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# Molecular Detection and Identification of an Enterovirus During an Outbreak of Aseptic Meningitis

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Stool samples from sixteen cases of children with meningitis originating from four different and geographically isolated parts of Greece were investigated for enteroviruses. The conventional method of cell culture in four different cell lines was initially used for the isolation of enteroviruses. The results showed a cytopathic effect (CPE) in all cases after two, or even more successive passages in only one cell line (RD), although a less-than-satisfactory CPE was obtained in many cases. Seroneutralization with RIVM mixed hyperimmune antisera followed and the isolates were typed as Coxsackie B viruses. The method of RT-PCR with enterovirus-specific primers targeted to the highly conserved 5′-UTR of the genome was initially used for the detection of enteroviruses from the inoculated cell cultures. A positive RT-PCR result was obtained for all of the clinical samples rapidly and accurately and the isolates were further characterized with the aid of Restriction Fragment Length Polymorphism (RFLP) analysis and Single Strand Conformation Polymorphism analysis (SSCP) of the amplicons. The RFLP analysis showed first of all that the isolates had an identical restriction pattern with Coxsackie B5 Faulkner reference strain with 4 out of 5 restriction enzymes and secondly, both RFLP and SSCP analysis indicated the epidemiological association of the isolates. The speed of the molecular methodology that was used in comparison with the conventional methods and its possible significance for the description of virus evolution and circulation in the populations is discussed. J. Clin. Lab. Anal. 15:87–95, 2001. 

**Key words:** meningitis; enteroviruses; RT-PCR; RFLP; SSCP

## INTRODUCTION

Non-polio enteroviruses constitute the leading recognizable cause of aseptic meningitis which, conversely, is the most commonly encountered illness associated with enterovirus infections (1). In temperate climates, these infections occur during summer and fall months. Young children are the most common victims, because the number of enterovirus infections is inversely proportional to the age of the susceptible individuals. Neonates are at risk for severe systemic illness such as hepatic necrosis, myocarditis, and necrotising enterocolitis (2,3), of which meningitis, or meningoencephalitis, is commonly a part (4). The incidence of morbidity and death caused by perinatal enterovirus infections may not be precisely known, but it is estimated that they could be as high as 74 and 10% respectively, depending on the infecting serotype (2). Clinical cases of enterovirus-induced meningitis appearing beyond the immediate neonatal period are rarely associated with severe disease or poor outcome. Nevertheless, according to the available data, neurological, cognitive, and developmental/language abnormalities have been reported in children in the long term following enteroviral meningitis during their infancy, although not more than 10% of patients have neurological abnormalities (5). The clinical features of the resulting illness may be indistinguishable from meningitis caused by bacterial infection. The course of meningitis does not usually last for more than one week.

Conventional diagnosis of aseptic meningitis caused by enteroviruses relies on the use of appropriately developed cell lines for the virus isolation from cerebrospinal fluid. Nevertheless, despite the increased availability of different continuous cell lines for routine culturing of enteroviruses, some enterovirus serotypes do not grow at all in cell culture (6). More important is the fact that 25–35% of specimens from patients with characteristic enterovirus infections are negative by cell culture due to the intrinsic insensitivity of the cell lines, to antibody neutralisation of the virus in situ, and to inadequate collection, handling, and processing of the samples (7), although the authors could not have examined at that time

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the cell lines that did not show cytopathic effect (CPE) by RT-PCR. Inoculation of suckling mice is too cumbersome and not significantly more sensitive than cell-culture isolation and enteroviruses grow slowly in tissue culture (1). Following isolation in cell culture, the method of seroneutralization with pools of equine, mixed hyperimmune antisera for the typing of enteroviruses is recommended by the World Health Organization. Intersecting pools have been developed at the National Institute of Public Health and the Environment (RIVM) in the Netherlands, which may allow for the identification of only 42 different enterovirus serotypes, and they are steadily replacing the initially developed LBM pools (8) in research and enterovirus reference laboratories. Apart from the reduced range of enterovirus identification, typing efforts with the seroneutralization method may frequently fail because it is a labor-intensive, time-consuming procedure and due to isolates that cannot be typed. The high evolution rates of the antigenic sites result in the appearance of so-called prime strains, which consist of an antigenic continuum with already known serotypes, and cannot be typed with the available antisera. It is not possible to identify previously unknown serotypes by seroneutralization, and typing efforts also frequently fail due to the lack of laboratory standardization (6).

Several alternative methods have been elaborated for the clinical identification of enteroviruses. These methods rely on the use of fluorescent antibodies directly on specimen material, on enzyme immunoassays with type-specific antisera (9), on the use of immunoelectron microscopy with polyvalent and type-specific antisera (10) and on the use of monoclonal antibodies in group-reactive (11–13), or type-specific antisera (12,14,15). However, these techniques are time-consuming and laborious, they are disadvantaged by the absence of a widely reactive virus antigen, and they have a reduced specificity.

Because of the disadvantages of the methods based on cell-culture amplification and/or immunological detection, there is a growing tendency towards the use of genetic information for the isolation and characterization of viruses and microorganisms in general. One such method is spot hybridization using cDNA probes, RNA probes, or oligomeric probes representing several different enterovirus subgroups (16–18). However, these tests have a limited diagnostic value in rapid and accurate detection and identification of enteroviruses from clinical material, as their sensitivities with actual clinical specimens is only 33% or less (13). Contributing to this limitation is the low titre of enteroviruses in many clinical specimens, particularly in cerebrospinal fluid (CSF) samples from patients with aseptic meningitis (1), whereas stool samples remain the most sensitive means for isolation of enteroviruses (19,20).

During the last 12 years, numerous reverse transcription/polymerase chain reaction (RT-PCR) assays have been applied for the detection of the RNA of most if not all all enterovirus serotypes—including those that cannot readily be isolated or typed in cell culture—in an attempt to improve speed, sensitivity, and specificity (21–25). These assays are based on the detection of extremely conserved genomic sequences amongst the different serotypes, such as the 5′-Untranslated Region (5′-UTR), offering an effective means for efficient and rapid isolation of the majority of enteroviruses infecting humans. A limitation, however, of most RT-PCR methods is their inability to provide information on the serotype or other sub-classification of enteroviruses. It is therefore necessary to supplement RT-PCR with methods for the assessment of differences in the sequence of the PCR products, including Restriction Fragment Length Polymorphism (RFLP) analysis (26–28), hybridization with type-specific probes (29), or single-strand conformational polymorphism (SSCP) (30). Nevertheless, serotype-specific, or serotype-group-specific RT-PCR have been described (31,32). Nucleotide sequences of RT-PCR products would also be quite helpful (33), at least for research purposes concerning the evolution and epidemiology of the viruses, although not for routine diagnosis of clinical isolates.

This paper describes the isolation of a non-polio enterovirus from 16 cases of aseptic meningitis by cell culture and RT-PCR and its identification by seroneutralization, RFLP, and SSCP analysis of the RT-PCR amplicons. The clinical and epidemiological significance of the results in conjunction with the different conventional and molecular methods that were used is discussed.

## MATERIALS AND METHODS

### Clinical Samples

Sixteen stool samples from respective cases of young children with meningitis were sent to the enterovirus reference center at the Hellenic Pasteur Institute in order to determine whether an enterovirus is implicated as the etiological agent. No CSF samples were available from any of the children. The samples originated from four very distant and isolated geographical sites: Ioannina in Northern Greece, Heraklion on the island of Crete, Distomo in the Central Greece district of Viotia, and Patra, Peloponissos, South Continental Greece. Table 1 summarizes the details of the patients. Two gr of each stool sample were added to a suspension containing 10ml PBS, 5 gr of glass beads and 0.5 ml chloroform. Following centrifugation at 3,000*g* for 30 minutes, the supernatant was removed and used for the inoculation of the cell cultures (34).

### Cell Cultures

Four different cell lines were used for the initial isolation of enterovirus from the sixteen clinical stool samples in an attempt to maximize isolation efficiency. Specifically, the cell lines RD (rhabdomyosarcoma), Hep-2 (human epidermoid carcinoma), Vero (African Green Monkey kidney cells), and $L20_B$ (derived from genetically modified mice) were used in

**TABLE 1. Details of the children showing the symptoms of meningitis that were examined for enteroviruses**

| Isolate number | Sex | Age | Location | Data sample received |
|---|---|---|---|---|
| 74337 | F | 7 years | Heraklion, Crete | 2/11/1999 |
| 74440 | M | 5 years | Ag. Ioannis, Crete | 5/11/1999 |
| 74395 | M | 6 years | Alikarnassos, Crete | 4/11/1999 |
| 74339 | F | 9 years | Ioannina | 2/11/1999 |
| 74439 | M | 9 years | Heraklion, Crete | 5/11/1999 |
| 74499 | M | 12 years | Patra | 9/11/1999 |
| 74340 | M | 12 years | Ioannina | 2/11/1999 |
| 74249 | M | 8 years | Ioannina | 29/10/1999 |
| 74252 | M | 12 years | Ioannina | 29/10/1999 |
| 74251 | F | 7 years | Ioannina | 19/10/1999 |
| 74106 | F | 8 years | Heraklion, Crete | 20/10/1999 |
| 74108 | M | 8 years | Heraklion, Crete | 20/10/1999 |
| 73904 | M | 3 months | Heraklion, Crete | 11/10/1999 |
| 74335 | F | 2 years | Heraklion, Crete | 2/11/1999 |
| 73888 | F | 1 month | Distomo, Voiotia | 11/10/1999 |
| 73903 | F | 3 months | Heraklion, Crete | 11/10/1999 |

tubes (Becton Dickinson, Franklin Lakes, NJ) containing 2 ml of D-MEM. Four hundred µl of inoculum per tube were added. The inoculated tubes were then incubated in a roller at 37°C for a period of 1 to 7 days, until a complete cytopathic effect (CPE) was observed under ordinary light microscope. Uninfected cells were used as negative control.

## Seroneutralization

The method of seroneutralization with mixed, equine antisera pools (RIVM/National Institute of Public Health and the Environment, Bilthoven, The Netherlands) was used for the typing of the clinical isolates, following the available instructions by the World Health Organization (34). In brief, these high-titred polyclonal antisera were mixed with 100 $TCID_{50}$ of the virus isolate of unknown identity. A back titration of the isolate was included in each assay, something that allowed the titre of the virus actually present in a sample to be calculated. The serum/virus mixtures were incubated for two hours at 37°C. Following this incubation, suspensions of RD cells were added to the microtitre plates and these were examined daily for the presence of CPE for up to five days. The antiserum or the combination of antisera that prevented the development of CPE indicated the identity of the virus.

## RNA Extraction

When a greater than 75% CPE was observed, the infected cells were frozen at –80°C and thawed three times; 350 µl of the cell culture were taken and used for RNA extraction with the phenol-based RNAwiz commercial kit by AMBION Inc. (Austin, TX) according to the manufacturer's instructions.

## Reverse Transcription-Polymerase Chain Reaction

The primers $UC_{53}$ (anti-sense, with the sequence 5′-TTGTCACCATAACCAGCCA-3′) and $UG_{52}$ (sense, with the sequence 5′-CAAGCACTTCTGTTTCCCCGG-3′) that were used for the enterovirus-specific RT-PCR were selected so as to be homologous to respective parts within the highly conserved 5′-UTR region. They were purchased from Genosys Biotechnologies, Europe (Cambridge, UK). The antisense primer is two bases shorter than primer 3 described by Zoll et al. (35), whereas the sense primer is three bases shorter than primer 1 used by the same authors. Table 2 shows the relative position of the target sequences of these primers on the genome of enteroviruses with known sequences, according to the GenBank sequence database. These primers yield amplicons approximately 435-bp long; they were adjusted to a concentration of 7 pmol/µl in sterile, RNase-free distilled water (Sigma Aldrich, St. Louis, MO), and were stored at

**TABLE 2. The relative position of the primers $UC_{53}/UG_{52}$ and $P_3/P_2$ on the genome of coxsackie viruses with known sequences**

| Species | Strain | Position $UC_{53}/UG_{52}$ | Position $P_3/P_2$ |
|---|---|---|---|
| Coxsackie A9 | Griggs | $UC_{53}$: 577-595 | $P_3$: 585-604 |
| | | $UG_{52}$: 162-182 | $P_2$: 441-460 |
| Coxsackie A16 | G-10 | $UC_{53}$: 578-596 | $P_3$: 587-606 |
| | | $UG_{52}$: 162-182 | $P_2$: 452-471 |
| Coxsackie A21 | Coe | $UC_{53}$: 580-598 | $P_3$: 576-595 |
| | | $UG_{52}$: 164-184 | $P_2$: 441-460 |
| Coxsackie A24v | EH24/70 | $UC_{53}$: 586-603 | $P_3$: 585-604 |
| | | $UG_{52}$: 168-188 | $P_2$: 450-469 |
| Coxsackie B1 | Japan | $UC_{53}$: 582-599 | $P_3$: 581-600 |
| | | $UG_{52}$: 165-185 | $P_2$: 446-465 |
| Coxsackie B2 | Ohio | $UC_{53}$: 583-600 | $P_3$: 582-601 |
| | | $UG_{52}$: 166-186 | $P_2$: 448-467 |
| Coxsackie B3 | Nancy | $UC_{53}$: 582-599 | $P_3$: 581-600 |
| | | $UG_{52}$: 165-185 | $P_2$: 446-465 |
| Coxsackie B4 | JVB | $UC_{53}$: 584-601 | $P_3$: 583-602 |
| | | $UG_{52}$: 167-187 | $P_2$: 448-467 |
| Coxsackie B5 | Faulkner | $UC_{53}$: 584-601 | $P_3$: 583-602 |
| | | $UG_{52}$: 167-187 | $P_2$: 448-467 |
| Coxsackie B6 | Schmidt | $UC_{53}$: 584-601 | $P_3$: 583-602 |
| | | $UG_{52}$: 166-186 | $P_2$: 448-457 |

–20°C. The isolated RNA was then converted into cDNA with the aid of Reverse Transcription; 20 units of RNase inhibitor (Promega Corporation, Madison, WI), 2 μl anti-sense primer and 5 μl extracted RNA from each sample were initially mixed and heated at 70°C for 5 minutes. The tubes were immediately transferred to ice and 5 μl of RT 5× buffer, 5 μl dNTPs 10mM, 100 units RTase M-MuLV (Promega Corporation) and 6 μl RNase-free water (Sigma Aldrich) were added to each tube, making up a total reaction mixture of 20 μl. This mixture was incubated at 42°C for 1 hour and the M-MuLV RTase was inactivated by heating at 95°C for 5 minutes. The produced cDNA was amplified by PCR using a reaction mixture of 50 μl /tube containing 5 μl 10× PCR buffer, 4 μl dNTPs 10 mM, 3 μl $MgCl_2$ 25 mM (yielding a final $MgCl_2$ concentration of 1.5 mM), 28 μl RNase-free water, 2 units Taq Polymerase (Minotech, Heraklion, Crete), 5 μl cDNA, 2 μl of each of the two primers $UC_{53}$ and $UG_{52}$ and a drop of paraffin oil in order to avoid evaporation of the samples. Forty cycles of denaturation (94°C, 15 sec), annealing (45°C, 15 sec) and extension (72°C, 15 sec), followed by incubation for 15 minutes at 78°C in order to complete the extension of the primers, were performed in a Techne Progene Thermal Cycler. Ten μl of each amplified product were analyzed by agarose gel electrophoresis in 2.5% agarose (Gibco BRL, ultra pure agarose, electrophoresis grade) containing 1μg/μl ethidium bromide in Tris-Boric acid-EDTA (TBE) buffer. The amplicons were then visualized through an UV transilluminator FOTO/PHORESIS I, FOTODYNE (Hartland, WI).

All procedures were carried out under conditions that minimized the risk of contamination from exogenous nucleic acid sources or carry-over of amplification products during RT-PCR. There was a physical separation of pre- and post-PCR procedures with separate rooms, and sets of pipettes with plugged, aerosol-resistant tips were allocated for each step of the PCR, i.e., reaction-mixture preparation, template addition, and amplified product electrophoretic analysis. RNA from uninfected cells was used as a negative control in each amplification assay and was always RT-PCR-negative, indicative of the efficiency of these preventative measures.

### Restriction Fragment Length Polymorphism Analysis of $UC_{53}/UG_{52}$-produced RT-PCR Amplicons of the Clinical Isolates

Twenty μl of the amplicons of the clinical strains were studied with the restriction enzymes: HpaII, DdeI (New England Biolabs, Beverly, MA), HaeIII, StyI (Promega Corporation) and NcoI (Minotech). The appropriate buffer and distilled, RNase-free sterile water (Sigma Aldrich) were added to each sample to a final volume of 30 μl. The samples were then incubated at 37°C for 2 hours and the products were subjected to electrophoresis in 3% gels made from high-resolution agarose (Metaphor FMC Bioproducts, Rockland ME) containing 1μg/ml ethidium bromide and visualized through an UV transilluminator. The results were analyzed with the aid of GelPro Analyzer software (Media Cybernetics, Silver Spring, MD.

### RFLP Analysis of $UC_{53}/UG_{52}$-produced RT-PCR Amplicons of CBV Reference Strains

When seroneutralization showed that the isolates belong to the CBV group, RFLP analysis with the same five restriction endonucleases used for the clinical isolates was conducted on $UC_{53}/UG_{52}$-produced RT-PCR amplicons of all the CBV reference strains and the results were compared with those obtained for the enterovirus isolates. The six different Coxsackie B virus reference strains used in this study, kindly provided by the National Institute for Public Health and the Environment (RIVM) in Holland, are CBV1 Japan, CBV2 Ohio, CBV3 Nancy, CBV4 JVB, CBV5 Faulkner, CBV6 Schmidt.

### Single Strand Conformation Polymorphism (SSCP)

The SSCP analysis of the PCR amplicons is based on the principle that the electrophoretic mobility of a particle in a gel depends on both size and shape. Under nondenaturing conditions, single-stranded DNA has a folded conformation that is determined by intra-strand complementarity and, therefore, by its sequence. In the present SSCP analysis, 5 μl of the PCR products of the different viral isolates were added to 20 μl of SSCP buffer (95% formamide and 5% bromophenol blue), were converted into single-stranded molecules, and were then subjected to vertical electrophoresis in a 12% polyacrylamide gel (49/1 acrylamide/bis) at 17°C ±1°C. The reduction in the buffer temperature was made in an attempt to increase the resolution efficiency of the gel (36). The single-stranded PCR products were then visualized by treating the polyacrylamide gel with silver staining using the commercial kit GelCode™ by Pierce (Rockford, IL). The differences in the PCR amplicon sequence of different viruses is detected by the corresponding differential mobility of these single-strand amplicons. The sensitivity of SSCP tends to decrease with increasing fragment length (37); it has been reported that it detects >90% of all single-base substitutions in 200-nucleotide fragments and >80% in 400-nucleotide fragments (36), which led to the use of the primers $P_3$ (anti-sense, with the sequence 5′-ATTGTCACCATAAGCAGCCA-3′, i.e., the same with $UC_{53}$ but only two bases longer) and $P_2$ (sense, with the sequence 5′-TCCTCCGGCCCCTGAATGCG-3′), as originally used by Zoll et al. (35) (primers 3 and 2 respectively), for the production of 155-bp-long amplicons that were analyzed by SSCP. Table 2 shows the relative position of the target sequences of these primers on the genome of enteroviruses with known sequences, according to the GenBank sequence database.

## RESULTS

Sixteen enterovirus strains were isolated initially by cell culture. Presumably due to the problems of reduced efficiency that are encountered with conventional cell culture systems, or, perhaps, to reasons that are concerned with the low initial titre of the isolated enteroviruses and their phenotype with respect to their adaptation to the isolation system used, these viruses grew very slowly. Only after two or three successive passages did a CPE appear, and that after 5 days in one cell line only (RD), which in some cases was not greater than 50% (Table 3).

A positive RT-PCR result was obtained for all sixteen clinical cases, implicating an enterovirus as the most plausible etiologic agent for the respective cases of meningitis. The method of seroneutralization with the RIVM hyperimmune antisera pools showed that the isolated enteroviruses belong to the Coxsackie B virus (CBV) group. The RT-PCR amplicons from the respective isolates that were produced with the primers $UC_{53}/UG_{52}$ were further studied with RFLP analysis using the five restriction enzymes mentioned before. All the isolates had the same restriction pattern, shown in Fig. 1, leading to the conclusion that all the patients had been infected by the same type of enterovirus.

The comparison of the RFLP data of the clinical isolates with the respective data for the CBV reference strains showed that the restriction pattern of the $UC_{53}/UG_{52}$-produced RT-PCR amplicons of the isolates was found to be identical with the restriction pattern deduced from the respective $UC_{53}/UG_{52}$-produced RT-PCR amplicons of the prototype strain Coxsackie B5 Faulkner using four of the five restriction enzymes (excluding DdeI). The results for reference strains CBV5 Faulkner are shown in Fig. 2. Figure 3 shows the results for CBV1 Japan as an example of another reference strain which had a different restriction profile with that of the isolates. The genetic relationship and, consequently, the possible identity of the isolates as Coxsackie B5 is therefore implied.

Figure 4 shows the results of the SSCP analysis of the denatured $P_3/P_2$-produced RT-PCR amplicons from four different isolates and from CBV5 Faulkner. The four isolates had the same electrophoretic pattern. However, CBV5 had a different electrophoretic pattern although, as shown in Fig. 3, the difference in the electrophoretic mobility was not large in comparison with that of the isolates, leading to the conclusion that there is, perhaps, a small difference between the sequence of the amplicons of CBV5 and that of the respective amplicons of the isolates. The rest of the 16 isolates that were also analyzed with this method showed the same electrophoretic pattern with that of the isolates shown in Fig. 2, leading to the same conclusion that the isolated enteroviruses are of the same species.

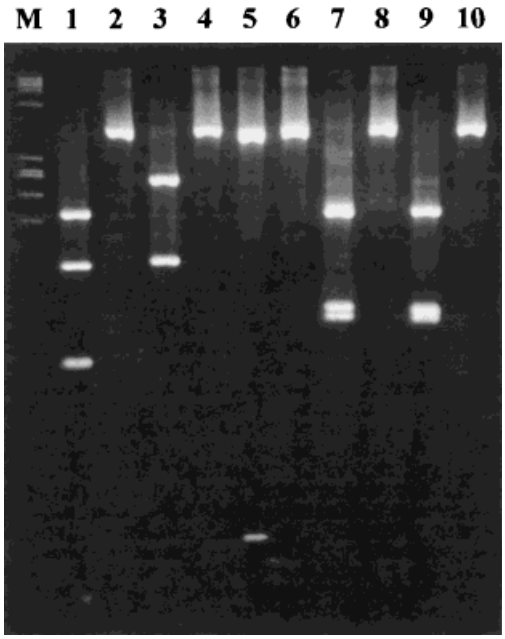


**Fig. 1.** The results of the RFLP analysis of $UC_{53}/UG_{52}$-produced RT-PCR amplicons from each of the 16 isolated enteroviruses with 5 different restriction enzymes. Lanes 1, 3, 5, 7, and 9 show the restriction pattern for each of the isolates with the restriction enzymes HaeIII, HpaII, DdeI, StyI, and NcoI respectively, whereas lanes 2, 4, 6, 8, and 10 show the RT-PCR product that was not treated with any restriction endonuclease. M shows the molecular weight marker ($\phi\chi$174 RF DNA/HaeIII Fragments/Gibco BRL). According to the analysis of this figure by GelPro Analyzer software, the following genomic fragments were obtained: HaeIII—205, 141, and 78 base pairs; HpaII—270 and 155 base pairs; DdeI—410 and 25 base pairs; StyI—209, 118, and 109 base pairs, and NcoI produced the same restriction fragments with StyI.

**TABLE 3. Results of isolation and propagation of the 16 clinical isolates in the four available cell lines**

| Clinical sample | Cell lines | | | |
|---|---|---|---|---|
| | RD | Vero | Hep–2 | $L_{20}$ |
| 74337 | ++++[3] | – | – | – |
| 74440 | +++[3] | – | – | – |
| 74395 | ++[3] | – | – | – |
| 74339 | ++++[3] | – | – | – |
| 74439 | ++[3] | – | – | – |
| 74499 | ++[3] | – | – | – |
| 74340 | ++++[1] | – | – | – |
| 74249 | ++++[2] | – | – | – |
| 74252 | +++[2] | – | – | – |
| 74251 | +++[2] | – | ++[22] | – |
| 74106 | ++++[3] | – | – | – |
| 74108 | ++[2] | – | – | – |
| 73904 | ++++[2] | – | – | – |
| 74335 | ++++[3] | – | – | – |
| 73888 | ++++[2] | – | – | – |
| 73903 | ++++[3] | – | – | – |

++:50% CPE; +++:75% CPE; ++++:100% CPE.
[1]CPE in 1st cell culture passage.
[2]CPE in 2nd passage.
[3]CPE in 3rd passage.

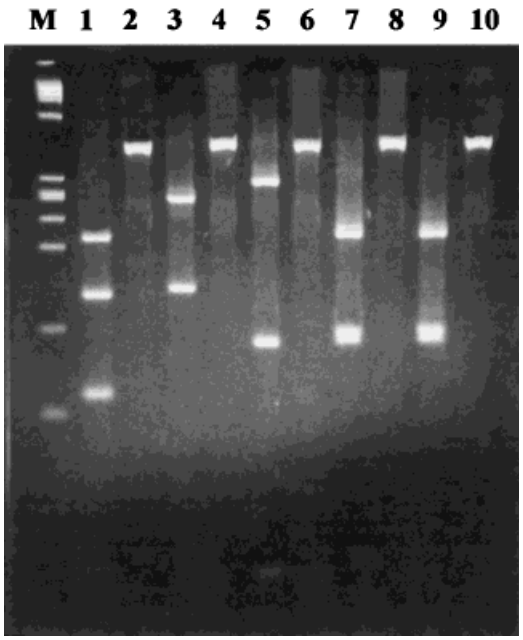


**Fig. 2.** The results of the RFLP analysis of $UC_{53}/UG_{52}$-produced RT-PCR amplicons from CBV5 Faulkner reference strain. Lanes 1, 3, 5, 7, and 9 show the restriction pattern with the restriction enzymes HaeIII, HpaII, DdeI, StyI, and NcoI respectively, whereas lanes 2, 4, 6, 8, and 10 show the RT-PCR product that was not treated with any restriction endonuclease. M shows the molecular weight marker (ϕχ174 RF DNA/HaeIII Fragments/Gibco BRL). According to the analysis of this figure by GelPro Analyzer software, the following genomic fragments were obtained: HaeIII—205, 141, and 85 base pairs; HpaII—270 and 155 base pairs; DdeI—300, 103, and 29 base pairs; StyI—209, 117, and 109 base pairs, and NcoI produced the same restriction fragments with StyI.

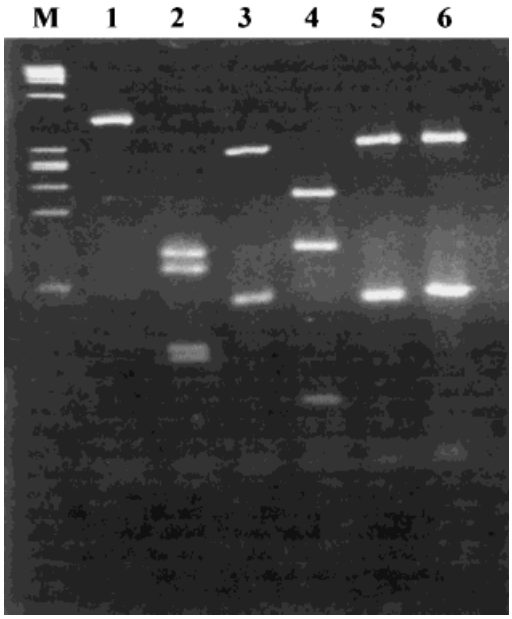


**Fig. 3.** The results of the RFLP analysis of $UC_{53}/UG_{52}$-produced RT-PCR amplicons from CBV1 Japan reference strain. Lanes 2, 3, 4, 5, and 6 show the restriction pattern with the restriction enzymes HaeIII, DdeI, HpaII, StyI, and NcoI respectively, whereas lane 1 shows the RT-PCR product that was not treated with any restriction endonuclease. M shows the molecular weight marker (ϕχ174 RF DNA/HaeIII Fragments/Gibco BRL). According to the analysis of this figure by GelPro Analyzer software, the following genomic fragments were obtained: HaeIII—146, 132, 81, and 76 base pairs; DdeI—300, 103, and 29 base pairs; HpaII—218, 152, and 57 base pairs; StyI—331 and 104 base pairs and NcoI produced the same restriction fragments with StyI.

## DISCUSSION

Clinical observation on its own may not provide satisfactory clues about the epidemiologic significance of meningitis cases, as the symptoms of enterovirus-instigated aseptic meningitis are generally indistinguishable from those of meningitis with a bacterial etiology. For this reason, the pathologic agent has to be isolated, identified, and characterized. And of equal importance is the fact that the determination of enterovirus meningitis early in the course of the illness will greatly facilitate a better patient management, by excluding, for instance, unnecessary treatment with antibiotics. For these reasons, accuracy and speed constitute a prerequisite for the purposes of clinical and epidemiological investigations. The fact that the isolates grew very slowly in only one of the available cell lines (RD) indicated the general shortcoming of virus isolation by cell culture (Table 3) and, subsequently, the possible inability and delay of typing the isolates via seroneutralization based on cell line propagation of the isolates, which may hamper clinical and epidemiological investigations. Viral culture has been reported to detect the etiologic agent in only 60% of cases of acute aseptic meningitis in young children (20). The inability to isolate enteroviruses in cell

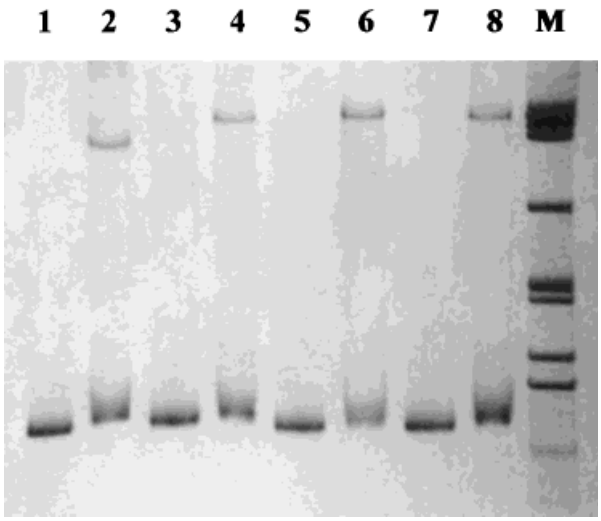


**Fig. 4.** The results of SSCP analysis of single-stranded $P_3/P_2$-produced RT-PCR amplicons of CBV5 Faulkner reference strain (lane 2) and 3 isolated enteroviruses (lanes 4, 6, and 8). Lanes 1, 3, 5, and 7 show the respective non-denatured, double-stranded RT-PCR amplicons. M shows the molecular weight marker (ϕχ174 RF DNA/HaeIII Fragments/Gibco BRL).

culture is partly attributable to the low concentration of virus in clinical specimens (38). When a CPE was observed, it was not possible via seroneutralization to indicate the precise identity of the isolates due to the intrinsic inability of RIVM mixed antisera pools, as mentioned before, but it was only possible to define that the isolates belong to the group B of Coxsackie viruses.

The increased specificity, sensitivity, and speed of the PCR assays significantly facilitate the derivation of correct information with a great impact in all aspects of virus biology, pathogenesis, and epidemiology. The 5′-UTR of enteroviruses was chosen as the genomic region to be studied because it is highly conserved among the enteroviruses due to its important role in translation and replication of the positive sense RNA genome, increasing therefore the probabilities of universal detection of wild-type enteroviruses circulating in the population. Secondly, by choosing this genomic region the risk of intratypic variation interfering with a reliable genotypic characterization of the isolates is reduced, and at the same time, the detection of any intertypic differences between the viruses becomes plausible. Nevertheless, a limitation of many RT-PCR methods that have been described so far, including that of the present study, is their inability to provide information for the sub-classification of the enteroviruses examined, losing in this way significant biological and epidemiological information. For this reason, the RT-PCR assay was supplemented with the molecular genotyping methods that are described in this study: RFLP and SSCP analysis. Most published data on biological/taxonomic studies and clinical investigations have described the use of genomic regions within the 5′-UTR of enteroviruses (21,23,27,32,35,39,40, and others), as was done in the present study, although other genomic regions of the enteroviruses have also been used (22,26,31,41).

Five different restriction endonucleases were used for the RFLP analysis in an attempt to optimize the detection of genetic differences between the different isolates. This method has been suggested and used in the past for the rapid identification of different enterovirus serotypes. Balanant et al. (26) showed the natural genomic variability of polioviruses analyzed by restriction-fragment-length-polymorphism analysis of genomic fragments originating from the N-terminal half of the 1D region. Schweiger et al. (42) used RFLP analysis for the differentiation between vaccine and wild-type polioviruses, whereas Jung et al. (43), studied with the same method genomic fragments from the 5′-UTR/VP2 region of reference non-polio enteroviruses and clinical isolates associated with aseptic meningitis. Mulders et al. (44) identified the Sabin-derived character of a field isolate of poliovirus type 1 displaying aberrant genetic and phenotypic features with the aid of PCR-RFLP, along with Sabin-specific PCR and cRNA-probe hybridization.

However, there is a very important drawback that has to be resolved regarding this method. Inherent to the RNA genome of enteroviruses and the lack of proofreading activity of the virus-encoded RNA polymerase are: (1) the genome's highly mutable nature, due to point mutations; (2) intraspecific or interspecific recombination events with members of the same group of viruses or with members from a different group respectively; or (3) even possible recombination with genetic material of cellular origin (45). For this reason, enteroviruses exist in a form of "quasispecies" populations, i.e., members of the same species in the same population are not completely identical. Another shortcoming of the method is the possibility of patients being infected by more than one enterovirus at the same time. The RFLP analysis in this case would be very complicated, although such a problem may not be frequently encountered in normal situations if one virus strain comes to predominate within an organism in a competitive manner against any other strains. It seems that the latter was the case since the sum of the length of the restriction fragments produced was approximately 435 bp—the expected length of the $UC_{53}/UG_{52}$-produced RT-PCR amplicons—indicating the isolation of only one enterovirus. Berlin et al. (20) studied 274 clinical cases of aseptic meningitis in infants less than 2 years of age; in only 2 cases was an additional isolate found.

When mutation does significantly alter the restriction pattern, we are presented with another reason for the use of as many restriction endonucleases as possible. Even when mutation/recombination events lead to inconclusive for the identity of isolates restriction patterns, the RFLP analysis would provide significant biological and epidemiological information by showing genetic variability between members of the same species or between viruses with a varying degree of genetic relatedness between them. For instance, Hierholzer et al. (46) showed that restriction-enzyme analysis of adenoviruses from AIDS patients had only limited usefulness for typing but was helpful in identifying groups of isolates with similar properties.

In this study, the sixteen enteroviruses isolated from the respective cases of aseptic meningitis had identical restriction profile, and consequently, the experimental results showed the epidemiologic relatedness of the clinical cases, since they were the result of infection by a single type of enterovirus. Nevertheless, for the reasons mentioned here, inferences can only be made concerning the precise identification of the isolates as Coxsackie B5 with respect to their restriction profile. In one study examining aseptic meningitis in infants younger than 2 years old over a 5-year period, more than 90% of the cases were due to group B Coxsackie viruses and echo viruses (20), with serotypes CBV 2, 4, and 5 and ECV 4, 6, and 11 being the most frequently isolated. This correlated with data from a 25-year study of the Center for Disease and Control (47). Despite the fact that the isolates originated from four very distant and extremely isolated topographically areas, it seems that factors pertaining to human activity—such as the evolution of transportation and overcrowding in small, closed areas—increased the possibility of bringing a large

number of susceptible individuals into close contact. These factors provide the basis for a propagated epidemic (48).

The SSCP analysis method was applied successfully for denatured PCR products from wild-type and mutant genomes by Hayashi (37). But Fujioka et al. (30) used single-strand conformation polymorphisms of PCR products from the 5′-UTR using the same set of primers with the primer pair $P_3/P_2$ described in this study, for the analysis of genotypes of 14 enteroviruses. The applicability of this to the detection of mutation and recombination events with an epidemiological and clinical value is sustained. However, due to this technique's power in the identification of genomic fragments differing by only a few nucleotides, it is doubtful whether it could be used for the typing of wild-type isolates; members of the same serotype exist in "quasispecies" populations differing slightly in their genetic constitution, even in highly conserved areas like the 5′-UTR. The diagnostic value of this method should be further assessed and developed, if possible, but, like the results of the RFLP analysis in this study, it was possible to draw useful conclusions concerning the epidemiologic relationship between the different isolates. For instance, Maisonneuve et al. (49) applied the method of RT-PCR-SSCP for the study of 154-bp-long genomic fragments from the 5′-UTR of different echovirus 30 isolates which were responsible for a meningitis outbreak in France and they managed to show the existence of 2 dominant clones of the serotype responsible for the epidemic.

Another important issue in this study is the isolation of the enteroviruses from fecal samples. It is generally supported that isolation of an enterovirus from affected organs and associated body fluids provides the strongest evidence of an enteroviral etiology for disease (6). Therefore, in the case of infection of the Central Nervous System (CNS) isolation of enteroviruses from CSF would provide the strongest causal relationship with the ongoing illness. No CSF samples were available for the present study and detection of enterovirus in the alimentary tract is also said to provide only circumstantial evidence of etiology, because shedding of virus particles at these sites may occur even in the absence of clinical symptoms, especially in infants and during epidemic seasons (6). Nevertheless, the use of stool samples for enterovirus isolation remains the most sensitive method. Glimaker et al. (19) detected enteroviral RNA in stool samples from 74 patients with aseptic meningitis. The rates of isolation of enteroviruses from stool samples in a study over 5 years of aseptic meningitis in infants younger than 2 years old were the highest in comparison with rectal swabs, urine, throat swabs, and CSF (20). Other previous studies of patients with aseptic meningitis have also shown that enterovirus yield has been higher from fecal samples (29–95%) than from CSF (13–70%) (50,51,52). The detection of enterovirus in stool samples from a patient with aseptic meningitis may not constitute an unmistakable criterion for the implication of the enterovirus for the specific clinical symptoms, but it significantly aids diagnosis in such cases and has proven to be highly accurate.

In conclusion, the RT-PCR assay described in this report was particularly useful for the isolation of enteroviruses from stool samples and their incrimination as the etiologic agents for the respective cases of aseptic meningitis. The advantages of this molecular technique over conventional virus isolation is sustained. Despite the fact that the applicability of the other molecular techniques that were used in identifying the isolates must be further assessed, their usefulness for the rapid and precise epidemiological association of the different meningitis cases was significant. In this way, inferences can be made for the plausible modes of enterovirus circulation in the populations and the correct management of patients and epidemiological episodes is greatly facilitated. With the aid of the molecular techniques used in this study, it was possible to describe here an outbreak of enterovirus-induced meningitis rapidly and accurately, something which seems quite useful given society's modern social and behavioural factors pertaining to human activity, which provide a fertile ground for the successful evolution and spread of viruses in the populations.

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