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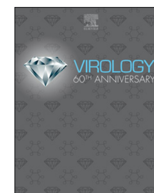

An emerging and expanding clade accounts for the persistent outbreak of Coxsackievirus A6-associated hand, foot, and mouth disease in China since 2013

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ABSTRACT

Enterovirus (EV)-A71 and Coxsackievirus (CV)-A16 have historically been the major pathogens of hand, foot, and mouth disease (HMFD) in China; however, CV-A6 , which had previously received little attention, became the predominant pathogen in 2013, and has remained one of the common pathogens since then. In this work, we conducted a molecular epidemiology study of CV-A6-associated HFMD in Xiamen from 2009 to 2015. The data showed CV-A6 pandemics had a certain periodicity rather than occurring randomly. Evolution analysis based on near-complete VP1 nucleotide sequences showed subgenotype D5 lineage 4 strains account for the persistent outbreak of CV-A6-associated HFMD in China since 2013. Alignment analysis revealed eight candidate amino acid substitutions in VP1, which may provide useful information for the research of CV-A6 virulence enhancement. This study contributed to elucidating the circulation patterns and genetic characteristics of CV-A6 in China; however, further surveillance and intervention in CV-A6 epidemics is recommended.

1. Introduction

Hand, foot, and mouth disease (HFMD) is a very common infectious disease, usually experienced by children younger than 5 years of age. It is characterized by the clinical symptoms of fever, sores, or ulcers in the mouth, and rashes on the hands or feet. Usually, the disease is mild and self-limiting. The majority of cases fully recover within 1 week, although a few present with neurological or/and cardiopulmonary complications, which may even result in death (Xing et al., 2014). Large outbreaks in the Asia-Pacific region have been described since 1997. In China, a large-scale outbreak of HFMD occurred in 2008 and is ongoing; hence, HFMD has become a significant public health issue (Liu et al., 2015; Xing et al., 2014). From 2008–2016, a total of 16,234,339 cases of HFMD in mainland China, including 3600 fatalities, were reported to the Chinese Center for Disease Control and Prevention (CDC: http://www.chinacdc.cn/tjsj_6693/fdcrbbg/).

Historically, the most common etiologic agents for HFMD have been enterovirus 71 (EV-A71) and Coxsackievirus A16 (CV-A16), both of which are human enterovirus A (human EV-A) species (Liu et al., 2015; Xing et al., 2014). CV-A6 rarely attracted clinical attention before 2008, as the associated infections were typically mild or asymptomatic. However, it caused a HFMD outbreak in Finland in 2008, and was responsible for HFMD outbreaks in France, Spain, Singapore, Taiwan, Japan, Thailand, the USA, and Cuba from 2009 to 2013 (Abedi et al., 2015; Ang et al., 2015; Fonseca et al., 2014; Fujimoto et al., 2012; Mirand et al., 2012; Montes et al., 2013; Osterback et al., 2009; Puenpa et al., 2013; Wei et al., 2011). In mainland China, EV-A71 and CV-A16 accounted for the majority of cases from 2008 to 2012 (Xing et al., 2014); however the prevalence of CV-A6 in HFMD cases has increased since late 2012, and CV-A6 has replaced EV-A71 and CV-A16 to become

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the main causative agent of HFMD outbreaks in many provinces and cities in 2013 (Han et al., 2014; Li et al., 2016; Tan et al., 2015; Zeng et al., 2015).

Phylogenetic analysis has revealed that the predominant CV-A6 strains circulating in China in 2013 were similar to strains isolated in Finland, France, Spain, and Japan since 2008 (Huang et al., 2015; Li et al., 2014). Moreover, a novel recombinant lineage of CV-A6 was identified, which accounted for 21.9% of HFMD-associated CV-A6 strains from 2012 to 2013 in Shanghai, China (Feng et al., 2015). However, there have been a few reports on the prevalence of CV-A6 in HFMD cases in China after 2013, and few attempts to systematically investigate the molecular epidemiology of CV-A6 in China over the past decade.

As CV-A6 became the most common agent of the ongoing HFMD outbreak in China in 2013, it is important to explore the prevalence of CV-A6-associated HFMD in the subsequent years and its long-term molecular epidemiology in China. In this study, CV-A6, which caused large number of HFMD cases in Xiamen, a city of 3.8 million inhabitants located in southeast China, during 2009–2015, was analyzed to investigate its prevalence in China after 2013. In addition, phylogenetic analysis based on nearly complete VP1 nucleotide sequences was conducted to explore the molecular epidemiology of CV-A6 in Xiamen and across China since 2006.

2. Materials and methods

2.1. Patients, specimens, and data collection

HFMD cases were clinically diagnosed using the Guidelines on the Diagnosis and Treatment of HFMD published by the National Health and Family Planning Commission of the People's Republic of China (http://www.nhfpc.gov.cn/yzygj/s3593g/201306/6d935c0f43cd4a1fb46f8f71acf8e245.shtml). As HFMD is a class C notifiable infectious disease in China, demographic and epidemiologic data from cases were collected and reported online to Xiamen CDC via the China Information System for Disease Control and Prevention (as for class C notifiable infectious disease, once a case is identified, medical and health institutions need to report Class C pathogens to China Information System for Disease Control and Prevention from direct reporting of network operation in 24 h. Decision departments could identify unusual cases early and respond to them in time. Medical and health institutions handle cases according to the guidelines for management or the latest instructions.). Throat swab specimens from clinically diagnosed HFMD patients were routinely collected and placed in sterile viral transport medium, then sent to Xiamen CDC for pathogen identification. Because of a large increase in the number of cases in 2010, sample collection was divided into two periods: from January 2009 to June 2010, and from July 2010 to December 2015. Specimens from all reported HFMD cases were collected in the former period, and specimens from randomly selected cases in 12 sentinel hospitals were collected in the latter period (samples of the first 10 clinically diagnosed patients who visited the outpatient department of the sentinel hospitals were collected every month, and if there were fewer than 10 patients, all the patients were sampled). As this was a public health surveillance study, ethical review was not required.

2.2. Genotypic identification of enteroviruses

Viral RNA was extracted from clinical samples using a GenMag Viral DNA/RNA kit (GenMag Biotechnology, China) and stored at − 80 °C. Then, samples were examined by nested reverse-transcription polymerase chain reaction (nRT-PCR) as previously described (Ge et al., 2013). In brief, the extracted RNA was tested by simultaneously using sets of pan-EV primers (5′ untranslated region; 5′ UTR), and specific primers targeting partial EV-A71 VP1 and CV-A16 VP1. For 5′ UTR-positive human EVs only, amplicons of the 5′ UTR were sequenced and analyzed to initially determine their genotypes. Specific primers targeting non-EV-A71 and non-CV-A16 VP1 were then used and amplicons of the VP1 were also sequenced and analyzed to ultimately determine the genotypes.

2.3. Amplification and sequencing of CV-A6 VP1 genes

Near-complete VP1 genes (891 bp) for CV-A6-positive samples were amplified using a set of previously described nested primers (outer sense: GARGCTAACATYATAGCTCTTGGAGC; outer antisense: CCYTCATARTCHGTGGTGGTTATGCT; inner sense: GACACYGAYGARATYCAACAAACAGC; inner antisense: CGRTCRGTTGCAGTGTTWGTTATTGT (He et al., 2017b). The locations of the primers based on the CV-A6 prototype strain Gdula were 2343–2371, 3301–3326, 2406–2431 and 3271–3296, respectively.). The extracted RNA was firstly reverse transcribed by using the PrimeScriptII1st Strand cDNA synthesis kit (TaKaRa, Japan). After reverse transcription, the first round of PCR was performed by using TaKaRa Taq (TaKaRa, Japan) in a final volume of 20 μl containing 5 μl cDNA, with the following conditions: initial denaturation at 95 °C for 10 min, followed by 35 cycles at 94 °C for 40 s, 53 °C for 40 s, and 72 °C for 1 min, then final extension at 72 °C for 10 min. The second round of PCR was conducted using 2 μl products of the first round as templates, and was performed in a final volume of 50 μl with the same PCR conditions. The products of the second round were purified and sequenced using A BigDye Terminator Ready Reaction Cycle sequencing Kit on an automated sequencer ABI 3130XL (Applied Biosystems, Foster City, CA). The sequences determined in the study were submitted to the GenBank database (accession no. MF973581-MF974177).

2.4. Evolutionary analysis of CV-A6

Complete or near-complete VP1 gene sequences of CV-A6 from other regions around the world (including Finland, France, Spain, the UK, Russia, India, Singapore, Japan, Taiwan, and other parts of mainland China) were retrieved from GenBank (accession numbers of the reference strains were listed in supplement material). Retrieved sequences were combined with the CV-A6 VP1 sequences obtained in this study, and multiple sequence alignments were generated with the ClustalW program (www.ebi.ac.uk/clustalw). A phylogenetic tree was reconstructed using the Tamura-Nei model and the Maximum Likelihood method in the MEGA 5.1 software suite (www.megasoftware.net), and its robustness was evaluated by a bootstrap method with replicates.

For investigating amino acid substitutions in VP1, amino acid sequences were deduced from nucleotide sequence alignments in MEGA 5.1. Then, conserved amino acids and unique amino acid changes in given monophyletic lineages were analyzed.

3. Results

3.1. Epidemiological, etiological and clinical data in Xiamen

From January 2009 to December 2015, a total of 26,194 clinically diagnosed HFMD cases were reported to Xiamen CDC, with patient ages ranging from 1 month to 38 years. The mean age of the cases was 2.46 years (interquartile-range, 1.8). The number of HFMD cases per year increased from 2009 to 2014, and slightly decreased in 2015. 1318 and 4724 throat swabs were collected for pathogen determination during January 2009 to June 2010, and July 2010 to December 2015, respectively.

Among these, 4436 cases were laboratory confirmed as EV positive, and 4426 cases were successfully serotyped. The constitution of the serotypes was as follows: 30.9% were EV-A71, 41.3% were CV-A16, 16.1% were CV-A6, 4.6% were CV-A10, and 7.1% were other EVs. The prevalence of the two major pathogens, CV-A16 and EV-A71, showed

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