Molecular characteristics of hand, foot, and mouth disease for hospitalized pediatric patients in Yunnan, China

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Abstract
Hand, foot, and mouth disease (HFMD) is a common infectious disease caused by multiple enteroviruses (EVs) in China. To better understand the etiologic agents and clinical characteristics of HFMD, we conducted this study in Yunnan, China.

In this study, 1280 stool specimens were collected from pediatric patients hospitalized for treatment of HFMD in 2010. EV was detected with nested reverse transcription polymerase chain reaction and directly genotyped by gene sequencing of the viral protein 1 (VP1) region. Phylogenetic analysis was performed based on the VP1 partial gene and the clinical characteristics were analyzed using SPSS Software.

Of 1280 specimens, 1115 (87.1%) tested positive for EV. Seventeen different EV serotypes were detected. Coxsackievirus A16 (CA16) was the most frequently detected serotype (615/1115 cases, 55.1%), followed by enterovirus 71 (EV71; 392/1115, 35.2%), CA10 (45/1115, 4.0%), and CA4 (23/1115, 2.1%). Among the 709 severe cases, CA16, EV71, CA10, and CA4 accounted for 48.0%, 42.0%, 3.5%, and 2.3%, respectively. Of the 26 critical cases, 13 were caused by EV71, 9 by CA16, 2 by CA4, and 1 each were the result of CA10 and E9, respectively. All EV71, CA16, CA10, and CA4 isolates were highly homologous to the strains isolated from mainland China, and belonged to the C4a, B1a, G, and C genotypes, respectively.

Our study showed that EV71 and CA16 were the main causative agents for severe and critical HFMD, but other serotypes can also cause severe and critical cases.

Abbreviations: CA16 = coxsackievirus A16, EV = Enterovirus, EV71 = enterovirus 71, HFMD = hand, foot, and mouth disease, PCR = polymerase chain reaction, VP1 = viral protein 1.

Keywords: clinical features, Enterovirus, hand, foot, and mouth disease, pathogen, serotyping

1. Introduction

Hand, foot, and mouth disease (HFMD) is a common infectious disease in China, which mainly affects children younger than 5 years.[1,11] The symptoms are generally mild and self-limiting. A few patients may develop severe complications, including encephalitis, pneumonia, myocarditis, brain-stem encephalitis, and acute flaccid paralysis.[6,7]

HFMD is caused by more than 20 different enteroviruses (EVs), particularly enterovirus 71 (EV71) and coxsackievirus A16 (CA16). Other EVs including CA2, 4, 5, 6, 8, and 10, CB1–5, also result in HFMD pathogenesis with diverse clinical manifestations.[4,8,9] EV comprises more than 100 serotypes, all of which belong to the genus Enterovirus and the family Picornaviridae. These viruses are characterized by a single positive-strand 7.4 kb genomic RNA with a long open reading frame that encodes 4 structural proteins [viral protein 1 (VP1) through VP4] and 7 nonstructural proteins (2A, 2B, 2C, 3A, 3B, 3C, and 3D). VP1 is the immunodominant protein of the picornavirus capsid and contains serotype-specific information. Thus, the VP1 has been used for virus serotype identification and molecular epidemiology.[13,10,11]

Since 2008, more than 1 million cases have been reported annually in mainland China, with more than 100 deaths per year. Yunnan is a major tourism province located in Southwest China, with a typical subtropical plateau monsoon climate. More than 80,000 cases presenting with HFMD are reported each year. It, however, remains unclear whether the epidemic of HFMD in Yunnan is like that in the rest of Southwest China. Here we report the etiology and clinical characteristics of hospitalized children with HFMD in Yunnan, China. These results help further understand the molecular epidemiological features of HFMD and EV infection in Southwest China.
occurred during the year, a seasonal increase in HFMD cases was observed in May, accounting for 20.7% of all cases, and there was a small increase in November, with the greatest number of severe cases occurring in June (Fig. 1).

The examination of stool specimens from these 1280 patients revealed the presence of EV in 1115 cases (87.1%). Among these 1115 patients, there were 376 general patients, 709 severely ill patients, 26 critically ill patients, and 4 deaths. Further VP1 sequencing and molecular typing showed that all sequences obtained were assigned to 17 different EV serotypes (Table 1). CA16 was the most frequently detected serotype (615/1115, 55.1%), followed by EV71 (392/1115, 35.2%), CA10 (45/1115, 4.0%), and CA4 (23/1115, 2.1%). Among the 709 severe cases, CA16, EV71, CA10, and CA4 accounted for 48.0%, 42.0%, 3.5%, and 2.3%, respectively ($P < .0001$). In addition, another 13 EVs also resulted in severe clinical manifestations. Of the 26 critical cases, 13 were caused by EV71, 9 by CA16, 2 by CA4, and 1 each were the result of CA10 and E9. Two deaths were the result of EV71 infection, whereas CA16 and CB2 caused 1 death each.

EV71 infection more often results in meningoencephalitis and hyperarousal ($P < .0001$), whereas CA4 is more likely to cause fever, vomiting, and convulsion. For CA10, the male/female ratio of patients was highest, where it reached 2.21:1. Moreover, the 26 critical cases frequently showed coma and/or convulsions (Table 2).

Phylogenetic analyses showed that EV71 viruses belonged to the C4a subgenotype, CA16 viruses belonged to the B1a genotype, CA10 viruses belonged to the G genotype, and CA4 viruses were assigned to the C genotype (Fig. 2), and the EV71, CA16, CA10, and CA4 strains were highly homologous with the strains isolated from mainland China.

4. Discussion

In this study, the epidemiological characteristics of the HFMD epidemic in Yunnan province of China showed some similarities with previous reports.6,12,14 The occurrence of HFMD had apparent seasonal distribution in Yunnan, China. The number of HFMD cases reached a maximum in May; we also observed a small increase in November, which is a common phenomenon in the southern provinces.6,15–17 This may be due to the different climate conditions between the southern and northern provinces of China.6,15–16 In addition, the mean age of all hospitalized pediatric patients was 2.60 ± 1.41 years old and 76.87% of all reported HFMD severe and critical cases were younger than 3 years old, which is also consistent with the reports from other countries and regions.18–21 Most patients with HFMD need to be transferred to a comprehensive hospital, in which patients
with HFMD might be timelously diagnosed and treated, and thus decreasing the risk of severe HFMD.\textsuperscript{[22,23]} Thus, these pediatric patients should be targeted as the main population in any prevention program.

In addition to EV71 and CA16, other EVs, including CA4, CA6, CA12, and CB3, are also associated with HFMD and cocirculated in outbreaks and sporadic cases.\textsuperscript{[14,16,19,20,24]} In the present study, we identified 17 serotypes of EVs, including EV71, CA16, CA10, CA4, CA2, CA5, CA6, CA8, CA12, CA13, CA24, CB2, CB4, E9, E11, E16, and E30, from stool samples of patients hospitalized for HFMD in Yunnan, China. Of these, CA16 was the most frequently detected serotype, followed by EV71, CA10, and CA4. However, in Yunnan, during 2016, 11 serotypes were identified, followed by EV71, CA10, and CA4. As for disease severity across the EV strains and symptoms distribution of the children with HFMD, there were no significant difference among the positive rate of EV71, CA16, CA10, and CA4. For disease severity across the EV strains and symptoms in different age groups, there was also no statistically significance. We, however, found that fever (>38°C), oral ulcer, and hand and foot rash were the most frequent symptoms for hospitalized pediatric patients. And limb shaking, startle, and vomiting were the most frequent severe symptoms for children aged 1 to 2 years. Meningoencephalitis was frequently observed in patients infected by EV71. Thus, the duration of fever for 3 days (>38°C), EV71 infection, limb shaking, startle, and vomiting are risk factors for severe HFMD, which is also consistent with the previous report.\textsuperscript{[43]}

The prevalent serotypes were, however, different in hospitalized and not hospitalized children.\textsuperscript{[11]} In addition, the causative agents of HFMD often overlap with those of aseptic meningitis including CB3, CB5, E9, and E30, which are often associated with outbreaks of aseptic meningitis in China.\textsuperscript{[12,28-31]} This is not unexpected as EVs have long been identified as the main agents of aseptic meningitis.\textsuperscript{[32-34]} Thus, it is possible that some of these serotypes may become predominant in HFMD in the future and we should pay special attention to the EVs associated with aseptic meningitis.

EV71 has been identified as the most prevalent serotype causing severe and critical cases of HFMD, and CA16 is thought to be associated with mild HFMD and self-limited symptoms. There have been reports of CA16, CA10, and CA4 being associated with severe HFMD independently of EV71.\textsuperscript{[15,29,30,42]} In the present study, we found that CA16 was the prevalent serotype causing severe and critical cases of HFMD, rather than EV71, this kind of preference may be limited to specific populations. In addition, other serotypes including CA4, CA10, and E9, were also associated with severe and critical cases. In particular, CB2 and CA16 had a strong association with fatal cases. It, however, remains difficult to distinguish between these serotypes in the clinical context. Long-term attention should also be paid to preventing infections with serotypes other than EV71 and CA16 in the future. In addition, for the age distribution of the children with HFMD, there were no significant difference among the positive rate of EV71, CA16, CA10, and CA4. As for disease severity across the EV strains and symptoms in different age groups, there was also no statistically significance. We, however, found that fever (>38°C), oral ulcer, and hand and foot rash were the most frequent symptoms for hospitalized pediatric patients. And limb shaking, startle, and vomiting were the most frequent severe symptoms for children aged 1 to 2 years. Meningoencephalitis was frequently observed in patients infected by EV71. Thus, the duration of fever for 3 days (>38°C), EV71 infection, limb shaking, startle, and vomiting are risk factors for severe HFMD, which is also consistent with the previous report.\textsuperscript{[43]}

When the VP1 sequences from the predominant EV71, CA16, CA4, and CA6 strains isolated from other provinces of China where compared, there were no characteristic mutations and all Yunnan isolates belonged to the corresponding predominant genogroups, respectively. This indicated that those predominant genogroups are relatively stable and could form the basis for preventative programs in China.

Thus, surveillance of the etiology might help with predicting potential outbreak serotypes and aid in effectively developing related vaccines, except the EV71 vaccine. In addition to the host and natural factors, including young age and a confirmed

### Table 1

| No. (%) cases | Mild case | Severe case | Critical case | Death case | Total |
|--------------|-----------|-------------|---------------|------------|-------|
| Serotype     | n = 541   | n = 709     | n = 26        | n = 4      | n = 1280 |
| EV71         | 79 (14.60)| 206 (42.03)| 13 (50.00)    | 2 (50.00)  | 392 (30.63) |
| CA16         | 265 (48.99)| 340 (47.95)| 9 (34.63)     | 1 (25.00)  | 615 (48.05) |
| CA10         | 19 (3.51) | 25 (3.53)  | 1 (3.84)      | 0 (0.00)   | 45 (3.52)  |
| CA4          | 5 (0.92)  | 16 (2.26)  | 2 (7.69)      | 0 (0.00)   | 23 (1.80)  |
| CA2          | 0 (0.00)  | 1 (0.14)   | 0 (0.00)      | 0 (0.00)   | 1 (0.08)   |
| CA6          | 3 (0.56)  | 6 (0.85)   | 0 (0.00)      | 0 (0.00)   | 9 (0.70)   |
| CA6          | 1 (0.18)  | 5 (0.71)   | 0 (0.00)      | 0 (0.00)   | 6 (0.47)   |
| CA9          | 2 (0.37)  | 5 (0.71)   | 0 (0.00)      | 0 (0.00)   | 7 (0.54)   |
| CA12         | 0 (0.00)  | 2 (0.28)   | 0 (0.00)      | 0 (0.00)   | 2 (0.15)   |
| CA13         | 0 (0.00)  | 1 (0.14)   | 0 (0.00)      | 0 (0.00)   | 1 (0.08)   |
| CA24         | 0 (0.00)  | 1 (0.14)   | 0 (0.00)      | 0 (0.00)   | 1 (0.08)   |
| CB2          | 0 (0.00)  | 1 (0.14)   | 0 (0.00)      | 0 (0.00)   | 2 (0.15)   |
| CB4          | 0 (0.00)  | 1 (0.14)   | 0 (0.00)      | 0 (0.00)   | 1 (0.08)   |
| E9           | 0 (0.00)  | 3 (0.42)   | 1 (3.84)      | 0 (0.00)   | 4 (0.31)   |
| E11          | 0 (0.00)  | 1 (0.14)   | 0 (0.00)      | 0 (0.00)   | 1 (0.08)   |
| E16          | 2 (0.37)  | 2 (0.28)   | 0 (0.00)      | 0 (0.00)   | 4 (0.31)   |
| E30          | 0 (0.00)  | 1 (0.14)   | 0 (0.00)      | 0 (0.00)   | 1 (0.08)   |
| Total infection | 376 (69.50) | 709 (100.00) | 26 (100.00) | 4 (100.00) | 1115 (87.11) |
| Nonenterovirus | 165 (30.50) | 0 (0.00)     | 0 (0.00)     | 0 (0.00)   | 165 (12.89) |

CA16 = coxsackievirus A16, EV71 = enterovirus 71.
diagnosis at first visit to hospital, virus factors may also be responsible for the different clinical phenotypes observed in hospitals in China.[22,43]

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Table 2

Comparison of the demographic and the clinical data from patients infected with different enteroviruses.

|                | EV71 | CA16 | CA10 | CA4 | P     |
|----------------|------|------|------|-----|-------|
| No. positive cases | 392  | 615  | 45   | 23  | .0001 |
| No. severe cases  | 298  | 340  | 25   | 16  | .0001 |
| No. critical case  | 13 (3.3) | 9 (1.5) | 1 (2.2) | 2 (8.7) | .047 |
| Age (years)       | 2.70 ± 1.51 | 2.50 ± 1.26 | 2.39 ± 1.22 | 2.51 ± 1.26 | .057 |
| Male/female ratio | 1.58 | 1.67 | 2.21 | 1.86 | .771 |
| Fever (>38°C)     | 365 (93.1) | 523 (85.0) | 40 (88.9) | 22 (85.7) | .001 |
| Oral ulcer        | 382 (97.4) | 590 (85.3) | 42 (83.3) | 19 (82.6) | .010 |
| Hand and foot rash| 368 (93.9) | 582 (94.6) | 41 (91.1) | 19 (82.6) | .001 |
| Meningoencephalitis| 284 (72.4) | 277 (45.0) | 14 (31.1) | 11 (47.8) | .0001 |
| Startle           | 199 (50.8) | 222 (36.1) | 16 (35.6) | 9 (39.1) | .013 |
| Decreased mental status | 59 (15.1) | 59 (9.6) | 9 (20.0) | 4 (17.4) | .064 |
| Coma             | 9 (2.3) | 5 (0.8) | 1 (2.2) | 1 (4.3) | .046 |
| Limb shanking     | 103 (26.3) | 84 (13.7) | 5 (11.1) | 3 (13.0) | .0001 |
| Vomiting          | 79 (20.2) | 84 (13.7) | 6 (13.3) | 6 (26.1) | .022 |
| Headache         | 47 (16.1) | 46 (8.2) | 0 (0) | 1 (4.3) | .010 |
| Convulsion        | 13 (3.3) | 9 (1.5) | 1 (2.2) | 3 (13.0) | .009 |
| Death            | 2 (0.5) | 1 (0.2) | 0 | 0 |

CA16 = coxsackievirus A16, EV71 = enterovirus 71.

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