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ORIGINAL ARTICLE

Molecular epidemiology of the enteroviruses associated with hand, foot and mouth disease/herpangina in Dongguan, China, 2015

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Abstract Enteroviruses (EVs) are the etiological agents involved in most cases of hand, foot and mouth disease (HFMD) and herpangina (HA). Information on the epidemiology profiles of EVs in China is very limited, as the present surveillance system of China focuses on CAV16 and EV71, and no published data are available in Dongguan. The aim of this study is to determine the prevalence of EVs among patients with HFMD and HA in Dongguan, China, during 2015. A total of 271 clinical stool specimens that were clinically determined to be positive for enteroviruses were genotyped by semi-nested polymerase chain reaction (PCR) for the VP1 genes of EVs. The results showed that a total of 14 enterovirus genotypes were identified among HFMD and HA patients in this study. CVA6 was the most common genotype for HFMD, and CVA2 accounted for the majority of HA cases in this study. Sequence and phylogenetic analysis showed that all of the CVA6 and CVA2 strains identified in our study displayed a close genetic relationship to strains identified in other cities in China. This study also demonstrates that there are associations between particular causative enterovirus genotypes and some clinical symptoms, which may provide useful information for improving case prevention, diagnosis and treatment of HFMD and HA.

Introduction

Hand, foot and mouth disease (HFMD) and herpangina (HA) are common infectious diseases primarily affecting young children [1–3]. They are similarly characterized by lesions on the skin and oral mucosa [4, 5]. HFMD has become one of the most common infectious diseases in Asia, including in China. HFMD is characterized by fever, skin eruptions on the hands and feet, and vesicles in the mouth; severe neurological and systemic syndromes that can be fatal occur in some patients [4]. Herpangina is characterized by the production of multiple oral ulcers mainly affecting the posterior region of the oral cavity [5, 6]. Both diseases are associated with various enteroviruses [7].

Enteroviruses belong to the family *Picornaviridae*, which is very diverse. EVs including coxsackievirus A (CVA) 2, 5, 6, 10, 16; coxsackievirus B (CVB) 1, 2, 5; and enterovirus (EV) 71 are the etiological agents involved in most cases of both HFMD and HA [8–11]. Among them, CVA16 and EV71 cause most of the epidemics worldwide [12–14]. Thus, in the present surveillance system of China, pathogen detection is focused on EV71 and CVA16. However, clinical data from our hospital have shown that the proportion of non-EV71 and non-CVA16 cases has drastically increased during recent years in Dongguan, but information on the other EVs, including their epidemiological profiles, is very limited. Increasing numbers of other EV types have emerged as major pathogens in other

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areas, such as the circulation of coxsackievirus A10 and A3 in Shijiazhuang, China during 2010-2012 [15], the outbreak of coxsackievirus A6 in Taiwan [16], and the emergence of coxsackievirus A12 in Qingdao from 2008 to 2012 [17]. There are no published data available on the incidence of HFMD or HA in Dongguan. In this study, clinical samples from patients with HFMD and HA were investigated in order to identify the circulating EV genotypes in the HFMD and HA outbreaks in Dongguan city, China, during 2015. The clinical features and demographic data were analyzed in order to fully understand the epidemic and to develop appropriate control strategies.

Materials and methods

Sample collection

A total of 307 patients clinically presenting with HFMD or HA were enrolled between January 2015 and December 2015 in Dongguan Children's Hospital, Guangdong Province, located in southern China. Two hundred seventy-one stool samples from these children testing positive for enteroviruses by the clinical laboratory were collected. Information on the patients' demographics, clinical manifestations and laboratory results were recorded. All specimens were stored at −80 °C. This study was approved and conducted in accordance with the protocol of the Institutional Medical and Ethics Committee of Dongguan Children's Hospital. Written informed consent was obtained from the parents or legal guardians of the subjects.

Pretreatment of the samples and extraction of nucleic acids

Stool samples were dissolved in 2 ml of physiological saline (0.9 % NaCl) and vortexed for 10 min, followed by centrifugation at 13,000×g for 2 min at room temperature to remove the solids. The supernatant was collected in a fresh tube and used for RNA extraction. Viral RNA was extracted from 200 µl of stool suspension supernatants using a TakaRa MiniBEST Viral RNA/DNA Extraction Kit Ver 5.0 (TaKaRa, Japan) according to the manufacturer's recommendations. The extracts were eluted with 40 µl of elution buffer.

RT-semi-nested PCR and genotyping

RT-semi-nested PCR was performed on the partial 5′ region of the VP1 capsid protein as described by Nix et al. [18], with minor modifications. The first cycle of amplification was performed using a PrimeScript™ One Step RT-PCR Kit Ver 2 (Takara, Japan), 10 pmol of each reverse transcription primer (AN32, AN33, AN34 and AN35), and PCR primer (222, 224), 12.5 µl of 2× 1-Step Buffer, 1 µl of PrimeScript 1-Step Enzyme Mix, 2.5 of µl template and ddH_2O to 25 µl. The reaction conditions were as follows: incubation at 50 °C for 30 min; denaturation at 94 °C for 2 min; 30 cycles of denaturation at 94 °C for 30 s, annealing at 42 °C for 30 s, and extension at 60 °C for 45 s; 10 additional cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and extension at 68 °C for 45 s; and a final extension at 72 °C for 10 min. One microliter of the RT-PCR products was added to a second PCR for semi-nested amplification. The second PCR contained 40 pmol each of primers AN88 and AN89, 25 µl of Premix Taq (TaKaRa, Japan), and 16 µl of RNase-free water, at a final volume of 50 µl. The reaction conditions were as follows: 95 °C for 8 min; 40 cycles of denaturation at 95 °C for 40 s, annealing at 56 °C for 40 s, and extension at 72 °C for 40 s; and a final extension at 72 °C for 5 min. The reaction products were separated and visualized on 1.0 % agarose gels. Visible PCR products after gel electrophoresis were purified and subjected to nucleotide sequencing by Sangon Biotech Company (Shanghai, China). The sequencing results were used in a BLAST search against the GenBank database.

Statistical analysis

Descriptive statistics were performed, and a database was developed for the storage and analysis of the study data. The data were analyzed using SPSS 22.0. The measurement data were analyzed using the non-parametric rank sum test (Kruskal-Wallis Test). Significant differences between proportions were tested by chi-square test. All reported p-values were two-tailed, and the significance threshold was set at $p < 0.05$.

Phylogenetic analysis

Multiple sequence alignments of CVA6 and CVA2 were constructed using Clustal W (Molecular Evolutionary Genetics Analysis program, version 6.0, MEGA6.0). Phylogenetic trees based on a portion of the VP1 gene sequence were built by the neighbor-joining method implemented in the MEGA program, using the Kimura 2-parameter model for nucleotide substitution. Gaps were treated as a complete deletion. The branch lengths of the dendrogram were determined from the topologies of the trees and were obtained by majority rule consensus among 1000 bootstrap replicates. Bootstrap values greater than 70 % were considered to be statistically significant for grouping. All of the reference strains of CVA6 and CVA2, including two prototype stains, BrCr and G-10, were obtained from the GenBank database (US National Center for Biotechnology Information, NCBI).

Results

Patient demographics and enterovirus genotypes

There were 100 females and 171 males, with a gender ratio of 1.00 (female):1.71 (male). The age of these patients ranged from 2 months to 11 years old (mean age 1.8 years old), with 71.2 % of the patients (193/271) being less than three years old, and 24.0 % (65/271) being three to five years old (Table 1).

Among the 271 patients infected with ?human enteroviruses? (HEVs), 175 patients were clinically diagnosed with HFMD, and the other 96 were HA cases. In total, 244 samples had definitive identified genotypes, and 27 of the samples had unidentified genotypes. A total of 14 genotypes were identified in this study. The most prevalent genotypes were CVA6 (75, 27.7 %), followed by CVA2 (61, 22.5 %), CVA10 (38, 14.0 %), CVA16 (22, 8.1 %), and EV71 (19, 7.0 %). The detailed genotypes of the enteroviruses are shown in Fig. 1, and the monthly distribution of HEV-positive cases is shown in Fig. 2. However, the EV genotypes in the HFMD patients were mainly identified as CVA6 (63, 36 %), CVA10 (21, 12 %) and CVA16 (21, 12 %). The EV genotypes in herpangina patients were mainly identified as CVA2 (45, 46.9 %), CVA10 (17, 17.7 %) and CVA6 (12, 12.5 %) (Table 2). In temporal distribution, the HFMD outbreak peaked in June and October, while the HA outbreak only peaked in May (Fig. 3).

Comparison of the clinical features and laboratory results of patients with different enterovirus infections

Most of the patients showed mild clinical manifestations, except for two severe cases: one severe case of acute myelitis caused by EV71 and another fatal case in which the patient died from severe complication of brain stem encephalitis and acute pulmonary hemorrhage caused by CVA16 infection.

We compared the clinical features and laboratory data of patients infected with five common enteroviruses (CVA6, CVA2, CVA10, CVA16 and EV71) (Table 3). We found that some of the clinical manifestations varied depending on the EV genotype. Among the CVA6-infected patients, 42.7 % (32/75) of the patients demonstrated atypical clinical symptoms of HFMD, such as widespread skin eruption on the trunk, extremities, knees, elbows, neck or face, which was a significant difference from the other groups ($p < 0.05$). This is consistent with a previous study from Taiwan in which atypical cases of HFMD caused by CV-A6 were seen between 2004 and 2009 [16]. Moreover, desquamations of the palms and soles as well as nail abnormalities were also commonly found after the infectious episodes [10, 16]. This manifestation was not observed in our study due to the lack of complete follow-up data. Patients infected with CVA2 and CVA10 had a significantly higher mean fever spikes than the other groups, but no differences were found between these two groups. The EV71- and CVA10-infected patients had a much higher incidence of neurological signs, and CVA2-infected patients were more likely to have digestive signs. An elevated serum C-reactive protein (CRP) level of >40 mg/L was noted in a substantial proportion of the patients in the CVA6, CVA2 and CVA10 infection groups, which was significantly higher than in the EV71- and CVA16-infected patients.

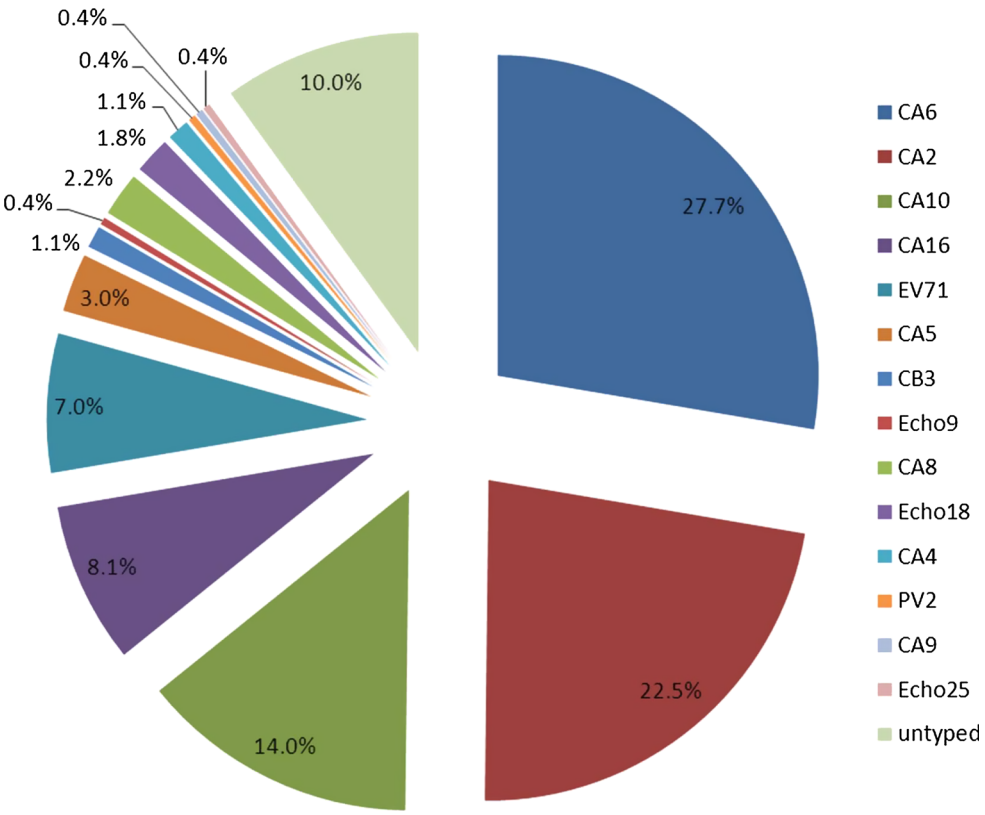
Table 1 Demographic information of patients with HFMD/HA in this study

Characteristic	Number (%)
Age (years)	
Mean age	1.8 ± 1.5
Range from	0.17-11
<1	57 (21.0)
1-3[#]	136 (50.2)
3-5	65 (24.0)
≥5	13 (4.8)
Gender	
Male	171 (63.0)
Female	100 (37.0)

[#] less than 3 years old

Sequence and phylogenetic analysis of enterovirus genotypes

Nucleotide sequence alignment of a portion of the VP1 gene was performed using 31 Dongguan CVA6 strains identified from the HFMD and HA patients in this study. Another 44 strains were excluded from this study for the same sequences. The partial VP1 genes of CVA6 strains in our study showed 92.3 %-99.6 % similarity, and the pairwise distances among them ranged from 0.004-0.077. Phylogenetic analysis showed that the CVA6 sequences were assigned to five groups (A-E) with clear temporal and geographical distribution (Fig 4a). Most of the Chinese CVA6 strains were segregated into groups A and E, and one strain was assigned to group D, which was isolated in Shandong province of China during 1996. Group A comprised earlier CV6 strains from Shandong province in 1992, Guangdong province during 2004-2007, Japan in 1996, and North Africa in 1994. Group E included strains from all over the world, such as France, Japan, Russia, India, Cuba, Finland, Taiwan and some cities of mainland China during 2000-2013. All of the CVA6 strains identified in our study were categorized into group E. In our results, the CVA6 strains displayed a close genetic relationship to



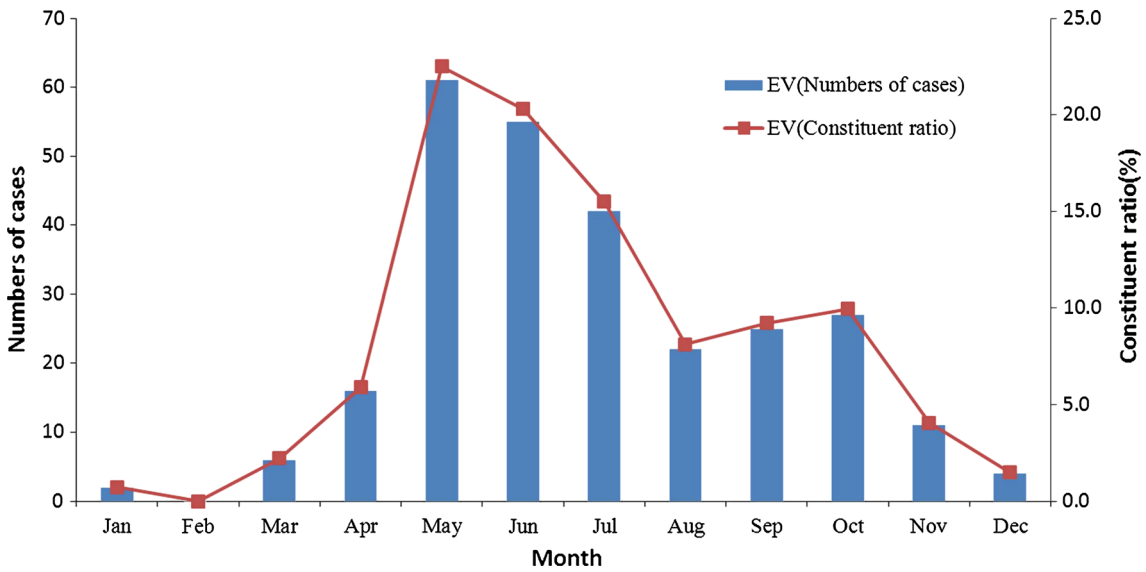


Fig. 1 The EV genotypes identified in HFMD and HA patients from January to December 2015, Dongguan, China. Echo indicates echovirus

Fig. 2 Monthly distribution of HEV-positive cases among 271 HFMD or HA patients

Chinese strains identified in other cities, including Beijing, Tianjin, Shanghai and Taiwan, which were isolated from 2008 to 2013. Moreover, they were also similar to two strains from Japan in 2010 and 2011.

Twenty-six Dongguan CVA2 strains (excluding 35 strains of the same sequences) were aligned with others and the reference strain BrCr. The CVA2 strains in the study displayed 90.5 %-100 % similarity, and the pairwise distance among them ranged from 0.000 to 0.095. According to the positions of the tree branches, CVA2 sequences were classified into five groups (A-E) with clear geographical and temporal distribution (Fig. 5). Most of the Chinese CVA2 strains in this study segregated into group E, and one strain was segregated into group D, which was isolated in Beijing

Table 2 Number of enteroviruses of each genotype identified from patients with HFMD/HA

Pathogen	HFMD (n = 175)		HA (n = 96)		*p*-value
	Number of cases	Percentage (%)	Number of cases	Percentage (%)	
CVA6	63	36.0	12	12.5	<0.05
CVA2	16	9.1	45	46.9	<0.05
CVA10	21	12.0	17	17.7	0.44
CVA16	21	12.0	1	1.0	<0.05
EV71	19	10.9	0	–	–
Others	15	8.6	14	14.6	–
Untypable	20	11.4	7	7.3	–

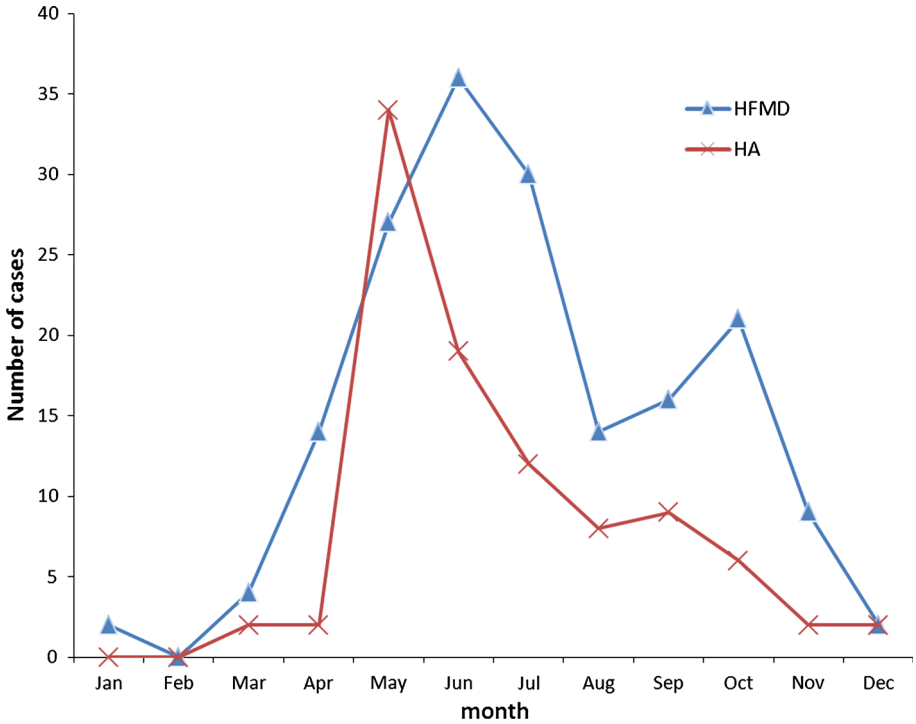


Fig. 3 Temporal distribution of enteroviruses isolated from patients with HFMD and herpangina

2007. In the phylogenetic tree, group A comprised one earlier strain from Japan in 2003; group B comprised one strain from Russia in 2005; groups C and D comprised strains from all over the world, including Russia, Japan, Korea, the Netherlands, Finland, Denmark, Italy, Germany and Taiwan during 2005-2011. However, all 26 strains in this study were assigned to group E, which comprised the strains in the study as well as others from regions in China, including Anyang, Wuhan, Yunnan, Shanghai and Beijing, during 2008-2013. They were also similar to two strains that were isolated in Japan in 2013 and 2014.

Discussion

We investigated the prevalence of enteroviruses among patients with HFMD and HA in Dongguan, an industrial city with a large migrant population in southern China. This is the first analysis of the comprehensive pathogenic enterovirus spectrum associated with HFMD and HA cases in Dongguan city using molecular epidemiological methods.

HFMD has become a major public health concern in most Asian countries, especially in China, because of the multiple large-scale outbreaks in the past few decades. Large outbreaks of EV71-associated HFMD with high morbidity and mortality have occurred in several Asian countries and regions, including Singapore [19], Malaysia [20], Japan [21], Thailand [22] and China [23]. This molecular epidemiological study showed that CVA6 was the most common pathogen causing HFMD, followed by CVA10 and CVA16, which indicated that they were the causative pathogens for HFMD in Dongguan during 2015. One of the most commonly known HFMD-associated EVs, EV71, was detected in a low proportion of the cases. Moreover, two prevalent peaks for HFMD were observed in this study; CVA10 was the main etiologic agent during

Table 3 Comparison of the clinical features and laboratory examination results of patients with different enterovirus infections

	Enterovirus genotype				
	CVA6 (n = 75)	CVA2 (n = 61)	CVA10 (n = 38)	CVA16 (n = 22)	EV71 (n = 19)
Mean age, years	1.5 ± 1.4	2.3 ± 1.9	1.6 ± 1.3	2.1 ± 1.3	2.0 ± 1.6
Male/female ratio	1.7	1.8	1.7	2.1	1.7
Duration of hospitalization, days	4.6 ± 1.2	4.4 ± 1.2	4.5 ± 1.1	5 ± 2.1	5.3 ± 2.7
Fever (axillary temperature >37.3 °C)	71 (94.7 %)	61 (100 %)	37 (97.3 %)	19 (86.4 %)	17 (89.5 %)
Fever spike, °C	39.1 ± 0.7	39.5 ± 0.7*	39.2 ± 0.5*	38.8 ± 0.7	38.7 ± 0.4
Duration of fever, days	2.4 ± 2.1	2.7 ± 1.6	2.3 ± 1	2.5 ± 1.7	3.8 ± 1.5
skin rashes beyond the typical sites	32 (42.7 %)*	4 (6.6 %)	5 (13.2 %)	1 (4.5 %)	1 (5.3 %)
Respiratory signs	21 (28 %)	17 (27.9 %)	12 (31.6 %)	9 (40.9 %)	9 (47.4 %)
Neurological signs	8 (10.7 %)	9 (14.8 %)	9 (23.7 %)*	2 (9.1 %)	7 (36.8 %)*
Digestive signs	8 (10.7 %)	17 (27.9 %)*	6 (15.8 %)	4 (18.2 %)	3 (15.8 %)
Random blood glucose (mmol/L)	5.4 ± 1.4	5.6 ± 1.1	5.4 ± 1.5	6.2 ± 2.8	5.9 ± 1.3
WBC ($\times 10^9$/L)	13.4 ± 5.6	13.5 ± 6.2	12.7 ± 5.5	10.6 ± 4.3	11.5 ± 4.7
WBC >12 × 10^9/L	43 (57.3 %)	32 (52.5 %)	19 (50 %)	6 (27.3 %)	10 (52.6 %)
Neutrophils ($\times 10^9$/L)	7.8 ± 4.9	8.8 ± 5	7.8 ± 5	6 ± 4.2	6 ± 1.9
Neutrophils >10×10^9/L	19 (25.3 %)	24 (39.3 %)	15 (39.5 %)	3 (13.6 %)	0
Neutrophils <1.5×10^9/L	6 (8 %)	4 (6.6 %)	4 (10.5 %)	0	0
Lymphocyte ($\times 10^9$/L)	4 ± 1.9	3.2 ± 2.2	3.5 ± 1.9	3.4 ± 1.5	5.8 ± 3.7
PLT ($\times 10^9$/L)	327.9 ± 109	287.7 ± 101.5	284.5 ± 94.9	292.5 ± 90	346.4 ± 121.9
PLT (>400×10^9/L)	13 (17.3 %)	7 (11.5 %)	2 (5.3 %)	3 (13.6 %)	3 (15.8 %)
CRP (mg/L)	16.1 ± 19.5*	24.7 ± 26*	16.1 ± 16.3*	5.2 ± 5.8	4 ± 4.6
CRP (>40 mg/L)	9 (12 %)	11 (18 %)	3 (7.9 %)	0	0
ALT (U/L)	22.9 ± 8	23.4 ± 9.3	29.5 ± 26.4	20.6 ± 7.2	30.6 ± 25.4
ALT (>40 U/L)	2 (2.7 %)	2 (3.3 %)	3 (7.9 %)	0	2 (10.5 %)
AST (U/L)	41.6 ± 8.5	36.8 ± 7.7	40.3 ± 10.8	37.5 ± 7.9	39.8 ± 9.7
AST (>40 U/L)	37 (49.3 %)	17 (27.9 %)	15 (39.5 %)	7 (31.9 %)	9 (47.4 %)
CK-MB (U/L)	32.1 ± 10.4	25.7 ± 9.4	31.7 ± 11.2	27.3 ± 8.2	33.2 ± 13.7
CK-MB (>40 U/L)	12 (16 %)	5 (8.2 %)	5 (13.2 %)	1 (4.5 %)	3 (15.8 %)
Number of fatal cases	0	0	0	1 (4.5 %)	0
Number of severe cases	0	0	0	0	1 (5.3 %)

Respiratory signs included cough, rhinorrhea and sore throat. Neurological signs included seizures, headache and startle response; Digestive signs included abdominal pain, diarrhea and vomiting

* Compared with the other enterovirus infection group, $P < 0.05$

the first peak in spring/summer, while CVA6 accounted for the majority of the cases during the second peak in autumn/early winter. A previous study from Taiwan indicated that CV6 and CVA10 were major pathogens causing HA, whereas CVA16 and EV71 were involved in HFMD cases [24]. However, our results revealed that CVA6 was more relevant to HFMD, and no significant difference between HFMD and HA was observed for CVA10. The epidemiological features of CVA10 and CVA6 as the major pathogens in Dongguan during 2015 could be very helpful in alerting the relevant control agencies for predicting epidemics in the future.

With regards to herpangina, few epidemiology data about HA are available, especially because the epidemiological study of HA in China is limited. Our study showed that CVA2 was the most prevalent genotype associated with HA patients, followed by CVA10 and CVA6, which was consistent with a previous study indicating that CVA2 was related to HA in Taiwan in 2008 [25]. However, Park et al. showed that CVA5 was the prevalent agent causing HA in Korea during 2009 [26]. Few relative epidemiology data are available about CVA2 infections, possibly because no large-scale outbreak has occurred. However, CVA2 was the second most frequently detected genotype in this study, indicating that it might be an emerging threat. This result suggests that the epidemiological surveillance of HA and changes in its etiological agent are worthy of further attention. The most prevalent period of HFMD and HA was observed in June/October and May, respectively. The temporal pattern of HFMD in Dongguan was similar to that

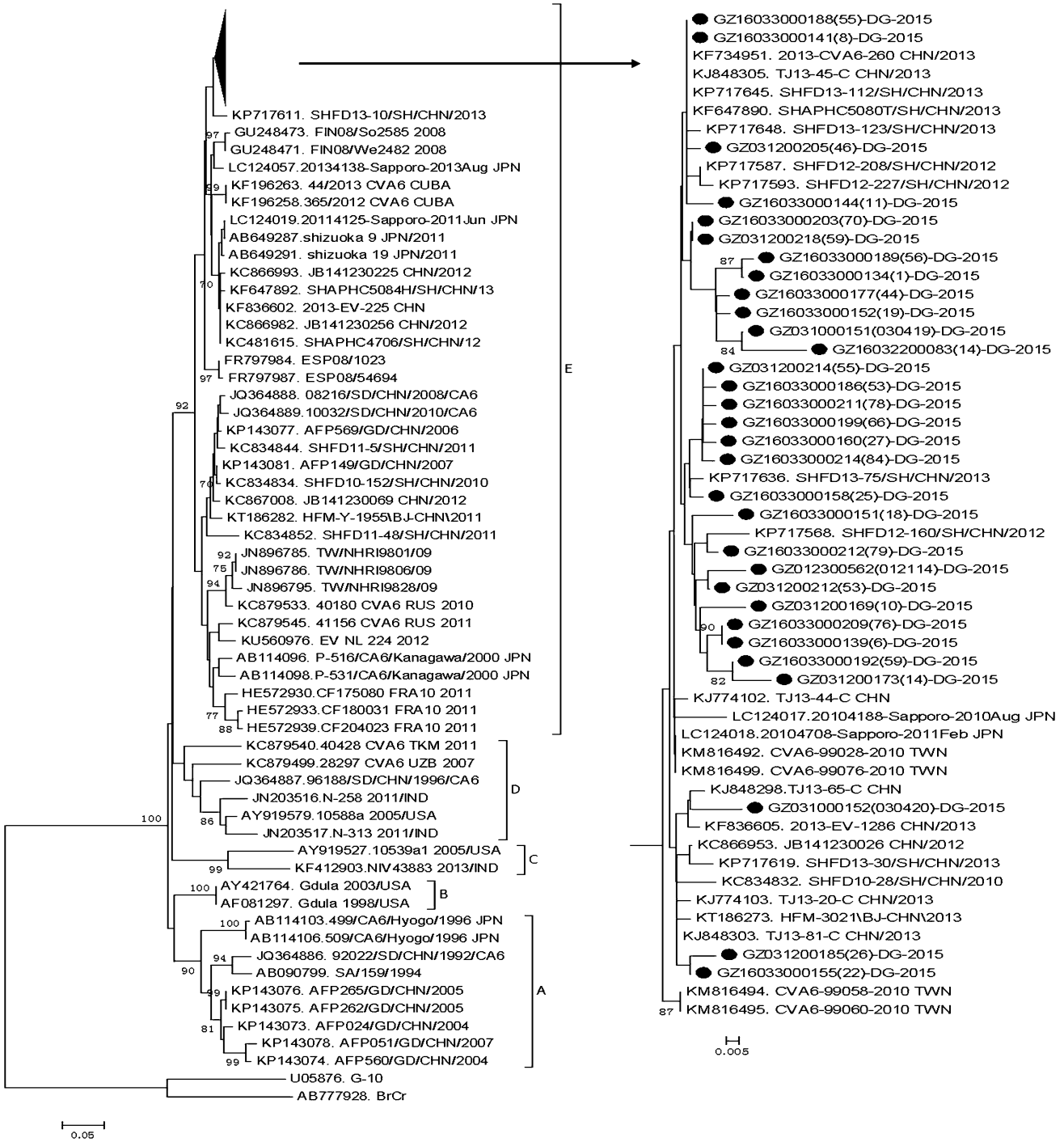


Fig. 4 Phylogenetic analysis by the neighbor-joining method with a 1000-bootstrap re-sampling based on the alignment of a partial VP1 gene sequence of 31Dongguan CVA6 strains. The strains indicated by ● were obtained in our study

in neighboring cities [27], but the pattern of HA was different from those observed in other studies, such as those in Korea in 2009 [26] and in Thailand in 2012 [6].

All CVA6 and CVA2 strains in Dongguan were closely related to other strains isolated from other cities in China and neighboring counties, such as Japan, which indicated that geographic factors may contribute to the prevalence of some pathogens. However, the strains in our study also showed distant genetic relationships to some strains isolated in China, such as the relationship between CVA6 strains in our study and a CVA6 strain isolated in Shandong in 1996, as well as between CVA2 strains in our study and a CVA2 strain in Beijing in 2007. Overall, analysis of geographical locations and temporal distributions would

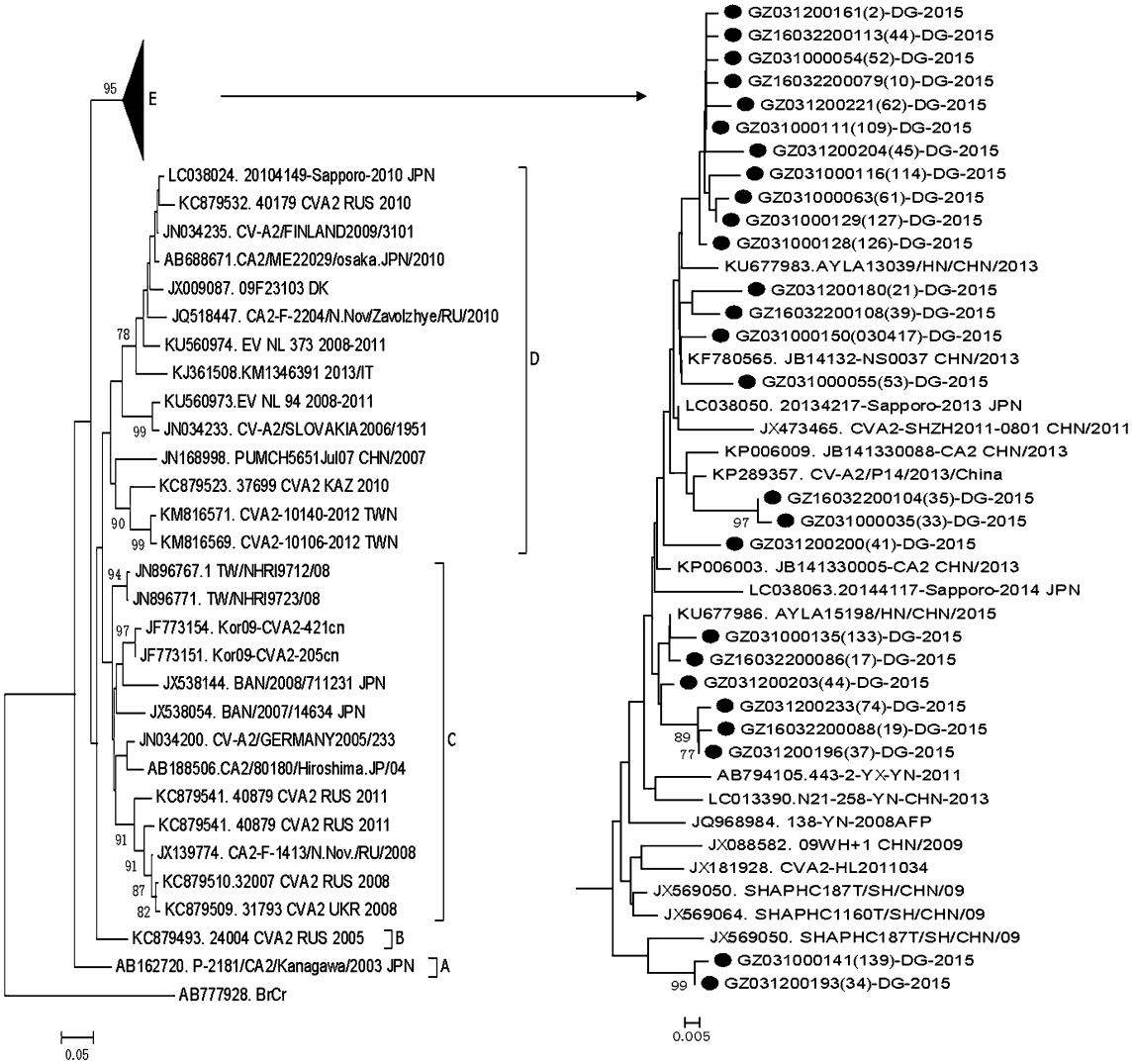


Fig. 5 Phylogenetic analysis by the neighbor-joining method with a 1000-bootstrap re-sampling based on the alignment of a partial VP1 gene sequence of 26 Dongguan CVA2 strains. The strains indicated by ● were obtained in our study

help to improve our understanding of the evolutionary relationships of these strains. Moreover, we found that both the CVA6 and CVA2 strains causing HFMD or HA were mixed throughout the phylogenetic trees, which indicates that differences in the VP1 gene might not account for different diseases. A previous report revealed that no notable difference existed in partial VP1 gene sequences between HFMD and HA [26]. Further studies are warranted to provide more insight into the pattern of HFMD epidemics.

Conclusion

Our study showed that CVA6 and CVA10 were the predominant HFMD pathogens, and CVA2 accounted for the majority of HA cases. This study provides strong evidence that the profile of prevalent EV strains is changing, and multiple non-EV71 and non-CVA16 enteroviruses are increasingly contributing to epidemics of HFMD and HA. Thus, our data suggest that health management organizations should pay attention to the necessity of comprehensive surveillance of more enterovirus genotypes causing HFMD in Dongguan and other parts of China in the near future, which will benefit vaccine development and clinical management.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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