Development and Evaluation of an Enterovirus D68 Real-Time Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) Assay

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ABSTRACT

We have developed and evaluated a real-time reverse transcription PCR (RT-PCR) assay for detection of human enterovirus D68 (EV-D68) in clinical specimens. This assay was developed in response to the unprecedented 2014 nationwide EV-D68 outbreak associated with severe respiratory illness in the United States. As part of our evaluation of the outbreak, we sequenced and published the genome sequence of the EV-D68 virus circulating in St. Louis, Missouri. 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 VP-1 gene with an analytic limit of detection of 4 copies per reaction, and 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.

INTRODUCTION

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 has been designated the Fermon strain. Subsequently, only small numbers of EV-D68 cases were reported until the early 2000s (2). However, from 2008-12 outbreaks in Japan, the Philippines, the Netherlands, and the USA (Georgia, Pennsylvania, and Arizona) have revealed EV-D68 as an emerging pathogen capable of causing severe respiratory illness (2-5, 7, 9). 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 at least 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 (6). Infected individuals were primarily children, and resided in 49 states and the District of Columbia (5). The CDC has also reported 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 (7). We also observed this pattern at St. Louis Children's Hospital in St. Louis, Missouri. 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, specific RT-PCR assay. We began by sequencing the genome of a representative EV-D68 isolate from St. Louis to

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obtain the sequence information required to define an assay with optimal sensitivity and specificity (8). EV-D68 causes respiratory illness and the virus can be found in an infected person's respiratory secretions, such as saliva, nasal mucus, or sputum (9). Therefore, an appropriate assay would primarily focus on evaluating respiratory disease due to EV-D68 by targeting nasopharyngeal and other respiratory specimens.

Development goals for our EV-D68 RT-PCR assay included: 1) avoiding false-positive detection of closely related enteroviruses and rhinoviruses, 2) increasing clinical and analytical sensitivity compared to other available assays, and 3) 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 (7), clinical specimens testing positive for enterovirus/rhinovirus with the BioFire FilmArray Respiratory Virus Panel (BioFire Diagnostics, Inc., Salt Lake City, Utah) were provided for further testing by the Diagnostic Virology Laboratory at St. Louis Children's Hospital, consistent with a protocol for testing of de-identified 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 100µl aliquots of original specimen using a

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bioMerieux NucliSENS® easyMAG® automated extractor (bioMerieux Durham, NC).

Challenge panel from New York State Department of Health. We received a challenge panel from the New York State Department of Health (courtesy of Kirsten St. George and Daryl Lamson). Viruses included are shown in **Supplemental Table S1**. This panel included nucleic acid extracts prepared using the NucliSENS® easyMAG® automated extractor from clinical specimens containing the following viruses, identified at the Wadsworth Laboratory by VP1 sequencing: Coxsackie A16 (n=2) and 21 (n=2), echovirus 18 (n=2) and 30, and enterovirus 71 (n=2). The panel also included a collection of 20 EV-D68 viruses selected to represent a range of sequence variants. A review of the VP1 sequences from this panel showed 93.8%-99.4% sequence identity when compared to the St. Louis 2014 strain. In comparison, the 1962 Fermon strain (see below) had 84.4% identity to the St. Louis 2014 strain in the sequenced VP1 region.

Challenge set from Children's Hospital Colorado. We also received a challenge set from Children's Hospital Colorado (courtesy of Christine Robinson), consisting of frozen aliquots of cultures positive for the following viruses: Coxsackie A7 and 9; Coxsackie B 1-5; echoviruses 1,3,4,5,6,11,19, and 30; and enteroviruses 68 (Fermon), 70, and 71. Most of these viruses were obtained originally from the American Type Culture Collection (ATCC®). Others were

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derived from clinical specimens that had been typed by the Centers for Disease Control (personal communication from Christine Robinson). All viruses received are shown in **Supplemental Table S1**. Total nucleic acid extracts were prepared at Washington University.

Washington University samples. Our Special Projects Laboratory at Washington University provided an additional panel of challenge viruses. These viruses had been detected in patient specimens from research projects carried out in the past five years (11). Viruses in this panel had been typed based on sequencing a region of the 5'-nontranslated region (10). Total nucleic acid extracts were prepared using either the NucliSENS easyMAG automated extractor or Roche MagNA Pure Compact System (Roche Diagnostics GmbH, Germany). Viruses included echovirus 14, Coxsackie A16, and 59 rhinoviruses from species A-C. The rhinovirus types and extraction methods are shown in **Supplemental Table S1**. We verified that our assay could amplify EV-D68 from total nucleic acid prepared on both extraction platforms.

EV-D68 St. Louis 2014 genome sequence. As previously described (8), we used high-throughput sequencing on the Illumina HiSeq 2500 to obtain one complete and eight partial sequences (GenBank: KM881710.2, BioProject: PRJNA263037) from specimens obtained during the 2014 outbreak in St. Louis. This genome sequence, along with other concurrently sequenced/published 2014

EV-D68 genomes, was used as a baseline for circulating EV-D68 sequence specificity.

PCR amplicon sequence selection. To create an assay with specificity for EV-D68, we performed comprehensive *in silico* analysis of all viruses in NIH's GenBank genetic sequence database using a *k*-mer approach described below to identify unique, contiguous sequences for candidate RT-PCR primers and probes. *K*-mer frequency-based methods were originally used in whole genome shotgun assembly algorithms to remove reads containing frequently occurring subsequences of length *k* during genome assembly (12, 13). We started by creating a consolidated viral sequence database by collecting all FASTA nucleotide sequences from viruses that infect vertebrate or invertebrate hosts, as found in the following areas of GenBank: RefSeq, Genome Neighbors, and Influenza Virus Resource. The database contained sequences from 34 viral families, which consisted of 190 annotated viral genera and 337 species. By design, this database contained only a single, complete EV-D68 reference genome (STL 2014 strain, GenBank: KM881710.2). Comprehensive *k*-mer analysis was performed on the database by indexing and reporting all 20-mer subsequences using Tallymer software (14). We eliminated 20-mers that were not unique in the *k*-mer pool, thus leaving 20-mers that were unique to EV-D68 as well as those unique to other viral species. EV-D68-unique 20-mers were collected by using BLAST (15) to align all unique 20-mers to the EV-D68 reference genome, requiring 100% identity. The EV-D68-specific 20-mers were

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consolidated into contiguous sequences by merging overlapping sequences with the BEDTools suite of utilities (16). Contiguous sequences ≥60 base pair (bp) were identified as promising regions for RT-PCR primer and probe design. Of these, a 141-bp region was selected based on its uniqueness, length, and relative conservation among available EV-D68 nucleotide sequences. Notably, this region was within the VP1 gene that is considered the "gold standard" for enterovirus typing (17, 18).

Design of oligonucleotide primers and probes. In addition to the VP1 gene sequence represented by our candidate 141-bp region from the St. Louis 2014 strain of EV-D68, we also collected 396 other unique EV-D68 VP1 sequences from GenBank. These nucleotide sequences were mapped and visualized online using MUSCLE (19) at the NIAID Virus Pathogen Database and Analysis Resource (ViPR) (http://www.viprbrc.org) website to produce a multiple sequence alignment (MSA). Focusing on the candidate 141-bp region within the MSA, we evaluated single nucleotide polymorphism (SNP) frequencies and identified conserved segments appropriate for primer and probe placement. The GenScript Real-time PCR Primer Design application (URL: https://www.genscript.com/ssl-bin/app/primer) was used to evaluate primer/probe options. Criteria for ideal amplicon selection included: primer sequences no shorter than 20 bp, PCR amplicons <100 bp in length, and Tm values within a +55 to +70 °C range.

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Based on this procedure, we selected an RT-PCR set consisting of two primers and a single probe with complete sequence identity to the 2014 outbreak virus (WashU Design 1). To broaden the detection of EV-D68 viruses, we made modifications based on SNP frequencies that included the addition of degenerate bases and a second reverse primer (WashU Design 2). Both designs are shown in **Table 1** and **Figure 1**.

Additional specificity analysis. The selected RT-PCR primer and probe sequences were aligned to GenBank nt database while excluding EV-D68 taxon (txid 42789) sequences, to evaluate possible homology to non-EV-D68 sequences. Using the NCBI's online BLAST interface (20, 21) for highly similar sequence alignment (megablast), fewer than 20 alignments (90-100% identity) were produced with all having identity to EV-D68 partial coding sequences that had been submitted to the database without full EV-D68 taxon designation (txid 1193974). Using discontiguous megablast, the top alignments that were not related to EV-D68 had between 70-83% sequence identity to EV-D70.

Washington University EV-D68 RT-PCR procedure. Primers and probes for the WashU assays were ordered from Applied Biosystems® at Life Technologies (Grand Island, NY). Other reagents included low EDTA TE, AgPath-ID One Step RT-PCR kit (Life Technologies), and H_2O for negative controls. Master mixes consisting of 10X primer/probe (4 µM primers/2 µM probe) were produced for each assay and 20 µL of master mix was added to each well of a 96-well PCR

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plate. For the clinical specimens and controls, 5 µL of each sample was added to the reaction. ROX™ Passive Reference Dye was included in the RT-PCR buffer to normalize well-to-well differences. Reactions were run on the Applied Biosystems® 7500 Real-Time PCR System and analyzed using accompanying Ct (threshold cycle) analysis software. Thermal cycling conditions were: 45 °C for 10 minutes, followed by 95 °C for 10 minutes, followed by 45 cycles of 95 °C for 15 seconds and 60 °C for 45 seconds.

Modification of the CDC-published EV-D68 assay. In mid-October 2014, the CDC Picornavirus Laboratory made a new EV-D68-specific RT-PCR assay available (personal communication from Steve Oberste at the Centers for Disease Control and Prevention, Atlanta, Georgia). 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 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) (personal communication from Rangaraj Selvarangan, Children's Mercy Hospital, Kansas City, MO). Primers and probes for the CDC assay were ordered from Integrated DNA Technologies, Inc. (Coralville, Iowa).

Commercial and laboratory-developed assay testing. Commercial multiplex panels that detect enteroviruses/rhinoviruses were tested according to the manufacturers' instructions. These assays included: Luminex 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 only for research purposes) (Luminex, Austin, TX), GenMark Dx eSensor® Respiratory Viral Panel (GenMark Diagnostics, Inc., Carlsbad, CA), BioFire FilmArray® Respiratory Panel IVD (BioFire Diagnostics, Inc., Salt Lake City, Utah), Cepheid GeneXpert® EV IVD (Cepheid, Sunnyvale, CA), and Focus Enterovirus Primer Pair Analyte Specific Reagent (ASR) (Focus Diagnostics, Inc., Cypress, California).

We also evaluated two laboratory developed tests (LDTs), the pan-enterovirus 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 the detection of 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 un-extracted specimen, a series of 10-fold dilutions of the original specimen was made using Universal Transport Medium (UTM) (Diagnostic Hybrids, Athens, Ohio) as diluent. 300µl of each dilution was then tested in the BioFire assay and 140µl in the GeneXpert® assay according to the manufacturers' instructions. For the LDTs and the GeneMark and Luminex xTAG® assays, which require extracted nucleic acids, total nucleic acids were extracted from 100µl of original specimen using a bioMerieux NucliSENS® easyMAG® automated extractor (bioMerieux Durham, NC). A series of 10-fold dilutions of the extract was then made using low EDTA

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TE as diluent, and each dilution was tested in each assay. For the Focus Enterovirus ASR assay, 5µl of reaction mix and 5µl of EasyMag nucleic acid extract was added to the wells of a 3M™ Integrated Cycler Universal Disc, and the amplification assay was run using standard Focus Diagnostics assay parameters and 3M™ Integrated Cycler. For the pan-enterovirus assay, we used the AgPath-ID 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-VP1-2325-fwn GGRTTCATAGCAGCAAAAGATGA and EV68-VP1-3121-rvni TAGGYTTCATGTAAACCCTRACRGT, 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 *Spe*I 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 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 less than 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. substitution of FAM with Cy5) enabled detection of all 14 samples. However, the Ct values were higher for the modified CDC assay when compared to 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 (**Figure 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 Ct of the WashU assay: Ct < 22; Ct = 22-27; Ct = >27-32; Ct > 32. Twenty samples negative for EV-D68 were also tested.

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In this test the modified CDC assay detected all of the samples with Ct values ≤32, but failed to detect those with Ct values >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. 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 Coxsackie A viruses, 5 different Coxsackie 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-C. All viruses tested are shown in **Supplemental Table S1**. The presence of viral RNA was confirmed for each of these samples by amplification of the nucleic acid extract with an alternative pan-enterovirus/rhinovirus real-time RT-PCR assay. The WashU assays did not amplify any of the of the test panel viruses.

Comparison with laboratory-developed and commercial assays. We compared 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 in detecting Fermon when compared to the WashU Design 2 assay; however, it had higher Ct values overall when compared to the WashU assays in detecting the 2014 outbreak strain, and was 10-fold less sensitive in serial dilution testing. Only the pan-enterovirus 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 0.14 $x10^0$ to $1x10^2$ copies per reaction and tested with the WashU Design 1 assay. Up to ten 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 3.1-6.6 copies.

DISCUSSION

During the summer and fall of 2014, enterovirus D68 circulated at an unprecedented level in the United States (4-5, 7). 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-VP2 or

VP1 (17, 18), a much more cumbersome procedure than a specific real-time RT-PCR assay. The lack of a rapid molecular assay resulted in vast under-recognition and under-reporting 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 detection of respiratory viruses detect enteroviruses, but these systems are broadly reactive and do not distinguish between enteroviruses and rhinoviruses; results are typically reported as *human rhinovirus/enterovirus*.

In response to the 2014 nationwide enterovirus D68 outbreak and associated increase in severe respiratory illness presentations, we developed and evaluated a real-time reverse transcription PCR assay for detection of EV-D68 in clinical specimens. 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's 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, 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.

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367 The CDC released the design and protocol for an EV-D68-specific RT-PCR on
368 their website as a diagnostic resource for clinicians and health care professionals
369 in mid-October 2014. As noted within the CDC's protocol, the amplicon size of
370 272 bp is larger than ideal for a real-time RT-PCR assay. Furthermore, their
371 selected TaqMan® probe had a guanine (G) at the 5'-end linked to the
372 fluorophore FAM, potentially incurring unwanted fluorescence quenching.
373 Replacement of FAM with Cy5 significantly improved the CDC assay's ability to
374 detect EV-D68 (**Table 2**).

375

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

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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 could cross-react with another subtype, although that is not likely based on *in silico* analysis of genome sequences. It is also possible that the assay could cross-react 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.

Development of another EV-D68-specific RT-PCR by Piralla, et al. was communicated in March 2015 (24). 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. Comparison of the assay to the CDC's 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 when compared to the WashU assays. Furthermore, the WashU assays detected the undiluted outbreak specimen 7 cycles before the 5'-nontranslated-targeting assay reached detection. Because these assays detect completely different segments of the viral genome, they may have complementary value in future applications.

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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 development of EV-D68 testing capability by authorizing emergency use of new *in vitro* diagnostics for EV-D68 detection (http://www.gpo.gov/fdsys/pkg/FR-2015-02-27/html/2015-04121.htm).

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FIGURE LEGENDS

Figure 1. WashU and CDC RT-PCR design comparison. WashU and CDC RT-PCR primers and probe locations are illustrated within the VP1 gene of the 2014 outbreak EV-D68 St. Louis (GenBank: KM881710.2) reference genome.

Figure 2. Amplification plot showing WashU RT-PCR assay EV-D68 sensitivity. 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

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548 fluorescence of a passive reference dye. ΔRn is Rn minus the baseline and is
549 plotted against PCR cycle number. The light green and light purple lines show
550 detection of the 2014 EV-D68 outbreak strain using the WashU Design 1 and
551 Design 2 assays, respectively. The brown and dark purple lines show detection
552 of the more distant 1962 Fermon EV-D68 type-strain using the WashU Design 2
553 and Design 1 assays, respectively. The incorporation of degenerate bases and
554 mixed primers in WashU Design 2 shows a significant increase in sensitivity (6.7
555 cycles earlier detection) for the Fermon type-strain (brown line), with minimal
556 decrease in sensitivity to the 2014 outbreak strain (light purple) (<0.5 cycles
557 difference).
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TABLE 1. WashU EV-D68-specific RT-PCR assay primers and probes

Designation	ID	Sequence (5'-3')	Strand	Location[c]	Tm	Modification
WashU Design 1[a]	L1-1	CACTGAACCAGAAGAAGCCA	forward	2475-2494	59.01	n/a
WashU Design 1[a]	R1-1	CCAAAGCTGCTCTACTGAGAAA	reverse	2551-2572	58.93	n/a
WashU Design 1[a]	P1-1	TCGCACAGTGATAAATCAGCACGG	forward	2502-2525	68.39	5' Fam & 3' Tamra
WashU Design 2[b]	L1-2	CAC(T/C)GAACCAGA(A/G)GAAGCCA	forward	2475-2494	58.38-59.01[*]	n/a
WashU Design 2[b]	R1-2	CCAAAGCTGCTCTACTGAGAAA	reverse	2551-2572	58.10-59.75[*]	n/a
WashU Design 2[b]	R2-2	CTAAAGCTGCCCTACTAAG(G/A)AA	reverse	2551-2572	58.10-59.75[*]	n/a
WashU Design 2[b]	P1-2	TCGCACAGTGATAAATCAGCA(T/C)GG	forward	2502-2525	68.39-69.21[*]	5' Fam & 3' Tamra

n/a: not applicable
[a] Distinct, single paired-primer design. Amplicon size is 98 bp.
[b] Degenerate bases and mixed primers included in design. Amplicon size is 98 bp.
[c] EV-D68 STL 2014 (GenBank: KM881710.2) subregion positions, 5'-3' orientation.
[*] Tm ranges span all combinations of degenerate bases and mixed primers.

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TABLE 2. Comparison of WashU and CDC assays using 2014 EV-D68 outbreak specimens and the Fermon strain

Test Material	Ct values:				ΔCt:	
	WashU Design 1[a]	WashU Design 2[b]	CDC[c]	Modified CDC[d]	WashU Design 1[a] and WashU Design 2[b]	WashU Design 2[b] and Modified CDC
EV-D68 specimens:						
WU-EV-1	21.0	21.3	neg	23.7	0.3‡	2.4
WU-EV-2	24.2	25.4	neg	28.7	1.2	3.3
WU-EV-3	20.0	20.7	41.0	22.7	0.7	2.0
WU-EV-4	20.7	20.8	neg	22.5	0.1‡	1.7
WU-EV-5	22.2	22.7	34.6	24.4	0.5‡	1.7
WU-EV-6	20.9	21.2	25.9	23.9	0.3‡	2.7
WU-EV-7	20.5	20.0	neg	23.4	-0.5‡	3.4
WU-EV-8	27.3	27.3	neg	30.8	0‡	3.5
WU-EV-9	17.3	17.5	27.7	20.5	0.2‡	3.0
WU-EV-10	21.4	22.1	37.2	23.8	0.7	1.7
WU-EV-11	26.3	26.8	neg	30.8	0.5‡	4.0
WU-EV-12	24.1	24.5	38.5	27.5	0.4‡	3.0
WU-EV-13	11.2	11.0	23.9	14.7	-0.2‡	3.7
WU-EV-14	20.3	18.5	32.7	20.6	-1.8‡	2.1
Fermon	22.7	15.9	neg	neg	-6.8‡	n/a
water	neg	neg	neg	neg	n/a	n/a

Ct: Crossing threshold; n/a: not applicable
[a] Distinct, single paired-primer design.
[b] Degenerate bases and mixed primers included in design.
[c] CDC published design with FAM.
[d] Modification of CDC assay by replacement of FAM with Cy5.
‡ ΔCt <= 0.5

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

Ct value range (WashU Design 1 defined)	# Samples tested	Positive tests:	
		WashU Design 1	Modified CDC
<22	10	10	10
22-27	10	10	10
>27-32	10	10	10
>32	10	10	0
neg	20	20	20

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TABLE 4. Comparison of detection of EV-D68 using laboratory-developed and commercial assays

Test Material	Laboratory-developed assays:					Commercial assays:				
	WashU Design 1[a]	WashU Design 2[a]	Modified CDC[a]	5'-nontranslated[a,b]	Pan-entero[a,c]	Luminex xTAG RVP[d]	GenMark Dx eSensor RVP[e]	Biofire FilmArray IVD	Cepheid GeneXpert[a]	Focus Enterovirus ASR[a]
EV-D68[‡] dilutions:										
10^{-1}	21.3	22.9	23.5	30.0	27.1	4415	10.5	pos	28.1	28.2
10^{-2}	24.0	25.5	28.0	33.0	30.1	5112	3.4	pos	31.2	31.6
10^{-3}	28.5	29.9	34.2	36.1	33.7	5405	6.9	pos	34.1	35.9
10^{-4}	31.8	33.1	neg	41.0	38.1	1132	neg	pos	neg	38.1
10^{-5}	36.2	37.0	neg	neg	37.1	neg	neg	neg	nt	neg
10^{-6}	neg	neg	neg	neg	neg	neg	neg	neg	nt	neg
Fermon[*]	20.0	15.4	neg	15.2	18.5	4775	neg	nt	nt	20.7
EV-D70[*]	neg	neg	neg	neg	14.5	3023	6.8	nt	nt	13.7
water	neg	neg	neg	neg	neg	neg	nt	nt	nt	neg

nt = not tested

[a] Ct (cross threshold) values.

[b] Protocol as described by Piralla, et al.

[c] Protocol as described by Nijhuis, et al. Modifications described in Methods.

[d] Luminex MFI (Mean Fluorescence Index) values: negative <150; equivocal 150-300; positive >300.

[e] GenMark nanoampere (nA) values: positve >3 with >100 being strong postive.

[‡] Nucleic acid extracted from nasopharyngeal swab from EV-D68-positive patient. See Methods section for details.

[*] ATTC® strains; total nucleic acid extracted from infected cell culture.

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Figure 1

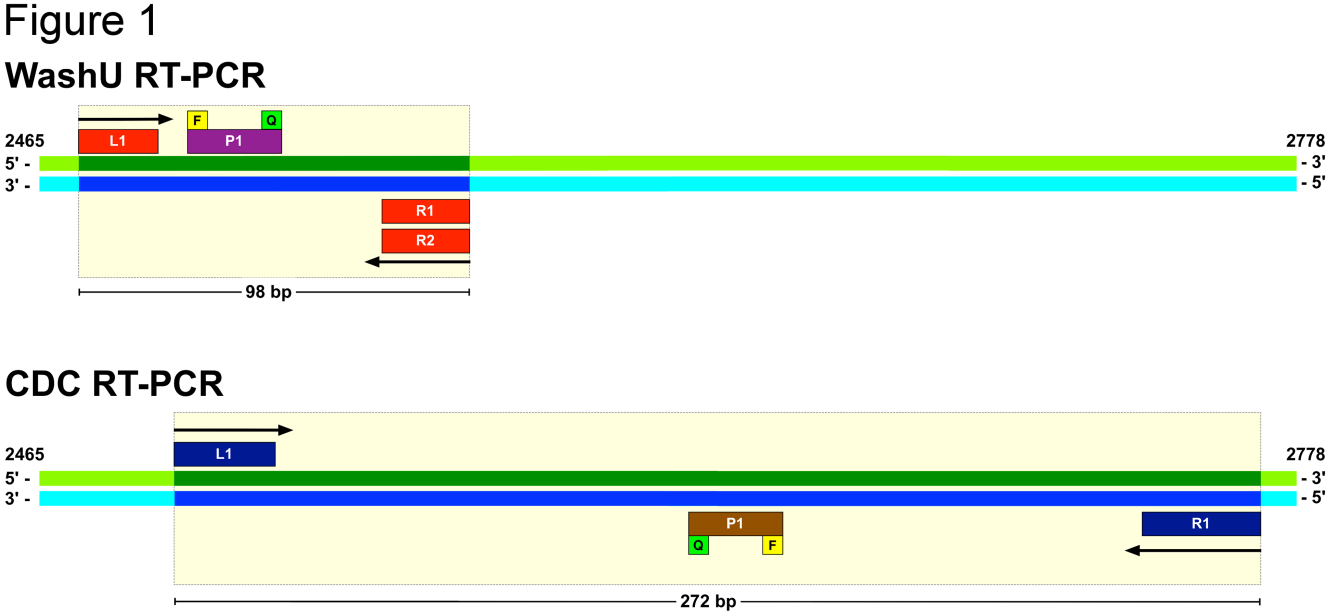

Figure 2