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Detection of Enterovirus D68 in Canadian Laboratories

Todd F. Hatchette,[a,b] Steven J. Drews,[c,d] Elsie Grudeski,[e] Tim Booth,[e] Christine Martineau,[f] Kerry Dust,[g] Richard Garceau,[h] Jonathan Gubbay,[i] Tim Karnauchow,[j] Mel Krajden,[k] Paul N. Levett,[l] Tony Mazzulli,[m] Ryan R. McDonald,[l] Alan McNabb,[k] Samira Mubareka,[n] Robert Needle,[o] Astrid Petrich,[p] Susan Richardson,[p] Candy Rutherford,[q] Marek Smieja,[q] Raymond Tellier,[r] Graham Tipples,[c,s] Jason J. LeBlanc[a,b]

Division of Microbiology, Department of Pathology and Laboratory Medicine, Capital District Health Authority (CDHA),[a] and Department of Pathology,[b] Dalhousie University, Halifax, Nova Scotia, Canada; Provincial Laboratory for Public Health (ProvLab)[c] and Department Laboratory Medicine and Pathology,[d] University of Alberta, Edmonton, Alberta, Canada; Enterovirus and Enteric Virus Laboratory, National Microbiology Laboratory (NML), Winnipeg, Manitoba, Canada[e]; Laboratoire de Santé Publique du Québec (LSPQ)/Institut National de Santé Publique du Québec (INSPQ), Québec, Canada[f]; Cadham Provincial Laboratory, Winnipeg, Manitoba, Canada[g]; Centre Hospitalier Universitaire George L. Dumont, Moncton, New Brunswick, Canada[h]; Public Health Ontario and University of Toronto, Toronto, Ontario, Canada[i]; EORLA Regional Virology Laboratory, Children's Hospital of Eastern Ontario (CHEO), University of Ottawa, Ottawa, Ontario, Canada[j]; Public Health Microbiology and Reference Laboratory, British Columbia Centre for Disease Control (BCCDC), Vancouver, British Columbia, Canada[k]; Saskatchewan Disease Control Lab (SDCL), Ministry of Health, Regina, Saskatchewan, Canada[l]; Department of Microbiology, Mount Sinai Hospital and University Health Network, and Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada[m]; Sunnybrook Health Sciences Centre, Toronto, Ontario, Canada[n]; Public Health Laboratory and Microbiology, St-John's, Newfoundland, Canada[o]; The Hospital for Sick Children, Toronto, Ontario, Canada[p]; St-Joseph's Healthcare, Hamilton, Ontario, Canada[q]; Provincial Laboratory for Public Health (ProvLab), University of Calgary, Calgary, Alberta, Canada[r]; Medical Microbiology and Immunology University of Alberta, Edmonton, Alberta, Canada[s]

The recent emergence of a severe respiratory disease caused by enterovirus D68 prompted investigation into whether Canadian hospital and provincial laboratories can detect this virus using commercial and laboratory-developed assays. This study demonstrated analytical sensitivity differences between commercial and laboratory-developed assays for the detection of enterovirus D68.

Enterovirus type D68 (EV-D68) was first isolated from children with respiratory disease in 1962 (1, 2). While outbreaks have occurred sporadically worldwide since 2008, EV-D68 was infrequently reported in the United States and Canada (3). In the late summer and early fall of 2014, widespread EV-D68 activity was described across North America, with severe cases described in Illinois and Missouri (4–6). Of particular concern, EV-D68 infection has been associated with acute flaccid paralysis, and fatal cases have been documented (7, 8). In most cases of respiratory infection, clinical specimens test positive for enterovirus (or rhinovirus) using nucleic acid amplification tests (NAATs), and the specimens are subsequently identified as EV-D68 using sequence-based typing (4–6).

While NAATs have become the method of choice for the detection of respiratory viruses, there is evidence that assays vary in their performance characteristics, including analytical sensitivity (9–11). Given the diversity of laboratory tests utilized, it is important to verify the ability of laboratory-developed tests (LDTs) and commercially available NAATs to accurately detect emerging pathogens (9–14). This study compared the analytical sensitivities of LDTs and commercial NAATs used in hospital-based and provincial public health laboratories across Canada for the detection of EV-D68.

The lower limit of detection (LoD) of each method was determined by testing serial dilutions of RNA extracted from the prototypic Fermon strain of EV-D68 (ATCC VR1076). To reduce interlaboratory variability, specimen preparation, nucleic acid extraction, and dilutions were carried out at the National Microbiology Laboratory (NML) in Winnipeg (Manitoba, Canada). The virus was cultured on human rhabdomyosarcoma cells (Centers for Disease Control and Prevention, Atlanta, GA), and the viral stock titer was determined by a 50% tissue culture infective dose ($TCID_{50}$) assay and the Kärber-Spearman calculation method. The viral stock (200 μl) was extracted using the NucliSENS easyMAG (bioMérieux, Canada), as recommended by the manufacturer. A 10-fold dilution of RNA was prepared in RNA storage solution (Ambion) as a single batch. Five replicates of each dilution were shipped on dry ice to participating sites in volumes sufficient to ensure that there would be only a single freeze-thaw cycle for each of the five independent experiments. The viral RNA was maintained at −70°C until tested. All commercial assays were performed as recommended by the respective manufacturers, and the various LDTs were performed as summarized in Table 1. Using StatPlus 2009 professional version 5.7.8, the estimated LoD was defined by Probit analysis (15) using a probability of 95%, and values were expressed as the number of target copies per milliliter (Table 1).

Our data show that different assays have variable sensitivities for the detection of EV-D68 (Table 1). The LoDs for most commercial NAATs and LDTs spanned a 2-log_{10} range. Of particular

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Address correspondence to Jason J. LeBlanc, jason.leblanc@cdha.nshealth.ca.

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TABLE 1 Protocol summaries and analytical sensitivity for LDTs and commercial NAATs

Test name[a]	Methods[b]		Target	Instrument	Estimated LoD at 95% CI[c]		Reference
	Type	Reagents			Copies/ml	$\log_{10}$ copies/ml	
LDT							
LDT- ON1	Real-time RT-PCR	QuantiTect RT-PCR kit (Qiagen, Inc.)	VP1	Corbett Rotorgene 6000 (Qiagen, Inc.)	306	2.49	NA
LDT- ON2	Real-time RT-PCR	QuantiTect RT-PCR kit	5′ NTR	Corbett Rotorgene 6000	306	2.49	NA
LDT- MB1	Conventional two-step seminested RT-PCR	SuperScript Vilo (cDNA), Platinum *Taq* Polymerase (Life Technologies)	VP1	GeneAMP PCR system 9700 (Life Technologies)	479	2.68	20
LDT- MB2	Real-time RT-PCR	AgPath-ID One-Step RT-PCR kit (Ambion)	5′ NTR	ABI 7500 Fast (Life Technologies)	562	2.78	21
LDT-BC2	Real-time RT-PCR	TaqMan Fast Virus 1-Step master mix kit (Life Technologies)	VP1	ABI 7500 Fast	562	2.78	NA
LDT- NS	Conventional RT-PCR	One-Step RT-PCR kit (Qiagen, Inc.)	5′ NTR	Dual Engine (Bio-Rad Laboratories)	562	2.78	25
LDT- SK1	Real-time RT-PCR	QuantiTect Probe RT-PCR kit (Qiagen, Inc.)	5′ NTR	ABI 7500 Fast	562	2.78	16
LDT- ON3	Real-time RT-PCR	Superscript III Platinum One-Step qRT-PCR w/ROX (Life Technologies)	5′ NTR	ABI 7900 HT Fast (Life Technologies)	1,175	3.07	21
LDT-AB	Real-time RT-PCR	TaqMan Fast Virus 1-Step master mix kit	5′ NTR	ABI 7500 Fast	5,495	3.45	17
LDT- SK2	Real-time RT-PCR	QuantiTect Probe RT-PCR kit	5′ NTR	ABI 7500 Fast	3,162	3.50	16
LDT-BC1	Real-time RT-PCR	TaqMan Fast Virus 1-Step master mix kit	5′ NTR	ABI 7500 Fast	6,026	3.78	23
LDT- ON4	Real-time RT-PCR	AgPathID One-Step RT-PCR kit	5′ NTR	ABI 7500 Fast	6,166	3.79	26
LDT- ON5	Conventional RT-PCR	One-Step RT-PCR kit	5′ NTR	Biometra Professional Thermocycler (MBI Lab Equipment)	11,749	4.07	24
LDT- SK3	Real-time RT-PCR	QuantiTect Probe RT-PCR kit; QuantiTect Probe PCR kit	5′ NTR	BioMark HD (Fluidigm Corp.)	24,266	4.39	16
LDT-NL	Real-time RT-PCR(4-plex)	SuperScript III One-Step RT-PCR	5′ NTR	LightCycler 480 I/II (Roche Diagnostics)	54,954	4.74	16
LDT- ON6	Real-time RT-PCR	Qiagen QuantiTect RT-PCR kit	5′ NTR	Corbett Rotorgene 6000	306,196	5.49	NA
LDT- ON7	Conventional RT-PCR	One-Step RT-PCR kit	5′ NTR	Biometra Professional Thermocycler	1,148,153	6.06	24
LDT- SK4	Real-time RT-PCR	QuantiTect Probe RT-PCR kit; QuantiTect Probe PCR kit	5′ NTR	BioMark HD	1,584,893	6.20	16
Commercial NAAT							
RVP v2 (QC)	Conventional RT-PCR	xTag RVP fast v2 (Luminex Corp.)	NA	ABI GeneAmp 9700 PCR System (Life Technologies) and LiquiChip 200 Workstation (Qiagen, Inc.)	1,202	3.08	NA
RVP v2 (ON1)	Conventional RT-PCR	xTag RVP fast v2	NA	MagPix (Luminex Corp.)	1,202	3.08	NA
RVP classic (AB)	Conventional RT-PCR	RVP classic (Luminex Corp.)	NA	Applied Biosystems 2720 (Life Technologies)	2,692	3.43	NA
AnDiaTec (MB)	Real-time RT-PCR	AnDiaTec Enterovirus real-time RT-PCR kit (Quidel Germany GmBH)	NA	CFX96 (Bio-Rad Laboratories)	6,166	3.79	NA
Artus (ON)	Real-time RT-PCR	artus Enterovirus LC RT-PCR kit (Qiagen, Inc.)	NA	LightCycler 2.0 (Roche Diagnostics)	31,623	4.5	NA
RVP v2 (ON2)	Conventional RT-PCR	Luminex RVP fast v2	NA	LX-200 (Luminex Corp.)	48,978	4.69	NA
AnDiaTec (NB)	Real-time RT-PCR	AnDiaTec Enterovirus real-time RT-PCR kit	NA	LightCycler 2.0	54,954	4.74	NA
RV15 (MB)	Conventional RT-PCR	Seeplex RV15 Ace One-Step Detection (Seegene, Inc.)	NA	ABI Veriti (Life Technologies)	ND[d]	ND[d]	NA
RV15 (NS)	Conventional RT-PCR	Seeplex RV15 Ace One-Step Detection	NA	Dual Engine	ND[d]	ND[d]	NA
RV16 (ON)	Conventional RT-PCR	Anyplex II RV16 Detection v1.01 (Seegene, Inc.)	NA	CFX96	ND	ND	NA

[a] AB, Alberta; BC, British Columbia; LDT, laboratory-detected test; MB, Manitoba; ND, not detected; NL, Newfoundland; NS, Nova Scotia; ON, Ontario; QC, Québec.

[b] RT-PCR, reverse transcription-PCR; NTR, nontranslated region; VP1, viral capsid protein 1.

[c] The estimated limits of detection (LoD) are ranked in descending order of sensitivity as determined by Probit analysis at a probability of 95%. CI, confidence interval; NA, not available; ND, not detected.

[d] For the two laboratories using the Seeplex RV15 Ace One-Step Detection (Seegene, Inc.), EV-D68 was detected at very high viral concentrations as a rhinovirus.

interest, the Anyplex II RV16 detection assay failed to detect EV-D68 at any viral concentrations except those exceeding 10^9 copies/ml. Similarly, both laboratories using the Seeplex RV15 OneStep ACE detection assay were unable to detect EV-D68 with the enterovirus-specific primers in the kit, but a positive amplicon was observed in the human rhinovirus reaction at high viral concentrations ($>10^8$ copies/ml). The results for the RV15 assays are consistent with the experience in the Netherlands (11). Compared to other enteroviruses, EV-D68 shares many characteristics with rhinoviruses, including acid lability and optimal growth conditions (1, 16), and cross-reactivity among enterovirus and rhinovirus is known for many molecular detection assays (17–19). Genetically, rhinovirus and enteroviruses are also quite similar, and the NAAT targets influence whether or not the two viruses can be differentiated. For example, the xTAG respiratory viral panel (RVP) classic and fast assays cannot differentiate between the two viruses. Previous reports suggest that both the classic and fast RVP formats offer good sensitivity for these viruses compared to other respiratory viruses, but the ability to detect EV-D68 was not specifically addressed (18, 19). In the present study, the LoD for the RVP classic assay displayed good sensitivity for EV-D68 compared to other NAATs, and the results were comparable to those achieved by two laboratories using the fast version (Table 1). Interestingly, a third laboratory using the RVP fast assay obtained a much higher LoD; the reasons for this reduced sensitivity are still under investigation (Table 1). It should be noted that differences were also observed between two laboratories using the AnDiaTec enterovirus kit (Table 1). Variation between laboratories using the same method would not be surprising if the specimen processing and nucleic extraction techniques differed (11); however, this study used normalized quantities of template RNA which was sent to all laboratories for amplification. Some degree of difference in LoD might be attributed to the different instrumentation used for nucleic acid amplification and detection (Table 1).

A limitation of this study was that the analytical sensitivity of LDTs and commercial NAATs was only assessed using a prototypic strain of EV-D68 that was commercially available (1, 11). This study was initiated and completed prior to the identification of multiple lineages within North America (4, 12–14). It is possible that the performance characteristics of molecular assays differ with genetic differences between the currently circulating lineages of EV-D68, and studies are under way to investigate this possibility. However, when comparing the prototypic Farmon strain and currently circulating EV-D68, little sequence differences were observed in the 5′ nontranslated region (NTR) and viral capsid protein 1 (VP1) target regions (see Fig. S1 in the supplemental material). Previous studies reported poor sensitivity for the detection of enterovirus with some molecular methods, but the reason was not specifically addressed (5, 18). In 2006, a quality assurance study, including 125 participating laboratories, demonstrated that only 54% of enterovirus-specific NAATs could detect EV-D68 (11). Interestingly, the performance was improved to 82% if the virus concentration was increased 100-fold, suggesting that the analytical sensitivity is affected by the degree of primer mismatch.

In this study, no conclusions could be drawn from a comparison of the molecular targets used by the commercial NAATs used in this study, since the target sequences are proprietary. The LDTs targeted sequences within the highly conserved regions of the 5′ NTR or VP1 that showed no or little sequence mismatches (see Fig. S1 in the supplemental material) (3, 11, 16–20). It should be noted that the LDT-SK3 target was much less sensitive than LDT-SK4 (Table 1). The difference in sensitivity between these targets is expected, as the primers and probe for LDT-SK4 are pan-enterovirus/rhinovirus designs with sequence mismatches in comparison to EV-D68, whereas LDT-SK3 is identical to EV-D68 (see Fig. S1). Interestingly, when using the same targets with a different assay and a different instrument (LDT-SK2 and LDT-SK1, respectively), both assays were much more sensitive. Based on the EV-D68 genomic sequences available in the GenBank database, two LDTs were also optimized by using modified primers, and they showed better sensitivity for EV-D68 detection (compare LDT-ON6 with LDT-ON2 and LDT-ON with LDT-ON5) (Table 1). A third laboratory optimized sequences targeting the VP1 region of EV-D68 (LDT-BC2) and increased the sensitivity approximately 10-fold over that of their previous assay targeting the 5′ NTR (LDT-BC1). While EV-D68 is more readily detected with these optimized assays, the performance characteristics for other enteroviruses and specificity analyses have yet to be reported. Overall, the reason for the analytical sensitivity differences between laboratory methods is likely multifactorial and is the subject of further investigation.

It is important to recognize that, regardless of the method used to identify enterovirus in clinical specimens, positive results require supplemental sequence-based analyses for confirmation and characterization of the virus as EV-D68. Such analyses are available at reference facilities like the National Microbiology Laboratory (NML). Of note, the two-step seminested reverse transcriptase (RT)-PCR used by the NML prior to sequencing was no more sensitive than other methods used in this study (Table 1). This suggests that some enterovirus-positive results obtained with NAATs or LDTs may not be confirmed by the NML (11, 20). Failure to confirm or type the positive enterovirus results could also be due to differences in the performance characteristic for enteroviruses other than EV-D68 or, possibly, cross-reactions with closely related rhinoviruses (1, 20). Ideally, the development of sensitive and specific assays for the direct detection and typing of EV-D68 would reduce turnaround times for the identification of this emerging pathogen and eliminate the need for sequence-based typing.

Overall, this study demonstrated the considerable variability in performance characteristics of assays used to detect EVD68 across Canada. While a clear definition of molecular assay performance is important for the laboratory, a broader understanding of the comparability of assays offered by reference-testing services is essential in the context of emerging agents of disease with public health importance. As with the Seegene RV15 and RV16 assays which failed to detect EV-D68, it is important to understand the limitations of each molecular assay for the detection of this emerging pathogen. Coordinated surveillance and detection algorithms are keys to the understanding of the scope of spread and spectrum of EV-D68 disease, and this study provides the first report comparing the analytical sensitivities of LDTs and commercial NAATs used in Canadian laboratories.

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