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Isolation of Polioviruses and Other Enteroviruses in South Greece Between 1994 and 1998

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During the five-year period between 1994 and 1998, a total of 217 clinical samples were assessed for the isolation of enteroviruses at the Enterovirus Reference Centre for South Greece. Fourteen enterovirus strains belonging to different serotypes were isolated. These field strains were detected by cell culture in appropriate cell lines. They were subsequently identified by neutralizing antibodies with the LBM (Lim-Benyesh Melnick) mixed antisera pools up to 1995 and RIVM (National Institute of Public Health and the Environment, Bilthoven, The Netherlands) pools from 1996 onwards. The isolated viruses included two strains of poliovirus type 2 Sabin-like, three strains of poliovirus type 1 non-Sabin-like, one Coxsackie B2 (CBV2) strain, one Coxsackie B5 (CBV5) strain, one Echo 5 (ECV5) strain, one Echo 7 (ECV7) strain, three Coxsackie A16 (CAV16) strains, and two currently enteroviral strains unidentified by RIVM pools. Reverse transcription-polymerase chain reaction (RT-PCR) using poliovirus-specific primers or poliovirus type-specific primers and enterovirus specific primers from the highly conserved 5′-UTR, the latter followed by RFLP, was also applied in 6 clinical isolates (3 strains of poliovirus type 1 non-Sabin-like, 1 polio type 2 Sabin-like, and 2 non-identified by RIVM pools enteroviruses). The advantages and the drawbacks of these assays against the conventional ones are discussed here. The isolations and the subsequent identification of the strains were carried out from fecal samples of clinical cases that included hand-foot-and-mouth disease, meningitis, and acute flaccid paralysis. The reappearance of non-Sabin-like poliovirus strains in Greece in 1996 after 14 years is considered to have an important medical and clinical value. J. Clin. Lab. Anal. 14:157–163, 2000.

Key words: polioviruses; enteroviruses; RT-PCR; RFLP

INTRODUCTION

The genus of enteroviruses is the most important of the picornavirus family in terms of human pathogenicity. It consists of polioviruses (three reference strains), Coxsackie A (23 reference strains), Coxackie B viruses (6 reference strains), Echoviruses (Enterovirus Cytopathic Human Orphan-28 reference strains), and the most recently discovered enteroviruses which have not yet been classified (although they have been given numbers in the order of their discovery: enteroviruses 68–71) (1). There are in total 64 immunologically distinct serotypes that infect humans. Infections from the members of this group of viruses, even the most pathogenic of them, are characterized by a significant percentage of subclinical manifestations (2). Most of them are asymptomatic. Nevertheless, enteroviruses have been known to be responsible for serious or even fatal diseases. They are responsible for a wide range of acute symptoms as a result of infection of the central nervous system, cardiac and skeletal muscles, pancreas, skin, and mucous membranes (2). A very well-known disease is paralytic poliomyelitis, which is perhaps the oldest recorded viral disease and its viral etiology was demonstrated in the early 20th century when epidemics of this disease increased in frequency and severity (3,4). Today, it causes significant disability in many parts of the world. The subsequent characterization in the 1950s of polioviruses as the causal agents of this disease led to the development of inactivated (Salk) and live attenuated (Sabin) poliovaccines, the only ones available against any enteroviral disease (3). Enteroviruses are the most commonly implicated agents of acute myocarditis and aseptic meningitis. Congenital infections also may occur, although their frequency is unknown and infection of neonate is frequently life-threatening (5). There is some evi-

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dence that enteroviruses are the possible etiological agents that cause or contribute to chronic diseases like dilated cardiomyopathy, one of the most common indications for heart transplantation, insulin-dependent diabetes mellitus, chronic fatigue syndrome and more, although such a role is still under debate (6–8).

Data from intense scientific research on mice have led to the conclusion that chronic enteroviral infections are characterized by limited replication and expression of the genome, despite the persistence of arguments concerning the precise role that the enteroviruses play in this kind of disease (1). Immunocompromised individuals are especially susceptible to chronic enteroviral infections, particularly the elderly, young children, individuals undergoing chemotherapy or transplantation, or those with disorders of their immune system (AIDS, systemic erythematosus lupus, etc.).

The necessity for detailed and organized scientific research on all the aspects of the biology of enteroviruses and the diseases they cause is therefore understandable. The purpose of this report is the recording of all the enteroviruses that were isolated at the Hellenic Pasteur Institute between 1994 and 1998 in order to extract useful conclusions for the epidemiology of enteroviruses and the ways that it should be studied.

MATERIALS AND METHODS

Clinical Samples

Between 1994 and 1998, 217 samples were sent to the virology department at the Hellenic Pasteur Institute in order to be examined for the detection of enteroviruses, according to the medical history of each case. The samples comprised of pharyngeal washings, vesicular fluid, and pleuritic fluid, which were used directly for the inoculation of the cell cultures, and feces. They were first processed as follows: 2 g of each stool sample were added to a suspension containing 10 ml PBS, 5 g of glass beads, and 0.5ml chloroform. Following centrifugation at 3,000 rpm for 10 minutes, the supernatant was removed and used for the inoculation of the cell cultures (9).

Cell Cultures

Since no single cell line currently in use supports the growth of all known enteroviruses, four different cell lines were used, according to the instructions given by the World Health Organization (9). These cell lines are RD (rhabdomyosarcoma), Hep-2 (human epidermoid carcinoma), Vero (African Green Monkey kidney cells), and $L20_B$ mouse cells in test tubes (Becton Dickinson, Franklin Lakes, NJ) containing 2 ml of D-MEM. Between 100 and 400 µl of inoculum per tube were used. 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 an ordinary light microscope. Uninfected cells from each of the four cell lines were used as the negative control.

Seroneutralisation

The method of seroneutralisation with mixed, equine antisera pools (LBM/Lim-Benyesh Melnick up to 1995, and RIVM/National Institute of Public Health and the Environment, Bilthoven, The Netherlands, from 1996 on), provided by the World Health Organization, was used for the typing of the clinical isolates, following the available instructions (10). In brief, these high-titred polyclonal antisera were used and mixed with approximately 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 one hour at 37°C. Subsequently, suspensions of cells were added to the microtitre plates and these were examined daily for the presence of CPE for up to five days. The antiserum that prevented the development of CPE indicated the identity of the virus.

RNA Extraction

Due to the frequently encountered low specificity and sensitivity of the currently available cell culture systems, the method of an enterovirus-specific reverse transcription-polymerase chain reaction was also used for the detection of enteroviruses in the clinical samples (5). After the observation of CPE, 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 TRIzol commercial kit by Gibco BRL (Life Technologies Ltd., Paisley, UK) according to the manufacturer's instructions.

Reverse Transcription-Polymerase Chain Reaction

The isolated RNA was converted into cDNA with the aid of reverse transcription; 25 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 RT 5× buffer, 5 µl dNTPs 10 mM, 100 units RTase M-MuLV (Promega) and 6 µl RNase-free water (Sigma Aldrich, St Louis, MO) 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 [$MgCl_2$] = 1.5mM), 28 µl RNase-free water, 2 units Taq Polymerase (Minotech, Heraklion, Crete, Greece), 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 a 15-minute incuba-

tion at 78°C 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% ultra-pure, electrophoresis-grade agarose gel (Gibco BRL) containing 1 µg/µl ethidium bromide in Tris-Boric acid-EDTA (TBE) buffer. The amplicons were then visualised 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 during the RT-PCR. Separate rooms and sets of pipettes with plugged tips were allocated for each step of the PCR, i.e., the 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, something which was indicative of these preventative measures.

Poliovirus-Specific RT-PCR

The clinical isolates that had been identified by seroneutralization as polioviruses were also studied with the primer pair UC_1/UG_1 which had been chosen so as to fit the criterion of universality for poliovirus strains, as previously described (11). The downstream primer (UC_1) has the sequence 5′-GAATTCCATGTCAAATCTAGA and the upstream primer (UG_1) has the sequence 5′-TTTGTGTCAGCGTGTAATGA. Table 1 shows the positions of these primers on the genome of poliovirus type 1, strain Mahoney, and the produced fragment which was a 480-nucleotide sequence coding for the N-terminal half of the capsid protein VP1, including antigenic site 1 (11). They were adjusted to a concentration of 50 pmol/µl in RNase-free, sterile distilled water and stored at –20°C. The RT-PCR was carried out as previously described with the primers UC_{53}/UG_{52}.

Polio Serotype-Specific RT-PCR

All the clinical samples identified by seroneutralisation as polioviruses were examined by the method of RT-PCR using primers containing mixed-base or deoxyinosine residues at positions of codon degeneracy, as has previously been described (12). According to this method, three sets of serotype-specific primers (PV1A/PV1,2S for the detection of polio type 1, yielding 70 bp-long amplicons, PV2A/PV1,2S for the detection of polio type 2, yielding 79 bp-long amplicons, and PV3A/PV3S for the detection of polio type 3, yielding 140 bp-long amplicons) were designed so as the antisense primers (PV1A, PV2A, and PV3A) pair with codons of VP1 amino-acid sequences that are conserved within but that differ across the three serotypes, whereas the sense polarity primers (PV1S, PV2S, and PV3S) match codons of more conserved capsid sequences. The reverse transcription and the PCR were carried out as described above, with the exception that the primer concentration was 80 pmol per tube and the amplification cycles were 30 with denaturation at 94°C for 1min, annealing at 42°C for 1 min and extension at 60°C for 1 min, as has been previously described (12).

TABLE 1. The relative position of the primers UC_{53}/UG_{52} and UC_1/UG_1 on the genome of enteroviruses with known sequences

Species	Strain	Position
Polio Sabin type 1	Ls-c, 2ab	UC_{53}: 577–595, UG_{52}: 162–182
Polio Sabin type 2	P712, Ch, 2ab	UC_{53}: 578–596, UG_{52}: 162–182
Polio Sabin type 3	Leon, 12 a, b	UC_{53}: 580–598, UG_{52}: 164–184
Polio wild type 1	Mahoney	UC_{53}: 578–596, UG_{52}: 162–182
Coxsackie B2	Ohio	UC_{53}: 582–600, UG_{52}: 166–186
Coxsackie B3	Nancy	UC_{53}: 580–598, UG_{52}: 165–185
Coxsackie B6	Schmidtt	UC_{53}: 584–602, UG_{52}: 166–186
Polio wild type 1	Mahoney	UC_1: 2761–2881, UG_1: 2402–2421

Enterovirus-Specific RT-PCR/Restriction Fragment Length Polymorphism Analysis

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 have been selected so as to be homologous to respective parts within the highly conserved 5′-UTR region; they were synthesised and purchased from Genosys Biotechnologies (Cambridge, UK). Table 1 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 440-bp long; they were adjusted to a concentration of 50 pmol/µl in sterile distilled water and were stored at –20°C.

Twenty µl of the amplicons from the last enteroviruses isolated in 1998 (one polio type 2 Sabin-like and two nonidentified by RIVM pools) were studied with the following restriction enzymes: HpaII, DdeI (New England Biolabs, Beverly, MA), HaeIII, StyI (Promega), and NcoI (Minotech). The appropriate buffer and distilled water were added in each sample to a final volume of up to 30µl. 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), containing 1 µg/µl ethidium bromide, and were visualised through a UV transilluminator. The results were processed with the aid of GelPro Analyzer software (Version 3.0, Media Cybernetics, Silver Spring, MD).

RESULTS

Fourteen enterovirus strains, twelve of which belonged to different serotypes and two that cannot be serotyped by RIVM pools, were isolated during the five-year period between 1994 and 1998 (Table 2).

The first of the two polio type 2 Sabin-like viruses was isolated in October 1994 from the feces of a four-year-old

TABLE 2. Isolation of enteroviruses at the Enterovirus Reference Centre for South Greece between 1994 and 1998

1994	1995	1996	1997	1998
3 CAV16	1 CBV2	3 polio type 1 Non-Sabin like	1 ECHO 7	1 polio type 2 Sabin-like
1 polio type 2 Sabin-like	1 ECHO 5			2 nonidentified enteroviruses by RIVM pools
	1 CBV5			

gypsy child with the clinical symptoms of acute flaccid paralysis (AFP); this child had never received any vaccination. The second polio type 2 Sabin-like virus was isolated in November 1998 from a 26-year-old woman also showing the symptoms of AFP; this woman developed the symptoms one and a half months after the vaccination of her three-month-old child with Sabin OPV and she had not been vaccinated before. The results of the polio-type-specific RT-PCR for this isolate led to the conclusion that the isolated strain is a poliovirus type 2. Figure 1 shows the results of the RFLP analysis for the UC_{53}/UG_{52}-produced amplicons of this poliovirus and Figure 2 shows the respective results for the Sabin polio type-2 reference strain, the comparison with which also led to the

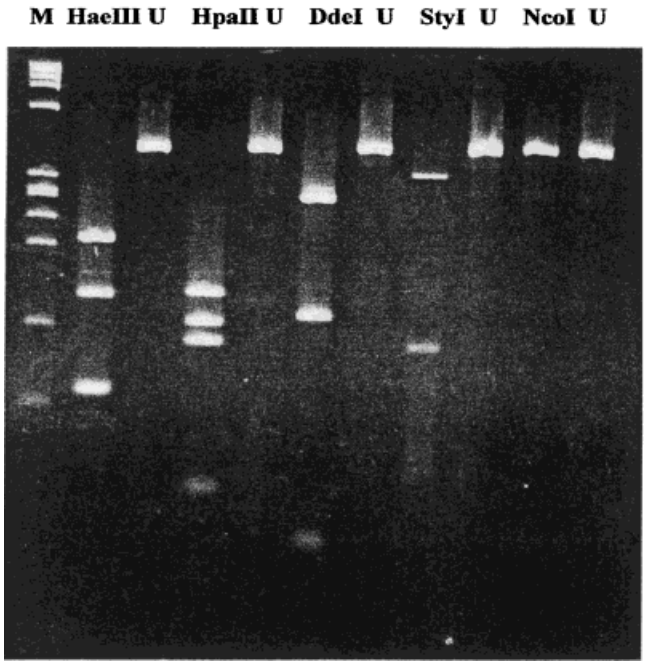

Fig. 1. The results of the RFLP analysis with five different restriction enzymes of RT-PCR amplicons (approx. 440 bp long) produced with the primer pair UC_{53}/UG_{52} from a poliovirus isolated from a clinical sample of an AFP case. M, molecular weight marker ($\phi\chi$174 RF DNA/HaeIII Fragments/Gibco BRL); U, nontreated with restriction endonucleases RT-PCR amplicons. According to the analysis of this figure by GelPro analyzer software, the restriction profile was identical to that of poliovirus Sabin type 2, shown in Figure 2.

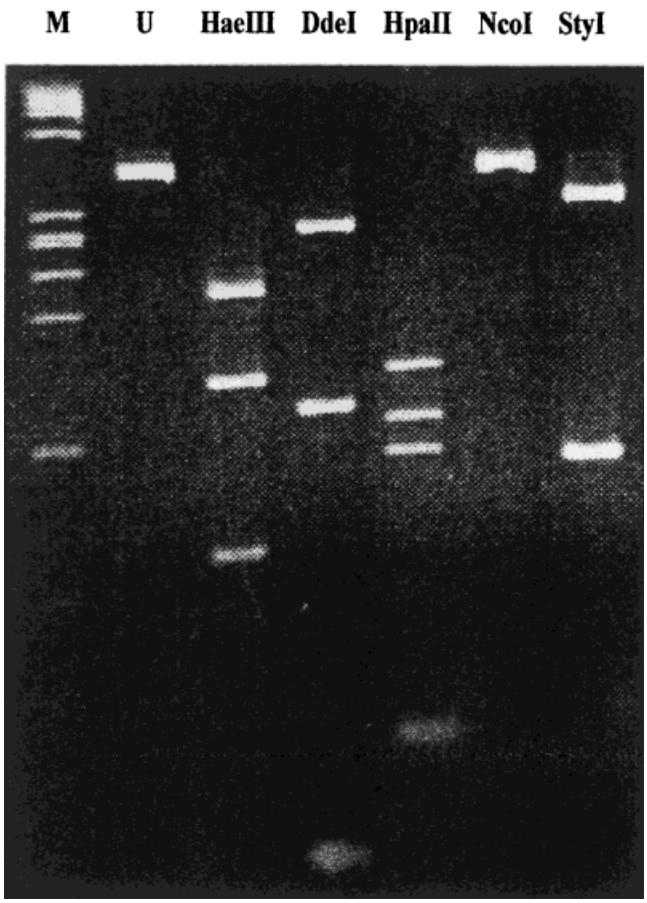

Fig. 2. The results of the RFLP analysis with five different restriction enzymes of RT-PCR amplicons (approx. 440 bp long) produced with the primer pair UC_{53}/UG_{52} from poliovirus Sabin type 2. M, molecular weight marker ($\phi\chi$174 RF DNA/HaeIII Fragments/Gibco BRL); U, "uncut" PCR amplicons. 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: 145, 119, 107, and 42 base pairs; DdeI: 262, 126, and 30 base pairs; StyI: 315 and 103 base pairs; NcoI produced no restriction fragment.

conclusion for the type of the isolated poliovirus. The comparison of the data shown in Figures 1 and 2, apart from indicating the type of the poliovirus, also clarified whether the clinical isolate is a wild-type or a Sabin-like strain. The three non-Sabin-like type 1 polio viruses were isolated by cell culture from the feces of three gypsy children, aged 7 months old, 9 1/2 months old, and 2 1/2 years old, respectively; the reported clinical symptoms for these cases were AFP again and there was not any reported vaccination with OPV. The type of the isolated polioviruses was defined by seroneutralization with the RIVM hyperimmune antisera pools and by polio type-specific RT-PCR (Fig. 3). The identity of the first poliovirus type 2 isolated in 1994 and that of the three polioviruses type 1 with respect to whether they were vaccine strains or not was determined in the molecular epidemiology department at the Pasteur Institute in Paris (Dr. R. Crainic). The three non-Sabin-like polioviruses were PCR-

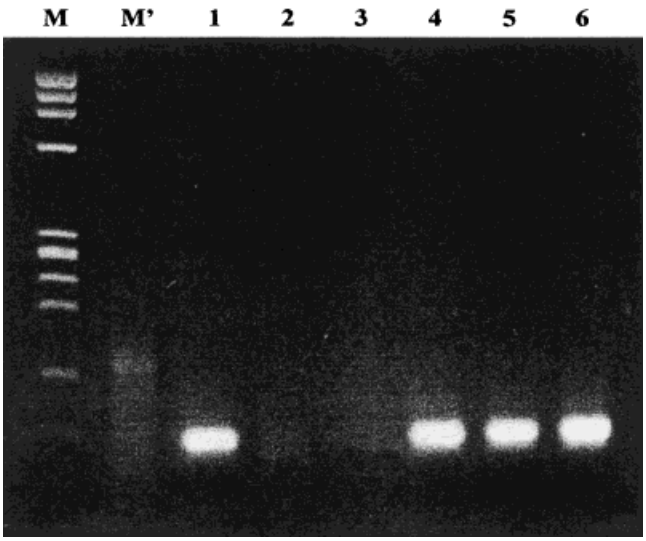

Fig. 3. The results of the polio-type 1-specific RT-PCR with the primer set PV1A/PV1,2S which yield 70 bp-long amplicons for three clinical isolates. Sabin type 1 (lane 1), Sabin type 2 (lane 2), and Sabin type 3 (lane 3) polioviruses were used as controls. M, molecular weight marker ($\phi\chi$174 RF DNA/HaeIII Fragments/Gibco BRL); M′, negative control, derived from RNA isolated from noninoculated cell cultures. The results for the examined clinical samples correspond to lanes 4, 5, and 6.

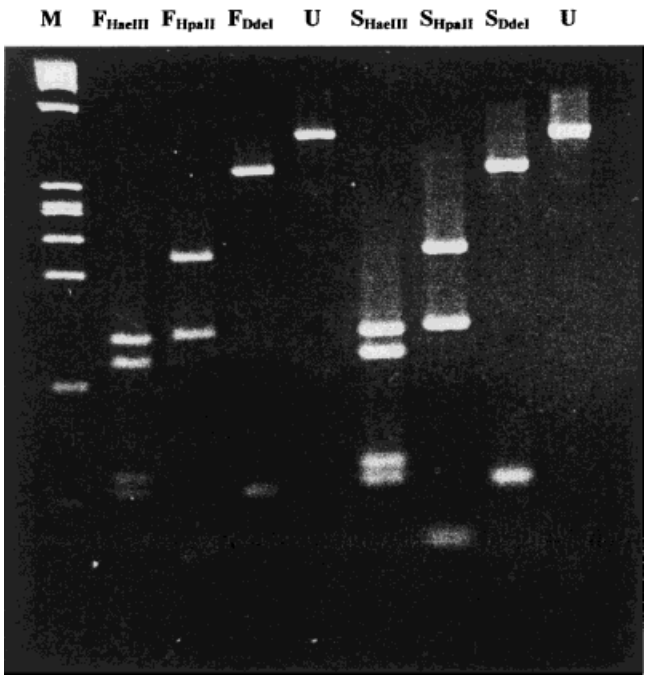

Fig. 4. RFLP analysis of UC_{53}/UG_{52}-generated, approximately 440 bp-long RT-PCR amplicons of enteroviral isolates from a man and his son with the restriction enzymes HaeIII, HpaII, and DdeI. M, molecular weight marker ($\phi\chi$174 RF DNA/HaeIII Fragments/Gibco BRL); F, virus isolated from the father; S, virus isolated from the son; U, respective, "uncut" PCR product. The isolated viruses from both father and son had the same restriction profile and, according to the analysis of this figure by GelPro analyzer software, the following genomic fragments were obtained—HaeIII: 146, 132, 81, and 76; HpaII: 213, 149, and 59; DdeI: 354 and 77.

negative with the set of primers UC_1/UG_1, despite the reported universal character that these primers have in terms of poliovirus-detection specificity (11).

The three Coxsackie A16 (CAV16) viruses were isolated from the fluid of skin vesicles of three children with the hand-foot-and-mouth disease, aged between two and ten years old in the spring of 1994. These strains were verified at the Enterovirus Reference Centre for South France (Prof. M. Aymard). The three Coxsackie B strains (CBV1, CBV2, and CBV5) and the two ECHO strains (ECV5 and ECV7) were isolated and identified by seroneutralization in the summer of 1995, 1996, and 1997 from pharyngeal washings and feces of children, aged between 4 and 7 years old, that showed the clinical symptoms of meningitis.

The two thus far unidentified enteroviruses were isolated from fecal samples of an adult and his 10-year-old son; the father had fever and diarrhea, whereas his son developed the symptoms of meningitis. Figures 4 and 5 show the results of the RFLP analysis of the UC_{53}/UG_{52}-produced amplicons for these isolated enteroviruses, from which it is apparent that both father and son had been infected by the same virus.

DISCUSSION

The enteroviruses have always been interesting due to the wide range of the infections and diseases for which they are responsible. Enteroviral infections are not commonly encountered in Greece, but there have been a few reported cases during the last three decades. The previously mentioned three CAV16 strains that were isolated in 1994 were the first ever observed at the Hellenic Pasteur Institute, although six CAV16 strains were isolated elsewhere in Greece from respective clinical cases of hand-foot-and-mouth disease in the spring of 1974 (13). CAV16 isolations have been generally reported to be distributed world wide from similar cases of hand-foot-and-mouth disease and cases of myocarditis as well (14).

Enteroviruses have become increasingly more important in the last decade, during which wild-type strains reappeared and epidemics of AFP occurred. Such epidemics were observed in Holland in 1992–93, in Israel in 1988, in Oman in 1988–89, and in Bulgaria in 1991 (14); nonvaccinated individuals were especially affected (15). The worst epidemic ever in recent years was in Albania, which started in April, 1996 and lasted until December, 1996. During that epidemic, 138 cases of AFP were reported and 16 of them were fatal (16,17). The three non-Sabin-like type-1 polio viruses that were isolated and presented in this report were verified by the molecular epidemiology department at the Pasteur Institute in Paris (Dr. Radu Crainic) as being similar to the serotypes which circulated in the epidemic in Albania and also in Yu-

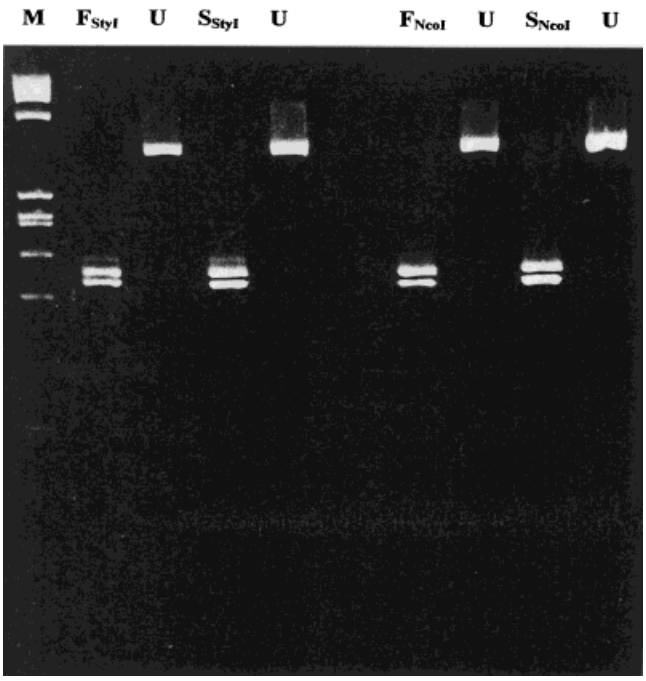

Fig. 5. RFLP analysis of UC_{53}/UG_{52}-generated, approximately 440 bp-long RT-PCR amplicons of enteroviral isolates from a man and his son with the restriction enzymes NcoI and StyI. M, molecular weight marker ($\phi\chi174$ RF DNA/HaeIII Fragments/Gibco BRL); F, virus isolated from the father; S, virus isolated from the son; U, respective, "uncut" PCR product. The isolated viruses from both father and son had the same restriction profile and, according to the analysis of this figure by GelPro analyzer software, the following genomic fragments were obtained—StyI: 221 and 214; NcoI produced the same fragments.

goslavia and Kosovo (16). It is remarkable that this was the first time that wild-type polio virus strains were isolated at the Enterovirus Reference Centre of the Hellenic Pasteur Institute during the 15 years of its operation. It is believed that these strains found their way to Europe from Asia.

Also, it is very interesting that it was not possible to detect these isolates with the aid of the UC_1/UG_1 primer set, something that quite plausibly implies the existence of mutations at the N-terminal half of the VP1 capsid protein in the genome of the isolated viruses. During the same period, two other type-1 non-Sabin-like polioviruses were isolated at the department of virology of the Aristotle University in Thessaloniki (16). The previously encountered incidents of poliovirus infections from cases of paralytic disease in our laboratory were those in 1981–82; the patients were children that had been recently vaccinated and the isolated viruses were indeed Sabin-like, leading to their identification as the etiologic agents for this plausible VAPP (Vaccine-Associated Paralytic Poliomyelitis).

In conclusion, enterovirus infections are not commonly encountered in Greece. The whole project, though, could be considered from two different points of view.

First, how effective are the current means for the correct, rapid, and reliable identification of circulating enteroviruses? Seroneutralization by equine, mixed hyperimmune sera is currently the most widely accepted by WHO-appointed laboratory methodologies for this purpose but, nevertheless, there are several drawbacks. These include the failure to identify all of the known enteroviruses and the so-called "prime" strains (antigenic variants of existing strains), the possible inaccuracy due to the high mutable nature of the enterovirus antigenic sites, and the inability to identify newly encountered viruses. Apart from this restricted reliability there are problems of a more practical nature as well; this technique is time-consuming and laborious. Modern molecular techniques should provide a more efficient means of typing clinical isolates in terms of speed, accuracy, and potential. In this report, three enteroviruses were presented for study by RT-PCR/RFLP of amplicons originating from the 5′-UTR. This highly conserved region of the genome due to its significant biological importance was chosen in order to reduce the risk of intratypic variation but to allow for any intertypic differences to be plausibly detected (1,18). Other researchers have reported the development of a nested PCR with specific primers derived from the 5′-UTR and consensus region of the human enterovirus genome in order to differentiate enterovirus RNA from different serotypes of prototypic strains (19). Furthermore, they used restriction fragment-length polymorphism analysis of 5′-UTR amplicons as an alternative and determined that 14 out of 16 enterovirus-infected specimens exhibited restriction patterns identical to those of the corresponding prototypes. The examination of the genomic variability of poliovirus by analyzing the restriction fragment-length polymorphism analysis of genomic fragments from the N-terminal half of the capsid protein VP1, including antigenic site 1 has also been reported (11). It was concluded in this study that such an RFLP analysis may be used to identify and characterize poliovirus genotypes circulating in nature. Nevertheless, the value of this method has yet to be determined due to its disadvantages: its inability to correctly identify enterovirus mixture—something that can be resolved by hybridization and gene cloning techniques—and the possible existence of mutations and rearrangements in the region, such as the 100-nucleotide duplication discovered in a downstream region of poliovirus 5′-UTR involved in the translational control (20). However, the advantages of this modern molecular technique over the conventional ones are highly significant, suggesting that they provide more accurate and rapid results and the potential to study in more detail the epidemiology and the evolutionary biology of enteroviruses.

Secondly, will the WHO target of achievement of zero poliomyelitis cases caused by wild poliovirus and the absence of wild poliovirus in all clinical and environmental samples obtained throughout the world be met by the end of the year 2000? This will depend on the crucial role that laboratories play in the effective surveillance of poliomyelitis and AFP cases, on the correct identification of any circulating wild

poliovirus, on the vaccination schemes against polio, and on the political dedication to the poliomyelitis eradication of all nations. In a number of developed countries where vaccination is applied effectively, no poliomyelitis cases, or only a few, have been reported for several years—those that were have been found to be of vaccine origin, like the Sabin-like strains presented in this report. The eradication of wild polioviruses has been reported in several European, American, Asian, and Western Pacific countries but, nevertheless, many cases of polio are still reported each year, with many more being unreported, although the number of cases is steadily decreasing. In the 1980s, more than 200,000 cases a year were reported on average, whereas 3,896 cases were reported in 1996, and only 136 cases were reported during the first months of 1997 (21,22). The recent epidemic in Albania is a warning to all countries, and more effective surveillance, research, and immunization practices should become the focus before the international goal of polio eradication can become a reality.

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