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External Quality Assessment for Enterovirus 71 and Coxsackievirus A16 Detection by Reverse Transcription-PCR Using Armored RNA as a Virus Surrogate▽†

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Three armored RNAs (virus-like particles [VLPs]) containing target sequences from enterovirus 71 (EV71) and coxsackievirus A16 (CA16) and a pan-enterovirus (pan-EV) sequence were constructed and used in an external quality assessment (EQA) to determine the performance of laboratories in the detection of EV71 and CA16. The EQA panel, which consisted of 20 samples, including 14 positive samples with different concentrations of EV and either EV71 or CA16 armored RNAs, 2 samples with all 3 armored RNAs, and 4 negative-control samples (NaN_3-preserved minimal essential medium [MEM] without VLPs), was distributed to 54 laboratories that perform molecular diagnosis of hand, foot, and mouth disease (HFMD) virus infections. A total of 41 data sets from 41 participants were returned; 5 (12.2%) were generated using conventional in-house reverse transcription-PCR (RT-PCR) assays, and 36 (87.8%) were generated using commercial real-time RT-PCR assays. Performance assessments of laboratories differed; 12 (29.3%) showed a need for improvement. Surprisingly, 4 laboratories were unable to detect EV71 RNA in any samples, even those containing the highest concentration of 10^7 IU/ml. Furthermore, the detection sensitivity for EV71 among all laboratories (82.1%) was substantially lower than that for EV (97.4%) or CA16 (95.1%). Overall, the results of the present study indicate that EQA should be performed periodically to help laboratories monitor their ability to detect HFMD viruses and to improve the comparability of results from different laboratories.

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

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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)-2601b (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.nibsc.ac.uk/documents/ifu/06-100.pdf]), and the other 2 armored RNAs (EV71-VLP and CA16-VLP) were quantified using calibrated EV-VLP as a standard. Here, the armored RNA (EV-VLP) content data are expressed in international units per milliliter based on 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 NaN_3-preserved minimal essential medium (MEM) to yield 10^3 and 10^5 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, 8 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 10^3 IU/ml and 10^5 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 2:1:1]) were spiked in serial 10-fold dilutions into MEM and pooled negative throat swab fluid, respectively, at dilutions from 10^3 IU/ml to 10^6 IU/ml. Two panels that included 7 positive samples and 2 negative controls were prepared. After nucleic acid extraction using a QIAmp 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 (C_T) 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 NaN_3-preserved MEM. The final panel consisted of a set of 16 positive samples and four NaN_3-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.

TABLE 1. EQA panel description and composite laboratory score for each panel member

Sample code	Sample content (VLP)[a]	Concn (IU/ml)[b]	No. of correct results reported/total no. of results (%)
HFMD02	EV-EV71	10^7	37/41 (90.2)
HFMD10	EV-EV71-CA16	10^6	37/41 (90.2)
HFMD14	EV-EV71	10^5	36/41 (87.8)
HFMD15	EV-EV71	10^5	36/41 (87.8)
HFMD06	EV-EV71	10^4	35/41 (85.4)
HFMD17	EV-EV71	10^4	36/41 (87.8)
HFMD08	EV-EV71	10^3	28/41 (68.3)
HFMD19	EV-EV71	10^3	19/41 (46.3)
HFMD12	EV-CA16	10^7	41/41 (100)
HFMD20	EV-EV71-CA16	10^6	37/41 (90.2)
HFMD04	EV-CA16	10^5	41/41 (100)
HFMD05	EV-CA16	10^5	41/41 (100)
HFMD07	EV-CA16	10^4	40/41 (97.6)
HFMD16	EV-CA16	10^4	37/41 (90.2)
HFMD09	EV-CA16	10^3	36/41 (87.8)
HFMD18	EV-CA16	10^3	31/41 (75.6)
HFMD01	None		38/41 (92.6)
HFMD03	None		36/41 (87.8)
HFMD11	None		39/41 (95.1)
HFMD13	None		39/41 (95.1)

[a] Equal copy numbers of EV-VLP were mixed with EV71-VLP or CA16-VLP as indicated, except for EV-EV71-VLP and EV-CA16-VLP, which were mixed with EV71-VLP and CA16-VLP, respectively, at a copy number ratio of 2:1:1. The EV-EV71 and EV-CA16 samples were designed as specificity controls for each other.

[b] The target concentrations of the panel samples were selected to range from 10^3 to 10^7 IU/ml, closely matching the concentration ranges that were detected in clinical samples as reported by Chang et al. (6).

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 improvable (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 NaN_3-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°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°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°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/n272442/n272530/n3479265/n3479308/appendix/fujian1%20shouzukoubiaobencaijijijiancejishufangan.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, 2 using 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 1×10^3 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

TABLE 2. Performance of 41 laboratories participating in the EQA study

Laboratory no.	Extraction method[a]	RT-PCR assay[b]	No. of correct samples/total no. of samples (%)	Classification
32	QIAamp	A	0 (100)	Optimal
17	RNeasy	A	1 (95)	Acceptable
20[e]	QIAamp	A	1 (95)	Acceptable
21	QIAamp	A	1 (95)	Acceptable
24[d]	QIAamp	A	1 (95)	Acceptable
37[d]	QIAamp	A	1 (95)	Acceptable
9	QIAamp	A	2 (90)	Acceptable
13	QIAamp	A	2 (90)	Acceptable
26[d]	QIAamp	A	2 (90)	Acceptable
29[d]	QIAamp	A	2 (90)	Acceptable
10	TRIzol	A	3 (85)	Improvable
11[e]	QIAamp	A	3 (85)	Improvable
30[d]	QIAamp	A	3 (85)	Improvable
31[d]	QIAamp	A	3 (85)	Improvable
7	QIAamp	A	9 (55)	Improvable
8[c]	QIAamp	A	9 (55)	Improvable
14[c,d]	QIAamp	A	10 (50)	Improvable
2	QIAamp	B	0 (100)	Optimal
4	QIAamp	B	0 (100)	Optimal
25	QIAamp	B	0 (100)	Optimal
27	QIAamp	B	0 (100)	Optimal
33	QIAamp	B	0 (100)	Optimal
34	QIAamp	B	0 (100)	Optimal
35	QIAamp	B	0 (100)	Optimal
38	QIAamp	B	0 (100)	Optimal
41	QIAamp	B	0 (100)	Optimal
12	QIAamp	B	1 (95)	Acceptable
40[d]	QIAamp	B	1 (95)	Acceptable
18	QIAamp	B	3 (85)	Improvable
28	QIAamp	C	0 (100)	Optimal
39	QIAamp	C	2 (90)	Acceptable
3[e]	QIAamp	C	5 (75)	Improvable
15[c]	QIAamp	D	9 (55)	Improvable
6[c,e]	QIAamp	D	14 (30)	Improvable
5	QIAamp	E	2 (90)	Acceptable
1	QIAamp	F	1 (95)	Acceptable
16	RNeasy	In-house	0 (100)	Optimal
36	QIAamp	In-house	0 (100)	Optimal
19[d]	TRIzol	In-house	1 (95)	Acceptable
23[e]	QIAamp	In-house	2 (90)	Acceptable
22	TRIzol	In-house	3 (85)	Improvable

[a] QIAamp, QIAamp viral RNA minikit (Qiagen) ($n = 36$ [87.8%]); RNeasy, RNeasy minikit (Qiagen) ($n = 2$ [4.9%]); TRIzol (Invitrogen) ($n = 3$ [7.3%]).

[b] A, B, C, D, E, and F represent 6 commercial TaqMan real-time RT-PCR kits for the detection of hand, foot, and mouth disease. A, Kinghawk Pharmaceutical, Beijing, China; B, Guangzhou Daan Gene Co., Ltd., Guangzhou, China; C, Promega Corporation, Fitchburg, WI; D, Bioperfects Technologies, Shanghai, China; E, Beijing Ipe-bio Technologies Co., Ltd., Beijing, China; F, Beijing Sunbiostar Gene Technologies Co., Ltd., Beijing, China. A to E are monoplex assays, and F is a triplex assay. In-house, in-house conventional RT-PCR assay for HFMD recommended by the Chinese CDC.

[c] The laboratory ($n = 4$) failed to detect EV71 in any of the samples.

[d] The laboratory ($n = 14$) returned one false-positive result.

[e] The laboratory ($n = 5$) returned two false-positive results.

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

Assay[a]	No. of laboratories using the indicated assay	EV detection		EV71 detection		CA16 detection	
		No. of correct positive results/total no. of positive results (% sensitivity)	No. of correct negative results/total no. of negative results (% specificity)	No. of correct postivie results/total no. of postve results (% sensitivity)	No. of correct negative results/total no. of negative results (% specificity)	No. of correct positive results/total no. of postve results (% sensitivity)	No. of correct negative results/total no. of negative results (% specificity)
A	17	265/272 (97.4)	65/68 (95.6)	114/153 (74.5)[b]	181/187 (96.8)	146/153 (95.4)	185/187 (98.9)
B	12	191/192 (99.5)	48/48 (100)	106/108 (98.1)	131/132 (99.2)	106/108 (98.1)	132/132 (100)
C	3	44/48 (91.7)	10/12 (83.3)	24/27 (88.9)	33/33 (100)	25/27 (92.6)	33/33 (100)
D	2	28/32 (87.5)[b]	7/8 (87.5)	0/18 (0)[b]	22/22 (100)	14/18 (77.8)	21/22 (95.5)
E	1	15/16 (93.8)	4/4 (100)	7/9 (77.8)	11/11 (100)	9/9 (100)	11/11 (100)
F	1	16/16 (100)	4/4 (100)	8/9 (88.9)	11/11 (100)	9/9 (100)	11/11 (100)
In-house	5	80/80 (100)	19/20 (95)	44/45 (97.8)	53/55 (96.4)	42/45 (93.3)	55/55 (100)
Total	41	639/656 (97.4)	157/164 (95.7)	303/369 (82.1)	442/451 (98)	351/369 (95.1)	448/451 (99.3)

[a] A, B, C, D, E, F, and In-house represent the same assays as described for Table 2.
[b] Statistically significant result compared to in-house assay result ($P < 0.0083$). The criteria of test α were adjusted using the Bonferroni method (22a). $\alpha = 0.05/(\text{group no.} - 1) = 0.0083$.

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 sensitivity 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 4. Summary of results for the 4 types of target specimens in the EQA

Sample[a]	Concn (IU/ml)	No. of replicates per laboratory[b]	No. of positive samples/no. of samples tested (%)	Total no. of positive samples/total no. of samples tested (%)
EV	10^7	2	82/82 (100)	639/656 (97.4)
	10^6	2	82/82 (100)	
	10^5	4	164/164 (100)	
	10^4	4	163/164 (99.4)	
	10^3	4	148/164 (90.2)	
EV71	10^7	1	37/41 (90.2)	303/369 (82.1)
	10^6	2	74/82 (90.2)	
	10^5	2	72/82 (87.8)	
	10^4	2	71/82 (86.6)	
	10^3	2	49/82 (59.8)	
CA16	10^7	1	41/41 (100)	351/369 (95.1)
	10^6	2	82/82 (100)	
	10^5	2	82/82 (100)	
	10^4	2	77/82 (93.9)	
	10^3	2	69/82 (84.1)	
Negative	0	4	12[b]/164 (7.9)	12/164 (7.3)

[a] EV71 and CA16 were used as specificity controls for each other. False-positive results for EV ($n = 7$) and EV71 ($n = 6$) were present in 12 negative samples. Negative sample 11 was simultaneously contaminated by EV and EV71 in laboratory 20. No false-positive CA16 results were reported for negative samples.
[b] Because positive samples contained two or three targets, the total number of replicates exceeds the number of samples tested ($n = 20$).

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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