

Retrospective Characterization of a Vaccine-Derived Poliovirus Type 1 Isolate from Sewage in Greece[∇]

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Retrospective molecular and phenotypic characterization of a vaccine-derived poliovirus (VDPV) type 1 isolate (7/b/97) isolated from sewage in Athens, Greece, in 1997 is reported. VP1 sequencing of this isolate revealed 1.87% divergence from the VP1 region of reference strain Sabin 1, while further genomic characterization of isolate 7/b/97 revealed a recombination event in the nonstructural part of the genome between a vaccine strain and a nonvaccine strain probably belonging to *Enterovirus* species C. Amino acid substitutions commonly found in previous studies were identified in the capsid coding region of the isolate, while most of the attenuation and temperature sensitivity determinants were reverted. The ultimate source of isolate 7/b/97 is unknown. The recovery of such a highly divergent derivative of a vaccine strain emphasizes the need for urgent implementation of environmental surveillance as a supportive procedure in the polio surveillance system even in countries with high rates of OPV coverage in order to prevent cases or even outbreaks of poliomyelitis that otherwise would be inevitable.

Poliovirus is a positive single-stranded RNA virus whose genome is approximately 7,500 bases long. It contains four regions: a 5' untranslated region (5' UTR), a coding region, a 3' UTR, and a poly(A) tail. After translation of the coding region, a polyprotein is produced, which is then enzymatically cleaved, yielding all the viral proteins (VP4, VP2, VP3, VP1, 2A, 2B, 2C, 3A, 3B, 3C, 3D).

Poliovirus is the pathogen responsible for paralytic poliomyelitis and is classified into three serotypes (PV1, PV2, and PV3) based on the pattern of neutralization by monoclonal antibodies specific for each serotype (56).

The initiative for eradication of poliomyelitis worldwide was launched by the World Health Organization (WHO) in 1988. By the end of 2005, the reported polio-associated cases had declined from 350,000 in 1988 to less than 2,000. The two basic cornerstones of the initiative for poliomyelitis eradication are massive OPV immunization and sensitive poliovirus surveillance (31). Surveillance for poliovirus consists of investigation of acute flaccid paralysis (AFP) cases and virologic studies of isolates originating either from clinical specimens or from the environment (environmental surveillance) (33).

Environmental surveillance is a powerful and highly sensitive tool for detection of poliovirus circulation in high-risk areas in which complete and effective AFP surveillance cannot be accomplished. Environmental poliovirus surveillance is implemented mainly through investigation of wastewater samples. A previous study proved that environmental poliovirus surveillance is capable of detecting a poliovirus strain even if it

is excreted by only 1 of 10,000 people (30). Furthermore, there are examples of detection of poliovirus strains in the environment even in the absence of reported AFP cases in the population (5, 41, 53).

The genomes of most RNA viruses are characterized by high evolution rates, which are many orders of magnitude greater than those of DNA viruses (18). Poliovirus is considered one of the most rapidly evolving RNA viruses (22, 39, 42). The mechanisms underlying rapid poliovirus evolution are mutation and recombination events.

OPV consists of live attenuated poliovirus strains (Sabin strains) of the three poliovirus serotypes. OPV proved to be a very effective vaccine for interrupting wild poliovirus transmission. However, due to its genetic instability there are two major disadvantages of its use. The most commonly recognized adverse effect is vaccine-associated paralytic poliomyelitis, whose clinical symptoms are identical to those of paralytic poliomyelitis caused by wild poliovirus (33). During multiplication of Sabin strains in the human intestine, mutations at specific sites of the genome are associated with the attenuated phenotype of Sabin strains and recombination between Sabin strains may result in the loss of the attenuating phenotype of OPV strains and the acquisition of traits characteristic of wild poliovirus, such as increased neurovirulence and loss of temperature sensitivity (1, 10, 21). The rate of vaccine-associated paralytic poliomyelitis worldwide is 1 case per 750,000 primary vaccinees (13).

In addition, OPV is the potential source of vaccine-derived polioviruses (VDPVs). An OPV-related isolate is considered to be a VDPV if the VP1 nucleotide sequence has diverged more than 1% from the VP1 nucleotide sequence of the reference OPV strain (57, 59). The $\geq 1\%$ threshold implies that replication and/or circulation of the strain has occurred for at least 1 year after administration of the OPV dose, which is

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substantially longer than the 4 to 6 weeks which is the normal period of virus replication in vaccinees.

VDPVs are subdivided into three categories: immunodeficient VDPVs (iVDPVs), circulating VDPVs (cVDPVs), and ambiguous VDPVs (aVDPVs). iVDPVs have been isolated from patients with primary B-cell immunodeficiencies and occasionally have caused paralytic disease in vaccinees (9, 29, 34, 42). cVDPVs emerge in communities with gaps in OPV coverage, where circulation of wild poliovirus has already ceased. The most important properties of cVDPVs are sustained person-to-person transmission and an increased capacity to cause poliomyelitis (14). Furthermore, the vast majority of cVDPV isolates have been found to recombine with *Enterovirus* group C in the nonstructural region of the genome. In recent years, polio outbreaks in which the causal agent was a cVDPV strain have been documented. The polio outbreak in Hispaniola in 2000 and 2001 was caused by a type 1 cVDPV (32). Similarly, in 2001 in The Philippines a type 1 cVDPV was implicated in three AFP cases (52), and a type 2 cVDPV was the underlying cause of five AFP cases in Madagascar in 2002 (51), while three AFP cases were connected to a type 1 cVDPV in China in 2004 (36). aVDPVs are a more recent category which contains isolates excreted from vaccinees with no known immunodeficiencies and environmental isolates whose ultimate source has not been identified. aVDPV strains with highly divergent genomes have been isolated from sewage in Israel (53) and Estonia (5) in the absence of reported AFP cases, revealing the importance of environmental surveillance even in adequately immunized populations.

In this paper we report in retrospect the isolation of a type 1 VDPV isolated from sewage in Athens, Greece, in 1997. Almost complete genomic sequencing revealed the recombinant vaccine/nonvaccine nature of this isolate. The ultimate source of the isolate was not known. The results of this study emphasize the need for enhanced environmental surveillance in order to identify highly divergent isolates which could be potential sources of epidemics, even in regions where the rates of OPV coverage are high.

MATERIALS AND METHODS

Environmental sampling. Environmental samples were collected from the sewage treatment plant of Athens from April to October 1997. Fourteen 1-liter samples were collected and stored at -80°C . The samples were stored at the Hellenic Pasteur Institute. Environmental samples were transported in dry ice to the Biochemistry & Biotechnology Department in Larissa, Greece, in 2004, where processing of the samples took place.

Processing of samples: virus isolation. Processing of the environmental samples was carried out using the method proposed by the WHO (58). A concentrate of every environmental sample was added to $10\times$ minimal essential medium (MEM) (9 ml of concentrate and 1 ml of $10\times$ MEM) and inoculated into 96-well plates. Fifty microliters of the concentrate was inoculated into the 96-well plates containing confluent HEP-2 cells in 100 μl of Eagle MEM supplemented with 3% fetal calf serum (FCS). Following absorption of the virus for 2 h at 37°C , the medium in each well was replaced by 100 μl of Eagle MEM supplemented with 1% FCS, and the 96-well plates were incubated at 37°C for 4 to 5 days. Then the plates were frozen and thawed three times before a second passage in cell culture. This procedure was repeated. The second passage lasted for approximately 5 to 6 days. The wells in which cytopathic effects (CPE) were observed were marked, and their contents were inoculated into 25-cm² flasks containing HEP-2 cells in 3 ml of Eagle MEM and 2% FCS in order to confirm the CPE. RNA extraction (12), reverse transcription, and PCR with primer pair UG52/UC53 (24) were performed in each flask in which CPE were observed in order to confirm the enterovirus CPE. Following confirmation of enterovirus CPE, serial 10-fold dilutions were prepared in order to avoid viral mixtures. The last

dilution in which CPE was observed was inoculated into a 25-cm² flask containing HEP-2 cells, and the presence of enterovirus was retested as described above.

Preliminary characterization of the isolates. For preliminary characterization of the isolates, RNA extraction (12), reverse transcription, and PCR with primer pair 292/222 (48) were performed. PCR amplicons were sequenced at Macrogen Inc. (Seoul, Korea). After sequencing the identified poliovirus isolates were subjected to further characterization.

Complete VP1 sequencing of poliovirus isolates. The primer pairs used for complete VP1 gene sequencing were UG1/222 (2, 47) for the first half of the VP1 gene and serotype-specific primer pairs S₁37/S₁688, S₂30/S₂688, and S₂26/S₂651 for Sabin 1, Sabin 2, and Sabin 3 isolates, respectively, for the second half of the VP1 gene (17). CLUSTALW (www.ebi.ac.uk/clustalw) was used for alignment of VP1 sequences of the isolated poliovirus Sabin strains with sequences of the reference poliovirus Sabin strains. The accession numbers of the reference Sabin strains that were used in this study were AY184219, AY184220, and AY184221 for Sabin 1, Sabin 2, and Sabin 3 strains, respectively. Isolate 7/b/97 exhibited high sequence divergence from the reference Sabin 1 strain and was studied further.

Genomic sequencing of isolate 7/b/97. Reverse transcription and PCRs for isolate 7/b/97 were performed in an Eppendorf Mastercycler personal. In brief, 5 μl of extracted RNA and 2 μl of random d(N6) primers (New England Biolabs, United States) were incubated for 5 min at 70°C . The reaction mixture contained 5 μl of $5\times$ Moloney murine leukemia virus (M-MLV) reaction buffer, 200 U M-MLV reverse transcriptase (Promega Corporation, Madison, WI), 5 μl deoxynucleoside triphosphates (1 mM), 40 U RNase inhibitor (Promega Corporation, Madison, WI), and enough RNase-free distilled water to bring the final volume to 18 μl . The 18- μl reaction mixture was added to the annealed template-RNA solution and incubated for 1 h at 37°C for cDNA synthesis and then at 94°C for 5 min for M-MLV reverse transcriptase inactivation.

The primer pairs used in this study are shown on Table 1. Primer pairs S₁23/S₁976, S₁82/S₁542, S₁350/S₁693, S₁476/S₁154, and S₁013/S₁669 were designed using the reference Sabin 1 strain (accession number AY184219), while primer pair CVD1/CVD2 was designed using the sequence of the 2C region of isolate 7/b/97 in order to cover a 50-base gap. For design of all other primer pairs the program Primer 3 (www.genome.wi.mit.edu/genomesoftware/other/) was used.

For a PCR, 3 μl of cDNA of isolate 7/b/97, 2 μl of each primer pair, 5 μl of deoxynucleoside triphosphates (10 mM), 2 μl of MgCl_2 (50 mM), 0.5 μl (2.5 U) of *Taq* DNA polymerase (Biotaq, Moscow, Russia), and enough double-distilled H₂O to bring the final volume to 50 μl were added to a tube. For primer pairs S₁23/S₁976, S₁82/S₁542, S₁350/S₁693, S₁476/S₁154, S₁013/S₁669, and CVD1/CVD2 40 cycles were performed. Each cycle consisted of 20 s of denaturation at 95°C , 20 s of annealing at 55°C , and 20 s of extension at 72°C . The final extension step was carried out at 78°C for 15 min. For the other primer pairs the PCR conditions were the same as those described in the original papers (Table 1). Analysis of PCR products was performed by agarose gel electrophoresis (2% agarose, 1 $\mu\text{g/ml}$ ethidium bromide in Tris-boric acid-EDTA buffer), and the *Hae*III ϕ X174 ladder (Invitrogen, Life Technologies, Paisley, United Kingdom) was used. Where necessary, PCR products were cleaned up with a PCR gel extraction kit (QIAGEN GmbH, Hilden, Germany). All PCR products were sequenced at Macrogen Inc. (Seoul, Korea).

Computational analysis. CLUSTALW was used for complete alignment of the genomes of isolate 7/b/97 and the Sabin 1 reference strain (accession number AY184219). Amino acid sequences of isolate 7/b/97 were obtained by using Gene-Runner V 3.05, and alignment of amino acid sequences of isolate 7/b/97 and the Sabin 1 reference strain (accession number AY184219) was also performed by using CLUSTALW. Isolate 7/b/97's amino acid substitutions in the capsid region were depicted with RasMol V.2.7.1.1 (www.umass.edu/microbio/rasmol). For visualization of amino acid substitutions of isolate 7/b/97, the capsid protomer structure of the poliovirus type 1 Mahoney strain (Protein Data Bank accession number 1ASJ.pdb) was used (55). Finally, the Simplot program (version 2.5) was used for depiction of the recombination event.

RCT marker. In an attempt to determine the reproductive capacity at different temperatures (RCT assay) the reproductive capacity of viral isolate 7/b/97 was tested at two different temperatures. The titers of the viral stock in HEP-2 cells were calculated and expressed as the 50% tissue culture infective dose TCID₅₀/0.1 ml at 36 and at 39.5°C . Two different incubators were used. The temperature of one incubator was adjusted to 36°C (optimal temperature for virus reproduction), while the temperature of the other incubator was adjusted to 39.5°C (supraoptimal temperature for virus reproduction). Viral isolates with differences between the titers at 36 and 39.5°C greater than 2 logarithms were considered temperature sensitive.

TABLE 1. Primers used for complete genomic sequencing of isolate 7/b/97

Primer	Polarity	Sequence (5'→3') ^a	Reference
72437	Sense	001-TTAAACAGCTCTGGGGTTG-020	47
216616	Antisense	545-GAAACACGGACACCCAAAGTA-565	6
S ₁ 23	Sense	323-GAGTCTGGACATCCCTCAC-342	This study
S ₁ 976	Antisense	957-TACCCGCAAGCCTCTATGTT-976	This study
S ₁ 82	Sense	882-CTCAAGACCCTTCCAAGTTCA-902	This study
S ₁ 542	Antisense	1523-ATTATCTGGTGC GGGAACAC-1542	This study
S ₁ 350	Sense	1350-GGGATAGCAACACCACTACCA-1370	This study
S ₁ 693	Antisense	1675-TGGAATCTCTGGGGAGGAC-1693	This study
S ₁ 476	Sense	1476-CGGTGGATTACCTTTTGA-1495	This study
S ₁ 154	Antisense	2136-AGTTTGCCAGTTGCCATCA-2154	This study
S ₁ 013	Sense	2013-TCTGCCTGCTACTCTCTCCA-2032	This study
S ₁ 669	Antisense	2648-TGGTTTGCCTGTATCAGAAGG-2669	This study
UG1	Sense	2402-TTTGTGTCAGGTAAATG-2421	2
222	Antisense	2982-CICCIGGIGGIAYRWACA-2999	48
S ₁ 37	Sense	2837-AGGAAATTGGAGTTCTTCACC-2857	17
S ₁ 688	Antisense	3468-ACATGACGTTCACTGCGTTTT-3488	17
71935	Sense	3206-GTCAATGATCACAAACCC-3222	46
EUC2	Antisense	4454-TTTGCACTTGAAGTGTATGTA-4474	11
UG23	Sense	4169-AAGGGATTGGAGTGGGTGTC-4188	25
UC15	Antisense	4948-CATCTCTTGAAGTTTGCT-4965	25
CVD1	Sense	ACGACCTGAACCAAAATCCA	This study
CVD2	Antisense	CAATCCACCCCTTCTTCTCAC	This study
S ₁ 42	Sense	4962-GATGCTGTCCTTTAGTGTGTGG-4984	49
S ₁ 952	Antisense	5853-GATGCTGTCCTTTAGTGTGTGG-5873	49
S ₁ 70	Sense	5769-TATGTATGTTCTGTCCGGTGCT-5791	49
S ₁ 299	Antisense	5977-ACTGGATTTCACCTTGACTCTG-5999	49
S ₁ 450	Antisense	6851-ATTGAAGTGCCTGAGCAACC-6871	49
S ₁ 198	Sense	6728-GCACTAAAGATGGTGCTTGAGAAA-6752	49
S ₁ 885	Antisense	7391-CTACAACAGTATGACCAATCCAA-7415	49

^a Primer positions are based on those of the Sabin 1 reference strain (accession number AY184219).

Nucleotide sequence accession number. The almost complete genomic sequence of isolate 7/b/97 (from nucleotide 48 to nucleotide 7406) has been deposited in the GenBank library under accession number EF456706.

RESULTS

Preliminary characterization of the isolates: complete VP1 sequencing. Partial sequencing of the VP1 region of the isolates identified five poliovirus isolates. Two of the isolates were of Sabin 2 vaccine origin, two were of Sabin 3 vaccine origin, and one was of Sabin 1 vaccine origin.

We further studied the poliovirus isolates by performing complete sequencing of the VP1 capsid protein. Complete sequencing of the VP1 genomic region revealed that the isolates of Sabin 2 and Sabin 3 vaccine origin were very closely related to the corresponding OPV reference strains (levels of homology, >99.5%). In contrast, isolate 7/b/97, which was found to be of Sabin 1 vaccine origin, exhibited 1.87% divergence in the VP1 region from the corresponding OPV reference strain. Thus, it was characterized as a type 1 VDPV and was studied further by sequencing a large part of the genome.

Genomic sequencing of isolate 7/b/97. The almost complete genome (from base 48 to base 7406) of isolate 7/b/97 was sequenced and was aligned with the genome of the reference Sabin 1 strain. Several mutations were identified in the 5' UTR and in the capsid coding region, and a recombination event was identified in the 2A region of the genome.

In the 5' UTR two mutations were identified, a transition mutation (C→T) at nucleotide 471 and a transversion mutation (C→G) at nucleotide 583 (Table 2). Furthermore, the

A→G transition mutation at nucleotide 480, which is a well-known determinant of loss of the attenuated phenotype of Sabin 1 strains, was not identified (7).

The mutational pattern of the capsid was characterized by a preponderance of synonymous mutations over nonsynonymous mutations. Of the 34 mutations identified in the capsid region, 27 were synonymous (~80%) and only seven were nonsynonymous (Table 2). Nonsynonymous mutations identified in the capsid are shown in Fig. 1.

In VP4 and VP2 10 synonymous mutations were identified. In the VP3 capsid protein, only two of seven mutations that were identified resulted in amino acid substitutions. Specifically, an A→C transition at nucleotide 1944 resulted in a lysine-to-threonine substitution at amino acid 60 of VP3, while an A→T transversion at nucleotide 2438 resulted in a methionine-to-leucine change at amino acid 225 of VP3.

In the VP1 genomic region, 12 of the 17 nucleotide changes identified were substitutions at synonymous positions (~70%), and only five nucleotide changes resulted in amino acid substitutions (Table 2). A G→A mutation at nucleotide 2645 resulted in a valine-to-isoleucine substitution at residue 56 of the VP1 capsid protein. Substitutions at nucleotides 2749 (A→G), 2774 (A→G), and 2795 (A→G) resulted in amino acid substitutions at residues 90 (isoleucine to methionine), 99 (lysine to glutamic acid), and 106 (threonine to alanine), respectively. A mutation at nucleotide 3188 (T→A) resulted in a phenylalanine-to-isoleucine amino acid substitution at residue 237.

Genomic sequencing revealed a recombination event in the

TABLE 2. Nucleotide mutations and amino acid substitutions identified in the 5' UTR and P1 coding region of isolate 7/b/97

Region	Nucleotide			Amino acid			Amino acid location (reference[s])
	Position	Sabin 1	7/b/97	Position	Sabin 1	7/b/97	
5' UTR	471	C	T				
	583	C	G				
VP4	790	A	G				
	793	T	C				
	814	C	T				
	856	T	C				
VP2	1060	T	C				
	1174	G	A				
	1354	T	C				
	1357	C	T				
	1477	G	A				
	1624	T	C				
VP3	1864	T	C				
	1942	A	G				
	1944	A	C	60	Lys	Thr	N-AgIII (4, 43)
	2243	C	T				
	2332	C	T				
	2438	A	T	225	Met	Leu	Protomer interface (16, 40)
	2476	A	G				
VP1	2542	A	G				
	2617	A	G				
	2623	A	G				
	2645	G	A	56	Val	Ile	Capsid interior (15)
	2749	A	G	90	Ile	Met	N-AgI (43)
	2767	T	C				
	2774	A	G	99	Lys	Glu	N-AgI, VP1 BC loop (20, 26, 27, 44)
	2779	T	C				
	2789	C	T				
	2795	A	G	106	Thr	Ala	VP1 BC loop (16, 26, 36)
	2905	C	T				
	3067	A	G				
	3070	G	A				
	3133	G	A				
	3157	C	T				
	3188	T	A	237	Phe	Ile	Canyon floor (4)
	3349	G	A				

2A region of isolate 7/b/97. Isolate 7/b/97 was the product of recombination between Sabin 1 and non-Sabin viruses. Simplot analysis revealed that in most genomic regions the strain most closely related to isolate 7/b/97 was CAV15, with levels of homology fluctuating between 72 and 92%. Sabin 1 and CAV18 were the strains with the highest levels of homology (~85%) in parts of the 2C and 3D genomic regions (Fig. 2a and 2b). After a comprehensive search of the GenBank database, no strain with a level of homology greater than 95% was found. Furthermore, analysis with the MEGA program (version 3.1) revealed that our strain is more closely related to *Enterovirus* species C than to *Enterovirus* species A, B, or D (data not shown) (35). These results suggest that the donor of noncapsid sequences of isolate 7/b/97 was an unidentified strain probably belonging to *Enterovirus* species C.

RCT marker test. The RCT marker assay showed that isolate 7/b/97 had lost the temperature-sensitive phenotype. Specifically, the titer of isolate 7/b/97 was the same at 36°C and at 39.5°C (4.7 log TCID₅₀/0.1 ml), which means that the virus can efficiently replicate at elevated temperatures. For the Sabin 1

reference strain, the difference between the titers at 36°C (4.5 log TCID₅₀/0.1 ml) and at 39.5°C (1.5 log TCID₅₀/0.1 ml) was greater than 2 logarithms, a result consistent with the temperature-sensitive phenotype of Sabin strains.

DISCUSSION

In the current study retrospective isolation and characterization of a type 1 VDPV isolate, 7/b/97, isolated from sewage in Athens, Greece, in 1997 is reported. Genomic sequencing revealed that this isolate originated from recombination between Sabin 1 and an unidentified enterovirus belonging to species C. Moreover, amino acid substitutions frequently found in previous studies were scattered throughout the capsid. The important findings of the present report are that highly divergent vaccine derivatives can be found even in communities with high immunization levels and that environmental surveillance for poliovirus should be permanent even in countries considered polio free.

Two mutations were identified in the 5' UTR. Nucleotide

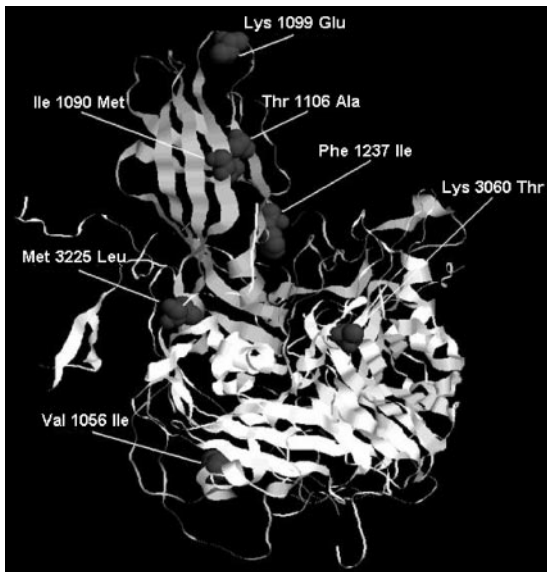


FIG. 1. Amino acid substitutions in the capsid protomer of isolate 7/b/97. Amino acids of the VP1 and VP3 capsid proteins are numbered starting at positions 1001 and 3001, respectively. For this visualization the capsid protomer structure of poliovirus type 1 Mahoney strain was used (Protein Data Bank accession number 1ASJ.pdb) (55).

471, where a C→T transition was identified, is located in a single-stranded part of domain V, while nucleotide 583, where a C→G transversion was observed, is located in a double-stranded region of domain VI (50). The C→G substitution at nucleotide 583 was not accompanied by a substitution at nucleotide 616 of domain VI in the frame of covariation in the 5' UTR.

Molecular characterization of strain 7/b/97 in the capsid coding region revealed amino acid substitutions in key spots of the virion. Amino acid substitutions were clustered in spots affecting the virion stability (Val-56-Ile of VP1 and Met-225-Leu of VP3), in virus antigenic sites (Lys-60-Thr of VP3 in N-AgIII and Ile-90-Met and Lys-99-Glu of VP1 in N-AgI), and in residues affecting virus-receptor interactions (Thr-106-Ile in the VP1 BC loop and Phe-237-Ile in the canyon floor).

Amino acid 56 of VP1 is located at the internal side of the capsid and probably participates in hydrophobic interactions between the VP3 and VP1 capsid proteins. These interactions are crucial for the stability of the capsid, as proposed by Cherkasova et al. (15). Amino acid 225 of VP3 is situated in the internal part of the virion at the interface between protomers, a region important for the regulation of virus-receptor interactions and for virion assembly (16, 40).

Amino acid 60 of VP3 is located at the exterior of the virion, at antigenic site III of the virus (44). Furthermore, amino acid 60 is located in an insertion in β -strand B of VP3, a part of the viral surface which is in contact with the D1 domain of the CD155 receptor during virus cell entry (4). Residues 90 and 99 of VP1 are highly exposed in the capsid exterior and are located at antigenic site I (44). Residue 99 of VP1 is localized in the VP1 BC loop, a region which is involved in virus-receptor interactions, as part of the north rim of the canyon (26, 27, 45). Furthermore, the Lys→Glu amino acid substitution at residue

99 eliminates a trypsin cleavage site, which is characteristic of Sabin 1 strains (20).

Residue 106 of VP1 is also located in the VP1 BC loop and participates in virus-receptor interactions. Amino acid 237 leans towards the inner surface of the capsid and is located next to the GH loop of VP1, a region which participates in formation of the canyon floor (4, 16, 26, 37).

It has been proved that certain amino acids located in the antigenic sites of Sabin 1 strains (amino acid 60 of VP3 and amino acids 106, 99, and 90 of VP1) change very rapidly once the viruses replicate in the human intestine into the corresponding wild-type amino acids or into amino acids homotypic to the corresponding residues of the wild-type virus. The very early fixation of these substitutions probably indicates that they are not the result of immune evasion and they might reflect an effort of the virus to adapt in the human intestine (60). Considering the fact that many of these substitutions were identified in the capsid coding region of isolate 7/b/97 and also in many previous studies (3, 15, 17, 23, 34, 43) in distinct evolving lineages of OPV strains, we suggest that they might confer a selective advantage to isolate 7/b/97. It can be hypothesized that these changes, which occur in the virus antigenic sites, may facilitate the virus-receptor interactions. Our hypothesis is reinforced by the fact that a large part of the region in contact with the poliovirus receptor CD155 comprises simultaneously all the virus antigenic sites. This is especially the case for residue 60, which is located in β -strand B of the VP3 part of the viral surface in contact with the D1 domain of receptor CD155, and for residues 99 and 106 of the VP1 BC loop, which are implicated in the formation of the north rim of the canyon (4, 26, 27, 28). Furthermore, the limitation of proteins' conformations in regions crucial for capsid stability is reflected in the conservative nature of amino acid substitutions observed in our isolate, especially the substitutions in residues 56 (Val→Ile) of VP1 and 225 (Met→Leu) of VP3. Both of these residues participate in hydrophobic interactions between the VP3 and VP1 capsid proteins, which contribute to capsid stabilization (15).

Mutations that result in loss of the attenuated and temperature-sensitive phenotype of Sabin 1 strains have been identified in the genome of strain 7/b/97. Mutations at nucleotides 2438 (A→T [Met-225-Leu]) and 2795 (A→G [Thr-106-Ala]) are well-known determinants of attenuation (7), and both were identified in the capsid coding region of isolate 7/b/97. Reversion of one of the major determinants of attenuation, located at nucleotide 480 of the 5' UTR, was not identified. Mutations at nucleotides 1944 (A→C [Lys-60-Thr]) and 2438 (A→T [Met-225-Leu]) and an amino acid change at residue 73 of 3Dpol from histidine to tyrosine were also present in isolate 7/b/97 and contributed to the loss of the temperature-sensitive phenotype (7), as confirmed by the RCT marker assay.

Genomic sequencing revealed a recombination event in the 2A region of isolate 7/b/97 between a Sabin type 1 strain and an unidentified strain probably belonging to enterovirus species C. Recombination provides evidence of circulation of isolate 7/b/97 in the community. This notion is in agreement with the fact that all circulating VDPVs isolated so far have been found to be recombinants with recombination in the nonstructural region of their genomes with enteroviruses belonging to species C (32, 36, 51, 52). Circulating wild polioviruses containing

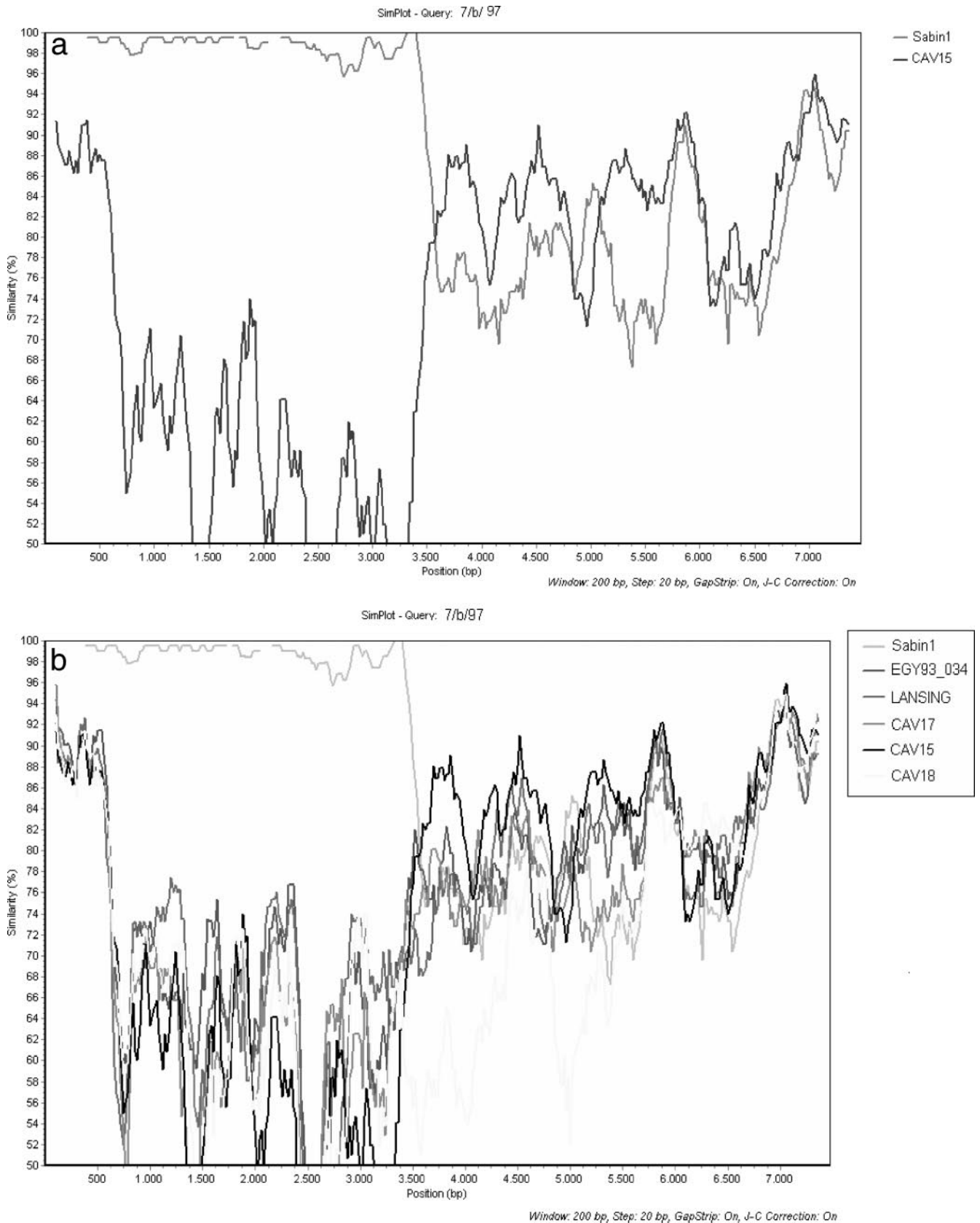


FIG. 2. (a) Simplot analysis of the complete genome of isolate 7/b/97 compared with the genome of isolate Sabin 1 (accession number AY184219) and CAV15 strain G-9 (accession number AF465512). (b) Simplot analysis of the complete genome of isolate 7/b/97 compared with the genomes of isolate Sabin 1 (accession number AY184219), CAV15 strain G-9 (accession number AF465512), CAV17 strain G12 (accession number AF499639), CAV18 strain G-13 (accession number AF465513), and strain EGY93_034 (accession number AF448783).

a block of Sabin 1-derived sequences have also been isolated (38, 39). Furthermore, we suggest that recombination may increase the fitness of isolate 7/b/97 by substitution in the temperature sensitivity determinants mapped to the 3Dpol and 3' UTR of the isolate. This is actually true for 3Dpol, since amino acid 73 was changed from histidine in the wild-type virus to tyrosine (unfortunately, we have no data for the 3' UTR since we obtained the genomic sequence of the isolate from nucleotide 48 to nucleotide 7406). This suggestion is also supported by recent phylogenetic studies which proposed that polioviruses and species C enteroviruses are distinct capsid sequences in quest of noncapsid sequences with the highest fitness (8).

Based on WHO criteria, isolate 7/b/97 is characterized as an aVDPV. Nevertheless, genomic evidence presented here could place isolate 7/b/97 in the cVDPV category. First, the variability among antigenic sites is limited despite the high degree of nucleotide divergence of the isolate, and second and most important, the recombination in the nonstructural part of the genome with an unidentified species C enterovirus strain provides evidence of circulation of the isolate in the population (33, 52, 61). However, there were no polio epidemics or suspected poliomyelitis cases, no gaps in OPV coverage, and no other conditions that may have benefited cVDPV emergence and spread in Greece during the time period that the virus was isolated. We hypothesize, therefore, that strain 7/b/97 is more likely to be an iVDPV strain that was excreted from an unidentified source.

Although AFP surveillance is one of the two major cornerstones in poliovirus eradication, environmental surveillance has been proven to be a highly sensitive tool in some situations. Environmental surveillance has the potential to identify poliovirus in the environment, even in the absence of paralytic poliomyelitis cases. For example, highly divergent VDPVs were isolated in Finland, Israel, and Estonia in the absence of suspected poliomyelitis cases (5, 41, 53). Furthermore, indigenous type 1 wild poliovirus was identified in environmental samples in Egypt even after OPV immunization campaigns (19). High-sensitivity environmental surveillance should be implemented during and after poliovirus eradication to prevent cases or even outbreaks of paralytic poliomyelitis that otherwise would be inevitable (54).

In conclusion, we report genomic and phenotypic characterization of an environmental aVDPV isolate (7/b/97). Extensive genomic sequencing revealed 1.87% VP1 sequence divergence and a vaccine/nonvaccine recombination event in the 2A genomic region of isolate 7/b/97. The genomic features of isolate 7/b/97 may place this strain in the cVDPV category, but as strain 7/b/97 was not connected with any polio case, it is more likely that it belongs in the iVDPV category.

The preponderance of countries certified to be polio free have switched from the OPV vaccine to the IPV vaccine. However, the question now is what would happen after importation of a highly divergent VDPV isolate in a population. Taking into consideration the fact that IPV does not induce the same immunity levels as OPV, importation of a highly virulent strain would have serious implications for public health. For this reason, increased laboratory surveillance is necessary even after eradication of poliomyelitis. Environmental surveillance should also be implemented as a supportive tool in the polio

surveillance system in communities that can support this procedure both financially and professionally. Furthermore, extensive genomic characterization of such evolved derivatives of vaccine strains would help to further elucidate the genomic properties of these isolates, since there is a significant risk of prolonged circulation in populations with a low immunity level and subsequent conversion of the isolates into epidemic strains.

Intensive research for optimal vaccination strategies for the final stages of polio eradication and the posteradication era should be urgently promoted.

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