Virus-virus interactions of enteroviruses and impact of the coronavirus disease 2019 interventions on the incidence of hand, foot and mouth disease in Nanchang, China

Xianfeng Zhou[1], Ke Qian[2], Chunlong Zhu[3], Liu Yi[2], Junling Tu[2], Shu Yang[2], Yanxia Zhang[2], Yanglin Zhang[3], Wen Xia[2], Xiansheng Ni[2], Tielong Xu[1], Fenglan He[2], and Hui Li[2]

[1]Jiangxi University of Traditional Chinese Medicine
[2]Nanchang Center for Disease Control and Prevention
[3]Third Hospital of Nanchang

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Abstract

Pathogen spectrum of Hand, foot and mouth disease (HFMD) has substantially changed in the past decade. How do the co-circulating pathogens interact and the coronavirus disease 2019 (COVID-19) intervene the incidence of HFMD remain unclear. Here, we evaluated the virus-virus interaction (VVI) of EVs using Spearman's Correlation in Nanchang, China. And the impact of COVID-19 intervention on HFMD incidence was estimated using seasonal autoregressive integrated moving average (ARIMA) models. Enterovirus (EV) serotypes were determined by RT-PCR. From 2019 to 2022, 1321 (57.5%) out of 2296 HFMD cases were EV-positive, in which coxsackievirus A6 (CVA6) and CVA16 were the major pathogens, accounting for 34.0%-59.6% and 14.9%-31.4%, respectively. Our analyses provide strong statistical support for the existence of VVIs among enteroviruses, in which CVA6 negatively interacted with CVA16 and EV-A71, and positive VVI between CVA16 and EV-A71 was observed. While CVA6 has a (albeit inconsistent) seasonal pattern in Nanchang, typically peaking in fall-winter months before COVID-19 epidemic, CVA16 and EV-A71 contemporaneously peaks around May, supporting the epidemiological VVIs among these strains. During the COVID-19 epidemic, the seasonal HFMD epidemic peak was restrained, indicating the COVID-19 intervention had mitigated EV transmission. Moreover, we first figured out the serotypes from other enteroviruses, among them CVA4, CVA2, CVA5 and CVB3 were the major agents accounting for 34.8%, 23.9%, 23.9% and 10.9%, respectively. Taken together, CVA6 and CVA16 were currently the most predominant pathogens negatively interacted with each other in Nanchang, while NPIs of COVID-19 outbreaks interfered the interactions by mitigating their incidence and transmission.

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[1] Cancer Research Center, Jiangxi University of Chinese Medicine, Nanchang, China.

[2] The Collaboration Unit for Field Epidemiology of State Key Laboratory for Infectious Disease Prevention and Control, Nanchang Center for Disease Control and Prevention, Nanchang, China.

[3] Clinical Laboratory, Third Hospital of Nanchang, Nanchang, China

[4] School of Life Science, Jiangxi University of Chinese Medicine, Nanchang, China.

[5] These authors contributed equally to this article

*Correspondence authors

Correspondence to FH (he_495380@163.com) and HL (nccdcyjb@163.com)

Address: 833 Lijing Road, Nanchang, 330038, China

Running title: VVI and COVID-19 intervention on HFMD

Abstract

Pathogen spectrum of Hand, foot and mouth disease (HFMD) has substantially changed in the past decade. How do the co-circulating pathogens interact and the coronavirus disease 2019 (COVID-19) intervene the incidence of HFMD remain unclear. Here, we evaluated the virus-virus interaction (VVI) of EVs using Spearman's Correlation in Nanchang, China. And the impact of COVID-19 intervention on HFMD incidence was estimated using seasonal autoregressive integrated moving average (ARIMA) models. Enterovirus (EV) serotypes were determined by RT-PCR. From 2019 to 2022, 1321 (57.5%) out of 2296 HFMD cases were EV-positive, in which coxsackievirus A6 (CVA6) and CVA16 were the major pathogens, accounting for 34.0%-59.6% and 14.9%-31.4%, respectively. Our analyses provide strong statistical support for the existence of VVIs among enteroviruses, in which CVA6 negatively interacted with CVA16 and EV-A71, and positive VVI between CVA16 and EV-A71 was observed. While CVA6 has a (albeit inconsistent) seasonal pattern in Nanchang, typically peaking in fall-winter months before COVID-19 epidemic, CVA16 and EV-A71 contemporaneously peaks around May, supporting the epidemiological VVIs among these strains. During the COVID-19 epidemic, the seasonal HFMD epidemic peak was restrained, indicating the COVID-19 intervention had mitigated EV transmission. Moreover, we first figured out the serotypes from other enteroviruses, among them CVA4, CVA2, CVA5 and CVB3 were the major agents accounting for 34.8%, 23.9%, 23.9% and 10.9%, respectively. Taken together, CVA6 and CVA16 were currently the most predominant pathogens negatively interacted with each other in Nanchang, while NPIs of COVID-19 outbreaks interfered the interactions by mitigating their incidence and transmission.

Keywords: enteroviruses; HFMD; seasonality; virus-virus interaction; COVID-19; ARIMA

Introduction

Hand, foot and mouth disease (HFMD) is a highly contagious disease in children caused by several human enteroviruses (EV), particularly enterovirus A71 (EV-A71) and coxsackievirus A16 (CVA16) and CVA6 (1-3). Enteroviruses belong to the family *Picornaviridae* , genus *Enterovirus* . Their positive single-strand RNA genome is about 7,500 nucleotides, and is composed of a large open reading frame (ORF) flanked by 5' and 3' untranslated regions (UTRs). The 5' part of the ORF encodes the structural proteins that form the capsid, while the 3' part of the ORF encodes the non-structural proteins (4). HFMD primarily affects children younger than 5 but can infect teenagers and adults (1, 2). Clinical manifestations of HFMD include mild to severe rash, herpangina, pulmonary edema, circulatory disturbances, meningitis, aseptic encephalitis, and even death (5, 6). Since 2012, outbreaks associated with Coxsackievirus A6 (CVA6) have been frequently reported in Europe, Japan and some developed regions of China (7-11). From then on, CVA6 has been gradually predominant in most areas of China since 2013 (9, 12). Our former study found D3 sub-genotype CVA6 (D3-CVA6) increasingly predominated in local HFMD cases, and EV-A71 was no longer detected from HFMD surveillance after 2-year EV-A71 vaccine promotion and implementation(12).

In early 2020, non-pharmaceutical interventions (NPIs) were implemented in China to reduce and contain the coronavirus disease 2019 (COVID-19) transmission. A national-scale investigation found that these NPIs have substantially reduced the incidence of HFMD in the first wave of COVID-19 (13). Since the first outbreak in most cities of China, NPIs, vaccines and dynamic Zero-Covid policy were implemented to combat SARS-CoV-2 around mainland China till the end of 2022 (14). However, the impact of these measures on epidemiological and etiologic characteristics of HFMD has not been well studied. In this study, we retrospectively analyzed the epidemiological and etiological characteristics of HFMD in the southeastern capital city of Nanchang before and during the COVID-19 pandemic. And for the first time, the virus-virus interactions among the major serotypes CVA6, CVA16, EV-A71 were systematically analyzed in Nanchang,

China. Our analyses provide strong statistical support for the existence of interactions among enteroviruses. Furthermore, we clarified the serotypes of other un-serotyped EVs using a reverse transcription-nested PCR targeting 5'UTR, facilitating to see the whole picture of the dynamics of pathogen spectrum. These findings will help improve disease forecasting and evaluation of HFMD control interventions in the future.

Materials and Methods

The aim and design of this study

This study aims to explore the interlinks among the circulating EVs that demonstrate a long-term regular seasonality of prevalence. And whether the interactions are interfered by outer interventions such as NPIs of COVID-19. Therefore, seasonal ARIMA models were used to forecast the incidence of HFMD in order to analyze the potential impact of COVID-19 interventions on mitigating EV transmission. Spearman's rank correlation was conducted for the major serotypes to figure out the potential VVIs.

Data collection

Nanchang, a city located at 115°27'-116°35' E longitude and 28°10'-29°11' N latitude (Figure 1). Nanchang had a population of 6.4 million as of 2022, accounting for 12.5% of the population of Jiangxi Province. Since June 2009, clinical specimens must be collected from all severe HFMD cases, and the first 5 mild cases reported per month in each county or district are tested for enteroviruses using real time RT-PCR by the local CDCs (1, 15).

During 2019-2022, a total of 2296 clinically diagnosed HFMD cases were collected from 9 hospitals in Nanchang, including Jiangxi Provincial Children's Hospital and 8 county and district sentinel hospitals. A total of 2296 clinical specimens (1737 pharyngeal/throat swabs, 381 anal swabs and 178 stools) were collected.

The study was approved by the ethics committee of the Nanchang Center for Disease Control and Prevention, and the procedures were performed according to the approved guidelines (Approval No. NCCDC-20100701). Prior informed consent was obtained from patients or their parents for sample collection.

Clinical diagnosis criteria

Patients with the following symptoms were defined as cases of HFMD: fever; papules and herpes on the mucous membranes of the hands, feet, or mouth; rash on the buttocks or knees with inflammatory flushing around the rash, and blisters with a small amount of fluid. Severe cases of HFMD were defined as the presence of additional neurologic, cardiogenic, or pulmonary disease.

Serotype identification of enteroviruses

Swabs were stored in a dedicated Universal Transport Medium (UTM) (Yocon, Beijing, China) for transport. Stool samples were diluted to a 10% suspension using MEMs. After thorough mixing, 200 μl of clinical samples were used to extract RNA using the QIAamp Viral RNA Mini Kit (Qiagen, CA) according to the manufacturer's instructions. EV, EV-A71, CVA16, CVA6 and CVA10 were confirmed using commercial real-time RT-PCR Kits (BioPerfectus technologies, Jiangsu, China). Samples positive for universal EV but negative for the above serotypes were named as un-serotyped EV (UEV).

Serotyping of UEV

A reverse transcription-nested PCR was used for UEV serotyping. Simply, pan-enterovirus specific primer sets targeting the 5'UTR for nested RT-PCR to give a 389-bp-long product was as follows: the first round PCR using primers Out-F 5'-CYTTGTGCGCCTGTTTT-3' and Out-R 5'-ATTGTCACCATAAGCAGCC-3' (530bp), then the second round PCR using primers Inner-F 5'-CAAGYACTTCTGTMWCCCC-3 and Inner-R 5'-CCCAAAGTAGTCGGTTCC-3'(389bp) (16). For the first RT-PCR, PrimeScript One Step RT-PCR Kit Ver.2 (Takara) was used with the following reaction conditions: 50 reverse transcription for 30 min, 94 pre-denaturation for 2 min, 40 cycles of 94 15 s, 55 30 s, 72 1 min. For the second PCR, TaKaRa Taq HS Perfect Mix (Takara) was used according to the manufacturer's protocol. The final PCR products were subjected to 2.0% agarose gel electrophoresis for analysis. PCR products of the second PCR were purified

using the QIAquick PCR Purification Kit (Qiagen), and then amplicons were sequenced in both directions using the ABI 3730 Genetic Analyser (Applied Biosystems).

Sequence analysis and phylogeny

SeqMan software (DNASTAR Inc., Madison, WI, USA) was used to assemble the obtained sequences, which were uploaded to the NCBI database for blast. The ClustalW method (MEGA X, www.megasoftware.net) was used for the sequence comparison using the neighbor-joining method to construct phylogenetic trees after estimation of genetic distance (Kimura two-parameter method). A bootstrapping test was performed 1,000 times.

Geographical mapping

Maps in this study were generated by ArcGIS software (Version 10.7, ESRI Inc., Redlands, CA, USA; https://www.esri.com/software/arcgis/arcgis-for-desktop, accessed on 23 March 2023).

NPI data collection during COVID-19 epidemic

Local COVID-19 outbreaks were updated by official websites of Nanchang Municipal Health Commission: http://hc.nc.gov.cn/ncwjw/index.shtml. The time span of each outbreak and level of public health emergency response (PHER) were determined according to the official announcement. According to the documents issued by the local governments based on the Joint Prevention and Control Mechanism about COVID-19, the start and end dates of the lockdown of districts and other public places were collected.

Analysis of the impact of COVID-19 intervention on HFMD incidence in Nanchang, China

Since HFMD has strong seasonality, a seasonal ARIMA model (p,d,q) (P,D,Q)s was used for modeling, where p and P are autoregressive order and seasonal autoregressive order respectively, and q and Q are the moving average and seasonal moving average respectively. d and D are the difference order and seasonal difference order respectively, and s is the seasonal period. Based on the monthly onset number of HFMD cases, we fit autoregressive integrated moving average (ARIMA) models for the pre-COVID-19 period (2010-2019) and used these models to predict the prevalence of HFMD in 2020. This was done by ARIMA Forecasting (v1.0.11) in Free Statistics Software (v1.2.1), Office for Research Development and Education, URL http://www.wessa.net/rwasp_arimaforecasting.wasp/.

Statistical analysis

Spearman's rank correlation coefficients were computed and tested between all pairs of virus infection prevalence (the proportion positive among those tested) in each month using GraphPad Prism 8. Mann Whitney U test was used for statistical analysis of Ct values. The difference was statistically significant with a P value of <0.05.

Results

Epidemic pattern of HFMD and the impact of COVID-19 intervention

From January 2019 to December 2022, a total of 2296 suspected HFMD cases were collected from sentinel hospitals for EV screening in Nanchang. Among the HFMD cases, 1321 (57.5%) were laboratory-confirmed (EV-positive) HFMD determined by real time RT-PCR. The pathogen spectrum of HFMD had a substantial change after the launch of EV-A71 vaccination in mid-2016, the typical biennial outbreak pattern gradually developed into less volatile mode as EV-A71-associated cases substantially decreased (Figure 2A, Supplementary Figure 1A). The monthly distribution of EV-positive cases during 2019-2022 were shown in Supplementary Figure 1B. CVA6 was the predominant agent accounting for 34.0-59.6% of EV-positive cases, followed by CVA16 (14.9-31.4%) and sporadic CVA10-associated cases, and the proportion of UEV reached to as high as 33.2% in 2019 (Supplementary Figure 2). To assess the potential impact of local COVID-19 outbreaks on EV transmission, a long time-span (2010-2022) etiology surveillance curve was displayed, and two predicted peaks was inserted to follow the epidemic pattern-biennial peak around May (Figure 2A). During the first local COVID-19 outbreak from January to March 2020, the HFMD incidence plunged and the

expected epidemic peak vanished as result of a city-level lockdown along with the first-level PHER (Figure 2B). The prediction was supported by ARIMA forecasting in the absence of NPIs (Figure 2C-D). From then on, the dynamic Zero-Covid policy continued until the 3rd outbreak associated with Omicron variants in the end of 2022 (Figure 2B) (14). Apparently, the predicted peak of HFMD didn't occur around May 2022 but a low-volatility plateau of HFMD incidence, which might be associated with the 2nd outbreak lasting for 2 months from March to May 2022.

Epidemiology and etiology of HFMD in Nanchang from 2019 to 2022

From 2019 to 2022, CVA6 and CVA16 were two major pathogens, accounting for 34.0%-59.6% and 14.9%-31.4% EV-positive cases, respectively. However, other un-serotyped enteroviruses (UEV) accounted for 13.7-%-33.2% EV-positive cases during 2019-2022. Overall, the UEV accounted for a larger proportion in 2019 (33.2%) and 2021 (31.3%) compared with that in 2020 (20.8%) and 2022 (13.7%). On the contrast, proportion of CVA6-associated cases increased in 2020 (59.6%) and 2022 (57.0%) compared with that in 2019 (43.7%) and 2021 (34.0%) (Supplementary Figure 2). This result indicated that the COVID-19 outbreaks and the associated measures had different levels of impact on EVs. In geographical level, we analyzed and compared the positive rate (Figure 3A-D) and serotype proportion (Figure 4) in different districts and counties, in which their population densities were displayed in Figure 3E. Overall, the EV-positive rate of the HFMD cases was high in urban and suburb areas. In northwest areas AY and XJ, the positive rate had a downtrend, while it was an uptrend in the suburb area NC from 2019 to 2022. During this period, Proportion of CVA6-infected cases maintained in a low volatility in different areas apart from a very low proportion in QSH but high proportion in DH and JX in 2022. This data supports the predominance of CVA6 in Nanchang regions in the past few years. As for CVA16, a relatively high volatility was observed in outskirt areas like JX and AY. The 0% proportion of CVA16 in some areas might be due to the COVID-19 intervention. In addition, UEV proportion in QSH district was the highest reaching up to 60%.

Characterization of UEV in Nanchang

As the dominance of CVA6 becomes more pronounced in HFMD cases, the proportion UEV tends to decline. However, there is an average 24% UEV cases with unspecified serotypes during 2019-2022. The serotypes and their proportion in UEV cases were determined using a nested RT-PCR, and an expected PCR product (˜389-bp) was obtained as shown in 2% agarose gel electrophoresis (Figure 5A). A total of 46 UEV cases were randomly selected during 2019-2022 for serotyping described above, among them 6 serotypes were determined by sequencing and then online blast in NCBI. Among them CVA4, CVA2, CVA5 and CVB3 were the major agents accounting for 34.8%, 23.9%, 23.9% and 10.9%, respectively (Figure 5B). And a partial 5' UTR-based phylogenetic tree was constructed for the representative strains of 6 serotypes CVA4, CVA2, CVA5, CVB3, CVA9 and Echovirus 9 (ECHO9) (Figure 5C). After pairwise sequence identity analysis, we found that the partial 5'UTR region was conservative in CVB3 but hypervariable in CVA2, CVA4 and CVA5 (Figure 5D). According to the proportion of these serotypes in UEV, the estimated proportions for CVA4, CVA2, CVA5, CVB3, CVA9 and ECHO9 in EV-positive cases were 8.6%, 5.9%, 5.9%, 2.7%, 1.1% and 0.5%, respectively. This result suggests that enhanced etiological surveillance is essential, especially when the proportion of UEV rises.

Virus-virus interactions of different serotypes and impact of COVID-19

Increasing evidence suggests that virus–virus interactions are common and may be critical to understanding viral pathogenesis in natural hosts. Currently, CVA6 and CVA16 are the major causative agents circulating in Nanchang, while these two viruses demonstrated a staggered prevalence pattern during 2019-2022 as shown in figure 6A, the spearman correlation analysis showed that monthly cases of CVA6 and CVA16 were negatively correlated (r =-0.459, p =0.001). To further determine this hypothesis, we conducted a pair correlation analysis for CVA16, CVA6 and EV-A71 before the COVID-19 epidemic. As expected, CVA6 versus CVA6 was negatively interacted (r =-0.578,p <0.001) during 2013-2019 and similar prevalence pattern was observed year by year (figure 6B). As EV-A71 vaccination initiated since mid-2016 and EV-A71 was no longer detected via laboratory-based surveillance after 2018, correlations of EV-A71 versus CVA16 and EV-

A71 versus CVA6 during 2010-2016 and 2013-2016 were respectively analyzed to rule out the interference of EV-A71 vaccination. It's found that EV-A71 and CVA16 displayed a strong positive interaction (r =0.644, p <0.001) (figure 6C), while EV-A71 and CVA6 were negatively interacted (r =-0.609,p <0.001) (figure 6D). However, the negative correlation between CVA6 and CVA16 was interfered by three local COVID-19 outbreaks occurred in 2020 and 2022 (r_{2020}=-0.400,p =0.196; r_{2022}=-0.178, p =0.574), while in 2019 (pre-COVID-19) and 2021 (without local COVID-19 cases), these two viruses were negatively correlated (r_{2019}=-0.747, p =0.007;r_{2021}=-0.734, p =0.009) (Figure 7A-D). These results indicated that infection exclusion type of negative interaction between CVA6 and CVA16 might exist, and NPIs for combating COVID-19, to some degree, mitigated their transmission as ARIMA forecasting indicated (Figure 7E-F). Taken together, in the absence of intervention from NPIs, the peak-shifting epidemic characteristics of CVA6, CVA16 and EVA71 sustained their seasonal regularities year by year, while the interactions might be shortly broken by strict prevention measures during COVID-19 pandemic. In addition, EV-A71 vaccination did not interfere prevalence of CVA6 and CVA16 and their interactions as figure 6B indicated.

Gender and age distributions of HFMD

To explore gender differences, we found male cases were 1.34 times more common than females, and the male-to-female ratios of cases associated with CVA6, CVA10 and CVA16 were 1.30, 1.59 and 1.40, respectively. Children aged 0-3 years accounted for 73.71% of laboratory-confirmed cases, with an average of 7.21% of children under 1 year of age. Children aged 4-5 years accounted for 18.60%, while only 5.61% of cases occurred in children aged 6-9 years. Infections in people aged 10 years and older were rare, accounting for approximately 1.14% (10-17 years) and 0.99% (>17 years). The results indicated that HFMD generally affects pre-school children and children under 3 years of age were at the highest risk of enterovirus infection. Besides, among the reported 9 severe cases, all of them (100%) were less than 3 years and their major neurological signs were convulsion or/and startle as described by clinicians.

Sample types and their reliability for etiology surveillance

During 2019-2022, a total of 2296 samples were collected from HFMD cases for etiology surveillance, clinicians tended to collect throat swabs (75.7%) other than anal swabs (16.6%) and stool (7.7%) (Figure 8A). We found comparable positive rates of EVs from throat swabs (46.6-68.8%), anal swabs (57.9-80.0%) and stool (58.0-71.1%) (Figure 8B). Generally, throat swabs or anal swabs had higher viral loads than stool, and viral loads of CVA6- or CVA10-infected cases were higher than that of CVA16- or UEV-infected cases (Figure 8C-F). Considering the convenience of sampling, storage, ages of the patients and seasonality of HFMD, throat swab seems convenient and reliable enough for etiology surveillance. However, it's worthy to continuously collect multiple types of samples from inpatient cases to explore dynamics of virus shedding in the future.

Discussion

From 2008-2012, EV-A71 was the major pathogen causing HFMD and was responsible for most severe and fatal cases in mainland China (1). Our previous studies have shown that locally circulating EV-A71 strains belong to the C4a sub-genotype, which is a dominant genotype causing most of severe cases and death in mainland China during 2008-2012 (2, 17-19). However, the pathogen spectrum of HFMD substantially changed as CVA6 increasingly replaced EV-A71 to be predominant in many cities of China, particularly after the launch of EV-A71 vaccination since 2016 (20). We formerly observed that the proportion of EV-A71 continued to decrease as vaccination rates increased, at 22.5% in 2017, 0.5% in 2018 and disappeared from 2019, suggesting that immune protection in children is quite efficient after programmed EV-A71 vaccination (12). Although prevalence and phylogenetic analysis of the predominant strains were extensively explored, the virus-virus interactions (VVIs) of EVs were not well studied.

VVI is a measurable difference in the course of infection of one virus as a result of a concurrent or prior infection by a different species or strain of virus (21). In this study, we demonstrated the presence of VVI in HFMD cases at the epidemiological level by 13-year continuous surveillance of EV serotypes. To our knowledge, this study provides the first-hand evidence of different types of VVIs among the main causative agents CVA6, CVA16 and EV-A71. It's appealing to find that CVA16 and EV-A71 interacted in a positive

way, which in turn boosted the large concentrated HFMD outbreaks with resonance of EV-A71 and CVA16 infection during the second quarter of each year during 2010-2016 (Figure 6C). As frequent CVA6 outbreaks occurred after 2013 in the city of Nanchang, and it prevailed in a staggered epidemic pattern negatively interacted with CVA16 and EV-A71. To our knowledge, EV-A71 and CVA16 share the same entry receptor SCARB2, while KREMEN1 was proven as an entry receptor for most of the coxsackie type A viruses including CVA2-CVA6, CVA10, and CVA12 (22, 23). Whether the VVIs among EVs are interlinked with the entry receptors is unknown (Supplementary Figure 3A). Therefore, it will be appealing to explore the potential mechanism behind it *in vitro* and *in vivo* in the future.

In this study, impact of EV-A71 vaccination on the interactions between CVA6 and CVA16 was not observed, suggesting little potential cross-immunization between EV-A71 and CVA6 or CVA16 (Supplementary Figure 3B). However, during the past three local COVID-19 outbreaks spanning 3 years, the VVIs were interfered in the coming months of each outbreak and subsequently the epidemic peak of HFMD was restrained (Figure 2A-2B). This result was also supported by a national-scale (covering 31 provincial capitals in mainland China) impact of NPIs on EV transmission in 2020 (13). It will be more convincing to use computer simulations to obtain improved understanding of how the epidemiology of viral infections is interlinked, which can help improve disease forecasting and evaluation of HFMD control interventions in the future.

Children age less than 5 years are still highest-risk population of EV infection regardless of serotypes and pathogen spectrum fluctuation, and male cases were more common than females in all serotypes of EVs. It's still unclear what is behind the proneness of gender. Formalin-inactivated EV71 vaccines are currently available for children of 6-59 months in China and substantially mitigated EV-A71 transmission (12, 24). The ARIMA models based on HFMD cases and EV-A71 cases effectively present the protective effect of EV-A71 vaccines against EV-71 infections in 2017, leading to lower incidence of HFMD and EV-A71-infection in Nanchang (Supplementary Figure 4A-B). However, these vaccines fail to confer cross-protection against CVA16 (Supplementary Figure 4C), highlighting the necessity of developing a multivalent HFMD vaccine. Although access to EV-A71 vaccine is convenient in Nanchang, we observed a downward trend of EV-A71 vaccination that was likely due to the COVID-19 intervention (Supplementary Figure 5). However, follow-up of vaccination rate and public health education are necessary to consolidate the achievements of elimination of EV-A71 infection. Although CVA6, CVA10, CVA16 and EV-A71 are routinely detected for suspected HFMD cases, there is still a quite proportion (24.8%) of UEV-associated HFMD. We first clarified serotypes and identified top four causative agents CVA4, CVA2, CVA5 and CVB3 from local UEV cases, which helps us get a clear view on the vicissitude of pathogen spectrum. Whether these strains epidemiologically interlink with each other or not remains unknow (Supplementary Figure 3C). Previous evidence suggests that CVA6 began sporadically spreading in China from late 2012 before turning dominant in 2013 (7, 15, 25, 26). Despite the lack of publicly available CV-A6 surveillance data after 2015, our survey observed a dominant trend of CV-A6 in Nanchang (Supplementary Figure 2). Nevertheless, CVA16 has been sustaining a stable proportion and low-volatility pattern since 2010. Thus, we assume that CVA6 and CVA16 will regain the negative VVI in the coming years without external intervention such as vaccines or NPIs. Moreover, an enhanced monitoring is necessary for neglected serotypes CVA2, CVA4, CVA5 and CVB3 that were "hidden" in UEV under current surveillance system. Therefore, serum neutralizing antibody assay for pre-school children will be helpful to comprehensively understand the etiology features of HFMD.

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Author Contributions

XZ and FH conceived and designed the study. XZ, CZ, KQ, JT, LY and FH performed experiments. XZ, LY, FH, YZ, WX, XN and TX collected data. XZ, FH, SY, YZ and HL analyzed and interpreted the data. XZ and HL wrote the manuscript. All authors approved the manuscript.

conflicts of interest

None

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Figure legends

Figure 1. Geographical location of Nanchang City, China

Figure 2.Pathogen spectrum of HFMD in Nanchang during 2010-2022. (A) Monthly curve of EV-positive HFMD cases, black arrows indicate biennial peak around May from 2010 to 2022, red dotted peaks are inserted as possible prediction; (B) Time line of three waves of local COVID-19 outbreaks and monthly curve of CVA6-, CVA10-, CVA16- and UEV-infected cases in Nanchang from 2019 to 2022. 1st outbreak: January 24 to March 11, 2020; 2nd outbreak: March 16 to May 5, 2022; 3rd outbreak: December 2022; (C) Observed HFMD case counts in Nanchang from 2010 to 2020, compared with the fitted (2010-2019) and predicted (2020) case counts obtained using the ARIMA models in the absence of COVID-19 outbreaks. The light-purple shaded part indicates the observed (black) and estimated (white) case counts from early January to the end of December 2020; (D) Zoom-in view of the estimated case counts of 2020. The dotted line indicates 95% CI.

Figure3. Proportion of different serotypes in each district or county of Nanchang, China from 2019-2022 (A-D).

Figure 4. Positive detection rates of enteroviruses in each district or county of Nanchang, China from 2019-2022 (A-D)**.**(E) Population density (data of 2020 census) of each district or county of Nanchang.

Figure 5. Serotyping of UEV using nested RT-PCR and sequencing.(A) 2% agarose gel electrophoresis (M: DNA marker; N: negative control; Lane 1: CVA6; lane 2-5: UEV samples); (B) Proportions of different serotypes during 2019-2022; (C) Phylogenetic tree of CVA2, CVA4, CVA5, CVA9, CVB3 and ECHO9 based on partial 5'UTR sequences; (D) Pairwise nucleotide similarity of CVA2, CVA4, CVA5, and CVB3, respectively.

Figure 6. Comparative prevalence of monthly EV infections detected among HFMD cases in Nanchang, China, 2010 to 2022. (A) Comparative prevalence of CVA6 and CVA16 during the COVID-19 epidemic. (B-C) Asynchronous seasonality, explained by negative epidemiological interactions. (D) Synchronous seasonality, explained by positive epidemiological interactions. r : correlation coefficients;p : p value.

Figure 7. Heatmap and Spearman correlation analysis of monthly HFMD cases associated with CVA6 or CVA16 from 2019-2022 (A-D) and observed monthly cases associated with CVA6 (E) and CVA16 (F) in Nanchang from 2015 to 2020, compared with the fitted (2015-2019) and predicted (2020) case counts obtained using the ARIMA models in the absence of COVID-19 intervention. r : correlation coefficients;p : p value. The light-purple shaded part indicates the observed (black) and estimated (white) case counts from early January to the end of December 2020. The dotted line indicates 95% CI.

Figure 8. Comparative analysis of sample types. (A) Numbers of throat swab, anal swab and stool during 2019-2022; (B) Positive rate of EVs from throat swab, anal swab and stool during 2019-2022; (C-F) Ct values of real time RT-PCR targeting CVA6, CVA10, CVA16 and UEV, respectively. dashed line: median; dotted line: 95% CI, *:p <0.05; **: p <0.01.