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Original Article

Dynamic Constitution of the Pathogens Inducing Encephalitis in Hand, Foot and Mouth Disease in Kunming, 2009–2011

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SUMMARY: Hand, foot and mouth disease (HFMD), caused by various viral pathogens, is an emerging infectious disease in children in Asia. Understanding the composition of these pathogens is necessary to prevent and control this disease. In the present study, the pathogens in 436 HFMD patients (from 2009 to 2011) with concurrent clinical indications of encephalitis, meningoencephalitis, or both, were defined using the semi-nested PCR. A systematic analysis of the composition of these pathogens was performed. Various enteroviruses that are capable of inducing central nervous system (CNS) damage in HFMD patients were identified, including enterovirus 71, coxsackievirus A16, and Echovirus 9. Most of these pathogens were found co-infecting the patients. The composition of the pathogens that induced CNS damage in the HFMD patients was dynamically modulated in the cases.

INTRODUCTION

In recent years, hand, foot and mouth disease (HFMD) has emerged as one of the major epidemic communicable diseases in children in Asian-Pacific countries (1–5). It manifests as vesicles on the hands, feet, and mouth; herpangina and other cold-like symptoms, and it is occasionally associated with severe encephalitis and cerebromeningitis, which pose a major threat to patients (6–9). Neurological injuries frequently lead to subsequent neurogenic pulmonary edema and abnormal lung function in HFMD patients (10–12). Pathogenic studies have demonstrated that HFMD is usually caused by infections with enteroviruses such as enterovirus 71 (EV-A71) and coxsakievirus A16 (CV-A16), which constitute 60%–80% of the causative agents of HFMD (13–15). Viruses such as coxsakievirus A9 (CV-A9), coxsakievirus B4 (CV-B4), and other echoviruses have been reported as other potential causative agents of HFMD (16–19). Although EV-A71 has frequently been identified as the major pathogen that causes HFMD-related deaths (20,21), to the best of our knowledge, there has been no systematic analysis undertaken of the pathogens responsible for neurological injuries in HFMD patients.

Hence, this study describes a pathogenic analysis of HFMD patients treated at Kunming Municipal Children's Hospital, Kunming, Yunnan, China during 2009–2011. The results showed a clear dynamic association between the different pathogens responsible for the neurological injuries in HFMD patients and the prevalence of their circulation in children and an increase in the echovirus 9 (E-9) infection.

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MATERIALS AND METHODS

Patients: Using the diagnostic standard for HFMD in children, published by the Ministry of Health (MOH) (22), a clinical diagnosis of HFMD was confirmed in 3,600 patients of an average age of 2.6 years, during the period from March 2009 to October 2011 in Kunming Municipal Children's Hospital. Thereafter, on the basis of clinical manifestations and examination of the cerebrolspinal fluid (CSF), 436 patients were further confirmed to have developed encephalitis and cerebromeningitis (Table 1). Throat swabs and stool specimens were collected from these patients for subsequent pathogenic examinations.

Ethical standards: The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008.

Molecular diagnosis of collected specimens: To identify the enteroviral genome, a semi-nested-PCR (S-PCR) was performed on stool specimens from the patients. Briefly, 1 g of stool specimen was suspended in 5 mL of phosphate buffered saline (PBS), and the suspensions were centrifuged at 4°C, 3,000 × g for 30 min. The supernatant was transferred into a new tube and stored at −80°C. Viral RNAs were extracted using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) to perform the S-PCR. cDNA was prepared using the PrimeScript One-Step RT-PCR Kit ver.2 (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. Semi-nested primers were used for the detection of enterovirus species A, B, and C (Table 2), based on the standard protocol (23). The 25-μL reaction system was composed of 1 μL of RT-PCR mix,

Table 1. The clinical characteristics of 436 HFMD patients with severe symptom during 2009–2011, Kunming, China

Characteristic	Value			*P* value
	2009 *n* = 94	2010 *n* = 305	2011 *n* = 37	
Age (yr); mean ± SD	2.59 ± 1.31	2.61 ± 1.59	2.66 ± 1.47	0.96
Sex; male/femele (%)	63/31 (67.02)	183/122 (60.00)	21/16 (56.75)	0.36
Clinical category; *n* (%)				
HFMD	50 (53.19)	131 (42.95)	12 (32.43)	0.07
Herpangina	70 (74.47)	173 (56.72)	25 (67.57)	0.01
Meningoencephalitis	44 (46.81)	191 (62.62)	9 (24.32)	<0.01
Presenting symptom; *n* (%)				
Fever (>38°C)	62 (65.96)	238 (78.03)	30 (81.08)	0.05
Headache	45 (47.87)	201 (65.90)	10 (27.03)	<0.01
Vomiting	32 (34.04)	76 (24.92)	4 (10.81)	0.02
Neck stiffness	5 (5.32)	15 (4.92)	5 (13.51)	0.10
Sore throat	7 (7.45)	30 (9.84)	10 (27.03)	<0.01
Abdominal pain	9 (9.57)	7 (2.30)	2 (5.41)	0.01
Diarrhea	3 (3.19)	15 (4.92)	3 (8.11)	0.53
Decreased mental status	35 (37.23)	130 (42.62)	7 (18.92)	0.02
Hand and foot ulcer	93 (98.94)	270 (88.52)	30 (81.08)	<0.01
Oral ulcer	80 (85.11)	165 (54.10)	21 (56.76)	<0.01

Table 2. Characterizations of primers used for semi-nested-PCR amplification

Name	Nucleotide sequence[1] (5′→3′)	Position[2]	Product size (bp)
Species A 1st round			
EntAF	TNCARGCWGCNGARACNGG	2571–2589	387
EntAR0	ANGGRTTNGTNGMWGTYTGCCA	2957–2936	
2nd round semi-nested			
EntARi	GGNGGNACRWACATRTAYTG	2898–2879	328
Species B 1st round			
EntBF	GCNGYNGARACNGGNCACAC	2610–2629	397
EntBR0	CTNGGRTTNGTNGANGWYTGCC	3006–2985	
2nd round semi-nested			
EntBRi	CCNCCNGGBGGNAYRTACAT	2970–2951	361
Species C 1st round			
EntCF	TNACNGCNGTNGANACHGG	2612–2630	395
EntCR0	TGCCANGTRTANTCRTCCC	3006–2988	
2nd round semi-nested			
EntCRi	GCNCCWGGDGGNAYRTACAT	2972–2953	361

[1]: D: A, G or T; M: A or C; N: A, C, T, or G; R: A or G; W: A or T; Y: C or T.
[2]: Nucleotde position number.

12.5 μL of 2× Reaction Buffer, 20 pmol of F and R0 primers, and 8 μL of RNA. The amplification conditions were as follows: reverse-transcription at 50°C for 40 min and pre-denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for 1 min. Subsequently, the second-round PCR setup was performed with the former PCR products and the F and Ri primers, as described above. The sequence analyses were performed using an ABI 3730XL automatic sequencer (Applied Biosystems, Foster City, CA, USA) by Sangon (Shanghai, China).

Identification of virus genome: For EV-A71 and CV-A16 genotyping, nested PCRs were performed using the RT-PCR products with EntAF and EntAR0 primers and the VP1 gene-specific primers for EV-A71 (EV71AF [5′-TCCAAGCTGCTGAAATTGG-3′, 2580–2598] and EV71ARi [5′-GGTGGCACAAACATATA TTG-3′, 2913–2894]) and for CV-A16 (CA16AF [5′-TACAAGCCGCGGAGACAGG-3′, 2580–2598] and CA16ARi [5′-GGTGGGACATACATGTACTG-3′, 2913–2894]). For E-9 genotyping, nested PCR was performed using the RT-PCR products with EntBF and EntBR0 primers and the specific primers for E-9 (Ech9F [5′-ACTGGRCACACCTCACAAGT-3′, 2555–2574] and Ech9Ri [5′-CCGCCAGGTGGTATGTACAT-3′, 2901–2882]). The PCR conditions were the same as described above.

Phylogenetic analysis: The sequences were determined by using BLAST <http://www.ncbi.nlm.nih.gov/Blast> and uploaded to the NCBI database (GenBank accession numbers: E-9, KF142191–KF142253; EV-A71, KF142254–KF142298; and CV-A16, KF142299–KF142379). Phylogenetic trees were constructed using the Kimura two-parameter algorithm and the neighbor-joining method, using the MEGA 5.0

software.

Statistical analysis: The mean values of the variables and the proportions of the categorical variables were compared by using a one-way ANOVA test and χ^2 tests, as appropriate. Two-tailed P values of less than 0.05 were considered significant. The data analysis was performed using the SPSS (Statistical Package for the Social Sciences) 12.0 software (Chicago, IL, USA).

RESULTS

Of the 436 HFMD patients with confirmed encephalitis and cerebromeningitis, 367 patients showed the presence of different individual enterovirus pathogens directly through repeated pathogenic examinations and verifications of the collected throat swabs and stool specimens. There were no enterovirus pathogens in the remaining 69 specimens (Table 3). Among the 367 molecularly confirmed enterovirus-positive specimens, EV-A71, CV-A16, and E-9 were identified as the sole pathogen in 45 (12.26%), 81 (22.07%), and 63 (17.17%) patients, respectively. Other enteroviruses, including CV-A1, CV-A8, CV-A9, CV-A10, CV-B2, CV-B4, E-1, and E-3 were also identified (Table 3).

It should be noted that the proportion of specimens infected with at least two different types of viruses was as high as 43.32% (159 specimens), and the proportion of specimens co-infected with EV-A71–CV-A16, EV-A71–CV-A16–E-9, CV-A16–other viruses, and E-9–other viruses was 12.26% (45 specimens), 3.27% (12 specimens), 4.36% (16 specimens), and 25.07% (92 specimens), respectively (Table 3). All these results are sufficient to suggest that EV-A71, CV-A16, and E-9 account for the vast majority of the cases of encephalitis and cerebromeningitis in HFMD patients.

Regardless of the distinct differences in individual pathogens each year, either single or mixed infections were typically found. In the year 2009, the majority of single infections were caused by EV-A71 (28.74%) or CV-A16 (36.78%) (Table 3); furthermore, the prevalence of co-infections with EV-A71–CV-A16, or E-9–other viruses or infection with the sole E-9 virus was 12.64%, 12.64%, and 4.60%, respectively. In the year 2010, the prevalence of single infections with CV-A16 was 19.60%, with E-9 viruses was 23.20%, and with EV-A71 was 4.00%. However, the prevalence rates of co-infections with EV-A71–CV-A16, EV-A71–CV-A16–E-9, and E-9-other viruses were at 12.40%, 4.00%, and 29.60%, respectively (Table 3). In the year 2011, the prevalence rates of single infections with CV-A16 or E-9 were reduced to 0% and 3.33%, respectively, with a virtual decline in total HFMD cases. In contrast, there was an increase of 33.33% in the prevalence of single infections with EV-A71 and increases of 10.00% and 23.33% in the prevalence rates of co-infections with EV-A71–CV-A16 and E-9–other viruses, respectively (Table 3).

On the basis of the above-described pathogens isolated in our study, we performed VP1 sequence analysis on the isolated EV-A71, CV-A16, and E-9 viruses. Twenty-five of the 45 EV-A71 strains isolated from the HFMD patients with encephalitis and cerebromeningitis complications in Kunming Municipal Children's Hospital were not co-infected with other enterovriuses, These 25 strains were identified to belong to genotype C4 of EV-A71 (Fig. 1). In light of the diverse distribution of genetic distances, they were close to the isolates from the Chinese mainland in recent years and exhibited a tendency toward clustering with a uniform distribution (Fig. 1). Twenty-seven of the 81 CV-A16 strains

Table 3. The pathogens identified in HFMD patients with severe symptom during 2009 to 2011 in Kunming, China

Serotype[1)]	Number of cases (%)			
	2009 n = 94	2010 n = 305	2011 n = 37	Total n = 436
Enterovirus single infection, n (%)[2)]	63 (72.41)	132 (52.80)	13 (43.33)	208 (56.67)
CV-A1	0	0	1 (3.33)	1 (0.27)
CV-A8	0	2 (0.80)	0	2 (0.54)
CV-A9	1 (1.15)	5 (2.00)	0	6 (1.63)
CV-A10	0	2 (0.80)	0	2 (0.54)
CV-A16	32 (36.78)	49 (19.60)	0	81 (22.07)
CV-B2	0	2 (0.80)	1 (3.33)	3 (0.82)
CV-B4	0	3 (1.20)	0	3 (0.82)
E-1	0	1 (0.40)	0	1 (0.27)
E-3	1 (1.15)	0	0	1 (0.27)
E-9	4 (4.60)	58 (23.20)	1 (3.33)	63 (17.17)
EV-A71	25 (28.74)	10 (4.00)	10 (33.33)	45 (12.26)
Enterovirus co-infection, n (%)[2)]	24 (27.59)	118 (47.20)	17 (56.67)	159 (43.32)
EV-A71–CV-A16	11 (12.64)	31 (12.40)	3 (10.00)	45 (12.26)
EV-A71–CV-A16–E-9	2 (2.30)	10 (4.00)	0	12 (3.27)
CV-A16–other virus	2 (2.30)	12 (4.80)	2 (6.67)	16 (4.36)
E-9–other virus	11 (12.64)	74 (29.60)	7 (23.33)	92 (25.07)
Total infection, n (%)[3)]	87 (92.55)	250 (81.97)	30 (81.08)	367 (84.17)
Non-enterovirus, n (%)	7 (7.45)	55 (18.03)	7 (18.92)	69 (15.83)

[1)]: CV-A, coxsackievirus A; CV-B, coxsackievirus B; E, echovirus; EV, enterovirus.
[2)]: The number of patients (percentage) with each pathogens among total enterovirus infected cases.
[3)]: The number of patients (percentage) with enterovirus among total HFMD cases.

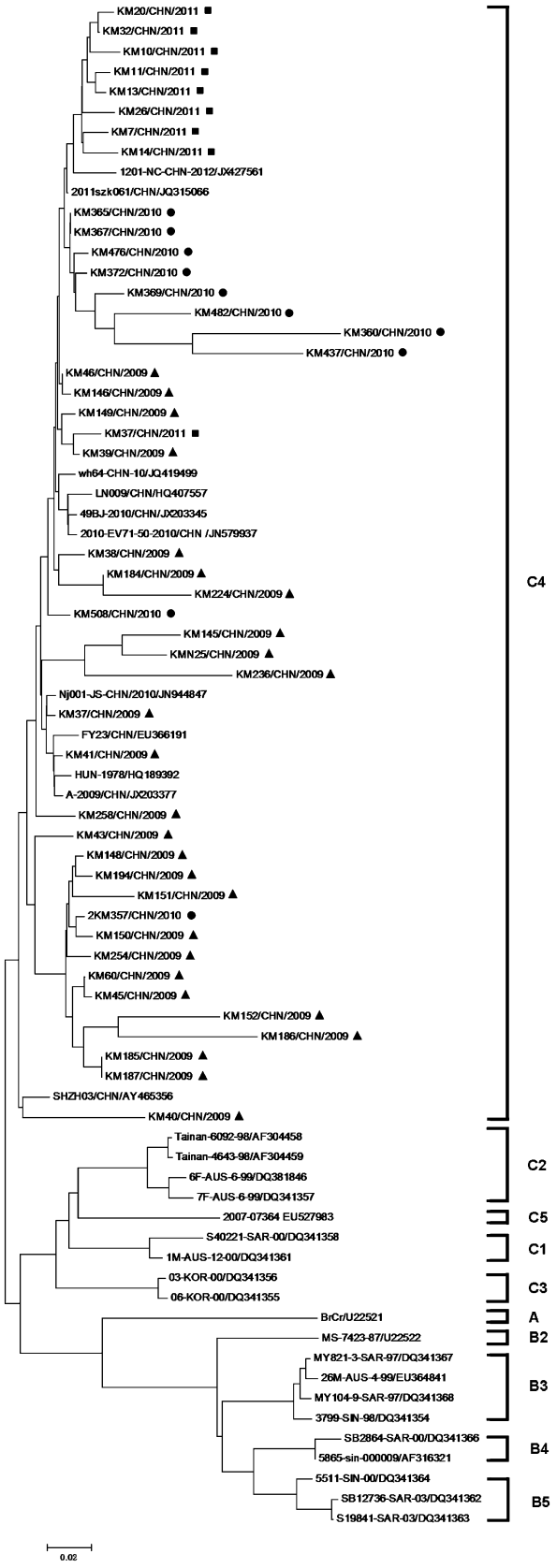

Fig. 1. Phylogenetic tree based on the VP1 gene sequence of EV-A71.
The nucleotide sequences retrieved from GenBank and investigated in this study (marked by a black triangle [▲, 2009], circle [●, 2010], and rectangle [■, 2011]). A, B (B2, B3, B4, and B5), and C (C1, C2, C3, C4, and C5) are the genotypes of EV71. The strains in this study are identified by abbreviations based on the following: name of city (KM, Kunming), sample number, name of country (CHN, China), and year of illness. The scale bar denotes the number of nucleotide substitutions per site.

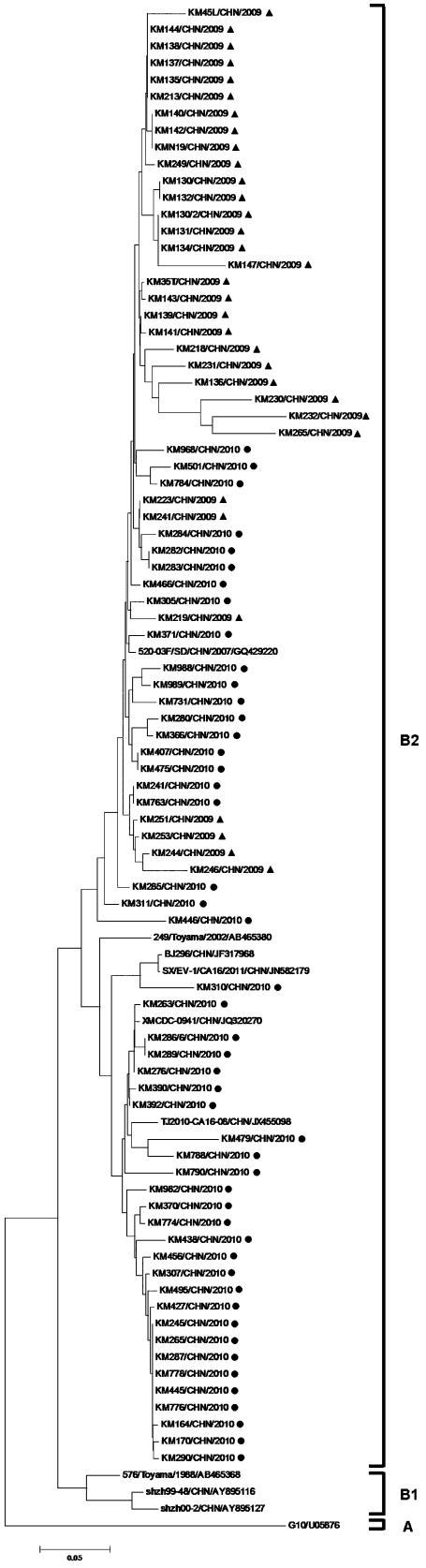

Fig. 2. Phylogenetic tree based on the VP1 gene sequence of CV-A16.
The nucleotide sequences retrieved from GenBank and investigated in this study (marked by a black triangle [▲, 2009] and circle [●, 2010]). A and B (B1 and B2) are the genotypes of CV-A16. The strains in this study are indicated by abbreviations based on the following: name of city (KM, Kunming), sample number, name of country (CHN, China), and year of illness. The scale bar denotes the number of nucleotide substitutions per site.

Fig. 3. Phylogenetic tree based on the VP1 gene sequence of E-9.
The nucleotide sequences retrieved from GenBank and investigated in this study (marked by a black triangle [▲, 2009], circle [●, 2010], and rectangle [■, 2011]). The strains in this study are indicated by abbreviations based on the following: name of city (KM, Kunming), sample number, name of country (CHN, China), and year of illness. The scale bar denotes the number of nucleotide substitutions per site.

were identified to belong to genotype B2 (Fig. 2). It is likely that these 27 strains were not co-infected with other enteroviruses, and they exhibited a tendency toward clustering with a uniform distribution (Fig. 2). Thirty of the 63 E-9 strains were not co-infected with other enteroviruses and had genetic distances similar to the clusters of isolates from the Chinese mainland in recent years (Fig. 3); they exhibited a tendency toward clustering with a uniform distribution as well.

DISCUSSION

HFMD has become an important communicable disease caused by enteroviruses in children in recent years, and related studies have proposed a variety of presumptions about the disease pathogenesis (15,16,24). In the present study, based on molecular analyses of the pathogenesis in HFMD patients with encephalitis and cerebromeningitis in Kunming Municipal Children's Hospital, the dynamic constitution of the pathogens responsible for HFMD was revealed. As the only hospital for children in the city, Kunming Municipal Children's Hospital serves approximately 2 million children residing in the downtown and suburban districts, with an outpatient capacity of over 3,000 children. On the basis of the diagnostic standards for HFMD in children published by the MOH, approximately 3,600 child HFMD patients were diagnosed and treated between 2009 and 2011. Of these, 367 enterovirus-positive patients were identified with clinical symptoms of encephalitis and cerebromeningitis and a variety of CSF characterizations. Pathogenic examination of the specimens collected from these patients indicated that the enteroviruses inducing neurological injuries in HFMD patients were primarily EV-A71, CV-A16, and E-9. Of these viruses, a single infection of either EV-A71 or CV-A16 and co-infections of EV-A71–CV-A16 were found to be the major causes of HFMD. Nevertheless, the detection of E-9 implies that there might be more enteroviruses that could mostly lead to HFMD, occasionally to serious meningitis and encephalitis, and even to death. Specifically, E-9 was identified in many specimens with single infections and mixed infections.

Importantly, the variability of the VP1 genes of EV-A71, CV-A16, and E-9 tended to be consistent with the distribution of their genetic distance, which might become one of the leading causes or the virus to circulate wildly each year. In summary, such characterizations and the identified ratios of prevailing pathogens every year suggest a potential scenario in which population infections are gradually generated from immune pressures caused by the interaction between EV-A71, CV-A16, E-9, and other pathogens with similar mechanisms and the host cells.

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Conflict of interest None to declare.

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