Nucleotide variation in Sabin type 2 poliovirus from an immunodeficient patient with poliomyelitis

Gabriele Buttinelli,1 Valentina Donati,1 Stefano Fiore,1 Jill Marturano,1 Alessandro Plebani,2 Paolo Balestri,3 Anna Rosa Soresina,2 Rossella Vivarelli,3 Francis Delpeyroux,4 Javier Martin5 and Lucia Fiore1

1Laboratory of Virology, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy
2Department of Pediatrics, University of Brescia, Italy
3Pediatrics Unit, University of Siena, Italy
4Epidemiologie Moleculaire des Enteurovirus, Institut Pasteur, Paris, France
5NIBSC, Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QG, UK

The molecular and antigenic properties of a Sabin-like type 2 poliovirus, isolated from the stool samples of a 2-year-old agammaglobulinaemic child who developed paralysis 1 year after receiving the third dose of oral poliovirus vaccine, were analysed. The virus revealed 0–88 % genome variation in the VP1 region compared with the standard reference strain, compatible with replication of the virus in the intestine over approximately 1 year. The typical mutations in the 5′NCR and VP1 associated with reversion to neurovirulence for Sabin type 2 poliovirus were found. Despite this, the virus was characterized by both PCR and ELISA tests as Sabin-like and showed temperature sensitivity and neurovirulence in transgenic mice typical of the Sabin type 2 vaccine strain. Gammaglobulin replacement therapy led rapidly to virus clearance, which, when combined with treatment with the antiviral drug pleconaril, stopped virus excretion; no further virus shedding occurred. This is the first case of poliomyelitis and long-term excretion from an immunodeficient patient to be reported in Italy through the active ‘Acute Flaccid Paralysis’ surveillance system.

INTRODUCTION

As global poliomyelitis eradication approaches, Europe is moving towards stopping poliovirus vaccination with oral polio vaccine (OPV). Whereas disease caused by wild poliovirus is decreasing, vaccine-associated paralytic poliomyelitis (VAPP), although rare, is causing increasing concern. Even more worrying is the fact that VAPP outbreaks, reported recently in the Caribbean and the Philippines, have been caused by highly mutated and neurovirulent Sabin-like type 1 polioviruses (Kew et al., 2002; CDC, 2002a). This emphasizes the need for an appropriate strategy to dismiss OPV after wild poliovirus eradication (Hull et al., 1997). Long-term excretors of poliovirus, like immunodeficient patients, are an additional concern, as they may ensure poliovirus persistence and new infections in the unvaccinated population in the post-eradication era. Prolonged intestinal replication of the virus in these subjects could increase the chances of reversion to neurovirulence and transmissibility characteristics typical of wild poliovirus strains.

In Italy, OPV vaccination was made compulsory for 3-month-old infants in 1966 and high immunization coverage has been achieved thereafter. The Ministry of Health (Italy) has documented more than 95 % immunization coverage for children of less than 5 years of age. The last two cases of paralytic disease due to indigenous wild poliovirus were reported in 1982 (Novello et al., 1987); since then, three imported poliomyelitis cases from Iran, India and Libya (the last in 1988) due to wild virus have been reported and investigated. With the exception of the cases cited above, only VAPP cases (two of which were contacts of vaccines) have occurred in Italy (Fiore et al., 1999, 2000).

Active surveillance of acute flaccid paralysis (AFP) was established in Italy in 1997 following WHO indications. To comply with an active surveillance system, hospitals are contacted by the local Regional Reference Centres every 15 days and asked to provide information on AFP cases admitted. The incidence rate found over the 5 years of activity was good and performance indicators improved with time (Fiore et al., 2000).

In Italy, a combination of inactivated polio vaccine (IPV) and OPV was adopted in May 1999 to reduce the number of VAPP cases, while keeping high levels of protection against possible wild virus importation, also related to the constant, often illegal, immigration flow from endemic countries.
The last VAPP case in Italy occurred in the year 2000 in an immunodeficient child (born in August 1998) who received three doses of OPV (last dose in July 1999). The child developed paralysis in April, several months after vaccination. A Sabin-like type 2 poliovirus was isolated from stool samples. Immunological assessment showed a condition of agammaglobulinaemia, which, unfortunately, was undiagnosed when the child was a year old.

Here, we report the complete nucleotide sequence of the Sabin type 2 poliovirus isolated from this patient soon after the onset of paralysis; the finding of the typical nucleotide changes of the wild-type genotype and the accumulation of other mutations suggested a prolonged replication of the virus in the patient’s gut. The virus isolate, ITA00-014, showed antigenic characteristics, neurovirulence and temperature-sensitivity typical of the Sabin type 2 vaccine strain.

The efficacy of treatments with gammaglobulins and the drug pleconaril in the clearance of the virus is also discussed.

**METHODS**

**Patient history.** The female patient (identified as ITA00-014 and hereon referred to as such) was born on August 9, 1998, after a normal delivery from non-consanguineous parents. Family history was negative for primary immunodeficiencies and early deaths due to proven or suspected infections.

The child was vaccinated according to the vaccination schedule in use in Italy before 1999; thus, three doses of OPV were administered in November 1998 and in both January and July 1999. In October 1999, she was admitted to a local hospital with clinical symptoms of irritability, drowsiness, muscular weakness, hypotony and right hemiparesis. Blood and cerebrospinal fluid (CSF) analysis, serological investigation as well as viral and bacterial cultures were normal. A presumed diagnosis of meningocencephalitis was performed and treatment with acyclovir was started, followed by clinical improvement. The child was discharged and admitted again to the hospital in Siena, Italy, in April 2000 with irritability, muscular weakness and flaccid paralysis. Stool samples, CSF and blood were collected and sent to the Istituto Superiore di Sanità, Italy, the WHO regional reference laboratory for polio. A type 2 poliovirus was isolated only from the faeces. Serum antibody titres to polioviruses were less than 1:4.

Immunological evaluation performed at this time showed absence of serum immunoglobulin levels (IgG, <33 mg dl$^{-1}$; IgA, <5 mg dl$^{-1}$; IgM, <15 mg dl$^{-1}$), absence of circulating B cells and normal count and function of T lymphocyte subsets. Because the patient was female, a tentative diagnosis of an autosomal recessive form of agammaglobulinaemia was made. Another course of acyclovir treatment was started with clinical improvement.

In May 2000, the child was admitted to the Department of Pediatrics of University of Brescia, Italy, for further immunological evaluation that confirmed the diagnosis of agammaglobulinaemia. On May 7, immunoglobulin substitution therapy was administered intravenously at a dosage of 400 mg kg$^{-1}$ for 4 consecutive days followed by the administration of the same dosage every week for the first 4 months and then every 3 weeks. Poliovirus excretion stopped after this treatment. On May 18, a 1-week treatment with pleconaril (Viopharma) at a dosage of 5 mg kg$^{-1}$ per dose 3 times a day was started with no side effects. The child was discharged with signs of improvement of the neurological symptoms. Nonetheless, she had residual paralysis at the lower right limb. Stool samples collected 1 year after treatment showed absence of poliovirus.

**Cells, viruses and animals.** HEP-2C and RD cells were obtained from the National Institute of Public Health and the Environment (RIVM, Bilthoven, The Netherlands), as were L20B cells, a mouse cell line carrying the human poliovirus receptor. Buffalo green monkey (BGM) cells were obtained from the ATCC. Strain S139C6, derived from a VAPP case, is a highly neurovirulent Sabin type 1 vaccine-derived strain, which has been shown to be as neurovirulent in Tg21 mice and also in monkeys as the wild type 1 poliovirus Mahoney reference strain (Christodoulou et al., 1990; Georgescu et al., 1997). Type 2 poliovirus strains 6735 and 102050 were isolated from two long-term excretor immunodeficient (ID) individuals, whereas strain 117 was derived from another VAPP case. Type 2 poliovirus strain P712 CH2ab was obtained from the ATCC. Two sets of transgenic mice were used: TgPVR21 (Koike et al., 1991) and Tg21-Bx (Martin et al., 2002).

**Virus isolation.** RD, L20B and HEP-2C cell monolayers, grown in Dulbecco’s and Eagle’s MEM, respectively, supplemented with 10% foetal calf serum, were inoculated with 0.2 ml of 10% treated stool suspension or CSF in Hanks’ balanced salts solution. Cell monolayers were incubated at 37°C for 7 days or until development of CPE. A total of three blind passages were performed before a culture was considered negative.

**Virus typing and characterization.** Virus isolates were identified by micro neutralization assay using serum pools and type-specific poliovirus antisera, kindly provided by the RIVM, according to the standard methods recommended by the WHO (WHO, 1997, 2001). Serotyping was also confirmed by immunostaining analysis (Di Lardono et al., 2002). Intratypic differentiation was performed by PCR, using Sabin-specific primers (CDC, Atlanta, USA) (Kilpatrick et al., 1998), and ELISA, with cross-absorbed type-specific polyclonal antibodies (van der Avoort et al., 1995).

**Temperature sensitivity.** Temperature sensitivity was assayed by observation of plaque formation on BGM cells at 35, 39.5 and 40.1°C, as described previously (Minor, 1980). BGM cells are more suitable than other cells for type 2 poliovirus growth at higher temperatures.

**Neutralization of virus isolate with mAbs.** Neutralization of virus infectivity with mAbs specific for type 2 poliovirus, either wild or vaccine strains, was performed as described previously (Crainic et al., 1984). IgG antibody 2b is directed toward antigenic site 2b and is designated ‘W’ (specific for wild-type strains). IgG antibodies 1a and 1b directed towards site 1a are classified ‘K’ (capable of neutralizing both wild and vaccine strains). IgG antibodies 2a and 2b are classified ‘S’ (specific for Sabin-like strains) (Crainic et al., 1984). As antibody 1G6, an IgA directed against site 2a (Buttinelli et al., 2001) neutralized both Sabin and wild-type virus, it was considered a ‘K’ antibody. Plates were scored up to day 5 for CPE. Neutralization indexes, determined as the difference of log virus titres in the absence and the presence of antibody, were used to quantify virus neutralization by each mAb.

**Nucleotide sequencing.** The viral genome was sequenced using the DyeDeoxy Terminator Cycle Sequencing kit (Perkin-Elmer) after reverse transcription and cDNA amplification with a panel of synthetich oligonucleotides as primers. The sequences obtained were aligned with the corresponding sequences of Sabin type 2 poliovirus reference strain (Pollard et al., 1989).

**Neurovirulence testing in transgenic mice.** Neurovirulence tests were carried out in two laboratories using different types of mice. The first laboratory used TgPVR21 mice (kindly provided by...
Characterization of isolate ITA00-014

Antigenic and molecular analyses performed by ELISA with cross-adsorbed antisera and PCR characterized the type 2 isolate ITA00-014 as a Sabin-like virus. Additional antigenic analysis of the strain was performed by studying reactivity with a panel of mAbs of known specificity. No difference was observed between the isolate and the reference Sabin type 2 poliovirus. The Sabin-specific epitope was present in the isolated strain and no reactivity with the mAbs directed against wild epitopes was evidenced, suggesting that the virus was antigenically indistinguishable from the Sabin type 2 vaccine reference strain. (Table 1).

Analysis of growth sensitivity at temperatures of 35, 39-5 or 40-1 °C showed Sabin-like characteristics for the isolated virus; growth was severely impaired at the higher temperatures (four-log differences in titre), as shown by the reference strain.

Nucleotide and amino acid sequence

Comparison of the full genome (approximately 7400 bases) of strain ITA00-014 with the reference Sabin type 2 poliovirus showed 0-54 % variation; mutations were distributed throughout the genome (Table 2).

Two mutations were found in the 5’NCR, at positions 189 (U→C) in domain III and 481 (A→G) in domain V (Ehrenfeld & Semler, 1995). The latter is associated typically with reversion to neurovirulence in Sabin type 2 vaccine strain.

Most of the mutations in the P1 region were clustered in VP1, leading to 0-88 % variation; the nucleotide change in VP2 did not produce any amino acid substitution, whereas one of the three mutations in VP3 lead to replacement of His97 with Tyr.

Of the eight mutations in VP1, six gave rise to amino acid substitutions. The mutation at nucleotide 2909 (t→c), causing substitution of Ile143 with Thr, is known to be associated with reversion to neurovirulence (Pollard et al., 1989; Equestre et al., 1991).

As no crystallographic structure is available for Sabin type 2 poliovirus to localize the observed amino acid substitutions in the three-dimensional structure of the virus, the Lansing strain was used (Lentz et al., 1997). These two strains have identical sequences in the positions where mutations in the ITA00-014 isolate were found, except for residue 143, which is in loop D–E. Amino acids at positions 166 and 169 of VP1 are localized in loop E–F and residues 231 and 236 are part of loop G–H. Amino acid 202 is in a short loop interrupting the G β-sheet. Although none of these amino acids is located in the antigenic sites described so far for type 2 poliovirus, all are exposed on the virion surface.

Mutations in the P2 region leading to amino acid replacement were found only in protein 2B. A mutation rate of 0-57 % was found in P3; protein 3A carried three nucleotide mutations, resulting in two amino acid changes, and six of the eight mutations of the 3D polymerase gene gave rise to amino acid replacements (Table 2).

Neurovirulence in transgenic mice

The ITA00-014 type 2 strain isolated appeared to be very attenuated following either intracerebral or intraperitoneal injections (Table 3). For a more accurate evaluation, in both neurovirulence tests performed in TgPVR21 mice, 20 animals were inoculated with ITA00-014, whereas six mice were used for neurovirulent control strain S139C6 and 10 for reference Sabin type 2 poliovirus. Only 1 of 40 mice inoculated was found dead 3 days post-intracerebral inoculation; since no paralysis was observed, death could be the...
Table 2. Nucleotide and amino acid substitutions in type 2 poliovirus ITA00-014 (GenBank AY177685)

<table>
<thead>
<tr>
<th>Region</th>
<th>Protein</th>
<th>Nucleotide change</th>
<th>No. of mutations/total no. of changes (%)</th>
<th>Amino acid change</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’NCR</td>
<td></td>
<td></td>
<td>2/746 (0.27)</td>
<td></td>
</tr>
<tr>
<td>P1</td>
<td>VP2</td>
<td>t189→c</td>
<td>1/813 (0.12)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>VP3</td>
<td>a481→g</td>
<td>3/714 (0.42)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VP1</td>
<td></td>
<td></td>
<td>8/903 (0.88)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P2</td>
<td>2A</td>
<td></td>
<td>12/1725 (0.69)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P3</td>
<td>3A</td>
<td></td>
<td>13/2259 (0.57)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3D</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3’NCR</td>
<td></td>
<td></td>
<td>1/78 (1.3)</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Pathogenesis of virus isolate ITA00-014

Intracerebral (IC) or intraperitoneal (IP) inoculation in TgPVR21 mice.

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Mean ± SEM</th>
<th>TgPVR21 mice paralysed/inoculated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC</td>
<td>IP</td>
</tr>
<tr>
<td>Type 1 poliovirus S139C6</td>
<td>3.5 ± 0.3</td>
<td>3.5 ± 0.3</td>
</tr>
<tr>
<td>Type 2 poliovirus Sabin</td>
<td>14.0 ± 0.0</td>
<td>ND</td>
</tr>
<tr>
<td>Type 2 poliovirus ITA00-4</td>
<td>13.5 ± 0.5</td>
<td>14.0 ± 0.0</td>
</tr>
</tbody>
</table>

ND, Not done.
consequence of an injury caused by the needle during injection. Thus, this strain appeared to be as attenuated as the Sabin type 2 reference strain; in fact, neither disease nor death during the 14 days following inoculation (MHT = 14.0 ± 0.0) was recorded.

Results confirming the attenuated phenotype of the virus in mice were obtained also using Tg21-Bx. Only 2 of 40 mice inoculated with strain ITA00-014 developed paralysis: of these (both males), one was from the 10^8.2 group and one was from the 10^7.2 group (Table 4). The PD50 dose was therefore estimated to be above 10^8.2 p.f.u. (Table 5).

**DISCUSSION**

Nearly four decades of experience have shown that OPV is very safe and effective in preventing poliomyelitis. However, it is also known that upon replication in the human gut, attenuated viruses may revert to the neurovirulent phenotype (Minor & Dunn, 1988). OPV vaccination is therefore not advisable for immunodeficient people, as these people are also known to excrete poliovirus for long periods of time (Kew et al., 1998; Bellmunt et al., 1999; Minor, 2001).

In this work, we have characterized a type 2 poliovirus strain isolated from an immunodeficient child who developed paralysis 1 year after receiving the third dose of OPV. The strain isolated from this case had most of the characteristics of type 2 vaccine strain, such as sensitivity to temperature, reactivity in ELISA with cross-adsorbed sera (van der Avoort et al., 1995) and PCR profile (Kilpatrick et al., 1998).

Despite this, genome sequencing showed 0·54 % nucleotide variation (particularly 0·88 % in capsid protein VP1) and 0·81 % amino acid substitutions. Based on the nucleotide change in VP1 between the isolate and the Sabin type 2 vaccine reference strain, the time between vaccine assumption and virus isolation was compatible with the mutation rate observed (Kew et al., 1995). The rate of VP1 evolution was assumed to be constant over the period of replication and similar to the rates observed for types 1 and 3.

Antigenic analysis of the type 2 isolate performed with a panel of mAbs did not show any difference with the Sabin type 2 reference strain, even though most of the amino acid mutations were in exposed regions of the capsid proteins. Prolonged excretion of poliovirus in immunodeficient patients who are receiving immunoglobulin therapy has been reported to lead to accumulation of changes and divergence in the antigenic sites from Sabin reference strains (Kew et al., 1998; Martin et al., 2000). Viruses replicating in the guts of these individuals were most probably subjected to selective antibody pressure, although lower than that encountered in immunocompetent individuals. In fact, a delay in the accumulation of antigenic changes in these subjects has been observed (Martin et al., 2000). Patient ITA00-014 had virtually no antibodies, which could explain the absence of mutations in antigenic sites of virus ITA00-014 and therapies were started after isolation.

Results of the neurovirulence test performed on TgPVR mice for this virus are contradictory. Even though the patient has permanent paralysis and mutations in positions 481 of the 5'NCR and 2908 of VP1 (amino acid 143), known to be correlated with the neurovirulent phenotype (Minor & Dunn, 1988; Pollard et al., 1989; Equestre et al., 1991), were present in the genome, the virus did not cause paralysis when tested in two different transgenic mouse strains.

It is difficult to explain this contradiction: (i) paralysis in the patient may not have been caused by the isolated poliovirus, but the onset of symptoms and the time of sample collection and isolation argue against this hypothesis; (ii) there might have been a mixed virus population in the gut and the strain causing paralysis was not isolated from stool samples. Both Kew et al. (1998) and Bellmunt et al. (1999) have reported cases of immunodeficient children from whom two type 1 poliovirus variants have been isolated simultaneously from stool specimens; in the first case, one of the variants vanished during the time of observation, whereas in the second study, excretion of both variants stopped spontaneously. In our case, a bottle-neck evolution process (Muller’s ratchet) could have led to the selection of an attenuated variant, probably better adapted to replication in the gut, from a more neurovirulent virus population (Domingo & Holland, 1997); (iii) determinants that allow either high multiplication in the digestive tract or circulation of the virus do not strictly correlate with those

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**Table 4. Pathogenesis of virus isolate ITA00-014**

Intramuscular inoculation in Tg21-Bx mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Inoculum titre (p.f.u. per mouse)</th>
<th>Paralysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10^8.2</td>
<td>1/8</td>
</tr>
<tr>
<td>2</td>
<td>10^7.2</td>
<td>1/8</td>
</tr>
<tr>
<td>3</td>
<td>10^6.2</td>
<td>0/8</td>
</tr>
<tr>
<td>4</td>
<td>10^5.2</td>
<td>0/8</td>
</tr>
<tr>
<td>5</td>
<td>10^4.2</td>
<td>0/8</td>
</tr>
</tbody>
</table>

**Table 5. Pathogenesis of virus isolate ITA00-014**

Intramuscular inoculation in Tg21-Bx mice

<table>
<thead>
<tr>
<th>Type 2 poliovirus strain*</th>
<th>Tg21-Bx mice (PD50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sabin</td>
<td>&gt;7·9</td>
</tr>
<tr>
<td>117 (VAPP)</td>
<td>7·0 (6·6–7·4)</td>
</tr>
<tr>
<td>6735 (ID)</td>
<td>5·6 (5·2–5·9)</td>
</tr>
<tr>
<td>102050 (ID)</td>
<td>5·2 (4·9–5·5)</td>
</tr>
<tr>
<td>ITA00-014</td>
<td>&gt;8·2</td>
</tr>
</tbody>
</table>

*VAPP, vaccine-associated paralytic poliomyelitis; ID, immunodeficient patient.
Implicated in neurovirulence. The supposed parental strain of the Sabin type 2 strain was partially attenuated (Sabin & Boulger, 1973). However, a highly divergent derivative of the Sabin type 2 OPV strain was recovered from environmental samples during routine screening for wild polioviruses and was shown to be highly neurovirulent in TG-PVR mental samples during routine screening for wild polioviruses and was shown to be highly neurovirulent in TG-PVR mice (Shulman et al., 2000); (iv) there is not always an exact correlation between poliovirus neurovirulence in animal models. Different animals such as monkeys and mice and various strains of normal and transgenic mice showed different sensitivity to certain attenuating mutations of Sabin type 1 poliovirus (reviewed by Bouchard et al., 1995). It should, however, be mentioned that a doubt concerning the role of mutation in position 481 of the 5’UTR in neurovirulence of type 2 poliovirus has been raised in a recent investigation on mutagenized poliovirus in a monkey neurovirulence test (Rezapkin et al., 1999).

Immunoglobulins may have had an important role in clearing the virus from the intestine of patient ITA00-014. Enteroviruses appear to be cleared from the host mainly by antibody-mediated mechanisms, unlike most other viruses, which are contained largely by innate and cellular immunity. Availability of intravenous preparations of immunoglobulins and early recognition of enterovirus infections have led to a decrease in the number of chronic enterovirus infections (such as meningocencephalitis) among patients suffering from humoral immune deficiencies (McKinney et al., 1987). The antiviral drug pleconaril was also administered with immunoglobulins to patient ITA00-014. Pleconaril is an agent with activity against all enteroviruses (Pevear et al., 1999) and works by inhibiting the uncoating and release of infectious viral RNA, therefore blocking production of progeny virions (Rotbart, 1999). It has been used recently in clinical trials in enterovirus-induced diseases and has so far yielded promising results (Rotbart & Webster, 2001). Clinical improvement was observed in the patient after this treatment; improvement could have been due to the natural resolution of infection or be the effect of the therapeutic regimen. Therapy with pleconaril was well tolerated by the patient and there were no side effects.

The discovery of prolonged excretion of poliovirus from people affected by immunodeficiency (congenital or acquired) has raised increasing concern in the WHO (Kew et al., 1998; Martin et al., 2002). In the last phases of eradication, such people could be reservoirs for poliovirus, not only wild but also vaccine-derived strains. Indeed, more than one outbreak of poliomyelitis caused by a highly mutated vaccine strain has been reported (Kew et al., 2002; CDC, 2002a, b).

OPV is the vaccine of choice for the eradication of wild poliovirus. However, maintenance of high immunization coverage is crucial to protect against imported wild polioviruses and to prevent person to person transmission of OPV-derived viruses. It is also important that all countries maintain high quality AFP and poliovirus surveillance and that a global strategy is developed for the cessation of immunization with OPV after global certification of polio eradication (WHO, 1997); before that, however, an adequate strategy to interrupt immunization with OPV must be developed.

The presence of a mixed schedule of vaccination in Italy and the adoption of a full IPV in August 2002 should avoid future cases of VAPP in immunodeficient subjects and the spread of possibly reverted highly neurovirulent strains in the population.

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Mutated PV2 from immunodeficient polio case


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