Hand, foot, and mouth disease (HFMD) is an infectious disease commonly diagnosed in young children and characterized by mucocutaneous papulovesicular rashes on the hands, feet, mouth, and buttocks. Human enterovirus 71 (EV71) and coxsackievirus A16 (CA16) are the principal causative agents of HFMD. EV71 is of special concern, because it is more often associated with major outbreaks and causes complications of greater severity and higher rates of mortality than other enteroviruses. In contrast, CA16-associated HFMD has a milder outcome and is accompanied by a much lower incidence of severe complications (7, 16, 21).

HFMD is an important public health concern worldwide (20). Since 1997, several large epidemics of HFMD have been reported in the Asia-Pacific region, especially in Southeast Asia, including Malaysia (2), Taiwan (24), and Singapore (5). In March 2008, an EV71-associated HFMD outbreak occurred in Fuyang City, Anhui Province, China, and subsequently spread rapidly and extensively across the entire country; a total of 488,955 HFMD cases, 126 of which were fatal, were reported nationwide in 2008 (28).

As no vaccine or antiviral drug is currently available, early and rapid detection is critical for HFMD prevention and control (30). At present, the causative agents of HFMD can be effectively diagnosed by the detection of infectious virus, viral antigens, viral genomic RNA, or antiviral antibodies (11, 17, 25, 27). Due to their speed, sensitivity, and specificity, reverse transcription-PCR (RT-PCR)-based molecular diagnostic assays are increasingly used to detect EV71 and CA16 RNA for HFMD diagnosis (8, 14, 15). Various commercial and in-house assays are currently available. Due to the limited availability of well-characterized reference reagents, most HFMD detection assays lack critical standardization of performance, which causes difficulties in comparing results between laboratories and consequently complicates the clinical interpretation of laboratory results for HFMD.

To date, many biological materials, including live virus, inactivated virus, and naked RNA, have been used as controls for the molecular diagnosis of RNA viruses (1, 19). However, each of these materials has some inherent disadvantages, such as the biosecurity risk of live virus, the risk of residual activity of inactivated virus, and the inability of clinicians to evaluate sample processing and RNA extraction and the relative instability of naked RNA. A potential alternative is armored RNA, a noninfectious and quantifiable synthetic substitute for live or inactivated RNA virus that can be spiked into clinical specimens without risking degradation, thus enabling simultaneous monitoring of nucleic acid extraction efficiency and the amplification process of the detection assay (3, 12, 18).

In this study, we constructed 3 types of armored RNAs carrying genomic sequence fragments from EV71 and CA16: one RNA specific for EV71, one RNA specific for CA16, and...
one RNA designed to detect all EV serotypes. Moreover, a pioneering national external quality assessment (EQA) study was organized within the nationwide HFMD reference laboratory network to evaluate the accuracy of EV71 and CA16 PCR assays used in laboratories in China.

**MATERIALS AND METHODS**

**Preparation of armored RNAs.** Three gene fragments were amplified by RT-PCR using EV71 and CA16 RNA as templates. Both EV71 RNA and CA16 RNA were kindly provided by the Beijing Center for Disease Prevention and Control (CDC). The EV71 5′ untranslated region (5′UTR) packaged into virus-like particles (EV-VLPs) is a pan-enterovirus sequence that is conserved among all enteroviruses. The primers used in this study are described in Table S1 in the supplemental material. As described in previous studies (13, 26), inclusion of a hepatitis C virus (HCV) 5′UTR in the chimeric armored RNA sequence allows the ready assignment of an international unit value to the chimeric armored RNA for quantitative detection. The HCV 5′UTR sequence was amplified from a pNCCL-HCV plasmid (constructed by our laboratory) and ligated to the EV 5′UTR by overlapping-extension PCR. Therefore, 3 armored RNAs, AR-HCV-EV(UTR)-994b (EV-VLP), AR-EV71(VP)-2557b (EV71-VLP), and AR-CA16(VP)-260lb (CA16-VLP), were generated by the conventional armored RNA technique (18) and further optimized as previously described in a report from our laboratory (29). The three armored RNAs were confirmed to contain the target sites for all PCR assays evaluated in this study.

EV-VLP armored RNA was calibrated using the WHO HCV RNA International Standard (version 3.0 [http://www.ncbi.nlm.nih.gov/documents/df/06-100.pdf]), and the other 2 armored RNAs (EV71-VLP and CA16-VLP) were calibrated using calibrated EV-VLP as a standard. Here, the armored RNA (EV-VLP) and the other 2 armored RNAs (EV71-VLP and CA16-VLP) were quantified three armored RNAs were confirmed to contain the target sites for all PCR optimized as previously described in a report from our laboratory (29). The three armored RNAs were confirmed to contain the target sites for all PCR assays evaluated in this study.

**Performance verification of VLP in two different matrices.** Two of the VLP mixtures (EV-EV71-VLP and EV-CA16-VLP [EV-VLP mixed with EV71-VLP and with CA16-VLP, respectively, at a copy number ratio of 2:1]) were spiked in serial 10-fold dilutions into MEM and pooled negative throat swab fluid, respectively, at dilutions from 10^0 to 10^7 IU/ml. As expected, all 3 types of armored RNAs were successfully calibrated using the WHO HCV RNA International Standard (22).

**Temperature and time stability assessments** were performed for the three armored RNAs prepared in the study. The quantified armored RNAs were diluted with NaN3-preserved minimal essential medium (MEM) to yield 10^0 and 10^12 IU/ml dilutions. For each dilution, a single batch was separated into 50 aliquots in individual time point samples of 0.5 ml. Two samples were stored at –80°C as controls. The remaining 48 samples were divided into four groups (12 samples/group) and incubated at 37°C, 25°C, 4°C, and –20°C, respectively. At each of the time points of 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, and 6 months, two samples of each group were removed and stored at –80°C until the completion of the experiment. All of the 50 samples were quantified in a single procedure using an enterovirus nucleic acid detection kit (real-time RT-PCR assay; Guangzhou Daan Gene Co., Ltd., Guangzhou, China).

In addition, two aliquots of the 10^0 IU/ml and 10^12 IU/ml dilutions were subjected to 5 freeze-thaw cycles and quantified using the enterovirus nucleic acid detection kit.

**Performance verification of VLP in two different matrices.** Two of the VLP mixtures (EV-EV71-VLP and EV-CA16-VLP [EV-VLP mixed with EV71-VLP and with CA16-VLP, respectively, at a copy number ratio of 2:1]) were spiked in serial 10-fold dilutions into MEM and pooled negative throat swab fluid, respectively, at dilutions from 10^0 to 10^12 IU/ml. Two panels that included 7 positive samples and 2 negative controls were prepared. After nucleic acid extraction using a QIAamp viral RNA minikit (Qiagen, Hilden, Germany), the two panel samples were tested for each of the aforementioned three targets in duplicate experiments in a single run by using the enterovirus nucleic acid detection kit. Finally, the cycle threshold (Ct) values determined for each target in the two panels were compared by a paired t-test (see Table S2 in the supplemental material).

**Participants.** The laboratories that perform HFMD molecular diagnosis in China were invited to participate in the EQA study. The invitees are all members of the Chinese Laboratory Network of HFMD Diagnosis, which is composed of municipal and provincial laboratories. These laboratories receive training and evaluation from the national Chinese Centers for Disease Control (CDC) laboratory annually, take responsibility for testing and surveillance of HFMD in their respective districts, and submit the surveillance data and 10 positive strains to the national laboratory each month during periods of peak HFMD prevalence.

**Proficiency panel.** The panel samples were designed and coded as indicated in Table 1. Tenfold serial dilutions of the 3 armored RNAs were made using NaN3-preserved MEM. The final panel consisted of a set of 16 positive samples and four NaN3-preserved MEM samples containing no VLPs for use as negative controls. Aliquots of 500 µl were assigned code numbers and stored at –20°C until distribution. Before distribution to the participants, the panel samples were tested using an in-house RT-PCR assay recommended by the Chinese CDC (http://www.chinacdc.cn/n272442/n272530/n3479265/n3479308/appendix/fujian1%20shouzukoubiaobencaijijijiancejishufangan.pdf) to confirm the positivity or negativity of the sample results.

Since the armored RNA was stable at higher temperatures, samples and detailed instructions were sent by express shipping at ambient temperature (approximately 10°C to 20°C) to 54 laboratories, and it took less than 1 week for them to reach those destinations. The recipients were requested to submit the results and other information on the assay details (RT-PCR method and RNA extraction procedure) within 6 weeks of receiving the panel.

**Evaluation of results.** Results were scored as optimal (20/20 [100%] correct responses), acceptable (at least 18 [≥90%] correct responses), or improbable (fewer than 18 (<90%) correct responses). Scored results were released to participants in an anonymous manner.

**Statistical analysis.** Data collected were entered into a spreadsheet in Microsoft Excel (Microsoft Corp., Bellingham, WA) and analyzed using SPSS 13.0 for Windows. The rates of correct responses for EV-, EV71-, and CA16-positive samples were compared using Pearson's chi-square test, which, together with Fisher's exact test, was also used to compare the sensitivity and specificity of participating laboratory PCR assays versus those of the Chinese CDC RT-PCR assay performed in the laboratory of one of the coauthors (J.I.) of the present study. Paired t-tests were performed to compare the cycle threshold values of VLPs determined using two different specimen matrices. A P value of <0.05 was considered statistically significant.

**RESULTS**

**Construction of armored RNAs.** Armored RNAs for EV, EV71, and CA16 were constructed successfully and were confirmed by sequencing to contain the corresponding full-length target sequence of all available assays for HFMD. Quantification using the WHO HCV international RNA standard yielded a concentration of 1.5 × 10^12 IU/ml. As expected, all 3 types of
armored RNA exhibited strong resistance to both RNase and DNase treatment.

At a concentration of \(10^5\) IU/ml, the three armored RNAs were stable in NaF-preserved MEM for 2 weeks at 37°C, 4 weeks at 25°C, 2 months at 4°C, and more than 6 months at \(20^\circ C\). At a concentration of \(10^3\) IU/ml, reduced stabilities were observed (1 week at 37°C, 2 weeks at 25°C, 4 weeks at 4°C, and more than 2 months at \(20^\circ C\)). Stability was defined as a decrease of no more than 0.5 log\(_{10}\) IU/ml compared to the control tube concentrations stored at \(-80^\circ C\) for the entire duration. The current stability findings are consistent with those of prior reports (3, 12, 18, 26, 29).

Performance comparison between armored RNAs in MEM and clinical sample matrices. There were no significant differences between the two panels in the cycle threshold (\(C_T\)) values determined for each of three targets (\(P > 0.05\)) (see Table S2 in the supplemental material), consistent with previous studies (4, 12).

Laboratory PCR methods and EQA performance. A total of 54 laboratories included in the national HFMD detection laboratory network were invited to participate in this EQA study. Among them, 41 laboratories replied with their results as requested, with 1 data set from each participant. The response rate was 75.9%.

As shown in Table 2, laboratories used various methods, including five monoplex commercial real-time RT-PCR kits (PCR assays A to E), one triplex commercial real-time RT-PCR assay (PCR assay F), and one in-house conventional RT-PCR assay recommended by the Chinese CDC (http://www.chinacdc.cn/n7/27442/n727530/n3479265/n3479308/appendix/fujian%20shouzoukoubiaojibenjiancejishufangan.pdf), to detect EV71 and CA16. Of the 41 data sets, 5 (12.2%) were generated using conventional in-house assays and 36 (87.8%) were produced by commercial real-time RT-PCR assays. The performance results differed substantially among all participating laboratories and among the laboratories using the same PCR assay (Table 2). Generally, laboratories using commercial assay B and the in-house PCR performed better than laboratories using assay A or C. Laboratories using assay D performed very poorly. Four laboratories, using 2 assay A (of the total of 17 laboratories using assay A) and both of the laboratories using assay D, failed to detect EV71 in any samples. Evaluations of the cumulative data according to comparisons of assay results (Table 3) support these findings, showing that the sensitivity of assay A for detection of EV71 was 74.5% (\(P < 0.05\)) and that assay D failed to detect any samples positive for EV71. Additionally, assay D showed decreased sensitivity for CA16. Only 31.7% (13/41) of the laboratories met criteria for optimal performance; 16 (39%) of the 41 participants showed acceptable performance, and the results from 12 (29.3%) of 41 laboratories indicated a need for improvement in HFMD diagnosis.

We next assessed the sensitivities of detection for the 3 targets (EV, EV71, and CA16) (Table 4). The sensitivity for EV71 (82.1%) was much lower than that for EV (97.4%) or CA16 (95.1%) (\(P < 0.05\)), but no obvious differences between the sensitivities for EV and CA16 were observed (\(P > 0.05\)). As expected, assay accuracy declined with decreasing concentration, and most of the detection failures occurred at the \(1 \times 10^5\) IU/ml target concentration. At that target concentration, the rates of detection were 90.2% for EV, 59.8% for EV71, and 84.1% for CA16.

All of the false-negative results were reported by 23 laboratories, and the overall proportion of false-negative results was 101/1,394 (7.3%). Considering that over 40% (17/41) of laboratories in the EQA program used assay A, the lower sensitivity of that assay for EV71 may contribute to false-negative results during routine testing of suspected diseases.
HFMD cases. The performance of assay D was unacceptable during this EQA, as the two laboratories that used that assay had scores of 30 and 55 (Table 2) and the assay failed to detect any samples containing EV71 and also had lower sensitivities for CA16 (Table 3).

A total of 19 (1.8%) false-positive results (of the total of 1,066 results) were reported in the study. The individual specificities of detection for EV, EV71, and CA16 were 157/164 (95.7%), 442/451 (98%), and 448/451 (99.3%), respectively, with no significant differences found among the 3 targets. There were also no statistical differences observed in specificity comparisons of each commercial assay versus the in-house assay (Table 3).

### TABLE 3. Comparison of sensitivity and specificity data between different commercial and in-house assays

<table>
<thead>
<tr>
<th>Assay</th>
<th>No. of laboratories using the indicated assay</th>
<th>EV detection</th>
<th>EV71 detection</th>
<th>CA16 detection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of correct positive results/total no. of positive results (% sensitivity)</td>
<td>No. of correct negative results/total no. of negative results (% specificity)</td>
<td>No. of correct positive results/total no. of positive results (% sensitivity)</td>
<td>No. of correct negative results/total no. of negative results (% specificity)</td>
</tr>
<tr>
<td>A</td>
<td>17</td>
<td>265/272 (97.4)</td>
<td>65/68 (95.6)</td>
<td>114/153 (74.5)</td>
</tr>
<tr>
<td>B</td>
<td>12</td>
<td>191/192 (99.5)</td>
<td>48/48 (100)</td>
<td>106/108 (98.3)</td>
</tr>
<tr>
<td>C</td>
<td>3</td>
<td>44/48 (91.7)</td>
<td>10/12 (83.3)</td>
<td>24/27 (88.9)</td>
</tr>
<tr>
<td>D</td>
<td>2</td>
<td>28/32 (87.5)</td>
<td>7/8 (87.5)</td>
<td>0/18 (0)</td>
</tr>
<tr>
<td>E</td>
<td>1</td>
<td>15/16 (93.8)</td>
<td>4/4 (100)</td>
<td>7/9 (77.8)</td>
</tr>
<tr>
<td>F</td>
<td>1</td>
<td>16/16 (100)</td>
<td>4/4 (100)</td>
<td>8/9 (88.9)</td>
</tr>
<tr>
<td>In-house</td>
<td>5</td>
<td>80/80 (100)</td>
<td>19/20 (95)</td>
<td>44/45 (97.8)</td>
</tr>
<tr>
<td>Total</td>
<td>41</td>
<td>639/656 (97.4)</td>
<td>157/164 (95.7)</td>
<td>303/369 (82.1)</td>
</tr>
</tbody>
</table>

### DISCUSSION

This study was the first national EQA program designed to evaluate molecular diagnosis of EV71 and CA16 by the use of armored RNAs as virus surrogates. Our data demonstrate that armored RNA serves as a robust and stable alternative to infectious or inactivated virus in proficiency programs. The 41 laboratories that participated in this EQA used six different TaqMan RT-PCR assays and one conventional RT-PCR assay. Our results in this study were not completely in agreement with those of previous EQA studies (9, 10), which reported that commercial real-time PCR technologies exhibited better sensitivity than conventional PCR. The less-than-perfect scores observed in this EQA study were likely due to a combination of poor assay performance (in particular, commercial assays A and D) and poor laboratory proficiency (contamination observed in 14 laboratories). However, several commercial assays performed well and represent a standardized method that would facilitate interpretation and comparability of results among different laboratories.

In this EQA, a triplex commercial real-time RT-PCR assay (kit F) was used by only one participant. Our communications with all participants revealed that most laboratories prefer single assays over multiplex methods, as they indicated that single assays customarily produce more sensitive and reliable results. The inclusion of multiple templates in a single reaction mixture has the potential to reduce the sensitivity of multiplex RT-PCR assays due to competition of reagents. Recently, accurate multiplex RT-PCR assays that simultaneously distinguish among EV71, CVA16, and other enteroviruses have been described (8, 23).

Laboratory use of commercial RT-PCR assay A in this EQA resulted in a wide range of scores, from 50 to 100%. Poor sensitivity for EV71 was the primary factor in less-than-perfect scores (Table 3). Further investigation of assay A revealed that higher rates of false-negative results were associated with specific lots of the EV71-specific reagents. While assay D was used by only two participants in this study, its false-negative rate for the EV71 samples reached a startling 100%. More studies are required to determine whether there are some subgenotypes of EV71 virus that are out of the detection scope of this assay. The lower sensitivity of some commercial assays for EV71...
indicated an urgent need to improve kit performance characteristics, internal quality control by the manufacturers, and external quality assurance in laboratories. In addition, false-positive results in some of the laboratories indicated the need for improved laboratory practices and quality management. The present report emphasizes that EQA is a very important tool for assessing the quality of diagnostic laboratory tests.

In summary, 3 armored RNAs for HFMD viruses were successfully constructed and used in a nationwide EQA in China. The results of this first nationwide EQA not only verified the feasibility of the use of those armored RNAs to serve as control samples but also highlighted a series of problems regarding HFMD diagnosis, such as the low sensitivity exhibited by some commercial assays and the overall poor performance of some participating laboratories. An EQA should be performed periodically to help laboratories monitor their ability to detect HFMD viruses and to improve the concordance of results from different laboratories. In addition, given the adaptability of RNA viruses, more HFMD viruses with a wider range of genotypes should be included in future EQA.

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