Results on Pathogen Detection of Foot and Mouth Disease in Guangxi China and Analysis on Its Popular Spectrum

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Abstract

Objective: The study aims to understand the characteristics and epidemic trend of the pathogen of hand, foot and mouth disease (HFMD) in Guangxi regions, China. Besides, it aims to analyze the differences of intestinal virus detection rate between anal swab and pharyngeal swab samples.

Methods: Anal swab and pharyngeal swabs of suspected HFMD children were collected in our hospital from 2012 to 2015. Real-time fluorescent PCR (Polymerase Chain Reaction) was used to detect enterovirus 71 (EV71), coxsackie virus type 16 (CA16), and universal intestinal virus nucleic acid (EV). Composition and conversion of predominant pathogens were analyzed, and paired samples’ test results of swabs anal and pharyngeal swab were statistically analyzed.

Results: There are 681 cases with enterovirus in 2351 cases of patients. Among those who got enterovirus, there are 501 cases of EV71, 102 cases of CA16 and 79 cases of EV. From 2012 to 2015, the total proportion of the virus detection is 46.47%, 16.23%, 41.02% and 15.33% respectively in each year, while the proportion of predominant epidemic virus is 93.93% of EV71, 66.12% of CA16, 89.30% of EV71 and 98.73% of EV, non-EV71, non-CA16 EV (from October to December in 2015). It’s obvious that the total virus detection rate in 2012 and 2014 is significantly higher than that in 2013 and 2015. There is statistical significance.

Conclusion: The main HFMD pathogens are EV71 from 2012 to 2015 in Guangxi regions. In 2012 and 2014, the predominant epidemic pathogens were EV71, while in 2013 and 2015, the predominant epidemic pathogens turn to be CA16 and non-EV71, non-CA16 EV respectively. What’s more, collecting anal swab and pharyngeal swab virus at the same time for nucleic acid detection is of great significance to improve the HFMD laboratory diagnostic.

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1. Introduction

Hand, foot and mouth disease (HFMD) is a kind of common acute infectious disease in children, with fever and rash in areas such as the hand, foot and mouth, herpes, or herpangina as the main characteristics. HFMD can turn into other kinds of severe diseases or even cause death. Small RNA (Ribonucleic Acid) virus’s intestinal virus genera (Enterovirus, EV) can cause HFMD pathogen, including coxsackie virus in group A (Coxsachievirus A, CA) of 2, 4, 5, 6, 7, 10, 16 type, coxsackie virus B (Coxsachievirus B, CB) of 1, 2, 3, 4, 5 type, Enterovirus 71 type (EV71 Enterovirus 71), and the virus of ECHO (Echovirus, ECHO) etc. In recent years, HFMD has been widespread in China. According to the data in national report from 2008 to 2014, there are 118,370,670,000 patients with HFMD, among which, there are 3230 cases of death [1], which become the largest public health emergencies of children in recent decades. Before 2012, EV71 and CA16 were the most common pathogens of HFMD in China, while the severe cases were caused by EV71 infection [2]. Over these years, because of the transformation of the pathogens, non-CA16 and non-EV71 EV are rapidly rising, and even become the predominate epidemic pathogens in some areas. Moreover, the proportion of non-EV71 has been increasing in severe or fatal cases [3], and CA6 has become one of the important pathogens of hand, foot and mouth disease. In addition to that, it has been confirmed that CA6 can lead to some serious complications such as pulmonary hemorrhage and acute flaccid paralysis, similar to the infection caused by EV71 [4]. The species of HFMD pathogen is numerous, and the prevalence of pathogen has regional characteristics and time difference. Moreover, children are susceptible to HFMD and they have no cross immunity to infection of different serotypes [5]. Therefore, it’s estimated that in several years, HFMD in China is still in a high incidence. For the treatment of HFMD, at present, it is lack of effective targeted intervention measures to control the spread. Therefore, the primary and effective measures to control the source of infection, block transmission and reduce the case fatality rate are to research HFMD epidemic regularity of pathogens and improve pathogen detection rate. In this paper, meaningful conclusions have been obtained after statistically analyzing the detection results of intestinal virus’s nucleic acid of HFMD patients in our hospital from 2012 to 2015. The results will be shown as follows.

2. Materials and Methods

2.1. The Objects of Study

A total of 2351 patients with HFMD treated in our hospital were selected from 2012 to 2015, among which 1473 cases were male, female were 877 cases. The ratio of male and female is 1.68:1, ages from 4 months to 9 years old. All specimens were collected after child guardian informed consent, and testing data of patients as well as patients’ information are under the standard management in our hospital ethics committee. The basis for the diagnosis of HFMD as follows. First and foremost, in the warm seasons of spring, fall and early summer, it’s early for some children to get fever, with rash (maculopapular rash, papules, herpes) in their hands, feet and mouths, buttocks or other parts of bodies. Secondly, those children have cough, runny nose and loss of appetite. Thirdly, there are some symptoms will appear in those patients, such as poor spirit, lethargy, easily startled, headache, vomiting, irritability, limb jitter, acute limb weakness, stiff neck, meningitis, encephalitis and polio syndrome. Fourthly, the heart rate and respiration turn to be faster. They will have a cold sweat, skin patterns, cold limbs, and high blood. Meanwhile, there is higher blood glucose, peripheral blood leukocyte, and abnormal heart ejection fraction. Finally, their hearts beats too fast or too low. And they have shortness of breath, cyanosis, and cough pink frothy sputum or bloody fluid as well as continuous decrease of blood pressure or shock.

2.2. Reagent and Instrument

PCR fluorescent probe method is used to detect EV71, CA16 and EV nucleic acid, and American ABI7500 PCR is used for amplification. Reagent is from Guangzhou Daan Biological Products Co., LTD. Kit with reagent is
applied to extract RNA. The items for the detection of intestinal virus subtypes include polioviruses, Coxsackie virus, ECHO virus and new enterovirus. Total number of serotypes is 71.

2.3. Research Methods

2.3.1. Specimen Collection
Pharyngeal swab or anal swab in children with HFMD were selected and then put in a sampling tube with 1.0 ml saline, storing them at minus 20°C and testing them weekly.

2.3.2. Nucleic Acid Extraction and Result Judgment
Specimens were removed from the refrigerator and thawed at room temperature, oscillating them with high speed for 2 minutes. Moisture in swab was extruded and then transferred to 1.5 ml centrifuge tube. Next, take 100 ul for nucleic acid extraction, and the remaining specimens preserved at minus 80°C. RNA (Ribonucleic Acid) was extracted with kits, operating in accordance with the instructions strictly. Reaction conditions of PCR amplification are as follows: from 50°C for 15 minus to 95°C for 15 minus (94°C for 15 S, 55°C for 45 S). Fluorescence detection was carried out under the temperature of 55°C, and FAM (Fast Auxiliary Memory) channel was used for testing. The judgment condition to positive of EV71, CA16 and EV is that there is obvious amplification curve of the FAM channels. Besides, Ct value was 35.1, 34.8, and 34.9 respectively. After analyzing the results of EV positive, it was found if EV positive combined with positive of EV71 or CA16, it would be judged to be EV71 or CA16 infection, while if EV is positive, but CA16 or EV71 is negative, it would be judged to be non-CA16 or non-EV71 EV infection.

2.4. Quality Control
Patients’ negative and positive samples were tested under the same experimental conditions. If the amplification results of negative materials are no typical s-shaped amplification curve or Ct value, while the positive materials present the typical s-shaped amplification curve and the Ct value is of 30 or less, it will show the success of the experiment.

2.5. Statistics Processing
SPSS 16.0 statistical software was used to analyze statistics. Chi-square was used to test composition ratio, and the McNemar was put into use for comparison of paired data. If P is less than 0.05, the difference was statistically significant.

3. Results

3.1. Virus Detection Results
From 2012 to 2015, there were 2351 cases of children with HFMD, among which 681 cases were detected to have detection enterovirus, including 501 cases of EV71, CA16 501 cases and 78 cases of EV. According to the statistical years, from 2012 to 2015, the total proportion of the virus detection is 46.47%, 16.23%, 41.02% and 15.33% respectively in each year, while the proportion of predominant epidemic virus is 93.93% of EV71, 66.12% of CA16, 89.30% of EV71 and 98.73% of EV excluding the EV71, CA16 and (from October to December in 2015). It’s obvious that the total virus detection rate in 2012 and 2014 is significantly higher than that in 2013 and 2015 (χ²2012:2013 = 63.394, χ²2012:2015 = 94.334, χ²2014:2013 = 75.201, χ²2014:2015 = 136.923, P = 0.000), while in 2012 and 2014, χ² = 2.140, P = 0.143, and in 2013 and 2015, χ² = 0.155, P = 0.693, which shows that the detection rate has no statistical significance. The results can be showed as follows.

3.2. Pathogen Distribution
In 2012 and 2014, the virus of EV71 took up the virus composition of 93.93% and 89.30% respectively, while in 2013, CA16 accounted for 66.12% in the composition of the virus. On October 2015, after testing EV, non-V71 and non-CA16 EV accounted for 98.73% of the virus pose at the same period. In the detection of pathogens, EV71 accounted for 73.56%. The results are shown in Table 1.
Table 1. Pathogen distribution of enterovirus from 2012 to 2015 [n(%)].

<table>
<thead>
<tr>
<th>Year</th>
<th>Cases</th>
<th>Total Positive Cases</th>
<th>Type of Virus</th>
<th>EV (+)</th>
<th>CA16 (+)</th>
<th>Non-EV71 and Non-CA16 EV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>EV71 (+)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CA16 (+)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2012</td>
<td>213</td>
<td>99 (46.47)</td>
<td>N.D.</td>
<td>93 (93.93)</td>
<td>6 (6.06)</td>
<td>N.D.</td>
</tr>
<tr>
<td>2013</td>
<td>382</td>
<td>62 (16.23)</td>
<td>N.D.</td>
<td>21 (33.87)</td>
<td>41 (66.12)</td>
<td>N.D.</td>
</tr>
<tr>
<td>2014</td>
<td>980</td>
<td>402 (41.02)</td>
<td>N.D.</td>
<td>359 (89.30)</td>
<td>43 (10.70)</td>
<td>N.D.</td>
</tr>
<tr>
<td>Total</td>
<td>1575</td>
<td>563 (35.74)</td>
<td>N.D.</td>
<td>473 (84.01)</td>
<td>90 (15.98)</td>
<td>N.D.</td>
</tr>
<tr>
<td>2015 (Jan.-Sept.)</td>
<td>670</td>
<td>39 (5.82)</td>
<td>N.D.</td>
<td>28 (71.79)</td>
<td>11 (28.20)</td>
<td>N.D.</td>
</tr>
<tr>
<td>2015 (Oct.-Dec.)</td>
<td>106</td>
<td>79 (74.52)</td>
<td>N.D.</td>
<td>0</td>
<td>1 (1.27)</td>
<td>78 (98.73)</td>
</tr>
<tr>
<td>Total</td>
<td>776</td>
<td>118 (15.20)</td>
<td>N.D.</td>
<td>28 (23.72)</td>
<td>12 (10.46)</td>
<td>78 (66.10)</td>
</tr>
<tr>
<td></td>
<td>2351</td>
<td>681 (29.00)</td>
<td>N.D.</td>
<td>501 (73.56)</td>
<td>102 (14.79)</td>
<td>78 (11.45)</td>
</tr>
</tbody>
</table>

*: N.D. Not Detected; Among 79 cases of positive EV, CA16 type is of 1 case, non-EV71 and non-CA16 type are 78 cases.

3.3. Virus Detection Results of Anal Swab Paired with Pharyngeal Swab Samples

Anal swab and pharyngeal swab were collected at the same time from 2033 patients. In all cases, EV71 and CA16 were examined, while 102 cases of EV were tested. EV71, CA16 and EV in the detection rate of anal swab was followed by 20.56%, 4.03% and 60.78%, while in the detection rate of pharyngeal swab was 14.36%, 3.54% and 64.70% respectively. The detection rate of EV 71 in the pharyngeal swab was significantly lower than that in anal swab ($\chi^2 = 72.16, P = 0.000$), while in two kinds of specimens the test results of CA16 and EV has no statistical difference ($\chi^2_{CA16} = 2.78, P = 0.094$; $\chi^2_{EVU} = 0.727, P = 0.394$). Comparing with two samples, missing rate of EV71 and CA16 and EV excluding the EV71, CA16 in single sample test of anal swab or pharyngeal swab is not less than 10%. The missing rate of EV71 in swabs detection is as high as 37.20%. Further details as showed in Table 2 and Table 3.

According to the reports of literature, severe HFMD patients are mainly caused by EV71 infection [2]. From 2012 to 2015, in the detection of HFMD pathogens, EV71 stood first on the list. In 2012 and 2014, EV71 accounted for about 90% of the constituents of the pathogen, which is the highest detection rate at this period. Maybe that’s because symptoms of EV71 patients are serious and the clinical visit rate is relatively high. Liu and other scholars [6] have done some statistical analysis on pathogen distribution of 10,714,237 cases of HFMD patients from 2008 to 2014 in mainland of China, which found that EV71, CA16 and other intestinal virus accounted for 43.73%, 22.04%, 43.73% of the total virus respectively. For the recent four year, the composition of HFMD pathogen in our hospital presented that EV71 accounted for 73.46%, which was higher than the national average. That’s the reason why the fatality rate of HFMD in Guangxi is higher than the national level of in recent years [7]. According to the features of HFMD pathogens recurrence every year and the research data in this study, it suggests that there is the possibility of EV71 HFMD breaking out again in the Guangxi region.

Virus of CA6 the main pathogens cause the disease of infantile herpangina. Since 2008, CA6 HFMD has taken place in the United States, Japan, Finland, Spain and other countries. In recent years, China’s report on HFMD indicated that CA6 gradually took the main part between all the virus [4] [8] [9]. It’s not until from October 2015 that EV was tested. In all the positive cases, there were almost the type of non-CA16 and non-EV71 EV. According to the analysis on predominant epidemic virus of HFMD from Guangdong, China and its surrounding provinces in 2013 [4], it’s speculated that in 2013 and 2015, the advantage of HFMD epidemic pathogen is the type of EV excluding EV71 and CA16 in Guangxi regions. Before October 2015, EV testing hasn’t been carried out, as a result, the detection rate of HFMD pathogen in 2013 and 2015 was significantly lower than that in 2012 and 2014, which indicated that there is on the rise of EV in China. Therefore, in order to avoid this kind of virus breaking out, great efforts should be made to strengthen detection and prevention to it.

Detecting intestinal virus nucleic acid is the preferred laboratory method to confirm HFMD recommend by the Hand and Feet Disease Diagnosis and Treatment Guidelines. At present, a standard operating procedure of intestinal virus nucleic acid detection has not been established in our country. And there is a great difference of
### Table 2. Comparison of virus detection rate of anal swab paired with pharyngeal swab samples.

<table>
<thead>
<tr>
<th>Specimen Type</th>
<th>Pharyngeal Swab</th>
<th>Anal Swab</th>
<th>Two-Sided Test (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>EV71</td>
<td>245</td>
<td>47</td>
<td>0.000*</td>
</tr>
<tr>
<td></td>
<td>173</td>
<td>1568</td>
<td></td>
</tr>
<tr>
<td>CA16</td>
<td>59</td>
<td>13</td>
<td>0.096</td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>1938</td>
<td></td>
</tr>
<tr>
<td>EV</td>
<td>53</td>
<td>13</td>
<td>0.394</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>27</td>
<td></td>
</tr>
</tbody>
</table>

*: Two-sided test of McNemar: if P is less than 0.05, there will be statistical differences.

### Table 3. Missing rate of anal swab paired with pharyngeal swab samples.

<table>
<thead>
<tr>
<th>Virus Type</th>
<th>Cases</th>
<th>AS+</th>
<th>PS+</th>
<th>AS− PS+</th>
<th>AS− PS−</th>
<th>Total positive cases</th>
<th>AS+</th>
<th>PS+</th>
<th>Missing rate of AS</th>
<th>Missing rate of PS</th>
</tr>
</thead>
<tbody>
<tr>
<td>EV71</td>
<td>2033</td>
<td>245</td>
<td>173</td>
<td>47</td>
<td>465</td>
<td>418</td>
<td>292</td>
<td>10.10 (47/465)</td>
<td>37.20 (173/465)</td>
<td></td>
</tr>
<tr>
<td>CA16</td>
<td>2033</td>
<td>61</td>
<td>23</td>
<td>13</td>
<td>97</td>
<td>84</td>
<td>74</td>
<td>13.40 (13/97)</td>
<td>23.71 (23/97)</td>
<td></td>
</tr>
<tr>
<td>EV</td>
<td>102</td>
<td>53</td>
<td>9</td>
<td>13</td>
<td>75</td>
<td>62</td>
<td>66</td>
<td>17.33 (13/75)</td>
<td>12.0 (9/75)</td>
<td></td>
</tr>
</tbody>
</table>

Note: AS+: positive samples of anal swab nucleic acids; PS+: positive samples of pharyngeal swab nucleic acids; AS−: negative samples of anal swab nucleic acids; PS−: negative samples of pharyngeal swab nucleic acids.

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detection, which is difficult to completely avoid detection leaking. In order to ensure timely diagnosis, there is much urgent to collect anal swab and pharyngeal swab at the same time and carry out conventional detection, especially at the period of EV71 dominating, it is not suitable to only collect pharyngeal swabs for virus detection.

4. Conclusion

From 2012 to 2015, the main HFMD pathogens were EV71 in Guangxi regions. In 2012 and 2014, the predominant epidemic pathogens were EV71, while in 2013 the predominant epidemic pathogens were CA16, and non-EV71 and non-CA16 VA in 2015. What’s more, collecting anal swab and pharyngeal swab virus at the same time for nucleic acid detection is of great significance to improve the HFMD laboratory diagnostic.

5. Limitations of the Study

In recent years, the predominant epidemic pathogens of HFMD have been gradually replaced by non-EV71, non-CA16 and other enteroviruses in China. In this study, further classification has not been carried out to detect other intestinal virus, which could not provide more valuable data for HFMD epidemiology, and prevention and control of infectious research.

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