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Development and Evaluation of an Enterovirus D68 Real-Time Reverse Transcriptase PCR Assay

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We have developed and evaluated a real-time reverse transcriptase PCR (RT-PCR) assay for the detection of human enterovirus D68 (EV-D68) in clinical specimens. This assay was developed in response to the unprecedented 2014 nationwide EV-D68 outbreak in the United States associated with severe respiratory illness. As part of our evaluation of the outbreak, we sequenced and published the genome sequence of the EV-D68 virus circulating in St. Louis, MO. This sequence, along with other GenBank sequences from past EV-D68 occurrences, was used to computationally select a region of EV-D68 appropriate for targeting in a strain-specific RT-PCR assay. The RT-PCR assay amplifies a segment of the VP1 gene, with an analytic limit of detection of 4 copies per reaction, and it was more sensitive than commercially available assays that detect enteroviruses and rhinoviruses without distinguishing between the two, including three multiplex respiratory panels approved for clinical use by the FDA. The assay did not detect any other enteroviruses or rhinoviruses tested and did detect divergent strains of EV-D68, including the first EV-D68 strain (Fermon) identified in California in 1962. This assay should be useful for identifying and studying current and future outbreaks of EV-D68 viruses.

A human enterovirus D68 (EV-D68) was first isolated from samples obtained in California in 1962 from four children with pneumonia and bronchiolitis (1). The type strain isolated from one of these children was designated the Fermon strain. Subsequently, only small numbers of EV-D68 cases were reported until the early 2000s (2). However, from 2008 to 2012, outbreaks in Japan, the Philippines, the Netherlands, and the United States (Georgia, Pennsylvania, and Arizona) have revealed EV-D68 as an emerging pathogen capable of causing severe respiratory illness (2-7). During the 2014 enterovirus/rhinovirus season in the United States, EV-D68 circulated at an unprecedented level (5). From August 2014 to January 2015, Centers for Disease Control and Prevention (CDC) and state public health laboratories confirmed a total of 1,153 cases of respiratory illness caused by EV-D68, with ≥14 deaths. The spectrum of disease was diverse. Cases of flaccid paralysis have been reported in association with EV-D68 infection, but as of the time of this report, a causal relationship has not been proven (8). The infected individuals were primarily children and resided in 49 states and the District of Columbia (5). The CDC also reported that there were likely millions of EV-D68 infections in which the etiology was not determined (5).

In mid-August of 2014, hospitals in Missouri and Illinois noticed an increased number of patients with severe respiratory illness and reported the presence of EV-D68 (6). We also observed this pattern at St. Louis Children’s Hospital in St. Louis, MO. Because efforts to define the outbreak were hampered by the lack of a test for EV-D68 that did not require nucleotide sequencing, we undertook the development of a rapid and specific reverse transcriptase PCR (RT-PCR) assay. We began by sequencing the genome of a representative EV-D68 isolate from St. Louis to obtain the sequence information required to define an assay with optimal sensitivity and specificity (9). EV-D68 causes respiratory illness, and the virus can be found in respiratory secretions, such as saliva, nasal mucus, or sputum (7), of an infected person. Therefore, an appropriate assay would primarily focus on evaluating respiratory disease due to EV-D68 by targeting nasopharyngeal and other respiratory specimens.

The development goals for our EV-D68 RT-PCR assay included (i) avoiding false-positive detection of closely related enteroviruses and rhinoviruses, (ii) increasing clinical and analytical sensitivity compared to those of other available assays, and (iii) retaining capability for sensitive detection of all known EV-D68 variants.

MATERIALS AND METHODS

Local specimens. After the EV-D68 outbreak was identified in August 2014 (6), clinical specimens testing positive for enterovirus/rhinovirus with the BioFire FilmArray respiratory virus panel (BioFire Diagnostics, Inc., Salt Lake City, UT) were provided for further testing by the Diagnostic Virology Laboratory at St. Louis Children’s Hospital, consistent with a protocol for testing of deidentified residual clinical specimen material approved by the Washington University Human Research Protection Office. Fourteen enterovirus/rhinovirus-positive specimens from the 2014 season were identified as containing EV-D68 by sequencing of the 5’-nontranslated region of each virus (10). Extracts of total nucleic acid were prepared from 100-µl aliquots of original specimens using a bioMérieux NucliSSENS easyMAG automated extractor (bioMérieux, Durham, NC).
During the 2014 outbreak in St. Louis, the CDC Picornavirus Laboratory made a new EV-D68-specific RT-PCR assay available (Steve Oberste, Centers for Disease Control and Prevention, Atlanta, GA, personal communication). We tested the CDC EV-D68-specific RT-PCR according to the procedure available at that time on the CDC website. In addition, we tested the same assay with Cy5 replacing 6-carboxyfluorescein (FAM) as the probe reporter dye (modified CDC assay) because of concerns for quenching of FAM by the guanine base located at the 5' end of the probe (22) (Rangaraj Selvarangan, Children’s Mercy Hospital, Kansas City, MO, personal communication). The primers and probes for the CDC assay were ordered from Integrated DNA Technologies, Inc. (Coralville, IA).
Commercial and laboratory-developed assay testing. Commercial multiplex panels that detect enteroviruses/rhinoviruses were tested according to the manufacturers’ instructions. These assays included Lu- 
mxin xTAG respiratory viral panel (CE cleared-EU/ROW, analyzed with 
the IS software version 2.3, which includes the FDA-approved Luminex 
targets plus additional targets for coronaviruses and parainfluenzavirus 
type 4 to be used for research purposes only) (Luminex, Austin, TX), the 
GenMark Dx eSensor respiratory virus panel (GenMark Diagnostics, Inc., 
Carlsbad, CA), BioFire FilmArray respiratory panel (RP) (BioFire Diag-
nostics, Inc., Salt Lake City, UT), Cepheid GeneXpert EV IVD (Cepheid, 
Sunnyvale, CA), and Focus enterovirus primer pair analyte-specific re-
agent (ASR) (Focus Diagnostics, Inc., Cypress, CA).

We also evaluated two laboratory-developed tests (LDTs), the panen-
tervirus assay described by Nijhuis et al. (23), and an assay described by 
Piralla et al. (24) that targets the 5'-nontranslated region of EV-D68. To 
determine the relative sensitivities of the different LDTs and commercial 
molecular assays for detecting EV68, material from the original specimen 
that yielded the full-length sequence of the St. Louis EV-D68 strain was 
used. For the Cepheid GeneXpert and BioFire FilmArray assays, which 
require raw unextracted specimen, a series of 10-fold dilutions of the 
original specimen were made using universal transport medium (UTM) 
(Diagnostic Hybrids, Athens, OH) as diluent. Three hundred microliters 
of each dilution was then tested in the BioFire assay and 140 
l was used in 
the GeneXpert assay, according to the manufacturers’ instructions. For 
the LDTs and the GenMark and Luminex xTAG assays, which require 
extracted nucleic acids, total nucleic acids were extracted from 100 
ml of original specimen using a bioMérieux NucliSENS easyMAG automated 
extractor (bioMérieux, Durham, NC). A series of 10-fold dilutions of the 
extract were then made using low-EDTA TE as a diluent, and each dilu-
tion was tested in each assay. For the Focus enterovirus ASR assay, 5 
ml of reaction mix and 5 
l of EasyMAG nucleic acid extract were added to the 
wells of a 3M integrated cycler universal disc, and the amplification assay 
was run using standard Focus Diagnostics assay parameters and a 3M 
integrated cycler. For the panenterovirus assay, we used the AgPath-ID
An analysis was carried out using the IBM SPSS Statistics Desktop (version 22) to linearize the cloned VP1-containing plasmid on two separate days. Probit analysis was used to determine the analytic limit of detection. The plasmid was linearized with SpeI prior to its use as a template in the real-time RT-PCR assay. The analytic limit of detection was 6.7 RT-PCR cycles earlier than WashU design 1 assay, and 6.8 cycles earlier than WashU design 2 assay, for the WashU EV-D68 assays but not the CDC assays detected the Fermon strain.

Comparison of WashU and CDC assays using 2014 EV-D68 outbreak specimens and the Fermon strain.

<table>
<thead>
<tr>
<th>Test material</th>
<th>WashU design 1</th>
<th>WashU design 2</th>
<th>CDC assay</th>
<th>Modified CDC assay</th>
<th>∆C_T WashU design 1 and WashU design 2</th>
<th>∆C_T WashU design 2 and modified CDC assay</th>
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<tr>
<td>EV-D68 specimens</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WU-EV-1</td>
<td>21.0</td>
<td>21.3</td>
<td>Neg</td>
<td>23.7</td>
<td>0.3b</td>
<td>2.4</td>
</tr>
<tr>
<td>WU-EV-2</td>
<td>24.2</td>
<td>25.4</td>
<td>Neg</td>
<td>28.7</td>
<td>1.2</td>
<td>3.3</td>
</tr>
<tr>
<td>WU-EV-3</td>
<td>20.0</td>
<td>20.7</td>
<td>41.0</td>
<td>22.7</td>
<td>0.7</td>
<td>2.0</td>
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<tr>
<td>WU-EV-4</td>
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<td>20.8</td>
<td>Neg</td>
<td>22.5</td>
<td>0.1c</td>
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<td>22.7</td>
<td>34.6</td>
<td>24.4</td>
<td>0.5c</td>
<td>1.7</td>
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<td>WU-EV-6</td>
<td>20.9</td>
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<td>25.9</td>
<td>23.9</td>
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<td>2.7</td>
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<td>20.0</td>
<td>Neg</td>
<td>23.4</td>
<td>−0.5b</td>
<td>3.4</td>
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<td>30.8</td>
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<td>0b</td>
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<td>20.5</td>
<td>0.2b</td>
<td>3.0</td>
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<td>WU-EV-10</td>
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<td>30.8</td>
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<td>27.5</td>
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<td>14.7</td>
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<td>WU-EV-14</td>
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<td>32.7</td>
<td>20.6</td>
<td>−1.8b</td>
<td>2.1</td>
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<tr>
<td>Fermon strain</td>
<td>22.7</td>
<td>15.9</td>
<td>Neg</td>
<td>Neg</td>
<td>−6.8b</td>
<td>NA’</td>
</tr>
<tr>
<td>Water</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>NA</td>
<td>NA’</td>
</tr>
</tbody>
</table>

* C_T threshold cycle; Neg, negative. WashU design 1 was a distinct single paired-primer design, and WashU design 2 had degenerate bases and mixed primers included in the design. The CDC assay had a published design with FAM. The modified CDC assay was modified by replacement of FAM with Cy5.

b ∆C_T ≤ 0.5

a NA, not applicable.

One-step RT-PCR kit and recommended cycling conditions, using an Applied Biosystems 7500 real-time PCR system. For the assay targeting the 5’-nontranslated region of EV-D68, we followed the authors’ recommended procedures and cycling conditions, using an Applied Biosystems 7300 real-time PCR system.

Analytic limit of detection. A 791-bp region of VP1 containing the amplicon of the WashU assays was reverse transcribed, amplified, and cloned from a clinical sample from the 2014 season from St. Louis using the primers EV68-VP1-3121-rvni (TAGGYTCATGTAAACCCTRACRGT), which were previously described (25). The product was cloned using a TOPO TA cloning kit (Life Technologies, Grand Island, NY). Sequence was verified by dideoxy sequencing of the plasmid insert. The plasmid was linearized with SpeI prior to its use as a template in the real-time RT-PCR assay. The analytic limit of detection (LOD) was determined by testing up to 10 replicates of dilutions of the linearized cloned VP1-containing plasmid on two separate days. Probit analysis was carried out using the IBM SPSS Statistics Desktop (version 22) software.

RESULTS

Comparison of WashU and CDC assays. We tested our two assays and the two versions of the CDC assay on a set of clinical samples from the 2014 outbreak (Table 2). We also included the Fermon strain of EV-D68 obtained from the Children’s Hospital Colorado. The two WashU assays performed similarly on the samples, with <1 cycle difference between the two assays for 12 of the 14 samples. The published CDC assay (FAM reporter) performed less well, failing to detect 6 of the 14 samples. However, the modified CDC assay (i.e., with the substitution of FAM with Cy5) enabled the detection of all 14 samples. However, the C_T values were higher for the modified CDC assay than those for the WashU assays. The WashU assays but not the CDC assays detected the Fermon strain. Strikingly, the WashU design 2 assay detected Fermon 6.7 RT-PCR cycles earlier than WashU design 1 assay, and the amplification curve indicated improved amplitude and amplification efficiency (Fig. 2).

To follow up on this observation, additional clinical samples from the 2014 season that had been tested with WashU design 1 were identified for comparison with the modified CDC assay (Table 3). Only the modified assay was used because of its greater sensitivity. The samples were selected to include 10 from each of 4 categories based on the C_T of the WashU assay: < 22, 22 to 27, > 27 to 32, and > 32. Twenty samples negative for EV-D68 were also tested. In this test, the modified CDC assay detected all of the samples with C_T values of ≤ 32 but failed to detect those with C_T values of > 32.

Other EV-D68 viruses. The WashU assays were used to test an additional 20 specimens positive for EV-D68 from the New York State Department of Health. Both WashU assays detected EV-D68 in each sample.

Analysis of specificity. The specificity of the WashU assays was evaluated using test panels provided by the New York State Department of Health, the Children’s Hospital Colorado, and our own Special Projects Laboratory. These panels included 4 different coxsackievirus A viruses, 5 different coxsackievirus B viruses, 9 different echoviruses, 3 enteroviruses, including EV-D70, which is the enterovirus that is most closely related to EV-D68, and 59 rhinoviruses representing species A to C. All viruses tested are shown in Table S1 in the supplemental material. The presence of viral RNA was confirmed for each of these samples by amplification of the nucleic acid extract with an alternative panenterovirus/rhinovirus real-time RT-PCR assay. The WashU assays did not amplify any of the test panel viruses.

Comparison with laboratory-developed and commercial assays. We compared the sensitivity of the WashU EV-D68 assays with that of 5 commercial enterovirus assays and 2 LDTs that
detect enteroviruses and/or rhinoviruses but do not specifically distinguish subtypes (Table 4). We prepared 10-fold serial dilutions of a clinical sample from the 2014 St. Louis outbreak and tested each of the assays in parallel. We found that the WashU assays were able to detect EV-D68 at a dilution of $10^{-5}$, which was 10- to 100-fold more sensitive than the commercial Luminex xTag, GenMark Dx eSensor, Biofire FilmArray, Cepheid GeneXpert, and Focus enterovirus assays. The LDT targeting the 5'-nontranslated region of EV-D68 showed equivalent sensitivity for detecting Fermon to that of the WashU design 2 assay; however, it had higher CT values overall than those of the WashU assays for detecting the 2014 outbreak strain and was 10-fold less sensitive in serial dilution testing. Only the panenterovirus LDT had comparable sensitivity to the WashU assays.

**Analytic sensitivity.** In order to determine the limit of detection (LOD) of the WashU EV-D68 assay, the cloned 791-bp fragment of VP1 was serially diluted in a range of $10^{-1}$ to $10^2$ copies per reaction and tested with the WashU design 1 assay. Up to 10 replicates were carried out at each dilution on two separate days. The resulting 95% LOD determined by probit regression analysis was 4 copies per reaction, with a 95% confidence interval of 3.1 to 6.6 copies.

**DISCUSSION**

During the summer and fall of 2014, enterovirus D68 circulated at an unprecedented level in the United States (4–6). Because no molecular test was available for EV-D68-specific identification, laboratories were forced to rely on amplification and partial sequencing of the structural protein genes, VP4 to VP2 or VP1 (17, 18).  

![Amplification plot showing WashU RT-PCR assay EV-D68 sensitivity. The PCR amplification cycle number is displayed on the x axis while log(ΔRn) is shown on the y axis. Rn is the fluorescence of the reporter dye divided by the fluorescence of a passive reference dye. ΔRn is Rn minus the baseline and is plotted against PCR cycle number. The light green and light purple lines show detection of the 2014 EV-D68 outbreak strain using the WashU design 1 and design 2 assays, respectively. The brown and dark purple lines show detection of the more distant 1962 Fermon EV-D68 type strain using the WashU design 2 and design 1 assays, respectively. The incorporation of degenerate bases and mixed primers in WashU design 2 shows a significant increase in sensitivity (6.7 cycles earlier detection) for the Fermon type strain (brown line), with minimal decrease in sensitivity to the 2014 outbreak strain (light purple) (<0.5 cycles difference).](http://jcm.asm.org/)

**TABLE 3 Comparison of sensitivities of WashU design 1 and modified CDC assays using 2014 EV-D68 outbreak specimens**

<table>
<thead>
<tr>
<th>C&lt;sub&gt;T&lt;/sub&gt; value range (WashU design 1 defined)</th>
<th>No. of samples tested</th>
<th>No. of positive test results for:</th>
<th>WashU design 1</th>
<th>Modified CDC assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;22</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>22–27</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>&gt;27–32</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>&gt;32</td>
<td>10</td>
<td>10</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Neg&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Neg, negative.
TABLE 4 Comparison of detection of EV-D68 using laboratory-developed and commercial assays

<table>
<thead>
<tr>
<th>Test material</th>
<th>C&lt;sub&gt;T&lt;/sub&gt; for laboratory-developed assays</th>
<th>Commercial assays</th>
</tr>
</thead>
<tbody>
<tr>
<td>EV-D68 dilutions&lt;sup&gt;f&lt;/sup&gt;</td>
<td>WashU design 1 WashU design 2</td>
<td>Modified CDC 5′-nontranslated region&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>10&lt;sup&gt;−1&lt;/sup&gt;</td>
<td>21.3</td>
<td>22.9</td>
</tr>
<tr>
<td>10&lt;sup&gt;−2&lt;/sup&gt;</td>
<td>24.0</td>
<td>25.5</td>
</tr>
<tr>
<td>10&lt;sup&gt;−3&lt;/sup&gt;</td>
<td>28.5</td>
<td>29.9</td>
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<td>10&lt;sup&gt;−4&lt;/sup&gt;</td>
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<td>10&lt;sup&gt;−6&lt;/sup&gt;</td>
<td>Neg</td>
<td>Neg</td>
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<tr>
<td>Fermon strain&lt;sup&gt;f&lt;/sup&gt;</td>
<td>20.0</td>
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<tr>
<td>EV-D70&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>Water</td>
<td>Neg</td>
<td>Neg</td>
</tr>
</tbody>
</table>

<sup>a</sup> Protocol was as described by Piralla et al. (24).
<sup>b</sup> Protocol as described by Nijhuis et al. (23). The modifications are described in Materials and Methods.
<sup>c</sup> Luminex mean fluorescence index (MFI) values: negative, <150; equivocal, 150 to 300; positive, >300.
<sup>d</sup> GenMark nanoampere (nA) values: positive, >3, with >100 being strong positive.
<sup>e</sup> Pos, positive; Neg, negative; NT, not tested.
<sup>f</sup> Nucleic acid extracted from nasopharyngeal swab from EV-D68-positive patient. See Materials and Methods for details.

18), a much more cumbersome procedure than a specific real-time RT-PCR assay. The lack of a rapid molecular assay resulted in vast underrecognition and underreporting of cases of EV-D68 infection, because the majority of clinical laboratories did not have the ability to test specifically for EV-D68. Specific identification of EV-D68 was primarily from the CDC and state labs. Several FDA-approved multiplex assays for the detection of respiratory viruses detect enteroviruses, but these systems are broadly reactive and do not distinguish between enteroviruses and rhinoviruses; the results are typically reported as human rhinovirus/enterovirus.

In response to the 2014 nationwide enterovirus D68 outbreak and the associated increase in severe respiratory illness presentations, we developed and evaluated a real-time reverse transcriptase PCR assay for the detection of EV-D68 in clinical specimens. The development of this assay was informed by sequencing the complete genome of the EV-D68 virus circulating in St. Louis, MO, during the outbreak. Our RT-PCR primer and probe sequences were derived computationally by k-mer-mediated filtering of potentially cross-reactive non-EV-D68 viral sequences. Broad detection of EV-D68 was achieved through multiple-sequence alignment review using all published EV-D68 VP1 regions available through GenBank. Reduced sensitivity for the older and more distant Fermon EV-D68 type strain, which has only 87.9% identity to the genome sequence of the St. Louis virus, led us to modify the assay, which then proved capable of efficiently amplifying more divergent EV-D68 viruses as well.

The CDC released the design and protocol for an EV-D68-specific RT-PCR on their website as a diagnostic resource for clinicians and health care professionals in mid-October 2014. As noted within the CDC protocol, the amplicon size of 272 bp is larger than ideal for a real-time RT-PCR assay. Furthermore, their selected TaqMan probe had a guanine (G) at the 5′ end linked to the fluorophore FAM, potentially incurring unwanted fluorescence quenching. The replacement of FAM with Cy5 significantly improved the ability of the CDC assay to detect EV-D68 (Table 2).

We evaluated the CDC assay alongside our own, testing against EV-D68-positive clinical samples (n = 35). Based on serial dilution testing of the 2014 outbreak virus, the WashU RT-PCR assays were 100-fold more sensitive than the published CDC assay, and the CDC assay failed to detect the Fermon strain. In addition, the WashU assays were ≳10-fold more sensitive for detecting EV-D68 than the FDA-approved commercial assays (i.e., Luminex xTAG RVP, GenMark Dx eSensor RVP, Biofire FilmArray RP, and Cepheid GeneXpert) for enterovirus/rhinovirus detection (Table 4), with the further advantage of specific identification of EV-D68. The WashU assays showed no evidence of amplification of other enteroviruses, including the relatively closely related EV-D70 virus, or rhinoviruses.

There are two limitations of this study. First, we were not able to test the specificity of this assay against every known enterovirus or rhinovirus subtype. It is possible that the assay cross-reacts with another subtype, although that is not likely based on in silico analysis of the genome sequences. It is also possible that the assay cross-reacts with a subtype that has yet to be discovered. Second, although we have tried to show that our assay evaluated a broad range of EV-D68 strains, EV-D68 strains may exist or emerge with mutations in the PCR target region that cause the assay to miss that strain of the virus.

The development of another EV-D68-specific RT-PCR by Piralla et al. (24) was communicated in March 2015. This underscores the international interest in EV-D68 detection stimulated by the global reemergence of the virus in 2014. The assay targets a 60-bp region of the 5′-nontranslated region of EV-D68. A comparison of the assay to the CDC RT-PCR and commercially available enterovirus/rhinovirus clinical assays was not reported in their paper. In our dilution tests, the assay was 10-fold less sensitive in detecting the 2014 outbreak strain of EV-D68 than the WashU assays. Furthermore, the WashU assays detected the undiluted outbreak specimen 7 cycles before the 5′-nontranslated region-targeting assay reached detection. Because these assays detect completely different segments of the viral genome, they may have complementary value in future applications.
While there are no specific treatments for EV-D68 and currently no antiviral targets available, rapid and accurate diagnosis of current and future EV-D68 infections is of great concern to clinicians and public health authorities. The EV-D68-specific RT-PCR assay we have developed can be used for epidemiological studies of the EV-D68 outbreak and for virus monitoring in subsequent seasons. It is unclear at this time whether typing EV-D68 will be useful for patient management. However, some FDA-approved multiplex respiratory panels may not detect EV-D68 optimally or at all. In laboratories using those assays, an additional assay that detects EV-D68 will be useful for laboratory documentation of EV-D68 infection, which may help with prognosis, antibiotic use, and appropriate isolation. The ongoing importance of improved diagnostic capability for EV-D68 is underscored by the recent decision by the Department of Health and Human Services to encourage the development of EV-D68 testing capability by authorizing the emergency use of new diagnostics for EV-D68 detection (http://www.gpo.gov/fdsys/pkg/FR-2015-02-27/pdf/2015-04121.htm).

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