Diagnosis of human enterovirus A71 infection in Malaysia using a commercial IgM-capture enzyme-linked immunosorbent assay and an IgM-colloidal gold immunochromatographic assay

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Abstract. Hand, foot and mouth disease (HFMD) is a common childhood infection caused by many enteroviruses, including enterovirus A71 (EV-A71). As EV-A71 is associated with severe neurological disease, early diagnosis is critical for clinical and public health management. In developing countries such as Malaysia, laboratory capacity to carry out EV-A71 IgM detection is greater than that of the gold standard methods of virus culture or molecular detection. This study evaluated two diagnostic kits, EV-A71 IgM-capture enzyme-linked immunosorbent (ELISA) and EV-A71 IgM-colloidal gold immunochromatographic assay (GICA), which had previously only been assessed in China. The assays were tested with 89 serum samples from patients with suspected HFMD. The sensitivity, specificity, positive predictive value, and negative predictive value rates were 78.4%, 80.8%, 74.4%, and 84.0%, respectively, for the IgM-capture ELISA, and 75.7%, 76.9%, 70.0%, and 81.6% for the IgM GICA. These performance measures were similar between the two assays. Concordance between the two assays was 91.1%. The sensitivity rates were lower than those previously reported, likely because the multiple circulating EV-A71 genotypes in Malaysia differ from the C4 subgenotype found in China and used in the assays. Both assays had low false positive rates (12.5% and 16.7% for ELISA and GICA, respectively) when tested on sera from patients confirmed to have enteroviruses. Both diagnostic kits are suitable for early diagnosis of HFMD caused by EV-A71 in Malaysia, but confirmation with culture or PCR is still important.

INTRODUCTION

Hand, foot, and mouth disease (HFMD) is a common viral infection in children. HFMD usually results in mild and self-limiting illness, characterised by fever, vesicular lesions on the palms and soles and oral ulcers. The causative agents of HFMD are viruses from the genus of Enterovirus within the family of Picornaviridae, with enterovirus A71 (EV-A71) and coxsackievirus A16 (CV-A16) being the most commonly detected viruses. Over the last decade, EV-A71 was responsible for several large epidemics in the Asia-Pacific region, raising serious public health concerns (Solomon et al., 2010; Chan et al., 2011). EV-A71 infection can cause severe and potentially fatal neurological complications such as aseptic meningitis, brainstem encephalitis and acute flaccid paralysis, especially in children below 5 years old (Ooi et al., 2010; Solomon et al., 2010). Currently there are no effective antivirals and vaccines against EV-A71 (Tan et al., 2014), hence early diagnosis of EV-A71 infection is critical for prompt public health measures to control the spread of virus and minimize the risk of fatality.
Traditionally, standard laboratory diagnosis of EV-A71 was achieved by immunofluorescence assay or virus culture of samples from the lesions, throat or rectum (Muir et al., 1998). However, immunofluorescence is insensitive, and viral culture is time-consuming and labour intensive. Additionally, the yield of enteroviruses from clinical specimens may be low, as some enteroviruses, particularly the coxsackievirus A group, do not readily grow in cell culture (Lipson et al., 1988). Neutralization test is used to detect specific antibodies that inhibit viral-induced cytopathic effect (CPE) (Muir et al., 1998), but is not widely used as it is difficult to perform, requires the use of infectious virus in a biosafety level II laboratory, and availability of paired patient sera to confirm seroconversion. Molecular diagnosis methods such as reverse transcription polymerase chain reaction (RT-PCR) are far more sensitive and quicker, and are the new “gold standard” for enterovirus detection (Hamilton et al., 1999; Robinson et al., 2002). However, it may not be widely available throughout developing countries where EV-A71 is more common, as it requires specialized equipment and well-trained personnel. Hence there is a need for a more accessible, reliable and rapid diagnostic assay for EV-A71 in laboratories without access to RT-PCR. A serological test such as enzyme-linked immunosorbent assay (ELISA) is an alternative as results can be obtained in 2-3 hours and most diagnostic laboratories will have access to the equipment, and IgM can be detected early in EV-A71 infection (Zhao et al., 2011). The use of two commercially available EV-A71 IgM kits, EV-A71 IgM-capture ELISA and EV-A71 IgM-colloidal gold immunochromatographic assay (GICA), have been reported in China (Xu et al., 2010; Yu et al., 2012; Wang et al., 2015) but it is critical to evaluate assays in different geographical settings, where there may be potential differences in circulating EV-A71 genotype and patient immune responses. In this study, we evaluated the performances of these two commercial assays in serum samples collected from HFMD patients in Malaysia.

MATERIALS AND METHODS

Serum specimens
Three panels of human sera (n=136) were used for the evaluation of EV-A71 IgM ELISA and IgM GICA, and were obtained from the diagnostic virology laboratory, University Malaya Medical Centre, in Kuala Lumpur, Malaysia. Panel A consisted of 36 residual serum samples obtained during a HFMD outbreak in 2000, and panel B consisted of 53 sera prospectively collected during an outbreak in 2012-2013. All patients from panels A and B showed clinical signs of HFMD (including ulcers in the mouth/tongue, rash/vesicles on the palms and soles, with/without fever), and were considered positive for EV-A71 or non-EV-A71 enteroviruses according to the virus cultured from throat swabs, vesicle swabs and/or rectal swabs. Swabs from patients in panel B were also tested by PCR for enteroviruses. Panel C, the non-HFMD (negative control) samples, consisted of 47 residual serum samples tested positive for IgM for other viral infections, such as dengue, chikungunya, measles, herpes simplex virus and varicella-zoster virus. This study was approved by the hospital's Medical Ethics Committee (reference number: 932.17). Our institution does not require informed consent for retrospective studies of anonymised samples.

Enterovirus detection and genotyping
Viral RNA was extracted using QIAamp Viral RNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. To detect enterovirus, the 5'-untranslated region (5’UTR) was amplified with primers CoxbanS (5’-GTAMCYTTTGGTRCGCCWGT3’ ) and CoxbanR (5’-GAAACACGGACACCCAAAA GTA-3’, Arola et al., 1995) using Access RT-PCR system (Promega, USA). The reaction was subjected to reverse transcription at 42°C for 60 min and reverse transcriptase inactivation at 94°C for 2 min, followed by 40 cycles at 94°C for 30 sec, 50°C for 1 min, and 68°C for 1 min, and final extension of 68°C for 7 min. The expected PCR products of 502 bp were visualized with 1.5% agarose gel stained with GelRed stain (Biotium Inc, Hayward, USA) under ultraviolet trans-
illumination. The purified amplicons were then sequenced with a 3730xl DNA Analyzer (Applied Biosystems). Sequencing results were subjected to BLAST search to identify the enterovirus serotypes.

Virus isolation
Clinical specimens (throat swabs, vesicle swabs and/or rectal swabs) were used for virus isolation. Specimens were inoculated into human rhabdomyosarcoma (RD) cell cultures in a 24-well plate. Viral cultures showing CPE were harvested and immunofluorescence assay was subsequently performed.

Immunofluorescence assay
Viral cultures showing CPE were harvested and centrifuged at 250 x g for 10 min. Cell pellets were resuspended with phosphate-buffered saline (PBS) and coated on poly-D-lysine treated microscope slides. After air drying, the cells were fixed with 3.7% paraformaldehyde and incubated for 10 min. The fixed cells were washed and permeabilized using 0.25% Triton X-100 (Sigma, USA) for 5 min. The cells were subsequently blocked with Image-iT FX Signal Enhancer (Invitrogen, USA) for 1 h. Enterovirus-infected cells were immunostained with Light Diagnostics Pan-Enterovirus Blend (Millipore, USA) as the primary antibody and FITC-labeled anti-mouse IgG conjugate (Millipore, USA) as the secondary antibody for 1 h at 37°C in a humidified chamber. Immunofluorescence was detected with a fluorescence microscope. All enterovirus-positive samples were further tested with Light Diagnostics EV-A71 monoclonal antibody 3324 (Millipore, USA) to confirm EV-A71.

EV-A71 IgM-capture ELISA
EV-A71 specific-IgM antibody in serum specimens were detected using EV-A71 IgM-capture ELISA (Beijing Wantai, China) according to the manufacturer’s instructions. Briefly, aliquots of 100 µl/well of diluent and 10 µl/well of serum were sequentially added into microplates coated with anti-human IgM µ-chain, followed by 30 min incubation at 37°C. After washing five times with PBS, 50 µl of purified EV-A71 antigen and 50 µl of horseradish peroxidase (HRP)-conjugate anti-EV-A71 monoclonal antibody were added to the microplate, which was then incubated for 30 min at 37°C. The plate was washed five times with PBS, followed by addition of 50 µl of urea peroxide and 50 µl of TMB substrate, and further incubated for 15 min at 37°C in the dark. The reaction was terminated with 50 µl of 2.0 M sulfuric acid. Optical density (OD) of each well was read at 450 nm with a 630 nm reference filter with a microplate reader (BioTek Instruments, USA). The cut-off value was calculated as 0.1 + mean OD of the negative controls. If the mean OD of negative controls was lower than 0.05, this was treated as 0.05. A serum specimen was considered positive with a signal/cut-off (S/CO) value of ≥ 1.0.

EV-A71 IgM GICA
Serum EV-A71 specific-IgM was detected with EV-A71 IgM GICA kit (Beijing Wantai, China) according to the manufacturer’s instructions. An aliquot of 15 µl of serum was added to the specimen diluent and mixed well. Aliquots of 80 µl of diluted samples were pipetted into the sample wells of the EV-A71 IgM GICA cassette and the results were read within 30 min. EV-A71-specific IgM antibodies were captured by immobilized EV-A71 antigen and formed an antibody-antigen complex on the test line. Serum specimens were considered positive if bands appeared at the test line and control line.

Statistical analysis
The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of EV-A71 IgM-capture ELISA and EV-A71 IgM GICA were calculated, and compared using Fisher’s exact test. Statistical analyses were performed using GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA). Cohen’s kappa (κ) was used to estimate inter-assay concordance, with a value of 1 indicating complete agreement. A P-value of <0.05 was considered significant.
RESULTS

Sera grouping
A total of 89 HFMD sera (36 sera from panel A and 53 sera from panel B) and 47 non-HFMD sera (panel C) were used for the evaluation (Table 1). Of these, 37 HFMD sera were from EV-A71-positive patients (confirmed by RT-PCR or culture), and 24 HFMD sera were positive for non-EV-A71 enteroviruses, which consist of CV-A4 (n=1), CV-A6 (n=10), CV-A16 (n=2), CV-B (n=1), echovirus 7 (n=6), rhinovirus (n=1) and untyped enteroviruses (n=3). The remaining HFMD sera were enterovirus RT-PCR-/culture-negative (n=28).

Performance characteristics
Overall sensitivity and specificity rates were moderately good for EV-A71 IgM-capture ELISA and IgM GICA, with 78.4% and 75.7% sensitivity and 89.9% and 85.9% specificity, respectively (Table 2). NPV rates were >90%, but PPV rates were moderate for ELISA (74.4%) and GICA (66.7%). Performances of the assays were also determined just for the HFMD cases, as this is the most likely group to be tested for EV-A71 IgM. Specificity (ELISA, 80.8% and GICA, 76.9%) and NPV (ELISA, 84.0% and GICA, 81.6%) decreased, while PPV were similar. There were no significant differences in sensitivity, specificity, PPV and NPV rates between the two assays.

Concordance between the results of the two assays for each category of sera is shown in Table 3. The overall concordance was 91.1%, with a $\kappa$ value of 0.805. The concordance for testing the HFMD sera (panels A and B) was 92.1%, with a $\kappa$ value of 0.841. The concordance for testing samples from EV-A71-positive cases was 97.3%, with a $\kappa$ value of 0.924, whereas the concordance for testing EV-A71 negative cases was 91.9%, with a $\kappa$ value of 0.622.

False positive rates of IgM-capture ELISA and IgM GICA
In sera from patients with HFMD but no confirmed EV-A71, anti-EV-A71 IgM was detected in 10/52 (19.2%) and 12/52 (23.1%) with IgM-capture ELISA and IgM GICA, respectively. Of those with confirmed non-EV-A71 enterovirus infection, anti-EV-A71 IgM was detected in 3/24 (12.5%) and 4/24 (16.7%) with IgM-capture ELISA and IgM GICA, respectively. These false positives were detected in patients confirmed to have echovirus 7 (n=2) and CV-A16 (n=1) using the IgM-capture ELISA assay, whereas the false positives in the IgM-GICA assay were seen in patients with echovirus 7 (n=1), CV-A16 (n=1) and CV-A6 (n=2). Two samples (one each with echovirus 7 and CV-A16) were positive with both assays. For the non-HFMD sera, only the IgM GICA recorded false positives, in 2/47 (4.3%) samples. Overall, the mean S/CO value (0.76±2.37) of the false-

Table 1. Classification of serum samples used for the evaluation of EV-A71 commercial diagnostic kits

<table>
<thead>
<tr>
<th>Group</th>
<th>Panel A</th>
<th>Panel B</th>
<th>Panel C</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>EV-A71$^a$</td>
<td>24</td>
<td>13</td>
<td>N/A</td>
<td>37</td>
</tr>
<tr>
<td>Non-EV-A71 enterovirus$^a,b$</td>
<td>12</td>
<td>12</td>
<td>N/A</td>
<td>24</td>
</tr>
<tr>
<td>Enterovirus RT-PCR-/culture-negative$^a$</td>
<td>N/A</td>
<td>28</td>
<td>N/A</td>
<td>28</td>
</tr>
<tr>
<td>Non-HFMD</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>47</td>
</tr>
<tr>
<td>Total</td>
<td>36</td>
<td>53</td>
<td>47</td>
<td>136</td>
</tr>
</tbody>
</table>

$^a$tested on throat swabs, vesicle swabs and/or rectal swabs from the same patient; all patients had suspected HFMD

$^b$CV-A4 (n=1), CV-A6 (n=10), CV-A16 (n=2), CV-B (n=1), echovirus 7 (n=6), rhinovirus (n=1), untyped enteroviruses (n=3).

N/A, not applicable
Table 2. Comparative performances of the EV-A71 IgM-capture ELISA and EV-A71 IgM GICA in serum samples

<table>
<thead>
<tr>
<th></th>
<th>EV-A71 (n=37)</th>
<th>non-EV-A71</th>
<th>Sensitivity, % [95% CI]</th>
<th>Specificity, % [95% CI]</th>
<th>PPV, % [95% CI]</th>
<th>NPV, % [95% CI]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgM positive</td>
<td>IgM negative</td>
<td>IgM positive</td>
<td>IgM negative</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall analysis&lt;sup&gt;a&lt;/sup&gt;</td>
<td>EV-A71 IgM-capture ELISA</td>
<td>29</td>
<td>8</td>
<td>10</td>
<td>89</td>
<td>89.9 (89.99)</td>
</tr>
<tr>
<td></td>
<td>EV-A71 IgM-GICA</td>
<td>28</td>
<td>9</td>
<td>14</td>
<td>85</td>
<td>85.9 (85.99)</td>
</tr>
<tr>
<td>Analysis of HFMD cases&lt;sup&gt;b&lt;/sup&gt;</td>
<td>EV-A71 IgM-capture ELISA</td>
<td>29</td>
<td>8</td>
<td>10</td>
<td>42</td>
<td>80.8 (42/52)</td>
</tr>
<tr>
<td></td>
<td>EV-A71 IgM-GICA</td>
<td>28</td>
<td>9</td>
<td>12</td>
<td>40</td>
<td>76.9 (40/52)</td>
</tr>
</tbody>
</table>

<sup>a</sup>non-EV-A71 cases comprising non-EV-A71 enterovirus, enterovirus RT-PCR-/culture-negative, and non-HFMD cases (n=99) for overall analysis

<sup>b</sup>non-EV-A71 cases comprising non-EV-A71 enterovirus and enterovirus RT-PCR-/culture-negative cases (n=52), all of whom had suspected HFMD
positive samples was significantly lower than the mean S/CO value of 4.70±3.91 for the EV-A71-positive sera (data not shown).

**DISCUSSION**

In Malaysia, HFMD is endemic and outbreaks of EV-A71 infection occur every 2-3 years (NikNadia et al., 2016). Early social distancing resulted in reduced HFMD cases in Sarawak, Malaysia in 2006 (Solomon et al., 2010). Therefore, early diagnosis of EV-A71 would enable early interventions to curb the spread of infection and appropriately observe patients for complications. While PCR will detect the virus earliest, IgM has also been shown as a good early indicator for EV-A71 infection, as it may be detectable as early as 1 day of illness and peaks on day 5 (Zhao et al., 2011), and is detectable for up to 94 days (Wang et al., 2004). Serological detection of IgM would be suitable for most laboratories which do not have PCR or viral culture facilities. More importantly, the rapid and more convenient GICA assay showed comparable performance to the ELISA-based assay, suggesting that it can be used in clinics or rural settings with no laboratory facilities.

There are very few commercial EV-A71 IgM diagnostic kits available. In this study, we compared EV-A71 IgM-capture ELISA and IgM GICA assays in Malaysian patients, and found sensitivity rates (78.4% and 75.7%, respectively) that were lower than the 93.6% and 94.1% (IgM-capture ELISA), and 93.3% (IgM-GICA) reported earlier in China (Xu et al., 2010; Yu et al., 2012, Wang et al., 2015).

One possible reason might be the varying detection of antibodies resulting from different circulating EV-A71 genotypes found in Malaysia and China. In Malaysia, subgenotypes B4, B5 and C1 circulated in 2000, and only subgenotype B5 has been present after 2005, whereas the current predominant EV-A71 in China is subgenotype C4 (Chan et al., 2011; Chan et al., 2012). The purified EV-A71 antigen used in the diagnosis kits was subgenotype C4, hence this may explain the higher sensitivity reported in the China studies.

When testing serum from children with HFMD, the assays showed specificity rates of 80.8% and 76.9% for the IgM-capture ELISA and IgM GICA, respectively. These specificity rates were lower than the 88.6% reported by Xu et al. (2010), but higher than the 69.6% reported for the IgM-capture ELISA by Yu et al. (2012) and the 50% for the IgM GICA reported by Wang et al. (2015). The PPV rates of 74.4% (ELISA) and 70.0% (GICA) were moderate, although these rates would have been affected by the lower prevalence of EV-A71 in the HFMD outbreak in 2012-2013 (panel B), which had relatively more cases due to CV-A6. The false positives are likely due to detection of IgM which recognize common epitopes among enteroviruses (Xu et al., 2010; Yu et al., 2012). Increasing the cut-off values according to locally-
determined background seropositive rates may improve specificity and PPV.

PCR is known to be the most efficient diagnosis for EV-A71 (Ooi et al., 2010); yet, in the present study, IgM was detected in 25.0% (ELISA) and 28.6% (GICA) of serum samples from patients with negative enterovirus RT-PCR. One possibility is the presence of low levels of virus RNA, which may be resolved by the use of real-time RT-PCR rather than the less sensitive conventional RT-PCR used in the present study. In addition, our RT-PCR is only limited to amplification of 5'UTR, so targeting other gene regions such as VP4/VP2 and VP1 should be considered.

One of the limitations of the present study is that the timings of disease onset are not known, and cannot be correlated with the IgM response. However, the value in this study is the use of samples from patients with confirmed enterovirus infection, in a different geographical location with different circulating enteroviruses to other previously reported evaluations, which were limited to China. The circulation of different serotypes of EV-A71 in Malaysia likely explains the lower test sensitivities seen here. Our study also showed that both EV-A71 IgM-capture ELISA and EV-A71 IgM GICA had comparable performance characteristics and concordance, despite the fact that point-of-care tests are generally felt to be inferior to ELISA-based assays. Therefore, the IgM GICA can be used in clinics or rural settings with no laboratory or ELISA facilities. Both diagnostic kits may be useful and convenient for the screening for EV-A71 infection during HFMD outbreaks in Malaysia, but confirmation (of patients with both positive and negative IgM results) with either culture or RT-PCR remains essential.

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