Abstract A quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) method based on TaqMan technology was developed to determine the presence and amount of enterovirus RNA. In order to prevent false-negative results, a one-step multiplex RT-PCR was optimized. It contains two dual-labelled fluorogenic probes to quantify the 5’ noncoding region of enterovirus and detect an internal positive control. In the present study, 104 cerebrospinal fluid samples collected during an outbreak of enteroviral meningitis were analyzed using this method. Amplification of the internal positive control was effective in all but two specimens, confirming the absence of PCR inhibitors and allowing the results of amplification to be validated. The sensitivity of the RT-PCR was 96.8%, while that of cell culture was 34.9%. Genomic viral loads found ranged between 3.3 and 5.9 log_{10} copies per milliliter of cerebrospinal fluid (mean, 4.8 log_{10} copies/ml). This fluorogenic enterovirus RT-PCR allows large numbers of samples to be screened rapidly. Moreover, its sensitivity and reproducibility make it highly reliable. With these characteristics, the enterovirus RT-PCR can be a useful tool that may offer considerable benefit in the clinical management of patients with enteroviral infections.

Introduction

Human enteroviruses (EVs) of the Picornaviridae family are the most common etiologic agents of aseptic meningitis. It is estimated that they cause 70–90% of the cases of viral meningitis [1, 2]. Conventional laboratory diagnosis of EV meningitis relies on isolation of the virus in cell culture with several cell lines. Observation of cell cultures for the development of a cytopathic effect continues for about 15 days and is then followed by neutralization typing. Besides being time-consuming, this method is frequently unsuccessful because of the small volume of samples such as cerebrospinal fluid (CSF) and the difficulty of propagating certain EV types in cell culture [3]. As an alternative method, therefore, the use of molecular biology for the detection of EV genomes is of considerable interest.

Detection of EVs by amplification of viral RNA from CSF using reverse transcriptase-polymerase chain reaction (RT-PCR) assay has already been reported [4, 5, 6]. Some studies have described the amplification of EV RNA (by single or nested RT-PCR), followed by a variety of endpoint detection or quantification systems such as the colorimetric microwell hybridization assay [7, 8, 9, 10, 11]. This last method is also time-consuming because of the post-PCR manipulations required, and its results are subject to error due to the plateauing effect [12]. Finally, substances present in the specimen can inhibit nucleic acid amplification and thus cause false-negative responses [13, 14].

A new quantification method combining RT-PCR with a real-time detection system (ABI Prim 7700; PE Applied Biosystems, France) has recently been developed in our laboratory [15]. Up to now, false-negative responses have been averted by “spiking” all negative specimens with the RNA standard for a second run, an impractical, laborious, and very expensive solution. In addition, the low amount of CSF often available limits the ability to perform retesting. We therefore decided to improve our technique by incorporating an internal positive control (IPC) by which amplification and quantification results could be validated in a single run. The aim of the present study was to apply this technique by measuring the EV genomic viral load in CSF collected during an outbreak of aseptic meningitis.
Materials and Methods

Patients and Cerebrospinal Fluid Specimens

Between May and August 2000, an outbreak of aseptic meningitis occurred in Nantes, France. A total of 104 CSF specimens from young patients aged 6 days to 20.3 years (median, 5.2 years) admitted to the pediatric emergency department for neurological symptoms were collected in sterile containers and sent to our laboratory for diagnostic evaluation. CSF samples were stored at −20°C before being analyzed.

Cell Culture and Typing

CSF samples were cultured directly on two different cell lines (MRC-5 and Hep-2). Each type was inoculated with 100 µl of specimen and then incubated at 37°C. Cultures were observed daily for cytopathic effect characteristic of EVs for 21 days. Cultures showing a cytopathic effect were subpassaged, and, if positive, the virus was typed by neutralization with the Lim-Benyesh-Melnick equine antisera pools [16].

Extraction of Viral RNA

Enterovirus RNA was extracted from 140 µl of CSF using the Qiaamp viral RNA kit (Qiagen, France) according to the manufacturer’s instructions. EV RNA was eluted with 50 µl of sterile water.

Primers and Probes for Amplification

Primers and probes used for amplification of EVs were as already published: Ev1 [5′-GATTGTCACCACTAAGCAGC-3′], EV2 [5′-CCCTGTAATTGCGGCTAATC-3′] and Ev probe [5′-FAM-CGGAACCAGACTATTGGGTGTTCCGT-TAMRA-Phosphor-3′] [13].

For absolute quantification, an EV RNA standard representing the 5′-noncoding region of EV RNA was synthesized in vitro by plasmid cloning and in vitro transcription using complementary DNA from Mahoney type 1 poliovirus and primers Ev1Clon 5′-AAATTCGGAGGATCCGGC-3′ and Ev2Clon 5′-CTACATA-GGAACCACCTCGGCCG-3′ [13].

An exogenous IPC was introduced into each reaction well in order to prevent false-negative results. This was an RNA template transcribed from the plasmid pAW109 (pAW109 RNA), purchased from PE Applied Biosystems (France) and used as internal quality marker. We designed the primers and probe using the software Primer Express (PE Applied Biosystems) in order to use this RNA template as a reference (IPC) in the multiplex fluorogenic RT-PCR. The constructed EV RNA standard was used at concentrations of 50–5×10^6 copies/reaction and the IPC at 1,000 copies/reaction. The first results showed that the IPC was not amplified in the presence of EV RNA standard at quantities over 5×10^4 copies/reaction, and that the EV RNA detection limit was 5×10^3 copies/reaction. Different concentrations of each primer (100–400 nM for EVs and 50–100 nM for IPC) were thus tested systematically. Final primer concentrations (120 nM EV primer and 60 nM IPC primer) enabled a constant amplification level for the IPC in the presence of EV RNA standard at concentrations of 50–5×10^5 copies/reaction.

The detection limit, intra-assay variability, and inter-assay variability were determined in 10 replicates of 10-fold dilutions of the EV RNA standard from 50–5×10^6 copies/reaction and the IPC at 1,000 copies/reaction. The results of virus cell culture were analyzed in parallel. Age of the patients as well as laboratory findings are summarized in Table 1. Mean biochemical

Results

The optimal simultaneous amplification conditions were determined during the initial optimizing steps of the multiplex fluorogenic RT-PCR. The constructed EV RNA standard was used at concentrations of 50–5×10^6 copies/reaction and the IPC at 1,000 copies/reaction. The first results showed that the IPC was not amplified in the presence of EV RNA standard at quantities over 5×10^4 copies/reaction, and that the EV RNA detection limit was 5×10^3 copies/reaction. Different concentrations of each primer (100–400 nM for EVs and 50–100 nM for IPC) were thus tested systematically. Final primer concentrations (120 nM EV primer and 60 nM IPC primer) enabled a constant amplification level for the IPC in the presence of EV RNA standard at concentrations of 50–5×10^5 copies/reaction.

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parameters of CSF such as chloride ions, protein, and glucose remained normal, while a relatively high cell count (mean, 172 cells/mm³) was found in the majority of cases. The duration of hospitalization was short, ranging from 1 to 11 days, with a mean of only 2.2 days.

Validation of the results of the fluorogenic RT-PCR was performed by analyzing the multicomponent curves. Any possibilities for the validation of the EV RT-PCR reactions are shown in Fig. 1. No amplification took place in two cases; with C₇-EV and C₇-IPC of 45 cycles, thus indicating the presence of PCR inhibitors. Subsequent amplification of a 1:5 dilution of these samples made it possible to conclude that they were negative, with C₇-EV of 45 cycles and C₇-IPCs of 33.2 and 34.6 cycles.

A comparison between the results of virus culture and those of the fluorogenic RT-PCR is summarized in Table 2. Of the 104 CSF samples, 41 (39.4%) were found to be negative by both RT-PCR and viral culture. Twenty specimens were positive by both techniques. Forty-three discrepant results were obtained: RT-PCR was positive in 41 culture-negative samples, while 2 culture-positive specimens were negative by RT-PCR. Sixty-one of 104 (58.7%) specimens were positive by RT-PCR. Sixty-one of 104 (58.7%) specimens were positive by RT-PCR, and 22 (21.2%) were positive in culture. Of the 22 EV strains isolated in culture, 3 remained untypable, while 19 were typed and yielded 3 different EV serotypes (echovirus types 30, 13, and 6). Figure 2 shows the distribution of the different serotypes isolated in the 22 culture-positive CSF samples. The most common serotype in this outbreak was echovirus type 30. For the two culture-positive specimens that were negative by RT-PCR, one remained untypable and the other was typed as echovirus type 6.

Using a “consensus positive” for EV infection, described by Hadziyannis et al. [14], which was defined as either a culture-positive or RT-PCR-positive result and clinical evidence of enteroviral meningitis, the sensitivity and the negative predictive value of RT-PCR analysis were 96.8% and 95.3%, respectively, while these values for cell culture were 34.9% and 50%.

Viral load in the 61 RT-PCR-positive specimens was measured automatically in the ABI Prism software system and was found to range between 3.3 and 5.9 log, with a median of 4.5 log copies per milliliter of CSF. Mean viral loads calculated for the two groups RT-PCR-positive/culture-positive and RT-PCR-positive/culture-negative were 4.5 and 4.4 log, respectively. Statistical comparison of these two means showed no significant

### Table 1

| Age of patients | Minimum | Maximum | Mean |
|-----------------|---------|---------|------|
| CSF characteristics | 6 days | 20.3 years | 5.8 years |
| Chloride ions (mmol/l) | 115 | 212 | 121.1 |
| Protein (g/l) | 0.11 | 1.28 | 0.47 |
| Glucose (mmol/l) | 0.8 | 5.9 | 3.2 |
| Cell count (cells/mm³) | 1 | 1740 | 172 |
| Viral load (log copies/ml) | 3.3 | 5.9 | 4.8 |
| Duration of hospital stay in days | 1 | 11 | 2.18 |

### Table 2

| Fluorogenic RT-PCR | Virus culture |
|--------------------|---------------|
| Positive | Negative | Total |
| Positive | 20 | 41 | 61 |
| Negative | 2 | 41 | 43 |
| Total | 22 | 82 | 104 |
difference ($P>0.1$). We also compared mean cell counts, chloride ions, glucose, and protein in CSF from patients with a viral load of $<5 \times 10^4$ copies/ml and those with a viral load of $>5 \times 10^4$ copies/ml, without finding any significant difference between the two groups. Only duration of hospitalization showed a statistically significant decrease from 2.7 days for patients with negative PCR results to 1.8 days for patients with positive PCR results ($P=0.042$).

**Discussion**

The diagnosis of aseptic meningitis by RT-PCR requires a sensitive technique with an internal control, in view of the often low levels of virus and the problems associated with PCR inhibitors. During the optimization of our previous fluorogenic RT-PCR [15], we overcame problems of sensitivity related to the secondary structures present in the 5’ noncoding region of the EV genome and problems associated with PCR inhibitors by using PCR adjuvants such as T4 gene 32 protein and polyvinylpyrrolidone [15]. In order to detect PCR inhibitors without a second run, we decided to work on the incorporation of an internal control. Real-time PCR assays allow the choice of internal controls totally different from those of the amplification target, as it is possible to evaluate competition between the two reactions with the amplification kinetic. The two amplification reactions must be independent. Thus, our internal control is just an internal quality control of the amplification, and its amplification at the same level (CT=35 cycles) over a range of 50–5x$10^3$ copies of EVs RNA standard improves its usability. The intra- and inter-assay variability values of 2.2% and 5.5% obtained with low numbers of EV RNA standard copies agree with those found in work on other TaqMan RT-PCRs (3.4% and 6.2%) [17, 18].

The presence of PCR inhibitors in 2 of our 104 (1.9%) specimens confirms the importance of an internal control for RT-PCR assays in CSF, as already demonstrated by other authors [13, 14] who found amplification inhibitors in 2.6% and 0.4% of their CSF specimens, respectively, using the competitive RT-PCR Amplicor EV (Roche Molecular Systems, USA). Since the exact nature of these inhibitors is unknown, their effects cannot be excluded, even by using numerous amplification facilitators [19].

In this study, RT-PCR was compared with EV isolation, which is routinely practiced in our laboratory to detect EVs in young patients with suspected aseptic meningitis referred to the hospital during the period of an EV outbreak. Our comparison confirms that RT-PCR is more sensitive than cell culture [20, 21]. Discrepant results (RT-PCR-negative/culture-positive) were obtained for two strains: one remained untypeable, and for the second one, it is possible that problems occurred during RNA extraction. Retesting of the second strain, however, was prevented by the low quantity of CSF.

The nonsignificant difference found between mean viral load measured in patients with RT-PCR-positive/culture-positive and RT-PCR-positive/culture-negative results suggests that a negative culture result is not necessarily related to a low titre of virus and that other factors, related to the capacity of the virus to propagate in culture and, more particularly, to induce cytopathogenic effects, are more important.

Our method is relatively easy to perform, with results obtained in less time than that required for virus culture. Thus, the entire process of RT-PCR analysis of multiple samples can be carried out easily in less than 4 h, whereas virus culture of CSF typically requires 3–7 days for a CPE to develop. Furthermore, a real-time PCR assay eliminates the necessity for precautions that must be taken with amplified products to avoid contamination because the technique is performed in completely sealed wells. This is a great improvement over conventional PCR assays, which are associated with risks of carryover contamination and are more time-consuming.

The other advantage of this method is that EV genome can be easily quantified using the standard curve. The EV viral loads detected in CSF correspond with the infectious EV loads reported in the literature: \(4 \log_{10} \text{pfu per milliliter of CSF was found for persistent echovirus type 11 in a child with hypogammaglobulinemia} [22].\) Persistent EV infections in agammaglobulinemic patients also have been demonstrated in CSF by RT-PCR [23]. Our assay allowing quantification could be useful in detecting and monitoring the progression or eradication of EV infections in these patients, because cell cultures are often negative during antibody therapy, and monitoring virus eradication using cell culture is very difficult. If specific antiviral agents become available in the future, this method would be a powerful tool to evaluate their efficacy.

The implementation of such a rapid, sensitive, and specific RT-PCR test with high negative predictive value (95.3%) would have a considerable impact on patient care, especially during outbreaks [24]. Previous studies have shown that early diagnosis of enteroviral meningitis would provide potential cost savings by facilitating early patient discharge, discontinuation of inappropriate antibiotic therapy, and elimination of unnecessary investigations [25].
This fluorogenic enterovirus RT-PCR allows a large number of samples to be screened rapidly during an epidemic, and its sensitivity, simplicity, and reproducibility make it a highly reliable and suitable tool in the clinical laboratory.

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