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# Common and Diverse Features of Cocirculating Type 2 and 3 Recombinant Vaccine-Derived Polioviruses Isolated From Patients With Poliomyelitis and Healthy Children

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**Background.** Five cases of poliomyelitis due to type 2 or 3 recombinant vaccine-derived polioviruses (VDPVs) were reported in the Toliara province of Madagascar in 2005.

**Methods.** We sequenced the genome of the VDPVs isolated from the patients and from 12 healthy children and characterized phenotypic aspects, including pathogenicity, in mice transgenic for the poliovirus receptor.

**Results.** We identified 6 highly complex mosaic recombinant lineages composed of sequences derived from different vaccine polioviruses and other species C human enteroviruses (HEV-Cs). Most had some recombinant genome features in common and contained nucleotide sequences closely related to certain cocirculating coxsackie A virus isolates. However, they differed in terms of their recombinant characteristics or nucleotide substitutions and phenotypic features. All VDPVs were neurovirulent in mice.

**Conclusions.** This study confirms the genetic relationship between type 2 and 3 VDPVs, indicating that both types can be involved in a single outbreak of disease. Our results highlight the various ways in which a vaccine-derived poliovirus may become pathogenic in complex viral ecosystems, through frequent recombination events and mutations. Intertypic recombination between cocirculating HEV-Cs (including polioviruses) appears to be a common mechanism of genetic plasticity underlying transverse genetic variability.

The poliomyelitis eradication program is based on massive vaccination campaigns with oral polio vaccine (OPV; available at: <http://www.polioeradication.org/>). This vaccine is composed of 3 serotypes (Sabin 1–3

strains) of live attenuated virus, which multiply to high titers only in the gastrointestinal tract. Following massive immunization campaigns, the frequency of poliomyelitis due to wild-type viruses has greatly decreased worldwide. However, this disease has not been entirely eradicated, because of low vaccine coverage in some areas of the developing world. Low vaccine coverage is also thought to allow interhuman circulation and the genetic drift of OPV strains, leading to the emergence of pathogenic, vaccine-derived polioviruses (VDPVs) [1]. These circulating VDPVs have caused epidemics of poliomyelitis in many regions of the world, including Madagascar in 2001–2002 and 2005 [2–4]. Most VDPVs are recombinants between polioviruses and other human enteroviruses (HEVs) [1, 2, 5]. All have similar genomic features: the region encoding the capsid proteins is that from vaccine strains but with nucleotide substitutions, and some or all of the rest of the

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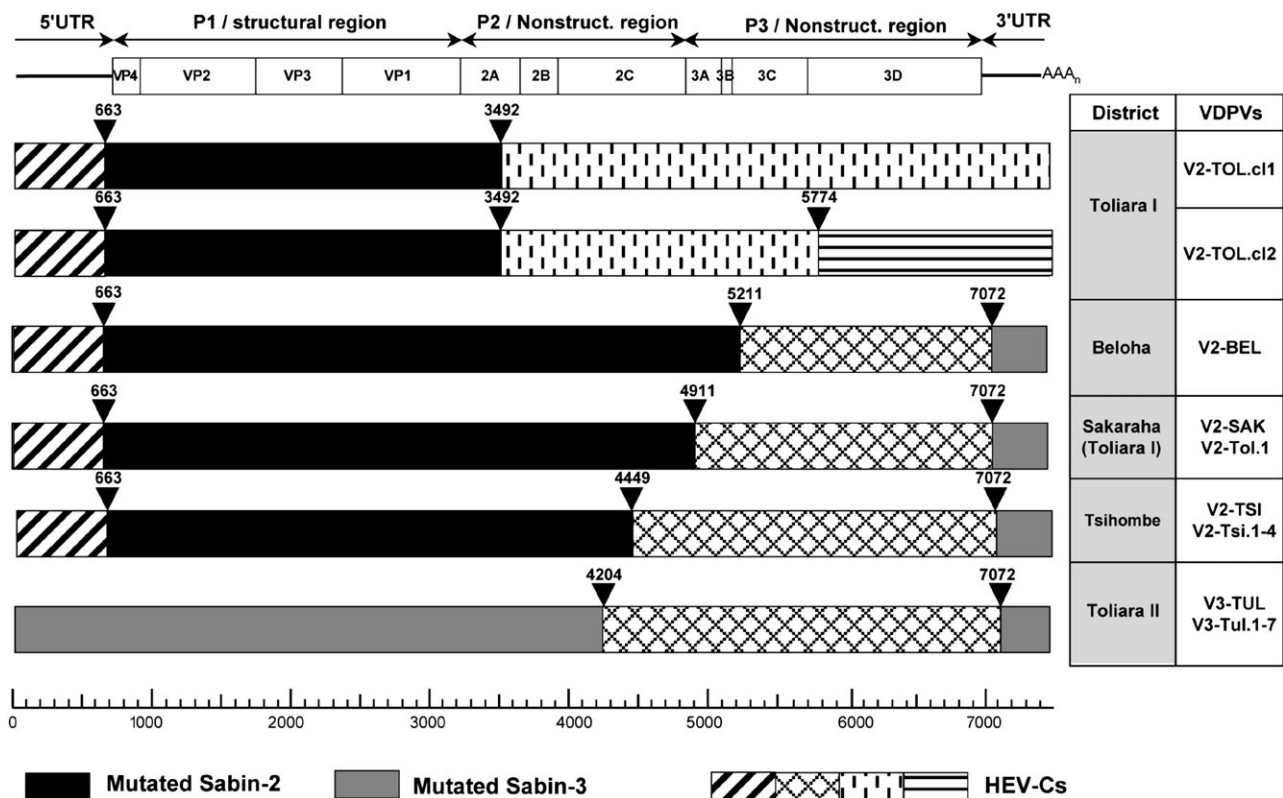
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**Figure 1.** Schematic diagram of the genomic structures of vaccine-derived polioviruses (VDPVs) isolated from patients and healthy children. The genetic organization of the poliovirus (PV) genome is shown (top row), including the 5' and 3' untranslated regions. Genomic regions encoding viral proteins (VP4 to 3D) and their precursors (P1–P3) are indicated. The presence of mutated Sabin 2 or Sabin 3 or nonpoliovirus sequences (species C human enteroviruses [HEV-Cs]) is indicated. Sequences labeled with the same motif differ by <3% (at the nucleotide level). Sequences with different motifs differ by more than 10%. Approximate locations of recombination sites are given. VDPVs with the same recombinant structure and derived from the same district are indicated. V2-TOL.c1 and V2-TOL.c2 are 2 plaque-purified viruses obtained from the stool sample of a single patient. Isolates recovered from patients with acute flaccid paralysis are shown in capital letters; isolates recovered from healthy children (contacts) are shown in lower case letters.

genome, including the region encoding nonstructural proteins in particular, originates from other HEVs.

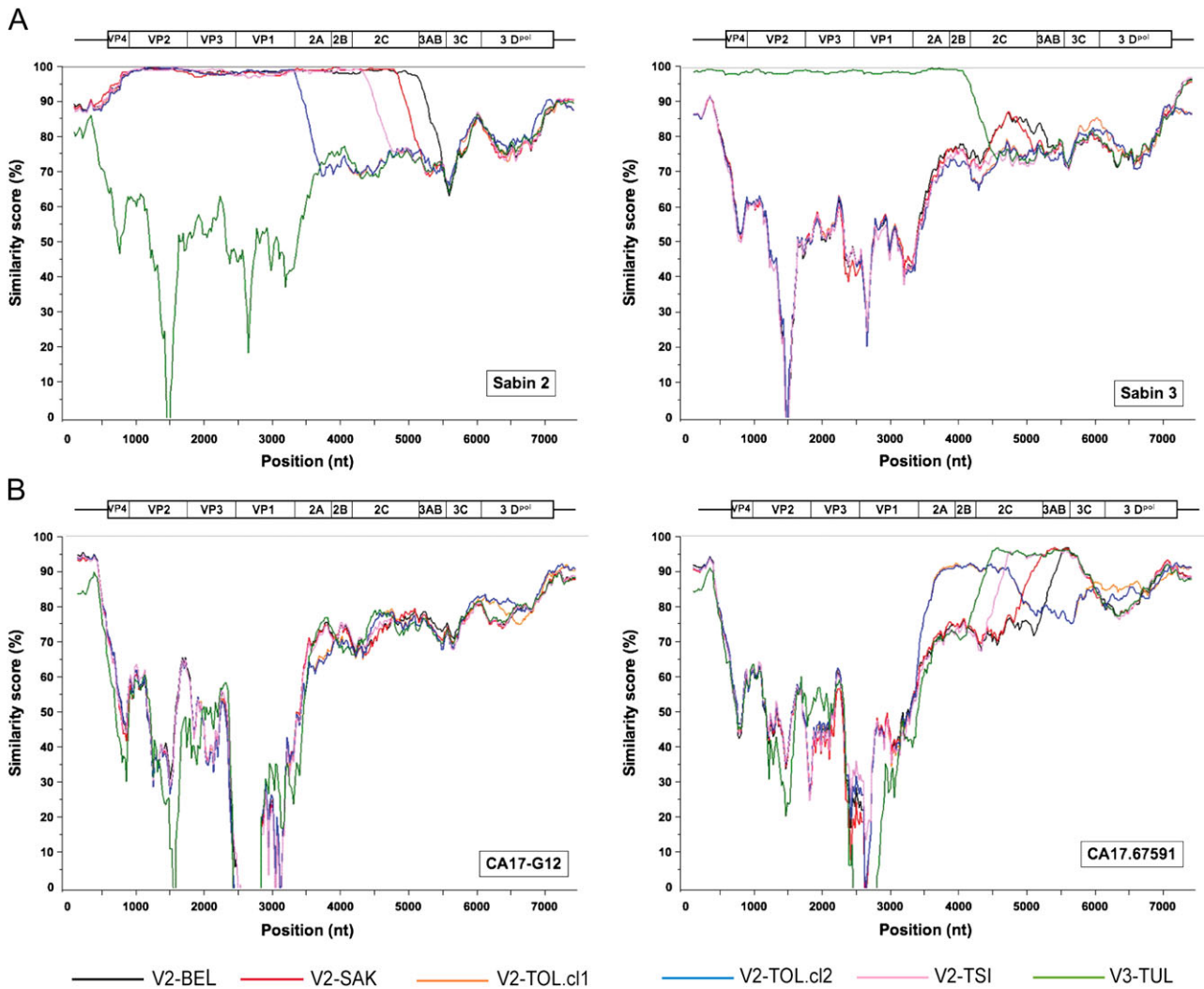
HEVs are small viruses consisting of a single-stranded RNA genome (about 7.5 kb long) of positive polarity surrounded by an icosahedral capsid composed of 4 proteins: VP1–4. The single large coding region of the genome is flanked by 5' and 3' untranslated regions (UTRs) [6]. HEVs constitute a large genus of viruses classified into 4 species of HEVs (HEV-A, -B, -C, and -D) and 3 species of rhinoviruses (available at: <http://www.ictvonline.org/>). The 3 serotypes of poliovirus belong to the species HEV-C, which also includes several types of coxsackie A viruses (CAVs).

Five cases of acute flaccid paralysis associated with type 2 VDPVs (VDPV2) were reported in 2001–2002 in the Toliara province of southern Madagascar [2, 4]. These VDPV2 belong to 2 independent recombinant lineages, with sequences from the Sabin 2 strain in the 5' half of the genome and sequences derived from nonpoliovirus HEV-C in the 3' half. Many types of CAV belonging to HEV-C were found in stool specimens collected from healthy children living in the small area in which

most of the acute flaccid paralysis cases occurred. Several CAVs of types 17 and 13 (CAV17 and CAV13) carried nucleotide sequences closely related to the 2C and the 3D<sup>pol</sup> coding regions of the VDPV2, respectively. The replacement of the 3' half of the VDPV2 genome with that of one of these CAV17 isolates had no significant effect on phenotypic characteristics, such as viral multiplication in infected cells or pathogenicity [7, 8].

From May to August 2005, 5 new polio cases due to VDPV2 and type 3 VDPVs (VDPV3) were reported in 5 districts of Toliara province [3]. In a preliminary study, virological investigations led to the isolation of 17 VDPVs, including 5 VDPVs from patients and 12 VDPVs from healthy contacts and other healthy children [3]. Partial genomic sequencing showed the VDPV genomes to consist of sequences derived from various different vaccine strain serotypes and non-poliovirus enteroviruses, probably from the HEV-C species [3].

With a view to increasing our understanding of VDPV emergence and the evolution of poliovirus with respect to other HEV-C in Toliara province, we sequenced the whole genome of the 17 VDPVs isolated in 2005, analyzed the relationships



**Figure 2.** Comparative analysis of the genomic sequences of vaccine-derived polioviruses, poliovirus vaccine strains Sabin 2 and Sabin 3, and coxsackie A virus strains CA17-G12 (prototype strain) and CA17.67591 (Madagascar isolate) [7]. The similarity plots were generated with 400 nucleotide (nt) windows, 20 nt increments, and the Kimura 2-parameter method with a transition-transversion (Ts/Tv) ratio of 10.

between them, and characterized certain phenotypic aspects. The VDPV genomes appeared to belong to 6 highly complex mosaic recombinant genotypes composed of OPV and HEV-C sequences closely related to some Madagascar CAV isolates. These results highlight the ways in which a VDPV strain may become pathogenic and the importance of intertypic recombination in the genetic variability of viruses in certain complex viral ecosystems.

## MATERIALS AND METHODS

### Cells and Viruses

VDPVs were isolated on human RD and murine L20B cells from stool specimens and amplified in RD or HEp-2c cells [2–4]. Viral stocks were titrated (50% tissue-culture infectious dose per

milliliter, TCID<sub>50</sub>/mL) and further experiments were carried out on HEp-2c cells.

The VDPV2 isolates V2-BEL, V2-SAK, V2-TOL ORI, and V2-TSI and the type 3 VDPV3 isolate V3-TUL were isolated from patients with acute flaccid paralysis, and VDPV isolates V2-Tol.1, V2-Tsi.1–4, and V3-Tul.1–7 were isolated from healthy children [3]. The poliovirus strain S2-4568 used is a neurovirulent and nontemperature-sensitive Sabin 2-derived strain isolated from sewage [9].

### Reverse-Transcription Polymerase Chain Reaction (RT-PCR) and Sequencing

Viral RNA was extracted with the QIAamp Viral RNA Mini Kit (QIAGEN). Reverse transcription was carried out with the P1c primer [10]. PCR and sequencing were carried out with

previously described primers and by the genome walking method [7, 11]. The 5' end of the viral genome was amplified with the 5'/3' RACE Kit (Roche), with the UC21, UC53, and oligo-dT primers [11].

Sequencing was performed with the BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems). Sequence electropherograms from both strands were obtained on an ABI3730XL automated sequence analyzer (Applied Biosystems) with the PCR primers.

The sequences in this study have the following accession numbers: for VDPVs from this study, HQ738286–HQ738303; for Sabin 2, AY184220; for Sabin 3, X00925; for MAD04–MAD07, AM084223-4 and AM884184-5; for MAD029, AM084225; for CA17-G12, AF499639; and for CA17.67591, FM955278.

CodonCode Aligner software (CodonCode Corporation), CLC Main Workbench 5.0 software (CLC bio, Denmark) and SimPlot version 3.5.1 [12] were used for sequence comparisons.

### Seroneutralization

Seroneutralization tests were carried out with 13 sera from adults immunized with either the injectable inactivated polio vaccine (IPV) alone or IPV and OPV. Virus ( $2.0 \pm 0.3 \log_{10}$  TCID<sub>50</sub>/50 µL) was mixed with 50 µL of 2-fold serial dilutions of sera (from 20-fold to 10 240-fold) in 96-well plates and incubated at 37°C for 2 hours. HEp-2c cells ( $1.5 \times 10^4$  cells per well) were added, and the plates were incubated at 37°C for 7 days. Antibody titers were determined as the highest dilution of sera preventing a cytopathic effect of the virus.

### Assay of Neurovirulence in PVR-Tg Mice

Viral neurovirulence was determined in PVR-Tg21 mice genetically modified to express the human poliovirus receptor gene [13]. All animal studies reported here were approved by and conducted in accordance with the guidelines of the Office of Laboratory Animal Care at the Institut Pasteur and complied with French laws and regulations (study number 08188). Before intracerebral and intranasal inoculation, mice were anesthetized by intraperitoneal injection of 0.25 mg xylazine (BAYER) and 2.5 mg ketamine (MERIAL).

### Plaque-Size Assay and Temperature Sensitivity

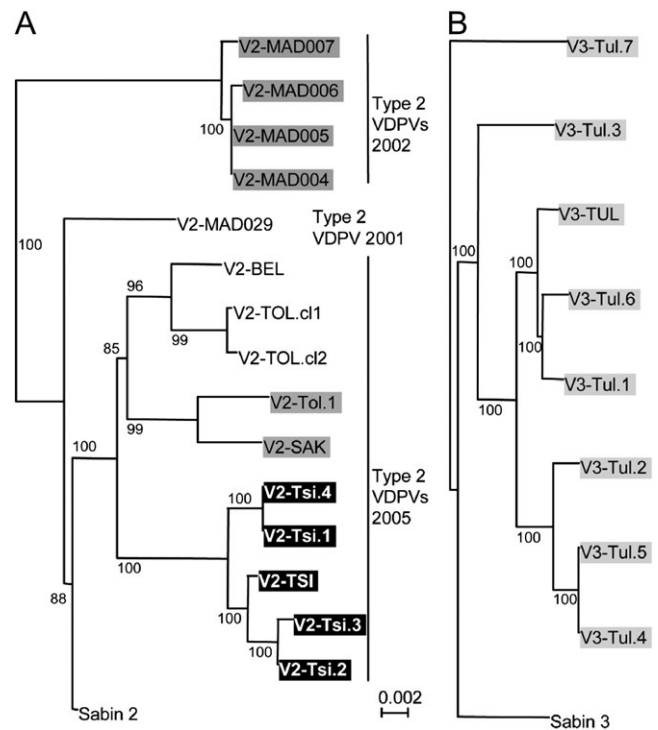
HEp-2c cell monolayers were infected and maintained in semisolid DMEM medium containing 1.2% Avicel [7]. Plates were incubated at 37°C (5% CO<sub>2</sub>) for 3 days before staining.

The temperature sensitivity of viruses was evaluated by titrating the same viral stock at 37.0°C and 40.0°C.

## RESULTS

### Analysis of the Sequences of the VDPV Genomes From Madagascar

Entire genome sequences were determined for the 17 VDPVs isolated in 2005 in Madagascar, except for a small fragment (35 nucleotides) at the 5' end of the genome, in some cases.



**Figure 3.** Phylogenetic trees depicting genetic relationships between the nucleotide sequences of vaccine-derived polioviruses (VDPVs) from Madagascar. Phylogenetic relationships between sequences (P1 regions) were inferred by the maximum likelihood method, with PUZZLE version 5.2, which uses QUARTET PUZZLING as the tree search algorithm [17]. The Hasegawa, Kishino, and Yano model of substitution for nucleotide with a Ts/Tv ratio of 10 was used. Trees were constructed by neighbor joining with PHYLIP (Phylogeny Inference Package) version 3.6, and branch length was determined by PUZZLE [18]. The genetic distance is indicated (bar). The numbers at nodes correspond to the percentage of 10 000 puzzle steps supporting the distal cluster. The nucleotide sequences of Sabin 2 and Sabin 3 were used as outgroups for trees (A) and (B), respectively. V2-MAD04–V2-MAD07 and V2-MAD29 are type 2 VDPVs isolated in Madagascar in 2001–2002 [2]. VDPVs with similar recombinant genomic structures are shaded with the same motif.

The VDPVs isolated from different patients from different districts had diverse mosaic recombinant genomic structures composed of sequences derived from vaccine polioviruses and from nonpoliovirus enteroviruses (Figure 1). A single recombinant genome was obtained from all but one of the viral isolates. Two viral populations differing in terms of the nonpoliovirus sequences present at the 3' end were distinguished following the sequencing of plaque-purified viruses from the isolate (V2-TOL ORI) from the patient in the district of Toliara I. The 5' parts of the genomes, up to nucleotide 5774, were mostly identical, with only 9 nucleotide differences (0.2%). By contrast, the 3' parts (1670 nucleotides) of the genomes differed at 173 nucleotides (10.4%), suggesting a recent recombination event. Two plaque-purified viruses (V2-TOL.cl1 and V2-TOL.cl2)

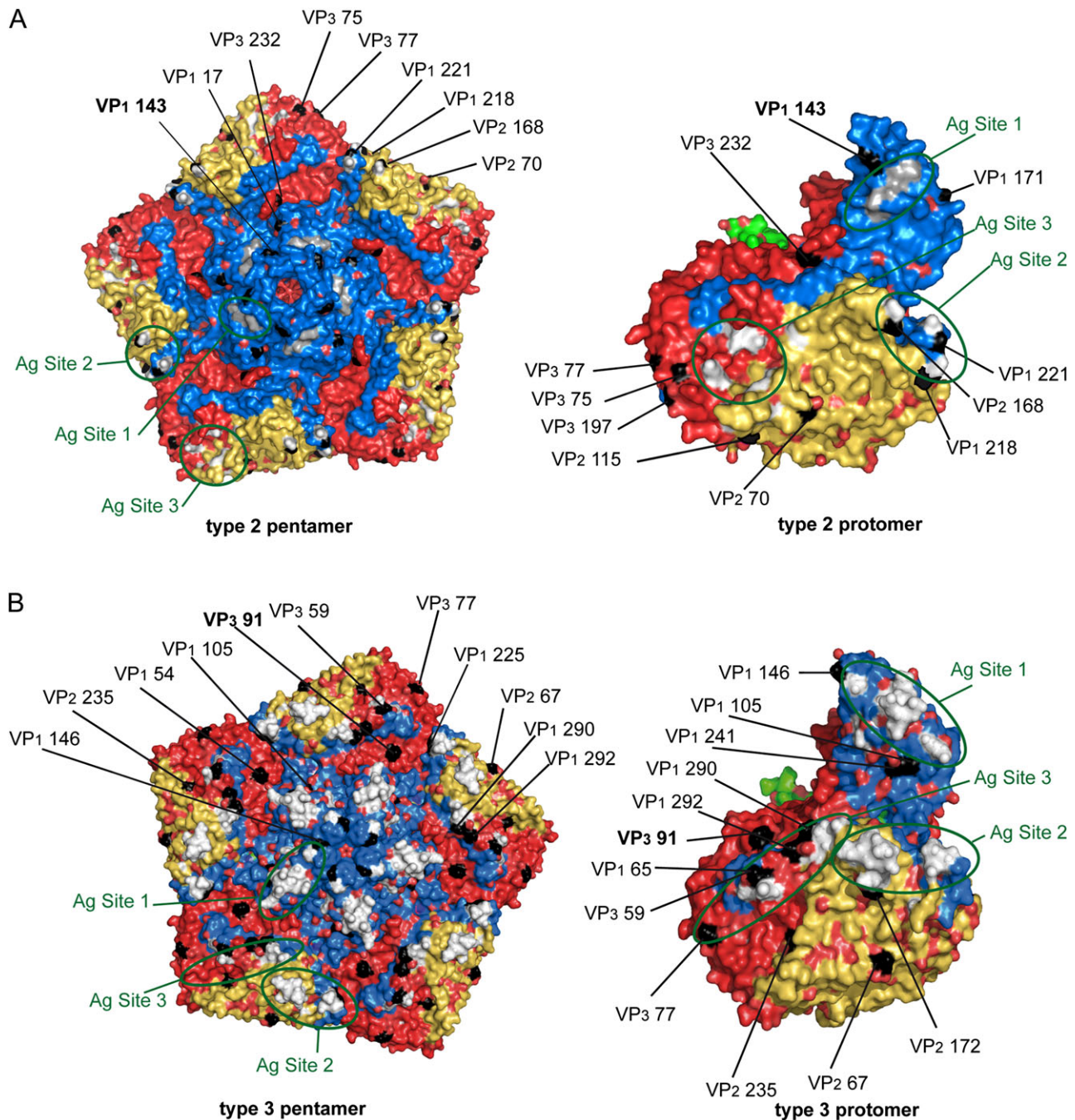


**Table 1. Modified Residues in the Capsid Proteins of Type 2 and Type 3 Vaccine-Derived Poliovirus (VDPV) Isolates**

| VDPV Type                             |                       |            |            |            |            |            |            |            |            |            |            |
|---------------------------------------|-----------------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|
| VDPV2 <sup>a</sup>                    |                       |            |            |            |            |            |            |            |            |            |            |
| Protein                               | Position <sup>b</sup> | S2         | V2-TSI     | V2-Tsi.1   | V2-Tsi.2   | V2-Tsi.3   | V2-Tsi.4   | V2-SAK     | V2-Tol.1   | V2-BEL     | V2-TOL.c11 |
| VP2                                   | 70                    | Thr        | Thr        | Thr        | <b>Ala</b> | <b>Ala</b> | Thr        | Thr        | Thr        | Thr        | Thr        |
|                                       | 168                   | Asn        | Asn        | Asn        | Asn        | Asn        | Asn        | <b>Ser</b> | <b>Ser</b> | Asn        | Asn        |
| VP3                                   | 75                    | Thr        | Thr        | Thr        | <b>Ala</b> | <b>Ala</b> | Thr        | <b>Ala</b> | <b>Ala</b> | <b>Ala</b> | <b>Ala</b> |
|                                       | 77                    | His        | His        | His        | His        | His        | His        | His        | His        | <b>Arg</b> | His        |
|                                       | 197                   | Arg        | <b>Lys</b> | Arg        | <b>Lys</b> | <b>Lys</b> | Arg        | Arg        | Arg        | Arg        | Arg        |
|                                       | 232                   | Ser        | Ser        | Ser        | Ser        | Ser        | Ser        | Ser        | Ser        | <b>Gly</b> | Ser        |
| VP1                                   | 5                     | Met        | <b>Leu</b> | <b>Leu</b> | <b>Leu</b> | <b>Leu</b> | <b>Leu</b> | Met        | Met        | Met        | Met        |
|                                       | 9                     | Ala        | <b>Val</b> | <b>Val</b> | <b>Val</b> | <b>Val</b> | <b>Val</b> | Ala        | Ala        | Ala        | Ala        |
|                                       | 10                    | Val        | Val        | Val        | Val        | Val        | Val        | <b>Ile</b> | <b>Ile</b> | Val        | Val        |
|                                       | 13                    | Ile        | Ile        | <b>Val</b> | Ile        | Ile        | <b>Val</b> | Ile        | Ile        | Ile        | Ile        |
|                                       | 35                    | Pro        | Pro        | Pro        | Pro        | Pro        | Pro        | Pro        | <b>Ser</b> | Pro        | Pro        |
|                                       | 36                    | Ala        | Ala        | Ala        | <b>Thr</b> | <b>Thr</b> | Ala        | Ala        | Ala        | Ala        | Ala        |
|                                       | <b>143</b>            | Ile        | <b>Thr</b> | <b>Thr</b> | <b>Thr</b> | <b>Thr</b> | <b>Thr</b> | <b>Thr</b> | <b>Thr</b> | <b>Thr</b> | <b>Thr</b> |
|                                       | 171                   | Asn        | Asn        | Asn        | Asn        | Asn        | Asn        | Asn        | Asn        | <b>Asp</b> | <b>Asp</b> |
|                                       | 221                   | Ala        | Ala        | <b>Thr</b> | Ala        | Ala        | <b>Thr</b> | Ala        | Ala        | Ala        | Ala        |
|                                       | 257                   | Ile        | Ile        | Ile        | Ile        | Ile        | Ile        | Ile        | Ile        | <b>Val</b> | Ile        |
| No. of amino acid differences/Sabin 2 |                       |            | 4          | 5          | 7          | 7          | 5          | 4          | 5          | 6          | 3          |
| No. of nucleotide differences/Sabin 2 |                       |            | 38         | 37         | 42         | 45         | 39         | 37         | 40         | 31         | 32         |
| % Nucleotide divergence/Sabin 2       |                       |            | 1.4        | 1.4        | 1.6        | 1.7        | 1.5        | 1.4        | 1.5        | 1.2        | 1.2        |
| VDPV3 <sup>a</sup>                    |                       |            |            |            |            |            |            |            |            |            |            |
| Protein                               | Position <sup>b</sup> | S3         | V3-TUL     | V3-Tul.1   | V3-Tul.2   | V3-Tul.3   | V3-Tul.4   | V3-Tul.5   | V3-Tul.6   | V3-Tul.7   |            |
| VP4                                   | 34                    | Lys        | <b>Arg</b> | <b>Arg</b> | <b>Arg</b> | <b>Arg</b> | <b>Arg</b> | <b>Arg</b> | <b>Arg</b> | <b>Arg</b> |            |
| VP4                                   | 61                    | Leu        | Leu        | Leu        | Leu        | <b>Phe</b> | Leu        | Leu        | Leu        | Leu        |            |
| VP2                                   | 45                    | Asp        | Asp        | Asp        | Asp        | <b>Gly</b> | Asp        | Asp        | Asp        | Asp        |            |
|                                       | 67                    | Asp        | Asp        | <b>Glu</b> | Asp        | Asp        | Asp        | Asp        | Asp        | Asp        |            |
|                                       | 172                   | Glu        | Glu        | Glu        | <b>Lys</b> | Glu        | <b>Lys</b> | <b>Lys</b> | Glu        | Glu        |            |
|                                       | 235                   | Pro        | Pro        | Pro        | Pro        | Pro        | Pro        | Pro        | Pro        | <b>Ser</b> |            |
| VP3                                   | 59                    | Ser        | Ser        | Ser        | Ser        | Ser        | Ser        | Ser        | Ser        | <b>Asn</b> |            |
|                                       | 77                    | Asp        | Asp        | Asp        | Asp        | Asp        | Asp        | Asp        | <b>Ser</b> | Asp        |            |
|                                       | <b>91</b>             | Phe        | <b>Ser</b> | <b>Ser</b> | <b>Ser</b> | <b>Ser</b> | <b>Ser</b> | <b>Ser</b> | <b>Ser</b> | <b>Ser</b> |            |
| VP1                                   | 2                     | Ile        | Ile        | Ile        | <b>Val</b> | Ile        | <b>Val</b> | <b>Val</b> | Ile        | Ile        |            |
|                                       | 7                     | Ser        | Ser        | Ser        | <b>Thr</b> | Ser        | Ser        | Ser        | Ser        | Ser        |            |
|                                       | 54                    | Ala        | <b>Val</b> | <b>Val</b> | <b>Val</b> | <b>Val</b> | <b>Val</b> | <b>Val</b> | <b>Val</b> | <b>Val</b> |            |
|                                       | 65                    | Val        | Val        | Val        | Val        | Val        | Val        | Val        | Val        | <b>Ile</b> |            |
|                                       | 105                   | Met        | <b>Thr</b> | <b>Thr</b> | <b>Thr</b> | <b>Thr</b> | <b>Thr</b> | <b>Thr</b> | <b>Thr</b> | <b>Thr</b> |            |
|                                       | 143                   | Ala        | <b>Ser</b> | <b>Ser</b> | <b>Ser</b> | <b>Ser</b> | <b>Ser</b> | <b>Ser</b> | <b>Ser</b> | <b>Ser</b> |            |
|                                       | 223                   | Ile        | Ile        | Ile        | Ile        | <b>Val</b> | Ile        | Ile        | Ile        | Ile        |            |
|                                       | 239                   | Leu        | <b>Met</b> | <b>Met</b> | Leu        | Leu        | Leu        | Leu        | <b>Met</b> | Leu        |            |
|                                       | 288                   | Asn        | Asn        | Asn        | Asn        | <b>Asp</b> | Asn        | Asn        | Asn        | Asn        |            |
| 290                                   | Asp                   | <b>Glu</b> | <b>Glu</b> | <b>Glu</b> | <b>Glu</b> | <b>Glu</b> | <b>Glu</b> | <b>Glu</b> | <b>Glu</b> |            |            |
| No. of amino acid differences/Sabin 3 |                       |            | 7          | 8          | 9          | 10         | 8          | 8          | 8          | 9          |            |
| No. of nucleotide differences/Sabin 3 |                       |            | 35         | 36         | 42         | 35         | 42         | 42         | 38         | 41         |            |
| % Nucleotide divergence/Sabin 3       |                       |            | 1.3        | 1.4        | 1.6        | 1.3        | 1.6        | 1.6        | 1.4        | 1.6        |            |

<sup>a</sup> Residues modified in the capsid proteins (P1 region) of the type 2 VDPV (VDPV2) and type 3 VDPV (VDPV3) isolates with respect to the Sabin 2 (S2) and Sabin 3 (S3) strains, respectively. Isolates recovered from acute flaccid paralysis cases are indicated in capital letters; isolates recovered from healthy children (contacts) are indicated in lower case letters. Modified amino acid residues are shown in bold.

<sup>b</sup> Residues involved in attenuation are indicated in bold in this column.

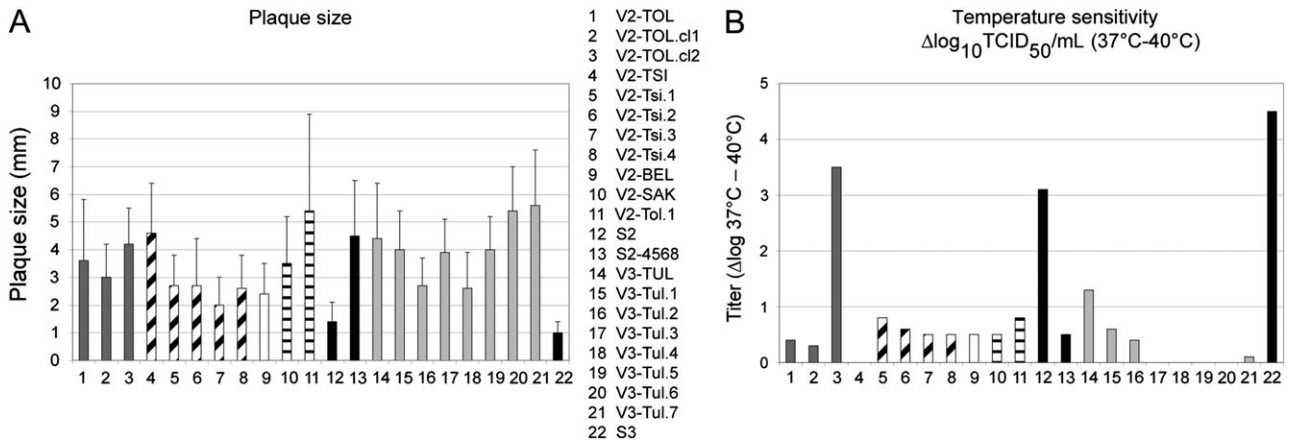


**Figure 4.** Localization of the modified residues of vaccine-derived polioviruses on the capsid shell. External sides of pentamers and protomers of the type 2 (Lansing strain) and type 3 (Sabin 3) vaccine strains. Illustrations of the molecular structures of the type 2 and 3 viral capsids were generated with PyMOL Molecular Graphics System version 1.3 (Schrödinger, LLC). The PV2/Lansing and PV3/Sabin3 coordinates were obtained from the Protein Data Bank under identification numbers 1EAH and 1PVC, respectively [26, 27]. Proteins VP1–VP4 are labeled in blue, yellow, red, and green, respectively. The modified residues indicated in Table 1 are shown in black. Residues known to be implicated in antigenic neutralization sites are shown in white (shadowed in gray) [28–30]. Capsid protein and residue number are indicated for all modified residues.

were chosen as representative of these 2 viral populations and used for further studies.

Most VDPVs isolated from the same district, from either patients or healthy children, had similar recombinant structures.

Only V2-Tol.1, isolated from a healthy child in the district of Toliara I, had a recombinant structure similar to that of an isolate from another district, V2-SAK, a VDPV2 isolated from a polio case in the neighboring district of Sakahara (Figure 1).



**Figure 5.** Phenotypic characteristics of vaccine-derived polioviruses (VDPVs) and parental vaccine strains. *A*, Plaque size. Plaque assays were performed on infected HEP-2c cell monolayers incubated at 37°C for 72 h in semisolid medium. The original Sabin 2 and Sabin 3 strains (S2 and S3) and a temperature-insensitive neurovirulent type 2 VDPV isolated from sewage (S2-4568) were used. V2-TOL ORI is the original isolate, containing a mixture of V2-TOL.cl1 and V2-TOL.cl2. Mean plaque diameters are given, with standard deviations. VDPVs with similar recombinant genomic structures are indicated by identical motifs. *B*, Temperature sensitivity. The differences between the  $\log_{10}$  virus titer of a viral stock measured after 5 d at optimal temperature (37.0°C) and that measured at supraoptimal temperature (40.0°C) are shown.  $\text{TCID}_{50}$ , 50% tissue-culture infectious dose.

The 5' UTRs of all VDPV2 were derived from closely related nonpoliovirus sequences (>97.8% nucleotide identity) and had identical sites of recombination with Sabin 2 genomes. Most VDPV2 (V2-BEL, V2-SAK, V2-TSI, and related VDPVs from healthy children) and all VDPV3 recombinant genotypes had a similar recombinant 3' end of the genome composed of similar nonpoliovirus and Sabin 3 sequences (>97.8% and 96.9% nucleotide identities, respectively), with identical sites of recombination between these sequences. However, these VDPV2 differed in their site of recombination between the Sabin 2 and nonpoliovirus sequences (P2 and P3 nonstructural regions). A comparison of the whole-genome sequences of the VDPV with those of OPV strains (Figure 2A) confirmed the genomic structure described above for poliovirus and nonpoliovirus sequences.

The nonpoliovirus sequences present in the VDPVs were compared with the partial sequences of HEV-C collected in 2002 in Toliara province (CAV11, -13, -17, -20 and -24 isolates described in [2]). The nonpoliovirus 5' UTRs of the VDPV2 (nucleotides 183–543) were closely related (98% nucleotide identity) to a few CAV isolates identified as CAV13 on the basis of VP1 nucleotide identities [14–16] and less related ( $\leq 96\%$ ) to the other CAV isolates. The 2C genomic regions (nucleotides 4128–4389) of V2-TOL.cl1 and cl2 were related to sequences from CAV17 isolates (90.4%–91.6% nucleotide identity;  $\leq 86\%$  for the other CAV). The nonpoliovirus 3D sequences (nucleotides 6145–6731) of these 2 VDPV2 were related to those of CAV11 or CAV13 isolates (90–93% nucleotide identity;  $\leq 88\%$  for other CAV), and the 3D sequences of V2-BEL, V2-SAK, V2-TSI, V3-TUL, and isolates with similar genomic structures were related to those

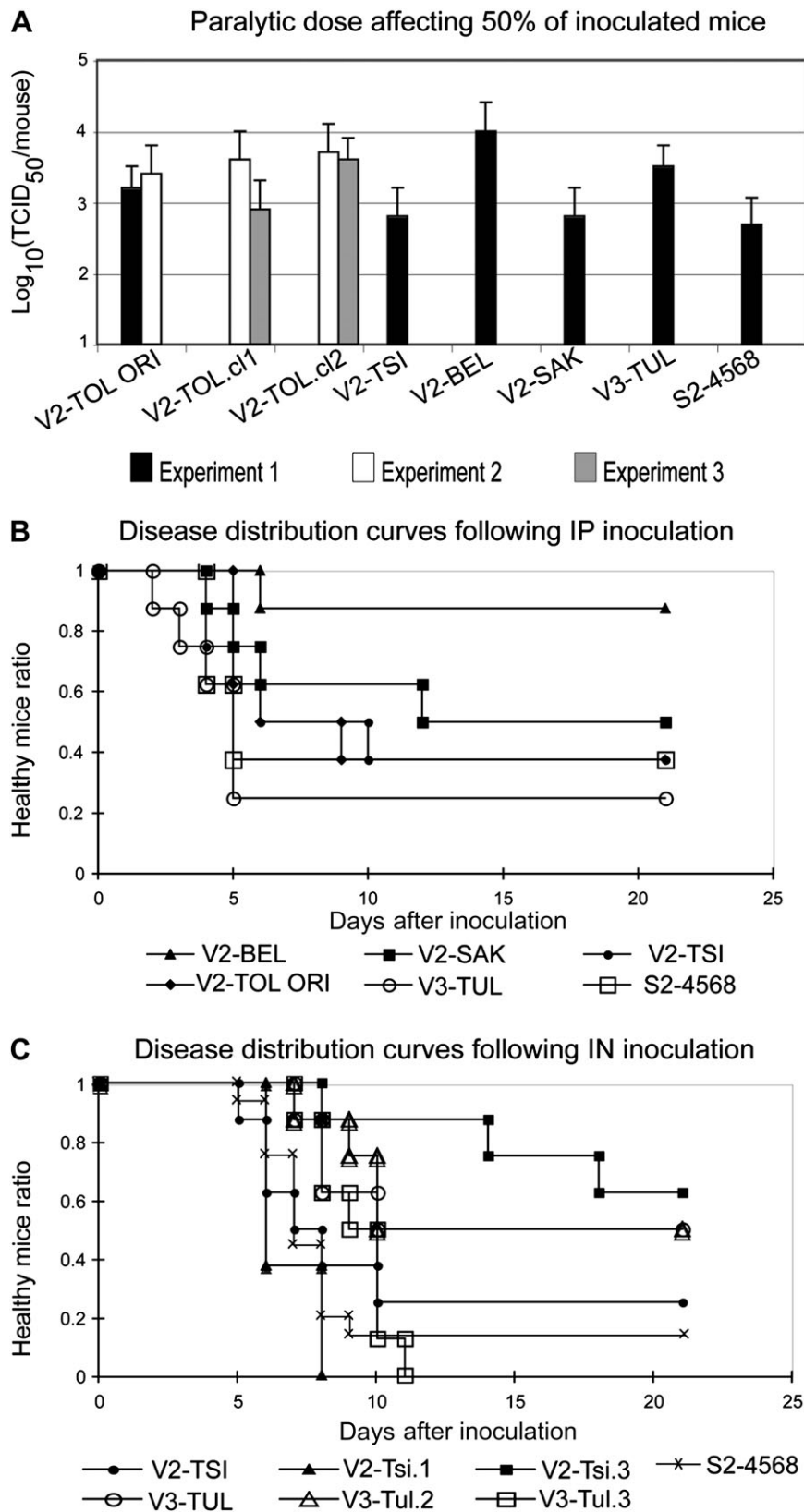
of CAV17 isolates (94% nucleotide identity;  $\leq 90\%$  for the others). In addition, a comparison of whole genomes (Figure 2B) confirmed that parts of the P2 and/or P3 regions of the genomes of all VDPVs were closely related to those of CA17-67591 and to one of the CAV17 isolates from Toliara province but not to those of the CAV17 prototype strain CA17-G12 [2, 7].

The VDPVs differed from each other, for at least some nucleotide positions. A comparison of the nucleotide sequences of the P1 region on the basis of molecular phylogenetic tools indicated that there were subclusters of VDPV2 and VDPV3 supported by high reliability values, suggesting that VDPVs with similar recombinant structures (named V2-TSI and V3-TUL series, respectively) spread along different transmission chains (Figure 3). The nucleotide differences between the P1 regions of the VDPV of the V2-TSI series (up to 0.8% nucleotide difference) or the V3-TUL series (up to 1.7% nucleotide difference) indicated that they had been multiplying in the same host and/or circulating independently for many months before isolation, according to the molecular clock of poliovirus of about 1% nucleotide substitutions per year [19–21].

Vaccine-derived genomic regions present in VDPVs systematically displayed mutations affecting the major attenuation determinants of the OPV strains, at position A481G in the 5' UTR and at Ile143Thr of VP1 for VDPV2 and T472C in the 5' UTR and Phe91Ser of VP3 for VDPV3 [22–25].

The P1 regions also contained many other missense mutations with respect to the original OPV strains (Table 1). The locations of these mutations were identified on the 3-dimensional structure of protomers and pentamers of the type 2 poliovirus Lansing strain and the Sabin 3 strain (Figure 4).





**Figure 6.** Neurovirulence of viruses used to inoculate transgenic PVR-Tg21 mice. *A*, Viral doses inducing paralysis or death in 50% of mice. Four groups of 6 PVR-Tg21 mice (3 males and 3 females) were challenged intracerebrally with a single dose of virus (10-fold dilutions of  $10^3$ – $10^6$  50% tissue-culture infectious doses [TCID<sub>50</sub>]/mouse, respectively). Inoculated mice were monitored for 21 d for paralysis and death, and the PD<sub>50</sub> was then calculated as described by Reed and Muench, with standard errors determined according to the formula of Pizzi [31]. *B*, A given viral dose (500  $\mu$ L containing

Many of the modified residues seemed to be present at the surface of the shell. Several were part of antigenic neutralization sites or were located close to these sites.

We evaluated the capacity of anti-poliovirus antibodies from vaccinated individuals to neutralize 4 VDPVs with putative antigenic modifications, by studying 13 serum samples from adults immunized with IPV or OPV plus IPV (last vaccine dose was received 1–16 years before sample collection). In all cases, the neutralization titers of individual sera were between 20 and 5120. Titers against V2-TSI, V2-Tsi.1 differed from those obtained against the original Sabin 2 vaccine strains by a factor of  $\leq 2$ , and titers against V3-TUL, V3-Tul.3 differed from those obtained with Sabin 3 by a factor of  $\leq 4$  (data not shown).

### Phenotypic Characteristics

We characterized this series of viruses by determining plaque size and temperature sensitivity in HEp-2c cells (Figure 5). All VDPV isolates had larger plaques than the Sabin 2 and Sabin 3 strains (Figure 5A), and plaque size varied with the VDPV isolate considered. In particular, plaque size differed between the isolates of the V2-TSI and those of the V3-TUL series, which had similar recombinant genomic structures.

The temperature sensitivity of the VDPVs was assessed by measuring virus titers for the same viral stock at 37.0°C and 40.0°C (Figure 5B). The difference in titer between the 2 temperatures was small for most isolates ( $< 1.3 \log_{10} \text{TCID}_{50}/\text{mL}$ ) except for the Sabin 2 and Sabin 3 reference strains and the V2-TOL.cl2 isolate ( $> 3 \log_{10} \text{TCID}_{50}/\text{mL}$ ). These results indicate that most VDPVs have lost the temperature-sensitive phenotype characteristic of the original OPV strains.

The pathogenic characteristics of VDPVs were evaluated in PVR-Tg21 mice after intracerebral, intraperitoneal, and intranasal inoculations (Figure 6). Unlike the Sabin 2 and Sabin 3 reference strains, all VDPVs induced paralysis or death in at least some of the mice inoculated. Previous studies have reported the existence of a good correlation between evaluations of the degree of neurovirulence of isolates for the 3 routes of inoculation [7, 8, 32].

The dose causing paralysis in 50% of the mice ( $\text{PD}_{50}$ ) was determined after intracerebral inoculation with serial dilutions of viral stocks (Figure 6A). All VDPVs from patients with poliomyelitis appeared to be highly neurovirulent, with a  $\text{PD}_{50}$  between 2.8 and 4.0  $\log_{10} \text{TCID}_{50}$  obtained. Some were

found to be as pathogenic as the highly neurovirulent positive control S2-4568 ( $\text{PD}_{50} = 2.7 \log_{10} \text{TCID}_{50}$ ), a VDPV isolated from sewage [9]. No difference was found between the original V2-TOL ORI isolate and the 2 plaque-purified recombinant variants V2-TOL.cl1 and V2-TOL.cl2 present in this isolate. All the VDPVs isolated from patients were neurovirulent in mice after intraperitoneal inoculation. No differences were found between these VDPVs when mice were inoculated with a single dose of  $10^8 \log_{10} \text{TCID}_{50}$  (data not shown). V2-BEL appeared to be slightly less neurovirulent than the other isolates after intraperitoneal inoculation with  $10^7 \log_{10} \text{TCID}_{50}$ , ( $P < .04$  by the log-rank test, except for V2-SAK ( $P = .11$ ); Figure 6B).

VDPVs were also neurovirulent after intranasal inoculation ( $10^6 \log_{10} \text{TCID}_{50}$ ). We compared the pathogenicity of 2 of the VDPVs isolated from patients with that of related VDPVs isolated from healthy children in the same district (Figure 6C). V2-Tsi.3, isolated from a healthy child, appeared to be less neurovirulent ( $P \leq .05$ ) than V2-Tsi.1 and V2-TSI, which were isolated from another healthy child and from the patient, respectively. V3-Tul.2 and V3-Tul.3, both isolated from healthy children, differed significantly from each other ( $P < .05$ ). Thus, VDPV isolates with the same recombinant genomic structure, circulating in the same district, at the same time, appeared to have acquired different levels of neurovirulence.

### DISCUSSION

Sequencing of the genomes of the VDPVs isolated in Toliara province showed that there were 6 highly complex mosaic recombinant lineages differing in terms of their OPV and HEV-C sequences and recombination sites. However, there was a clear genetic relationship between the VDPV2 genomes, which had similar recombinant 5' parts, and between some of the VDPV2 and the VDPV3, which had similar recombinant 3' parts (Figure 1). The similarity of the features of these recombinant parts of the genome strongly suggests that they originated from single recombination events and from common recombinant ancestors. In addition, on the basis of common recombinant features and other nucleotide markers (data not shown), it appears likely that VDPV2 with both recombinant 5' and 3' parts (ie, V2-BEL, V2-SAK, and V2-TSI series) originated from recombination between a VDPV2 ancestor with

*Figure 6 continued.*  $10^7 \text{TCID}_{50}$  was used for the intraperitoneal (IP) inoculation of groups of PVR-Tg mice expressing the human poliovirus receptor (8 mice per virus). The proportions of healthy mice following inoculation with parental or chimeric viruses or the neurovirulent positive-control virus S2-4568 are shown. C, A given viral dose ( $2 \times 5 \mu\text{L}$  containing  $10^6 \text{TCID}_{50}$ ) was used for the intranasal (IN) inoculation of groups of PVR-Tg mice (8 mice per virus). The curve for S2-4568 was generated from cumulative data previously obtained following the IN inoculation of PVR-Tg21 mice with identical doses of virus [7, 8]. No symptoms were observed following IC and IP inoculation with the highest doses of Sabin 2 and Sabin 3 used. Similar results were obtained for IN inoculation with Sabin 2 (Sabin 3 was not tested). Disease distribution curves were determined by the Kaplan-Meier method and compared by log-rank tests carried out with XLSTAT software (Addinsoft).

the 5' part and a VDPV3 ancestor with the 3' part. Several complex scenarios might account for the subsequent diversification of the recombinant VDPV2 lineages. Two recombinant VDPVs (V2-TOL.c11 and V2-TOL.c12) differing in terms of their 3' nonpoliovirus sequences and a few other nucleotides were found in 1 patient. We cannot rule out the possibility that the patient was infected with a mixture of 2 VDPVs, but it appears more likely that recombination occurred in his gut. Nevertheless, attempts to isolate the parental HEV-C donor in the presence of anti-PV antibodies were unsuccessful (data not shown). These results indicated that there was intense circulation of enterovirus strains throughout Toliara province and that genetic variation was common and established rapidly through recombination between poliovirus and endogenous nonpoliovirus HEV-C strains. Recombination is well-known to contribute to the genetic diversity of enteroviruses, including HEV-C [33]. The diverse and complex "mosaicism" of VDPV genomes, due to genetic recombination, revealed by this study highlights the considerable genetic plasticity of poliovirus and other HEV-Cs and shows that intertypic recombination is a common mechanism generating widespread genetic variability.

Given the frequency and diversity of these recombination events, the similarity of the recombinant genomic structures of the VDPVs isolated in the same district from healthy children and from patients is quite surprising. This similarity suggests that a given recombinant genome may display relative genetic stability during circulation. In particular, the recombinant VDPV3 lineage appeared to be stable despite evidence from the various transmission chains that it had been circulating for some months.

Our findings also indicate that all the VDPVs isolated in Toliara province in 2001, 2002, and 2005 contained nonpoliovirus sequences (mostly from the P2 region) closely related to those of enteroviruses isolated from the same province in 2002 and identified as CAV17 on the basis of sequence comparisons [2, 7]. Sequences from CAV13 isolates were also found to be closely related to sequences from the 5' UTR and P3 regions of some VDPVs. CAV11 sequences may also be involved in recombination. However, it should be borne in mind that 5' or 3' UTR and P3 regions are highly conserved among HEV-Cs, making it more difficult to draw firm conclusions from sequence comparison data [34]. Nevertheless, direct relationships have been demonstrated between a recombinant VDPV3 from Cambodia and indigenous CAV17 strains displaying similarities in the P2 region and a CAV13 isolate with a similar P3 region [5]. These findings indicate that CAV17 and CAV13 isolates may be ideal partners for the transfer of nucleotide sequences through recombination with polioviruses.

The presence of nonpoliovirus sequences related to CAV17 and CAV13 isolates is the only factor common to all the VDPV2 isolated in Madagascar in 2001, 2002, and 2005. Exhaustive analysis of Sabin 2 sequences clearly indicated that VDPV2

lineages emerged independently during these 3 periods (Figure 3). The recurrent presence of sequences related to CAV17 and, to some extent, CAV13 in VDPVs from Toliara province and the relative stability of the recombinant genomic structures of VDPVs circulating during 2005 in some Toliara districts strongly suggest that recombination mechanisms and/or functional or structural constraints regulate and limit intertypic genetic exchanges, as previously shown for intertypic recombinant OPV strains isolated from vaccinated subjects [35, 36].

A comparison of some of the phenotypic characteristics of the VDPVs isolated in 2005 showed that most had lost the temperature-sensitive and attenuated phenotypes of the original vaccine strains. However, these VDPVs displayed heterogeneity in terms of plaque-size phenotype. This diversity can be accounted for by differences in the features of the recombinant genome and/or nucleotide differences, because this heterogeneity was observed between VDPVs with similar recombinant genomic structures (eg, the V2-TSI and V3-TUL series). Similarly, different levels of pathogenicity in PVR-Tg mice were observed for different VDPVs, even between VDPVs of the same series (Figure 6C). We cannot rule out the possibility that the process of isolation from stool samples may have selected variants with a particular phenotype, but our results suggest that the circulation and genetic drift of VDPVs due to recombination and/or nucleotide substitutions may generate phenotypic diversity.

Nucleotide substitutions affecting the vaccine-derived capsid may result in amino acid changes within or close to antigenic sites involved in neutralization (Figure 4), potentially affecting the reactivity of the VDPVs with vaccine-induced antibodies. Nevertheless, neutralization experiments with some VDPVs and serum samples from vaccinated subjects showed that modified viral capsids reacted well with human antibodies [37]. These results are consistent with the absence of additional poliomyelitis case detection after the immunization campaigns organized in response to the 2005 outbreak, suggesting that the subsequent immune status of the population was sufficient to eradicate the circulating VDPVs.

In conclusion, this is the first detailed study of VDPV3 implicated in an outbreak of poliomyelitis. It confirms the genetic relationship between VDPV2 and VDPV3 and suggests that the 2005 outbreak in Madagascar was a single event involving both types of VDPV. This study also highlights the diverse ways in which an OPV-derived strain may become pathogenic, through recombination and mutations, providing support for the continuation of studies to elucidate the viral factors shaping viral genetic diversity and contributing to the emergence of VDPVs.

## Notes

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