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Quantitative real-time RT-PCR assay for research studies on enterovirus infections in the central nervous system

Romain Volle[a,b,c], Céline Nourrisson[b], Audrey Mirand[a,b,c], Christel Regagnon[b,c], Martine Chambon[a,b,c], Cécile Henquell[b,c], Jean-Luc Bailly[a,c], Hélène Peigue-Lafeuille[a,b,c], Christine Archimbaud[a,b,c,*]

[a] Clermont Université, Université d'Auvergne, EA4843, BP 10448, F-63000 Clermont-Ferrand, France
[b] Centre Hospitalier Universitaire de Clermont-Ferrand, Service de Virologie, F-63003 Clermont-Ferrand, France
[c] Centre National de Référence des Entérovirus et Parechovirus, laboratoire associé: CHU Clermont-Ferrand, Service de Virologie, F-63003 Clermont-Ferrand, France

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ABSTRACT

Human enteroviruses are the most frequent cause of aseptic meningitis and are involved in other neurological infections. Qualitative detection of enterovirus genomes in cerebrospinal fluid is a prerequisite in diagnosing neurological diseases. The pathogenesis of these infections is not well understood and research in this domain would benefit from the availability of a quantitative technique to determine viral load in clinical specimens. This study describes the development of a real-time RT-qPCR assay using hydrolysis TaqMan probe and a competitive RNA internal control. The assay has high specificity and can be used for a large sample of distinct enterovirus strains and serotypes. The reproducible limit of detection was estimated at 1875 copies/ml of quantitative standards composed of RNA transcripts obtained from a cloned echovirus 30 genome. Technical performance was unaffected by the introduction of a competitive RNA internal control before RNA extraction. The mean enterovirus RNA concentration in an evaluation series of 15 archived cerebrospinal fluid specimens was determined at 4.78 $\log_{10}$ copies/ml for the overall sample. The sensitivity and reproducibility of the real time RT-qPCR assay used in combination with the internal control to monitor the overall specimen process make it a valuable tool with applied research into enterovirus infections.

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1. Introduction

Human enteroviruses (EVs) are members of the *Picornaviridae* family, which includes small non enveloped RNA viruses. With more than 100 serotypes, EV strains are classified into four species designated from HEV-A to HEV-D, and the reference poliovirus serotypes are included in the HEV-C species (Stanway et al., 2005). EVs are associated with a number of common illnesses such as hand foot and mouth disease (HFMD), myocarditis and acute hemorrhagic conjunctivitis, but the most vexing clinical syndromes caused by EVs are seen in patients with neurological infections, which result in an inflammatory reaction. The inflammatory process initiated by strains of the different EV serotypes can affect the subarachnoid space and lead to a meningitis syndrome, brain parenchyma (encephalitis), or both (meningoencephalitis) (Archimbaud et al., 2003; Rhoades et al., 2011; Robinson et al., 2002; Tauber and Moser, 1999). Encephalitis is a well-recognized but infrequent manifestation of central nervous system (CNS) infection by EVs (Tunkel et al., 2008). The most common neurological syndrome caused by EV infections is aseptic meningitis, which requires surveillance in both children and adults (Huraux et al., 2003; Peigue-Lafeuille et al., 2002; Ramers et al., 2000). EV meningitis occurs in sporadic forms and outbreaks of variable size, from local community outbreaks to large regional epidemics (Bailly et al., 2002; Khetsuriani et al., 2006).

The advent of molecular diagnosis over the last decade has allowed the sensitive, specific and rapid detection of EV genomes in cerebrospinal fluid (CSF) specimens. Previous studies suggested that the use of rapid molecular diagnosis of EV in children with meningitis has a clinical benefit by reducing antibiotic or antiviral

Abbreviations: EV, enterovirus; IC, internal control; EV-RNA QS, enterovirus-RNA quantitative Standards; RT-qPCR, reverse transcriptase quantitative polymerase chain reaction; Ct, threshold cycle; CSF, cerebrospinal fluid; EU-QCMD, European Union Quality Control for Molecular Diagnostics Proficiency Panel.

* Corresponding author at: Centre Hospitalier Universitaire de Clermont-Ferrand, Service de Virologie, F-63003 Clermont-Ferrand, France. Tel.: +33 4 73 75 48 50; fax: +33 4 73 75 48 51.

E-mail addresses: romain.volle@u-clermont1.fr (R. Volle), celine.nourrisson@laposte.net (C. Nourrisson), amirand@chu-clermontferrand.fr (A. Mirand), cregagnon@chu-clermontferrand.fr (C. Regagnon), m_chambon@chu-clermontferrand.fr (M. Chambon), chenquell@chu-clermontferrand.fr (C. Henquell), J-Luc.BAILLY@u-clermont1.fr (J.-L. Bailly), hlafeuille@chu-clermontferrand.fr (H. Peigue-Lafeuille), carchimbaud@chu-clermontferrand.fr (C. Archimbaud).

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therapy, avoiding ancillary tests, reducing hospital related costs, and allowing earlier discharge (Archimbaud et al., 2009; Hamilton et al., 1999; Ramers et al., 2000; Robinson et al., 2002; Stellrecht et al., 2002). As several serotypes are involved in meningitis, the laboratory of virology of Clermont-Ferrand Hospital has also developed genotyping techniques to rapidly identify EV strains in biological samples (Bailly et al., 2002; Mirand et al., 2006). The feasibility of direct genotyping in CSF was shown in a large prospective study of patients with meningitis, which provided a more complete picture for the circulation patterns of EV strains than genotyping of virus strains after isolation in cell culture (Mirand et al., 2008).

Although qualitative molecular detection of EV in CSF is sufficient for diagnosis, a quantitative approach will be a valuable tool with research applications in the investigation of pathological processes during EV infections. Specifically, the determination of EV load in the CSF of patients with neurological manifestations would prove helpful in elucidating further the history of diseases caused by these infections. The quantitative genome detection of other viral pathogens in CSF specimens has been reported in patients with neurological disease and showed a clear association between a high viral load and the severity of neurological symptoms caused by Varicella-Zoster or Epstein-Barr Viruses (Aberle et al., 2005; Weinberg et al., 2002). With regard to EVs, one study in neonatal systemic infection showed a strong association of higher viral load in the blood with younger age and greater disease severity of neonatal Coxsackievirus B3 infection (Yen et al., 2007). However, few molecular amplification assays have been developed and used for the quantitation of EVs in CSF specimens (Monpoeho et al., 2002; Dalwai et al., 2009). The aim of this study was to upgrade a qualitative real-time RT-PCR described previously (Archimbaud et al., 2004) to a quantitative assay, including TaqMan hydrolysis probes, a competitive internal control to assess the quality of the analysis and quantitative RNA standards to assess EV loads in patients' CSF.

2. Material and methods

2.1. Enterovirus strains and cerebrospinal fluid specimens

The EV strains used in the study comprised 23 reference strains and 60 clinical strains that were originally isolated in cell cultures from biological specimens of CSF, throat, or stools at the virology laboratory of the University Hospital of Clermont-Ferrand. The virus samples were composed of one strain of each of the following serotypes: coxsackievirus (C) CA4, CA8, CA9, CA13, CA15, CA16, CB4, echovirus (E) E1, E9, E11, E15, E16, E17, E19, E20, E21, E24, E26, E27, E29, E31, E32, E33, EV69, EV70, EV71, and EV77; two strains of CB1, CB2, CB6, E2, E3, E5, E7, and E14; three strains of CB3, CB5, E4, E18, E25; seven strains of E13; and nine strains of E6 and E30. The E25 prototype strain (E25/JV-4) titrated at 1.3×10^7 tissue culture infective dose 50% ($TCID_{50}$) per ml was serially diluted 10-fold in sterile water and used to evaluate internal control competition with EV template, and inter- and intra-assay variability.

Samples from the 2010 and 2011 European Union Quality Control for Molecular Diagnostics Proficiency Panel for enterovirus (EU-QCMD; Glasgow, UK) were also used in the study. They contained different amounts of various EV serotypes (CA9, CA16, CA21, CA24, CB3, E11, E30, EV71, and EV68). Three samples contained human parechovirus HPeV-3, and one sample human rhinovirus HRV-16.

Thirty nine CSF specimens collected from patients hospitalized for suspicion of aseptic meningitis were obtained from the collection of the laboratory. All the CSF specimens were conserved at −80 °C until analysis. The CSF samples comprised 19 specimens tested positive for EV by nucleic acid sequence-based amplification (NASBA) with the NucliSens EasyQ® Enterovirus kit (bioMérieux, Marcy l'Etoile, France) and 20 tested negative for EV with the same technique. EV genotyping in CSF specimens was done by nucleotide sequencing of gene 1D (the viral gene encoding the VP1 capsid protein) as described previously (Mirand et al., 2008).

2.2. Molecular design of primers and TaqMan probes

Real-time RT-qPCR primers EV1 (forward) and EV2 (reverse) are described elsewhere (Archimbaud et al., 2009, 2004). TaqMan hydrolysis probes EVPb (EV RNA template) and ICPb (internal control RNA template), and 5′ overhanging end primers EV1pBR and EV2pBR (see Section 2.3), were designed using Primer 3 Input program (Rozen and Skaletsky, 2000). The specificity of all primers and probes was validated by BLAST analyses (Altschul et al., 1990). TaqMan probes (EVPb and ICPb) were respectively 5′-labelled with the fluorescent dye 6-carboxy-fluorescein (FAM) and dimethoxy-fluorescein (JOE) and respectively 3′-labelled with the quenching dye carboxytetramethylrhodamine (Tamra) and black hole quencher 1 (BHQ1). Sequences are given in Table 1.

2.3. Internal control construction

Internal control (IC) is an RNA chimera synthesized using a previously described method with minor modifications (Naze et al., 2009). Briefly, EV1pBR and EV2pBR primers were used to amplify a 217-bp segment chosen in the plasmid vector pBR322. Primer EV1pBR contained in 5′- to -3′order a *Sal*I restriction endonuclease site, the sequence of the EV1 primer, and 20 bp homologous to bp 1655–1675 in pBR322. Primer EV2pBR contained in 5′- to -3′order a *Bam*HI site, the sequence of the EV2 primer and 20 bp homologous to bp 1795–1815 in pBR322. Amplification conditions were: 2 min at 94 °C followed by 40 cycles with 15 s at 95 °C, 50 s at 60 °C and 30 s at 72 °C and a final elongation step 5 min at 72 °C. The resulting hybrid PCR product (IC DNA fragment) was purified with NucleoSpin® extract II kit (Macherey-Nagel) and cloned (*Sal*I/*Bam*HI) into the T7 expression vector p0p24 (Michel et al., 2000). The sequence was checked by nucleotide sequencing. The *Eco*RI linearized recombinant p0p24 plasmid DNA was used as a template for *in vitro* transcription with RiboMAX™ Large Scale RNA production System-T7 kit (Promega, Mannheim, Germany). Transcripts were treated with DNase I, purified with NucleoSpin® RNA Virus kit (Macherey-Nagel), and quantified by fluorimetry using the Quant-iT™ Assays kit (Invitrogen). Internal control RNAs were then serially diluted 10-fold in RNase-free water from 2×10^5 to 2 copies/μl, and stored in single-use aliquots at −80 °C until use. Copy number of transcripts (cp) was calculated by using the equation: $cp = (C/M) \times N$, where C is the fluorimetry measured mass concentration, M is the molecular weight calculated for the transcripts (372,570 g mol^{-1}) and N is the Avogadro's number (6.02×10^{23} mol^{-1}).

2.4. Preparation of the EV RNA quantitation standard

A synthetic EV RNA quantitation standard (EV-RNA QS) was constructed by cloning 576 nucleotides (nucleotide positions 66–642) from the highly conserved 5′ untranslated EV region of the prototype echovirus 30 strain (Bastiani) into the T7 expression vector p0p24. An *in vitro* transcription reaction was performed following the procedure described above for IC construction. Concentration of EV-RNA QS was obtained using Quant-iT™ Assays kit (Invitrogen). In order to obtain a standard curve for the EV RT-qPCR assay, the EV-RNA QS was serially diluted 10-fold in RNase-free water from 6×10^5 to 6 copies/μl and stored in single-use aliquots at −80 °C. Copy number of the transcripts was calculated using the above equation ($M = 501,270$ g mol^{-1}).

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