Rare natural type 3/type 2 intertypic capsid recombinant vaccine-related poliovirus isolated from a case of acute flaccid paralysis in Brazil, 2015

Klécia M. S. M. Cassemiro, Fernanda M. Burlandy and Edson E. da Silva

Enterovirus Laboratory, Oswaldo Cruz Institute, Oswaldo Cruz Foundation, Rio de Janeiro, Brazil

A natural type 3/type 2 intertypic capsid recombinant vaccine-related poliovirus was isolated from an acute flaccid paralytic case in Brazil. Genome sequencing revealed the uncommon location of the crossover site in the VP1 coding region (nucleotides 3251–3258 of Sabin 3 genome). The Sabin 2 donor sequence replaced the last 118 nt of VP1, resulting in the substitution of the complete antigenic site IIIa by PV2-specific amino acids. The low overall number of nucleotide substitutions in P1 region indicated that the predicted replication time of the isolate was about 8–9 weeks. Two of the principal determinants of attenuation in Sabin 3 genomes were mutated (U472C and C2493U), but the temperature-sensitive phenotype of the isolate was preserved. Our results support the theory that there exists a PV3/PV2 recombination hotspot site in the tail region of the VP1 capsid protein and that the recombination may occur soon after oral poliovirus vaccine administration.

Polioviruses (PV) are in a constant process of evolution, driven by their high mutational rate and genomic recombination (Savolainen-Kopra & Blomqvist, 2010). RNA recombination facilitates the exchange of genetic information between genomes, granting evolutionary advantages by the generation of variability and reduction of mutational load (reviewed by Simon-Loriere & Holmes, 2011). The Sabin Oral Poliovirus Vaccine (OPV types 1, 2 and 3 strains) provides optimal conditions for multiple infections of human intestinal target cells, favouring the occurrence of intermolecular recombination between heterotypic genomes (Egger & Bienz, 2010).

The recombination junctions appear to follow a systematic distribution throughout the PV genome, since they are preferably correlated with RNA secondary structure elements identical to both recombination partners (Dedepsidis et al., 2010). The majority of the crossover sites are located in the nonstructural coding regions of the genome (P2 and P3 regions), with recombination hotspots located in the 2C and 3D genomic regions for recombination junctions of type 3/type x and type 2/type x, respectively (Cuervo et al., 2001; Karakasiliotis et al., 2004; Paximadi et al., 2006). The natural occurrence of intertypic capsid recombinants is very rare in nature, and seems to be a consequence of structural constraints that maintain the integrity of the capsid shell (Simmonds & Welch, 2006). Previous studies have shown that capsid recombinants chimeras are usually nonviable (Kohara et al., 1988) or unstable in comparison to parental strains (Kohara et al., 1985).

Very little is known about the capsid regions susceptible to undergoing recombination that maintain viral viability. There are few descriptions of S3/S2 recombinants in the literature (Blomqvist et al., 2003, 2010; Dedepsidis et al., 2008; Martin et al., 2002; Mueller et al., 2009; Tao et al., 2010; Zhang et al., 2010). In all cases, the recombination junctions were placed in the tail of the VP1 coding region, with Sabin 2 donated sequences ranging from 28 nt (Blomqvist et al., 2010) to 136 nt (Zhang et al., 2010) in the P1 region of Sabin 3 genomes, as seen in Fig. 1.

In this study we investigated the genome and the phenotypic characteristics of a natural type3/type2 capsid recombinant poliovirus isolated from a faecal specimen of an 8-month-old boy with acute flaccid paralysis symptoms.

Faecal samples were collected for the purpose of epidemiologic surveillance in São Paulo state, Brazil, in January 2015, four days after the onset of paralytic symptoms. Virus isolation attempts were performed in L20B [National Institute for Biological Standards and Control (NIBSC) accession no. 081102] and RD (NIBSC accession no. 081003) cell lines, according to the WHO algorithm for poliovirus isolation (World Health Organization, 2006), and intratypic differentiation and preliminary characterization were determined by real-time PCR...
assay and VP1 sequencing, according to standard protocols (CDC, 2009; World Health Organization, 2004).

Viral RNA was extracted from an aliquot of 140 μl L20B cell culture supernatant presenting cytopathic effect, using a QIAamp Viral RNA Mini Kit (Qiagen). cDNA synthesis was performed by using the specific primer S2_7439R (5'-TCGA TAAGGTTGTTTTTTTTTTTTTTTTTCCCGAA TT-3') and Superscript II Reverse Transcriptase (Invitrogen). The entire VP1 coding region (900 nt) was amplified and sequenced using the primers Y7 and Q8 (Rico-Hesse et al., 2009).
The complete genomic sequence of isolate 45507 was amplified from two overlapping PCR products of 3.57 kb and 5.28 kb, respectively, using the Expand Long Template PCR System (Roche) and two sets of sense–antisense primers: 001F_Hind (5¢-TCGATGTCGACTAATACGACTCACTATAGGTTAAAACAGCTCTGGGG-3¢) – Q8 and Y7 – S2_7439R. The two fragments were purified using a QIAquick Gel Extraction Kit (Qiagen), and cycle sequencing reactions were carried out using BigDye terminator chemistry version 3.0 (Applied Biosystems) on an ABI 3730XL instrument. Sequence analyses were performed using BioEdit (Hall, 1999) and MEGA 6.0 software package (Tamura et al., 2013). Recombination events were evaluated by the Simplot program v3.5.1 (Lole et al., 1999). The evolution time of isolate 45507 was estimated by calculations of synonymous substitutions per synonymous sites (Ks) and non-synonymous substitutions per non-synonymous sites (Ka) by using a modified version of the Goldman–Yang codon model of evolution (Goldman & Yang, 1994) as implemented in MBEToolbox v.3.0 (Cai et al., 2005, 2007), and the total number of substitutions per site (Kt) was estimated using the Tamura 92 (3-parameter) model of nucleotide evolution (Tamura, 1992), also available in MBEToolbox v.3.0.

Cross-reactivity using serotype-specific hyperimmune sera and reproductive capacity at high temperatures (RCT marker) were performed in order to determine the antigenic characteristics of isolate 45507. The cross-reactivity was tested in a microneutralization assay in RD cells, according to WHO protocols (World Health Organization, 2004). The reproductive capacity of isolate 45507 was tested at different temperatures (RCT test for 36.5°C and 40°C) at 8, 24 and 48 h post-infection in RD cells, in comparison to Sabin-2 (NIBSC code 01/530) and Sabin-3 (NIBSC code 01/532) reference strains. The RCT value is defined as the difference between the log_{10} virus titre of the viral stock measured at the optimal temperature 36.5°C and supraoptimal temperature 40°C. The values are expressed as log_{10} TCID_{50}/0.1 ml.

Viruses were considered thermosensitive (ts sensitive phenotype) if the ΔRCT value was greater than or equal to 2, and

Table 1. Nucleotide and amino acid substitutions in the 45507 recombinant virus compared to Sabin 3 (GenBank accession no. AY184221) and Sabin 2 (AY184220) reference sequences

The nucleotide and amino acid positions are related to the 45507 genome (GenBank accession no. KU763188). Mutations that resulted in reversion to the respective wild-type sequences Poliovirus type 3 Leon/37 (GenBank accession no. K01392) and Poliovirus type 2 Lansing (GenBank accession no. M12197) are indicated. Mutations in the determinants of attenuation for Sabin 3 genomes are underlined.

<table>
<thead>
<tr>
<th>Sabin origin</th>
<th>Region</th>
<th>Nucleotide position*</th>
<th>Nucleotide substitution</th>
<th>Amino acid position*</th>
<th>Amino acid substitution</th>
<th>Reversion to wild-type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 3</td>
<td>5’-UTR</td>
<td>121</td>
<td>U-to-C</td>
<td>190</td>
<td>C-to-U</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>472</td>
<td>U-to-C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>VP2</td>
<td>1035</td>
<td>C-to-U</td>
<td>29</td>
<td>A-to-V</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1158</td>
<td>U-to-C</td>
<td>70</td>
<td>M-to-T</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1455</td>
<td>C-to-A</td>
<td>169</td>
<td>P-to-Q</td>
<td></td>
</tr>
<tr>
<td></td>
<td>VP3</td>
<td>1852</td>
<td>C-to-U</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1991</td>
<td>G-to-A</td>
<td>76</td>
<td>D-to-N</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2001</td>
<td>A-to-G</td>
<td>80</td>
<td>Q-to-R</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2033</td>
<td>U-to-A</td>
<td>91</td>
<td>E-to-I</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2188</td>
<td>C-to-U</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>VP1</td>
<td>2188</td>
<td>C-to-U</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2493</td>
<td>C-to-U</td>
<td>6</td>
<td>T-to-I</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2509</td>
<td>G-to-A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2749</td>
<td>C-to-U</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 2</td>
<td>2A</td>
<td>3531</td>
<td>C-to-A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2C</td>
<td>4533</td>
<td>U-to-C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3A</td>
<td>5265</td>
<td>A-to-U</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3B</td>
<td>5391</td>
<td>A-to-C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3C</td>
<td>5676</td>
<td>C-to-U</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3D</td>
<td>5925</td>
<td>U-to-C</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*The figures are for isolate 45507 (GenBank accession no. KU763188).
Table 2. Reproductive capacity of S3/S2 capsid recombinant 45507, Sabin-3 and Sabin 2 strains at different temperatures (RCT marker)

Data from representative experiment. Tests carried out in triplicate showed similar results.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Times p.i. (h)</th>
<th>Titres at 36.5°C</th>
<th>Titres at 40°C</th>
<th>ARCT</th>
</tr>
</thead>
<tbody>
<tr>
<td>45507</td>
<td>8</td>
<td>7.8</td>
<td>3.7</td>
<td>4.1</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>7.7</td>
<td>4.8</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>6.9</td>
<td>4.5</td>
<td>2.4</td>
</tr>
<tr>
<td>Sabin</td>
<td>8</td>
<td>7.9</td>
<td>3.6</td>
<td>4.3</td>
</tr>
<tr>
<td>2</td>
<td>24</td>
<td>7.8</td>
<td>3.8</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>7.7</td>
<td>3.7</td>
<td>4.0</td>
</tr>
<tr>
<td>Sabin</td>
<td>8</td>
<td>7.4</td>
<td>2.7</td>
<td>4.7</td>
</tr>
<tr>
<td>3</td>
<td>24</td>
<td>7.1</td>
<td>3.5</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>6.9</td>
<td>3</td>
<td>3.9</td>
</tr>
</tbody>
</table>

Isolate 45507 was characterized as a type 3 Sabin-like poliovirus by real-time PCR assay. VP1 sequence analysis revealed a crossover junction placed in the 3'-end of the capsid coding region, at nucleotide positions 3251–3258 of the Sabin 3 genome (Fig. 1). The last 118 nt of the VP1 gene were found to have 100% identity with the Sabin-2 reference strain, which characterizes the virus 45507 as a natural intertypic S3/S2 vaccine-related capsid recombinant. The recombination event resulted in replacement of six capsid amino acid residues from the Sabin 3 genomic background to the Sabin 2 donor sequence: VP1-279, VP1-286, VP1-287, VP1-288, VP1-290 and VP1-293, which are located on the surface of the virion, as part of the antigenic site IIIa (Nag IIIa) of poliovirus capsid (Minor et al., 1986), with implications for receptor binding and determination of virus serotype.

The nucleotide and amino acid changes between isolate 45507 and the respective Sabin strains are presented in Table 1. The entire genome of isolate 45507 comprises 7431 nucleotides plus the polyA tail, with an open reading frame of 2207 amino acids. No other recombination junction was encountered throughout the genome (Fig. 1a). There were 22 nucleotide substitutions throughout the genome. A total of seven amino acid substitutions were noted, all placed before the crossover site, in the Sabin 3 background.

There are three main determinants of the attenuated phenotype in Sabin 3 genomes: nucleotides (nt) 472 (5'UTR), 2034 (VP3aa91) and 2493 (VP1aa6) (Westrop et al., 1989). Importantly, two of them in isolate 45507, nt 472 in the domain V of the 5'-UTR (U-to-C) and nt 2493 in the VP1 coding region (C-to-U), which leads to a Thr-to-Ile amino acid substitution in VP1aa6, had reverted to sequences present in the neurovirulent precursor of Sabin 3, P3/Leon/USA/1937 (Minor et al., 1993; Westrop et al., 1989). These mutations are associated with the increased neurovirulence of Sabin 3 strains (Minor, 1992; Rezapkin et al., 1995). The U-to-C reversion in the determinant for attenuation nt 2034, which leads to a Phe-to-Ser mutation in VP3aa91, was not found in isolate 45507. However, isolate 45507 contains a U-to-A mutation in nt 2033, resulting in a Phe-to-Ile mutation in VP3aa91, which is not found in the neuroviral precursor P3/Leon/USA/1937 nor in Sabin 3 sequences. The Phe-to-Ser mutation in residue VP3aa91 is known to restore a defect in virus assembly (Macadam et al., 1989). More studies are needed to understand the impact of the Phe-to-Ile substitution described in VP3aa91 of isolate 45507. Additionally, isolate 45507 presented a mutation placed inside the NAg3b of isolate 45507 (G1991A), leading to the amino acid substitution Asp-to-Asn at position VP3aa76.

The evolution time of isolate 45507 was estimated from the differences presented in the P1/capsid sequence between virus 45507 and the Sabin 3 reference strain (the last 118 nt in the VP1 coding region, after the crossover site, were not considered for analysis). For the P1/capsid region of isolate 45507, the corrected proportion of synonymous substitutions per synonymous sites (Ks) and total substitutions (Kt) were 0.77% and 0.5%, respectively. By assuming the constant nucleotide substitution rates of 3.2% synonymous substitutions per synonymous sites per year, and 1.1% total substitutions per site per year in the P1/capsid region (Jorba et al., 2008), we estimated the ages of evolution for isolate 45507 to be 87 days from the Ks clock and 165 days for the Kt clock. Since isolate 45507 has been replicating for a short time, it is suggested that the recombination event occurred soon after OPV administration, most probably during poliovirus replication in the primary vaccinee.

Isolate 45507 was fully neutralized with PV type 3 antisera, but polyclonal sera specific for PV type 1 or 2 did not neutralize its infectivity. Though alterations in antigenicity had not been noted with polyclonal serum, it is possible that insertion of the type 2 sequences of antigenic site IIIa in the capsid of virus 45507 might provide benefits for escaping serotype-specific antibodies during replication in the human gut, which could favour the enrichment of polio strains with chimeric VP1 capsid protein.

When compared to Sabin 3 and Sabin 2 strains regarding its capacity to replicate at elevated temperatures (RCT marker), the isolate maintained the ts phenotype characteristic of attenuated Sabin strains, presenting a greater than 2 log titre difference at 36.5°C and at 40°C (Table 2). Tests carried out in triplicate showed similar results. This result was similar to previous reports (Tao et al., 2010; Zhang et al., 2010), which also found capsid recombinants that had maintained the temperature sensitivity, suggesting that the recombination event has no direct impact on the RCT marker. However, it is noteworthy that, although the titre reduction of isolate 45507 at 8h post-infection was quite significant, the ΔRCT at 24 or 48h post-infection were...
slightly lower than Sabin strains, suggesting that during evo-
olution of isolate 45507, its temperature sensitivity was some-
what weakened. It had been reported that the temperature
sensitivity phenotype of type 3 poliovirus is strongly attrib-
utable to a difference in residue 91 of the VP3 capsid pro-
tein (Minor et al., 1989). Residue 91 of VP3 in the 45507
isolate does present an amino acid substitution (Phe-to-Ile),
but it is not a return to the serine residue in the neurovirus
lent precursor P3/Leon/USA/1937. It is suggested that the
mutation at residue 91 of VP3 acts with one or more unidenti-
cified additional mutations to generate the full ts phenotype
of the Sabin type 3 vaccine strain (Minor et al., 1989).

This is supported by the observation that the tail of the
VP1 coding region works as a capsid hotspot for recombina-
tion sites. The authors believe that, as was previ-
ously described by Martin et al. (2002) in isolate 31043. It
represents the second larger Sabin 2 donated sequence to a Sabin
3 genome. Also, strain 45507 was simpler in that it had only
one crossover site throughout the entire genome, represent-
ing the longest Sabin 2 sequence ever reported to be natu-
ally inserted in a Sabin 3 background.

Besides the crossover junction 3251–3258 nt, only the cross-
over sites 3233–3240 nt, 3275–3285 nt and 3314–3321 nt
have been described in more than one S3/S2 capsid recom-
binant isolate (four isolates, four isolates and three isolates,
respectively). The recurrent report of these regions as cross-
over junctions in S3/S2 recombinants suggests that the tail
region of VP1 capsid protein is a viable site for S3/S2 capsid
recombination events, and that these are the probable recombina-
tion sites. The authors believe that, as was previously
proposed by Blomqvist and colleagues (Blomqvist et al., 2010) and is reinforced by the present study, the
tail of the VP1 coding region works as a capsid hotspot for
the formation of viable capsid recombinants.

Acknowledgements
This work was supported by the National Council for Scientific and
Technological Development in Brazil (CNPq) and the Oswaldo Cruz
Foundation. The authors would like to thank Amanda Neves, Cri-
stiane Souza, Jaqueline das Graças, Valdemar de Paula, Elaine Silva
and Silas Oliveira for their technical assistance.

References
tion of a recombinant type 3/type 2 poliovirus isolated from a healthy
vaccinee and containing a chimeric capsid protein VP1. J Gen Virol 84,
573–580.

Blomqvist, S., Savolainen-Kopra, C., Paananen, A., El Bassioni, L., El
Maamoon Nasr, E. M., Firstova, L., Zamiatina, N., Kutaleiadze, T. &
Roivainen, M. (2010). Recurrent isolation of poliovirus 3 strains with
chimeric capsid protein VP1 suggests a recombination hot-spot site

MATLAB toolbox for sequence data analysis in molecular biology and
evolution. BMC Bioinformatics 6, 64.

an enhanced version of a MATLAB toolbox for molecular biology and
evolution. Evol Bioinform Online 2, 179–182.

Centers for Disease Control and Prevention (CDC) (2009). Labora-
tory surveillance for wild and vaccine-derived polioviruses - worldwide,
959.

Cuervo, N. S., Guillot, S., Romanenkova, N., Combiescu, M., Aubert-
Combiescu, A., Seghier, M., Caro, V., Crainic, R. & Delpeyroux, F.
(2001). Genomic features of intertypic recombinant sabin poliovirus

Dedepsidis, E., Pliaka, V., Kyríakopoulou, Z., Brakoúlias, C.,
Levidiotou-Stefanou, S., Pratti, A., Mamuris, Z. & Markoulatos, P.
(2008). Complete genomic characterization of an intertypic Sabin
3/Sabin 2 capsid recombinant. FEMS Immunol Med Microbiol 52,
343–351.

Dedepsidis, E., Kyríakopoulou, Z., Pliaka, V. & Markoulatos, P. &
Markoulatos, P. (2010). Correlation between recombination junctions
and RNA secondary structure elements in poliovirus Sabin strains.

Egger, D. & Bienz, K. (2002). Recombination of poliovirus RNA pro-
ced in mixed replication complexes originating from distinct repli-

substitution for protein-coding DNA sequences. Mol Biol Evol 11,
725–736.

ment editor and analysis program for Windows 95/98/NT. Nucleic
Acids Symp Ser 41, 95–98.

tiple poliovirus molecular clocks covering an extended evolutionary

Karakasiliotis, I., Markoulatos, P., Katsorchis, T., Markoulatos, P. &
Katsorchis, T. (2004). Site analysis of recombinant and mutant polio-
virus isolates of Sabin origin from patients and from vaccinees. Mol
Cell Probes 18, 103–109.

Kohara, M., Omata, T., Kameda, A., Semler, B. L., Itoh, H., Wimmer, E.
recombinant constructed from infectious cDNA clones of the neuro-
virulent Mahoney strain and the attenuated Sabin 1 strain. J Virol 53,
786–792.

Kohara, M., Abe, S., Komatsu, T., Tago, K., Arita, M. & Nomoto, A.
(1988). A recombinant virus between the Sabin 1 and Sabin 3 vaccine
strains of poliovirus as a possible candidate for a new type 3 polio-

Lole, K. S., Bollinger, R. C., Paranjape, R. S., Gadkari, D.,
(1999). Full-length human immunodeficiency virus type 1 genomes
from subtype C-infected seroconverters in India, with evidence of

Macadam, A. J., Arnold, C., Howlett, J., John, A., Marsden, S., Taffs, F.,
of the attenuated and temperature-sensitive phenotypes of the Sabin

Martin, J., Samoilovich, E., Dunn, G., Lackenby, A., Feldman, E.,
Heath, A., Svirchevskaia, E., Cooper, G., Yermalovich, M. & other
authors (2002). Isolation of an intertypic poliovirus capsid recombi-
nant from a child with vaccine-associated paralytic poliomyelitis.

Minor, P. D., Ferguson, M., Evans, D. M., Almond, J. W. & Icenogle, J. P.
(1986). Antigenic structure of polioviruses of serotypes 1, 2 and 3.


