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Detection and identification of viral pathogens in patients with hand, foot, and mouth disease by multilocus PCR, reverse-transcription PCR and electrospray ionization mass spectrometry

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ABSTRACT

Background: Rapid detection and identification of viruses are important for early diagnosis and effective surveillance of hand, foot, and mouth disease (HFMD). We described a novel assay using multilocus PCR and reverse transcription-PCR coupled with electrospray ionization mass spectrometry (RT-PCR/ESI-MS) to simultaneously detect and identify human enterovirus A–D, adenovirus A–F, human herpesvirus 1–8, parvovirus B19 and polyomavirus.
Objectives: To evaluate the accuracy and efficacy of the RT-PCR/ESI-MS method, to detect and type enterovirus from specimens of clinical diagnosed HFMD patients.
Study design: In this study, 152 specimens of clinically diagnosed HFMD patients were studied by the assay using RT-PCR/ESI-MS method. The detection and typing of enterovirus by RT-PCR/ESI-MS were compared with results from reference molecular methods.
Results: The assay detected enteroviruses in 97 (63.8%) specimens, resulting in a sensitivity of 93.8% (95% CI: 91.8–95.7%) and a specificity of 87.5% (95% CI: 84.8–90.2%) compared with a reference clinical diagnostic test. Most enterovirus genotypes (65/84; 77%) determined by the platform were consistent with 5′ UTR sequence analysis, and most misidentifications resulted from the virus library, which could be further improved by updating the enterovirus database. In addition to enteroviruses, herpesviruses, polyomaviruses, adenoviruses and human rhinoviruses were detected and identified in 55 (36%) HFMD specimens by RT-PCR/ESI-MS.
Conclusion: With the capability of high throughput and detection and typing of multiple clinically relevant viruses simultaneously, RT-PCR/ESI-MS can be a rapid and robust laboratory tool for identifying viral pathogens.

1. Background

Hand, foot, and mouth disease (HFMD) is a common acute infectious disease that usually affects infants and young children. Although most HFMD infections are mild and self-limiting, some patients develop significant morbid complications, including herpangina, myocarditis, encephalitis, acute flaccid paralysis, and pulmonary edema or hemorrhage, which can result in death. HFMD is caused by enteroviruses, which belong to the *Picornaviridae* family of single-stranded RNA, non-enveloped viruses. Human enterovirus 71 (EV71) and the closely related coxsackievirus A16 (CVA16) are the main causes of HFMD [1,2], while other enteroviruses including CVA4, CVA6, CVA10, CVA12, CVB and echovirus (ECHO) also cause sporadic infections [3–8].

Molecular diagnostic assays have a great impact on infectious disease diagnosis and clinical management, particularly for viral infections. Rapid molecular virological diagnosis is also mandatory to quickly implement proper public health measures to limit the

Abbreviations: RT-PCR/ESI-MS, reverse transcription-PCR coupled with electrospray ionization mass spectrometry; HFMD, hand, foot and mouth disease; EV, enterovirus; CVA, coxsackievirus A; ECHO, echovirus; CSF, cerebrospinal fluid; HSV, herpes simplex virus; VZV, varicella zoster virus; EBV, Epstein–Barr virus; CMV, cytomegalovirus; HHV, human herpesvirus.

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extension of the outbreak of HFMD. Conventionally, enterovirus (EV) is detected and serotyped by labor- and time-intensive assays such as indirect immunofluorescence, cell culture and neutralization assays. The current molecular methods – which amplify, sequence and phylogenetically analyze viral genomes – are effective for detecting and typing EV [9–12].

Multilocus PCR and reverse transcription-PCR followed by electrospray ionization mass spectrometry (RT-PCR/ESI-MS) rapidly detect, identify and semi-quantify multiple pathogens simultaneously. This method has successfully identified multiple respiratory viruses [13,14], influenza viruses [15], adenovirus [16], orthopoxvirus [17], flaviviruses [18] and other common viruses [19]. Severe HFMD or post-HFMD complications are sometimes due to multiple virus co-infections with enterovirus and norovirus or rotavirus, for example [20,21]. By allowing broad-range, simultaneous and multiplexed pathogen detection in public healthcare settings, RT-PCR/ESI-MS could aid the early diagnosis of HFMD.

2. Objectives

The aim of the study is to evaluate the accuracy and efficacy of the multilocus RT-PCR/ESI-MS method to both detect and identify enterovirus from specimens of clinical diagnosed HFMD patients.

3. Study design

3.1. Study population and clinical specimens

This study was conducted during the period of March 1, 2010 to June 30, 2011 and included 146 patients in a consecutive fashion with clinical diagnoses of HFMD (86 males and 60 females, aged from 4 months to 5 years, with median age of 3 years). Totally 152 specimens were obtained, including 48 throat swabs, 22 stool, 80 serum specimens and 2 cerebrospinal fluid (CSF), in cases of encephalitis. The specimens were taken following clinical examination. The diagnostic criteria for clinical HFMD cases were listed in "Guide for HFMD diagnosis and treatment (2010)" by the Chinese Ministry of Health. In short, diagnosis was based on age and symptoms including fever in infants and young children, vesicular rash and cutaneous vesicles on the hands, feet, mouth, tongue, inside of the cheeks, or buttocks. Throat swabs were transferred to viral transportation medium immediately after collection.

3.2. Nucleic acid extraction and cDNA synthesis

Approximately 0.5 g stool was suspended in 1 mL of PBS. Total nucleic acids were extracted from 200 μL of throat swab, sera or stool supernatants with a QIAamp MinElute Virus Spin Kit (Qiagen). cDNA was synthesized with a SuperScript III reverse transcriptase kit according to manufacturer's protocol (Invitrogen).

3.3. Viral pathogen detection and identification by RT-PCR/ESI-MS

A novel Broad Virus I assay (Abbott, USA) using RT-PCR/ESI-MS was used to detect and identify viruses in clinically diagnosed HFMD patients, which included human enterovirus A–D, adenovirus A–F, herpes simplex virus 1, 2 (HSV-1, -2), varicella zoster virus (VZV), Epstein–Barr virus (EBV), cytomegalovirus (CMV), human herpesvirus (HHV) 6–8, parvovirus B19 and polyomavirus. In this assay, 14 primer pairs detected DNA targets, RNA targets, and control DNA were distributed in a 96-well plate (Supplemental Table 1). The same total nucleic acid extracted above was added to 8 wells and amplified by multiplex RT-PCR and PCR. Automated post-PCR desalting, ESI-MS signal acquisition, spectral analysis, and data analysis were performed by a previously described mass spectrometry platform (Abbott, USA) [15,22,23]. Semi-quantitative results were obtained by comparing the peak heights with the internal PCR calibration controls present in each well.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jcv.2013.11.007.

3.4. Detection of enterovirus and typing of enterovirus by reference diagnostic tests

The detection and typing of enterovirus by RT-PCR/ESI-MS were evaluated by comparing with reference molecular diagnostic tests.

A reference diagnostic nested PCR currently used in a clinical laboratory detecting EV was performed on cDNA from all specimens. For the first round of PCR, primers MD91 and ENTD were used and in the nested PCR, primers MD91 and MD90 [2] were used (Supplemental Table 1). The presence of a 154-bp band indicated successful amplification of EV [2,24].

A 480-bp fragment of the EV 5′ UTR was amplified in all EV-positive specimens using primers ENTD and CA160 (Supplemental Table 1) [24,25]. The purified amplicons were ligated into the pMD18T vector (Takara, Japan), transformed into *Escherichia coli* competent cells and sequenced with a universal primer by ABI 3730. The consensus sequences were compared by BLAST with EV sequences available in GenBank [26]. Each sequence was classified according to its highest BLAST identity score.

3.5. Statistics

EV detection results from RT-PCR/ESI-MS and reference assays were compared for all specimens. Kappa values were calculated by Cohen's kappa coefficient measurement. Values greater than 0.75 indicated a strong association. A p value of <0.05 was considered statistically significant.

4. Results

4.1. Enterovirus detection by RT-PCR/ESI-MS and reference tests

As shown in Table 1, 97 of 152 samples (64%) were EV-positive by RT-PCR/ESI-MS. By the reference test, 96 (63%) specimens were EV-positive (Table 1). Thus, RT-PCR/ESI-MS and the reference test had equivalent EV detection rates of 100% in stool, 40–41% in sera and 85–87% in throat swabs. Both tests had statistically significant concordance in EV detection ($\kappa = 0.816 \pm 0.049$, $p < 0.001$). For detecting EV, the sensitivity and specificity of the RT-PCR/ESI-MS assay were 93.8% (95% CI: 91.8–95.7%) and 87.5% (95% CI: 84.8–90.2%), respectively. For different types of specimens, the sensitivity of EV detection was 100%, 98% and 87% in stool, throat swabs and sera, respectively.

4.2. Enterovirus typing by RT-PCR/ESI-MS

All EV specimens ($n = 97$) positively detected by the ESI-MS platform were successfully typed. Of the 97 specimens, 84 EV specimens were successfully typed by the reference method, whereas 7 specimens were EV-negative by the reference method and 6 were unable to yield the typing PCR fragments. Therefore, EV typing methods were only compared in these 84 positive specimens. The prevalent viruses in our study were EV71 (56/84, 67%) and CVA16 (15/84, 18%), showing the typical distribution of EV infections in HFMD patients from China [27] (Table 2). Other viruses including CVA10, CVA6, CVA12, CVA9, CVB3, ECHO9 and ECHO30 were also present. Of these, 65 (77%) EV genotypes identified by