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ORIGINAL ARTICLE

A large outbreak of hand, foot, and mouth disease caused by EV71 and CAV16 in Guangdong, China, 2009

Wu De · Ke Changwen · Li Wei · Corina Monagin · Yan Jin · Ma Cong · Zen Hanri · Su Jun

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Abstract A large outbreak of hand, foot and mouth disease (HFMD) occurred in Guangdong, China, in 2009. A total of 92,749 cases were officially reported to the Center for Disease Control and Prevention of Guangdong (GDCDC). To clarify the pathogen causing the outbreak, 600 specimens, including stool, rectal swabs, vesicular swabs, cerebrospinal fluid, and throat swabs, were collected from 541 patients and subjected to one-step RT-PCR. Four hundred eighty-nine of 541 patient samples were positive for enterovirus. All positive samples were cultured on RD and Hep2 cells; 307 specimens displayed CPE. Sequence analysis of PCR fragment and typing real-time PCR indicated that these isolates included EV71 (56%), CAV16 (35.5%), CAV6 (2.0%), CAV10 (1.0%), CAV2 (0.7%), CAV4 (1.3%), Echo30 (0.7%), Echo25 (1.0%), Echo4 (0.3%), CBV5 (1.0%) and human rhinovirus (0.7%). 100% (12/12) of fatal cases and 97.2% (140/144) of severe cases carried EV71 and CAV16. The results implied that EV71 and CAV16 were mainly responsible for the outbreak. Comparison with the three global types of EV71 and the five clusters of genotype C showed that the sequences from mainland China (not including the Hong Kong region) are located in subgenogroup C-4 and originate from isolates from the Shenzhen area of Guangdong Province. Results from this study show that the C-4 genotype has been a prevalent pathogen in mainland China since 1998.

W. De (✉) · K. Changwen · L. Wei · Y. Jin · M. Cong · Z. Hanri · S. Jun
Institute of Microbiology, Center for Disease Control and Prevention of Guangdong, No.176, Xingang Xilu, Guangzhou, Guangdong, People's Republic of China
e-mail: wude68@yahoo.com.cn

C. Monagin
Global Viral Forecasting Initiative, San Francisco, CA 94104, USA

Introduction

Hand, foot and mouth disease (HFMD) is characterized by multiple oral, hand, and foot lesions lasting roughly 7–10 days, with an incubation period ranging from 5 to 7 days. HFMD is a common viral disease, caused by many kinds of viruses, including enterovirus 71 (EV71), coxsackievirus A (CAV), coxsackievirus B (CBV), and echovirus (Echo) [1, 2]. Of these, EV71 and CAV16 are the most frequent etiologic agents causing outbreaks of HFMD [1–3]. These viruses are transmitted among children mainly by hand contact; young children are the highest risk group. The majority of infections are mild and self-limited, with recovery taking only a short time, although previous data have shown that EV71 infections can result not only in febrile disease and HFMD herpangina but also in serious complications, such as aseptic meningitis, encephalitis, myocarditis, poliomyelitis-like paralysis, and even death [4, 5]. To control outbreaks of HFMD, prevention methods (e.g., frequent hand washing, environment and toy sterilization) should be targeted to high-risk groups.

The Asia-Pacific region has encountered frequent and widespread outbreaks of enterovirus 71 infections within the past decades [6]. Outbreaks of HFMD were first documented in 1980 in mainland China and have been reported frequently in the area since that time. A large-scale outbreak caused by an emerging recombinant EV71 occurred in the Fuyang region of China in 2008 [6], affecting most of the 34 districts, including Anhui, Guangdong, Zhejian, Beijing, and Hong Kong [7]. Data from several early reports indicated that EV71 was the most prevalent

pathogen responsible for the outbreak [8, 9]. Another large outbreak of HFMD occurred recently in the Guangdong region in 2009. During the outbreak, many individuals with HFMD suffered central nervous system (CNS) complications that resulted in death. The aim of this study was to identify which pathogen caused this outbreak and which resulted in severe and fatal cases by using RT-PCR and phylogenetic analysis. We collected specimens from numerous mild cases as well as specimens from all of the severe and fatal cases from monitoring hospitals.

Materials and methods

Specimen collection, preparation and RNA extraction

In total, 398 specimens were collected from patients with mild symptoms. These cases were mainly from students at schools with HFMD outbreaks in 19 cities of the Guangdong region. Two hundred two specimens were obtained from 155 severe and 13 fatal cases that were admitted to monitoring hospitals due to serious complications. Specimens collected included 318 stool samples, 122 rectal swabs, 30 vesicular swabs, 125 throat swabs and 5 cerebrospinal fluid (CSF) samples. Rectal swabs were taken with a gentle circular motion on the rectal wall. Throat swabs were taken, with the aid of a tongue depressor, by carefully swabbing the lateral and posterior pharynx without touching the tongue or buccal mucosa. For vesicle swabs, the skin was gently cleaned with 0.9% sterile normal saline. Alcohol was not used, as it can destroy the virus. A sterile 24-gauge needle was used to rupture the vesicle, and a swab was used to absorb the fluid. Alternatively, the swab was gently rolled over the vesicle to squeeze out fluid. Mouth ulcers were sampled by rolling the swab over the floor of the ulcer [10].

Stool and rectal swabs were processed using the following standard operating procedure: One millilitre of chloroform was mixed with 10 ml PBS containing penicillin (1000 U/ml) and streptomycin (1000 µl/ml). Two grams of stool or rectal swabs and 10 pills of glass beads were added, mixed by vortexing for 3-5 min, and then centrifuged at 3000 rpm for 30 min, after which 4.5 ml of prepared supernatant was mixed with 0.5 ml PBS containing penicillin/streptomycin. The prepared supernatants were stored at 4°C for 12 hours. Throat and vesicle swabs were processed immediately by using 4 ml Hanks′ medium containing penicillin/streptomycin and mixing by pulse-vortex for 15 s. These prepared specimens were then used for RNA extraction and virus isolation.

RNA was extracted from clinical specimens and isolated with a QIAamp MinElute Virus Spin Kit (QIAGEN Inc., Valencia, Calif). In summary, 200 µl of prepared specimen and virus culture were treated with 200 µl of guanidine hydrochloride extraction buffer containing 28 µg/ml of carrier RNA, followed by alcohol precipitation. The precipitates were applied to the QIAamp MinElute column, and the viral nucleic acids were absorbed onto the silica gel membrane. The pellet was then resuspended in 50 µl of RNase-free water.

One-step RT-PCR assay and virus isolation

One-step RT-PCR amplification reactions for 5′UTR and 5′UTR-VP4-VP2 were performed using a QIAGEN One Step RT-PCR Kit (QIAGEN, Hilden, Germany) [11, 12]. The reaction system consisted of 5 µl 5× PCR buffer containing 12.5 µM $MgCl_2$, 1 µl 10 mM each of dATP, dGTP, dCTP, and dTTP, 3 µl 5× Q-Solution, 1 µl One-step Enzyme Mix, 0.4 µM forward primers (OL26 or MD91) and reverse primers (OL27 or OL68-1) for the 5′ UTR and 5′ UTR-VP4-VP2. 5 µl of extracted RNA was added to a final volume of 25 µl (Table1). The cycling conditions for the RT-PCR were as follows: an initial cycle at 50°C for 30 min and 94°C for 15 min; followed by 35 cycles at 94°C for 30 s, 50°C for 5′ UTR, or 55°C for 5′ UTR-VP4-VP2 for 30 s, and 72°C for 1 min; with a final incubation at 72°C for 10 min. The RT-PCR reaction was performed in a thermocycler (GeneAmp PCR system 2700; Amplied Biosystems, CA, USA). The reaction products were analyzed by electrophoresis in a 1% agarose gel, stained with 0.5 mg/ml ethidium bromide and observed under UV light.

All of the 5′ UTR RT-PCR-positive specimens were subjected to virus enrichment in the RD and Hep2 cell lines. The prepared specimens were inoculated onto fresh RD cells and allowed to adsorb for 1 hour at 36°C before adding 1 ml of fresh MM supplemented with 2% fetal calf serum. The cultures were incubated at 36°C and observed daily for cytopathic effects (CPE), with medium replacement every 7 days. Two blind passages were performed when no CPE was observed. Specimens for which no CPE was observed were subjected to virus enrichment on Hep2 cell line [13].

Nucleotide sequencing and genetic analysis

The 5′ UTR-VP4-VP2 region PCR products were separated in a 1% agarose gel by electrophoresis. PCR products of the appropriate size were subsequently excised from the gel and purified using a QIAGEN gel extraction kit (QIAGEN, Germany). Nucleotide sequencing reactions were performed with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and resolved on an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The reaction system consisted of 1.75 µl

Table 1 Primers used in this study

Primer	Region	Nucleotide sequence (5′→3′)	Nucleotide position	Amplimer (bp)	Reference
OL26	5′ UTR	GCACTTCTGTTTCCCC	172–187	392	[11]
OL27	5′ UTR	CGGACACCCAAAGTAG	548–563		
OL68-1	5′ UTR-VP4-VP2	GGTAAYTTCCACCACCAN	1,178–1,197	749	[12]
MD91	5′ UTR-VP4-VP2	CCTCCGGCCCCTGAATGCGGCTAAT	444–468		
CA16-F	VP1	GATCCTATTGCAGACATG	2314–2331	119	This study
CA16-R	VP1	GTGCCTAATCTGTGACTA	2562–2545		
CA16-P	VP1	FAM- CTGTGAATAATCAAGTGAACCGCTCC -BHQ1	2341–2366		
EV71-F	VP1	TGCTCCAATATATGTTTGTG	2886–2905	189	This study
EV71-R	VP1	CTCCGAATGTGGGATATC	3074–3057		
EV71-P	VP1	FAM- AACCATTGATAAGCACTCGCAGG-TAMRA	3048–3026		

5× sequencing buffer, 0.5 μl BigDye mix, and 1 μl 3.2 μM primer (OL68-1 or MD91), 1 μl purified RT-PCR products. One microliter of water was added to a final volume of 10 μl. Sequencing reactions were subjected to initial denaturation at 96°C for 2 min and 30 cycles consisting of 96°C for 10 sec, 50°C for 5 sec, and 60°C for 4 min in a Gene Amp PCR system 2700 (Applied Biosystems). The products were purified using an illustra Autoseq G-50 kit (Amersham Biosciences, Buckinghamshire, UK).

To investigate divergence and to infer genetic relationships between the PCR products, sequence analysis was performed using version 4.1 of the MEGA sequence analysis package. Phylogeny was analyzed, and trees were constructed using the neighbor-joining method of phylogeny reconstruction [14].

Molecular serotyping of enterovirus

Typing and serotyping of enterovirus were done by comparing partial sequences of the 5′ UTR-VP4-VP2 region with sequences from the GenBank database, which contains the complete sequences of all known human enteroviruses. The comparison of sequences was performed using the BLAST program (http://www.ncbi.gov/BLAST/) from NCBI.

Nucleotide sequence accession numbers

The partial sequences of 5′ UTR-VP4-VP2 from this study are deposited in GenBank with the following accession numbers: HQ005427-HQ005470.

Results

The outbreak

HFMD has been monitored due to the frequent occurrence of fatalities in Guangdong since May 2008. The Center for Disease Control and Prevention of Guangdong Province (GDCDC) uses the case definition of HFMD defined by Zhang (2008) and Ho et al. (1999) [6, 15]. Following this definition, physicians diagnosed HFMD and reported to the local CDC through the surveillance network.

A large outbreak occurred in 21 cities from March to December, 2009, in the Guangdong region of China. An epidemiological curve of HFMD based on the surveillance network data is shown in Fig. 1. The outbreak was characterized by a large and a small peak. The large peak reached a maximum number of cases in April. The baseline fluctuated from 575 to 596 cases between January and February. A total of 92,749 patients were diagnosed by physicians, including 306 severe cases and 23 fatal cases. A total of 60,743 male and 32,006 female cases were reported to GDCDC through the surveillance network. The overall male-to-female ratio was 1.9 to 1. The ages of patients ranged from 2 months to 75 years. Of the total cases, 87,496 (94.3%) were below 6 years of age, 4,871 (5.3%) were between 6 and 15 years old, and 382 (0.4%) were over 16 years of age.

RT-PCR tests and virus isolation

All 600 specimens, including 318 stools, 122 rectal swabs, 30 vesicular swabs 125 throat swabs and 5 CSFs, were

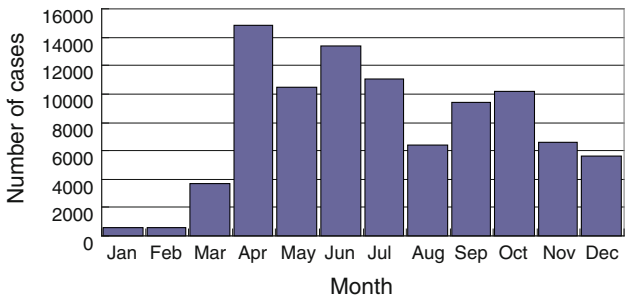

Fig. 1 Number of reported cases of HFMD in Guangdong, China, in 2009

subjected to real-time RT-PCR for the detection of EV71 and CA16. Negative specimens were tested by one-step RT-PCR with universal primers OL26 and OL27 for other enteroviruses. RT-PCR assays were positive for enteroviruses in 549 (91.5%) specimens from 489 cases. Of the 549 positive specimens, 312, 111, 108, 15 and 3 were from stool, rectal swabs, vesicular swabs, throat swabs and cerebrospinal fluid, respectively (Table 2). In these 489 cases, 12 were fatal cases with an acute onset of fever, sore throat, and myalgia, deteriorating to tachypnoea, cyanosis and seizures. Many cases were initially diagnosed as severe pneumonia, and some patients died within 1-5 days after onset. Severe cases (144) had the following clinical manifestations: brainstem encephalitis or aseptic meningitis; continuous high fever (temperature of at least 38°C); weakness, vomiting, irritability, myoclonus and acute flaccid paralysis; pulmonary edema or hemorrhage; and heart and lung failure. Of these, 333 were mild, with fever and vesicular lesions on the palms, feet and mouth. All were tested for EV71 and CAV16 using real-time RT-PCR. Of the cases, 50.1% were positive for HEV71, including 12 of the fatal cases, 128 of the severe cases and 105 of the mild cases, and 37.4% were identified as CAV16, including 11 severe cases and 173 mild cases. Dual infection was observed in two severe cases, and 12.07% of the cases were positive for other enteroviruses (Table 3).

Five hundred forty-nine positive specimens were subjected to virus enrichment in RD cells and were monitored daily. After 3-7 days of incubation, CPE in RD cells was observed with 282 of them. CPE was observed with an additional 18 specimens after one blind passage on RD cells. All of the specimens that were CPE negative were incubated with Hep2 cells. After incubation, 7 specimens produced CPE in Hep2 cells. Real-time RT-PCR for EV71 and CA16 revealed 172 isolates of EV71, 109 isolates of CA16, and 26 isolates of other enteroviruses (Table 2).

Molecular typing of enterovirus and sequencing

All amplified RT-PCR products for 5' UTR-VP4-VP2 from isolates were purified and sequenced. Three hundred seven PCR products were successfully sequenced. The comparison and phylogenetic analysis of the 5' UTR-VP4-VP2 sequences were performed by using the BLAST (NCBI) and Mega 4.1 software packages. The result of sequence analysis showed that 109, 172 and 26 isolates were CAV16, EV71 and other enteroviruses (one Echo4, two Echo30s, two rhinoviruses, two CAV2s, three Echo25s, three CBV5s, three CAV10s, four CAV4s, and six CAV6s), respectively. Eight EV71, eight CAV16 representatives, and 26 other enteroviruses were used for phylogenetic analysis. The results showed that all of the isolates that had been sequenced could be divided into three groups: HEV-A, HEV-B and rhinovirus. HEV-A consisted of EV71 and coxsackievirus A sequences, and HEV-B consisted of coxsackievirus B and echovirus sequences. Two sequences clustered in the rhinovirus group (Fig. 2).

Thirty-two sequences belonged to the HEV-A group, eight to the HEV-B group, and a further two to the

Table 2 Specimen source and number positive for enterovirus by RT-PCR and virus isolation

Specimen	RT-PCR	Virus isolation	Virus classification		
			EV71	CAV16	Other enteroviruses
Stool	312	228	115	93	20
Rectal swab	111	44	33	9	2
Throat swab	108	31	21	7	3
Vesicular swab	15	4	3	0	1
Cerebrospinal fluid	3	0	0	0	0
Total	549	307	172	109	26

Table 3 Case classification and enterovirus-positive samples (constituent rate) identified by RT-PCR

Case classification	EV71		CAV16		EV71+CAV16		Other enteroviruses		Total
	No.	Ratio (%)	No.	Ratio (%)	No.	Ratio (%)	No.	Ratio (%)	
Mild	105	31.53	173	51.95	0	0.00	55	16.52	333
Severe	128	88.89	10	6.94	2	1.39	4	2.78	144
Fatal	12	100.00	0	0.00	0	0.00	0	0.00	12
Total	245	50.10	183	37.42	2	0.41	59	12.07	489

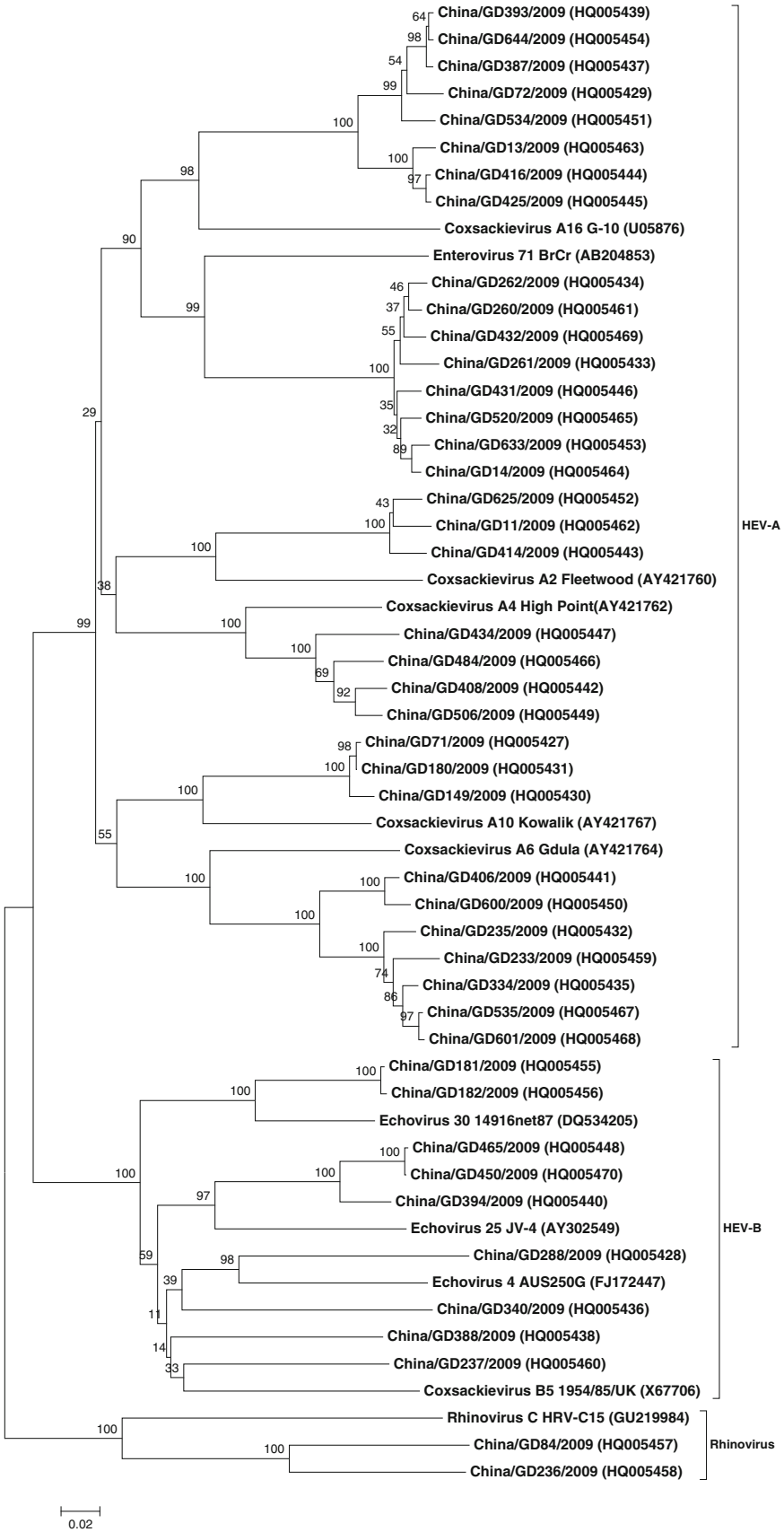

Fig. 2 Phylogenetic tree depicting the relationship of the 42 isolates from the 2009 HFMD outbreak and 11 different prototypes from around the world based on 594 nt of the 5′UTR-VP4-VP2 region. This tree was constructed by the neighbor-joining method with the Mega 4.1 software package. The bootstrap values for 1,000 pseudoreplicates for major lineages within the dendrogram are shown as percentages. The marker denotes a measurement of relative phylogenetic distance. Accession numbers are given in parentheses

rhinovirus group (Fig. 2). The eight (8/42) sequences belonging to HEV-A displayed nucleotide sequence identity in the range of 77–80% with coxsackievirus A16 prototype G-10 (U05876), with a mean of 78.6% among the eight sequences. Another eight (8/42) sequences belonging to HEV-A displayed nucleotide sequence identity in the range of 79–80% with enterovirus 71 prototype BrCr (AB204853), with a mean of 79.4% among the eight sequences. The four, three, three and six sequences were classified as HEV-A, which displayed 85-88%, 80-81%, 76%, and 82% nucleotide sequence identity with prototypes AY421762 (CAV4), AY421760 (CAV2), AY421767 (CAV10), AY421764 (CAV6), respectively. Eight sequences assigned to HEV-B displayed 90%, 80%, 81%, and 81-83% identity with prototypes DQ534205 (Echo30), X67706 (CBV5), FJ172447 (Echo4) and AY302549 (Echo25), respectively. Two sequences were placed in the rhinovirus group, which displayed 69% identity with prototype GU219984 (human rhinovirus C).

Phylogenetic analysis of EV71

A total of 54 EV71 5′ UTR-VP4-VP2 gene sequences, including 8 representative sequences from the 2009 outbreak and 46 sequences from GenBank, were used for phylogenetic analysis. The construction of the dendrogram was based on the neighbor-joining method, with the results shown in Fig. 3. All sequences were divided into three distinct branches: A, B and C (Fig. 3). Branches A and B consisted of 2 and 17 sequences, respectively. Branch C, which included 35 sequences, was further divided into five clusters: C-1, C-2, C-3, C-4 and C-5. To test the reliability of the branches defined by the tree, one thousand replicates of bootstrap were performed using the Phylip and Mega software packages.

Prototype BrCr-ts and BrCr-tr were the strains for genotype A. Genotype B included strains isolated from Singapore, Japan, Malaysia, Australia, the United Kingdom, the Hong Kong region of China, and other countries. Most of the strains were isolated during the 1982–2003 time period. Genotype C consisted mostly of strains (34/35) isolated from South East Asia, including China, Korea, Singapore, Australia, Malaysia and Japan, and was further divided into five clusters. Fifteen sequences, including 8 sequences from this study were located in subgenogroup C-4, which displayed nucleotide sequence identity in the range of 93-99%. These sequences, located in subgenogroup C-4, were all from mainland China. The earliest circulating isolates observed in subgenogroup C-4 were from the Shenzhen area (AF302996) of Guangdong province in 1998. Eight sequences in this study appeared to be closely related to the strains circulating in the Guangdong, Zhejiang and Anhui provinces of mainland China in 2008. Therefore, it can be inferred that the EV71 strain causing this outbreak was a local circulating strain from the Shenzhen area of Guangdong.

Discussion

HFMD is a common viral illness of infants and children, who are often infected in kindergartens and elementary schools. The Asia-Pacific region has had increasingly frequent and widespread outbreaks of HFMD in the past decades. HFMD has been a prevalent disease in China for a significant amount of time, with a recent outbreak occurring in 2008. The first sign of the EV71 outbreak was from the Fuyang area of the Anhui province in March, 2008. After these first reports, additional outbreaks were reported in many provinces throughout China [6]. GDCDC then developed an HFMD surveillance system in 2008. Physicians were required to report diagnosed cases of HFMD to the local CDC through this surveillance network. As HFMD did not become a notifiable disease in China until May 2008, HFMD surveillance was not optimally performed in many hospitals, especially in small hospitals and private clinics, before then. In March, 2009, the number of HFMD cases rapidly increased in Guangdong, indicating an outbreak of HFMD in the region.

According to the surveillance data, a total of 92,749 cases were registered, including 23 deaths, but the number of actual cases was estimated at >100,000 during the 2009 outbreak. The outbreak spanned 10 months from March to December 2009, with the baseline being observed in January and February. We have made several conclusions regarding the recent outbreaks and epidemics of HFMD in Guangdong. Low herd immunity against EV71 and CAV16 infections has permitted the widespread transmission and periodic outbreaks of HFMD in children younger than 5 years old [16]. High population density was a contributing factor in the long period of transmission in the 2009 HFMD outbreak. Person-to-person transmission among children also played an important role in causing the 2009 epidemic. As the Guangdong region is in a subtropical zone with high humidity, the average yearly temperature is between 19°C and 24°C, and the average yearly rainfall is between 1300 mm and 2500 mm. January and February have the lowest average temperatures and humidity of the year. We can infer that humidity and high temperatures increase the likelihood of HFMD spread and transmission.

Notably, many adult cases were reported during surveillance. The overall male-to-female ratio was 1.9:1, which is higher than the ratios 1.3: 1 and 1.5:1 taken from reports from Singapore [17] and Taiwan [18]. This predominance has been observed in other enteroviral infections in which the male-to-female ratio ranged from 1.5:1

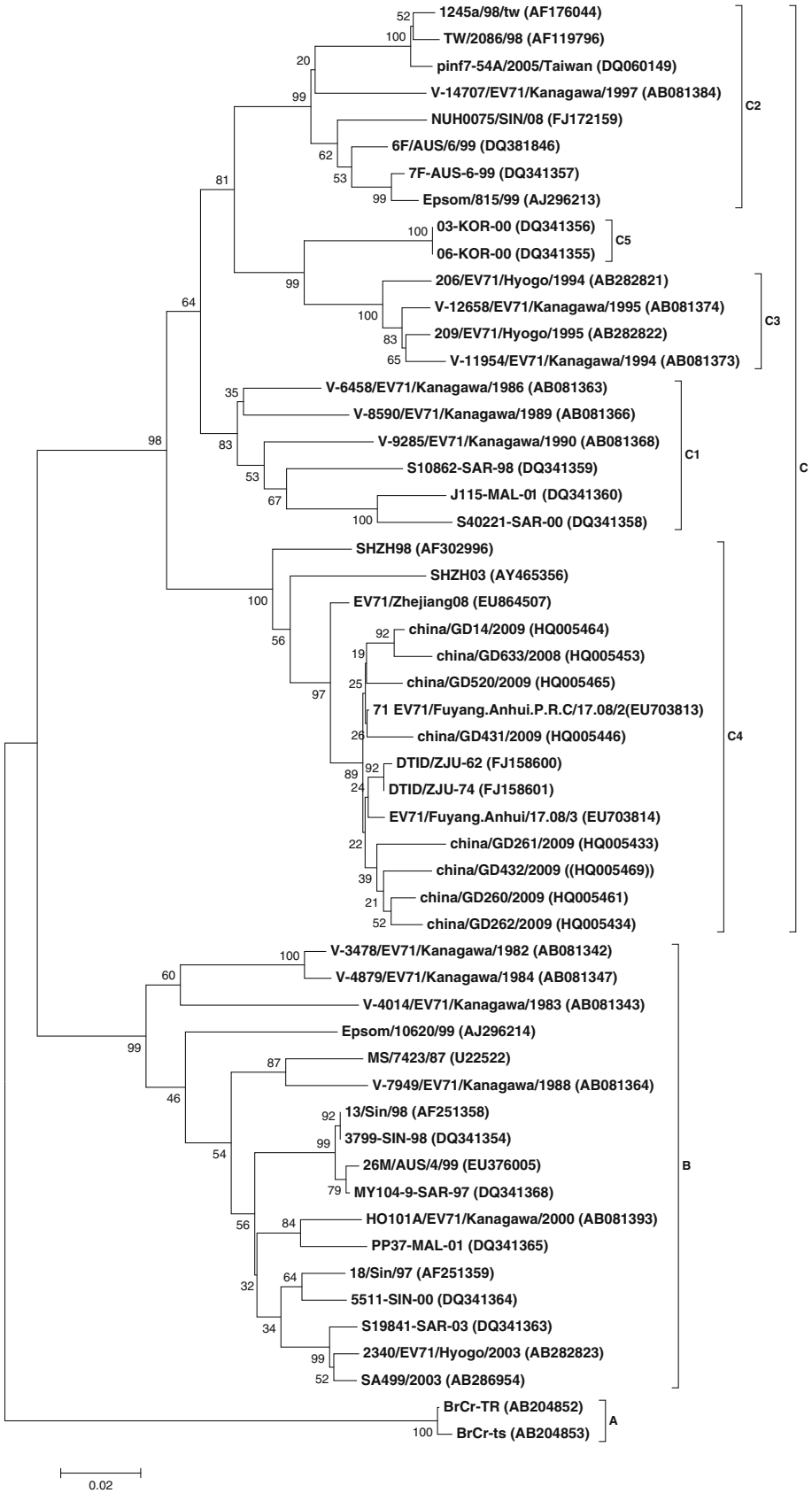

Fig. 3 Phylogenetic analysis of the genetic relationships of 54 EV71 strains worldwide, 8 EV71 sequences from this outbreak and 2 EV71 prototype strains, BrCr-TR and BrCr-ts, based on 594 bp of 5′UTR-VP4-VP2 region. The phylogenetic tree was constructed by the neighbor-joining method with the Mega 4.1 software package. The bootstrap values for 1,000 pseudoreplicates for major lineages within the dendrogram are shown as percentages. The marker denotes a measurement of relative phylogenetic distance. Accession numbers are given in parentheses

to 2.5:1 [19, 20]. These results cannot be reasonably explained at present, but they may suggest susceptibility at the host genetic level [18].

HFMD can be caused by various etiologic agents. To clarify which virus caused the outbreak of HFMD in 2009, we selected a pair of universal and sensitive primers for amplification of the 5′UTR region of enterovirus. The screen was performed by 21 municipal CDCs. Positive and susceptible specimens were sent to GDCDC for viral culture and typing detection. Multiple viruses were detected in this study, including EV71, CAV16, CAV4, CAV10, CAV2, CAV6, CBV5, Ech25, Echo30, Echo4 and rhinovirus. Similar to our study, multiple viruses were detected in the 2003 outbreak in Singapore [16], including EV71, CAV16, CAV24, CAV2, CBV3, CBV4, and untypable EV. EV71 and CAV16 were the two prominent causative agents of the 2009 HFMD outbreak. Worldwide, EV71 outbreaks are more frequent than CAV16 outbreaks, but outbreaks with patients being co-infected by EV71 and CAV16 are rarely documented. HFMD caused by EV71, CAV16, CAV10 [21], CAV2 [8], CAV4 [22], CAV6 [23], CBV5 [24], Echo4 [17], Echo25 [25] and Echo30 [26] have been reported in previous studies, but reports of HFMD caused by rhinovirus cannot be found in PubMed. Rhinovirus is a common virus that infects the pars nasalis pharyngis region of the body. Two specimens that were positive for rhinovirus were found in throat swabs in this study. Several studies have found that 12–22% of specimens from asymptomatic individuals were positive for rhinovirus [27, 28]. In addition, both rhinoviruses and enteroviruses belong to the family *Picornaviridae*. 5'UTR RT-PCR is designed for identification of picornaviruses, not only enteroviruses. According to these results, we conclude that rhinovirus is not an HFMD agent. Coinfection with rhinoviruses and other enteroviruses was observed in two cases.

Interestingly, two cases of coinfection with by EV71 and CAV16 were diagnosed by PCR techniques in this study. Both of these cases were severe, in a 1- and 0.7-year-old male child, respectively. Co-outbreaks caused by EV71 and CAV16 increase the risk of dual infection. Viral culture is usually considered to be the gold standard for viral diagnosis, but dual infection is difficult to diagnose by viral culture alone [29]. Dual infections of EV71 with other viruses are common [30], which increases the likelihood of enterovirus gene recombination. This is supported by the emerging recombinant EV71 enterovirus strain that was found in the Fuyang region of China in 2008 [6].

The VP4-VP2-based classification was established for the molecular typing of human enteroviruses and was also used for a more detailed molecular epidemiological analysis [10, 31, 32]. In this study, we chose 5′UTR-VP4-VP2 as the target region for the phylogenetic analysis of EV71 and endeavored to determine the origin of the EV71 outbreaks that occurred in Guangdong in 2009. The genetic relationship among 59 sequences worldwide was inferred by the neighbor-joining method based on the 594 nucleotides of the 5′UTR-VP4-VP2 gene in GenBank. Three genogroups, A, B, and C, were deduced in the phylogenetic tree. Genogroup C was divided further into five subgenogroups; the C-4 subgenogroup, observed since 1998, the major genotype of EV71 (C-4), was found in this outbreak. All isolates from the mainland of China were clustered at C-4 and originated from shzh03 and shzh98. This indicates that the C-4 genotype is a prevalent pathogen in the mainland China area.

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