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Original Article

Relationship between serologic response and clinical symptoms in children with enterovirus 71-infected hand-foot-mouth disease

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Abstract: This study aimed to explore the correlation between clinical symptoms, including rash and fever, and serum antibody reaction to enterovirus 71 (EV71) infection in children hospitalized due to hand-foot-mouth disease (HFMD). From May 2014 to July 2014, a total of 547 children hospitalized due to HFMD in Children's Hospital of Fudan University were enrolled retrospectively. RNA levels of EV71 and CA16 in fecal, serum, and cerebrospinal fluid specimens were measured using quantitative real-time RT-PCR, and EV71-IgM antibody in the serum was detected using immune colloidal gold assays. Of the 547 fecal specimens, 296 were EV71 RNA positive, 109 were CA16 RNA positive, and 8 were positive for both EV71 RNA and CA16 RNA. The total positive rate for either EV71 or CA16 in feces was 72.58% (397/547). Additionally, 544 serum specimens were collected, and 409 were EV71-IgM positive (75.18%). The duration of rash and fever was found to be correlated to the positive rate of serum EV71-IgM, and the positive rate of serum EV71-IgM plus EV71 RNA in feces. The positive rates of serum EV71-IgM and serum EV71-IgM plus EV71 RNA in fecal collected at day 3 of fever were 79.7% and 52.8%, respectively. In conclusion, EV71 and CA16 were found to be the major pathogens responsible for the epidemics of HFMD in children during May to July 2014 in Shanghai, China. There is a close relationship between the positive rate of serum EV71-IgM and the duration of fever and rash.

Keywords: Hand-foot-mouth disease, children, enterovirus 71, serum antibody, fever, rash

Introduction

In the past three years, hand-foot-mouth disease (HFMD) has been the most commonly accoutered infectious diseases in mainland China. According to the report of Chinese Center for Disease Control and Prevention in 2012, there were 2,168,737 individuals diagnosed as HFMD, and 567 of these cases were fatal (http://www.chinacdc.cn/tjsj/fdcrbbg/201303/t20130327_79057.htm). HFMD is a highly contagious infectious disease, and young children are the most sensitive population. The common clinical manifestations of HFMD include fever, rash, and oral herpes. Previous studies had reported that human enterovirus 71 (EV71) and coxsackievirus A16 (CA16) are the major pathogens responsible for the epidemics of HFMD, and subgenotype C4 was recognized as the predominant subgenotype of EV71 [1]. In addition, severe HFMD and death were mainly caused by EV71 infection [2, 3]. Unfortunately, at present there is no effective vaccine or approved antiviral medication against EV71. An inactivated alum-adjuvant enterovirus 71 vaccine is still in phase III clinical trial in children in China [4]. Despite the fact that there isn't strong evidence of general clinical efficacy, in combination with the concern of antibody dependent enhancement (ADE) of virus infection, intravenous immunoglobulin (IVIG) is still applied in severe cases [5, 6]. However, there is no consensus or guidance regarding its administration timing [7]. EV71 seroconversion was the most important evidence to indicate the application of IVIG treat-

ment in clinical practice. However, due to ethical concerns, clinically getting dynamic seroconversion data following EV71 infection in HFMD patients is difficult. Therefore, we retrospectively investigated the relationship between the positive rate of EV71-specific serum immunoglobulin (Ig) M antibody and the duration of fever and rash in HFMD children. It could provide further valuable referred information to IVIG treatment in patients with HFMD in clinical practice.

Materials and methods

Patients

A total of 547 children hospitalized patients due to HFMD from May to July 2014 in Children's Hospital of Fudan University were enrolled retrospectively. The demographic and clinical characteristics information, such as duration of fever and rash, vomiting, diarrhea, etc were collected. The information of magnetic resonance imaging (MRI) scan of head and spinal cord were collected either if available. In addition, the results of etiology tested in fecal, serum and cerebrospinal fluid specimens were collected.

Diagnosis of HFMD

Patients were diagnosed as HFMD according to the clinical diagnosis criteria proposed by China's Ministry of Health which are provided as below: (1) HFMD emerges mainly in summer and autumn in preschool children, especially in infants; (2) Patients have fever accompanied with rash on hands, feet, lips, or buttocks. Some patients may not have fever. (3) In the minority of severe patients with atypical rash who are difficult to diagnose, clinical diagnosis primarily depends on etiologic or serologic test result. (4) It is not recommended to diagnose HFMD for patients without rash.

In addition, patients with the following symptoms, such as altered mental status, tremor, startle, seizures, neck resistance, and with abnormality in cerebrospinal fluid or CNS imaging examination were diagnosed to have the complication of central nervous system (CNS) infection.

Detection of EV71 and CA16 RNA levels

The RNA levels of EV71 and CA16 in feces, serum, and cerebrospinal fluid specimens were detected using quantitative fluorescent real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis. Specific detection kits (DaAn Gene Co., Ltd. of Sun Yat-Sen University, Guangzhou, China) were used to do the detection. Primers used for the nested PCR were: forward EV1: 5'-GTGGCAGATGTGATTGAGAG-3'; reverse EV2: 5'-GTTATGTCTATGTCCCAGTT-3'; forward EV3: 5'-GTGGCAGATGTGATTGAGAG-3'; reverse EV4: 5'-CCYTCAAGAGGGAGRTCTAT-3'. Additionally, other known human enteroviruses were detected in cerebrospinal fluid specimens with standardized clinical nested-PCR procedure.

Detection of serum EV71-IgM antibody

Blood samples were clinically collected from fingertip when patients visited in clinic or at admission. EV71-IgM antibody in serum and cerebrospinal fluid specimens was detected using immune colloidal gold technique, according to the manufacturer's instruction. EV71-IgM antibody detection kit (Wantai Biological Pharmacy Enterprise Co., Ltd. Beijing, China) was used for the detection.

Statistical analysis

All statistical analyses were performed using SAS version 9.1 statistical software (SAS Institute Inc., Cary, North Carolina, USA). Categorical data were expressed as numbers of patients and percentage (%). Difference between the groups was compared with Chi-square test. A P value of < 0.05 was considered statistically significant.

Results

Clinical characteristics

From May to July 2014, a total of 547 patients with a mean age of 34 months (range, 4 months-10 years) were diagnosed as HFMD. At the time of 547 serum specimens collected, 511 patients had fever, 535 had rash. 190 of the 547 patients were clinically suspected to complicate with CNS infection, and they received the lumbar puncture examination. MRI was performed in 77 patients and 24 were found with imaging abnormality. Finally, a total of 158 patients were confirmed to be HFMD complicated with CNS infection, including meningitis (120 cases), meningoencephalitis (24

Table 1. Demographic and clinical characteristics

Variable	No. of patients (% total patients [n=547])	EV71-positive (%)
Gender		
Male	355 (64.9)	272 (76.6)
Female	192 (35.1)	156 (81.3)
Age, year		
< 1	48 (8.8)	35 (72.9)
1-2	175 (32.0)	118 (67.4)
2-3	122 (22.3)	101 (82.8)
3-5	151 (27.6)	130 (86.1)
> 5	51 (9.3)	44 (86.3)
Clinical examination		
Lumbar puncture	190 (34.7)	173 (91.1)
MRI	77 (14.1)	74 (96.1)
Complication		
CNS infection	158 (28.9)	153 (96.8)
Meningitis	120 (21.9)	117 (97.5)
Meningoencephalitis	24 (4.4)	24 (100)
Encephalitis	8 (1.5)	6 (75)
Brainstem encephalitis	4 (0.7)	4 (100)
Cerebrospinal meningitis	2 (0.4)	2 (100)
IVIG treatment	63 (11.5)	62 (98.4)

CNS, central nervous system; IVIG, intravenous immunoglobulin; MRI, magnetic resonance imaging.

Table 2. Detection of viral RNA and serum EV71-IgM antibody

Variable	No. of patients, n (%)
Total No. of patients	547
Viral RNA detection	
Fecal specimens	547
Total EV71- and CA16-positive	397 (72.6)
EV71-positive	296 (54.1)
CA16-positive	109 (19.9)
Positive for both EV71 and CA16	8 (1.5)
CSF specimens	190
Total EV71- and CA16-positive	1 (0.5)
EV71-positive	1 (0.5)
CA16-positive	0 (0)
Enterovirus-positive	24 (12.6)
Serum specimens	544
EV71-IgM-positive	409 (75.2)

EV71, enterovirus 71; CA16, coxsackievirus A16; IgM, immunoglobulin M; CSF, cerebrospinal fluid. There were 12 patients who had no sign of rash at the time of specimen collection and developed rash thereafter, but they were not included in the analysis.

cases), encephalitis (8 cases), brain stem encephalitis (4 cases), and cerebrospinal meningitis (2 cases). There were 56 patients who received IVIG treatment. The clinical characteristics are shown in **Table 1**.

Detection of EV71 and CA16 RNA

In the 547 fecal specimens collected clinically, 54.1% (296/547) of which were EV71 RNA positive and 19.9% (109/547) of which were CA16 RNA positive. 8 fecal specimens were found to be positive for both EV71 RNA and CA16 RNA. The total positive rate for either EV71 or CA16 in feces was 72.6%. It suggests that EV71 is the major pathogen for HFMD between May and July 2014 in Shanghai, China, followed by CA16. Of the 190 cerebrospinal fluid specimens, only one specimen was EV71 RNA positive, and 24 were positive for other known enteroviruses. CA16 was not detected in any of the cerebrospinal fluid specimens (**Table 2**).

Detection of serum EV71-IgM antibody

544 serum specimens were collected clinically. 409 of these specimens were EV71-IgM positive. The positive rate of EV71-IgM is 75.18% (409/544) (**Table 2**).

In the 409 cases with positive serum EV71-IgM, 277 cases were found to have positive EV71 RNA in fecal, and 21 cases were tested enterovirus positive in CSF (1 case was tested EV71 RNA positive in CSF). In the 135 cases with negative serum EV71-IgM, only 17 cases were found to have positive EV71 RNA in fecal specimen, and 2 cases were tested enterovirus positive in CSF (no EV71 RNA detected in CSF). Further analysis was done to investigate the correlation between serum EV71-IgM and EV71 RNA in fecal. It showed that there is significant correlation between positive serum EV71-IgM and positive EV71 RNA in fecal ($P < 0.05$).

Table 3. Positive serum EV71-IgM and positive serum EV71-IgM plus EV71 RNA in feces based on the duration of rash

Duration of rash, day	1	2	3	4	≥ 5	*P* value for trend
No. of patients, n	108	139	146	74	79	
EV71-IgM positive, n (%)	59 (54.6)	91 (65.5)	127 (87.0)	61 (82.4)	67 (84.8)	< 0.001
Positive EV71-IgM plus EV71 RNA, n (%)	38 (35.2)	57 (41.0)	87 (59.6)	43 (58.1)	52 (65.8)	< 0.001

Table 4. Positive serum EV71-IgM and positive serum EV71-IgM plus EV71 RNA in feces based on the duration of fever

Duration of fever, day	1	2	3	4	≥ 5	*P* value for trend
No. of patients, n	104	172	123	51	93	
EV71-IgM positive, n (%)	64 (61.5)	123 (71.5)	98 (79.7)	48 (94.1)	67 (76.3)	< 0.001
Positive EV71-IgM plus EV71 RNA, n (%)	48 (46.2)	80 (46.5)	65 (52.8)	33 (64.7)	71 (76.3)	< 0.001

Table 5. Positive serum EV71-IgM and positive serum EV71-IgM plus EV71 RNA in feces in patients complicated with CNS infection based on the duration of rash

Duration of rash, day	1	2	3	4	≥ 5	*P* value for trend
No. of patients, n	29	33	46	31	18	
EV71-IgM positive, n (%)	27 (93.1)	31 (93.9)	46 (100)	28 (90.3)	18 (100)	0.650
Positive EV71-IgM plus EV71 RNA, n (%)	23 (79.3)	31 (93.9)	41 (89.1)	22 (80.0)	13 (72.2)	0.126

Table 6. Positive serum EV71-IgM and positive serum EV71-IgM plus EV71 RNA in feces in patients complicated with CNS infection based on the duration of fever

Duration of fever, day	1	2	3	4	≥ 5	*P* value for trend
No. of patients, n	31	42	38	24	17	
EV71-IgM positive, n (%)	31 (100)	40 (95.2)	35 (92.1)	24 (100)	16 (94.1)	0.629
Positive EV71-IgM plus EV71 RNA, n (%)	29 (93.5)	36 (85.7)	33 (86.8)	18 (75.0)	13 (76.5)	0.051

Correlation between the positive rate of serum EV71-IgM antibody and the duration of fever and rash

In general, the positive rate of serum EV71-IgM antibody was correlated with the duration of rash and fever ($P < 0.05$). The positive rates of serum EV71-IgM collected at day 3 of rash and at day 4 of fever were 87.0% and 94.1% respectively (**Tables 3**, **4**). In the 157 patients who were diagnosed as HFMD complicated with CNS infection, the positive rate of serum EV71-IgM collected at any day of fever or rash was very high (all > 90%), even at the first day of symptoms (rash or fever) , but did not show any correlation with duration of symptoms (**Tables 5**, **6**).

Discussion

In mainland China, the subgenotype C4 is reported as the predominant subgenotype of EV71 strains and the major pathogen that causes severe HFMD. Our study also suggests that EV71 is the major pathogen responsible for the epidemics of HFMD during May to July 2014 in Shanghai, China, which is consistent with what previous studies have reported [8-11]. Epidemiology data from Children EV71 serum serology in mainland China showed that the number of subclinical infection was significantly greater than that of apparent infection [12-16]. Unfortunately, generally it's difficult to make a definite diagnosis for patients with atypical HFMD in clinical practice according to the clinical diagnostic criteria proposed by China's Ministry of Health. It has been reported that serum EV71-IgM antibody can be detected for a certain period in children with subclinical EV71 infection, and 11.4% to 31.1% of children with CA16 or other enteroviruses infection also can be tested as EV71-IgM antibody positive in serum [15, 16]. In our study, it

is also found that in some patients with positive CA16 RNA and negative EV71 RNA, EV71-IgM antibody was positive. These findings indicate that it might be impracticable to diagnose HFMD with EV71 infection through serum EV71-IgM as the only provided evidence. EV71 has been found to be carried in human body and excreted from the intestine for a long period [17]. It is therefore far from sufficiency to justify a conclusion that EV71 is the major pathogen for acute HFMD in patients with positive EV71 RNA in feces provided as the only evidence. The time period of viremia in blood/cerebrospinal fluid and virus existence in CNS lasts for only several days, and viral RNA level in blood and cerebrospinal fluid is much lower than that in feces. These evidences can be treated as rationale for acute infection, but the issue is the positive rate is extremely low in clinical practice. Therefore, we suggest to combine RT-PCR test result in feces and serum IgM antibody test result as the evidence of whether there is clinical acute infection with EV71 [18].

The seroconversion rate of EV71-IgG among children > 4 years of age was reported to be at least 40% [12, 13]. IVIG has been extensively used in clinical practice in the treatment of severe HFMD. However, to date the use of IVIG to treat severe HFMD still lacks of evidence support from randomized clinical trials [19]. Moreover, in addition to IgG1, the antibody dependence mediated by other IgG subclasses like IgG2, IgG3, and IgG4 has been shown to enhance EV71 infection [7, 20]. The clinical symptoms of EV71 infection are caused by direct viral attack as well as tissue and organ damage due to immune response. Following EV71 infection, EV71-IgM antibody is firstly produced by B cells, followed by subsequent production of IgG. Hence, the production of EV71-IgM plays a very important role in suppressing virus replication in acute phase in human body. However, whether the presence of EV71-IgM in EV71-infected patients indicates the capability of efficient immune response to remove virus and suppress virus replication has not been elucidated. It also remains unclear whether EV71-IgM antibody has a potential influence on the production of exogenous IgG by IVIG therapy. Due to ethical concern, the dynamic changes of specific serum IgM antibody and IgG antibody are very difficult to be obtained in clinical practice in EV71-infected patients. Moreover, the time curve of antibody seroconversion following EV71 infection remains unclear. Thus, it is difficult to determine the optimal administration schedule of IVIG treatment in EV71-infected patients. In our study, it suggests there is a close relationship between the serum EV71-IgM positive rate and the duration of fever and rash. This observation indicates that HFMD patients with 3 days of rash or 3-4 days of fever are generally with EV71 seroconversion already. Therefore, the clinical decision might be able to be simply dependent on these clinical symptoms in case of absence of virus RNA and serologic test. In addition, a very high positive rate of serum EV71-IgM (95.6%) was found in severe HFMD patients complicated with CNS infection during the early onset of fever or rash, indicating that the presence of endogenous EV71-IgM antibody may not be efficient to protect the body against the progression of HFMD caused by EV71 infection.

There are some limitations in this study. Firstly, we mainly recruited children hospitalized due to HFMD, whose clinical symptoms should be more severe than outpatients. Hence, patients with atypical symptoms of HFMD may be missed and not be recruited. Secondly, because the dynamic change of EV71-IgM antibody was not monitored in each patient, our result can only reflect the possible time relationship between the EV71-IgM production and duration of fever and rash. Thirdly, the study did not include the minority of HFMD patients without fever or rash, and the patients without evidence of EV71-IgM who actually have acute EV71 infection. Finally, because serologic test can only identify patients infected with the C4 subgenotype of EV71, whether there is serum cross-reactivity between C4 sub-genotype and other EV71 sub-genotypes remains unclear [21].

This study firstly tried to investigate the relationship between the positive rate of serum EV71-IgM antibody and the duration of fever and rash in HFMD children with clinical/laboratory-confirmed EV71 infection, which would provide valuable information and rationale to the clinical use of IVIG treatment in HFMD patients. Our results also indicate that the optimal time of IVIG treatment if needed is within the first three days of the onset of fever or rash, and IVIG should be used with caution after five days of symptom onset.

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Disclosure of conflict of interest

None.

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