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Research Article

Enterovirus 71- and CoxsackievirusA16-Associated Hand-Foot-Mouth Disease in Guangdong, China: A Retrospective Clinical Study

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Abstract

Background: Since 1997, the Asia-Pacific region had experienced epidemics of Enterovirus 71 (EV71)-associated Hand-Foot-Mouth Disease (HFMD), with cases at risk for severe illness and even death.EV71 and coxsackie virus A group 16 (CA16) are both major causative agents of HFMD and have similar early symptoms. These similarities hamper early diagnosis, making it difficult to identify potentially severe cases without genotyping. EV71-VP1 gene sequence are related to the severity of disease remains controversial.

Method: We performed a retrospective study of clinical cases in Guangdong Province, China, from April 2009 to December 2012, using real-time RT-PCR to detect EV71/CA16/pan-enterovirus. Viral isolation followed VP1 genes sequencing were performed for molecular epidemiological analyses.

Result: 395 and 156 hospitalized patients were confirmed to be infected with EV71 and CA16, accounting for 51.6% and 20.4% of all inpatients with enterovirus infections, respectively. Hyperpyrexia (≥39 °C, $P<0.001$), vomiting ($P<0.001$), headache ($P=0.03$), and neurological symptoms such as irritability ($P<0.001$), altered level of consciousness ($P<0.001$), tremors/trembling in the extremities ($P<0.001$), limb weakness/paralysis ($P=0.007$), and altered muscular tension ($P=0.02$) were significantly more common in EV71- than in CA16-infected patients. The incidence of neurological complications (62.8% VS 5.1%, P<0.001) and outcomes were significantly different in two infections. Logistic-regression analysis revealed four independent risk factors: chloride-ion concentration, LDH activity in the CSF, troponin I levels, and serum myoglobin. EV71 isolates were all C4a subgeno-group, sharing high identity with each other (94.4%–100%). Amino acid sequences of three EV71 strains from severe cases were identical to those from mild cases.

Conclusion: A comparative study of the differences in the clinical presentations of EV71 and CA16 infections in China yielded insights. Clinical features strongly associated with rapid progression and severe EV71 infections were identified. This retrospective study provides valuable information to primary-care doctors, permitting rapid intervention for high-risk patients. No difference in EV71-VP1 amino sequences were found between severe and mild cases.

Key Words:

Coxsackie virus A16, Clinical features, *Enterovirus* 71, Hand-Foot-Mouth disease, Molecular epidemiology

Introduction

Hand-foot-mouth disease (HFMD) is a pediatric illness that is especially common among children less than 5 years of age. The causative agents are a group of *enteroviruses* belonging to the genus *Enterovirus*, family Picornaviridae, including coxsackie virus A group (serotypes CA 2–8, 10, 12, 16) and *enterovirus* 71(EV71) [1] Although most cases are mild and self-limiting, HFMD associated with EV71 caused outbreaks with several severe cases with central nervous system (CNS) complications and deaths over the past 15 years in the Asia-Pacific region. In China, epidemics of HFMD have been reported every year since the national reporting system launched in 2008, following an outbreak in Anhui Province, in central China. As of 2012, 7,200,092 HFMD cases and 2,457 deaths had been reported in China [2]. Due to the increasing severity and distribution of EV71-associated HFMD, this disease has become a public-health concern.

EV71 and CA16 are the predominant agents of HFMD in China since 1999 [3,4]. EV71 and CA16 can cause diverse clinical symptoms, most of which are very similar, including fever, a characteristic rash, and possibly mouth ulcers; however, some differences presentations occur late in infection. EV71 infection is frequently associated with CNS complications that may develop into fulminant cardio-respiratory failure days or even hours after onset [5,6]. In contrast, CA16 and other species a *enteroviruses* seldom lead to neurological symptoms or death. Virological diagnostic methods are the most efficient ways to distinguish EV71 from CA16 infections during both causative agents circulating in HFMD epidemics [7-9]. Therefore, early identification is crucial to helping primary-care doctors institute timely and appropriate interventions for high-risk patients when the pathogens isolated from clinical samples cannot be genotyped in time. Previous studies have revealed that high fever, lethargy, vomiting, and myoclonic jerks indicate CNS involvement in HFMD [5,10] providing insights into clinical features associated with severe infections; however, these investigations took place in regions other than China, where epidemics have been caused by C4, a new lineage of EV71 [11]. Moreover, the northern and southern China have experienced different patterns of *enterovirus* due to their different climates and populations [2]. To date, the identities of the clinical features associated with severe cases in epidemics in China are unknown.

Even though EV71 and CA16 sharing significant genomic and antigenic similarity [12], the mechanism that EV71 infection frequently associated with neurologic symptoms and severe complications remains unclear. EV71 variants are classified into three genogroups, A, B (B1~B5), and C (C1~C5), based on the sequence of capsid protein VP1 gene [13]. EV71 strains isolated in mainland China were all C4 subgeno type since 1999 [11]. Three genetic lineages of CA16 (A,B,andC) have been found co-circulating in the epidemics in China [3,14,15]. The convincing evidence of the association between genogroups and the severity of disease was rarely reported [16].

To gain insight into the association of symptoms and epidemiological factors with the development of severe disease, we retrospectively analyzed detailed data associated with a large cohort of inpatients with HFMD confirmed to have EV71 and CA16 infections in Guangdong Province, southern China, from 2009 to 2012. We also compared the clinical manifestations of EV71 and CA16 infections, the different stages of EV71 infection, and the VP1 gene sequences of EV71 strains from varied severity cases. This study provides valuable insights into the factors causing severe complications and death to facilitate clinical decision-making and management of future epidemics.

Methods

Patient enrollment and data collection

Pediatric patients admitted to Zhujiang Hospital between April 2009 and December 2012 with a diagnosis of HMFD were enrolled in this study. The diagnosis was based on the clinical manifestations outlined in the Guidelines for HFMD: fever, characteristic rash with or without mouth ulcers, and/or symptoms of CNS infections. In most cases, HFMD is self-limiting and patients recover with treatment of the symptoms. Patients with systematic complications including autonomic-nervous-system dysregulation, cardiopulmonary failure, and/or central-nervous-system involvement such as meningeal irritability and meningitis were hospitalized. Moreover, patients at high risk of severe complications were hospitalized following clinical assessment according to the Guidelines for HFMD. Demographic data, clinical notes and laboratory findings for all inpatients were reviewed. Figure 1 shows the diagnostic tests used in this study.

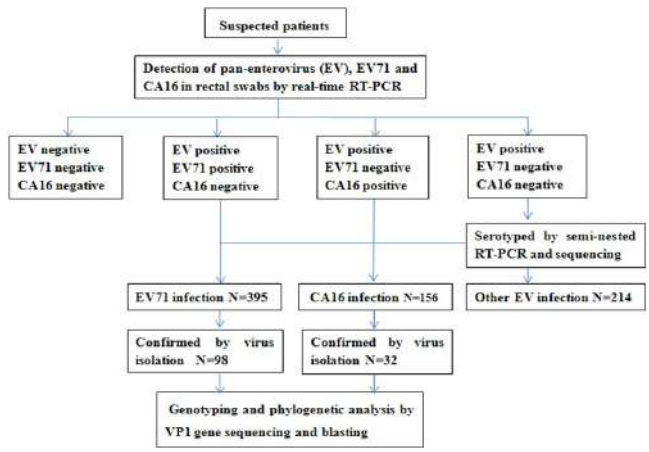

Figure 1: Schematic of the diagnostic tests and phylogenetic analysis conducted on rectal swabs with results obtained for each test.

The Ethics Committee of Zhujiang Hospital approved this study (Ratification No: ZJYY-2012-YXJYZX-001). The parents

of the enrolled patients gave their informed consent before rectal swabs were collected.

Etiological studies

Rectal swabs were collected from all suspected patients at admission and within 14 days after the onset of symptoms for further etiological examination. Only inpatients were included in this study. The rectal swab placed in a Virus Transport System (Copan, Via Perotti, ITA), then placed on ice and transported to the laboratory for real-time PCR or stored at -80°C for viral isolation. Viral RNA was extracted using a QIA amp Viral RNA Mini Kit (Qiagen, Hilden,GER), and a EV71/CA16/pan-*enterovirus* detection assay was conducted using real-time PCR (Shanghai ZJ, Shanghai, CHN). ACODEHOP PCR was performed on pan-enterovirus-positive and EV71/CA16-negative samples, and the resulting PCR products were sequenced.

Samples that were EV71/CA16-positive by real-time PCR were inoculated into human rhabdomyosarcoma (RD) and/or human larynx epidermoid carcinoma cells (HEp-2). Viruses were harvested upon appearance of complete cytopathic effects (CPE) and identified by real-time PCR, as described above.

Primers (5'-GCAGCCCAGAAGAACTTCAC and 5'-ACCACTCTAAAGTTGCCCAC for EV71, 5'-CTGGG-TACTTTGACTATTACACand 5'-GTTGTTATCTTGTCTC-TACTAGTG for CA16) bind to VP1 encoding regions with full-length of 891bp. Phylogenetic analysis was performed using the neighbor-joining method with MEGA5.1software. All reference sequences and other VP1 sequences were retrieved from Gen Bank with accession number: AF376072, AF376098, AF376101, AF376121, AM490143, AF009535, AY125973, AY895142, AB115493, AF135880, AB213625, EU913467, GQ487688, JN874547, U05876 for EV71, and GQ429266, GQ429230, GQ429246, GQ429223, AY895103, AY895095, AF177911, EU262658, AB465400, GQ429226, GQ429274, GQ429260, GQ429257, AB465370, AB4653661, AB4653671,AB465368-9, AB465402, AY895116, AY895127, U058761, AM292455, JN874547 for CA16. Those in our laboratory were: KC689916-KC68998. Homology of VP1 gene nucleic and amino acid sequences was determined in EV71 and CA16 isolates from the clinical samples.

Clinical definitions

Patients confirmed to have EV71 or CA16 infection were categorized into four groups according to a separate clinical staging system (1-4) based on the presence/absence and severity of CNS complications[3,4,6,10]. Mild CNS complications included cerebrospinal fluid (CSF) pleocytosis ($>5\times10^6$ leukocytes/L), headache, irritability, and neck stiffness without alterations in consciousness or focal signs. Diagnosis of severe CNS complications was based on evidence of altered consciousness, CSF pleocytosis, and/or poliomyelitis-like syndromes such as decreased reflexes and muscle strength. Neurogenic cardiopulmonary failure was defined by the necessity of inotropic agents, endotracheal intubation, and ventilator support 2-36 hours after the onset of CNS complications. Patients with independent pneumonia, myocarditis, or bacterial sepsis were excluded. Patients without CNS complications were classified in group 1, while those with less severe CNS complications (i.e., aseptic meningitis), more severe CNS complications and neurogenic cardiopulmonary failure and/or those who died were classed in groups 2,3 and 4, respectively.

Data analysis

Continuous variables with a normal distribution were assessed using the t-test. Nonparametric data were assessed using the Mann-Whitney U test and expressed as medians and ranges. Differences of clinical manifestation between EV71 and CA16 infections were assessed using the chi-square test. Unconditional logistic regression was used to estimate the odds ratios (ORs) and 95% confidence intervals (95% CIs) for the risk of neurological complications. All statistical analyses were performed using SPSS version 13.0 (SAS Institute Inc., Carey, NC, USA). $P< 0.05$ was considered statistically significant.

Results

Demographical characteristics of EV71 and CA16 infections

Of all 551 patients admitted from 2009 through 2012, 239 patients were local (Guangzhou City), while 269 were from 18 other cities in Guangdong Province and 43 were from other provinces. Most cases (76.2%, 301/395, EV71; and 67.3%, 105/156, CA16) occurred between April and July. A smaller peak was observed in October and November in 2010 and 2011. EV71 and CA16 infections were confirmed in 395 and 156 hospitalized patients, respectively, accounting for 51.6% and 20.4% of all *enterovirus*-infected inpatients. Additionally, 214 inpatients were infected with other *enteroviruses*.

Patients ranged in age from 1 month to 14 years (median: EV71, 2.08 years; CA16, 2.25 years). Young children (< 4 years) accounted for 88.0% (485/551), with peak incidence occurring at 1 year of age. Eight infants (<6 months) were infected with EV71, and two infants were infected with CA16. Critical infections were more prevalent in males, with male-to-female ratios of 2.0:1 (EV71) and 2.6:1 (CA16). No age or gender differences were observed in the prevalence of EV71 and CA16 (Figure 2A and 2B). The duration of hospitalization was slightly longer in EV71 than in CA16 infection (P=0.001, Figure 2C).

Comparison of the clinical manifestations between EV71 and CA16 infection

Clinical manifestations of EV71 and CA16 infection were summarized and compared (Figure 2D and 2E). Patients with both EV71 and CA16 presented with fever and acute rash;

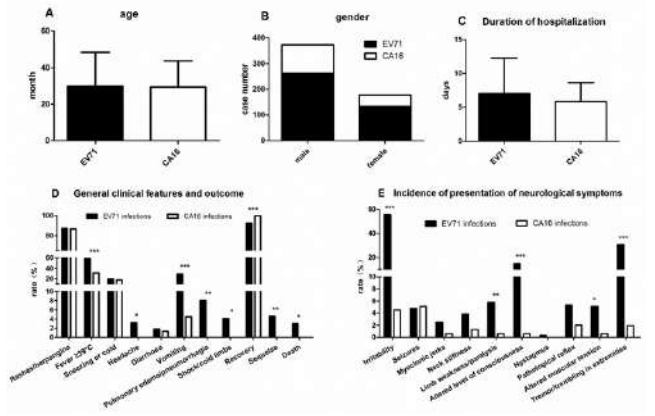

Figure 2: Comparison of demographical characteristics and clinical manifestations betweenEV71 and CA16 infections.(A and B): No age or gender differences were observed between EV71 and CA16 infections.(C): The duration of hospitalization was slightly longer in EV71 than CA16 infection. (D): general clinical features and outcomes. Fever, acute rash, sneezing, cough, and fever were very common in children with both EV71 and CA16. The peak body temperature in patient's withEV71infections was slightly higher than patients with CA16 infections. Hyperpyrexia (body temperature ≥ 39°C, P<0.001) and non-projectile vomiting (P<0.001) were significantly more common among patients with EV71infections. Pulmonary edema, pneumorrhagia and shock were observed only in EV71-infected inpatients. Patients with EV71 infection progressed to sequelae and death. (E): The incidence of neurological presentation. EV71-infected patients presented with a significantly higher incidence of neurological presentations, especially irritability (P<0.001), limb weakness/paralysis (P=0.007), altered level of consciousness, muscular tension (P=0.02), and tremors/trembling in the extremities (P<0.001). One asterisk: P<05; two asterisks: P< 0.01; three asterisks: P< 0.001.

sneezing, cough, and fever were also common symptoms of both infections. The peak body temperature in EV71 infections was slightly higher than in CA16 infections (38.9±0.73 vs. 38.2±0.96°C, *P*<0.001). Additionally, hyperpyrexia (body temperature ≥ 39 °C, *P*<0.001) and non-projectile vomiting (*P*<0.001) were significantly more common inEV71infections (Figure 2D). Thirdly, the incidence of neurological complications was higher among EV71-infected patients (62.8%) than among CA16-infected patients (5.1%). As shown in Figure 2E, irritability was the most common of these symptoms, followed by tremors/trembling in extremities (30.6%), altered consciousness (15.2%), limb weakness/paralysis (5.8%) and pathological reflexes (5.3%).Pulmonary edema, pneumorrhagia and shock were observed only in EV71-infected inpatients (Figure 2D). In addition, outcomes differed significantly between the two infections (*P*=0.003), compared by the Mann-Whitney rank-sum test (Figure 2D). All patients infected with CA16 recovered fully, but 16(4.1%) EV71-infected patients experienced various neurological sequelae at discharge, including varying degrees of paralysis (13 patients), central facial paresis (2 patients), and pseudo bulbar palsy (1 patient). Approximately 3.0% (12/395) of EV71-infected patients died of pneumorrhagia, shock or multiple-system organ failure within 1-14 (median 2.0) days of hospitalization; these patients ranged in age from 5 to 29 (median 19.0) months.

Comparison of the clinical characteristics of EV71 among four stages of the disease

As EV71 infections yielded more severe outcomes than CA16 infection's, we further analyzed the clinical symptoms of EV71 infection. According to a separate clinical staging system based on the presence and severity of CNS involvement (summarized in Figure 2E), all 395 EV71-infected patients were categorized into four groups (1-4). The results of the comparative analysis of demographic data and laboratory findings among the four groups of EV71-infected in patients are shown in Table 1.

Although patients in the group four were younger than those in the group one, no significant difference in age, gender or other demographic was observed among the four groups. Clinical manifestations and laboratory findings, including peak body temperature (r=0.16, *P*<0.001), altered level of consciousness (r=0.42, *P*<0.001), activity of lactate dehydrogenase (LDH) in the CSF(r=0.29, *P*<0.001), WBC count in the CSF(r=0.22, *P*<0.001), concentration of troponin I (cTnI; r=0.31, *P*<0.001) and myoglobin (MYO; r =0.23, *P*<0.001) in serum, were analyzed; however, these clinical features and outcomes were only weakly correlated. Logistic-regression analysis was performed to screen risk factors as potential predictors of the progression of severe EV71 infections and identify clinical characteristics independently associated with disease progression and death. The results indicated four independent risk factors: chloride-ion concentration, LDH activity in the CSF, cTnI levels and serum MYO. Lower chloride-ion concentration and higher LDH activity in the CSF, as well as the higher cTnI concentration and serum MYO indicated a poor prognosis (summarized in Table 1).

Twelve of 16 patients in Group 4 progressed rapidly and died in the hospital, and one died at home after ceasing treatment. Despite intensive treatments and supportive therapy, 12 patients developed progressive complications and died of cardio respiratory failure (100%, 12/12) with pulmonary bleeding (66.7%, 8/12), pulmonary edema (33.3%, 4/12), encephalitis (8.3%, 1/12) or gastrointestinal bleeding (8.3%, 1/12).

EV71/CA16 genetic subtypes and sequence homology

A total of 98 (EV71) and 32 (CA16) clinical strains were isolated and identified based on theVP1gene sequences. All EV71 strains were genotype C4 and subdivided as clade C4a (Figure 4A); CA16 strains were genotype B1 (B1a in 2009, 2010, 2012 and B1b in 2011).High identity of 94.4%–100% (EV71) and 91%–100% (CA16) was shared among full-length VP1 nucleotide sequences of the clinical isolates (the phylogenetic tree shown in Figure 3).

VP1 amino acid sequence alignment (Figure 4) showed an identical protein in all EV71 disease groups, including three isolates from severe cases (group 4), suggesting no relationship between disease severity and VP1 amino acid sequence. No obvious differences between strains from various years were observed. However, a Gln22 (Q) to His (H) amino acid substitution in two isolates from 2009, and an Asn31 (N) to

Characteristics	Group 1 (n = 147)	Group 2 (n = 176)	Group 3 (n = 56)	Group 4 (n = 16)
Demographics				
Age range, months	2 to 144	5 to 127	7 to 169	5 to 47
Mean±SD	33.7±20.6	29.0±17.9	31.6±23.6	20.4±11.0
Male, n (%)	277(67.2)	120(68.2)	37(66.1)	11(68.8)
Laboratory findings				
CSF WBC count($\times 10^6$ /L), n	-	74	49	10
Median (Q1 and Q3)	-	30.0 (10.0, 72.5)	44.0 (15.0,102.5)	95.0 (60.3, 197.5)
CSF glucose (mmol/L), n	-	72	49	10
Mean±SD	-	4.26±0.93	4.38±0.91	4.26±1.39
CSF protein (mg/L), n	-	72	49	10
Mean± SD	-	363±136	382±179	572±347
CSF Cl (mmol/L), n	-	72	49	10
Mean±SD	-	122.3±3.88	121.9±3.99	120.3±7.44
CSF LDH(IU/L), n	-	72	49	10
Mean±SD	-	22.0±9.68	28.2±13.8	60.3±56.5
Troponin I(μg/L), n	36	108	25	11
Median (Q1 and Q3)	0.01 (0.01,0.01)	0.01 (0.01,0.01)	0.01 (0.01,0.01)	1.59 (0.16,8.12)
MYO(μg/L), n	30	95	19	8
Median (Q1 and Q3)	14.3 (10.4,26.9)	16.5(10.1,26.7)	18.3 (8.55,25.5)	66.4 (38.0,122.8)
CK-MB(IU/L), n	138	172	45	19
Median (Q1 and Q3)	25.4 (19.4,38.4)	24.7(18.4, 31.4)	25.1 (20.4, 31.8)	29.7 (18.6, 54.9)

Table 1: Comparison of clinical characteristics in the four groups of EV71-infected inpatients.

Asp (D) amino acid substitution in two isolates from 2012 were observed. Lys98 (K) to Glu (E) amino acid substitution were commonly observed in isolates from each year. Figures of the amino acid sequence alignments of CA16-VP1 were not shown.

Discussion

HFMD has been recognized as a common childhood illness since its discovery in the UK in 1959 [17]. Because infections are usually mild and self-limiting, there was little public concern before the EV71-associated HFMD outbreak in the late 1990s in the Asia-Pacific region, in which many cases led to neurological disorders, severe sequelae and death. Early EV71 outbreaks in the Asia-Pacific regions were usually episodic, with the incidence rising rapidly within one or a few years, then decreasing before re-emerging again. The associated EV71 strain also changed frequently. More recent Chinese HFMD outbreaks have been different. Since 2008, the numbers of reported HFMD cases have been persistently high, and C4 has been the sole causative strain. The underlying reason for this shift remains unclear. As more than a million HFMD/herpangina cases have been reported each year in mainland China, including Guangdong [18], a thorough understanding of the clinical profile of HFMD and the molecular epidemics of the main causative agents are urgently needed.

We summarized the clinical characteristics of more than 500 hospitalized pediatric patients infected with EV71 and CA16 in Guangdong, China, over 4 consecutive years. The two major pathogens associated with HFMD/herpangina accounted for 51.6% (EV71) and 20.4% (CA16) of the pediatric patients hospitalized for *enterovirus* infections. Most *enterovirus* infections present similarly, are self-limiting and do not require hospitalization. We attempted to identify differences in symptoms between EV71- and CA16 infection that could be used by primary-care doctors to predict disease progression and prevent unnecessary hospitalizations. Peak body temperature ≥ 39°C presented more frequently in EV71-infected patients than that in CA16 patients (P<0.001), in agreement with Ooi [10], who found that a peak body temperature>38.5°C for at least 3 days predicts neurological involvement. In addition, we found that peak body temperature is correlated with outcomes of EV71 infection. Other symptoms that may be related to neurological involvement, such as vomiting and headaches, were more commonly observed in EV71 than CA16 infection (P<0.001 and P=0.03), respectively. Additionally, in Ooi's studies, a history of lethargy, which is one manifestation of altered level of consciousness, as well as irritability, limb weakness/paralysis, tremors/trembling in extremities, and altered muscular tension presented more frequently among EV71-infected patients (P<0.05) and were somewhat related to CNS infections in our study. Myoclonic jerks was more common in EV71 infection and were considered an early predictor of CNS involvement, particularly in the brainstem [19]. However, no such difference was observed in our study, partly because brainstem involvement is a rare complication of EV71 infections. Seizure was common in CA16 infections, which were generally seen in children younger than 2 years, and was more likely a consequence of febrile convulsions than of CNS involvement [20]. The incidence of CNS complications

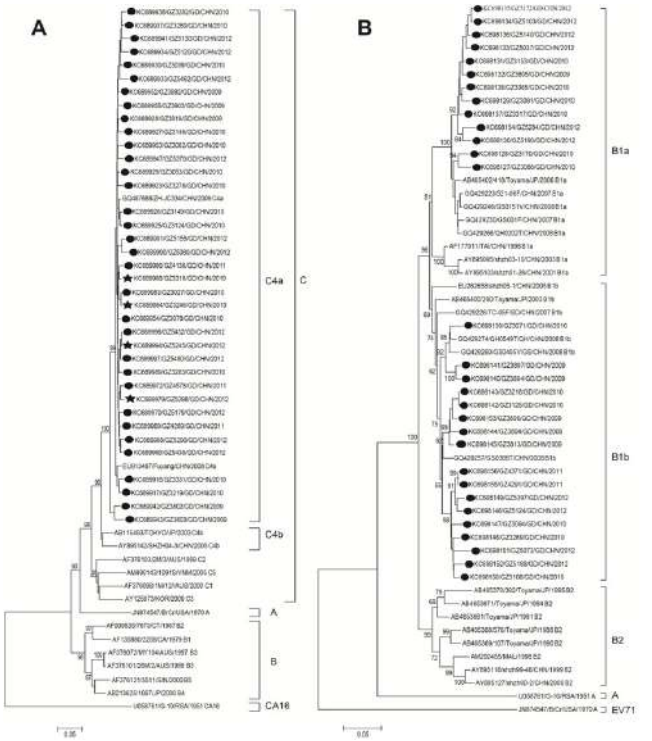

Figure 3: Phylogenetic tree based on a complete VP1 region sequence for 35 EV71.

(A) 30 CA16 (B) representative strains. Bootstrap values for 1,000 pseudo replicates of major lineages are shown as percentages. Markers denote relative phylogenetic distances.

●: strains from mild cases,

✶: strains from severe and fatal cases; other reference strains.

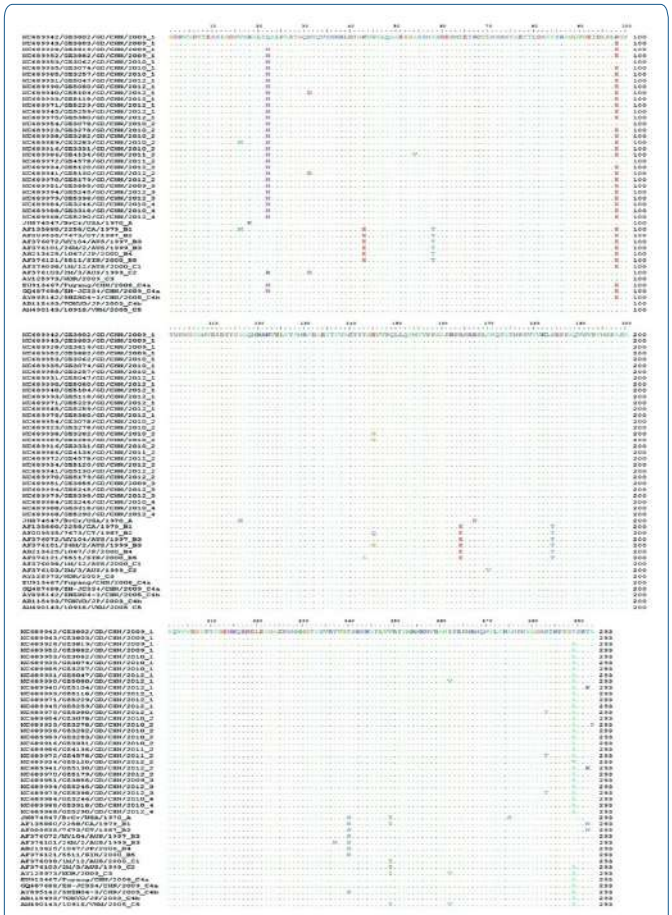

Figure 4: VP1 amino acid sequence alignments showing partially representative EV71 isolates from mild and severe CNS-complicated cases during 2009–2012. Characters mark amino acid substitutions. The last number of each strain code indicates the disease group. Genogroups A, B1–5, and C1–5 were downloaded from Gen Bank.

in this study was particularly high (63.2%) compared to that seen in outbreaks in Sarawak (10-30%) and Taiwan (32%) [10,21,22]. One possible reason for this difference is that these were cases in this study were transferred from primary hospitals all over Guangdong province to Zhujiang Hospital, which serves as a statutorily HFMD-designated hospital.

Several clinical features were correlated with the outcome of EV71-infections by statistical analysis. Four laboratory findings were found to be independent risk factors; however, these risk factors, such as the chloride-ion concentration and LDH activity in the CSF, require further evaluation because the odds ratios were approximately equal to 1.0.EV71 encephalitis, which typically manifests as brain-stem encephalitis with pulmonary edema, is the hallmark of EV71 CNS infection. Infected children develop acute and rapidly progressing cardio respiratory failure, presenting as shock and pulmonary edema or hemorrhages and death within 24 hours [6]. The elevated levels of MYO and cTnI, which were identified via cardiopulmonary dysfunction in stage 4, presented too late to be useful predictors of neurogenic pulmonary edema/hemorrhage. Necropsy indicated no evidence of EV71infection in the myocardium [23]. EV71infections with brain-stem involvement caused increased catecholamines, blood pressure, and tachycardia [24]. Patients with moderately increased cTnI levels have shown neither myocardial inflammation nor necrosis on pathological examinations [23]. Myocarditis has been associated with CA16 [25]. Peak activity of CKMB is slightly more elevated in CA16 than in EV71 infection (P=0.02) in mild HFMD cases (group 1), indicating injury to them yocardium caused by CA16 (data not shown). CKMB activity in blood was considered as the result of the myocardial necrosis. It was not confirmed the elevated levels of MYO and cTnI were the direct or indirect damage of cardio myocyte caused by virus infection. The effects of CA16 on myocardial cells should be further investigated.

Age is a critical factor in the development of HFMD. Our results are comparable with those of an eight-year study in Taiwan [26]. In our study, 74.1% cases occurred in children aged ≤ 4 years, versus 93% in the previous study. The peak incidence of all HFMD cases occurred at 1 year of age, as shown by a study in other regions [22]. Most of the children who died of EV71 infection were one year old, while in the Taiwan study, children younger than 1 year had the highest mortality rate [26]. The observed trend of infection and severe complications in children less than four years of age may be partly due to developmental changes in humoral immunity.

Lower pre-epidemic EV71-seroprevalence rates were associated with higher mortality rates and more severe disease during the EV71 epidemic in 1998 [27]. The three lowest EV71-seroprevalence rates occurred in children 6 months to 3 years of age, who also experienced the highest rates (86%) of fatal and severe (69%) cases. In contrast, high neutralizing-antibody titers specific for EV71 and CA16 were found among 10-to-14-year-old children [4,28]; however, broader factors such as immune states, host-pathogen relationships, increased probability of human-human transmission, and hygiene should also be considered, particularly in severe cases.

Molecular epidemiology of EV71and CA16 from serial samples within a 4-year period were preformed to find the relationship between genotypes and pathogenic properties, particularly among EV71 isolates from HFMD patients with severe CNS involvement. Geno group replacements documented in the Asia-Pacific region showed greater genetic diversity [22,29]. However, in mainland China, few subgeno groups was detected excepting for C4 genotype, which reportedly originated in China and differed from that found in Taiwan [30,31]. Our results consisted to the previous reports that EV71 circulating in Guangdong during 2009–2012 was the C4 genotype of mainland China, suggesting that viruses from adjacent regions had little impact on those in mainland China, even though co-circulation, mutation, and recombination of *enterovirus* populations are distinct mechanisms in *enterovirus* evolution. The relationship between EV71-VP1 amino acid sequences and the incidence of severe CNS complications remains controversial. High identity of VP1 sequences was found from varied cases (groups 1-4). In a previous study, amino acid position 145 of the structural protein VP1 was related to receptor specificity and virulence, and an E–Q substitution was observed in more than one virulent strain [32-34]. Most strains at this position were glutamic acid (E) instead of glycine (G) in two strains from mild cases (group 2) in 2010, suggesting the residue not directly related to disease severity. Severity of EV71 infections was reported not associated with the sequence variation in VP1s or VP4sin some studies [35]. Other positions were reported associated with EV71 virulent phenotypes, such as GlyP710/GlnP710/ArgP710 and GluP729 on the DE and EF loops of the 5'UTR of VP1s 2A and 3D [32,36-38]. More samples are required for whole genome sequencing and blasting for further investigation.

Retrospective studies like that presented here are limited by the systemic examination of clinical features, particularly laboratory tests, which may not be completed for each patient enrolled in the study. Data for most cases were based on the information from primary-care physicians throughout Guangdong and thus may not be comparable due to differences in procedure. Meanwhile, clinical features may not be sufficient for complete understanding of the reasons underlying severe development of HFMD. Moreover, transmission-electron microscopy [39], autopsy [23], and high-resolution virus structures from isolates [40-43] in different phases of the disease may provide further information on predictors of severe HFMD.

Conclusion

A comparative study of the clinical characteristics of EV71- and CA16-associated diseases in south China between 2009 and 2012 was conducted. Clinical profiles and independent risk factors were identified. This retrospective study provides valuable information to primary-care doctors, permitting rapid intervention for high-risk patients. No difference in EV71-VP1 amino sequences were found between severe and mild cases.

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