2015

Development and evaluation of an enterovirus D68 real-time reverse transcriptase PCR assay

Todd N. Wylie
Washington University School of Medicine in St. Louis

Kristine M. Wylie
Washington University School of Medicine in St. Louis

Richard S. Buller
Washington University School of Medicine in St. Louis

Maria Cannella
Washington University School of Medicine in St. Louis

Gregory A. Storch
Washington University School of Medicine in St. Louis

JCM
Journals.ASM.org

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

Todd N. Wylie,[a,b] Kristine M. Wylie,[a,b] Richard S. Buller,[a] Maria Cannella,[a] Gregory A. Storch[a]

Department of Pediatrics, Washington University School of Medicine, St. Louis, Missouri, USA[a]; McDonnell Genome Institute, Washington University School of Medicine, St. Louis, Missouri, USA[b]

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.

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 specimen using a bioMérieux NucliSENS easyMAG automated extractor (bioMérieux, Durham, NC).

Received 6 April 2015 Returned for modification 6 May 2015
Accepted 2 June 2015

Accepted manuscript posted online 10 June 2015

Citation Wylie TN, Wylie KM, Buller RS, Cannella M, Storch GA. 2015. Development and evaluation of an enterovirus D68 real-time reverse transcriptase PCR assay. J Clin Microbiol 53:2641–2647. doi:10.1128/JCM.00923-15.

Editor: M. J. Loeffelholz

Address correspondence to Gregory A. Storch, storch_g@kids.wustl.edu.

Supplemental material for this article may be found at http://dx.doi.org/10.1128/JCM.00923-15.

doi:10.1128/JCM.00923-15

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). The included viruses are shown in Table S1 in the supplemental material. 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: coxsackievirus 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% to 99.4% sequence identity 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: coxsackievirus A7 and 9; coxsackievirus B1 to 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 derived from clinical specimens that had been typed by the Centers for Disease Control (Christine Robinson, personal communication). All viruses received are shown in Table S1 in the supplemental material. 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 5 years prior (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, coxsackievirus A16, and 59 rhinoviruses from species A to C. The rhinovirus types and extraction methods are shown in Table S1 in the supplemental material. 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 (9), we used high-throughput sequencing on the Illumina HiSeq 2500 to obtain one complete and eight partial sequences (GenBank accession no. KM881710.2, BioProject no. 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 the NIH GenBank genetic sequence database using a *k*-mer approach described below to identify unique and contiguous sequences for candidate RT-PCR primers and probes. The *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 (St. Louis [STL] 2014 strain, GenBank accession no. KM881710.2). Comprehensive *k*-mer analysis was performed on the database by indexing and reporting all 20-mer subsequences using the 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 and those unique to other viral species. EV-D68-unique 20-mers were collected 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 consolidated into contiguous sequences by merging overlapping sequences with the BEDTools suite of utilities (16). Contiguous sequences of ≥60 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 National Institute of Allergy and Infectious Diseases (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 was used to evaluate primer/probe options. The criteria for ideal amplicon selection included primer sequences of ≥20 bp, PCR amplicons of <100 bp in length, and melting temperature (T_m) within a range of 55 to 70°C.

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 Fig. 1.

Additional specificity analysis. The selected RT-PCR primer and probe sequences were aligned to the GenBank nt database while excluding EV-D68 taxon (taxid 42789) sequences to evaluate possible homology to non-EV-D68 sequences. Using the NCBI online BLAST interface (20, 21) for highly similar sequence alignment (MegaBlast), <20 alignments (90 to 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 (taxid 1193974). Using discontiguous MegaBlast, the top alignments that were not related to EV-D68 had between 70 and 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 Tris-EDTA (TE) buffer, AgPath-ID one-step RT-PCR kit (Life Technologies), and H_2O for negative controls. Mastermixes consisting of 10× primer/probe (4 μM primers/2 μM probe) were produced for each assay, and 20 μl of mastermix was added to each well of a 96-well PCR plate. For the clinical specimens and controls, 5 μl of each sample was added to the reaction mixture. ROX passive reference dye was included in the RT-PCR buffer to normalize well-to-well differences. The reactions were run on the Applied Biosystems 7500 real-time PCR system and analyzed using accompanying threshold cycle (C_T) analysis software. The thermal cycling conditions were 45°C for 10 min, followed by 95°C for 10 min, and then 45 cycles of 95°C for 15 s and 60°C for 45 s.

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 (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).

TABLE 1 WashU EV-D68-specific RT-PCR assay primers and probes

ID by designation[a]	Sequence (5′–3′)	Strand	Location[b]	T_m (°C)[c]	Modification[d]
WashU design 1[e]					
L1-1	CACTGAACCAGAAGAAGCCA	Forward	2475–2494	59.01	NA
R1-1	CCAAAGCTGCTCTACTGAGAAA	Reverse	2551–2572	58.93	NA
P1-1	TCGCACAGTGATAAATCAGCACGG	Forward	2502–2525	68.39	5′-FAM and 3′-TAMRA
WashU design 2[f]					
L1-2	CAC(T/C)GAACCAGA(A/G)GAAGCCA	Forward	2475–2494	58.38–59.01*	NA
R1-2	CCAAAGCTGCTCTACTGAGAAA	Reverse	2551–2572	58.10–59.75*	NA
R2-2	CTAAAGCTGCCCTACTAAG(G/A)AA	Reverse	2551–2572	58.10–59.75*	NA
P1-2	TCGCACAGTGATAAATCAGCA(T/C)GG	Forward	2502–2525	68.39–69.21*	5′-FAM and 3′-TAMRA

[a] ID, identification.
[b] EV-D68 St. Louis (STL) 2014 (GenBank accession no. KM881710.2) subregion positions, 5′-3′ orientation.
[c] T_m ranges span all combinations of degenerate bases and mixed primers.
[d] NA, not applicable; FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine.
[e] Distinct single-paired primer design. Amplicon size is 98 bp.
[f] Degenerate bases and mixed primers included in the design. Amplicon size is 98 bp.

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 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 Diagnostics, Inc., Salt Lake City, UT), Cepheid GeneXpert EV IVD (Cepheid, Sunnyvale, CA), and Focus enterovirus primer pair analyte-specific reagent (ASR) (Focus Diagnostics, Inc., Cypress, CA).

We also evaluated two laboratory-developed tests (LDTs), the panenterovirus 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 μl 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 μl 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 dilution was tested in each assay. For the Focus enterovirus ASR assay, 5 μl of reaction mix and 5 μl 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

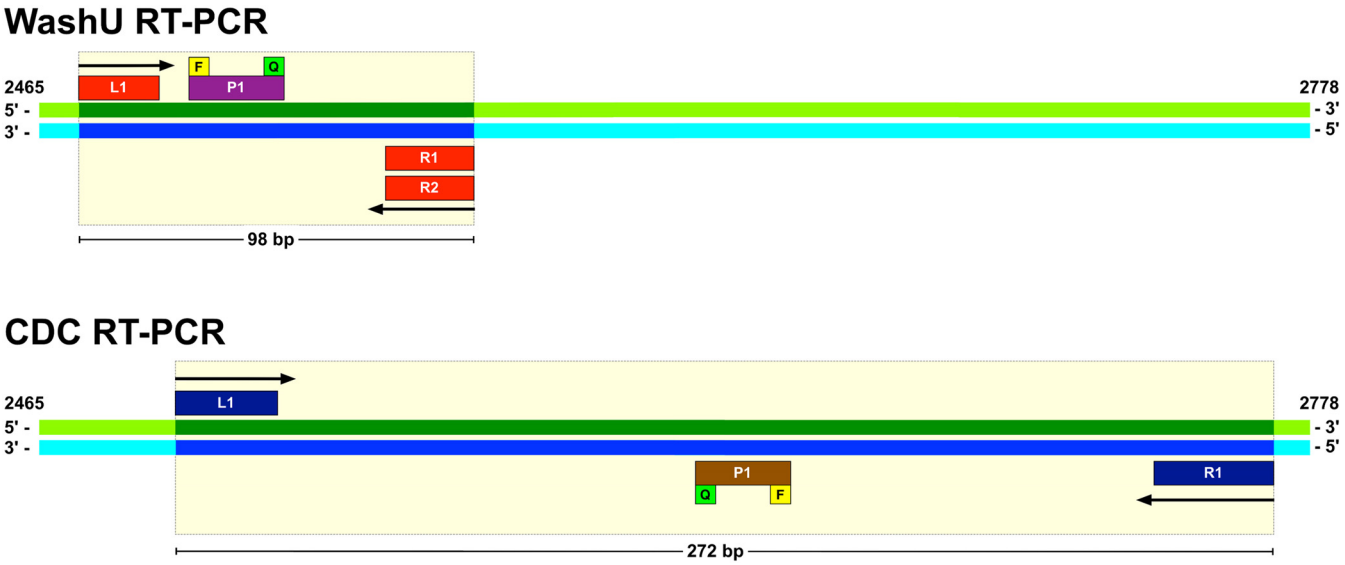

FIG 1 WashU and CDC RT-PCR design comparison. WashU and CDC RT-PCR primers and probe locations are illustrated within the VP1 gene of the 2014 outbreak EV-D68 St. Louis (GenBank accession no. KM881710.2) reference genome. The dark green and dark blue areas, as well as the light yellow bounding box, indicate the regions of the genome targeted by the respective assays and their associated PCR product lengths. Arrows indicate direction of priming for the left (L) and right (R) primers. The yellow squares labeled F (fluorescent reporter) and the green squares labeled Q (quencher) show the relative orientation of the fluorophores on the probes.

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

Test material	C_T values for[a]:				ΔC_T	
	WashU design 1	WashU design 2	CDC assay	Modified CDC assay	WashU design 1 and WashU design 2	WashU design 2 and modified CDC assay
EV-D68 specimens						
WU-EV-1	21.0	21.3	Neg	23.7	0.3[b]	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[b]	1.7
WU-EV-5	22.2	22.7	34.6	24.4	0.5[b]	1.7
WU-EV-6	20.9	21.2	25.9	23.9	0.3[b]	2.7
WU-EV-7	20.5	20.0	Neg	23.4	−0.5[b]	3.4
WU-EV-8	27.3	27.3	Neg	30.8	0[b]	3.5
WU-EV-9	17.3	17.5	27.7	20.5	0.2[b]	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[b]	4.0
WU-EV-12	24.1	24.5	38.5	27.5	0.4[b]	3.0
WU-EV-13	11.2	11.0	23.9	14.7	−0.2[b]	3.7
WU-EV-14	20.3	18.5	32.7	20.6	−1.8[b]	2.1
Fermon strain	22.7	15.9	Neg	Neg	−6.8[b]	NA[c]
Water	Neg	Neg	Neg	Neg	NA	NA

[a] 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] $\Delta C_T \leq 0.5$.
[c] 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-VP1-2325-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 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

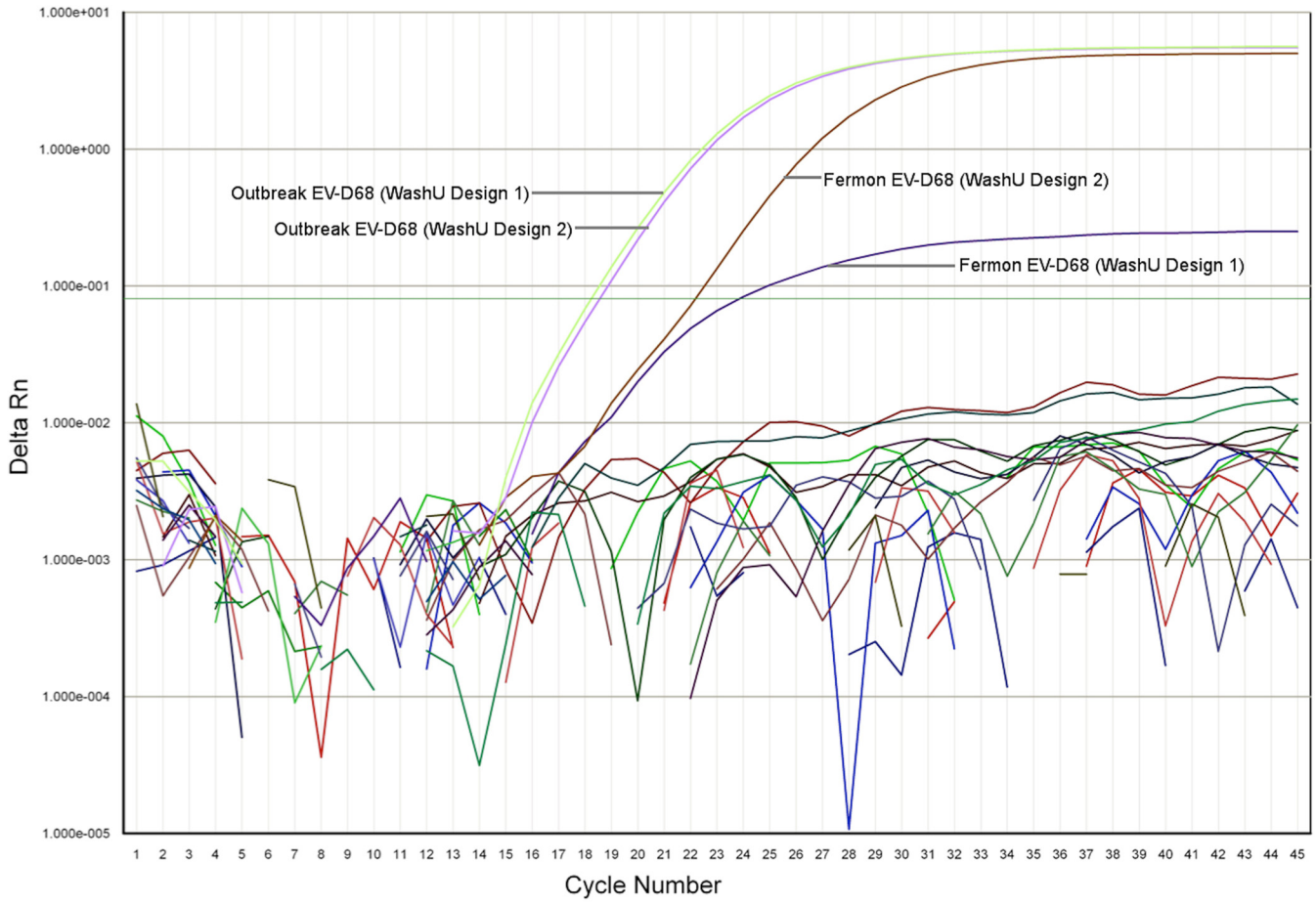

FIG 2 Amplification plot showing WashU RT-PCR assay EV-D68 sensitivity. The PCR amplification cycle number is displayed on the *x* axis while log(ΔRn) is shown on the *y* axis. Rn is the fluorescence of the reporter dye divided by the fluorescence of a passive reference dye. ΔRn is Rn minus the baseline 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).

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 C_T 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.

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

C_T value range (WashU design 1 defined)	No. of samples tested	No. of positive test results for:	
		WashU design 1	Modified CDC assay
<22	10	10	10
22–27	10	10	10
>27–32	10	10	10
>32	10	10	0
Neg[a]	20	20	20

[a] Neg, negative.

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 0.14×10^0 to 1×10^2 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,

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

Test material	C_T for laboratory-developed assays					Commercial assays				
	WashU design 1	WashU design 2	Modified CDC	5′-nontranslated region[a]	Panenterovirus[b]	Luminex xTAG RVP MFI[c]	GenMark Dx eSensor RVP nA[d]	BioFire FilmArray RP result[e]	Cepheid GeneXpert C_T	Focus enterovirus ASR C_T
EV-D68 dilutions[f]										
10^{-1}	21.3	22.9	23.5	30.0	27.1	4,415	10.5	Pos	28.1	28.2
10^{-2}	24.0	25.5	28.0	33.0	30.1	5,112	3.4	Pos	31.2	31.6
10^{-3}	28.5	29.9	34.2	36.1	33.7	5,405	6.9	Pos	34.1	35.9
10^{-4}	31.8	33.1	Neg	41.0	38.1	1,132	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 strain[g]	20.0	15.4	Neg	15.2	18.5	4,775	Neg	NT	NT	20.7
EV-D70[g]	Neg	Neg	Neg	Neg	14.5	3,023	6.8	NT	NT	13.7
Water	Neg	Neg	Neg	Neg	Neg	Neg	NT	NT	NT	Neg

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

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 *in vitro* diagnostics for EV-D68 detection (http://www.gpo.gov/fdsys/pkg/FR-2015-02-27/html/2015-04121.htm).

ACKNOWLEDGMENTS

We thank Daryl Lamson and Kirsten St. George from the New York State Department of Health and Christine Robinson from Children's Hospital Colorado for providing enterovirus/rhinovirus samples used in our testing; Rangaraj Selvarangan from Children's Mercy Hospital, Kansas City, MO, Anthony Orvedahl from the Department of Pediatrics at Washington University School of Medicine, and Stephanie Bledsoe from the Clinical Virology Laboratory at St. Louis Children's Hospital for providing EV-D68 residual specimen material; Brandi Herter from the Microbial Genomics Laboratory in the Department of Pediatrics at Washington University for assistance with cloning the fragment of the EV-D68 VP1 gene; and Elena Deych from the division of General Medical Sciences in the Department of Medicine at Washington University for calculating the assay limit of detection.

This study was supported in part by grant R01AI097213 from the National Institute of Allergy and Infectious Diseases awarded to G.A.S. and by institutional funds. Specimen collection was supported in part by grant U01AI077810 from the National Institute of Allergy and Infectious Diseases awarded to Stuart C. Sweet, Washington University School of Medicine, in support of the Clinical Trials in Organ Transplantation in Children (CTOT-C) research program. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

REFERENCES

1. **Schieble JH, Fox VL, Lennette EH.** 1967. A probable new human picornavirus associated with respiratory diseases. Am J Epidemiol **85:**297–310.
2. **Imamura T, Oshitani H.** 2015. Global reemergence of enterovirus D68 as an important pathogen for acute respiratory infections. Rev Med Virol **25:**102–114. http://dx.doi.org/10.1002/rmv.1820.
3. **Tokarz R, Firth C, Madhi SA, Howie SRC, Wu W, Sall AA, Haq S, Briese T, Lipkin WI.** 2012. Worldwide emergence of multiple clades of enterovirus 68. J Gen Virol. **93**(Pt 9)**:**1952–1958. http://dx.doi.org/10.1099/vir.0.043935-0.
4. **Centers for Disease Control and Prevention.** 2008. Clusters of acute respiratory illness associated with human enterovirus 68—Asia, Europe, and United States, 2008–2010. MMWR Morb Mortal Wkly Rep **60:**1301–1304).
5. **Centers for Disease Control and Prevention.** 2014. Enterovirus D68. Centers for Disease Control and Prevention, Atlanta, GA. http://www.cdc.gov/non-polio-enterovirus/outbreaks/EV-D68-outbreaks.html.
6. **Centers for Disease Control and Prevention.** 2014. Severe respiratory illness associated with enterovirus D68—Missouri and Illinois, 2014. MMWR Morb Mortal Wkly Rep **63:**798–799. http://www.cdc.gov/MMWR/preview/mmwrhtml/mm6336a4.htm?s_cid=mm6336a4_w.
7. **Oberste MS, Maher K, Schnurr D, Flemister MR, Lovchik JC, Peters H, Sessions W, Kirk C, Chatterjee N, Fuller S, Hanauer JM, Pallansch MA.** 2004. Enterovirus 68 is associated with respiratory illness and shares biological features with both the enteroviruses and the rhinoviruses. J Gen Virol **85:**2577–2584. http://dx.doi.org/10.1099/vir.0.79925-0.
8. **Greninger AL, Naccache SN, Messacar K, Clayton A, Yu G, Somasekar S, Federman S, Stryke D, Anderson C, Yagi S, Messenger S, Wadford D, Xia D, Watt JP, Van Haren K, Dominguez SR, Glaser C, Aldrovandi G, Chiu CY.** 2015. A novel outbreak enterovirus D68 strain associated with acute flaccid myelitis cases in the USA (2012–14): a retrospective cohort study. Lancet Infect Dis **15:**671–682. http://dx.doi.org/10.1016/S1473-3099(15)70093-9.
9. **Wylie KM, Wylie TN, Orvedahl A, Buller RS, Herter BN, Magrini V, Wilson RK, Storch GA.** 2015. Genome sequence of enterovirus D68 from St. Louis, Missouri, USA. Emerging Infect Dis **21:**184–186.
10. **Lee WM, Kiesner C, Pappas T, Lee I, Grindle K, Jartti T, Jakiela B, Lemanske RF, Jr, Shult PA, Gern JE.** 2007. A diverse group of previously unrecognized human rhinoviruses are common causes of respiratory illnesses in infants. PLoS One **2:**e966. http://dx.doi.org/10.1371/journal.pone.0000966.
11. **Colvin JM, Muenzer JT, Jaffe DM, Smason A, Deych E, Shannon WD, Arens MQ, Buller RS, Lee WM, Weinstock EJ, Weinstock GM, Storch GA.** 2012. Detection of viruses in young children with fever without an apparent source. Pediatrics **130:**e1455–e1462. http://dx.doi.org/10.1542/peds.2012-1391.
12. **Sutton G, White O, Adams M, Kerlavage A.** 1995. TIGR Assembler: a new tool for assembling large shotgun sequencing projects. Genome Sci Technol **1:**9–19. http://dx.doi.org/10.1089/gst.1995.1.9.
13. **Huson DH, Reinert K, Kravitz SA, Remington KA, Delcher AL, Dew IM, Flanigan M, Halpern AL, Lai Z, Mobarry CM, Sutton GG, Myers EW.** 2001. Design of a compartmentalized shotgun assembler for the human genome. Bioinformatics **17**(Suppl 1)**:**S132–S139. http://dx.doi.org/10.1093/bioinformatics/17.suppl_1.S132.
14. **Kurtz S, Narechania A, Stein JC, Ware D.** 2008. A new method to compute K-mer frequencies and its application to annotate large repetitive plant genomes. BMC Genomics **9:**517. http://dx.doi.org/10.1186/1471-2164-9-517.
15. **Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ.** 1990. Basic local alignment search tool. J Mol Biol **215:**403–410. http://dx.doi.org/10.1016/S0022-2836(05)80360-2.
16. **Quinlan AR, Hall IM.** 2010. BEDTools: a flexible suite of utilities for comparing genomic features. Bioinformatics **26:**841–842. http://dx.doi.org/10.1093/bioinformatics/btq033.
17. **Oberste MS, Maher K, Kilpatrick DR, Flemister MR, Brown BA, Pallansch MA.** 1999. Typing of human enteroviruses by partial sequencing of VP1 1. J Clin Microbiol **37:**1288–1293.
18. **Nix WA, Oberste MS, Pallansch MA.** 2006. Sensitive, seminested PCR amplification of VP1 sequences for direct identification of all enterovirus serotypes from original clinical specimens. J Clin Microbiol **44:**2698–2704. http://dx.doi.org/10.1128/JCM.00542-06.
19. **Edgar RC.** 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res **32:**1792–1797. http://dx.doi.org/10.1093/nar/gkh340.
20. **Johnson M, Zaretskaya I, Raytselis Y, Merezhuk Y, McGinnis S, Madden TL.** 2008. NCBI BLAST: a better Web interface. Nucleic Acids Res **36:**W5–W9. http://dx.doi.org/10.1093/nar/gkn201.
21. **Boratyn GM, Camacho C, Cooper PS, Coulouris G, Fong A, Ma N, Madden TL, Matten WT, McGinnis SD, Merezhuk Y, Raytselis Y, Sayers EW, Tao T, Ye J, Zaretskaya I.** 2013. BLAST: a more efficient report with usability improvements. Nucleic Acids Res **41:**W29–W33. http://dx.doi.org/10.1093/nar/gkt282.
22. **Xiao M, Kwok P-Y.** 2003. DNA analysis by fluorescence quenching detection. Genome Res **13:**932–939. http://dx.doi.org/10.1101/gr.987803.
23. **Nijhuis M, van Maarseveen N, Schuurman R, Verkuijlen S, de Vos M, Hendriksen K, van Loon AM.** 2002. Rapid and sensitive routine detection of all members of the genus enterovirus in different clinical specimens by real-time PCR. J Clin Microbiol **40:**3666–3670. http://dx.doi.org/10.1128/JCM.40.10.3666-3670.2002.
24. **Piralla A, Girello A, Premoli M, Baldanti F.** 2015. A new real-time RT-PCR assay for detection of human enterovirus 68 (EV-D68) in respiratory samples. J Clin Microbiol **53:**1725–1726. http://dx.doi.org/10.1128/JCM.03691-14.
25. **Rahamat-Langendoen J, Riezebos-Brilman A, Borger R, van der Heide R, Brandenburg A, Schölvinck E, Niesters HGM.** 2011. Upsurge of human enterovirus 68 infections in patients with severe respiratory tract infections. J Clin Virol **52:**103–106. http://dx.doi.org/10.1016/j.jcv.2011.06.019.