

A sensitive and quantitative single-tube real-time reverse transcriptase-PCR for detection of enteroviral RNA

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Abstract

Background: Enteroviruses (EVs) are significant human pathogens. Rapid and sensitive diagnostic techniques are desirable. **Objectives:** To develop a quantitative single-tube real-time reverse transcription-polymerase chain reaction (RT-PCR) for human enterovirus ribonucleic acid (RNA) (QPCR), with protection against amplicon contamination. **Study design:** The method was evaluated with serial dilutions of EV, 62 cerebrospinal fluid (CSF) specimens from meningitis patients, and the third and fourth European Union Concerted Action Enterovirus Proficiency Panels. A commercial EV PCR test was run in parallel. **Results:** Optimisations included RNA extraction procedure, design and concentrations of primers and probes from the 5' non-coding region as well as recombinant *Thermus thermophilus* polymerase (*rTth*), Mn(OAc)₂ and thermolabile UNG concentrations. Of 62 CSF samples from cases of meningitis submitted for QPCR testing, 34 (76%) and 21 (47%) were positive by QPCR and a commercial EV RNA detection kit, respectively. The detection limit of QPCR was 0.001 TCID₅₀/ml (50% tissue culture-infective dose per millilitre) for a coxsackievirus B2 preparation and <10 copies of a plasmid containing coxsackievirus B2 complementary deoxyribonucleic acid (cDNA). The relation between threshold cycle (*C_t*) and amount of virus was linear ($r = 0.99$) over a range of 10⁻³ to 10⁴ TCID₅₀/ml of coxsackievirus B2. **Conclusions:** The QPCR method allows a large number of samples to be screened rapidly. Its sensitivity, simplicity, and reproducibility make it a suitable tool for the routine laboratory.

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Keywords: Enterovirus; *rTth*; Real-time fluorescence PCR

1. Introduction

The enteroviruses (EVs) are ribonucleic acid (RNA) viruses which belong to the *Picornaviridae* family. They are among the most common viruses that infect humans. They consist of more than 70 serotypes and include coxsackievirus A and B, echoviruses, polioviruses and enterovirus types 68–73. They are associated with a broad spectrum of disease. Enteroviral infection accounts for 10–15 million cases of symptomatic infection each year in the USA alone. This includes infections such as respiratory disease, gas-

trointestinal disease, acute myopericarditis and CNS disease (aseptic meningitis and, rarely, encephalitis). Poliomyelitis remains a threat in a few parts of the world. EV infections account for a substantial number of aseptic meningitis and encephalitis patients requiring hospitalisation in the summer and fall (Moore, 1982). More than 80% of all cases of aseptic meningitis world-wide are estimated to be caused by EV. Severe infections occur in children and neonates (Modlin, 1997). EVs are also suspected of being involved in the genesis of human diabetes (Jaeckel et al., 2002).

Traditionally, the diagnosis of EV disease is based on isolation of the virus in cell culture, averaging 6–7 days for identification in routine assay and possibly 14 days for a negative report. Although cell culture is a reliable diagnostic method (Chonmaitree et al., 1988), molecular methods such as polymerase chain reaction (PCR) developed during the last decade offer more sensitive, highly specific and rapid test results for the support of an EV diagnosis (Chapman et al., 1990; Halonen et al., 1995; Lina et al., 1996; Watkins et al., 2002).

Abbreviations: CSF, cerebrospinal fluid; *C_t*, threshold cycle; cDNA, complementary DNA; DNA, deoxyribonucleic acid; FAM, 6-carboxy-fluorescein; PCR, polymerase chain reaction; RNA, ribonucleic acid; RT-PCR, reverse transcriptase polymerase chain reaction; UTR, untranslated region; *rTth*, recombinant *Thermus thermophilus* polymerase; Mn(OAc)₂, manganese acetate; TCID₅₀, 50% tissue culture-infective dose; EV, enterovirus

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Primer sets directed to highly conserved sequences in the 5' untranslated region (5'UTR) of the EV genome enable broad detection of EV (Romero, 2002). Many of these assays require gel electrophoresis to detect the amplified product. This implies considerable hands-on time, is laborious and poses a hazard for amplification product carryover (Rotbart et al., 1990). This should be guarded against by the enzyme uracil deoxyribonucleic acid (DNA) glycosylase (UNG) for amplicon contamination control (Taggart et al., 2002). Further simplification and shortening of analysis time may have a strong impact on the diagnosis and clinical management of viral meningitis (Romero, 1999). Rapid diagnosis is important for appropriate patient management, especially as antivirals such as Pleconaril® are becoming available for EV infection (Rotbart and Webster, 2001). An early positive EV diagnosis can lead to the avoidance of inappropriate treatment of suspected bacterial meningitis or herpes encephalitis (Foray et al., 1999) as well as reducing the duration of hospitalisation (Nigrovic and Chiang, 2000).

The recombinant *Thermus thermophilus* polymerase (*τTth*) DNA polymerase has the ability to reverse transcribe RNA in the presence of manganese acetate (Mn(OAc)₂) at elevated temperatures (Myers and Gelfand, 1991; Hofmann-Lehmann et al., 2000). By using it, we have developed a simple quantitative single-tube real-time PCR (QPCR) that can be used to detect and quantify human EV RNA.

2. Materials and methods

2.1. Serial dilution of EV strains

EV stocks used in our study were obtained from the routine laboratory at the Clinical Virology Section of the Clinical Microbiology Laboratory of the Uppsala Academic Hospital, Sweden. Infectivity was assayed in microplates in serial tenfold dilutions of coxsackieviruses A9, A16, B2 and B5, and echovirus types 6, 11 and 30, from 10,000 to 0.001 TCID₅₀ (50% tissue culture-infective dose), with four wells per dilution. TCID₅₀ titers were calculated according to the Kärber method (Cary, 1999). Further details regarding the optimisation of the PCR reaction are given under “the optimised QPCR procedure”, below.

2.2. Patient samples

Patient's ages ranged between 15 and 77 years. A total of 62 cerebrospinal fluid (CSF) specimens were obtained from patients with meningitis visiting the Infectious Diseases Clinic at the Uppsala University Hospital between 1997 and 2001. Complete patient records were analysed retrospectively for clinical signs and additional diagnostic markers of meningitis. Meningitis was defined for patients with fever and headache and more than 5000 leukocytes/ml in CSF. Cases with high CSF leukocyte values and positive bacterial culture were recorded in a separate category.

2.3. Quality control panels

Specificity and sensitivity was tested on freeze-dried quality control samples of viral culture supernatants from different EV. The third and fourth European Union Concerted Action Enterovirus Proficiency Panel, which contained different concentrations of coxsackievirus A9, coxsackievirus B5, echovirus types 6 and 11, enterovirus 71 and coxsackievirus A16 was used (Table 3a and b). Samples were reconstituted by using 1 ml of nuclease-free water as stated in the package insert and either used immediately or kept frozen at 70 °C until analysis. The reconstituted quality control samples were extracted as described below (Section 2.5) and tested in duplicate with the EV QPCR assay.

2.4. A commercial enterovirus PCR test

The EnteroVision™ PCR detection kit (DNA Technology A/S, Aarhus, Denmark) was used as described by the manufacturer. Viral RNA was purified from culture supernatants by using the QIAamp viral RNA extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, followed by a one-step reverse transcription (RT)-PCR. The PCR uses biotinylated primers targeted to the (5'UTR) of the viral genome, resulting in a 154 bp PCR biotinylated fragment. The PCR fragment is then captured through hybridisation to probe covalently bound in microtiter wells. This is followed by the addition of horse radish peroxidase-streptavidin (HRP-streptavidin). TMB substrate for HRP is added and a signal is detected from enterovirus positive wells at 450 nm by using an ELISA reader. The assay takes 5–7 h, not including the isolation of RNA.

2.5. RNA extraction

RNA was initially extracted according to the instructions in the QIAamp viral RNA extraction kit (Qiagen, Hilden, Germany); a sample of 140 µl of CSF or viral culture supernatant was passed through the silica column. RNA was then recovered in 60 µl of nuclease-free water. To increase the RNA concentration in the PCR the sample volume was increased to 280 µl of CSF. RNA was recovered in 70 µl of nuclease-free water. It was used immediately or stored at –70 °C. The RNA yield was then approximately doubled, doubling the sensitivity of the entire method (extraction and PCR). In both the original and the modified extraction procedure, 14 µl of RNA extract was added to the PCR reaction mix.

2.6. Real-time PCR system

Real-time PCR in the TaqMan® format is based on the incorporation of a dual-labelled fluorogenic TaqMan® probe into the PCR reaction. The probe is designed to anneal to the target sequence between the positions of the forward and reverse primer. Attached to the 5' end of the probe is a



Fig. 1. Alignment of nucleotides 330–482 of the 5' untranslated region of 23 human enterovirus sequences. The numbering is derived from the Coxsackie B2 genome sequence (Genbank accession no. AJ295199). The on-line software MultAlin (<http://www.prodes.toulouse.inra.fr/multalin/multalin.html>) was used.

fluorescent reporter dye and at the 3' end there is a quencher dye. The quencher has the ability to absorb the fluorescence emitted by the reporter. In this study RT and PCR were carried out in the same buffer mixture, *rTth* DNA polymerase acts as both a reverse transcriptase and as DNA polymerase in our single buffer system (TaqMan[®] one-step RT-PCR master mix reagents kit). The One-Step EZ RT-PCR TaqMan[®] reagent kit (Applied Biosystems, Sweden) was adapted for use with heat labile Uracil-*N*-Glycosylase (HKTM UNG; Cat. No. HU59100; Epicentre Technologies Corporation, Madison, WI) in a one tube format. Reaction mixtures containing viral RNA samples were first incubated at 50 °C for 2 min (heat labile UNG contamination protection), incubated at 70 °C for 10 min to activate *rTth* DNA polymerase and deactivate UNG, and then at 60 °C for 30 min to allow the downstream primer to anneal to the target RNA and initiate complementary DNA (cDNA) synthesis. The relatively high RT temperature 60 °C destabilises secondary structure associated with the EVs 5'UTR. The real-time quantitative PCR analysis was performed using the Corbett Research Rotor-Gene 2000 Real-time Amplification system (Corbett Research, Mortlake, NSW, Australia). The Rotor-Gene software version 4.4 was used for threshold selection and standard curve interpolation to derive RNA concentrations relative to the RNA standard.

2.7. Primers and probe for QPCR

Initially, the inner primer pair from a published method (Monpoeho et al., 2000) was used. However, we wanted to increase the sensitivity in our *rTth* system. We then modified these primers with the aim of: (i) having a similar *T_m* of both forward and reverse primers; (ii) minimising primer–primer and primer–probe interactions; (iii) while still operating within conservation restraints in the 5'UTR (Fig. 1). The above mentioned published primer pair has a *T_m* of 68 and 57 °C for forward and reverse primers, respectively. After redesign, the primers NMF1 and NMR1 have a *T_m* of 64 and 58 °C, respectively. The original probe was retained unmodified. Using a tenfold dilution series of coxsackievirus

B2, the redesigned primers gave a sensitivity increase of around tenfold over the published ones (data not shown).

The final TaqMan[®] system consisted of two primers; NMF1 (forward), 5'GCCCTGAATGCGGC-3' (positions 334–347 in the coxsackieviruses B2 sequence, GenBank accession number AJ295199), and NMR1 (reverse) 5'-AA-TTGTCACCATAAGCAGC-3' (positions 464–482). The dual-labelled fluorescent TaqMan[®] probe, here referred to as MP was; 5' (FAM)CGGAACCGACTACTTTGGGTGT-CCGT(DARK)3' (positions 416–441). It had the reporter FAM (6-carboxyfluorescein) and the “dark” (DABCYL) [4-((4-(dimethylamino) phenyl) azo) benzoic acid ester] quencher dyes attached to the 5' end and 3' ends, respectively. All were synthesized at SGS (Scandinavian Gene Synthesis AB, Köping, Sweden). The programs Oligo Analyzer, Primer3 (Rozen and Skaletsky, 1998) and Primer Express 1.0 (Applied Biosystems, Foster City, CA, USA), were used.

2.8. Carryover contamination containment

HKTM UNG hydrolyses the N-glycosidic bond between the deoxyribose sugar and uracil in amplimers which contain deoxyuridine in place of thymidine. The enzyme is fully active at 37–50 °C and is inactivated by 10 min incubation at 65 °C or higher. Different amounts of HKTM UNG, 0.01, 0.05, 0.1, 0.2, 0.5 and 1 units per PCR reaction, were tested in the temperature scheme presented under “optimised QPCR procedure”. Up to 0.1 units per reaction did not have any adverse effect on the PCR. Higher amounts gave a slight inhibition (data not shown). We therefore settled for 0.1 unit of HKTM UNG (and diethylpyrocarbonate treated nuclease-free H₂O) to diminish the risk of PCR carryover contamination. The negative control (water) was negative in all QPCR reactions.

2.9. The optimised QPCR procedure

The method (QPCR) uses the *rTth* polymerase in its RNA (reverse transcriptase) and DNA dependent DNA

Table 1
Optimisation of *rTth* DNA polymerase and Mn(OAc)₂ concentrations with RNA prepared from 0.01 TCID₅₀/ml of Coxsackievirus B2

<i>rTth</i> concentration (units/PCR reaction)	C _t out of 55 cycles		
	Mn(OAc) ₂ 2 mM	Mn(OAc) ₂ 3 mM	Mn(OAc) ₂ 4 mM
	5	0	0
7.5	0	0	45.7
10	0	43.1	36.6

polymerase modes. Different concentrations of each primer (100–900 μ M for [NMF1 and NMR1] and 100–300 μ M for the fluorogenic probe) were tested systematically. Final concentrations of 500 and 400 μ M for primers NMF1 and NMR1, respectively, and 100 μ M of the fluorogenic probe MP, gave an optimal sensitivity with EV RNA. A further increase in sensitivity of the assay was observed after increasing the Mn(OAc)₂ and *rTth* DNA polymerase concentrations (Table 1). An example is coxsackievirus B2 which at a dilution of 10⁻⁸ was not detected at the previous conditions (at the lowest concentration of *rTth* and Mn(OAc)₂ shown in Table 1). When we increased the concentrations of *rTth* up to 10 units and Mn(OAc)₂ to 4 mM (Auer et al., 1995), we detected virus at this dilution.

The final procedure was as follows; 14 μ l of RNA extract was added to the reaction mix, resulting in a final concentration of 500 nM forward primer NMF1, 400 nM reverse primer NMR1, 100 nM dual-labelled fluorogenic probe MP, 10 μ l of 5 \times EZ TaqMan[®] buffer; 4 mM Mn(OAc)₂; 400 μ M dATP, dCTP, dGTP and 800 μ M dUTP; 10 units of *rTth* DNA polymerase and 0.1 unit of HKTM UNG. Samples (clinical and quality control panels) were assayed in duplicate reactions in a total volume of 50 μ l. They were first incubated at 50 °C for 2 min (heat labile UNG contamination protection) and then at 70 °C for 10 min to activate *rTth* DNA polymerase and deactivate UNG. The RT step was performed at 60 °C for 30 min, immediately followed by an initial denaturation at 95 °C for 5 min. A total of 55 cycles were performed, each consisting of a denaturation step at 95 °C for 15 s and a combined annealing-extension step at 59 °C for 1 min. Fluorescence data were collected during each annealing-extension step. Negative and positive PCR run controls were included in each analytical round. Negative controls were RNase free water.

2.10. Preparation of a DNA standard from coxsackievirus B2

Directly following visualization of the PCR products on a 2% agarose gel, 20 μ l of the amplicon was purified using a QIAquick PCR purification kit (Qiagen, Santa Clara, CA) and resuspended in 30 μ l of water. Four microliters of the purified amplicon was then immediately used for cloning with the PCR 2.1 vector following instructions in the TOPO TA

Table 2
Comparison of threshold cycle value with tissue culture infectious dose

	Virus titrations	
	TCID ₅₀	C _t
Coxsackievirus B2	0.001	36.6
Coxsackievirus B5	0.001	33.2
Coxsackievirus A16	0.01	32.8
Coxsackievirus A9	1	45.7
Echovirus 11	1	30.9
Echovirus 6	10	31.1
Echovirus 30	0.1	38.0

The lowest positive dose is shown.

cloning kit (Invitrogen, Carlsbad, CA). Approximately, 3–10 white colonies per dish were picked and cultured 14–16 h in Luria-Bertani (LB) medium with yeast-tryptone (YT) containing 50 μ g/ml ampicillin. Plasmids were purified using the QIAprep[®] Minipreps plasmid DNA purification system, eluted in 50 μ l of water and quantified by spectrophotometric analysis using DyNa QuantTM 200 (Amersham Biosciences, Uppsala, Sweden). One plasmid was selected as a standard.

3. Results

3.1. Limit of detection, linearity and reproducibility

Coxsackievirus B2 was diluted 10-fold serially. The lowest detectable dilution factor of 10 corresponded to a threshold cycle C_t of 10.8, while the highest detectable dilution of 10⁻² TCID₅₀ corresponded to a C_t of 36.6. The minimum detectable amount of coxsackievirus B2 plasmid DNA was two and five molecules per PCR reaction in two determinations. Reproducibility was tested by analysing a positive sample repeated at least five times on different days. The mean was 364,804 RNA equivalents per reaction, with an S.D. of 19,457 (5.3%).

RNA extracts, prepared as described, from serial ten-fold dilutions (10⁴ to 10⁻² TCID₅₀/ml) of viral culture supernatants of coxsackieviruses A16, A9, B2 and B5, echovirus types 6, 11 and 30 were analysed in QPCR. The highest positive dilution of coxsackievirus A16 1 \times 10⁻⁸ (0.01 TCID₅₀/ml) corresponded to a C_t of 32.8, while the lowest dilution, 10,000 TCID₅₀/ml, corresponded to a C_t of 12.5 of the same virus. Each of the titrated viruses represents one member of a major EV group (Table 2). The minimum detectable amount of enteroviral RNA was equivalent to 0.001 TCID₅₀ of coxsackievirus B2, B5 and 0.01 TCID₅₀ of coxsackievirus A16. The data generated a log-linear regression plot that showed a strong linear relationship ($r^2 > 0.99$) between the log₁₀ of the starting copy number and the C_t values (Fig. 2b). The dynamic range was seven orders of magnitude, since the assay could discriminate template concentrations between 10⁴ and 10⁻³ TCID₅₀ equivalents in a single reaction.

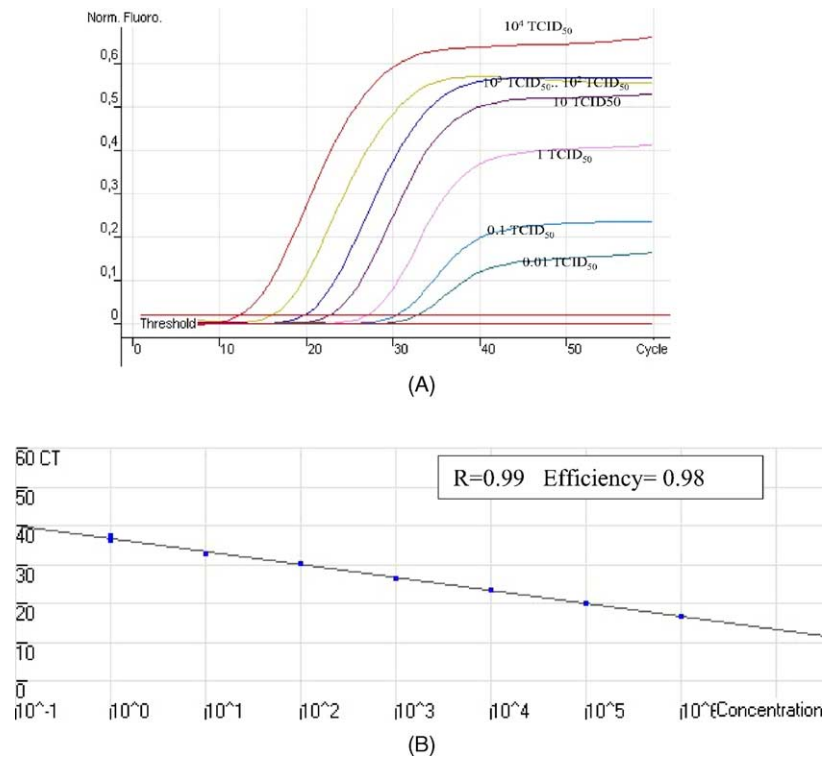


Fig. 2. (a) Amplification curves of QPCR for Coxsackie B2 (10^4 – 0.01 TCID₅₀/0.1 ml). Relative fluorescence units are plotted against cycle number (C_t). (b) Standard Curve of Coxsackie B2 (10^4 – 0.01 TCID₅₀/0.1 ml). Threshold cycle is plotted against TCID₅₀ of Coxsackie B2. The C_t value is the cycle number at which a positive amplification reaction was measured; the straight line is the regression line.

3.2. Sensitivity and specificity studied with quality control panels

EV serotype reactivity was tested to confirm that NMF and NMR primers and MP probe recognise various EV serotypes. The ability to amplify a range of EV was further tested by participating in the international Third (delivered in 2001; 11 samples) and Fourth (delivered in 2002; 12 samples) 'European Union Quality Control Concerted Action (EU-QCCA) EV proficiency panel'. In each panel, the reconstituted samples were processed identically to clinical CSF specimens (see RNA preparation). The positive samples at the panels (third and fourth) contained coxsackieviruses A9, A16, B5 and B6, echovirus types 6, 11 and 30 and enterovirus type 71. The C_t values from QPCR are shown in (Table 3a and b). In the third and fourth panels, all reconstituted samples containing EV were found to be positive by QPCR.

3.3. Comparison of QPCR with a commercial EV RNA detection kit

From a total of 62 CSF specimens of patients diagnosed as having meningitis, 34 were found to be positive when tested with the QPCR assay. Twenty one out of the 62 (Table 4) were positive in both QPCR and the EnteroVision™ PCR detection kit, whereas 13 samples was found to be positive

in the QPCR only. Twenty eight out of the 62 were negative in both assays. All samples were tested in duplicate with the same results; samples were tested without knowledge of clinical data.

3.4. Comparison of results with clinical data

According to the final clinical analysis of the 62 meningitis patients, 45 were classified as non-herpetic non-tick borne encephalitis (non-TBE) aseptic meningitides, 6 were diagnosed as herpes simplex type 2 meningitis infections, one was diagnosed as TBE, 7 were found to be bacterial meningitis, and 3 were diagnosed as "other diseases". The latter 17 samples are referred to as "non enteroviral meningitis cases", assuming that double infection with enterovirus and other agents is rare.

QPCR detected 34 out of the 45 non-bacterial, non-herpetic and non-TBE cases while the EnteroVision™ PCR detection kit detected 21 out of the 45 (Table 4). None of the 17 samples from non-enteroviral meningitis cases were positive in QPCR.

4. Discussion

The QPCR presented here is a modification of several previous EV PCR techniques (Monpoeho et al., 2002; Rabenau

Table 3
Result with EV PCR quality control panels

QC panel no.	Serotype	TCID ₅₀ /ml	C _t
(a) Third EUQCCA EV panel (2001)			
EV-C01	Coxsackievirus A9	0.036	32.2
EV-C02	Coxsackievirus A9	0.36	33.1
EV-C03	Coxsackievirus A9	3.6	29.2
EV-C04	No virus	0	0
EV-C05	Echovirus 6	20	29.5
EV-C06	Echovirus 11	25.2	31.8
EV-C07	Coxsackievirus B5	317	30.1
EV-C08	Enterovirus 71	56.4	29.9
EV-C09	Echovirus 11	25.2	28.6
EV-C010	No virus	0	0
EV-C011	Echovirus 11	252	32.9
QC panel no.	Serotype	Dilution	C _t
(b) Fourth EUQCCA EV panel (2002)			
EV-D01	Echovirus 11	1 × 10 ⁻⁶	30.5
EV-D02	Echovirus 11	1 × 10 ⁻⁵	27.3
EV-D03	Coxsackievirus A9	1 × 10 ⁻⁵	27.1
EV-D04	Negative	0	0
EV-D05	Coxsackievirus A9	1 × 10 ⁻⁷	34.4
EV-D06	Echovirus 11	1 × 10 ⁻³	27.7
EV-D07	Coxsackievirus A16	1 × 10 ⁻⁶	33.8
EV-D08	Coxsackievirus A9	1 × 10 ⁻⁷	38.3
EV-D09	Coxsackievirus A9	1 × 10 ⁻⁸	38.1
EV-D10	Negative	0	0
EV-D11	Coxsackievirus B5	1 × 10 ⁻⁶	31.4
EV-D12	Coxsackievirus A9	1 × 10 ⁻⁶	34.6

The TCID₅₀/ml of the original virus stock was 3.6 × 10⁶ for Coxsackievirus A9, 3.2 × 10⁷ for Coxsackievirus B5, 2.0 × 10⁸ for echovirus 6, 2.5 × 10⁷ for echovirus 11, and 5.6 × 10⁶ for enterovirus 71. C_t: threshold cycle.

et al., 2002; Rotbart, 1997; Verstrepen et al., 2001; Zoll et al., 1992). However, it is unique in that it can be performed in a single closed tube. No post-amplification steps are required and the calculation of the initial amount of starting material is performed automatically by the ThermoCycler software. The high temperature for reverse transcription 60 °C allows the use of (HKTM UNG) thermolabile uracil N-glycosylase for carryover prevention (Taggart et al., 2002). This is rational and minimises the risk of carry-over contamination. The lowest RNA concentration we detected was at 0.001 TCID₅₀ of coxsackievirus B2. In the same experiments, the same C_t values were obtained at concentrations of two to five molecules of coxsackievirus B2 plasmid DNA. Therefore, it is likely that the lower limit of enteroviral nucleic acid detection for the QPCR is in the neighbourhood of a few molecules.

Table 4
Comparison of the results of the QPCR and a commercial EV RNA detection kit in CSF specimens from meningitis cases

Method	Positive/total CSF	Negative/total CSF	Meningitis due to Herpes, TBE or bacteria	Remaining aseptic meningitides
QPCR	34/62	28/62	0/14	34/45 (76%)
Commercial EV RNA detection kit	21/62	41/62	0/14	21/45 (47%)

According to patient records, six patients were diagnosed as herpes simplex infection, seven were positive in bacterial culture; one case was tick borne encephalitis (TBE). All enterovirus PCR positive cases were in the remaining 45 patients.

In 62 CSF samples from cases initially diagnosed as meningitis and later re-evaluated, 45 were from cases of non-bacterial, non-herpetic and non-TBE origin. QPCR detected EV RNA in 76% of these cases while the EIA-based technique used for comparison detected 47%. This shows that QPCR is a sensitive technique, and confirms that EVs are major causes of aseptic meningitis. High sensitivity is important in establishing a diagnosis in patients with neurological disorders such as aseptic meningitis where only a few copies of virus may be present in CSF. The negative outcome of QPCR in the remaining 11 patients out of 45 cases may be explained by: (i) suboptimal time of sampling, (ii) that inadvertent freezing and re-thawing might have exposed the RNA in enteroviral particles to RNase degradation, and (iii) that some cases might be caused by unknown microbes or microbes not tested for.

Analysis of reference panels with various concentrations of EVs obtained from the EU-QCCA, demonstrated that our EV QPCR is as sensitive as most of the molecular detection assays used by other European laboratories, and that it can detect RNA from a broad variety of EVs. During the development we noted that *rTth* polymerase activity decreased during the long RT and PCR procedure. This was suggested by the amplification curves, which gave successively lower plateaus at higher cycle numbers. By increasing both polymerase and manganese concentration it was found that the amplification could go on unhindered even late in the process.

Meningitis is a common infection that often requires hospitalisation and antibiotic therapy. However, the majority of the cases are caused by viral rather than bacterial pathogens. Hence, the management of patients suspected of having meningitis is associated with significant health care interventions that could be eliminated with a rapid, definitive diagnostic tool for EV meningitis. Previously, the diagnosis of EV meningitis required the isolation of the virus in cell culture. Viral culture is a methodological standard for diagnosis of EV meningitis. Unfortunately, the sensitivity of culture is not always enough, as many serotypes grow relatively poorly in culture. Results take several days to weeks (Rotbart et al., 1994). A rapid diagnostic test will therefore have a strong impact on the diagnosis and clinical management of viral meningitis (Romero, 1999). Several authors have developed reverse transcription-PCR (RT-PCR) assays as a more convenient alternative to viral culture (Monpoeho et al., 2002; Verstrepen et al., 2001).

Confirmation of a suspected EV infection is important for patient management including prognosis, reducing hospitalisation, preventing nosocomial outbreaks, and exclusion of other infectious diseases. An EV diagnosis also prevents unnecessary antibiotic drugs. Clinically, it is difficult to differentiate between viral or bacterial meningitis on admission of the patient. Symptoms can be misleading.

In summary, the QPCR described here can be carried out in 4 h, including the RNA preparation step. A large number of samples can therefore be screened rapidly, and its sensitivity, simplicity, and reproducibility make it a suitable tool for the routine laboratory. This allows time for adequate clinical management and evaluation of antiviral therapy. The QPCR will also be a suitable tool for the study of chronic diseases associated with enteroviral infections.

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