.

**Location and characterization of rash:** Oral lesions were the most common (89.6%), followed by lesions on hands, feet, knees, buttocks, and elbows (85.4, 85.4, 35.4, 18.8, and 4.2%), respectively. All subjects presented with vesicular lesions but 3 also displayed maculopapular rash (1 generalized and 2 locally on the trunk). Clinical manifestations of the 5 patients with CA10 and CA16 were distinct from those of the remaining cases. As shown in Table 1, all 5 displayed vesicles in the oral cavity, 4 had lesions on their hands and feet, and 2 on their knees.

**Severity and complication:** The clinical course was mild with the majority of subjects except for 10 (20.8%)
who developed complications. Two patients (4.2%) PCR-positive for EV71 developed serious complications in the form of brainstem encephalitis to which 1 patient succumbed. Of the remaining 8 patients who required hospital admission due to dehydration, 3 were PCR-positive for EV71, 1 for CA16 while the others were negative. The clinical course in the other 3 cases of CA16 and 1 case of CA10 was mild and self-limited.

**History of contact case:** Seven subjects had contact with an index case and approximately 50% had acquired the infection by transmission among family members within a 5- to 7-day period.

**Molecular characterization:** The sensitivity of RT-PCR was tested by performing the titration of PCR-positive plasmid DNA and the PCR amplicon, the result gave 10 and 10^5, respectively. Positive samples of hepatitis B virus, respiratory syncytial virus (RSV), and bocavirus were used to check the specificity.

Genotype determination accomplished by BLAST/FASTA and phylogenetic analysis showed that 23 samples were EV71, with 9 samples clustered in the same lineage with sub-genotype C4b, 5 with C4q, 2 with B5, 2 with C2, and 5 with C1. Five samples were coxsackievirus, with 1 CA10 and 4 CA16 (Fig. 2).

**DISCUSSION**

A high number of samples (9/23, 39.1%) analyzed in this study exhibited sub-genotype C4b which circulated in Thailand in 2008 (unpublished); 5 of 23 (21.7%) clustered with C4q that circulated in China and Shanghai from 2008 to 2009; 2 samples (8.6%) displayed genotype B5 that had been detected in Malaysia; 2 samples (8.6%) clustered with C2 which had been found in Taiwan; 5 samples (21.7%) showed genotype C1 such as that isolated in the USA and the UK. Four samples of CA16 clustered with a group found in Finland and the 1 CA10 could be traced by BLAST/FASTA analysis to Japan in 2003 (CA10/80269/Hiroshima.JP/03 and 03-150NPC3/Fukuoka City). Based on this study, the predominant sub-genotype was C4b which circulated in Thailand in 2008, in China in 2005 and clustered in the same C4 lineage that had circulated in China from 2008 to 2009.

The majority of patients in the present study were below the age of 5 years (75.0%) with a median age of 3.0 years. Five patients (10.4%) were above the age of 10 years. The patients’ demographic details support the previous report that the majority of HFMD cases are under 5 years old (9,11). However, 1 patient in this study was above the age of 25 and had been infected via transmission among family members. In this patient E71 was detected in rectal swab, throat swab, and vesicle fluid as confirmed by directed sequencing. The patient had a mild and self-limiting clinical course. Tai et al. reported the disease in a healthy adult, aged 42,
from transmission of EV71 among family members (14).

This study has shown that the causative agent of HFMD in Thai children between 2008 and 2009 was EV71 in 47.9% of the patients, in contrast to other enteroviruses which were detected in 10.4%. According to the Annual Epidemiology and Surveillance Report of the Bureau of Epidemiology, Department of Disease Control, the ratio of EV71 to other enteroviruses was 12.9:15.1 in 2007 (13). This might indicate a possible shift of disease etiology among the Thai population.

Looking at the single case of CA10, the patient was a 7-month-old boy with indifferent clinical features from the others (vesicles in the oral cavity as well as on hands and feet). His illness spontaneously resolved without any complication.

Rectal swab specimens examined in this study were PCR-positive in 58.3% of cases, which was similar to the study of Zhang et al. which had a positive rate of 63.2% (11). Vesicle fluid specimens were positive in 60% of cases. The mortality rate of cases in this study was quite high (4.2%) compared to figures from other studies or the report by the Bureau of Epidemiology, Department of Disease Control (13,15). This may be because King Chulalongkorn Memorial hospital is a tertiary care and university hospital where the proportion of more serious cases and referred cases is higher. Additionally, the total number of subjects in our study was less than in other reports and thus, the mortality rate would appear higher than usual.

Risk factors for fatal and nonfatal HFMD cases, studied by Chong et al. (10) in 2000–2001 in Singapore include atypical physical findings, lymphocytosis, vomiting, and absence of oral lesions. Rash morphology did not differ between fatal cases and nonfatal cases, but maculopapular rash was more common than vesicles (10). The fatal case in this project also presented with ataxia with a small amount of lesions in the mouth and on the feet. All 3 cases with maculopapular rash had mild clinical symptoms and only 1 patient was PCR-positive for EV71.

In summary, the result of our study demonstrated a high prevalence of EV71 as the etiologic agent of HFMD in Thai children in 2008–2009 even though the number of subjects in this study was smaller than in other published projects (6–10). This could be due to the change of prevalence of etiologic agents year by year. However, caregivers should be more aware of potential serious complications caused by EV71 such as brainstem encephalitis, pulmonary edema, and death. In addition, virus transmission among family members, especially to healthy adults, resulting in potential complications should raise concern. Hence, surveillance of epidemiology and monitoring of severity of HFMD should be maintained among Thai children and adults.

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