TYPE 2 POLIOVIRUS RECOMBINANTS ISOLATED FROM VACCINE-ASSOCIATED CASES AND FROM HEALTHY CONTACTS IN BRAZIL

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Summary. – In a previous study (Friedrich *et al.*, 1995b) P2/Sabin-derived strains isolated in Brazil from vaccine-associated paralytic poliomyelitis (VAPP) cases and from healthy contacts were analyzed for the presence of mutations at nucleotide (nt) 481 in the 5'-noncoding region (5'NCR) and at the codon of amino acid (aa) 143 of the capsid protein VP1, that are known to increase neurovirulence. In the present study a part of the 3Dpol-coding region of these strains was sequenced (3Dpol seq.) with the aim to find recombinant strains. In the 3Dpol seq., four out of ten strains isolated from VAPP cases turned out to be recombinants: one had 3Dpol seq. from the P1/Sabin strain, while the second had a part of 3Dpol seq. both from the P2/Sabin and P1/Sabin strains; the third and fourth recombinants had 3Dpol seq. from non-vaccine strains. The strains isolated from healthy contacts of the two VAPP cases, from which type 2 vaccine/non-vaccine recombinant strains were isolated, also consisted from recombinant genomes with the same nt sequences as those of the isolates from VAPP cases, confirming the transmission of P2/Sabin-derived recombinants. Comparison of the aa sequence of the viral RNA polymerase of the P2/Sabin strain with the predicted aa sequences of these recombinants in 3Dpol seq. demonstrated that an aa 69 (Asp->Glu) substitution was observed in most of the recombinant genomes, while an aa 113 (Thr->Ser) substitution was observed in all the recombinant genomes. The possibility that the genomic recombination increased the neurovirulence of these strains cannot be excluded.

Key words: poliovirus; recombination; mutation, neurovirulence; vaccine-associated poliomyelitis

Introduction

Polioviruses are members of the *Enterovirus* genus, belonging to the *Picornaviridae* family and consist of an icosahedrical particle composed of 60 copies of capsid proteins VP1, VP2, VP3 and VP4 (Hogle *et al.*, 1985), surrounding a single-stranded positive-sense RNA genome of approximately 7500 nt (Kitamura *et al.*, 1981). The RNA molecule contains a 5'NCR of about 740 nt with a terminally linked protein VPg, a single open reading frame (ORF) coding for the structural and non-structural proteins, and a 3'NCR of about 70 nt followed by a poly(A)-tract (Kitamura *et al.*, 1981; Wimmer and Nomoto, 1993). Polioviruses are grouped into three serotypes (1, 2 and 3) on the basis of the antigenicity of the capsid. These viruses are the causative agents of poliomyelitis, a paralytic and occasionally fatal disease of humans.

Poliomyelitis has been efficiently controlled in Brazil by the use of the oral poliovirus vaccine (OPV) developed by A. Sabin, and wild poliovirus strains were not detected in this country during the last 5 years (Filippis *et al.*, 1994). Although the vaccine controlled the circulation of wild strains, rare VAPP cases have been detected in Brazil mainly in connection with type 2 and type 3 vaccine strains (Friedrich *et al.*, 1995b,c). Recently a type 1 vaccine strain was also isolated from a VAPP case in Brazil (Friedrich *et al.*, 1996). The isolation of Sabin-derived poliovirus vaccine strains (and also enterovirus 71) in Brazil from cases classified as Guillain-Barré syndrome, transverse myelitis and facial paralysis, suggested that these paralyses could be

Abbreviations: aa = amino acid; 3Dpol = 3Dpol coding region; 3Dpol seq. = the sequenced part of the 3Dpol; NCR = non-coding region; nt = nucleotide; OPV = oral poliovirus vaccine; ORF = open reading frame; VAPP = vaccine-associated paralytic poliomyelitis

caused in certain cases by enterovirus infections (Friedrich *et al.*, 1995a,c).

Molecular studies demonstrated that mutations are important for attenuation as well as for reversion of the Sabin vaccine strains to neurovirulence (Almond, 1987; Racaniello, 1988; Minor, 1992, 1993; Minor *et al.*, 1993; Macadam *et al.*, 1994). The A at nt 481 in the 5'NCR and the aa Ile at position 143 of the capsid protein VP1 are important for attenuation of the P2/Sabin strain (Ren *et al.*, 1991; Macadam *et al.*, 1991, 1993). Mutations in these two attenuating determinants were observed in most of the P2/Sabin-derived strains isolated from VAPP cases (Pollard *et al.*, 1989; Equestre *et al.*, 1991; Muzychenko *et al.*, 1991; Macadam *et al.*, 1991, 1993; Guillot *et al.*, 1994; Georgescu *et al.*, 1994, 1995a; Friedrich *et al.*, 1995b). Although mutations increase the neurovirulence of the P2/Sabin strain, other studies have suggested that genomic recombination could lead to the same effect (Lipskaya *et al.*, 1991; Furione *et al.*, 1993; Georgescu *et al.*, 1994, 1995a).

P2/Sabin-derived strains previously isolated from VAPP cases and from healthy contacts in Brazil, and analysed for the presence of mutations at nt 398 and 481 of the 5'NCR, and at the codon (nt 2908-2910) of aa 143 of the VP1 capsid protein coding region (Friedrich *et al.*, 1995b) were subjected in the present study to the analysis of their 3Dpol coding region with the objective of finding recombinant strains.

Material and Methods

Viruses and cells. Type 2 poliovirus strains, analyzed in the present study, were isolated from faecal samples collected in different Brazilian regions from ten paralytic poliomyelitis cases with sequels and from six healthy contacts (Friedrich *et al.*, 1995b). The following strains were isolated from the paralytic poliomyelitis cases: P2/10989, P2/13121, P2/1006, P2/1851, P2/998, P2/7790, P2/15517, P2/1400, P2/2645 and P2/1397. The strains P2/15561, P2/15562 and P2/15815 were isolated from three healthy contacts of the VAPP case from which the P2/15517 strain was isolated. The strains P2/1401 and P2/1402 were isolated from two healthy contacts of the VAPP case from which the P2/1400 strain was isolated. The P2/1398 strain was isolated from a healthy contact of the VAPP case from which the P2/1397 strain was isolated.

The type 2 poliovirus strains, isolated from VAPP cases and from healthy contacts, were previously characterized as P2/Sabin-derived ones (Friedrich *et al.*, 1995b). In the present study these P2/Sabin-derived strains were grown in RD cells at 37°C to increase their titer for sequencing purposes. The RD cell line was obtained from CDC, Atlanta, GA, USA.

Nucleotide sequence analysis. After virus purification and RNA extraction the latter was partially sequenced by the dideoxynucleotide chain termination technique (Sanger *et al.*, 1977; Zimmern and Kaesberg, 1978) using the avian myeloblastosis virus reverse transcriptase and synthetic oligonucleotide primer 5'-ATGTCTCTCTTTTTCTTTC-CCATTGCTAC-3' (nt 6370-6398).

Numbers in parentheses indicate genomic intervals (nt) that are complementary to the primer using the consensus nucleotide numbering system for the poliovirus genome (Toyoda *et al.*, 1984). This primer reacted with all the strains analyzed in this study.

Results

The 3Dpol coding region of the genome of P2/Sabin-related strains isolated from VAPP cases and healthy contacts was sequenced in the region of nt 6166-6360 (3Dpol seq.). The results are presented in Fig. 1 and any differences from the P2/Sabin sequence (Toyoda *et al.*, 1984; Pollard *et al.*, 1989) are indicated. The nt sequences of the strains P1/Sabin (Nomoto *et al.*, 1982; Toyoda *et al.*, 1984) and P3/Sabin (Stanway *et al.*, 1984; Toyoda *et al.*, 1984; Weeks-Levy *et al.*, 1991) are also shown.

The P2/2645 strain (isolated from a VAPP case) maintained a part of the 3Dpol seq. originating from the P2/Sabin strain, while the other part was derived from the P1/Sabin strain. It demonstrates that this strain is a recombinant having at least a genome segment derived from the P1/Sabin strain. Comparison of the aa sequence of the viral RNA polymerase of the P2/Sabin strain with the predicted one of the P2/2645 strain in the 3Dpol seq. demonstrated an aa 113 (Thr->Ser) substitution.

The P2/1397 strain (isolated from a VAPP case) showed that its 3Dpol seq. was derived from the P1/Sabin strain. It demonstrates that this strain is a recombinant having at least a part of the 3Dpol derived from the P1/Sabin strain. This 3Dpol seq. revealed three mutations: an A->G mutation at nt 6166 leading to an aa 53 (Asn->Asp) substitution in the viral RNA polymerase; an U->C mutation at nt 6183 maintaining aa 58 (Ile) in the viral RNA polymerase; and a C->U mutation at nt 6226 leading to an aa 73 (His->Tyr) substitution in the viral RNA polymerase. Comparison of the aa sequence of the viral RNA polymerase of the P2/Sabin strain with the predicted aa sequence of the P2/1397 strain in the 3Dpol seq. demonstrated aa 69 (Asp->Glu) and aa 113 (Thr->Ser) substitutions. The P2/1398 strain (isolated from a healthy contact of the case from which the P2/1397 strain was isolated) maintained the 3Dpol seq. from the P2/Sabin strain.

Both the strains P2/1400 (isolated from a VAPP case) and P2/1402 (isolated from a healthy contact of the case)

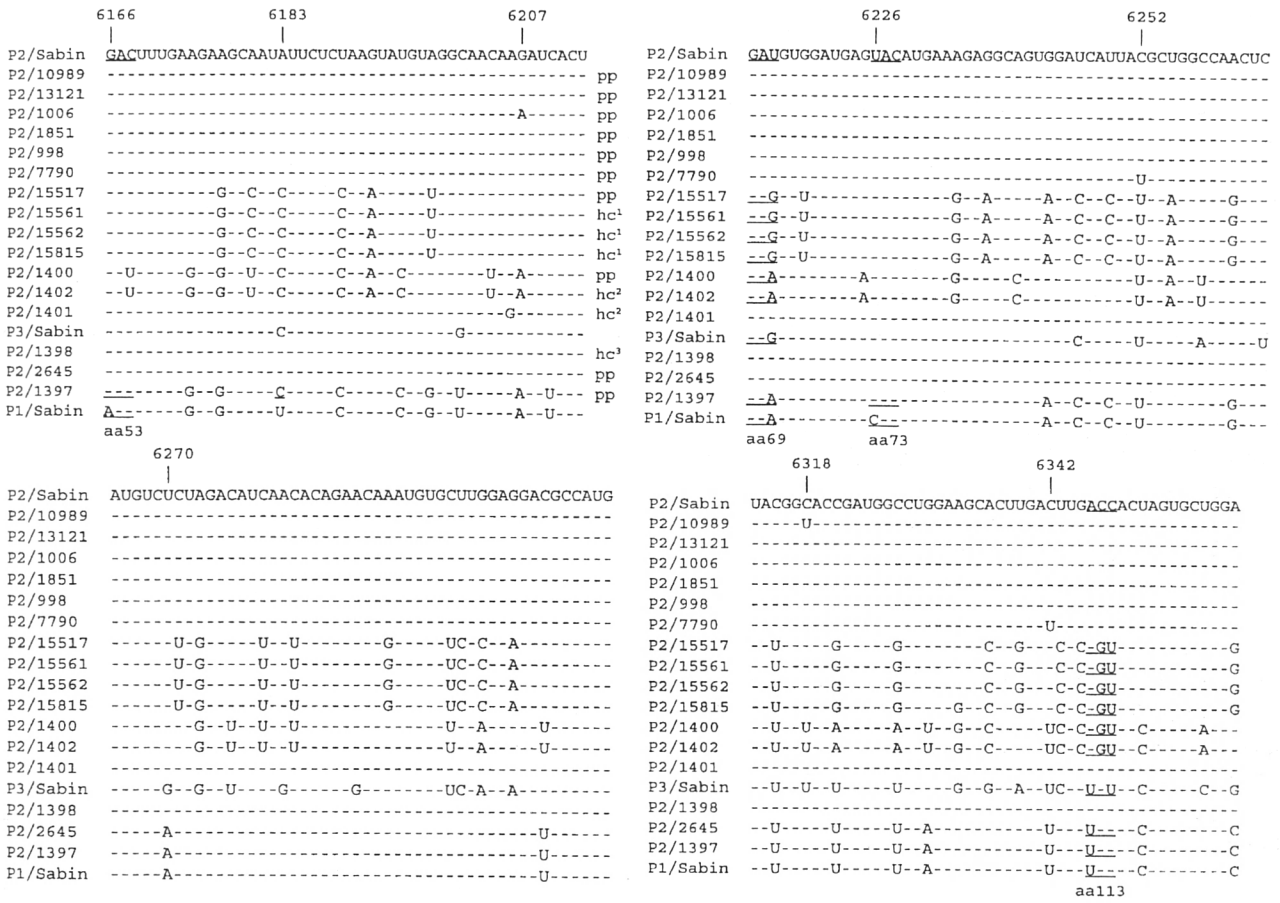

Fig. 1

Comparison of nucleotide sequences of a part of the 3Dpol coding region (3Dpol seq., nt 6166-6360) of P2/Sabin-related poliovirus strains isolated from VAPP cases and healthy contacts with those of the strains P1/Sabin, P2/Sabin, and P3/Sabin

Differences from the P2/Sabin strain are indicated. Codons of aa 53, 69, 73, and 113 are underlined. [1]Strains isolated from healthy contacts of the VAPP case from which the P2/15517 strain was isolated. [2]Strains isolated from healthy contacts of the VAPP case from which the P2/1400 strain was isolated. [3]Strain isolated from a healthy contact of the VAPP case from which the P2/1397 strain was isolated.
pp – strain isolated from a patient with persistent paralytic poliomyelitis; hc – strain isolated from a healthy contact of VAPP case.

showed that their 3Dpol seq. were derived from a non-vaccine strain with many nt differences as compared to vaccine strains P1/Sabin, P2/Sabin and P3/Sabin (Fig. 1). It demonstrates that these strains are recombinants having at least a part of 3Dpol derived from a non-vaccine strain (a wild strain or some other enterovirus strain). In the 3Dpol seq., the nt sequence of the P2/1400 strain showed a 100% homology with the nt sequence of the P2/1402 strain, confirming the transmission of the strain. The nt sequence of the 3Dpol seq. of the strains P2/1400 and P2/1402 showed an 80.51% homology with the P2/Sabin strain, while the predicted aa sequence of the viral RNA polymerase of these two strains in the 3Dpol seq. had a 96.92% homology with that of the P2/Sabin strain. Comparison of the aa sequence of the viral RNA polymerase of the P2/Sabin strain with the predicted aa sequences of the strains P2/1400 and P2/1402 in the 3Dpol seq. demonstrated aa 69 (Asp->Glu) and aa 113 (Thr->Ser) substitutions in these two strains. The P2/1401 strain (also isolated from a healthy contact of the case) maintained the 3Dpol seq. from the P2/Sabin strain with an A->G mutation at nt 6206, leading to an aa 66 (Lys->Arg) substitution in the viral RNA polymerase.

The P2/15517 strain (isolated from a VAPP case) and strains P2/15561, P2/15562 and P2/15815 (isolated from healthy contacts of the case) showed that their 3Dpol seq. was derived from a non-vaccine strain with many nt differences in comparison with the three Sabin vaccine strains (Fig. 1). It demonstrates that these strains are recombinants having at least part of 3Dpol derived from a non-vaccine strain (a wild strain or some other enterovirus strain). The nt sequence of the 3Dpol seq. of the strains P2/15517, P2/15561, and P2/15562 showed an 82.05% homology with the P2/Sabin strain, while the strains P2/15517,

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             53           66 69  73                          86                         113
             |            |  |   |                           |                          |
P2/Sabin     DFEEAIFSKYVGNKITDVDEYMKEAVDHYAGQL   P2/Sabin    MSLDINTEQMCLEDAMYGTDGLEALDLTTSAG
P2/10989     ---------------------------------   P2/10989    --------------------------------
P2/13121     ---------------------------------   P2/13121    --------------------------------
P2/1006      ---------------------------------   P2/1006     --------------------------------
P2/1851      ---------------------------------   P2/1851     --------------------------------
P2/998       ---------------------------------   P2/998      --------------------------------
P2/7790      ---------------------------------   P2/7790     --------------------------------
P2/15517     ----------------E----------------   P2/15517    ---------------------------S----
P2/15561     ----------------E----------------   P2/15561    ---------------------------S----
P2/15562     ----------------E----------------   P2/15562    ---------------------------S----
P2/15815     ----------------E----------------   P2/15815    ---------------------------S----
P2/1400      ----------------E----------------   P2/1400     ---------------------------S----
P2/1402      ----------------E----------------   P2/1402     ---------------------------S----
P2/1401      -------------R-------------------   P2/1401     --------------------------------
P3/Sabin     ----------------E----------------   P3/Sabin    -----S---------------------S----
P2/1398      ---------------------------------   P2/1398     --------------------------------
P2/2645      ---------------------------------   P2/2645     ---------------------------S----
P2/1397      ----------------E----------------   P2/1397     ---------------------------S----
P1/Sabin     N---------------E---H------------   P1/Sabin    ---------------------------S----
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Fig. 2

Comparison of amino acid sequences of a part of the viral RNA polymerase of P2/Sabin-related poliovirus strains isolated from VAPP cases and healthy contacts, deduced from nucleotide sequences of the 3Dpol coding region shown in Fig. 1, with those of the strains P1/Sabin, P2/Sabin and P3/Sabin

Differences from the P2/Sabin strain are indicated.

P2/15561 and P2/15562 had a 100% homology. Although the P2/15815 strain had the same nt sequence in the 3Dpol seq. as the strains P2/15517, P2/15561 and P2/15562, it had a G instead of A at nt 6333. In the 3Dpol seq., the nt sequence of the P2/15815 strain had a 99.49% homology with the P2/15517 strain (and the strains P2/15561 and P2/15562), and an 81.53% homology with the P2/Sabin strain. As all these four strains had the same nt sequence in the 3Dpol seq. the transmission of these strains appears to be confirmed. The predicted aa sequence of the viral RNA polymerase of these four strains in the 3Dpol seq. had a 96.92% homology with that of the P2/Sabin strain. Comparison of the aa sequence of the viral RNA polymerase of the P2/Sabin strain with the predicted aa sequence of the strains P2/15517, P2/15561, P2/15562, and P2/15815 in the 3Dpol seq. demonstrated aa 69 (Asp->Glu) and aa 113 (Thr->Ser) substitutions in the viral RNA polymerase of all these strains.

The P2/1006 strain (isolated from a VAPP case) maintained the 3Dpol seq. from the P2/Sabin strain with a G->A mutation at nt 6207. This mutation occured at the third nt of the codon of aa 66, maintaining Lys at this position. The P2/10989 strain (isolated from a VAPP case) maintained the 3Dpol seq. from the P2/Sabin strain with a C->U mutation at nt 6318. This mutation occurred at the third nt of the codon of aa 103, maintaining Gly at this position. The P2/7790 strain (isolated from a case classified as polio-compatible) maintained the 3Dpol seq. from the P2/Sabin strain with two mutations: a C->U mutation at nt 6252, and a C->U mutation at nt 6342. The mutation at nt 6252 occurred at the third nt of the codon of aa 81, maintaining Tyr at this position, while the mutation at nt 6342 also occurred at the third nt of the codon of aa 111, maintaining Asp at this position. The strains P2/13121, P2/1851 and P2/998 (isolated from VAPP cases) also maintained the 3Dpol seq. from the P2/Sabin strain.

Discussion

The P2/Sabin-derived strains isolated in Brazil from VAPP cases and from healthy contacts, studied in the present article, were previously analyzed for the presence of mutations at nt 481 of the 5'NCR and in the codon of aa 143 of VP1 (Friedrich *et al.*, 1995b) that are known to increase the neurovirulence (Ren *et al.*, 1991; Macadam *et al.*, 1991, 1993), and at nt 398 also suspected to play some role in increasing the neurovirulence. Most of these brazilian strains mutated at nt 481 and at aa 143 of VP1, and a half of them also mutated at nt 398 (Friedrich *et al.*, 1995b). In the 3Dpol segment analyzed in the present study (3Dpol seq.), four out of ten strains isolated from VAPP cases turned out to be recombinants; one had the 3Dpol seq. from the P1/Sabin strain; another had a part of the 3Dpol seq. from the

P2/Sabin strain, and the other part from the P1/Sabin strain; the two other strains had the 3Dpol seq. from non-vaccine strains. The strains isolated from healthy contacts of the two VAPP cases, from which the type 2 vaccine/non-vaccine recombinant strains were isolated, also consisted of recombinant genomes with the same nt sequences as those of the isolates from VAPP cases, confirming the transmission of P2/Sabin-derived recombinants. Other studies also identified P2/Sabin-derived recombinant strains isolated from VAPP cases having at least part of the genome derived from the P1/Sabin strain (Lipskaya *et al.*, 1991; Furione *et al.*, 1993; Georgescu *et al.*, 1994) or even from a non-vaccine strain (Furione *et al.*, 1993; Georgescu *et al.*, 1994, 1995a). Studies have also demonstrated the isolation of P3/Sabin-derived recombinant strains from VAPP cases (Macadam *et al.*, 1989; Furione *et al.*, 1993; Georgescu *et al.*, 1994). The possibility that genomic recombinantion increased the neurovirulence of these strains cannot be excluded.

One of the recombinants (P2/1397) isolated from a VAPP case in Brazil and having the 3Dpol seq. derived from the P1/Sabin strain, underwent mutations known or suspected to participate in reversion to neurovirulence. The C->U reverting mutation at nt 6226 that is equivalent in sequence to nt 6203 in the P1/Sabin strain (Nomoto *et al.*, 1982) is known to increase the neurovirulence of this strain (Christodoulou *et al.*, 1990; Tardy-Panit *et al.*, 1993; Georgescu *et al.*, 1995b; Baker *et al.*, 1995). This mutation that leads to an aa 73 (His->Tyr) substitution in the viral RNA polymerase, was previously identified in a P1/Sabin-derived strain isolated from a VAPP case (Otelea *et al.*, 1993), a P1/Sabin-derived strain isolated from a Guillain-Barré syndrome case (Friedrich *et al.*, 1995a), P1/Sabin-derived strains selected at elevated temperatures (Christodoulou *et al.*, 1990), and also in P2/Sabin-derived recombinant strains having this segment derived from the P1/Sabin strain (Lipskaya *et al.*, 1991; Furione *et al.*, 1993; Georgesco *et al.*, 1994). The A->G reverting mutation at nt 6166 of the P2/1397 strain in the 3Dpol segment derived from the P1/Sabin strain is equivalent in sequence to nt 6143 of the P1/Sabin strain (Nomoto *et al.*, 1982), and leads to an aa 53 (Asn->Asp) substitution in the viral RNA polymerase. A G at nt 6143 and an Asp at aa 53 of the viral RNA polymerase were found in the P1/Mahoney strain (Kitamura et al., 1981; Racaniello and Baltimore, 1981; see also Almond, 1987) which is the neurovirulent precursor of the P1/Sabin strain (Nomoto *et al.*, 1982). The possibility that this reverting mutation at nt 6143 also had some effect, even if minor, on the increase of the neurovirulence cannot be excluded. The comparison of the aa sequence of the viral RNA polymerase of the P2/Sabin strain with the predicted aa sequence of the P2/1397 strain in the 3Dpol seq. demonstrated aa 69 (Asp->Glu) and aa 113 (Thr->Ser) substitutions. The P2/2645 strain , isolated from a VAPP case, also had a part of the 3Dpol seq. from the P1/Sabin strain with an aa 113 (Thr->Ser) substitution.

Some of the P2/Sabin-derived recombinant strains isolated from VAPP cases (2) and healthy contacts (4) had the 3Dpol seq. from non-vaccine strains. Comparison of the nt sequences of these strains with that of the P2/Sabin strain demonstrated several nt differences and two aa substitutions in the viral RNA polymerase. The strains P2/1400 and P2/1402 had aa 69 (Asp->Glu) and aa 113 (Thr->Ser) substitutions, while the strains P2/15517, P2/15561, P2/15562 and P2/15815 had also aa 69 (Asp->Glu) and aa 113 (Thr->Ser) substitutions. Interestingly, the aa 69 (Asp->Glu) and aa 113 (Thr->Ser) substitutions were observed also in the P2/1397 strain, while the P2/2645 strain also had the aa 113 (Thr->Ser) substitution. It is possible that these aa substitutions in the viral RNA polymerase increased the neurovirulence of these strains.

Some of the strains that maintained the 3Dpol seq. from the P2/Sabin strain underwent silent mutations in this region. Mutations in the 3Dpol had also been observed in other non-recombinant P2/Sabin-derived isolates (Pollard *et al.*, 1989; Equestre *et al.*, 1991). The possibility that silent mutations increased the neurovirulence or had some positive effects on the replicaton of these strains cannot be excluded. This study demonstrates that genomic recombination also occurs in P2/Sabin-derived isolates from VAPP cases in Brazil, and confirms the capacity of transmission of P2/Sabin-derived strains with many genomic modifications.

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