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Detection of enteroviruses and parechoviruses by a multiplex real-time RT-PCR assay

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ABSTRACT

Detection of all enteroviruses while excluding cross-detection of rhinoviruses is challenging because of sequence similarities in the commonly used conserved targets for molecular assays. In addition, simultaneous detection and differentiation of enteroviruses and parechoviruses would be beneficial because of a similar clinical picture presented by these viruses. A sensitive and specific real-time RT-PCR protocol that can address these clinical needs would be valuable to molecular diagnostic laboratories. Here we report a multiplex nucleic acid based assay using hydrolysis probes targeting the 5′ non-translated region for the detection and differentiation of enteroviruses and parechoviruses without cross-detection of rhinoviruses. This assay has been shown to detect enteroviruses belonging to the different species in a variety of specimen types without detecting the different species of rhinoviruses. Laboratory validation shows the assay to be sensitive, specific, reproducible, easy to set up and uses generic cycling conditions. This assay can be implemented for diagnostic testing of patient samples in a high throughput fashion.

1. Introduction

The family *Picornaviridae* belongs to the order Picornavirales and currently consists of 46 species grouped into 26 genera including *Parechovirus* and *Enterovirus*. The genus *Parechovirus* can be divided into two species; *Human parechovirus* (HPeV) and *Ljungan virus*. The species HPeV currently consists of 16 types, HPeV-1 to 16. HPeV-1 and HPeV-2, were formerly classified in the *Enterovirus* genus as echovirus 22 (E-22) and 23, respectively. Another isolate, CT86-6760, originally classified as E-23/HPeV-2 (based on serological cross-reactions) has been re-classified as HPeV-5. The genus *Enterovirus* consists of 12 species of which Enterovirus A, Enterovirus B, Enterovirus C, Enterovirus D, Rhinovirus A, Rhinovirus B and Rhinovirus C cause human disease. The three poliovirus (PV) serotypes now belong to the species Enterovirus C (www.Piconaviridae.com; [1,7,20]). In this study, "enterovirus" (EV) will refer exclusively to the human enteroviruses, consisting of species A, B, C and D and rhinoviruses will be referred to as RV.

The majority of human enterovirus (EV) infections are asymptomatic, however they can cause a wide spectrum of acute diseases, including mild upper respiratory illness; hand, foot, and mouth disease and herpangina; pleurodynia, aseptic meningitis, encephalitis, acute flaccid paralysis, and neonatal sepsis-like disease. In addition to these acute illnesses, EVs have also been associated with severe chronic diseases [16]. Different EV types are associated with certain clinical manifestations such as coxsackievirus A16 (CV-A16), enterovirus 71 (EV-A71), and CV-A6 have strong associations with outbreaks of hand, foot, and mouth disease and CV-A24 and EV-D70 with hemorrhagic conjunctivitis. Severe EV-A71 outbreaks have involved cases of fatal encephalitis in infants and children in the Asia-Pacific region [12,15].

Human rhinoviruses (RVs) are responsible for many cases of common cold but are also frequently found in otitis media, sinusitis, bronchitis, pneumonia, and asthma exacerbations [18]. Several RV types circulate continuously and differentiation of RV and EV infections is often clinically important. Specific identification of these viruses can have implications for the supportive management of patients and can become more significant when specific antiviral drugs become available. This has been highlighted in the CDC health

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advisory on the recent outbreak of EV-D68, where samples that test positive by commonly used assays that are unable to distinguish between EVs and RVs have to be further tested for the confirmation of EV-D68 (http://www.bt.cdc.gov/han/han00370.asp). Real-time RT-PCR targeting the 5′non-translated region (5′NTR) can be used for sensitive and specific detection of EV and RV, however sequence similarity in this region creates difficulties for differentiation.

Human parechovirus infections have been described mostly in infants and young children, however they have been historically under-diagnosed because of the difficulty of detection by traditional methods such as virus isolation [7]. Infections often appeared to be asymptomatic or associated with mild gastrointestinal and respiratory symptoms, although severe neonatal diseases including sepsis, meningitis, encephalitis and hepatitis have been described [9,10]. More severe disease outcomes have been linked to infection with HPeV type 3 [10]. The epidemiology, natural history and prevalence of HPeVs in general have not been fully established.

Because of the similarity in the clinical picture caused by EVs and HPeVs, multiplex detection of the two viruses can be beneficial for a speedy and cost-effective diagnosis. Also the specific detection of EVs without cross-reactions with RVs can aid diagnosis and guide supportive treatment. The turn-around-time for the diagnosis of EV and HPeV infections can be improved by the use of molecular testing which can be beneficial for hospitalized children. Here we report the development and validation of a multiplex real-time RT-PCR assay (multiplex rtRT-PCR) targeting the 5′NTR for simultaneous detection of EVs and HPeVs using hydrolysis probes in a format that can be easily implemented for high throughput testing of patient samples in a diagnostic setting.

2. Methods

2.1. Design of primers and probes

Representative sequences of the 5′NTR from the different EV and RV serotypes were used for the design of primers (PanEV2_5′NTR_For and PanEV2_5′NTR_Rev) and probe (PanEV5_5′NTR_probe) for the detection of all EVs while avoiding cross reactivity with RVs. The alignment included representative sequences for all serotypes of the 64 “classical” EVs, namely polioviruses 1–3, Echoviruses, Coxsackie A viruses, Coxsackie B viruses, and numbered enteroviruses EV-D68, B69, D70 and A71. It also included sequences EV-B73, B74, B75, A76, B77, B79, B80, B81, B82, B83, B84, B85, B86, B87, B88, A89, B97, B100, B101 and RV 87 (now classified within EV-D68) and representative sequences from all the serotypes of Rhinovirus A and B. The 5′NTR from HPeVs 1–8 was available in Genbank and was used for the design of primers (ParechoV4_5′NTR_For and ParechoV4_5′NTR_Rev) and probe (Parecho_5′NTR_VIC) for their detection. Probes for the detection of EVs and HPeVs were designed as minor groove binding probes and purchased from Applied Biosystems (ABI, Foster City, California) and labelled with NED and VIC as the reporter dyes respectively. PanEV_clone_For and EVRV2a-rev (Thomas Briese, personal communication) were designed for amplification of a longer region of the 5′NTR from EVs including the detection region to generate a plasmid clone for the preparation of in-vitro RNA. Similarly Parecho_5′NTR_ClonFor and Parecho_5′NTR_ClonRev were used for amplification of the HPeV detection region to generate a plasmid. The sequences and source of all the oligonucleotides used in this study are provided in Table 1.

2.2. Real-time RT-PCR assay

A one-step RT-PCR method was used for the amplification and detection of EVs and HPeVs simultaneously. The TaqMan® Fast Virus One-Step RT-PCR Master Mix (ABI) was used with 0.8 μM each of sense and antisense primers and 0.2 μM of the probes. Five microlitres of the extracted RNA was combined with 15 μl of the master mix and the reverse transcription step was performed at 50 °C for 5 min followed by incubation at 95 °C for 20 s. Amplification included 45 cycles of denaturation at 95 °C for 3 s, followed by annealing, extension and data acquisition at 60 °C for 30 s on the 7500 Fast Real-Time PCR system (ABI).

2.3. Preparation of RNA transcripts for sensitivity studies

PanEV_clone_For and EVRV2a-rev were used for the amplification of a longer region of the 5′NTR to generate a plasmid clone with the detection region. This region was amplified from CV-A16, CV-B3, CV-A9, EV-D70 and PV-1, 2 and 3 to represent the different species. Similarly Parecho_5′NTR_ClonFor and Parecho_5′NTR_ClonRev were used for amplification of the detection region from HPeV-1 and 3. The PCR products were cloned using the TOPO® TA Cloning Dual Promoter Kit (Life Technologies, California, USA). The plasmid DNA was linearized using restriction enzyme Hind III and transcribed using the T7 RiboMAX™ Express (Promega, Madison, WI, USA) to synthesize negative-strand RNA in-vitro. The transcribed RNA was spectrophotometrically quantified for the calculation of copy numbers.

2.4. Sensitivity, specificity and reproducibility of RT-PCR

Ten-fold serial dilutions of quantified in-vitro transcribed RNA for EVs representing the different species and HPeVs were used to determine assay sensitivity using the assay in both singleplex and multiplex formats. End point sensitivity was assessed by testing the dilutions in triplicate on three independent runs using the multiplex assay for EV and HPeV detection.

Specificity for the singleplex and multiplex assays was determined by testing high copy number anonymized samples containing

Table 1
Primer and probe design.

Target	Primer/probe name	Sequence (5′ – 3′)	Source
EVs	PanEV2_5′NTR_For	CATGGTGCGAAGAGTCGATTGA	In-house
	PanEV2_5′NTR_Rev	CACCCAAAGTAGTCGGTTCCGC	In-house
	PanEV5_5′NTR_probe	NED-CCCTGAATGCGG-MGB/NFQ	In-house
HPeVs	ParechoV4_5′NTR_For	TGCAAACACTAGTTGTAAGGCCC	In-house
	ParechoV4_5′NTR_Rev	GCCCCAGATCAGATCCATAGTG	In-house
	Parecho_5′NTR_VIC	VIC-AAGGATGCCCAGAAGG-MGB/NFQ	In-house
EV cloning	PanEV_clone_For	CAAGCACTTCTGTTTCCCCG	In-house
	EVRV2a-rev	CCGGYAAYTTCCACCACCA	Thomas Briese, (Personal Communication)
HPeV cloning	Parecho_5′NTR_ClonFor	TGAAAGGGGTCTCCTAGAGAGC	In-house
	Parecho_5′NTR_ClonRev	GTTTGGCCCACTAGACG	In-house

common respiratory pathogens including different strains of influenza virus A and B, parainfluenza virus 1, 2, 3, 4A, and 4B, RSV A and B, human coronaviruses 229E, NL63, HKU1 and OC43, human bocavirus, herpes simplex viruses 1 and 2, varicella zoster virus, West Nile virus, human metapneumovirus, adenovirus serotypes 4, 10, 31 and 40, *Legionella pneumophila*, *Mycoplasma pneumoniae*, *Bordetella bronchiseptica*, *Bordetella holmesii*, *Bordetella parapertussis*, *Bordetella pertussis*, and *Chlamydophila pneumoniae*. In addition 51 RVs characterized by sequencing as belonging to species A (n = 25), B (n = 1) or C (n = 25) from anonymized patient samples were tested using the designed assay to thoroughly verify whether there was any cross-reaction of the primers and probe.

Reproducibility of the multiplex assay was evaluated using a culture of HPeV-1 spiked into a background matrix of an anonymized oral swab sample at a crossing threshold (Ct) of 23.2 and anonymized lip swab sample at a Ct of 30.06; HPeV-3 culture was spiked into a background of CSF at Ct values of 24.21 and 33.88. Spiked samples were used for HPeV testing due to the unavailability of appropriate positive patient specimens. Representatives of *Enterovirus A*, *B*, *C* and *D* were tested using an ulcer swab positive for CV-A6 at a Ct of 24.62, CSF positive for CV-B5 at a Ct of 34.80, an aliquot of PV-1 virus vaccine strain dilution at a Ct of 34.52 and EV-D68 at a Ct of 30.96. All samples were tested in triplicate on three independent runs.

2.5. Detection of different enterovirus species and co-infections

High and low viral loads of representative EVs from the different species including CV-A2, CV-A4, CV-A5, CV-A6, CV-A16 and EV-A71 for species A; CV-A9, CV-B2, CV-B3, CV-B4, CV-B5, E-4, E-9, E-18, E-25, E-30, E-82, EV-B86 and EV-B101 from species B; vaccine strains of PV-1, PV-2 and PV-3 from species C and EV-D68 and EV-D70 from species D were tested by the multiplex assay. Human PeVs tested by the assay included HPeV-1 and 3. All EVs were typed based on the partial sequence of the VP2 gene [4], or of VP1 for CV-A5 and CV-B2 [13].

To assess any competitive inhibition of target detection in cases with co-infections of EV and HPeVs, spiked samples were tested with different concentrations of both viruses. Cultured CV-B4 was spiked to give Ct values ranging from 20.17 to 32.47 and HPeV-3 was spiked to give Ct values ranging from 22.85 to 32.64 into universal transport media (UTM) and extracted using the using the easyMAG® automated extractor (BioMérieux, Durham, NC, USA). All extracts were tested by the single and multiplex assays.

2.6. Clinical specimens

Specimens that had previously tested positive for EVs by a nucleic acid sequence based amplification (NASBA) assay [8] which is an isothermal, transcription-based amplification method, were used for the validation of the multiplex assay. These included 20 positives (blood, CSF, feces and amniotic fluid) with Ct values ranging from 23.96 to 35.57; and 36 negative CSF samples. Viral RNA from the different specimen matrices was extracted using the easyMAG, according to manufacturer's instructions.

3. Results

3.1. Assessment of the RT-PCR assay performance; sensitivity, specificity, and reproducibility

These results are indicated in Table 2 including the number of replicates that tested positive at the end-point, the average Ct value at the end point and the %CV in the Ct value. The limit of detection for the multiplex assay was estimated around 7 copies of in-vitro transcribed RNA in 5 μl of template for HPeVs 1 and 3, CV-A16, CV-A9, and EV-D70, and CV-B3. The limit of detection ranged from 150 to 376 copies of in-vitro RNA for the vaccine strains of PV-1, PV-2 and PV-3 by the multiplex assay. Sensitivity using the singleplex assays was largely comparable at around 7 copies of in-vitro transcribed RNA in 5 μl of template for CV-A16, CV-B3, CV-A9, and EV-D70. The limit of detection for the singleplex assay was 7 copies of in-vitro RNA for the vaccine strain of PV-2 and less than 70 copies for HPeVs 1 and 3. Linear amplification of target was obtained over 6–7 logs of template concentration using in-vitro RNA for the different species. The efficiency for amplification ranged from 98.53% to 117.11%.

Table 2
Copy number sensitivity for the multiplex assay indicating variability in the crossing threshold values at the end-point.

Sample	Copy no.	Number of positive replicates	Average	SD	%CV
HPeV-1	6	8/9	36.61	0.64	1.76
HPeV-3	7	9/9	37.13	0.67	1.80
CV-A16	7	8/9	37.92	0.97	2.55
CV-B3	6	5/9	38.97	0.36	0.92
CV-A9	6	6/9	37.91	0.94	2.48
EV-D70	6	8/9	38.69	1.16	3.00
PV-1	150	8/9	39.66	0.75	1.88
PV-2	307	6/9	41.71	2.58	6.20
PV-3	376	7/9	39.20	0.66	1.69

Quantified in-vitro RNA was used to determine the end-point sensitivity.
The template copy number in 5 μl of extract used per reaction is indicated.
The average, standard deviation (SD) and coefficient of variation (%CV) indicate variability of the crossing threshold values at the end-point.

The singleplex and multiplex assays did not amplify other viral and bacterial respiratory pathogens that can potentially cause co-infections with EVs and HPeVs thus establishing 100% specificity. A total of 51 RVs characterized by sequencing as species A (n = 25), B (n = 1) and C (n = 25) tested negative by the singleplex and multiplex assays showing that there is no cross detection of RVs by the EV primers and probe. Rhinoviruses will continue to be tested by this assay as they become available in our laboratory to ensure specific detection of EVs.

Eight samples with Ct values ranging from 23.20 to 34.80 were tested in triplicate on three independent runs resulting in nine replicate values. The intra-assay variability (%CV) was calculated using the replicates within the same run. For the eight samples tested, this varied from 0.17 to 1.94%. The inter-assay variability was calculated using values obtained from the different runs, this ranged from 1.00 to 1.99% showing reproducible detection and good precision at different viral loads from the different specimen types. The Ct values, inter-assay and intra-assay variability are shown in Table 3.

The sensitivity of NASBA methodology is comparable to real-time RT-PCR for molecular-based diagnostic procedures for RNA viruses; in addition specimens tested for EV detection by the NASBA assay were readily available in our laboratory and were thus used for the validation. Retrospective positive and negative patient samples used as an accuracy panel for assay validation provided concordant results between the NASBA and multiplex real-time RT-PCR assay. These included 20 positives (blood, CSF, feces and amniotic fluid) with Ct values ranging from 23.96 to 35.57; and 36 negative CSF samples.

3.2. Testing of different enterovirus types and co-infections

High and low viral loads of representative viruses from *Enterovirus A*, *B*, *C* and *D* listed in the methods were detected by the multiplex assay at the expected Ct values.

Table 3
Intra and inter assay variability of the multiplex assay for the detection of high and low viral loads of different EVs and HPeVs.

		Intra-assay variability									Inter-assay variability		
		Run 1			Run 2			Run 3			Run1/2/3		
Target	Specimen type	Average	SD	%CV	Average	SD	%CV	Average	SD	%CV	Average	SD	%CV
HPeV-1	Oral swab	23.50	0.25	1.05	23.31	0.45	1.94	22.80	0.06	0.24	23.20	0.41	1.75
HPeV-1	Lip swab	30.16	0.05	0.17	30.44	0.59	1.92	29.57	0.16	0.53	30.06	0.49	1.63
HPeV-3	CSF	24.65	0.32	1.29	24.23	0.25	1.02	23.77	0.28	1.19	24.21	0.45	1.87
HPeV-3	CSF	34.47	0.51	1.47	34.05	0.34	1.00	33.12	0.10	0.30	33.88	0.68	1.99
CV-A6	Ulcer swab	24.93	0.07	0.29	24.65	0.05	0.21	24.26	0.16	0.64	24.62	0.30	1.24
CV-B5	CSF	35.08	0.19	0.54	34.95	0.28	0.81	34.36	0.11	0.32	34.80	0.38	1.08
PV-1	Carrier RNA	34.71	0.35	1.00	34.67	0.24	0.69	34.19	0.23	0.68	34.52	0.35	1.00
EV-D68	Carrier RNA	30.93	0.38	1.22	31.36	0.28	0.90	30.60	0.20	0.64	30.96	0.42	1.34

All samples were tested in triplicate on three independent runs and intra-assay variability was calculated for each of the runs. Inter-assay variability was calculated based on all three runs.
The average, standard deviation (SD) and coefficient of variation (%CV) indicate variability of the crossing threshold values at the end-point.

Results of the co-infection studies are indicated in Table 4. The Ct values obtained from specimens with a co-infection were comparable to those from specimens with a single target infection showing that there is no competitive inhibition for the detection of either target at the different viral loads tested.

4. Discussion

Enteroviruses are common and important human pathogens. Although the majority of infections are asymptomatic, EVs cause several severe illnesses. Molecular methods, especially real-time RT-PCR, have become the diagnostic modality of choice given the rapid turn around time and the high sensitivity provided by these methodologies as previously reported [2,3,6,14,19]. The 5′NTR has been the most commonly chosen target because of the high degree of conservation among different EV types; the origin of this high conservation stems from the secondary structure required for the internal ribosome entry site (IRES) function of the 5′NTR. Unfortunately, this high conservation also extends to a high homology with the 5′NTR of RVs, and many RT-PCR assays for EVs cross detect at least some of the RVs. In this study we designed and validated a set of primers and probes that do not cross-detect RVs, and can detect all the 64 “classical” EVs as well as numerous newly described EVs up to EV-B101, with a high sensitivity. Such lack of cross detection is particularly useful for specific laboratory diagnosis of respiratory samples since both rhinoviruses and enteroviruses commonly infect the respiratory tract. Infection of the respiratory tract by rhinoviruses is more prevalent than enteroviruses and to ensure the detection of rhinoviruses, the algorithm used in our laboratory includes initial testing by the respiratory viral panel (RVP) assay from Luminex Molecular Diagnostics (Austin, TX, USA). This assay can detect both enteroviruses and rhinoviruses, however it cannot distinguish them. The assay described in this manuscript is used for this differentiation when the clinical picture warrants an enterovirus-specific assay. The assay described in the manuscript is also used for the specific detection of enteroviruses and parechoviruses in samples such as blood and CSF as the front line assay.

Table 4
Co-infection studies with enteroviruses and parechoviruses.

CV-B4 singleplex Ct	HPeV-3 singleplex Ct	CV-B4/HPeV-3 multiplex Ct
20.17	22.85	20.05/22.35
20.17	32.64	20.35/32.12
32.47	22.85	32.12/22.94
32.47	32.64	32.27/32.77

Crossing threshold (Ct) values obtained when the samples were tested as single and co-infections are indicated.

As a consequence of the strong constraints against mutations in the 5′NTR, it is expected that the assay will also detect new or emerging EVs. Caution must however be exercised in that regard; already, several isolates of the newly designated EV-C104, EV-C105, EV-C109, EV-C117 and EV-C118, which have been shown to constitute a new clade within species C [5,11,17,21,22] display important differences in the sequence of their 5′NTR and several RT-PCR assays targeting the 5′NTR [7] have failed to detect them [17,21]. Based on sequence comparison of our primers and probes and the sequences of these isolates, we would predict that our assay would also fail to detect these viruses. The exact geographic distribution and clinical frequency of viruses in this new clade remains to be established. Given the difficulties of designing a comprehensive assay for the detection of EVs that does not cross react with RVs, we would argue that the best way to improve our current assay in order to detect this new clade would be to add primers and probe specific for this clade to the multiplex mix. Further work will be required towards this goal.

Human Parechoviruses constitute a new species within the *Picornavirus* genus; and relatively few laboratories are routinely testing for this group; thus the full natural history of these viruses still remains to be understood. Genome sequence data is available for HPeVs 1–8 for the region targeted by the reported primers and probe and based on in-silico analysis, these viruses can be detected with equal efficiency. Parechoviruses belong to a different genus and the 5′NTR region is quite distinct from that of EVs and RVs; it is thus quite feasible to design primers and probe that are specific for HPeVs, and to multiplex them with the primer and probes for EVs. The reported mutiplex assay was sensitive for the detection of the different species of EVs and HPeVs tested and was able to exclude the detection of RVs. This assay showed 100% specificity and excellent reproducibility making it suitable for implementation in a diagnostic setting. A variety of specimen types including blood, CSF, feces, swabs and amniotic fluid were used for viral detection.

Even though no antiviral drugs effective against EV and HPeV infections are currently available, testing can aid in patient management by providing a diagnosis and reducing antibiotic use. Sensitive nucleic acid based detection methodologies would contribute to a better understanding of the epidemiology and natural history of these agents. The reported multiplex real-time RT-PCR assay has been successfully implemented for diagnosis of EV and HPeV infections in a routine diagnostic laboratory.

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