ORIGINAL RESEARCH

# Profiles of Human Enteroviruses Associated with Hand, Foot, and Mouth Disease in Nanjing, China

Qian Chen, MS; Qihua Zhang, BS; Zheng Hu, BS

## ABSTRACT

**Objective:** Hand, foot, and mouth disease (HFMD) is a common infectious disease caused by a group of viruses. The causative viruses have changed over time, and there is a need for a more effective protective vaccine. In this study, we investigated the profiles of human enteroviruses that caused HFMD outbreaks in Nanjing in 2015, with the goal of guiding the future prevention and treatment of HFMD.

**Methods:** Specimens were collected from 1097 patients admitted to our hospital and diagnosed with HFMD. Enteroviruses in the specimens were identified by real-time polymerase chain reaction and epidemiological patterns were analyzed with the clinical data.

**Results:** Among the 1097 clinically diagnosed HFMD cases, 916 cases were confirmed by laboratory tests. The results showed that the main infectious virus was coxsackievirus A6 (CVA6) (41.75%), followed by enterovirus 71 (EV71) (27.48%), coxsackievirus A16 (7.43%), coxsackievirus A10 (6.84%), and others (16.51%). Further investigation indicated that CVA6 caused mild cases of HFMD, while EV71 caused severe cases. More enterovirus positive cases were reported from rural areas than from urban areas.

**Conclusions:** CA6 and EV71 were the chief pathogenic viruses of HFMD cases in the present study. Schools, childcare centers, and families from rural areas should be the major targets for prevention and awareness of HFMD. This study will provide information useful in the prevention and management of HFMD and the development of relevant vaccines for HFMD in the future. (Disaster Med Public Health Preparedness. 2019;13:740–744)

**Key Words:** hand, foot, and mouth disease, coxsackievirus A6, enterovirus 71, epidemiological monitoring

Hand, foot, and mouth disease (HFMD) is a relatively common viral illness that predominantly occurs in children less than 5 years old. This syndrome is characterized by fever and vesicular exanthema on hands, feet, and mouth. Generally, HFMD is mild and self-limited, but severe complications can occur including central nervous system and cardiopulmonary manifestations (such as encephalomyelitis, aseptic meningitis, acute flaccid paralysis, pulmonary edema, end-organ dysfunction, and even death).[1,2] Historically, outbreaks of HFMD have mainly been caused by various ratios of enterovirus 71 (EV71) and coxsackievirus A16 (CVA16).[1-3] Recently, a number of reports have demonstrated that additional pathogens are involved in HFMD outbreaks. Since 2008, other enteroviruses (EVs), such as CVA6 and CVA10, along with EV17 and CVA16, were also identified as the major pathogens in several HFMD outbreaks.[4-14]

HFMD has been a major public health concern in China since the first case report[15] in Shanghai in 1982. In 2008, a huge outbreak of HFMD caused by EV71 in FuYang City, China, had a significant impact on the health of local infants and young children.[16-18] Consequently, in order to reinforce the prevention and control of HFMD, the National Health Commission of China decided to include this disease in the list of infectious diseases that it manages. Currently, only one EV71 inactivated vaccine has been developed in China. Although this vaccine has been demonstrated to be of great value in protecting against EV71, it offers no protection against the other common pathogens[19] such as CVA6, CVA10, and CVA16. A safe, effective, and affordable multivalent vaccine targeting all types of HFMD viruses is needed. A database including real-time etiology and clinical manifestations of HFMD is essential for developing appropriate interventions for the prevention and control of HFMD. In this study, we provided an up-to-date profile of human EVs associated with HFMD outbreaks in Nanjing from a total of 1097 cases that occurred in 2015.

## METHODS AND MATERIALS

### Sample Collection

The Ethics Committee of Children's Hospital of Nanjing Medical University approved this study in



 DOI: 10.1017/dmp.2018.155

accordance with the Helsinki Declaration. A total of 1097 throat swab samples were obtained from patients with suspected HFMD at Children's Hospital of Nanjing Medical University from January 4, 2015, to December 30, 2015. All collected samples were stored at −80°C for later analysis.

### Viral Detection

Viral RNA was extracted from all samples using the QIAamp Viral RNA Mini Kit (Qiagen Inc., Hilden, Germany). Briefly, 200 μL eluted specimen was lysed at room temperature, viral RNA was efficiently captured by the columns, and then ultrapure RNA was eluted and stored at −80°C. A series of EV detection kits (Sansure Biotech, Inc, Changsha, China) were used to detect EV infection and EV types, including EV71, CVA16, CVA6, and CVA10. Reverse transcription and real-time polymerase chain reaction were performed with a CFX96 real-time polymerase chain reaction system (Bio-Rad Corporation, USA) and conditions were maintained in accordance to the manufacturer's instructions: 1 minute at 95°C, 30 minutes at 50°C, 1 minute at 95°C, and 45 cycles of 15 seconds at 95°C and 30 seconds at 55°C. The results with cycle threshold values lower or equal to 40 were considered positive.

### Statistical Analysis

All statistical analyses were performed using the SPSS 19.0 software package (SPSS Corporation, Illinois, USA). *P* values of less than .05 were regarded as statistically significant.

## RESULTS

### Description of Patients

From January 4, 2015, to December 30, 2015, a total of 1097 HFMD cases were reported in Children's Hospital of Nanjing Medical University. The sex ratio of the patients was 1.64:1 (681 boys and 416 girls) and their ages ranged from 1.44 to 144 months, with 1074 of the patients (97.903% of all cases) under 6 years old (Table 1). The majority of patients between 1 to 3 years old had been diagnosed with HFMD. Later, we analyzed the occupational composition of those HFMD cases, as shown in Table 2. Of them, 875 patients (875/1097, 79.76%) did not attend kindergarten or school, 199 patients (199/1097, 18.14%) were kindergarten students, and 23 patients (23/1097, 1.91%) were grade school students. All these children were clinically diagnosed with HFMD, including 246 severe cases and 851 mild cases. Of these, 97.15% (239/246) of the severe cases and 98.12% (835/851) of the mild cases were children under 6 years old.

### Laboratory Identification of Etiologic Pathogen of the Outbreak

Out of the total 1097 cases, 916 cases were confirmed as HFMD positive by EV kits. Because of the insufficient nucleic acid samples, 68 cases failed to be tested by CVA6 and CVA10 kits. From the 848 cases tested by EV, EV71, CVA16, CVA6, and CVA10 kits, we found the following major infectious pathogens: CVA6 (354/848, 41.75%), EV71 (233/848, 27.48%), CVA16 (63/848, 7.43%), CVA10 (58/848, 6.84%), and other EV pathogens (140/848, 16.51%) (Table 3). There were significant differences between all these pathogens ($\chi^2 = 462.24$, $P < .05$). Obviously, CVA6, rather than EV71, was detected most often among the 848 clinical HFMD patients ($\chi^2 = 38.14$, $P < .05$). Although CVA6 (340/663, 51.28%) was the leading causative pathogen in mild HFMD, EV71 (152/185, 82.16%) was still the major pathogen to cause severe HFMD accompanied by severe symptoms. The ratio of severe to mild cases infected with EV71 (1.88, 152:81) was much higher than that of cases infected with CVA6, CVA10, or CVA16 (only 0.04, 14:340; 0.12, 6:52; and 0.03, 2:61; respectively) (Tables 1 and 3).

**TABLE 1**

**Demographic Characteristics of Hand, Foot, and Mouth Disease Cases in Nanjing (2015) with Positive Test Results for Various Enteroviruses**

| | Enterovirus | | | | |
|---|---|---|---|---|---|
| **Demographics of Infected Patients** | **EV-71** | **CV-A6** | **CV-A10** | **CV-A16** | ***P*** |
| Median age (months) | 27.72 | 18.60 | 18.92 | 25.56 | Varies[a-d] |
| Male/female ratio | 1.59 | 1.90 | 1.76 | 1.17 | .326 |
| Severe/mild ratio[e] | 1.88 | 0.04 | 0.12 | 0.03 | <.0001 |
| Home/school ratio[f] | 2.15 | 8.08 | 10.6 | 3.50 | <.0001 |
| Urban/rural ratio | 1.22 | 2.54 | 2.10 | 1.54 | .0002 |

[a]There is no significant difference in median age between EV-71 positive and CV-A16 positive children ($P = .131$).

[b]The median ages of CV-A6 positive and CV-A10 positive children are significantly different than the median ages of EV-71 positive children ($P < .001$ and $P < .001$, respectively).

[c]There is no significant difference in median age between the CV-A6 positive children and the CV-A10 positive children ($P = .851$).

[d]The median age of CV-A16 positive children is significantly different than that of the CV-A6 positive and CV-A10 positive children ($P = .006$ and $P = .026$, respectively).

[e]Patients presenting with hand, foot, and mouth disease and with serious complications, including encephalitis, meningitis, acute flaccid paralysis, cardiorespiratory failure, or death, were considered to have severe cases. Patients without the abovementioned serious complications were classified as mild cases.

[f]School includes kindergarten children and school students.

### Epidemiological Data

Subsequently, we also investigated the regional distribution patterns of the detected EV types. As shown in Table 4, the main infectious viruses in both Nanjing's urban and suburban areas was CVA6 (43.18%, 19/44; 49.10%, 217/442), while the majority of infectious viruses from other cities in Jiangsu Province were found to be CVA6 and EV71 (37.04%, 50/135; 34.81%, 47/135). Cases from other provinces in China had EV71 infections (39.65%, 90/227).



https://doi.org/10.1017/dmp.2018.155 Published online by Cambridge University Press

## TABLE 2

**Living Environments of Patients with Hand, Foot, and Mouth Disease of Varying Severity**

| | n | No School | Kindergarten | Grade School |
|---|---|---|---|---|
| | | No. (%) | | |
| Severe | 246 | 177 (71.95) | 62 (25.20) | 7 (2.85) |
| Mild | 851 | 698 (82.02) | 137 (16.10) | 16 (1.88) |
| Total | 1097 | 875 (79.76) | 199 (18.14) | 23 (2.10) |

## TABLE 3

**Rate of Positive Enterovirus (EV) Detection Tests in 848 Clinical Hand, Foot, and Mouth Disease Cases of Varying Severity**

| | n | EV71 | CA16 | CA6 | CA10 | Other EVs |
|---|---|---|---|---|---|---|
| | | No. (%) | | | | |
| Severe | 185 | 152 (82.16) | 2 (1.08) | 14 (7.57) | 6 (3.24) | 11 (5.95) |
| Mild | 663 | 81 (12.22) | 61 (9.20) | 340 (51.28) | 52 (7.84) | 129 (19.46) |
| Total | 848 | 233 (27.48) | 63 (7.43) | 354 (41.75) | 58 (6.84) | 140 (16.51) |

## TABLE 4

**The Enterovirus (EV) Distribution of Hand, Foot, and Mouth Disease Cases from Different Regions of China**

| | n | EV71 | CA16 | CA6 | CA10 | Other EVs |
|---|---|---|---|---|---|---|
| | | No. (%) | | | | |
| Nanjing urban areas | 44 | 9 (20.45) | 1 (2.27) | 19 (43.18) | 7 (15.91) | 8 (18.18) |
| Nanjing rural areas | 442 | 87 (19.68) | 35 (7.92) | 217 (49.10) | 30 (6.79) | 73 (16.52) |
| Other cities in Jiangsu | 135 | 47 (34.81) | 8 (5.93) | 50 (37.04) | 7 (5.19) | 23 (17.04) |
| Other provinces | 227 | 90 (39.65) | 19 (8.37) | 68 (29.96) | 14 (6.17) | 36 (15.86) |

### Risk Factors for HFMD

The patients aged from 11.75 to 21.75 months and from 27 to 53 months had high risk for EV infections. Obviously, EV infection in children presented an age-associated pattern. Further investigations indicated that there were no dramatic differences between EV types in children of different ages, although the median age of EV71- and CVA16-infected children was older than that of CVA6- and CVA10-infected children Table 1).

The reported cases of HFMD included more boys (681 cases) than girls (416 cases). Boys were more likely to become infected by EV. Regarding the EV types, CVA6 has the highest male/female ratio of all the detected EVs (Table 1). We also explored the risk of infections based on the individual's living environment. Unexpectedly, our results suggested that children living in dispersed areas were more likely to acquire EV-associated HFMD, which indicates a possible relationship between the living environment and HFMD (Table 1).

### Onychomadesis

It has been reported that onychomadesis is a sequel of children infected by CVA6. We randomly chose 221 cases from the 1097 patients diagnosed with HFMD and investigated their nails after the infection. Among 145 cases with effective follow-up, 14 cases (14/145, 9.66%) had at least 1 fingernail or toenail falling off (the worst case had 10 fingernails and 2 toenails falling off), including 11 cases of CVA6, 1 case of EV71, and 2 cases of other common enteroviruses. Of the children who developed onychomadesis, 78.57% (11/14) were infected with CVA-6. Among the children infected with CVA6, 22.0% (11/50) developed onychomadesis. Among them, only 1 child had a severe case of EV71 infection, while all others had mild infections. Most of the onychomadesis developed within 1 month after infection and



https://doi.org/10.1017/dmp.2018.155 Published online by Cambridge University Press

the new nails had no sign of falling off in 1 year. Pain was not associated with falling nails and growth of new ones.

## DISCUSSION

HFMD has become a significant public health concern worldwide since 1998. More than 90% of HFMD cases were caused by Human enterovirus A and EV71. The main pathogens of HFMD are sometimes different in different epidemic periods and epidemic regions. Lack of information regarding the pathogenic role, geographic distribution, and epidemiology of EVs will lead to misdiagnosis or delayed treatment of HFMD. In China, the previous surveillance of HFMD has mainly focused on EV71 and CVA16, but recent reports revealed that the leading causative agents of HFMD have changed in the last few years. Though EV71 was a major pathogen, other EVs, especially CVA6 and CVA10, are prevalent and have become the predominant causative agents.[12-14] In Jiangsu province, EV71 and CVA16 were the major etiologic agents of HFMD[20] from 2009 to 2012. For example, a previous study[21] showed that EV71 was the main pathogen causing HFMD in Suzhou, Jiangsu Province from April to July 2013. However, another study[14] showed that CVA6 was already the main causative agent of HFMD in Nanjing, Jiangsu Province in 2013. In this study, we demonstrated the up-to-date profile of HFMD in Nanjing in 2015.

Compared with the reported profile of HFMD in Nanjing[14] in 2013, which showed CVA6 and EV71 as the 2 major pathogens for HFMD, our present study found that the percentage of CVA6 rose from 30.00% to 41.75%, while EV71 increased from 17.41% to 27.48% and CVA10 increased from 3.33% to 6.84%. Notably, the percentage of CVA16 increased from 0.74% to 7.43%. Previous studies provide evidence that EV71 is more likely to cause serious symptoms than other EVs are.[15,22] In this study, most patients infected with EV71 had severe symptoms while patients infected with the other EVs had more mild infections.

It has been reported that most HFMD patients are between 6 months and 5 years old.[23] In this study, however, children aged from 1.44 to 144 months were enrolled. According to our results, 2 age groups were more likely to be infected with HFMD. One age group was from 11.75 to 21.75 months; this is because maternal immunity decreases at that age and the infant innate immune system has not been established. Another age group was from 27 to 53 months. In this group, children are very active and did not have good hygienic practices. Besides, previous studies also showed no significant difference between the gender distributions of children with HFMD,[23] while other studies showed that boys were easily infected with HFMD.[24,25] In our study, we found a male/female ratio of 1.64 (681/416) in all HFMD cases, which is consistent with the previous report of Li Wei Ang and Kow-Tong Chen.[24,25] It might be that boys are more active than girls and consequently are more likely to become infected. Interestingly, different EVs showed different gender distributions. CVA6-infected children had the highest male/female ratio, 1.90, while CVA16-infected children had a ratio of 1.17, which was much lower than the average level. However, the mechanisms underlying such gender distribution patterns still needs to be addressed in future studies.

Nanjing is a big developed city in east China, and naturally, it has good medical and childcare/school facilities. Urban children have better medical facilities, but higher population density may lead to higher risk of infections. Children living in rural areas may not go to the hospital, leading to fewer reported cases. Interestingly, CVA6 and CVA10 had much higher urban/rural ratio than EV71 and CVA16. One possibility was that mild cases caused by CVA6 and CVA10 in rural areas were not reported or hospitalized. Alternatively, EV71 and CVA16 may still be still the predominant agents in rural areas, though EV71 and CVA6 appeared to be the major pathogens of Nanjing in 2015. Moreover, we found that the home/school ratio of EV71 and CVA16 were much lower than that of CVA6 and CVA10. When considering the more severe cases of EV71-infected children, it was essential for the government to pay more attention to children in rural areas who were less likely to be in school or childcare.

In conclusion, this study showed the seasonal pattern of HFMD outbreaks of Nanjing in 2015. Due to recent changes in the HFMD pathogen spectrum and the emergence of the CA6 pathogen, the control of HFMD has become more difficult. The presentation of CA6-associated HFMD was somewhat different from that of typical HFMD due to EV71 and CVA16. CA6 should receive more attention as one of the major pathogens responsible for causing HFMD. So, comprehensive surveillance of pathogens for HFMD needs to be carried out. Research on etiology, epidemiology, laboratory diagnosis, and animal models should be carried out to pave the way for developing a multivalent vaccine that combines EV71, CVA16, and CA6.

Children aged 11.75 to 21.75 months and 27 to 53 months had the highest risk of becoming infected with outbreaks of HFMD. So, more attention should be paid to children who have just been weaned and have bad schools and childcare in rural areas. Our results would help the medical professionals and public health officials to develop effective measures for strengthening health education and management of HFMD outbreaks.

## About the Authors

*Institute of Pediatrics, Children's Hospital of Nanjing Medical University, Nanjing, Jiangsu, China.*

*Correspondence and reprint requests to Zheng Hu, Institute of Pediatrics, Children's Hospital of Nanjing Medical University, No.72 Guangzhou Road, Nanjing, Jiangsu 210008, China (e-mail: hz18951769051@163.com).*



https://doi.org/10.1017/dmp.2018.155 Published online by Cambridge University Press

## Acknowledgements

We wish to acknowledge the support of the Nanjing Medical Science and Technology Development Foundation (No. QRX11323 and No. QRX17078).

## Competing Interests

The authors declare no competing interests.

## REFERENCES

1. Repass GL, Palmer WC, Stancampiano FF. Hand, foot, and mouth disease: identifying and managing an acute viral syndrome. *Cleve Clin J Med*. 2014;81(9):537–543.
2. Nassef C, Ziemer C, Morrell DS. Hand-foot-and-mouth disease: a new look at a classic viral rash. *Curr Opin Pediatr*. 2015;27(4):486–491.
3. Chang LY, Lin TY, Huang YC, et al. Comparison of enterovirus 71 and coxsackie-virus A16 clinical illnesses during the Taiwan enterovirus epidemic, 1998. *Pediatr Infect Dis J*. 1999;18(12):1092–1096.
4. Blomqvist S, Klemola P, Kaijalainen S, et al. Co-circulation of coxsackieviruses A6 and A10 in hand, foot and mouth disease outbreak in Finland. *J Clin Virol*. 2010;48(1):49–54.
5. Bracho MA, Gonzalez-Candelas F, Valero A, et al. Enterovirus co-infections and onychomadesis after hand, foot, and mouth disease, Spain, 2008. *Emerg Infect Dis*. 2011;17(12):2223–2231.
6. Wu Y, Yeo A, Phoon MC, et al. The largest outbreak of hand; foot and mouth disease in Singapore in 2008: the role of enterovirus 71 and coxsackievirus A strains. *Int J Infect Dis*. 2010;14(12):e1076–e1081.
7. Lo SH, Huang YC, Huang CG, et al. Clinical and epidemiologic features of Coxsackievirus A6 infection in children in northern Taiwan between 2004 and 2009. *J Microbiol Immunol Infect*. 2011;44(4):252–257.
8. Fujimoto T, Iizuka S, Enomoto M, et al. Hand, foot, and mouth disease caused by coxsackievirus A6, Japan, 2011. *Emerg Infect Dis*. 2012;18 (2):337–339.
9. Gopalkrishna V, Patil PR, Patil GP, et al. Circulation of multiple enterovirus serotypes causing hand, foot and mouth disease in India. *J Med Microbiol*. 2012;61(Pt 3):420–425.
10. Mirand A, Henquell C, Archimbaud C, et al. Outbreak of hand, foot and mouth disease/herpangina associated with coxsackievirus A6 and A10 infections in 2010, France: a large citywide, prospective observational study. *Clin Microbiol Infect*. 2012;18(5): E110–E118.
11. He YQ, Chen L, Xu WB, et al. Emergence, circulation, and spatiotemporal phylogenetic analysis of coxsackievirus a6- and coxsack-ievirus a10-associated hand, foot, and mouth disease infections from 2008 to 2012 in Shenzhen, China. *J Clin Microbiol*. 2013;51 (11):3560–3566.
12. Lu QB, Zhang XA, Wo Y, et al. Circulation of Coxsackievirus A10 and A6 in hand-foot-mouth disease in China, 2009-2011. *PLoS One*. 2012;7 (12):e52073.
13. Guan H, Wang J, Wang C, et al. Etiology of multiple non-EV71 and non-CVA16 enteroviruses associated with hand, foot and mouth disease in Jinan, China, 2009-June 2013. *PLoS One*. 2015;10(11):e142733.
14. Hu YQ, Xie GC, Li DD, et al. Prevalence of Coxsackievirus A6 and Enterovirus 71 in hand, foot and mouth disease in Nanjing, China in 2013. *Pediatr Infect Dis J*. 2015;34(9):951–957.
15. Xing W, Liao Q, Viboud C, et al. Hand, foot, and mouth disease in China, 2008-12: an epidemiological study. *Lancet Infect Dis*. 2014;14 (4):308–318.
16. Yang F, Ren L, Xiong Z, et al. Enterovirus 71 outbreak in the People's Republic of China in 2008. *J Clin Microbiol*. 2009;47(7):2351–2352.
17. Zhang Y, Tan XJ, Wang HY, et al. An outbreak of hand, foot, and mouth disease associated with subgenotype C4 of human enterovirus 71 in Shandong, China. *J Clin Virol*. 2009;44(4):262–267.
18. Zhang Y, Zhu Z, Yang W, et al. An emerging recombinant human enterovirus 71 responsible for the 2008 outbreak of hand foot and mouth disease in Fuyang city of China. *Virol J*. 2010;7:94.
19. Liu Q, Tong X, Huang Z. Towards broadly protective polyvalent vaccines against hand, foot and mouth disease. *Microbes Infect*. 2015;17 (2):155–162.
20. Liu WD, Wu Y, Liang Q, et al. Epidemiological characteristics and temporal-spatial clustering analysis on hand-foot-mouth disease in Jiangsu province, 2009–2011 [in Chinese]. *Zhonghua Liu Xing Bing Xue Za Zhi*. 2012;33(8):813–817.
21. Chen Z, Sun H, Yan Y, et al. Epidemiological profiles of hand, foot, and mouth disease, including meteorological factors, in Suzhou, China. *Arch Virol*. 2015;160(1):315–321.
22. Zhang Q, MacDonald NE, Smith JC, et al. Severe enterovirus type 71 nervous system infections in children in the Shanghai region of China: clinical manifestations and implications for prevention and care. *Pediatr Infect Dis J*. 2014;33(5):482–487.
23. Zou XN, Zhang XZ, Wang B, et al. Etiologic and epidemiologic analysis of hand, foot, and mouth disease in Guangzhou city: a review of 4,753 cases. *Braz J Infect Dis*. 2012;16(5):457–465.
24. Ang LW, Koh BK, Chan KP, et al. Epidemiology and control of hand, foot and mouth disease in Singapore, 2001-2007. *Ann Acad Med Singapore*. 2009;38(2):106–112.
25. Chen KT, Chang HL, Wang ST, et al. Epidemiologic features of hand-foot-mouth disease and herpangina caused by enterovirus 71 in Taiwan, 1998-2005. *Pediatrics*. 2007;120(2):e244–e252.



https://doi.org/10.1017/dmp.2018.155 Published online by Cambridge University Press