# Epidemiological and etiological characteristics of hand, foot, and mouth disease before and after introducing enterovirus 71 vaccines in Sichuan, China: a 6-year retrospective study

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*To the Editor*: Hand, foot, and mouth disease (HFMD) is well recognized as a pediatric infectious disease caused by a group of enteroviruses (EVs) with global distribution. Currently, the HFMD is still one among the major threats to the health of children in China, with estimated 20,537,199 cases, resulting in 3667 deaths in 2018.[1] Available data indicated that most of HFMD cases were caused by serotypes of enterovirus 71 (EV-71) and coxsackievirus A16 (CV-A16). EV-71 was found to be predominant among severe and fatal cases. Initiated from 2015, three inactivated monovalent EV-71 vaccines were licensed in China and proved with high protective efficacy against HFMD caused by EV-71 but no cross-protection for non-EV-71 serotypes,[2] and the EVs exhibit highly geographical diversity and serotype diversity. Recently, concerns have raised for the outbreaks of CV-A6 and CV-A10 with severe cases, as well as emerging of rare serotypes such as echo virus 11 and echo virus 30. The inadequate coverage of vaccine protection, the geographical diversity, and changing spectrum of HFMD etiology still challenge the comprehensive HFMD prevention and control in China. Information regarding the broad feature of HFMD epidemiology, and the shifting of viral genetic diversity and evolution dynamic as EV-71 vaccination progressing, are still limited. Thus, we conducted a comprehensive retrospective analysis on the epidemiological and etiological characteristics of HFMD in Sichuan Province from 2014 to 2019 before and after the introduction of EV-71 vaccines, which was expected to help better HFMD prevention and control in this area.

The study protocol was approved by the Ethics Committee of West China Fourth Hospital (No. 20200983). The written consents from the patients' guardians were waived, because this was a retrospective analysis and patients' records were anonymized and identified before analysis. The HFMD patients enrolled in this study were defined as probable and confirmed cases, as well as severe and mild cases, according to the Chinese guidelines for the diagnosis and treatment of HFMD.[3] From January 2014 to December 2019, probable HFMD cases were reported and recorded in the system of National Surveillance of Notifiable Infectious Disease Program, randomly confirmed by sentinel hospitals at each site or Centers for Disease Control and Prevention and enrolled in this study. The epidemiological and clinical data were extracted from the database. Viral RNA extraction kit (QIAamp, Qiagen, Valencia, CA, USA) and one-step real-time reverse transcription-polymerase chain reaction (RT-PCR) (Bio-Perfectus Technologies, Beijing, China; Cat. No. JC20303) was applied to detect the EVs. The entire VP1 (viral protein) region ($n = 907$) or partial VP4 region ($n = 246$) of virus was amplified using the method reported.[4,5] The PCR products were sent to a DNA sequencing company (BGI, Inc., Beijing, China) for further purification and sequencing. Sequence editing and serotyping worked through BioEdit software (v7.0.4.1, http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and BLAST algorithm (Basic Local Alignment Search Tool, https://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic analysis and Bayesian skyline plot (BSP) were performed using BEAST (Bayesian Evolutionary Analysis Sampling Trees, v1.10.4; http://beast.community/), Tracer (v1.5, http://beast.community/

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tracer), and Figtree (v1.4.4, http://beast.community/figtree). (Description of reference sequences are shown in Supplementary Table 1, http://links.lww.com/CM9/A662) Statistical analysis was conducted with the statistical software SPSS (v 22.0.0.0, IBM Corp., New York, USA). The epidemiological and etiological changes were analyzed and categorized into three periods as: (1) 2014 to 2015, before EV-71 vaccination; (2) 2016, transitional period of EV-71 vaccination; and (3) 2017 to 2019, after EV-71 vaccination, respectively. Pearson Chi-square test was conducted for analyzing categorical data with $P < 0.05$ considered as statistically significant. The VP1 and VP4 sequences had been submitted to the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) under the accession number MW178307-MW179459. The three EV-71 vaccines involved in this study were manufactured by Sinovac, Chinese Academy of Medical Science, and Sinopharm group (Wuhan Institute of Biological Products Co., Ltd., China).

A total of 565,408 probable HFMD cases were reported in Sichuan province during the study period. Of the 73,370 probable cases available for RT-PCR testing, 44,022 were identified as confirmed cases. Among the confirmed cases, other EVs (pan-enterovirus+) were the predominant groups (59.5%, 26,196/44,022), followed by CV-A16 with a rate of 26.5% (11,654/44,022) and EV-71 at 14.0% (6172/44,022). Moreover, compared with CV-A16 and other EVs, more HFMD cases caused by EV-71 (9.7%, 585/6172) were severe cases and 23 patients died. Of the selected samples for further amplification and sequencing, 907 VP1 and 246 VP4 sequences were obtained, including EV-71 (VP1, $n = 191$), CV-A16 (VP1, $n = 306$), and other EVs (VP1, $n = 410$; VP4, $n = 246$) [Supplementary Figure 1, http://links.lww.com/CM9/A662 and Supplementary Table 2, http://links.lww.com/CM9/A662].

During the study period, the annual incidence of probable cases represented fluctuations with biennial cycle and peaked in even-numbered years (2014, 2016, 2018) with an average incidence rate at 114.3/100,000, which indicated that the prevention and control of HFMD still needs attention in this area. The difference in epidemic fluctuation pattern may be due to the accumulation of immunologically immature preschoolers between epidemics until reaching a critical threshold level.[6] Furthermore, the demographical, cultural, and socio-economic diversity varied between different regions, which may also have impact on the transmission and incidence of HFMD. Compared with EV-71 vaccine pre-implementation period (2014–2015), the incidence rate of HFMD after vaccination significantly increased ($\chi^2 = 5317.400$, $P < 0.001$), especially in 2018 (the annual incidence was 164.6/100,000). Among the 565,408 HFMD probable cases, there were 2791 severe cases and 46 deaths, indicated that the proportion of severe cases sharply decreased and the numbers of deaths diminished following the introduction of EV-71 vaccines ($\chi^2 = 987.732$, $P < 0.001$) which was in accordance with previous study in Chengdu[2] [Supplementary Table 3, http://links.lww.com/CM9/A662].

Overall, the monthly distribution of probable cases mainly exhibited two epidemic peaks: (1) the first peak varied between April and June and (2) the second was in November, which may be associated with stronger semi-annual periodicity by increasing latitude.[7] And the seasonal pattern of HFMD epidemic was described to be significantly associated with meteorological factors.[8] It should be noticed that a unique pattern was observed in 2018, with only one peak between July and August. Interestingly, there was an explosion of other EVs peaked between July and August in 2018, which apparently distinguished from the characteristics of serotype monthly distribution of HFMD confirmed cases within other years. According to the data from the Sichuan Climate Center, the annual average temperature of Sichuan in 2018 was particular higher than usual years, potentially contributing to unusual season pattern in 2018. With regard to the monthly distribution of serotypes in 2018, remarkable amounts of other EVs cases between July and August may also correlate to the unusual season patter, however, which cannot be confirmed in our study [Supplementary Figure 2, http://links.lww.com/CM9/A662].

In our study, the obvious shifting of viral genetic diversity was observed following the introduction of EV-71 vaccines. During the study period, the main serotypes of HFMD virus were the group of other EVs in Sichuan province. The proportion of CV-A16 kept stable with little variation, while EV-71 drastically decreased from 27.5% (5513/20,016), between 2014 and 2017 in average, to 2.9% (270/9403) in 2018, and stayed at a low level in 2019 (4.6%, 389/8431). It can be observed that the proportion of EV-71 was significantly decreased in the period after EV-71 vaccination compared with the pre-EV-71 vaccination period ($\chi^2 = 999.374$, $P < 0.001$). After EV-71 vaccination, the CV-A16 still occupied a higher proportion of 26.7% (6800/25,529) in average between 2017 and 2019. Meanwhile, the other EVs became predominant with proportion of 66.3% (16,317/24,595) averagely after vaccination, and it even peaked at 80.1% (7746/9673) in 2018 [Supplementary Figure 3A, http://links.lww.com/CM9/A662]. Of the 1153 sequences obtained, the rates of EV-71, CV-A16, and the group of other EVs were 16.6% (191/1153), 26.5% (306/1153), and 56.9% (656/1153), respectively. Further in the group of other EVs, 16 serotypes were identified [Supplementary Figure 3B, http://links.lww.com/CM9/A662], which indicated a high etiological diversity in this area. And although EV-71 vaccination dramatically decreased the severity and fatality of HFMD, co-circulation of other EVs could still remain an issue for HFMD control.

For phylogenetic analysis, all five main serotypes of EVs (EV-71, CV-A16, CV-A4, CV-A6, and CV-A10) did not form unique clusters in Sichuan, mixing within main circulating strains in China [Supplementary Figure 4, http://links.lww.com/CM9/A662]. In brief, the genotypic characteristics of five main serotypes were as: (1) all EV-71 strains in Sichuan belonged to the C4 genotype, and further sub-grouped to the C4a [Supplementary Figure 4A, http://links.lww.com/CM9/A662]; (2) two major genotypes of CV-A16: B1a and B1b were found, 62 (20%) CV-A16 strains in our study belonged to type B1a, 243 (80%) categorized to subtype B1b [Supplementary Figure 4B, http://links.lww.com/CM9/A662]; and (3) the most prev-

alent strains of CV-A4 [Supplementary Figure 4C, http://links.lww.com/CM9/A662], CV-A6 [Supplementary Figure 4D, http://links.lww.com/CM9/A662], and CV-A10 [Supplementary Figure 4E, http://links.lww.com/CM9/A662] were D2, D3, and C subtypes, respectively.

BSP analysis was performed to analyze the population dynamics during period of EV-71 vaccines implementation of EV-71, CV-A16, CV-A4, CV-A6, and CV-A10 based on the VP1 sequences data [Supplementary Figure 3C, http://links.lww.com/CM9/A662]. It should be noticed that the viral population dynamic of five main serotypes notably changed following the EV-71 vaccines implementation. In the pre-EV-71 vaccination period (2014–2015), the VP1 genetic diversity of all five EVs presented nearly constant; however, following the implementation of the EV-71 vaccination program, (1) the BSP of EV-71 (C4a) showed that it presented a rapid population growth at the end of 2017; (2) for CV-A16, population of type B1a showed a tendency to decrease first and then increase in 2018, while an abrupt increase in genetic diversity was observed in B1b groups since transition year of 2016 and kept expansion in 2017 to 2019; (3) the population size of CV-A4 D2 genotype experienced an increase in late 2017 and a decrease in 2019; (4) the D3 group of CV-A6 showed a population expansion early in transition and kept increasing in the following years with peak at 2019; and (5) two relatively rapid expansion were found in 2018 and 2019 in the population size of CV-A10 (genotype C). One hypothesis was that after introduction of EV-71 vaccines, the expansion of EV-71 population may probably result from the response of EV-71 population to the selection pressure of vaccine. Although EV-71 infections declined after vaccination, high selective pressure may lead to the emerging of new mutants, which could potentially change the pathogenicity and have impact on protective efficacy of vaccines. In light of the incidence of HFMD maintaining after EV-71 vaccination, the group of other EVs may take advantages to reform the etiological spectrum with increasing cases. Then, it is understandable that the expansion of CV-A16 and other EVs were probably resulted from the increased infections by those etiologies.

There are several limitations in our study. Only 13.0% (73,370/565,408) probable cases were available to analyze the serotype distribution of three main classifications. Then, restricted by the strategy of routine etiological monitoring for HFMD in China, only a small proportion (7.8%, 44,022/565,408) of confirmed cases were sequenced for phylogenetic analysis. However, our findings could still draw a contemporary picture of HFMD's epidemiological and etiological characteristics in Sichuan, and shed light on describing the shifting of etiological spectrum as EV vaccination progressing.

In conclusion, this was the first study demonstrating the epidemiological and etiological characteristics of HFMD in Sichuan Province, before and after introduction of EV-71 vaccines. Such surveillance on the HFMD molecular characteristics should be continued to monitor the dynamic of viral population, helping in better HFMD prevention and control in this area.

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## Conflicts of interest

None.

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