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Rapid and Sensitive Routine Detection of All Members of the Genus *Enterovirus* in Different Clinical Specimens by Real-Time PCR

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We developed a rapid and sensitive method for the routine detection of all members of the enterovirus genus in different clinical specimens by using real-time TaqMan quantitative PCR. Multiple primer and probe sets were selected in the highly conserved 5′-untranslated region of the enterovirus genome. Our assay detected all 60 different enterovirus species tested, whereas no reactivity was observed with the viruses from the other genera of the picornaviridae family, e.g., hepatovirus and parechovirus. Weak cross-reactivity was observed with 7 of the 90 different high-titer rhinovirus stocks but not with rhinovirus-positive clinical isolates. Analysis of a well-characterized reference panel containing different enteroviruses at various concentrations demonstrated that the enterovirus real-time TaqMan PCR is as sensitive as most of the currently used molecular detection assays. Evaluation of clinical isolates demonstrated that the assay is more sensitive than the "gold standard" method, i.e., viral culture. Moreover, the PCR assay can be used on different clinical specimens, such as plasma, serum, nose and throat swabs, cerebrospinal fluid, and bronchoalveolar lavage, without apparent inhibition. Our data demonstrate that the real-time TaqMan PCR is a rapid and sensitive assay for the detection of enterovirus infection. The assay has a robust character and is easily standardized, which makes it an excellent alternative for the conventional time-consuming viral culture.

According to the most recent classification of viruses, the family of the *Picornaviridae* is divided in six genera, among which are the human enteroviruses, rhinoviruses, parechoviruses, and hepatoviruses. The genus enterovirus includes the following human viruses: the polioviruses and human enterovirus groups A to D (8).

These viruses cause a wide spectrum of clinical syndromes ranging from mild fever to respiratory infections, meningitis, encephalitis, and paralytic poliomyelitis and myocarditis. An estimated 10 to 30 million enterovirus infections occur annually in the United States, causing significant short-term morbidity and economic impact (18, 22, 23, 28). Life-threatening enterovirus infections may occur, especially in at-risk individuals such as immunocompromised patients. Recently, bone marrow transplantation patients have been found to be infected with enteroviruses, resulting in serious complications such as pneumonia (6, 19). The relatively immunodeficient neonate is also at risk for severe enterovirus infections, resulting in serious febrile illnesses in the first weeks of life (23, 26). In addition, enteroviruses may be associated with some chronic conditions, for instance, chronic fatique syndrome and amyotrophic lateral sclerosis (1, 4, 5, 17, 30).

Viral culture is the "gold standard" for the diagnosis of enterovirus infection in different clinical specimens such as feces, nose-throat (NT) swabs, bronchoalveolar lavage (BAL), and cerebrospinal fluid (CSF). However, viral culture is labor-intensive and takes several days to weeks to be conclusive. A rapid diagnostic test may therefore have a strong impact on management of patients with enterovirus infections, especially in light of the availability of a novel antiviral drug such as pleconaril (21, 25, 27).

Rapid diagnostic tests have been developed in recent years and are usually based on nucleic acid amplification technology, such as reverse transcription-PCR and nucleic acid sequence-based amplification (3, 7, 9, 12, 20, 24, 31). These assays have proven to be sensitive and specific but, unfortunately, are often in a (semi-)nested format or require specific detection methods, which poses serious hazards for amplification product carryover. In addition, they still require time-consuming sample handling and post-PCR handling and are therefore often difficult to implement in a routine diagnostic setting.

We describe here a novel TaqMan-based real-time PCR assay for rapid, sensitive, and specific detection of all members of the enterovirus genus in different clinical specimens. Moreover, this assay can be used for direct virus quantification. We compared this real-time PCR assay to conventional virus culture and to an in-house nested PCR assay. This novel robust assay can generate results within 4 to 5 h, is easily standardized, does not require any post-PCR handling, and can therefore be implemented in routine viral diagnostic testing.

MATERIALS AND METHODS

Virus stocks. The enterovirus, parechovirus, and hepatovirus stocks were kindly provided by the Laboratory for Virology, National Institute of Public Health and the Environment (RIVM, Bilthoven, The Netherlands). The rhinovirus stocks we kindly provided by T. Hovi, Department of Microbiology, National Public Health Institute (Helsinki, Finland). Coxsackievirus A9 (CVA9) and echovirus 11 (EV11) specimens were obtained from the EU-QCCA entero-

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TABLE 1. Primer and probe sequences used in the enterovirus real-time TaqMan PCR

Primer or probe	Sequence	Nucleotide position[a] (range)
Forward primer	5′-TCCTCCGGCCCCTGA	452–466
Reverse primer 1	5′-AATTGTCACCATAAGCAGCCA	607–587
Reverse primer 2	5′-GATTGTCACCATAAGCAGCCA	607–587
Probe 1	5′-CGGAACCGACTACTTTGGGTGTCCGT	541–566
Probe 2	5′-CGGAACCGACTACTTTGGGTGACCGT	541–566

[a] Primer and probe nucleotide positions are given according to the CVA16 sequence (U05876).

virus panel (i.e., the Quality Control Concerted Action for Nucleic Acid Amplification in Diagnostic Virology).

Clinical specimens. In the present study, detection of enterovirus infection by real-time TaqMan PCR was analyzed and compared to conventional virus culture in two groups of hospitalized patients.

The first group of patients consisted of 41 neonates who developed sepsis symptoms. Feces samples were obtained from all patients and were used for routine virus culture and real-time TaqMan PCR analysis. Serum or plasma samples were obtained from 39 of the 41 neonates and were used for real-time TaqMan PCR analysis. A lumbar puncture was performed in eight of the patients and was used for routine virus culture and real-time TaqMan PCR analysis.

The second group of patients consisted of 43 adults with hematologic cancer who had signs of pneumonia and radiographic pulmonary abnormalities and whose BAL samples had been stored. NT swabs taken within 1 week of the BAL sample were available from 17 of these 43 patients.

NT swabs, which were placed in viral transport media, as well as BAL, serum, plasma, CSF, and feces samples, were either transported directly to the laboratory or were stored at 4°C for a maximum of 24 h. In the laboratory, 0.5 ml of the BAL sample was added to 2.5 ml of virus transport medium, and the feces samples were extracted with chloroform. All of the different clinical specimens were divided, part of the material was directly used for viral culture, and the remainder was frozen and stored at −70°C for analysis by PCR.

Viral culture. Viral culture was performed on confluent layers of tertiary rhesus monkey kidney cells, human embryonic rhabdomyosarcoma cells, and human embryonic lung fibroblast cells grown in Eagle minimal essential medium supplemented with 0.01 M HEPES, 0.084% bicarbonate, 100 U of penicillin-streptomycin/ml, 0.625 μg of amphotericin B/ml, and 0.2 M glutamine (SVM; Foundation for the Advancement of Public Health and Environment, Bilthoven, The Netherlands). After inoculation of 0.25 ml of clinical specimen and absorption to the cells for 1 h, 1 ml of culture medium was added, and cells were maintained at 37°C on roller drums and examined daily during 14 days for a cytopathic effect.

Viral RNA isolation. RNA extraction was performed by using the NucliSens Isolation kit (Organon Teknika, Boxtel, The Netherlands) according to the method described by Boom et al. (2). Briefly, 100 μl of clinical specimen or virus stock was mixed with 900 μl of lysis buffer and 50 μl of silica and then incubated for 10 min at room temperature to allow binding of the nucleic acid to the silica particles. Unbound material was removed by several washing steps. Next, the RNA was eluted in either 100 μl of 40 ng of poly(A) RNA/μl and directly used for nested PCR analysis or eluted in 100 μl of nuclease-free water and directly used for cDNA synthesis and real-time TaqMan PCR.

Nested PCR. After viral RNA isolation, an equivalent of 10 μl of clinical specimen was used in a one-tube reverse transcription-PCR procedure, essentially as described by Nijhuis et al. (13), with 3 mM $MgCl_2$ and a 400 μM concentration of the oligonucleotides RHI-1 (5′-CGG TAA YTT TGT ACG CCA GTT-3) and RHI-2 (5′-ACA CGG ACA CCC AAA GTA-3′) and MET21C (5′-ATG TAC YTT TGT ACG CCT GTT-3′) (Pharmacia Biotechnology, Roosendaal, The Netherlands). Reactions were performed in a GeneAMP PCR system 9600 (Applied Biosystems International, Foster City, Calif.) according to the following procedure. cDNA synthesis was carried out for 30 min at 42°C, followed by reverse transcriptase inactivation for 5 min at 95°C. Subsequently, a total of 35 cycles were performed, consisting of a denaturation step for 30 s at 95°C, an annealing step for 30 s at 50°C, and an extension step for 2 min at 72°C. After this procedure, the amount of amplified product was further increased in a second (nested) amplification reaction, containing 3.5 mM $MgCl_2$ and a 400 μM concentration of the oligonucleotides RHI-3 (CAA GCA CTT CTG TTT CCC CGG-3′) and RHI-4 (CAT TCA GGG GCC GGA GGA-3′). The nested PCR was also performed in a GeneAMP PCR system 9600 according to the following procedure: 30 cycles of a denaturation step for 30 s at 95°C, an annealing step for 30 s at 64°C, and an extension step for 2 min at 72°C. PCR products were visualized on an ethidium bromide-stained agarose gel by using UV illumination. A 100-bp marker was used as a control for fragment length.

Real-time TaqMan PCR. The isolated viral RNA was reverse transcribed by using MultiScribe reverse transcriptase and random hexamers (TaqMan Reverse Transcription Reagents; Applied Biosystems International). Each 50-μl reaction contained 10 μl of eluted RNA (corresponding to 10 μl of clinical specimen), 5 μl of 10× RT buffer, 5.5 mM $MgCl_2$, a 500 μM concentration of each of the deoxynucleoside triphosphates, a 2.5 μM concentration of random hexamer, 62.5 U of MultiScribe RT, and 20 U of RNase inhibitor. cDNA synthesis was performed in a GeneAMP PCR system 9600 according to the following procedure: after an annealing step for 10 min at 25°C, reverse transcription was carried out for 30 min at 48°C, followed by reverse transcriptase inactivation for 5 min at 95°C. The cDNA was stored at −70°C before real-time TaqMan PCR.

Primers and probes for enteroviruses were selected by using Primer Express software version 1.0 (Applied Biosystems International) and were based on highly conserved regions in the 5′-untranslated region of the enterovirus genome. The exact primers and probes were chosen after sequence comparison of 143 different enterovirus sequences. The forward and reverse primers and the probe sequences are shown in Table 1. For enterovirus detection, two different reverse primers and two different probes were selected to ensure that all different members of the enterovirus genus could be detected. Both fluorogenic probes were labeled with the 5′-reporter dye 6-carboxyfluorescein (FAM) and the 3′-quencher dye 6-carboxytetramethylrhodamine (TAMRA). After optimization of the primer and probe concentrations, samples were assayed in duplicate in a 25-μl reaction mixture containing 5 μl of cDNA, 12.5 μl of 2× TaqMan Universal PCR Master Mix, a 900 nM concentration of forward primer, 300 nM concentrations of each of the reverse primers, and 100 nM concentrations of each of the probes. RNA extracts were substituted by RNase-free water in negative control reactions. The PCR mixture was incubated 2 min at 50°C for AmpErase uracil-*N*-glycosylase-mediated decontamination, followed by 10 min at 95°C to activate AmpliTaq Gold DNA polymerase. Subsequently, a total of 45 cycles were performed; these consisted of a denaturation step for 15 s at 95°C and a combined annealing-extension step for 1 min at 60°C. During the annealing-extension step, the ABI Prism 7700 SDS (Applied Biosystems International) monitored real-time PCR amplification by quantitatively analyzing fluorescence emissions. The reporter dye (FAM) signal was measured relative to the reference dye (ROX) as present in the TaqMan Universal PCR Master Mix to normalize for non-PCR related fluorescence fluctuations occurring from well to well. The threshold was set at 10 times the standard deviation of the mean baseline emission calculated between cycles 3 and 15. The threshold cycle number (Ct value) represented the refraction cycle number at which a positive amplification reaction was measured.

RESULTS

Comparison of real-time TaqMan PCR with enterovirus PCR on the third QCCA panel. The enterovirus real-time TaqMan PCR was compared to our in-house nested PCR and to the enterovirus PCR results obtained from 82 participants in the third EU-QCCA distribution (Table 2) (www.qcmd.org). The results with the 10-fold serial dilution of CVA9 and EV11

TABLE 2. Comparison of real-time TaqMan PCR and enterovirus PCR on part of the third EU-QCCA enterovirus proficiency panel

Virus stock	Virus titer ($TCID_{50}$)[a]	Real-time TaqMan PCR result	In-house nested PCR result	% Positive PCR result (n = 82)[b]
CVA9	0.036	Negative	Negative	17
CVA9	0.36	Positive	Equivocal	51
CVA9	3.6	Positive	Positive	87
EV11	25.2	Positive	Equivocal	72
EV11	252	Positive	Positive	87
EV11	25.200	Positive	Positive	95

[a] $TCID_{50}$, 50% tissue culture infectious dose.
[b] n = the number of participants.

TABLE 3. Analysis of the real-time TaqMan PCR on different picornaviridae

Picornaviridae	Species analyzed	Taqman PCR result
Enterovirus	Poliovirus: PV1 to -3	Positive
	Human enterovirus A: CVA2 to -4, -6 to -8, -10, -12, -14, and -16 and HEV71	Positive
	Human enterovirus B: CVB1 to -6, CVA9, ECV1 to -7, -9, -11 to -21, -24 to -27, and -29 to -33 and HEV69	Positive
	Human enterovirus C: CVA11, -13, -15, -17, -18, -20, -21, and -24	Positive
	Human enterovirus D: HEV68, -70	Positive
Rhinovirus	Human rhinovirus A, B, and unassigned (90 serotypes)	83 negative
Hepatovirus	Human hepatitus A virus	Negative
Parechovirus	Human parechovirus 1 (formerly ECV22) and 2 (formerly ECV23)	Negative

demonstrated that our novel real-time TaqMan PCR performed more consistently than our in-house nested PCR. Comparison of our data with the results of the different enterovirus detection methods used by the 82 participants of the QCCA distribution demonstrated that our assay reproducibly detected the dilution of CVA9, which in the QCCA panel was detected by 51% of the participants.

Detection of the different *Picornaviridae*. Viral culture supernatants of different members of the picornaviridae were analyzed in the real-time TaqMan PCR (Table 3). The data reveal that our PCR, with multiple primers and probes, detected all 60 different enterovirus species tested. Members from other genera of the *Picornaviridae* family, such as hepatovirus and parechovirus, were found to be negative. High-titer viral culture supernatants of 90 different rhinovirus serotypes were also evaluated. Seven of these isolates were tested weakly positive in our enterovirus TaqMan PCR. However, their Ct values were about 10 cycles higher than if tested in a rhinovirus real-time TaqMan PCR, a finding which indicated that the enterovirus TaqMan PCR is ca. 30,000-fold less susceptible for the detection of rhinoviruses than our rhinovirus TaqMan PCR. Clinical samples of patients with culture-positive rhinovirus infection tested negative in our enterovirus real-time TaqMan PCR, which also indicated that only high-titer rhinovirus samples will test positive in our enterovirus TaqMan PCR.

Comparison of real-time PCR and viral culture on different clinical specimens. To investigate whether the real-time TaqMan PCR can be used for the detection of enterovirus infection in different clinical specimens, spike experiments were performed. In these experiments, serial 10-fold dilutions of CVA9 were either added to virus transport medium (control) or to negative plasma, serum, feces, CSF or BAL samples. These experiments demonstrated no apparent inhibition in our clinical specimens, since there was no more than one Ct value increase between the various clinical specimens used.

In the present study, detection of enterovirus infection by real-time TaqMan PCR was also compared to conventional virus culture in two groups of hospitalized patients. The first group of patients consisted of 41 neonates with symptoms of sepsis. Feces were obtained from all patients and were used for routine virus culture and real-time TaqMan PCR analysis. Nine enterovirus infections were diagnosed by virus culture in 41 patients. All of these culture-positive clinical samples were also determined to be positive by real-time PCR. One additional feces sample was found to be positive by real-time PCR, which was further confirmed by analyzing the serum sample of the patient. In general, the PCR results of the feces samples were reflected by the outcome of the real-time PCR on serum or plasma samples. A lumbar puncture was performed in eight of the patients and was used for routine virus culture and real-time TaqMan PCR analysis. Three of the CSF samples tested as enterovirus positive in both virus culture and real-time TaqMan PCR, whereas one additional sample was found to be positive in the real-time TaqMan PCR only. All enterovirus PCR-positive CSF samples were also positive in the feces and the serum or plasma samples (Table 4).

The second group of patients consisted of 43 adults with hematologic cancer with signs of pneumonia and radiographic pulmonary abnormalities and whose BAL samples had been stored. Using real-time TaqMan PCR enterovirus could be detected in two samples, whereas no enterovirus could be detected by traditional virus culture. Analysis of 17 NT swabs that had been obtained within 1 week of the BAL sample demonstrated one enterovirus-positive swab in the PCR and none in the virus culture. This positive sample was obtained from one of the two patients with an enterovirus-positive BAL sample; unfortunately, no NT swab was available from the other patient. Five patients demonstrated a rhinovirus infection, and all of these samples were found to be negative in the enterovirus real-time TaqMan PCR.

Clinical specimens obtained from patients without a suspicion of sepsis ($n = 20$) or pneumonia ($n = 30$) were all found to be enterovirus negative in the real-time TaqMan PCR.

DISCUSSION

We developed a rapid and sensitive technique for the routine detection of all enteroviruses in different clinical specimens.

Confirmation of a suspected enterovirus infection is important for patient management including, prognosis, reducing

TABLE 4. Comparison of real-time TaqMan PCR and virus culture on different clinical specimens

Patient group	Clinical specimen	No. of enterovirus-positive samples/ total no.	
		Virus culture	Real-time TaqMan PCR
Neonates with sepsis	Feces	9/41	10/41
	Serum or plasma	ND[a]	8/39
	CSF	3/8	4/8
Hematologic cancer patients with pneumonia	BAL fluid	0/43	2/43
	NT swab	0/17	1/17

[a] ND, not determined.

hospitalization, preventing outbreaks, and excluding other infectious diseases, and therefore prevents unnecessary (antibiotic) drug usage. Moreover, for adequate use of novel antiviral drugs targeted against the picornaviridae, such as pleconaril (21, 25, 27), rapid and accurate diagnostic testing is needed.

The novel real-time TaqMan PCR described here allows rapid and accurate diagnosis of all enteroviruses in different clinical specimens. In addition to being rapid, this assay also has the advantage of a standardized protocol that can easily be applied to other viruses, since the TaqMan PCR is performed under uniform conditions. In addition, the procedure is less complicated than other RT-PCR methods and the chances of laboratory contamination are minimized because there is no post-PCR processing of the samples.

Analysis of a reference panel with various concentrations of enteroviruses obtained from the EU-QCCA demonstrated that our enterovirus real-time TaqMan PCR is as sensitive as most of the molecular detection assays used by the different participants in the present study. Moreover, analysis of clinical isolates also demonstrated that our assay is more sensitive than the current gold standard approach: viral culture. In contrast to two real-time TaqMan PCRs described previously for the detection of enterovirus (10, 29), we used multiple primer and probe sets to ensure the detection of all different enteroviruses. Members of the other genera of the *Picornaviridae* family, such as hepatovirus and parechovirus, could not be detected. However, some cross-reactivity occurred with high-titer rhinovirus stocks but not with rhinovirus-positive clinical isolates.

Enterovirus spike experiments demonstrated that different clinical specimens, such as plasma, serum, CSF, NT swab, and BAL samples, can be used in our assay without apparent inhibition. We demonstrated in neonates with symptoms of sepsis that all enterovirus culture-positive feces samples were also found to be positive in the real-time TaqMan PCR. Moreover, plasma and serum samples obtained from the same patient during enterovirus infection were also found to be positive in the real-time PCR. Since this body compartment is easily accessible, an enterovirus real-time TaqMan PCR analysis of serum or plasma may be a good alternative for the enterovirus culture of feces, at least in neonates with sepsis.

Typing of enteroviruses is important to firmly establish an epidemiologic link among cases during an outbreak and to recognize serotype-specific clinical illness, such as poliomyelitis. To determine enterovirus serotype, virus culture is followed by a serum neutralization test, the gold standard for enterovirus typing. This method is generally reliable but also labor-intensive, time-consuming, and costly. Furthermore, the supply of antisera is limited and the problem of "untypeable" enteroviruses is frequently encountered (11). If the traditional virus culture for the identification of enteroviruses is replaced by a molecular approach by using the real-time TaqMan PCR, an alternative for the conventional enterovirus typing should be developed. A preliminary molecular approach for genotypic enterovirus typing has been described (14–16).

In conclusion, we have developed a rapid and sensitive real-time TaqMan PCR for the detection of all enteroviruses in different clinical specimens. Results can be obtained within a few hours, thus allowing time for adequate clinical management and evaluation of antiviral therapy.

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