Original article

Intestinal detoxification time of hand-foot-and-mouth disease in children with EV71 infection and the related factors

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Background: **Hand-foot-and-mouth disease (HFMD) is a common pediatric infectious disease caused by a variety of intestinal viruses. Enterovirus 71 (EV71) is the primary pathogen that might cause severe symptoms and even death in children with HFMD. This study aimed to investigate the intestinal detoxification time of HFMD children with EV71 infection and its related factors.**

Methods: **Sixty-five HFMD children with EV71 infection were followed up. Their stool samples were collected once every 4 to 7 days. Viral nucleic acids were detected by fluorescent polymerase chain reaction until the results became negative. The positive rates of viral nucleic acids were analyzed by the Kaplan-Meier method. The Log-rank test and Cox-Mantel test were used to analyze factors affecting the HFMD children with EV71 infection.**

Results: **On the 2nd, 4th, 6th and 10th week, the positive rates of viral nucleic acids in stool samples of the 65 children were 94.6%, 48.1%, 17.2% and 0, respectively. Univariate analysis showed that the intestinal detoxification time of the children were related to gender, pre-admission disease course, severity of disease, and use of steroids or gamma globulin (P<0.05). Multivariate analysis showed that the severity of disease was an independent factor affecting the intestinal detoxification time (P<0.05), with a relative risk of 2.418.**

Conclusions: **The longest intestinal detoxification time of HFMD children with EV71 infection was 10 weeks. The severity of disease was an important factor affecting the intestinal detoxification time of HFMD children with EV71 infection. Severe HFMD children with EV71 infection had a longer intestinal detoxification time.**

World J Pediatr 2015;11(4):380-385

Key words: **detoxification time;**
enterovirus 71;
hand-foot-and-mouth disease;
relative factor

Introduction

Hand-foot-and-mouth disease (HFMD) is a common pediatric infectious disease caused by a variety of intestinal viruses, typically characterized by fever and rash or herpes over the hands, feet and oral cavity. Most cases can be treated successfully in a week, while a few cases may present with serious complications causing death, including aseptic meningitis, encephalitis, acute flaccid paralysis and neurogenic pulmonary edema (NPE). There are more than 20 types of intestinal viruses that can cause HFMD. Among them, enterovirus 71 (EV71) is prone to outbreak or to be prevalent because of its strong infectivity, complex route of transmission and fast transmission speed.[1] Studies[2,3] have confirmed that neurological complications which can cause NPE and circulatory collapse are particularly frequent after EV71 infection and that EV71 is the primary pathogen causing severe HFMD and death among children. Isolation of HFMD children with EV71 infection is an important measure to prevent the prevalence of HFMD. Detoxification time of HFMD children is a crucial reference for formulating HFMD isolation policies. However, so far there are few studies concerning the detoxification time of HFMD children with EV71 infection. In 1999, Chung et al[4] followed up 2 patients with EV71 infection and found that the stool specimen of one patient was still positive in pathogen detection in the sixth week after onset. In 2009, Pan et al[5] found that the detoxification time of EV71 virus through feces could persist for 10 weeks. Until now, the precise

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doi: 10.1007/s12519-015-0045-z

intestinal detoxification time of HFMD children with EV71 infection is not clear. Therefore, this study was undertaken to clarify the time and its inner rules and also to find the related factors affecting the intestinal detoxification time of HFMD children with EV71 infection. The results may be helpful for the prevention and control of the disease.

Methods

Patients

Sixty-five HFMD children with EV71 infection who had been hospitalized in our hospital from May to September in 2013 were enrolled in the study. All of them conformed to the diagnostic standards of HFMD in the Guidelines of Hand-Foot-and-Mouth Disease Diagnosis and Treatment 2010.[6] Fluorescence polymerase chain reaction (PCR) detection showed positive results of EV71 nucleic acid in stool samples of all patients, which confirmed EV71 infection. All patients were free from other enterovirus infections. This study was approved by the Hospital's Ethics Committee, and informed consents were obtained from the parents of all 65 children.

Study design

The 65 HFMD children with EV71 infection were followed up with regular tests in a fixed period at the hospital. Stool samples (5-8 g each) were collected every 4 to 7 days after the onset of the disease. The samples were put into sterile collection tubes at 4°C for temporary storage and sent to a laboratory within 12 hours and cryo-preserved at a temperature of -20°C. The laboratory tests were ended within 48 hours. Nucleic acids of EV71 were tested by fluorescent PCR until the result turned out to be negative. Meanwhile, clinical data of the cases were also collected, including age, gender, onset time, pre-admission disease course, visiting time, days of hospitalization, clinical manifestations, usage of drug, blood glucose, blood routine, immune globulin, cerebrospinal fluid routine and biochemical tests. All the patients were divided into ordinary HFMD and severe HFMD groups according to the clinical classification standards of HFMD in the Guidelines of Hand-Foot-and-Mouth Disease Diagnosis and Treatment 2010.[6] In addition, patients lost to follow-up were called and asked for reasons.

Laboratory assays

Stool samples (0.2 g or 200 μL) were added to centrifugation tubes, with 1.5 mL of normal saline. The samples were mixed and shaken for three times, 10 seconds each time, and then kept standing for 10 minutes. After that, the tubes were centrifuged at a speed of 8000 rpm/min for 5 minutes, and then 200 μL of supernatant was obtained. Nucleic acids were extracted by nucleic acid extraction KitII (Geneaid, Taiwan of China). The EV71 nucleic acids, coxsackievirus A16 (CA16) nucleic acids, and universal nucleic acids for enteroviruses were detected using RNA detection kit for HFMD enterovirus (Pfizer, Shanghai, China) by ABI7500 fluorescent quantitative PCR (AppliedBiology, USA). The quality control of the laboratory was ensured: one positive control and one negative control were provided in the kit. The corresponding cycle number (Ct value) at which fluorescence signal reached the threshold was called $Ct_{positive}$ and $Ct_{negative}$ respectively. If the reagent quality was intact and operation was proper, $Ct_{positive}<Ct_{negative}$, $Ct_{positive}<30$ and a typical S-shaped amplification curve should be plotted; otherwise the test was considered invalid. Reaction conditions were as follows: 50°C 30 minutes; 95°C 5 minutes; 95°C 10 seconds, 40 cycles; and 55°C 45 seconds, 40 cycles. Ct value <35 was assessed as positive; Ct value ≥38 as negative; and 35≤Ct value<38 as gray zone which required two extra tests. When both universal nucleic acid for enteroviruses and EV71 virus nucleic acid were positive, but CA16 nucleic acid was negative and the Ct value of universal nucleic acid for enteroviruses was equivalent to the Ct value of EV71 virus nucleic acid, EV71 infection can be confirmed, with no other enterovirus infections.

Statistical analysis

The data were analyzed using statistical package SPSS version 16.0 for Microsoft Windows. Continuous data were presented as mean±standard deviations in normal distribution, or median and interquartile range in abnormal distribution. Count data were presented as constituent ratio or rate. The Kaplan-Meier method was used to analyze the positive rate of viral nucleic acids in the follow-up period. The negative test results of EV71 nucleic acids were defined as outcome variables. Truncated variables were defined when negative test results were defined as outcome variables, and positive test results in patients who were lost to follow-up were defined as censored values. Calculation formula for a positive rate of P: $P(x>t)=\pi\rho=\pi[(n-d)/n]$ (π: continuous multiplication symbol; ρ: estimated value of positive rate; n: initial number of follow-up cases; d: negative number of tests). Continuous multiplication product of positive rate estimates at each time point was the positive probability of viral nucleic acids at that time point.

The log-rank test was used to sort out correlation factors affecting the intestinal detoxification time in children (univariate analysis), and COX analysis was used in multi-factor analysis (significant level: $\alpha=0.05$).

Results

Baseline characteristics

Among the 65 children, 36 were males and 29 females, with a male/female ratio of 1.24:1. The average onset age was 1.92 (1.25-3.46) years, including 9 children aged 0.3-1 years, 38 aged 1-3 years, 12 aged 3-5 years, and 6 aged 5-8 years. The average hospitalization ranged from 3 to 15 days with an average of (7.85±2.80) days. The children were classified clinically into two groups: 25 children with ordinary HFMD (38.5%) and 40 with severe HFMD (61.5%).

The 65 children were given antiviral treatment including injection of ribavirin and reduning. The 40 children with severe HFMD were further subjected to injection of mannitol to reduce intracranial pressure. Some of these severe children were treated with steroids appropriately [methylprednisolone injection (1-2 mg/kg per day) for 3 continuous days, 35 children] or intravenous gamma globulin (1 g/kg per day for 2 continuous days, 18 children).

Follow-up found viral nucleic acid was negative in 32 children and it was negative in stool within 26 days. In addition, 33 children (50.8%) were lost to follow-up: 16 children due to the interference from their parents; 3 children living far away from the hospital; 12 children reluctant to follow the procedures of examination; and 2 children out of contact.

Viral nucleic acids in stool of HFMD children with EV71 infection

The positive rate of viral nucleic acids decreased in stool of HFMD children. The positive rate in the second week was 94.6%, and the negative conversion rate was 5.4%. In the fourth week, the positive rate and the negative conversion rate were 48.1% and 51.9%, respectively. In the sixth week, the two rates of viral nucleic acids were 17.2% and 82.8%, respectively. In the tenth week, viral nucleic acids turned to be negative. These results suggested that the maximum intestinal detoxification time of HFMD children with EV71 infection was 10 weeks (Table 1 and Fig.).

Univariate analysis on the intestinal detoxification time of HFMD children with EV71 infection

The effect of age, gender, pre-admission disease course, severity of disease, and use of steroids or gamma globulin on intestinal detoxification time in children was detected when intestinal detoxification time was considered as a dependent variable. The results of log-rank test suggested that intestinal detoxification time was unrelated to age ($P>0.05$) but correlated with gender, pre-admission disease course, severity of disease, and use of steroids or gamma globulin ($P<0.05$) (Table 2).

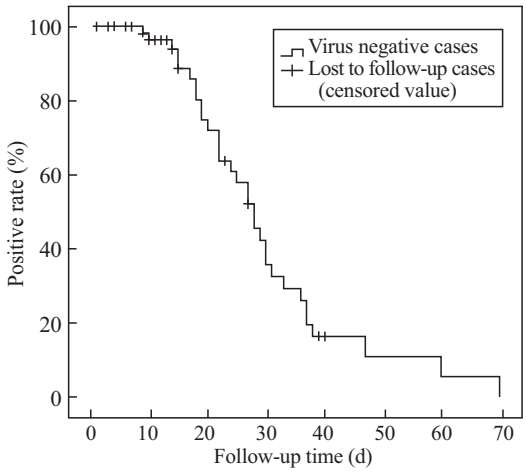

Fig. Positive rate of stool samples in HFMD patients with EV71 infection in different follow-up periods. HFMD: hand-foot-and-mouth disease; EV71: enterovirus 71.

Table 1. Viral nucleic acids test results of stool samples in HFMD patients with EV71 infection in different follow-up periods

Time (wk)	Initial cases	Lost to follow-up cases	Virus negative cases	Virus positive		Positive rate (%)
				Cases	Estimated positive rate (%)	
1	65	9	0	65	100.0	100.0
2	56	17	3	53	94.6	94.6
3	36	2	8	28	77.8	73.6
4	26	3	9	17	65.4	48.1
5	14	0	5	9	64.3	30.9
6	9	2	4	5	55.6	17.2
7	3	0	1	2	66.7	11.5
8	2	0	0	2	100.0	11.5
9	2	0	1	1	50.0	5.7
10	1	0	1	0	0	0
Total		33	32			

HFMD: hand-foot-and-mouth disease; EV71: enterovirus 71.

Table 2. Univariate analysis of intestinal detoxification time in HFMD patients with EV71 infection

Factors	Cases	Median intestinal detoxification time, d (95% confidence interval)	Log rank χ^2 value	*P* value
Age (y)				
0.3-1	9	22.00 (14.00-30.00)	0.882	0.830
1-3	38	28.00 (24.51-31.49)		
3-5	12	38.00 (17.05-58.95)		
5-8	6	22.00 (22.00-29.00)		
Gender				
Male	36	31.00 (23.90-38.10)	4.601	0.032
Female	29	24.00 (14.36-33.64)		
Pre-admission disease course	65	28.00 (23.75-32.25)	15.015	0.036
Severity of disease				
Ordinary	25	24.00 (16.93-31.07)	5.689	0.017
Severe	40	30.00 (27.18-32.82)		
Use of steroids				
Not used	30	25.00 (19.92-30.08)	5.219	0.022
Used	35	30.00 (28.13-31.87)		
Use of gamma globulin				
Not used	47	25.00 (18.56-31.44)	2.413	0.012
Used	18	31.00 (28.79-33.21)		

HFMD: hand-foot-and-mouth disease; EV71: enterovirus 71.

Multivariate analysis on the intestinal detoxification time of HFMD children with EV71 infection

Multivariate analysis was carried out on 5 selected factors affecting intestinal detoxification time including gender, pre-admission disease course, severity of disease, and use of steroids or gamma globulin. Stepwise forward method was used in regression analysis. The results of the analysis showed that the severity of disease was an independent factor affecting intestinal detoxification time ($P<0.05$, relative risk 2.418). This suggested that the severity of disease was an important factor affecting the intestinal detoxification time of HFMD children with EV71 infection, and severe HFMD children with EV71 infection had a longer intestinal detoxification time.

Discussion

EV71 is a member of enterovirus A of the picornavirus family. EV71 particle is an icosahedral symmetric sphere with no envelope and spikes. It is a single-stranded positive RNA with 7408 nucleotides. This virus has obvious dermatotropic and neurotropic characteristics and varying clinical manifestations. Mild patients may be characterized by only self-limited HFMD or herpangina, but severe patients may present with brainstem encephalitis, neurogenic pulmonary edema, pneumorrhagia or even cardiopulmonary failure. Moreover, exceptional severe patients may progress quickly and are more likely to die. In recent years, EV71 infection has been prevalent in quite a few areas of China and shown an increasing morbidity with deaths occurring constantly.[1-3,7] Therefore, the prevalence of EV71 infection has attracted more and more attention from physicians and researchers in this country.

EV71 mainly spreads through the gastrointestinal tract (fecal-oral route, water or food contamination) or respiratory tract (droplets, cough or sneeze), as well as through skin contact or mucosa herpes fluid. Children with HFMD and asymptomatic virus-carriers are the main sources of EV71 infection. This study demonstrated that EV71 existed for a long time in the feces of children with HFMD. The positive rate of EV71 nucleic acids in the second week reached 94.6%, and decreased as the time went on. EV71 could exist in feces for a longest period of 10 weeks. Pan et al[5] found that EV71 virus can be detoxified via excrement in the first week of the outbreak, and the longest detoxification period was 10 weeks. He et al[8] also found that the detoxification period of HFMD children with EV71 infection might last more than 6 weeks. The results of this study are consistent with the mentioned studies.

Since EV71 can exist for a long time in the feces of HFMD children and is contagious, HFMD children with EV71 infection should be isolated and their excreta need sterilization in order to reduce the possibility of transmission by the fecal-oral route. In addition, virus detection should be done before EV71-infected HFMD children are released from quarantine to determine whether the patients still carry the virus.

Univariate analysis in this study showed that except for age, intestinal detoxification time is correlated with gender, pre-admission disease course, severity of disease, and use of steroids or gamma globulin. Gender of children may be related to the intestinal

detoxification time of HFMD, with males having a longer intestinal detoxification time than females. The cellular immune function of HFMD children is dependent on genders, and the ability of males to remove virus is weaker than that of females. Thus male children need a longer intestinal detoxification time.

Multivariate analysis indicated that the severity of disease was the dependent factor affecting intestinal detoxification time. Hence, gender, pre-admission disease course and use of steroids or gamma globulin are not used for this analysis. This may be due to the poor influence of these factors or their correlation with the severity of disease, that is, their effects may be covered when analyzed together.

This study revealed that the severity of disease was an important factor affecting the intestinal detoxification time of HFMD children with EV71 infection. Intestinal detoxification time was longer in severe patients than in ordinary patients, as reported by others.[9] There should be different quarantine measures between severe and ordinary patients, and the quarantine period for severe patients should be prolonged appropriately.

In our study, the intestinal detoxification time of severe HFMD patients with EV71 infection was longer than that of ordinary patients. First, EV71 could infect T cells directly, resulting in apoptosis of T cells.[10] The reduction of regulatory T cells was positively correlated with the severity of EV71 disease.[11] As the severity of EV71 disease increased, the quantity of CD3+ T cells, CD4+ T cells, CD8+ T cells and natural killer cells declined gradually.[12] These findings suggested that cellular immune function and the ability to clear the viruses of severe HFMD patients with EV71 infection are reduced more significantly than those of ordinary patients, leading to a longer intestinal detoxification time. Second, experiments showed that the viral replication capacity of severe EV71 isolates was stronger than that of mild EV71 isolates.[13] Therefore, we speculate that the virus replication speed of severe EV71-infected HFMD children is faster than that of ordinary EV71-infected HFMD children, resulting in generation of more viruses and continuous detoxification. That is why severe EV71-infected HFMD children have a longer intestinal detoxification time. Third, comparison of a whole genome sequence of EV7l in fatal and non-fatal cases showed that there was a difference in the nucleotide sequence of 3C region of non-structural protein.[14] In a study on genome sequence analysis of 6 strains of EV71 isolates with different virulence from China, Chang et al[15] discovered that the only difference between 3 isolates causing mild clinical diseases and 3 isolates causing severe clinical diseases was the mutation of amino acids (3D region) at the 1994th loci, which changes from valine to isoleucine. Therefore, although further discussion is needed it is speculated that there might be nucleotide polymorphism in the viruses between ordinary and severe HFMD children with EV71 infection, resulting in a longer intestinal detoxification time in severe patients.

In conclusion, this study found that EV71 can be detoxified via excrement for a long time. The longest intestinal detoxification time of HFMD children with EV71 infection was 10 weeks. The severity of disease was an important factor that influenced the intestinal detoxification time of HFMD children with EV71 infection, and severe patients had a longer intestinal detoxification time. Therefore, HFMD children with EV71 infection should be isolated and the quarantine period for severe cases should be prolonged appropriately. Disinfection of secretion should be strengthened, and personal hygiene awareness of the population should be enhanced so as to effectively reduce EV71 infection and the risk of transmission. However, the study has some shortages such as limited sample size and a high rate of lost to follow-up. Since only qualitative detection of the virus was performed in this study, the conclusion was limited. For further studies, sample size should be increased, follow-up system should be improved, parents of children should be educated to support the follow-up of their children.

Funding: This study was supported by grants from the Health Science and Technology Program of Hangzhou (No. 2011A039) and the Medical Science and Technology Program of Zhejiang Province (No. 2012KYB159).
Ethical approval: This study was approved by the Ethics Committee of Hangzhou Children's Hospital.
Competing interest: None.
Contributors: Teng S wrote the first draft of this paper. All authors contributed to the intellectual content and approved the final version. Zhao SY is the guarantor.

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Received December 29, 2014
Accepted after revision April 7, 2015