D. Papaventsis,^{1,2,3} N. Siafakas,^{1,4} P. Markoulatos,⁴* G. T. Papageorgiou,⁵ C. Kourtis,⁵ E. Chatzichristou,² C. Economou,² and S. Levidiotou³

Department of Virology, National Reference Enteroviruses Center, Hellenic Pasteur Institute,¹ and Technological

Educational Institution,² Athens, Department of Microbiology, Medical School, University of Ioannina,

Ioannina,³ and Microbiology-Virology Laboratory, Department of Biochemistry and Biotechnology,

University of Thessaly, Larissa,⁴ Greece, and Microbiology Section, State

General Laboratory, Ministry of Health, Nicosia, Cyprus⁵

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We present a new approach for the detection and identification of enteroviruses concentrated and isolated from sewage. Samples were collected from two study sites located at Nicosia and Limassol sewage treatment plants in Cyprus. Viruses were adsorbed to cellulose nitrate membrane filters, cultured directly from the membrane filters by using the VIRADEN method, and identified by reverse transcription-PCR, followed by 5' untranslated region (5'-UTR) restriction fragment length polymorphism (RFLP) analysis and partial sequencing of the VP1 protein coding region. Initial subgrouping based on the HpaII restriction profile showed that all of the isolates except one belonged to the same genetic subcluster. Partial VP1 sequencing revealed that most isolates belonged to serotypes coxsackie B4 (42.5%) and coxsackie A9 (30%), whereas coxsackie B2 (17.5%) and coxsackie B1 (3%) isolates were less frequently observed. One poliovirus type 2 isolate (2.5%) of vaccine origin was also found. The HpaII digests predicted the genetic subcluster for all isolates. They also accurately differentiated the isolates as nonpolio or polio isolates. This approach seems to be very promising for environmental surveillance of enterovirus circulation and epidemiology, with all of the significant effects that this entails for public health. Partial VP1 sequencing is efficient for molecular serotyping of enteroviruses, while 5'-UTR RFLP analysis with HpaII can also be considered an asset for the initial subclassification of enterovirus isolates.

Approximately 140 types of viruses may contaminate water and wastewater, and enteroviruses are the most frequently detected viruses (30). The enterovirus genus, which is the most important genus of the *Picornaviridae* in terms of human pathogenicity (16, 36), contains 65 immunologically distinct human serotypes in five Human enterovirus species (Human enterovirus A to D and Poliovirus) (14). It consists of polioviruses (PV) (three serotypes), coxsackie A viruses (CAV) (23 serotypes), coxsackie B viruses (CBV) (six serotypes), echoviruses (28 serotypes), and enteroviruses 68 to 71 and 73. The clinical manifestations and diseases caused by human enteroviruses range widely from asymptomatic infections and the common cold to fatal cases of meningitis, encephalitis, and poliomyelitis. Central nervous system diseases are common manifestations of infections caused by several different types of enteroviruses, and aseptic meningitis is the most common such disease recorded (16). Other less common central nervous system diseases include encephalitis, Guillain-Barré syndrome, paralysis, cerebellar ataxia, and peripheral neuritis (16). Enterovirus infections of the myocardium are also extremely important, since they are the most common cause of acute myo-

* Corresponding author. Mailing address: Microbiology-Virology Laboratory, Department of Biochemistry & Biotechnology, University of Thessaly, Ploutonos 26 & Aeolou str., Larissa 41221, Greece. Phone: 30 2410 565274. Fax: 30 2410 565290. E-mail: markoulatos @bio.uth.gr. carditis and they have also been implicated in dilated cardiomyopathy (especially CBV serotype 1 to 5 infections), which is one of the most common cardiac diseases requiring heart transplantation.

Enteroviruses are transmitted by a fecal-oral cycle, multiply in the gastrointestinal tract, and are finally excreted in large numbers into the environment through feces. Although these viruses are readily found in fecally contaminated waters, waterborne enterovirus infection has only occasionally been documented because many infections caused by these agents are subclinical (30).

Identification of enterovirus isolates does not have a significant impact on the clinical management of infected individuals. Nevertheless, it can contribute significantly to the identification of various epidemics and to the subsequent effective surveillance of populations by determining the source of infection, the correlation between enterovirus serotype or strain and clinical symptoms, the characteristics of particularly virulent viruses, the possible means of transmission, and the emergence of new strains or the reemergence of older strains. Moreover, a means for accurate distinction between polio and nonpolio isolates is essential for public health polio surveillance programs that aim to eradicate wild-type polioviruses.

Environmental surveillance has been successfully used in monitoring enteric virus circulation and in assessing the extent or duration of epidemic poliovirus infection in specific conditions (3). The methods used for identification of enteroviruses from raw sewage and sewage effluents have many limitations, mainly due to the nature of the samples examined. Raw sewage usually contains organic compounds at high concentrations, which may interfere with either the adsorption of the virus on a membrane filter or the cultivation of the virus in tissue cultures. Moreover, a large proportion of viruses found in raw sewage are associated with solids, which leads to major technical limitations due to incomplete virus elution. A simple, low-cost, efficient method for concentrating and counting cytopathogenic viruses present in wastewater after the viruses are adsorbed to cellulose nitrate membrane filters has recently been described (26). This method, which is referred as VIRADEN (an acronym derived from virus adsorption enumeration), is suitable for testing raw sewage and secondary sewage effluents in volumes large enough to find significant numbers of enteroviruses.

Typing of concentrated enteroviruses is based mainly on (i) serotyping with equine type-specific hyperimmune sera that have been mixed to obtain intersecting pools (16) and (ii) use of molecular methods based on reverse transcription PCR (RT-PCR) of RNA extracted from preparations showing cyto-pathic effects on cell monolayers and application of other molecular techniques (notably restriction fragment length polymorphism [RFLP] analysis and sequencing) (20, 29). However, serotyping by seroneutralization is time-consuming, labor-intensive, and costly, the antiserum supply is limited, and there is still the problem of untypeable enteroviruses.

A new approach for molecular serotyping of enteroviruses concentrated and isolated from sewage samples by the VIRADEN method was developed in the present study. We performed both RFLP-based molecular typing of the 5' untranslated region (5'-UTR) of isolated enteroviruses in genetic clusters, using restriction endonuclease HpaII as previously described (35), and molecular serotyping of a portion of the VP1 gene, based on RT-PCR amplification and sequencing (22). The combination of these techniques was tested by using raw sewage collected at Nicosia and Limasol sewage treatment plants in Cyprus. This approach may be used as a rapid, lowcost, efficient method for environmental surveillance of circulating enteroviruses in sewage.

MATERIALS AND METHODS

Sampling sites. Six sewage samples were collected between April and December 2003 from two study sites located at the Nicosia and Limassol sewage treatment plants in Cyprus. The Nicosia sewage treatment plant is a waste stabilization pond plant that receives about 13,000 m³ of sewage effluents per day. The Limasol sewage treatment plant is one of the most sophisticated activated sludge sewage treatment plants in Cyprus. It is relatively new (it was built in the mid-1990s), serves a total population of 80,000 people, and receives 15,000 m³ of sewage effluents per day on average.

Collection of sewage samples. Trained local authorities collected samples in April, May, September, and December 2003. The existing sewage sample collection system was used for assessment of the microbiological and chemical quality of sewage effluents. One-liter grab samples were collected as recommended previously (3) during the peak morning flow. The samples were transported in clean, sterile, leak-proof containers at 4°C. Samples were transported and analyzed on the day of collection.

VIRADEN method. (i) Cells, media, and reagents. The Buffalo green monkey (BGM) continuous cell line was used for propagation of viruses. Cells were grown in Eagle's minimum essential medium with Earle's salts (Gibco, Invitrogen, Paisley, United Kingdom) containing 5% fetal bovine serum, 2 mM L-glutamine, 26.8 mM NaHCO₃, 100 U of penicillin per ml, and 100 μ g of streptomycin per ml.

The overlay medium used for the standard plaque assay was medium 199 with Earle's salts (Gibco) supplemented with 2% fetal bovine serum, 26.8 mM NaHCO₃, 100 U of penicillin per ml, and 100 mg of streptomycin per ml. The overlay medium was prepared as a $2\times$ stock and was mixed before use with an equal volume of 2% purified agar (Oxoid, Basingstoke, Hampshire, United Kingdom).

Penicillin (100 U/ml), streptomycin (100 mg/ml), gentamicin (50 mg/ml), nystatin (50 mg/ml), and ceftazidime (20 mg/ml) were added to the growth and overlay media. All of the antibiotics except ceftazidime were obtained from Gibco. Ceftazidime (Ceftazidime Fortum 1g) was obtained from GlaxoWellcome (Greenford, United Kingdom). Ceftazidime was diluted in sterile distilled water to a concentration of 10,000 mg per ml and was stored at -20° C. Defrosted ceftazidime was used within 7 days.

(ii) Membrane prefilters and filters. The suspended solids and the microbial load were removed from a sample by filtering it through 0.22- μ m-pore-size, 33-mm-diameter, syringe-driven, hydrophilic polyester sulfonate filter units (SLGP 033 RS; Millipore Corp., Bedford, Mass.) as previously described (18, 19). Cellulose nitrate filters with a pore size of 3 μ m and a diameter of 47 mm (Millipore) were used to concentrate viruses from the samples.

(iii) Virus adsorption on the membranes and detection by the VIRADEN method. Viruses present in the filtered raw sewage samples were first adsorbed to the cellulose nitrate membrane filters by using the VIRADEN method as previously described (26). Briefly, a sample was amended by adding $MgCl_2 \cdot 6H_2O$ to a final $MgCl_2$ concentration of 0.05 M. Then it was filtered through a 47-mm-diameter, 3-µm-pore-size cellulose nitrate membrane at a flow rate that did not exceed 200 ml per min. When the entire sample had been filtered, the membrane filter was washed by passing 100 ml of sterile 0.05 M MgCl_2 through it. The volumes examined for each sample water (20 to 30 ml) was added to the funnel before the sample was poured in order to make the flow rate more realistic and to evenly disperse the viruses on the filter. The VIRADEN method can be used for raw sewage samples with volumes up to 20 ml without a significant loss of recovery (19).

Finally, the viruses adsorbed on a membrane filter were detected and counted on a BGM monolaver as follows. The growth medium in a 60-mm-diameter petri dish with a confluent monolayer was discarded. Then 100 µl of a suspension of BGM cells in Eagle's minimum essential medium with Earle's salts supplemented with antibiotics containing $1.75 \times 10^7 \pm 0.25 \times 10^7$ cells per ml was placed in the center of the petri dish. The membrane filter with the adsorbed viruses was placed upside down on top of the suspension and the cell monolayer. Five milliliters of overlay medium was then poured slowly onto the center of the membrane filter and spread all over the plate. The whole procedure was performed under aseptic conditions. The agar was allowed to set protected from light, and the petri dishes were incubated at 37°C in the presence of 5% CO2 at a relative humidity of more than 80% for 48 to 72 h. We did not use neutral red in our overlay medium since the plaques were visible without it. Addition of neutral red (1 ml of a 0.01% solution in 200 ml of overlay agar medium) could enhance plaque visibility, but care had to be taken to protect the cultures from direct light. After incubation, both the agar and the membrane were carefully detached with a spatula. The plate was examined carefully from the top in order to observe any cytopathogenic effects on the cell monolayer in the form of plaques. Virus material was picked from each plaque after resuspension in 3 to 4 µl of sterile distilled water by using a sterile pipette tip. It was then used to inoculate growing BGM cells in 24-well tissue culture trays. Mixing of material from different plaques was avoided. The final products, subcultures in liquid medium, were kept at -20° C until they were examined further.

RNA extraction. Viral RNA extraction was carried out by the method described by Casas et al. (6), and it was applied directly to the VIRADEN final products. The RNA extraction technique involved the use of a lysis buffer, which contained the chaotropic agent guanidinium thiocyanate acid (GuSCN) buffer and did not contain organic solvents. Specifically, 100 µl of a VIRADEN final product was lysed in 400 µl of GuSCN buffer. Following addition of 50 µg glycogen, the mixture was incubated for 20 min at room temperature. Then 500 µl of isopropyl alcohol (-20°C) was added to precipitate the nucleic acids, and the preparation was incubated on ice for 20 min. The mixture was centrifuged at $14,000 \times g$ for 10 min at 4°C, the supernatant was discarded, and the pellet was washed with 1 ml of 70% ethanol. The centrifugation at 14,000 \times g for 10 min at 4°C was repeated, and the supernatant was discarded again. The pellet was left to dry at 70°C for 5 min and was finally dissolved in 50 µl of double-distilled H₂O. The efficiency of the RNA extraction procedure used and the presence of inhibitors of RNA amplification in each specimen were evaluated with α-tubulin primers in a separate PCR assay (15). Moreover, poliovirus Sabin type 1 was used as a positive control (33).

RT-PCR. Isolated RNA was converted to cDNA by reverse transcription; 40 U of RNase inhibitor (Promega Corporation, Madison, Wis.), 2 µl of d(N)9 primers (50 pmol/µl; Takara, Tokyo, Japan), and 5 µl of extracted RNA from each sample were initially mixed and heated at 70°C for 5 min. The tubes were immediately transferred to ice, and 5 μl of 5 \times RT buffer, 5 μl of a preparation containing each deoxynucleoside triphosphate at a concentration of 10 mM, 100 U of Moloney murine leukemia virus reverse transcriptase (Promega Corporation), and 6.5 µl of RNase-free water (Sigma Aldrich) were added to each tube; the total volume of the reaction mixture was 25 $\mu l.$ This mixture was incubated at 37°C for 1 h, and heating at 95°C for 5 min was used to inactivate the Moloney murine leukemia virus reverse transcriptase. The cDNA produced was amplified by PCR by using a reaction mixture (50 μ l/tube) containing 5 μ l of 10× PCR buffer, 5 µl of a preparation containing each deoxynucleoside triphosphate at a concentration of 10 mM, 2 µl of 50 mM MgCl₂ (resulting in a final MgCl₂ concentration of 2 mM), 32.6 µl of RNase-free water, 2 U of Taq polymerase (BIOTAQ, Moscow, Russia), 5 µl of cDNA, and 2 µl of each of the two primer pairs (either UC53 and UG52 [20 pmol/tube] or 292 and 222 [50 pmol/tube]). Forty cycles of denaturation at 94°C for 10 s, annealing at 42°C for 30 s, and extension at 74°C for 10 s, followed by incubation for 15 min at 78°C in order to complete the extension of the primers, were performed with a Perkin-Elmer 9600 thermal cycler. Ten microliters of each amplified product was analyzed by agarose gel electrophoresis by using 2.5% agarose (ultrapure; electrophoresis grade; Gibco BRL) containing 1 µg of ethidium bromide per ml in Tris-boric acid-EDTA buffer. The amplicons were then visualized with a FOTO/PHORESIS I UV transilluminator (Fotodyne, Hartland, Wis.).

The antisense primer UC₅₃ (5'-TTGTCACCATAACCAGCCA-3'; positions 583 to 601 in the genome of CAV9 reference strain Griggs) and the sense primer UG₅₂ (5'-CAAGCACTTCTGTTTCCCCGG-3'; positions 167 to 187 in the genome of CAV9 reference strain Griggs) were selected to be homologous to the corresponding parts in the highly conserved 5'-UTR; they were synthesized by and purchased from Genosys Biotechnologies (Cambridge, United Kingdom). These primers yielded amplicons that were 435 bp long; they were adjusted to a concentration of 10 pmol/µl in sterile distilled water and were stored at -20° C (11, 32, 33, 34).

The sense primer 292 (5'-MIGCIGYIGARACNGG-3'; positions 2612 to 2627 in the genome of PV1 reference strain Mahoney) and the antisense primer 222 (5'-CICCIGGIGGIAYRWACAT-3'; positions 2969 to 2951 in the genome of PV1 reference strain Mahoney) were used to amplify a portion of the gene encoding VP1 capsid protein (23, 24). These primers yielded amplicons that were approximately 340 bp long; they were adjusted to a concentration of 50 pmol/µl in sterile distilled water and were stored at -20° C.

All procedures were carried out under conditions that minimized the risk of contamination from exogenous nucleic acid sources or carryover of amplification products during RT-PCR. There was physical separation of the pre- and post-PCR procedures in 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). Negative controls were used in each amplification assay and were always RT-PCR negative, which indicated the effectiveness of these preventative measures.

Restriction fragment length polymorphism analysis of UC53-UG52-produced RT-PCR amplicons of the VIRADEN isolates. In a previous study it was shown that RFLP analysis of the 5'-UTR with the enzyme HpaII could be used to accurately classify both reference and wild-type strains into five different genetic subclusters with no intraserotypic variation in the HpaII-produced haplotypes (35). Therefore, an initial RFLP analysis of the UC53-UG52-produced RT-PCR amplicons with the restriction enzyme HpaII (New England Biolabs, Beverly, Mass.) was carried out as described previously. Briefly, 1 µl of the appropriate $10 \times$ buffer, 20 U of the restriction enzyme (2 µl), and 1 µl of distilled, RNasefree, sterile water (Sigma Aldrich) were added to 6 µl of each of the UC53-UG52-produced RT-PCR amplicons of the VIRADEN isolates to obtain a final volume of 10 µl. The samples were then incubated at 37°C for 2 h, and the products were subjected to electrophoresis in 3% agarose gels made from highresolution agarose (Metaphor FMC Bioproducts, Rockland Maine) containing 1 μg of ethidium bromide per μl and visualized with a UV transilluminator. The results were analyzed by using the GelPro Analyzer software (Media Cybernetics, Silver Spring, Md.).

Sequence and phylogenetic analysis of the 292-222-produced RT-PCR amplicons of the VIRADEN isolates. As there cannot be a direct correlation between the 5'-UTR and enterovirus serotype (35), the partial VP1 sequences of the environmental isolates were also obtained for serotypic identification of these isolates by using the model proposed by Oberste et al. (22, 24), since the VP1 gene contains important serotype-specific epitopes. The 292-222-produced amplification products of the VIRADEN isolates were extracted from the electrophoresis gels with a NucleoSpin extract isolation kit (Macherey-Nagel, Düren, Germany) and sequenced by Macrogen Inc. (Seoul, Korea). The serotypic identity of each isolate was deduced by comparison of the partial VP1 sequences with the sequences of the corresponding genomic regions of all human enteroviruses which are available in the GenBank database by using the program BLAST, version 2.2.8 (http://www.ncbi.nlm.nih.gov/BLAST) (1, 2).

A phylogenetic analysis was carried out by pairwise comparison of the partial VP1 sequences of the enterovirus isolates and the corresponding partial VP1 sequences of reference and wild-type human enterovirus strains of the same serotype, which are available from GenBank, by using ClustalW (www.ebi.ac.uk //ClustalW) (39). Construction of the phylogenetic tree was carried out with ClustalX (version 1.83) by using the alignment file obtained by analysis with ClustalW. By using this program the distances between all pairs of sequences were calculated, and this was followed by application of the neighbor-joining method to the distance matrix. Confidence values for the groups in the tree (bootstrap values on a scale from 1 to 1,000) were also calculated by using ClustalX. A dendrogram showing the phylogenetic relationships among the enterovirus isolates used in the present study and prototype strains was plotted in the PHYLIP format output by using the TreeView software (version 3.0), which was obtained from the website of the University of Glasgow (http:\\taxonomy .zoology.gla.ac.uk/rod/rod/html).

Nucleotide sequence accession numbers. The accession numbers of all the virus sequences obtained in this and previous studies that were used for the phylogenetic comparison are shown in Tables 1 and 2.

RESULTS

A total of 40 VIRADEN isolates were concentrated from six sampling procedures carried out at two sites, Nicosia and Limassol (Table 1). From sampling A (Nicosia, April 2003), six plaques were picked and characterized by RT-PCR. Similarly, seven plaques from sampling B (Nicosia, May 2003), six plaques from sampling C (Nicosia, September 2003), 10 plaques from sampling D (Limassol, September 2003), four plaques from sampling E (Nicosia, December 2003), seven plaques from sampling F (Limassol, December 2003) were examined.

All the samples produced cytopathogenic effects in the BGM cell line, which indicated the presence of propagating, potentially infectious viruses. The expected amplification products, which were approximately 435 nucleotides long with primers UC₅₃ and UG₅₂ and approximately 350 nucleotides long with primers 292 and 222, were successfully obtained (Fig. 1a). Finally, amplification of all the isolates with the α -tubulin-specific primers yielded positive results (data not shown), which proved the effectiveness of the GuSCN-based RNA extraction procedure for both the recovery of a sufficient quantity of RNA from the environmental samples and the elimination of PCR-inhibiting substances.

The results of the RFLP analysis of the initial subgrouping of the enterovirus isolates with restriction endonuclease HpaII are shown in Fig. 1b. All of the isolates except one (isolate A5) belonged to the same genetic subcluster, and the restriction profile comprised four HpaII-generated fragments with lengths of 213, 149, 55, and 18 nucleotides. This genetic subcluster included 38 different serotypes of the *Human enterovirus B* species, which was previously described in detail (35), and therefore, an initial assumption was made about the possible serotypic group that the isolates belonged to. Isolate A5 was the only isolate which belonged to a different genetic subcluster, and the restriction profile included five HpaII-generated fragments that were 148, 121, 108, 40, and 18 nucleotides long. This genetic subcluster contained three enteroviruses of the polio-like cluster on the basis of the 5'-UTR classification, the

Pk1pak92

BE00-117

48112fin98

9128net93

10199

10198

10197

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10195

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10192

10191

10190

TABLE	1.	Collection si	ites, da	ates, '	VIRADEN	isolates,	and
		GenBank	k acces	ssion	numbers		

 TABLE 2. Accession numbers of reference and wild-type human enterovirus strains used for the phylogenetic analysis

AF160019

AF521311

AF160025

AF160021

AY373132

AY373131

AY373130

AY373129

AY373128

AY373127

AY373126

AY373125

AY373124

AY373123

Location Date" VIRADE N solate sequence accession no. PV2 Lansing sequence accession no. M1207 Nicosia April A1 AY56332 W-2 M0055 Nicosia April A1 AY56332 W-2 M0057 Nicosia A2 AY563351 HEF-1 AY28473 A3 AY563353 HGV98-074 AP448783 A5 AY563353 HGV98-074 AP448783 A6 AY563355 HC920-0743 AP448783 B6 AY563355 H2200-0743 AP448783 B7 AY563356 Belnikue798 AP351889 B8 AY563359 Belnikue798 AP351889 B9 AY563361 AP551849 AP351849 B10 AY563364 H498 H1898 AP28978 Nicosia September LK1 AY563364 H498 H2897 Limassol September L1 AY563370 H498 H199-H224 AY34202 Limassol		D -		VP1	Serotype	Strain	Accession no.
Nicosia April A1 AY563382 A2 Salin Salin X0055 Nicosia April A1 AY563382 A2 W-2 D00625 A3 AY563351 MEF-1 AY28479 A4 AY26479 A75 AY28479 A4 AY27850 AY27850 A4 AY563353 ECV38-074 AY27850 A75 AY27850 A75 AY27850 A75 Nicosia May B1 AY563355 P2NMog65-1 AY27850 A7278550 B5 AY563355 B0055-2 AY27850 A7278550 A7551843 B6 AY563356 Benburg98 AF551843 B750 AY563350 Benburg98 AF551843 B9 AY563362 4486 L15898 A25807 Nicosia September LK1 AY563362 4486 L15898 A228075 Limassol September L1 AY563366 CAV9 GRIGGS O00627 Limassol December L1 AY563370 B101-2230 AY342746 L2 AY563370 B20	Location	Date ^a	VIRADEN isolate	sequence accession no	PV2	Lansing	M12197
Nicosia April A1 AY563352 W-2 D00053 A3 AY563351 MEF-1 AY228473 AF448750 A3 AY563352 ECY38-074 AF448750 A4 AY563353 ECY38-074 AF448750 A5 AY563355 ECY38-074 A7278551 A5 AY563355 ID2050 A7545135 B4 AY563357 Belnisue198 A7551835 B6 AY563357 Belnisue198 A7551836 B6 AY563357 Belnisue198 A7551836 B700050 A7551836 A623 115808 A725808 B70 AY563357 Belnisue198 A7551836 B70 AY563350 A808 1/51098 A128006 A7503361 A99 AY563362 A741/15808 A128006 LK1 AY563361 A99 A128066 A228067 LK2 AY563361 A99 A128066 A128067 LK1 AY563362 CAV9 G140763 A128066						Sabin	X00595
A2 A7563351 MEP-1 A728473 A3 A7563352 ECY98-074 AP448782 A4 A7563353 ECY98-074 AP448782 A5 A7563353 ECY98-074 AP448782 A5 A7563353 ECY98-074 AP448782 A6 A7563354 ECY98-074 AP278551 A6 A7563355 E00050 A7563355 PSNMog65-2 A7278551 PSNMog65-2 A7278551 B6 A7563355 Belbeira AP551838 B7 A7563350 Astatististististististististististististist	Nicosia	April	A1	AY563382		W-2	D00625
A3 AY563352 ECY98-074 AP448783 A4 AY563353 ECY93-024 AP448783 A5 AY563353 ECY93-024 AP448783 A5 AY563354 PENhog61 AY72851 Pishog62 AY27851 PENhog62 AY27851 Pishog62 AY27851 PENhog64 AY27851 Pishog62 AY27851 PENhog66 AY27851 Pishog62 AY27851 PENhog66 AY27851 B6 AY563357 Behicar97 AF51848 B7 AY563361 A8089 A28066 B9 AY563362 4741/15808 A28067 Kicosia September LK1 AY563362 4625/15898 A28067 Lika AY563361 A808/1/5808 A28067 A28067 Limassol September LK1 AY563360 GR10GS D00627 Limassol December L1 AY563364 4551 AY34274 Limassol December L1 AY5633			A2	AY563351		MEF-1	AY238473
A4 AY503353 A5 ECY93104 A75 AF44783 A75 AF44783 A75 AF44783 A75 AF44783 A75 AF44783 A75 AF44783 A75 AF44783 A7278551 B75 AF44783 A7278551 B75 AF44783 A7278551 B75 AF44783 A7278551 B75 AF44783 A7278551 B75 AF44783 A7278551 B75 AF44783 A7278551 B75 AF44783 A7278551 B75 AF44783 A7278551 B75 AF44783 B75 AF44783 A72785181 Nicosia B1 AY563357 Beleira/97 AF551838 AF551838 AF551839 AF551839 AF551839 AF551839 AF551840 AF551840 AF551840 AF551840 AF551840 AF551840 AF551840 AF551839 AF551840 AF551845 AF551840 AF5451879 AF54787 AF54787 AF547878 AF547878 <td< td=""><td></td><td></td><td>A3</td><td>AY563352</td><td></td><td>EGY88-074</td><td>AF448782</td></td<>			A3	AY563352		EGY88-074	AF448782
A5 AY563379 P25M0805-1 AY26339 Nicosia May B1 AY563355 P2SM0805-1 AY27855 Nicosia May B1 AY563355 P2SM0807-2 AY27855 B4 AY563355 B00250798 AP551838 AP551839 B5 AY563350 Beheira/97 AP551839 Beheira/97 AP551839 B6 AY563350 Beheira/97 AP551839 Behaira/98 AP551830 B9 AY563360 Alexandria/98 AP551840 BP51840 B9 AY563362 4488 L151808 AD28075 Nicosia September I.K1 AY563362 4488 L151808 AD28075 LK2 AY563363 GAUS 4538 AD28075 AD28075 LK3 AY563365 CAV9 GRIGOS D00627 AB167980 LK1 AY563364 4398 L151808 AD28066 AP380144 AP380144 AP380144 AP380144 AP38014575 AP342744 AP342744			A4	AY563353		EGY93-034	AF448783
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Nicosia May Bi AY563355 102050 AY563355 Nicosia May Bi AY563355 102050 AJ56451 Nicosia Bi AY563355 102050 AJ56451 Bi AY563357 Beheira/97 AJ56350 Bi AY563350 Sahag/00 AF551840 Bi AY563350 Sahag/00 AF551840 Bi AY563360 Alexant/inv08 AF551840 Bi AY563361 Alexant/inv08 AF551840 Bi AY563362 4408 LISR8 AJ28074 Alexant/inv08 AJ28074 AJ28074 AJ28074 LiX AY563361 4252 LISR8 AJ28074 LiX AY563365 CAV9 GRIGCS D00627 Lixia AY563361 BE0-4239 AY34274 Lixia AY563361 BE9-4750 AY34274 Lixia AY563351 BE0-4239 AY34274 Lixia AY563350 BE9-4750 AY3			A6	AY563354		P25/M0g05-1	AY2/8550
Nicosia May B1 AY563355 1000000000000000000000000000000000000						P25/M0g00-4 P25/Mog65-2	AY2/8551
Anom B4 AY563356 Henking (9) AF551837 B5 AY563357 Beheiray (9) AF55183 B6 AY563358 Sahag00 AF55183 B8 AY563359 Ackang (9) AF55183 B9 AY563350 Gizag/98 AF551841 B9 AY563360 Gizag/98 AF551830 B10 AY563361 Aswan (9) AF551841 B10 AY563361 Aswan (9) AF551830 B10 AY563362 4774 H/JSR98 AJ28807 LK2 AY563364 4625 H/JSR98 AJ288067 LK4 AY563366 CAV9 GR16056 D00627 LK14 AY563380 G3-141PC2 AB167990 Limassol September L1 AY563380 BE01-4075 AY342746 Li AY563380 BE01-4075 AY342746 AY342746 Limassol December L1 AY563370 BE01-4075 AY342746 L1 AY563370	Nicosia	May	B1	AY563355		102050	A 12/0332 A 15//513
B5 A Y563357 B6 Beheimung A Y563359 Beheimung A A231/SR08 A A25182906 A A251839 B6 A Y563350 Sahag00 A F551840 B9 A Y563350 Gitta Samper Sa	Neosia	intay	B4	AY563356		BeniSuef/98	AF551838
B6 AY563358 462 JISR98 AD28906 B8 AY563350 Salag00 AF531841 B9 AY563360 Gra/98 AF531841 B9 AY563361 Aswam/95 AF531840 B10 AY563361 Aswam/95 AF531840 Nicosia September LK1 AY563362 4774 JISR98 AD288067 LK2 AY563363 4662 JISR98 AD288067 LK1 AY563365 LK1 AY563365 LK1 AY563366 CAV9 GRIGGS D00627 AB167970 03-171FCR2 AB167970 AB167970 03-132NPR2 AB167970 AY342746 AY342745 AY342746 AY363370 BE01-4533 AY342746 AY342745 AY342745 AY342745 AY342745 AY342745 AY342745 AY342745 AY363370 BE01-4075 AY342745 AY342745 <td></td> <td>B5</td> <td>AY563357</td> <td></td> <td>Beheira/97</td> <td>AF551837</td>			B5	AY563357		Beheira/97	AF551837
Bit A Y56339 Saling00 A P551840 B9 A Y365361 Aexandria/98 A P551840 B10 A Y365361 Aexandria/98 A P551840 B10 A Y365361 Aexandria/98 AE551840 Kicosia September LK1 A Y563362 4708, L/ISR98 AL288074 LK2 A Y363364 4625, L/ISR98 AL288074 KIS AY263364 4588, L/ISR98 AL288074 LK3 A Y563364 LK3 AY563364 4588, L/ISR98 AL288074 LK4 A Y563366 CAV9 GRIGGS D00627 AB167979 Limassol September L1 A Y563360 BE01-2839 AY342745 L2 A Y563370 BE01-4075 AY342745 L5 A Y563370 BE99-8750 AY342628 L7 AY563370 P-2990(CB1/Kanagawa/2003 AB167375 L10 AY563371 P-2240(CB1/Kanagawa/2003 AB167375 L9 AY563371 P-2290(CB1/Kanagawa/2003 AB167374 <tr< td=""><td></td><td>B5 B6</td><td>AV563358</td><td></td><td>4623 I/ISR98</td><td>AJ288066</td></tr<>			B5 B6	AV563358		4623 I/ISR98	AJ288066
Bio A Y56330 Alexandria/98 A F551830 Nicosia September LK1 A Y563361 Aswan/95 AF551830 Nicosia September LK1 A Y563362 4774/1/18708 A1288075 LK2 A Y563363 4625 1/18708 A1288075 LK5 A Y563364 4588 1/18708 A1288067 LK5 A Y563365 GAV 4588 1/18708 A1288067 LK11 A Y563366 CAV9 GRIGGS D00627 AB167970 Limassol September L1 A Y563360 BE01-5294 A914746 Li A Y563363 BE01-5294 A7342746 A9167979 A914746 Li A Y563361 BE99-5750 A734274 A7342746 A7342745 L6 A Y563370 P-2199/CH1/Kanagawa/2003 AB167737 A914274 L12 A Y563371 L10 A Y563371 P-2199/CH1/Kanagawa/2003 AB167737 L19 A Y563371 L10 A Y563371 <td< td=""><td></td><td></td><td>BS</td><td>AV563350</td><td></td><td>Sahag/00</td><td>AF551841</td></td<>			BS	AV563350		Sahag/00	AF551841
B70 A 150300 Gizu/8 Ar551830 B10 A Y563361 Aswany5 AF551830 Nicosia September LK1 A Y563362 4776_11/18708 A2388075 LK2 A Y563363 4625_11/3808 A2388075 A2388075 LK5 A Y563364 4585_11/3808 A2388067 LK8 A Y563365 CAV9 GRIGGS D00637 LK14 A Y563367 01-171FCR2 AB167980 A3288064 Limassol September L1 A Y563366 CAV9 GRIGGS D00637 Limassol September L1 A Y563360 BED1-329 A342451 L3 A Y563360 BED1-4293 A Y34276 A342451 L5 A Y563363 BED9-5750 A Y34224 L6 A Y563373 BED9-5750 A Y34224 L10 A Y563373 BED9-5750 A Y34223 L10 A Y563373 P 2290(CB1/Kanagawa/2003 AB167379 L10 A Y563376 ID179			Do	AT 505559		Alexandria/98	AF551840
Dio A 150301 Aewan,95 AF551836 Nicosia September LK1 A Y563362 4408_1/USR98 A1288074 LK2 A Y563363 4625_1/USR98 A1288074 LK5 A Y563364 4585_1/USR98 A1288074 LK8 A Y563365 GRIGGS D00677 LK11 A Y563366 CAV9 GRIGGS D00677 Umassol September L1 A Y563366 GRIGGS D00677 Limassol September L1 A Y563380 BE01-2839 AY342746 L2 A Y563370 BE01-42075 A Y342745 L4 A Y563370 BE01-5294 A Y342628 L7 A Y563376 BE01-5294 A Y342628 L7 A Y563376 CBV1 Japan M16500 L9 A Y563376 CBV1 Japan A167735 Nicosia December LE1 AY563376 P-2290/CB1/Kanagawa/2003 AB167735 L10 AY563373 10165 AY3373			D9 D10	A 1 303300		Giza/98	AF551839
Nicosia September LK1 AY563362 4776_1/15R98 A1288075 LK2 AY563363 4625_1/15R98 A1288074 LK3 AY563365 4625_1/15R98 A1288074 LK1 AY563366 CAV9 GRIGGS D00627 LK11 AY563366 CAV9 GRIGGS D00627 Limassol September L1 AY563366 CAV9 GRIGGS D00627 Limassol September L1 AY563360 B501-5294 AY342746 L3 AY563320 BE01-4075 AY342746 L5 AY563330 BE9-3750 AY342746 L5 AY563330 BE9-3750 AY342746 L8 AY563373 BE9-3750 AY342746 L8 AY563373 BE9-3750 AY342746 L8 AY563370 P-2290(B1/Kanagawa/2003 AB162735 L10 AY563371 P-2240(CB1/Kanagawa/2003 AB162735 L10 AY563372 P-2240(CB1/Kanagawa/2003 AB162735			B10	A 1 303301		Aswan/95	AF551836
Nicosia September LK1 A Y56336 4774_1/TSR98 A128807 LK2 A Y563363 4625_1/TSR98 A1288067 LK5 A Y563364 4625_1/TSR98 A1288067 LK8 A Y563364 4625_1/TSR98 A1288067 LK14 A Y563366 CAV9 GRIGGS D00627 Limassol September L1 A Y563361 BE01-2839 A Y142746 L2 A Y563320 BE01-2839 A Y142746 A Y142746 L3 A Y563320 BE01-2839 A Y142746 L5 A Y563330 BE99-1823 A Y142746 L5 A Y563330 BE99-5750 A Y342628 L7 A Y563364 BE99-1823 A Y342628 L7 A Y563370 December L8 A Y563370 L8 A Y563371 P-2240/CB1/Kanagawa/2003 A B162735 Nicosia December LE1 A Y563372 D10429 A Y35373 L10 A Y563374 CBV2 Nanagy A Y3731	NT' '	0 / 1	T 171	13/5(22/0		4808_1/ISR98	AJ288075
Limassol September L1 AY563364 4625 //SR98 A2288064 LKS AY563364 4588_//SR98 A2288064 LK8 AY563366 CAV9 GRIGGS D00627 03-171FCR2 AB167978 12 AY563360 03-132NPR2 AB167978 12 AY563370 03-132NPR2 AB167978 12 AY563370 BE01-2839 AY342746 14 AY563370 BE01-2839 AY342746 15 AY563331 BE99-5750 AY342743 16 AY563331 BE99-5750 AY342743 16 AY563331 BE99-5750 AY342628 17 AY563369 CBV1 Japan M16560 19 AY563337 P-2264(CB1/Kanagawa/2003 AB162735 10 AY563371 P-2264(CB1/Kanagawa/2003 AB162735 10 AY563371 P-2264(CB1/Kanagawa/2003 AB162735 110 AY563371 P-2204(CB1/Kanagawa/2003 AB162735 110 AY563371 P-2204(CB1/Kanagawa/2003 AB162735 1159 AY563373 D1059 AY373092 Nicosia December LE1 AY563371 P-2204(CB1/Kanagawa/2003 AB162735 112 AY563373 D2-240(CB1/Kanagawa/2003 AB162735 1159 AY563375 D1159 AY373092 LE4 AY563376 D1159 AY373102 LE4 AY563377 D1159 AY373102 LM3 AY563378 D10165 AY373102 LM4 AY563378 D10175 AY373102 LM3 AY563378 D10175 AY373102 LM4 AY563378 D10176 AY373102 LM5 AY563378 D10175 AY373102 LM7 AY563378 D10175 AY373102 D175 AY373102 P2204(CB1/Kanagawa/2003 AP3162735 D175 AY373102 P2346(CB1/Kanagawa/2003 AP3162735 D175 AY373102 LM4 AY563378 D10176 AY373102 LM7 AY563378 D10175 AY373102 P2346(CB1/Kanagawa/2003 AP3162735 D175 AY373104 AY373104 AY373105 D175 AY373104 AY373106 D175 AY373104 D175 AY373104	Nicosia	September	LKI	AY563362		4774_1/ISR98	AJ288074
LKS AY56356 LK8 AY563365 LK11 AY563366 CAV9 GRIGGS D00627 LK14 AY563366 CAV9 GRIGGS D00627 LK14 AY563366 CAV9 GRIGGS D00627 LK14 AY563380 03-144PPC3 AB167980 03-144PPC3 AB167978 L2 AY563381 BE90-1823 AY342746 L3 AY563329 BE01-4075 AY342744 L5 AY56339 BE01-4075 AY342744 L5 AY563368 CBV1 Japan M16560 L7 AY563368 L8 AY563369 CBV1 Japan M16560 L9 AY563333 P-2199(CB1/Kanagawa/2003 AB162735 L10 AY563333 P-2294(CB1/Kanagawa/2003 AB162735 L10 AY563372 10159 A43437409 L2 AY563373 D4-290(CB1/Kanagawa/2003 AB162735 H2 A240(CB1/Kanagawa/2003 AB162735 H2 A240(CB1/Kanagawa/2003 AB162735 L10 AY563372 10159 A43437409 LE1 AY563373 10165 AY373098 LE4 AY563376 10179 A7373098 Limassol December LM1 AY563374 CBV2 Nancy AF08148 LM2 AY563376 10179 AY373112 LM4 AY563376 10179 AY373112 LM4 AY563376 10179 AY373112 LM4 AY563376 10179 AY373112 LM4 AY563378 10177 AY373108 LM7 AY563378 10177 AY373109 LM6 AY563378 10177 AY373109 LM7 AY563378 10177 AY373109 LM7 AY563378 10177 AY373109 LM7 AY563378 10177 AY37310 LM7 AY563378 10177 AY373109 LM7 AY563378 10177 AY373109 PV2 Sabin strain, CAV11, and CAV22, which also provided an indication of the possible serotypic identity of the isolate. The nexting VD1 sequences were publosenetically compared PUB			LK2	AY563363		4625_1/ISR98	AJ288067
Limassol September L1 AY563366 CAV9 GRIGGS D00627 LK14 AY563367 03-171FCR2 AB167979 03-144NPC3 AB167979 03-144NPC3 AB167979 1.1 AY563380 BE01-2839 AY342766 L2 AY563381 BE99-1823 AY34276 L3 AY563326 BE01-5294 AY342745 L4 AY563330 BE10-4075 AY342744 L5 AY56331 BE99-5750 AY342744 L5 AY563331 BE99-5750 AY342745 L6 AY563331 BE99-5750 AY342745 L8 AY563368 - L7 AY563368 - L9 AY563371 BE99-5726 AY342628 Nicosia December LE1 AY563371 P-2240/CB1/Kanagawa/2003 AB162735 L10 AY563371 P-229NGC1 AB167399 LE2 AY563371 P-2204/CB1/Kanagawa/2003 AB162735 P-219/CB1/Kanagawa/2003 AB162735 P-2240/CB1/Kanagawa/2003 AB162735 P-2240/CB1/Kanagawa/2003 AB162735 P-2240/CB1/Kanagawa/2003 AB162735 P-2240/CB1/Kanagawa/2003 AB162735 P-2240/CB1/Kanagawa/2003 AB162735 P-2240/CB1/Kanagawa/2003 AB162735 P-2240/CB1/Kanagawa/2003 AB162735 P-2200/CB1/Kanagawa/2003 AB162735 P-2200/CB1/Kanagawa/2003 AB162735 P-2200/CB1/Kanagawa/2003 AB162735 P-2200/CB1/Kanagawa/2003 AB162735 P-2200/CB1/Kanagawa/2003 AB162735 P-2200/CB1/Kanagawa/2003 AB162735 P-2200/CB1/Kanagawa/2003 AB162735 P-2200/CB1/Kanagawa/2003 AB162735 L10 AY563376 D10159 AY373092 LE4 AY563377 D10159 AY373108 LM7 AY563378 D10165 AY373108 LM7 AY563378 D1075 AY373108 LM7 AY563378 D1075 AY373108 LM7 AY563378 D1075 AY373108 PV2 Sabin strain, CAV11, and CAV22, which also provided an indication of the possible serotypic identity of the isolate. CBV4 IVB VS			LK5	AY563364		4588_1/ISR98	AJ288064
Limassol September L1 AY56336 CAV9 GRIGGS D00627 LK14 AY563367 03-171FCR2 AB167980 03-144PPC3 AB167978 12 AY563381 BE90-1823 AY342745 L2 AY563381 BE90-1823 AY342745 L4 AY563320 BE01-5294 AY342745 L4 AY56330 BE99-5750 AY342744 L5 AY56330 BE99-5750 AY342744 L5 AY563368 CBV L8 AY563368 CBV L8 AY563368 CBV L9 AY563332 P-2199(CB1/Kanagawa/2003 AB162735 P-2294(CB1/Kanagawa/2003 AB162735 P-2294(CB1/Kanagawa/2003 AB162735 P-2294(CB1/Kanagawa/2003 AB162735 P-2294(CB1/Kanagawa/2003 AB162735 P-2294(CB1/Kanagawa/2003 AB162735 P-2240(CB1/Kanagawa/2003 AB162735 L10 AY563370 P-2200(CB1/Kanagawa/2003 AB162735 P-2240(CB1/Kanagawa/2003 AB162735 P-2240(CB1/Kanagawa/2003 AB162735 LE2 AY563371 P-2240(CB1/Kanagawa/2003 AB162735 P-2240(CB1/Kanagawa/2003 AB162737 LE3 AY563373 10156 AY373098 Limassol December LM1 AY563374 CBV2 Nancy AF081485 LM2 AY563375 10180 AY373113 LM3 AY563376 10179 AY373113 LM3 AY563377 10178 AY373113 LM4 AY563377 10178 AY373113 LM5 AY563378 10176 AY373113 LM6 AY563377 10178 AY373113 LM7 AY563328 10176 AY373110 1016 AY373111 LM5 AY563377 10178 AY373110 10174 AY373110 247563378 10176 AY373110 10174 AY373110 10174 AY373110 10174 AY373110 10174 AY373110 10169 AY373110 10174 AY373110 10179 AY373104 10170 AY373109 PV2 Sabin strain, CAV11, and CAV22, which also provided an indication of the possible serotypic identity of the isolate. CBV4 IVB CS44 CBV2 IVB CS44 CBV2 IVB CS44 CBV4 IVB CS454 CBV4 IVB CS454 CBV4 IVB CS454 CBV4 IVB CS454 CBV4 IVB CBV4 IVB CBV			LK8	AY563365			
Limassol September L1 AY563367 03-171PCR2 AB167980 03-144NPC3 AB167978 L2 AY563380 BE01-2839 AY342746 L3 AY563326 BE01-3239 AY342746 L3 AY563326 BE01-3294 AY342746 L4 AY563320 BE01-4075 AY342746 L5 AY56330 BE99-5750 AY342746 L5 AY56330 BE99-5750 AY342746 L8 AY563369 CBV1 Japan M16560 L9 AY563333 P-226/(CB1/Kanagawa/2003 AB162735 L10 AY563333 P-2240/CB1/Kanagawa/2003 AB162735 L10 AY563371 P-2246/(CB1/Kanagawa/2003 AB162735 P-2264/CB1/Kanagawa/2003 AB162735 P-2246/CB1/Kanagawa/2003 AB162735 P-2240/CB1/Kanagawa/2003 AB162735 P-2240/CB1/Kanagawa/2003 AB162735 P-2240/CB1/Kanagawa/2003 AB162735 December LE1 AY563370 P-2200/CB1/Kanagawa/2003 AB162735 LE2 AY563371 P-2346/CB1/Kanagawa/2003 AB162735 LE4 AY563373 10155 AY37309 LE4 AY563376 10179 AY373102 LM3 AY563376 10179 AY37310 LM4 AY563377 10159 AY37310 LM5 AY563378 10176 AY37310 LM6 AY563378 10176 AY37310 LM7 AY563378 10176 AY37310 LM7 AY563378 10176 AY37310 PV2 Sabin strain, CAV11, and CAV22, which also provided an indication of the possible serotypic identity of the isolate. CBV4 JVB			LK11	AY563366	CAV9	GRIGGS	D00627
Limassol September L1 AY563380 U3-144/PC3 AB167979 Limassol September L1 AY563380 U3-132/PR2 AB167978 L2 AY563381 BE01-2839 AY342745 BE01-2839 AY342745 L4 AY563329 BE01-4075 AY342744 L5 AY563329 BE01-4075 AY342744 L5 AY563330 BE99-5750 AY342628 L7 AY563368 L8 AY563368 CBV1 Japan M16560 L9 AY563332 P-2199/(CB1/Kanagawa/2003 AB162735 P-2199/(CB1/Kanagawa/2003 AB162735 P-2199/(CB1/Kanagawa/2003 AB162735 P-2199/(CB1/Kanagawa/2003 AB162735 P-2240/(CB1/Kanagawa/2003 AB162735 L10 AY563370 P-2240/(CB1/Kanagawa/2003 AB162735 LE2 AY563371 P-2240/(CB1/Kanagawa/2003 AB162735 LE2 AY563371 P-2240/(CB1/Kanagawa/2003 AB162735 LE3 AY563373 U165 AY37302 LE4 AY563373 U165 AY37302 LE4 AY563373 U165 AY373112 AY373092 AY373113 LM3 AY563376 10179 AY373112 LM4 AY563377 10178 AY37311 LM5 AY563378 10176 AY37310 LM6 AY563378 10176 AY37310 LM6 AY563378 10176 AY37310 LM6 AY563377 10178 AY37310 PY2 Sabin strain, CAV11, and CAV22, which also provided an indication of the possible serotypic identity of the isolate. The partial VPL securepres were phylogenerget apple			LK14	AY563367		03-171FCR2	AB167980
Limassol September L1 AY563380 03-152NPR2 AB10/97 L2 AY563381 BE01-2339 AY342746 L2 AY563381 BE01-239 AY342746 L3 AY563326 BE01-5294 AY342746 L4 AY563329 BE01-4075 AY342744 L5 AY563330 BE99-1823 AY342746 L6 AY563331 BE99-5750 AY342745 L6 AY563330 BE99-5750 AY342628 L7 AY563368 CBV1 Japan M16560 L9 AY563332 P-2264/CB1/Kanagawa/2003 AB162735 P-2240/CB1/Kanagawa/2003 AB162735 D4-29NGC1 AB16793 P-2240/CB1/Kanagawa/2003 AB162735 D4-29NGC1 AB16793 D4-29NGC1 AA7373102 D4-29NGC1 AB16793 D4-29NGC1 AA7373102 D4-29NGC1 AA7373102 D4-29NGC1 AB16793 D4-29NGC1 AA7373102 D4-29NGC1 AA7373102 D4-29NGC1 AA7373102 D4-29NGC1 AA7373102 D4-29NGC1 AA7373102 D4-29NGC1 AA7373102 D4-						03-144NPC3	AB167979
Linassol December LM1 AY563374 LE2 AY563381 BE99-1823 AY342619 L3 AY563329 BE01-4075 AY342744 L5 AY563330 BE99-5750 AY342623 L6 AY563331 BE99-8726 AY342628 L7 AY563368 L8 AY563369 CBV1 Japan M16560 L9 AY563332 P-2264/CB1/Kanagawa/2003 AB162733 P-2240/CB1/Kanagawa/2003 AB162733 P-2340/CB1/Kanagawa/2003 AB162733 P-2340/CB1/Kanagawa/2003 AB162733 P-2340/CB1/Kanagawa/2003 AB162737 LE3 AY563371 P-2340/CB1/Kanagawa/2003 AB162737 LE4 AY563373 10159 AY373098 Limassol December LM1 AY563374 CBV2 Nancy AF081485 LM2 AY563375 10180 AY373112 LM3 AY563376 10179 AY373108 LM4 AY563377 10178 AY373107 AY373102 LM6 AY563378 10176 AY373107 Months in 2003. * Months in 2003. CBV4 JVB X7373102 PV2 Sabin strain, CAV11, and CAV22, which also provided an indication of the possible serotypic identity of the isolate. The partial VP1 securepres were pub/logompared	Limassol	September	L1	AY563380		03-132NPK2 DE01-2820	AB16/9/8
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L4 AY563329 BE01-2075 AY34274 L5 AY563330 BE99-5750 AY342623 L6 AY563331 BE99-5750 AY342623 L7 AY563368 BE99-5750 AY34274 L8 AY563369 CBV1 Japan M16560 L9 AY563332 P-2264/CB1/Kanagawa/2003 AB162735 L10 AY563333 P-2199/CB1/Kanagawa/2003 AB162735 L10 AY563370 P-2200/CB1/Kanagawa/2003 AB162735 LE2 AY563371 P-2200/CB1/Kanagawa/2003 AB162735 LE3 AY563372 10159 AY373098 Limassol December LM1 AY563375 10180 AY373112 LM3 AY563375 10180 AY373111 LM3 AY563377 10177 AY373111 LM4 AY563377 10176 AY373109 AY373109 LM4 AY563377 10176 AY373109 AY373109 LM4 AY563377 10176 AY373101 AY373101			L3	AY563326		BE99-1625 BE01 5204	A 1 342019 A V342745
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L7 AY563368 M16560 L8 AY563369 CBV1 Japan M16560 L9 AY56332 P-2264/(CB1/Kanagawa/2003) AB162735 L10 AY563333 P-2199/CB1/Kanagawa/2003 AB162735 Nicosia December LE1 AY563370 04-29NGC1 AB162735 LE2 AY563371 P-2340/CB1/Kanagawa/2003 AB162737 AB162737 LE3 AY563372 10159 AY373092 AB162737 LE4 AY563373 10165 AY373092 AB162737 Limassol December LM1 AY563373 10159 AY373092 Limassol December LM1 AY563375 10180 AY373112 LM2 AY563376 10179 AY373111 LM5 AY36332 10176 AY373111 LM5 AY563378 10176 AY373106 10172 AY373106 LM6 AY563328 10175 AY373106 10172 AY373106 LM6 AY563378 1			L6	AY563331		BE99-8726	AY342628
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L9 AY563332 P-2264/CB1/Kanagawa/2003 AB162736 L10 AY563333 P-2199/CB1/Kanagawa/2003 AB162735 Nicosia December LE1 AY563370 P-2200/CB1/Kanagawa/2003 AB162735 LE2 AY563370 P-2200/CB1/Kanagawa/2003 AB162735 AB162735 LE2 AY563371 P-2346/CB1/Kanagawa/2003 AB162735 LE3 AY563372 10159 AY373092 LE4 AY563373 10165 AY373092 Limassol December LM1 AY563375 10180 AY373113 LM2 AY563376 10179 AY373113 LM3 AY563376 10179 AY373110 LM3 AY563377 10178 AY373100 AY373100 AY373100 LM6 AY563377 10176 AY373100 AY373100 LM6 AY563327 10174 AY373107 AY373100 LM6 AY563327 10174 AY373100 AY373100 '* Months in 2003. LM7 AY563327			1.8	AY563369	CBV1	Japan	M16560
Lino AY563333 P-219/CB1/Kanagawa/2003 AB162733 P-2240/CB1/Kanagawa/2003 AB16783 P-2240/CB1/Kanagawa/2003 AB16789 P-2200/CB1/Kanagawa/2003 AB167734 LE2 AY563370 P-2200/CB1/Kanagawa/2003 AB162734 LE3 AY563372 10159 AY373092 LE4 AY563373 10165 AY373092 LE4 AY563373 10165 AY373098 Limassol December LM1 AY563374 CBV2 Nancy AF081485 LM2 AY563375 10180 AY373113 LM3 AY563376 10179 AY373112 LM4 AY563377 10178 AY373110 LM5 AY563378 10176 AY373110 LM6 AY563328 10176 AY373109 LM6 AY563327 10174 AY373107 " ^a Months in 2003. PV2 Sabin strain, CAV11, and CAV22, which also provided an indication of the possible serotypic identity of the isolate. The partial VP1 sequences were phylogenetically compared			1.9	AY563332		P-2264/CB1/Kanagawa/2003	AB162736
Lito Product P-2240/CB1/Kanagawa/2003 AB162735 Nicosia December LE1 AY563370 P-22200/CB1/Kanagawa/2003 AB162735 Nicosia LE2 AY563371 P-2346/CB1/Kanagawa/2003 AB162737 LE3 AY563372 10159 AY373092 LE4 AY563373 10165 AY373098 Limassol December LM1 AY563375 10180 AY373112 LM2 AY563376 10179 AY373112 LM4 AY563376 10179 AY373111 LM4 AY563376 10178 AY373110 LM5 AY563378 10176 AY373109 Mathematical Mathematic			L 10	AY563333		P-2199/CB1/Kanagawa/2003	AB162733
Nicosia December LE1 AY563370 04-29NGC1 AB167989 Nicosia LE2 AY563371 P-2200/CB1/Kanagawa/2003 AB162734 LE3 AY563372 10159 AY373092 LE4 AY563373 10155 AY373092 Limassol December LM1 AY563374 CBV2 Nancy AF081485 LM2 AY563376 10179 AY373112 LM3 AY563376 10179 AY373112 LM4 AY563376 10178 AY373110 AY373110 LM5 AY563377 10178 AY373109 LM6 AY563328 10175 AY373109 LM6 AY563327 10174 AY373107 " ^a Months in 2003. M6 has provided an indication of the possible serotypic identity of the isolate. CBV4 JVB AY373101 PV2 Sabin strain, CAV11, and CAV22, which also provided an indication of the possible serotypic identity of the isolate. CBV4 JVB AY373101 The partial VP1 sequences were phylogenetically compared CBV4 JVB AY3660018 </td <td></td> <td></td> <td>210</td> <td>1115055555</td> <td></td> <td>P-2240/CB1/Kanagawa/2003</td> <td>AB162735</td>			210	1115055555		P-2240/CB1/Kanagawa/2003	AB162735
Intension P-2200/CB1/Kanagawa/2003 AB162734 LE2 AY563371 P-2346/CB1/Kanagawa/2003 AB162737 LE3 AY563372 10159 AY373098 Limassol December LM1 AY563374 CBV2 Nancy AF081485 Limassol December LM1 AY563375 10180 AY373103 LM3 AY563376 10179 AY373113 LM4 AY563376 10178 AY373101 LM5 AY563378 10176 AY373103 LM6 AY563328 10175 AY373106 LM7 AY563327 10174 AY373107 ^a Months in 2003. Months also provided an 10170 AY373103 PV2 Sabin strain, CAV11, and CAV22, which also provided an 10168 AY373101 indication of the possible serotypic identity of the isolate. CBV4 JVB X05690 The a partial VP1 sequences were phylogenetically compared CBV4 JVB X05690	Nicosia	December	LE1	AV563370		04-29NGC1	AB167989
Limassol December LM1 AY563372 10159 AY373092 LE4 AY563373 10165 AY373098 Limassol December LM1 AY563374 CBV2 Nancy AF081485 LM2 AY563375 10180 AY373113 LM3 AY563376 10179 AY373112 LM4 AY563377 10178 AY373111 LM5 AY563378 10176 AY373109 LM6 AY563328 10175 AY373109 LM7 AY563327 10174 AY373100 LM7 AY563327 10174 AY373100 10172 AY373100 10172 AY373100 10173 AY373100 10172 AY373100 10174 AY373100 10175 AY373100 10175 AY373100 10176 AY373100 10177 AY373100 10178 AY373100 10178 AY373100 10179 AY373100 10179 AY373100 10170 AY373100 10168 AY369 10169 AY373100 10168 AY373100 10168 AY369 10168 AY369 10169 AY373100 10168 AY369 10168	Nicosia	Determoer		AV562271		P-2200/CB1/Kanagawa/2003	AB162734
Limassol December LM1 AY563373 10165 AY373092 Limassol December LM1 AY563374 CBV2 Nancy AF081485 LM2 AY563375 10180 AY373113 LM3 AY563376 10179 AY373110 LM4 AY563377 10178 AY373110 LM4 AY563378 10177 AY373110 LM5 AY563328 10176 AY373109 LM7 AY563327 10173 AY373109 LM7 AY563327 10173 AY373100 CBV2 Nancy AF081485 10179 AY373100 AY373107 a Months in 2003. 10172 AY373100 PV2 Sabin strain, CAV11, and CAV22, which also provided an indication of the possible serotypic identity of the isolate. CBV4 JVB X05690 The partial VP1 sequences were phylogenetically compared CBV4 JVB X05690				AV562272		P-2346/CB1/Kanagawa/2003	AB162737
Limassol December LM1 AY563374 CBV2 Nancy AF081485 LM2 AY563375 10180 AY373113 LM3 AY563376 10179 AY373112 LM4 AY563377 10178 AY373110 LM5 AY563378 10177 AY373100 LM6 AY563328 10175 AY373100 LM7 AY563327 10174 AY373107 ^a Months in 2003. 10172 AY373102 PV2 Sabin strain, CAV11, and CAV22, which also provided an indication of the possible serotypic identity of the isolate. CBV4 JVB The partial VP1 sequences were phylogenetically compared CBV4 JVB				A I 303372		10159	AY373092
Limassol December LM1 AY563374 CBV2 Nancy AF081485 LM2 AY563375 10180 AY373113 LM3 AY563376 10179 AY373112 LM4 AY563377 10178 AY373111 LM5 AY563378 10177 AY373109 LM6 AY563328 10175 AY373109 LM7 AY563327 10174 AY373107 ^* Months in 2003. 10172 AY373102 PV2 Sabin strain, CAV11, and CAV22, which also provided an indication of the possible serotypic identity of the isolate. CBV4 JVB X05690 The partial VP1 sequences were phylogenetically compared			LE4	A1303575		10165	AY3/3098
Linkissor December Linki LN12 Fully Fuly Fully Fully	Limassol	December	I M1	AY563374	CBV2	Nancy	A F081485
LM3 AY563376 10179 AY373112 LM4 AY563377 10178 AY373111 LM5 AY563378 10177 AY373100 LM6 AY563328 10176 AY373109 LM6 AY563327 10174 AY373107 " ^a Months in 2003. 10174 AY373107 ^a Months in 2003. 10172 AY373105 PV2 Sabin strain, CAV11, and CAV22, which also provided an 10168 AY373101 indication of the possible serotypic identity of the isolate. CBV4 JVB X05690 The partial VP1 sequences were phylogenetically compared CBV4 JVB X05690	Linusson	December	I M2	AV563375	0012	10180	AY373113
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LM4 AY50377 10177 AY373110 LM5 AY563378 10176 AY373108 LM6 AY563328 10175 AY373108 LM7 AY563327 10174 AY373107 "Months in 2003. 10172 AY373106 "Months in 2003. 10172 AY373104 PV2 Sabin strain, CAV11, and CAV22, which also provided an indication of the possible serotypic identity of the isolate. CBV4 JVB X05690 The partial VP1 sequences were phylogenetically compared CBV4 JVB X05690				AV562277		10178	AY373111
LND A1305378 10176 AY373109 LM6 AY563328 10175 AY373108 LM7 AY563327 10174 AY373107 ^a Months in 2003. 10173 AY373106 ^a Months in 2003. 10171 AY373106 10170 AY373104 10170 10170 AY373103 10169 PV2 Sabin strain, CAV11, and CAV22, which also provided an indication of the possible serotypic identity of the isolate. CBV4 JVB X05690 The partial VP1 sequences were phylogenetically compared CBV4 JVB X05690				A 1 505577		10177	AY373110
LM0 A 1303228 10175 A Y373108 LM7 AY563327 10174 AY373107 " Months in 2003. 10173 AY373106 " Months in 2003. 10173 AY373106 10172 AY373104 10170 PV2 Sabin strain, CAV11, and CAV22, which also provided an indication of the possible serotypic identity of the isolate. CBV4 JVB X05690 The partial VP1 sequences were phylogenetically compared CBV4 JVB X05690				A 1 303576		10176	AY373109
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PV2 Sabin strain, CAV11, and CAV22, which also provided an indication of the possible serotypic identity of the isolate. The partial VP1 sequences were phylogenetically compared CBV4 JVB X05690 The partial VP1 sequences were phylogenetically compared AE160018	inomin i	. 2000.				10172	AY373105
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PV2 Sabin strain, CAV11, and CAV22, which also provided an indication of the possible serotypic identity of the isolate. 10108 AY3/3101 The partial VP1 sequences were phylogenetically compared CBV4 JVB X05690 AF160018						10169	AY3/3102
The partial VP1 sequences were phylogenetically compared CBV4 JVB X05690	PV2 Sabin	strain, CAV11,	and CAV22, which al	so provided an		10108	A13/3101
The partial VP1 sequences were phylogenetically compared P23(pab02 AF160018	indication	of the possible	serotypic identity of t	he isolate.	CBV/	IVB	X05600
= 110 101101 VI = 304000104000000000000000000000000000000	The nar	tial VP1 sequen	ces were phylogenetic	cally compared	CDVT	P234pak92	AF160018

The partial VP1 sequences were phylogenetically compared with enterovirus sequences available from the GenBank sequence database, and the results are shown in Table 3. The levels of partial VP1 sequence identity with previously studied enterovirus reference and wild-type strains ranged from 82 to 98%. The second-highest levels of identity were less than 70% in all cases. All but one of the isolates were nonpolio enteroviruses; in particular, 17 of 40 isolates (42.5%) were CBV4, 12 of 40 isolates (30%) were CAV9, 7 of 40 isolates (17.5%) were CBV2, and 3 of 40 isolates (7.5%) were CBV1. Also, all isolates of the same serotype had very similar VP1 sequences (98 to 100%), indicating that they may have been the same strain



FIG. 1. (a) Representative results for UG_{52} - Uc_{53} (435-bp) amplicons and 292–222 (350-bp) amplicons for isolates A1, A2, A3, and A4. Lane M contained a molecular weight marker (ϕ X174 HaeIII-generated restriction fragments; Gibco BRL). (b) Representative results of RFLP analysis of UC_{53} - UG_{52} -produced RT-PCR amplicons of the enterovirus isolates with the HpaII restriction endonuclease (for a description, see the text). All of the isolates except one (isolate A5 [data not shown]) belonged to the same genetic subcluster, and the restriction profile comprised four HpaII-generated fragments with lengths of 213, 149, 55, and 18 nucleotides. The lane on the left contained a molecular weight marker (ϕ X174 HaeIII-generated restriction fragments; Gibco BRL).

of the specific serotype circulating in the population. It was observed that the HpaII digests predicted the genetic subclusters of the clinical isolates of all isolates when the five corresponding HpaII-generated subclusters of most human enterovirus serotypes studied by Siafakas et al. (35) were used as references. Specifically, each the CAV9, CBV4, CBV2, and CBV1 isolates had an HpaII-generated restriction profile that was identical to the profile of a cluster of enteroviruses that included the corresponding strains of the same serotype. One poliovirus strain (2.5%) was isolated, and the VP1 sequence revealed that it was a Sabin type 2 isolate. The HpaII-produced restriction profile placed this isolate in a subcluster that contained PV2 and two other CAVs of the polio-like cluster of 5'-UTR, as mentioned above; most importantly, this subcluster contained only the PV2 Sabin strain and no other wild-type PV2 prototype strain, providing clues about the possible vaccine origin of the isolate.

Finally, the phylogenetic comparison between these isolates

TABLE 3. Results of RFLP analysis, partial VP1 sequencing, and phylogenetic comparison of our 40 isolates with enterovirus sequences available in the GenBank sequence database.

Isolate ^a	VP1 Serotype	% Nucleotide sequence identity
A1	CAV9	90
A2	CAV9	91
A3	CAV9	90
A4	CAV9	91
A5	Sabin 2	98
A6	CAV9	92
B1	CAV9	92
B4	CAV9	91
B5	CAV9	90
B6	CAV9	91
B8	CAV9	92
B9	CAV9	90
B10	CAV9	91
LK1	CBV4	95
LK2	CBV4	94
LK5	CBV4	93
LK8	CBV4	93
LK11	CBV4	93
LK14	CBV4	93
L1	CBV2	87
L2	CBV2	87
L3	CBV1	83
L4	CBV2	87
L5	CBV2	87
L6	CBV2	86
L7	CBV4	93
L8	CBV4	94
L9	CBV2	87
L10	CBV2	87
LE1	CBV4	93
LE2	CBV4	95
LE3	CBV4	93
LE4	CBV4	93
LM1	CBV4	95
LM2	CBV4	94
LM3	CBV4	95
LM4	CBV4	96
LM5	CBV4	96
LM6	CBV1	82
LM7	CBV1	83

^{*a*} Most isolates belonged to HpaII genetic cluster I; the only exception was isolate A5, which belonged to cluster IV. In all cases there was agreement between HpaII classification and VP1 partial serotyping results.

and other enterovirus reference and wild-type strains showed the serotype-specific pattern of strain classification into single clusters (Fig. 2). It was also very interesting that the PV2, CBV4, and, to a lesser extent, CAV9 isolates showed the greatest levels of VP1 alignment with other enterovirus strains isolated elsewhere in Europe, in Asia, in northern Africa, and in the Middle East (Fig. 2), implying a possible epidemiological relationship of all these isolates. Specifically, the PV2 isolates were most closely related to PV2 strains of vaccine origin isolated in Israel in 2002 (unpublished data obtained from GenBank) and in Egypt from 1983 to 1993 (41), reinforcing the Sabin-like character of the isolates. CBV and CAV9 isolates were related to similar strains isolated in Belgium from 1999 to 2002 (38), and especially the CBV4 isolates also showed correlation with strains of the same serotype isolated during the last four decades in France, Finland, Romania, Denmark, North America, and Pakistan (21).



FIG. 2. Dendrogram showing the relationships among enterovirus environmental strains isolated in the present study and reference and other clinical strains, as determined by using the partially sequenced VP1-encoding region.

DISCUSSION

Environmental surveillance has been successfully used to assess the extent or duration of epidemic enterovirus circulation in specific populations (25, 31). Furthermore, screening of wastewater has been shown to be a more sensitive tool for detection of wild-type polioviruses in communities than surveillance for acute flaccid paralysis (AFP) (12), since wild-type polioviruses have frequently been isolated from sewage in the absence of cases of AFP (5, 17, 37, 40). It may also prove to be useful for monitoring the effectiveness of vaccination against poliomyelitis and for detecting the introduction of wild-type strains into communities previously considered polio-free (7, 27). All these things indicate the importance of environmental surveillance in prevention of a possible enterovirus outbreak.

An effective surveillance scheme is a prerequisite for epidemiological investigation of disease outbreaks, providing important information about the viruses circulating in susceptible populations and, consequently, guiding a reliable system of early warning and prevention (26). In the present study we attempted to contribute to this goal by developing a simple and effective method for detection and identification of infectious enteroviruses in sewage by the VIRADEN method (cellulose nitrate membrane adsorption and direct cell culture from the membrane) and RT-PCR. In our procedure virus plaques are produced on a cell monolayer while the virus grows on the membrane filter. To our knowledge, this is the only method which produces plaques directly from the membrane filter (the virus adsorption site) without a need for elution. No special equipment or decontamination procedures are necessary, and all isolates from environmental specimens can be identified within a few days after sampling. Moreover, since cell lines other than BGM have the advantage of growing on cellulose nitrate membrane filters and since viruses other than enteroviruses can adsorb to such filters, VIRADEN has the potential of being used for detection and characterization of a wide range of viruses with different cell lines (26).

Previous studies reported detection of enteroviruses by RT-PCR from culture-negative environmental samples, showing that cytopathogenic viruses are only a minor component of the enteroviruses present in water environments (12, 28, 31). Nevertheless, a combination of cell culture amplification and detection by RT-PCR still is more sensitive for detection of enteric viruses than either method alone (28, 29, 31). Cell culture amplification also has the advantage of increasing the frequently low virus titer in environmental samples and eliminating the action of substances that inhibit RT-PCR. Moreover, it is our view that although a culture-negative, RT-PCRpositive result indicates detection of nucleic acid from an intact capsid particle, what is really important from a clinical and epidemiological point of view is detection of cytopathogenic and consequently infectious and potentially hazardous viruses.

Partial sequencing of the VP1-encoding gene produced unambiguous results regarding serotyping of the isolates, showing the effectiveness of the specific serotyping system and also showing that the cultures that were obtained were pure and that each contained only one virus clone. Moreover, the distinction between nonpolio and polio-like enteroviruses was accurate, something which is extremely important for the eradication of wild-type polioviruses from the environment and for surveillance for vaccine-associated cases of AFP (4, 8, 9, 10, 11, 13). Thoelen et al. (38) reported a subtype-specific clustering pattern for enteroviruses on the basis of the 5'-UTR and proposed that the suggestive value of the 5'-UTR for enterovirus serotype determination and further investigation should not be underestimated.

An interesting finding of the present study was that the PV2, CBV4, and, to a lesser extent, CAV9 isolates showed the highest levels of VP1 alignment with other enterovirus strains isolated elsewhere in Europe, in North America, in Asia Manor (21, 38), in northern Africa, and in the Middle East (41) (Fig. 2), implying the possible epidemiological relationships of all these isolates. Cyprus is located at a crossing point between Europe, the Middle East, and northern Africa, and the fact that the molecular analysis of the environmental isolates in the present study provided some evidence about the possible introduction of these enteroviruses from individuals traveling between these geographic regions was very interesting. It would be very interesting to carry out a more detailed and combined genotypic analysis of all these strains in an attempt to follow enterovirus circulation and evolution in the populations. The possible epidemiological association also gave us confidence in the value of the environmental surveillance for enteroviruses that was carried out in the present and previous studies.

In conclusion, the combination of virus concentration by cellulose nitrate membrane adsorption and cell culture (VI-RADEN), followed by detection with RT-PCR and serotyping by partial VP1 sequencing, seems to be very promising for environmental surveillance of enterovirus circulation and epidemiology, with all the significant effects that this could have on public health. Nevertheless, the predictive value of the simple RFLP analysis of the 5'-UTR in this and previous studies can also be considered an asset for the initial subclassification of enterovirus isolates. It would also be interesting to determine the epidemiological relationship of the environmental isolates identified in the present study with viruses isolated elsewhere in Europe, in North America, in Asia, and in northern Africa in previous years. Finally, an effective means of environmental surveillance should facilitate the monitoring of potential sources for new recombinant enteroviruses and identification of newly discovered or previously unidentified strains.

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