

# Reemergence of Recombinant Vaccine-Derived Poliovirus Outbreak in Madagascar

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**Background.** After the 2001–2002 poliomyelitis outbreak due to recombinant vaccine-derived polioviruses (VDPVs) in the Toliara province of Madagascar, another outbreak reoccurred in the same province in 2005.

**Methods.** We conducted epidemiological and virological investigations for each polio case patient and for their contacts.

**Results.** From May to August 2005, a total of 5 cases of acute flaccid paralysis were reported among unvaccinated or partially vaccinated children 2–3 years old. Type-3 or type-2 VDPV was isolated from case patients and from healthy contacts. These strains were classified into 4 recombinant lineages that showed complex mosaic genomic structures originating from different vaccine strain serotypes and probably from human enterovirus C (HEV-C) species. Genetic relatedness could be observed among these 4 lineages. Vaccination coverage of the population was very low (<50%).

**Conclusions.** The broad distribution of VDPVs in the province and their close genetic relationship indicate intense and rapid cocirculation and coevolution of the vaccine strains and of their related HEV-C strains. The occurrence of an outbreak due to VDPV ~3 years after a previous outbreak indicates that a short period with low vaccination coverage is enough to create favorable conditions for the emergence of VDPV in this setting.

In 1988, the World Health Assembly adopted the goal of global eradication of poliomyelitis. Currently, with the extensive use of the trivalent oral polio vaccine (OPV), wild-type polioviruses are endemic in only 4 countries [1]. However, OPV strains frequently undergo mutations during replication in humans, which may occa-

sionally cause vaccine-associated paralytic poliomyelitis [2]; OPV strains also frequently undergo recombination, which contributes to the variability of polioviruses [3–5]. These 2 genetic characteristics seem to underlie the occurrence of poliomyelitis outbreaks associated with circulating VDPV (cVDPV) [6, 7].

Between 2000 and 2005, a total of 6 outbreaks due to cVDPV were reported in Haiti and the Dominican Republic, the Philippines, Madagascar, China, Cambodia, and Indonesia [8–12]. From 1988 to 1993, evidence of VDPV circulation was retrospectively identified in Egypt [13]. In all these outbreaks, the affected patients had not been vaccinated or had been only partially vaccinated against polioviruses, and except in China, the viruses implicated had undergone recombination with human enterovirus C (HEV-C) species. In addition, all indigenous wild polioviruses, or at least those of the same serotype as the VDPVs, had been eliminated in the countries or regions where those outbreaks occurred, suggesting a decrease in the natural immunity of the population. These factors—low population immunity,

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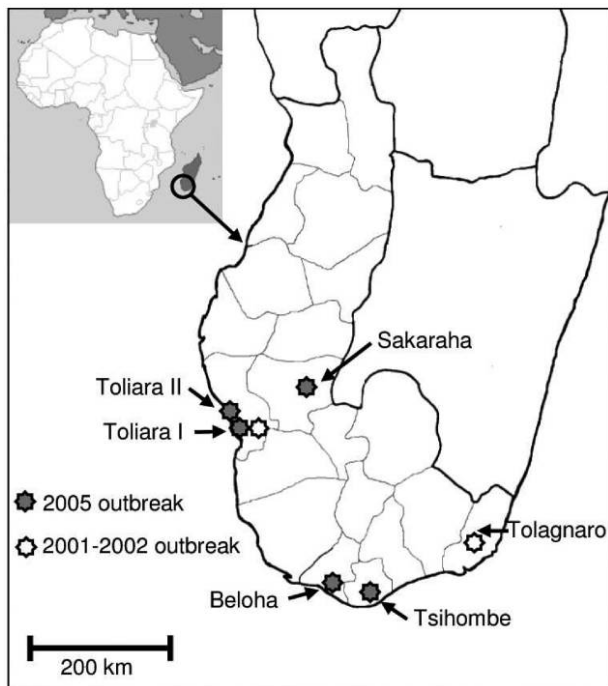
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**Figure 1.** Geographic distribution of cases in the outbreak due to circulating vaccine-derived poliovirus, Toliara province, Madagascar, April–August 2005.

HEV-C circulation, and the absence of wild poliovirus of the same VDPV serotype—thus appear to be associated, in most cases, with the emergence of vaccine-derived strains [6, 7].

In Madagascar, where the last wild-type poliovirus had been reported in October 1997 [14], an outbreak due to type-2 cVDPV occurred in the southern province of Toliara in December 2001 and in March–April 2002 [11]. To interrupt circulation of VDPV, the Ministry of Health of Madagascar conducted a nationwide mass vaccination campaign in September and October 2002. However, ~3 years later, a second outbreak again occurred in the same province, from April to August 2005.

In the present study, we examined the epidemiological and virological aspects of this new outbreak, which was associated with type-2 and type-3 cVDPVs. We also point out some important aspects of the evolution and circulation of VDPVs and speculate as to the possible cause of the outbreak.

## METHODS

**Epidemiological investigation.** To identify the agent responsible for the outbreak, we studied stool specimens collected from 5 patients who developed acute flaccid paralysis (AFP) in 2005. Clinical signs included paralysis of 1 or 2 lower limbs associated with fever. Figure 1 shows the geographic distribution of the cases. Between 21 April and 9 September 2005, there were 2 stool specimens collected from each case patient at 24–48 h intervals

(10 specimens), and 8 additional specimens were obtained at an approximately 1-month interval (for a total of 18 specimens).

To determine the epidemiology and extent of virus transmission, we conducted field investigations among close contacts of the case patients between 20 May and 28 August 2005. A baseline questionnaire was completed for each child; it included date of birth; sex; site of enrollment; and previous routine vaccinations, based on health cards. We collected 218 stool specimens on 20 May and 28 July, including 112 specimens from children who lived in the district of Toliara II; 22 specimens from children in the district of Toliara I, on 24 May and 28 August; and 84 from children in the district of Tsihombe, on 17 and 27 July. This investigation was conducted as part of national AFP surveillance, and the specimens were collected after obtaining informed consent from parents. The latest data on routine vaccination and AFP surveillance from Toliara province were also evaluated.

**Virus isolation and intratypic differentiation.** Extracts of stool specimens were treated with chloroform and cultured on rhabdomyosarcoma (RD) and HEp-2 cell lines used for enterovirus isolation and L20B cells used specifically for poliovirus isolation [15]. All L20B poliovirus isolates were identified by use of a microneutralization serotyping test. Isolates that showed cytopathic effect only on HEp-2 or RD cell lines were considered to be nonpolio enteroviruses (NPEVs) and were further analyzed only by molecular methods.

To determine whether the poliovirus isolates were from vaccine or of wild origin, we performed reverse-transcription polymerase chain reaction (RT-PCR) and ELISA that targeted the VP1 coding region [16]. In addition, to detect mutant and recombinant poliovirus vaccine strains, we performed restriction fragment length polymorphism (RFLP) assays, as described elsewhere [17], except that only 2 regions of the genome were amplified: the VP3-VP1 capsid region and the 3D polymerase-3' noncoding region (3D-3'-NC).

**Genetic analysis.** To analyze and compare the genetic structures and the genetic relationships among isolates, we sequenced different regions of the viral genomes. We extracted viral RNA from the supernatant of virus-infected cells by use of the QIAamp Viral Mini Kit (Qiagen). The primer P1C (5'agctgatcgggctaccatgcgtaccctttttttttttttttttttttc3') was used for the reverse transcription of the viral RNAs. Subsequently, poliovirus subgenomic regions were amplified by RT-PCR: the 5' noncoding region (5'-NC), the VP1-coding region, the VP1-2A region, the P2-coding region, and the 3D-3'-NC region [18]. All NPEVs were confirmed by RT-PCR, by use of generic primers that targeted the 5'-NC region [18], and characterized by partial sequencing in the VP1-coding region [19]. After purification of PCR products, cycling sequencing reactions were performed and nucleotide sequences were analyzed, by means of methods reported elsewhere [19].

**Table 1. Intratypic differentiation of virus isolates obtained from acute flaccid paralysis (AFP) case patients and their contacts during an outbreak due to recombinant type-2 and type-3 circulating vaccine-derived poliovirus, Toliara province, Madagascar, April–August 2005.**

| Isolate type, identification number | District   | Age, years | Sex    | RT-PCR and ELISA result | RFLP assay result, capsid (noncapsid) <sup>a</sup> | VP1 nucleotide divergence from Sabin strain, no. (%) |
|-------------------------------------|------------|------------|--------|-------------------------|--|--|
| <b>Type-2 isolate</b>               |            |            |        |                         |  |  |
| <b>V2-Tsi<sup>b</sup></b>           | Tsihombe   | 2.0        | Male   | Sabin 2–like            | Drift (recombinant)                                | 21 (2.3)   |
| V2-Tsi.1                            | Tsihombe   | 1.5        | Female | Sabin 2–like            | Drift (recombinant)                                | 22 (2.4)   |
| V2-Tsi.2                            | Tsihombe   | 3.0        | Female | Sabin 2–like            | Drift (recombinant)                                | 23 (2.5)   |
| V2-Tsi.3                            | Tsihombe   | 2.0        | Female | Sabin 2–like            | Drift (recombinant)                                | 24 (2.7)   |
| V2-Tsi.4                            | Tsihombe   | 6.0        | Female | Sabin 2–like            | Drift (recombinant)                                | 23 (2.5)   |
| <b>V2-Tol<sup>b</sup></b>           | Toliara I  | 2.0        | Male   | Sabin 2–like            | Drift (recombinant)                                | 10 (1.1)   |
| V2-Tol.1                            | Toliara I  | 3.0        | Male   | Sabin 2–like            | Drift (recombinant)                                | 12 (1.3)   |
| <b>V2-Bel<sup>b</sup></b>           | Beloha     | 3.0        | Female | Sabin 2–like            | Drift (recombinant)                                | 10 (1.1)   |
| <b>V2-Sak<sup>b</sup></b>           | Sakaraha   | 2.0        | Male   | Sabin 2–like            | Drift (recombinant)                                | 16 (1.8)   |
| <b>Type-3 isolate</b>               |            |            |        |                         |  |  |
| <b>V3-Tul<sup>b</sup></b>           | Toliara II | 2.0        | Male   | Sabin 3–like            | Drift (recombinant)                                | 13 (1.4)   |
| V3-Tul.1                            | Toliara II | 2.0        | Male   | Sabin 3–like            | Drift (recombinant)                                | 11 (1.2)   |
| V3-Tul.2                            | Toliara II | 4.0        | Male   | Sabin 3–like            | Drift (recombinant)                                | 14 (1.6)   |
| V3-Tul.3                            | Toliara II | 1.8        | Female | Sabin 3–like            | Drift (recombinant)                                | 11 (1.2)   |
| V3-Tul.4                            | Toliara II | 4.0        | Female | Sabin 3–like            | Drift (recombinant)                                | 16 (1.8)   |
| V3-Tul.5                            | Toliara II | 3.0        | Female | Sabin 3–like            | Drift (recombinant)                                | 15 (1.7)   |
| V3-Tul.6                            | Toliara II | 0.7        | Male   | Sabin 3–like            | Drift (recombinant)                                | 12 (1.3)   |
| V3-Tul.7                            | Toliara I  | 4.0        | Male   | Sabin 3–like            | Drift (recombinant)                                | 9 (1.0)  |
| S3-Tul.1                            | Toliara II | 0.5        | Male   | Sabin 3–like            | Sabin 3–like (. . .)                               | 1 (0.1)  |
| S3-Tul.2                            | Toliara II | 2.0        | Male   | Sabin 3–like            | Sabin 3–like (. . .)                               | 2 (0.2)  |
| S3-Tul.3                            | Toliara II | 0.3        | Female | Sabin 3–like            | Sabin 3–like (Sabin 2–like)                        | 0  |
| S1-Tul                              | Toliara II | 0.5        | Male   | Sabin 1–like            | Drift (Sabin 1–like)                               | 3 (0.3)  |

**NOTE.** RFLP, restriction fragment length polymorphism; RT-PCR, reverse-transcription polymerase chain reaction.

<sup>a</sup> Mutations and recombination were screened by RFLP in the VP3-VP1 capsid region and in the 3D polymerase 3' noncoding region, respectively.

<sup>b</sup> Isolates recovered from AFP case patients are in bold type; isolates recovered from case patients' close contacts are in regular type.

Phylogenetic relationships of all sequences isolates were done with Puzzle software (version 5.0) [20], which uses quartet puzzling as the tree search algorithm (maximum likelihood method). The sequences generated by our study were deposited in GenBank (accession numbers, EF628377–EF628473 and EF634001–EF634021).

## RESULTS

### Epidemiological Investigations

All 5 cases of AFP reported in this study occurred in patients who either were not vaccinated (Toliara I and II, Beloha, and Tsihombe districts) or were incompletely vaccinated (Sakaraha district) with OPV. Toliara province had the lowest reported OPV coverage, compared with the other 5 provinces in Madagascar. In 2004, the reported coverage with 3 OPV doses was <50% in 12 of the 21 districts of Toliara province, and coverage was 50%–79% in the other 9 districts. All but 1 case of AFP from Toliara I district occurred in rural villages.

Detailed clinical histories were available for 2 AFP case patients. The patients from Tsihombe and Toliara II, both 2-year-old boys, developed paralysis of the right and left leg, respectively, on 13 June and 9 April 2005. During the month preceding the onset of paralysis, the patient from Tsihombe had received oral corticoid medication and multiple intramuscular injections of antibiotic in both legs. The patient from Toliara II was reported to have been chronically ill since the age of 3 months. The other 3 patients with AFP, aged 2–3 years, had onset of paralysis on 26 June (Beloha), 13 July (Toliara I), and 14 August (Sakaraha) 2005. Among the 5 patients with AFP, only the patient from Beloha was known to have residual paralysis 60 days after onset. Only the patient from Toliara II had a history of travel outside the area in which he resided. Among the 218 close contacts, 156 (72%) had not been vaccinated with OPV, 31 (14%) had received 1–2 doses, and 31 (14%) had received 3–4 doses.

### Virus Identification

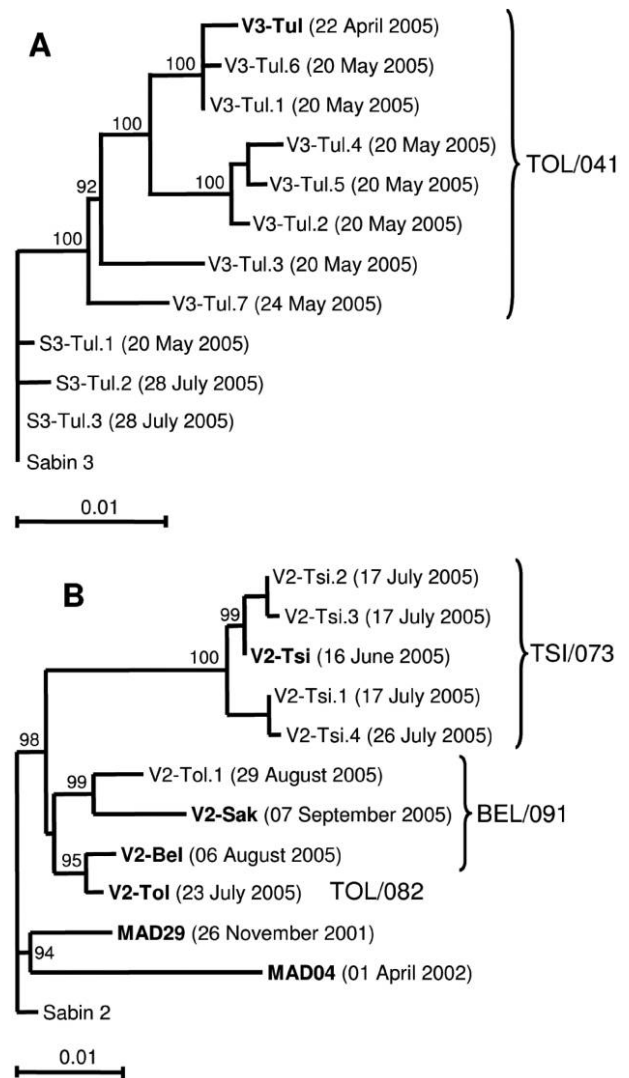
**Viruses from AFP case patients.** A total of 9 polioviruses were isolated on L20B cells from the 18 stool specimens. One type-3

poliovirus was isolated from the second specimen obtained from the patient in Toliara II (V3-Tul), and 8 type-2 polioviruses were isolated from the first 2 specimens from patients in Tsihombe (V2-Tsi), Beloha (V2-Bel), Toliara I (V2-Tol), and Sakaraha (V2-Sak). For each case patient, no virus was isolated in the stools collected >1 month after the first stool sample positive for virus. Because viruses isolated from the same patient were found to have the same features, we consider only 1 isolate per patient below.

**Viruses from contacts.** A total of 16 polioviruses (1 type-1 virus, 5 type-2 viruses, and 10 type-3 viruses) were isolated from the 218 stool specimens obtained from close contacts of 3 of the 5 AFP case patients. There were 48 isolates recovered on RD and/or HEp-2 cells, but not on L20B cells; 45 of the 48 isolates were confirmed as NPEV by RT-PCR that targeted the 5'-NC region, and 3 were identified as adenovirus by immunofluorescence assay. To determine the NPEV serotypes, we sequenced a 321-bp fragment in the VP1-coding region from 22 isolates. Using the FASTA program [21], we identified 3 human enterovirus B species and 19 HEV-C species (data not shown).

**Intratyptic differentiation and primary characterization.** When tested by RT-PCR and ELISA, all poliovirus isolates ( $n = 21$ ) were found to be OPV-like (1 type-1 virus, 9 type-2 viruses, and 11 type-3 viruses) (table 1). However, we found some modifications in the RFLP pattern of the VP3-VP1 coding region of 18 isolates, suggesting that they could be mutated polioviruses [17]. Partial sequencing of the VP1-coding region confirmed that 17 of the 18 isolates were actually VDPVs with  $\geq 1\%$  nucleotide sequence divergence from their respective vaccine strain. The remaining 4 isolates were OPV type-1 and type-3 strains (Sabin 1 and 3). In addition, when the RFLP profiles in the 3D-3'-NC regions of these 17 VDPVs were analyzed, results strongly suggested that they were recombinant with NPEV. To characterize the 21 poliovirus isolates further, they were partially sequenced and different regions of the genomes were compared.

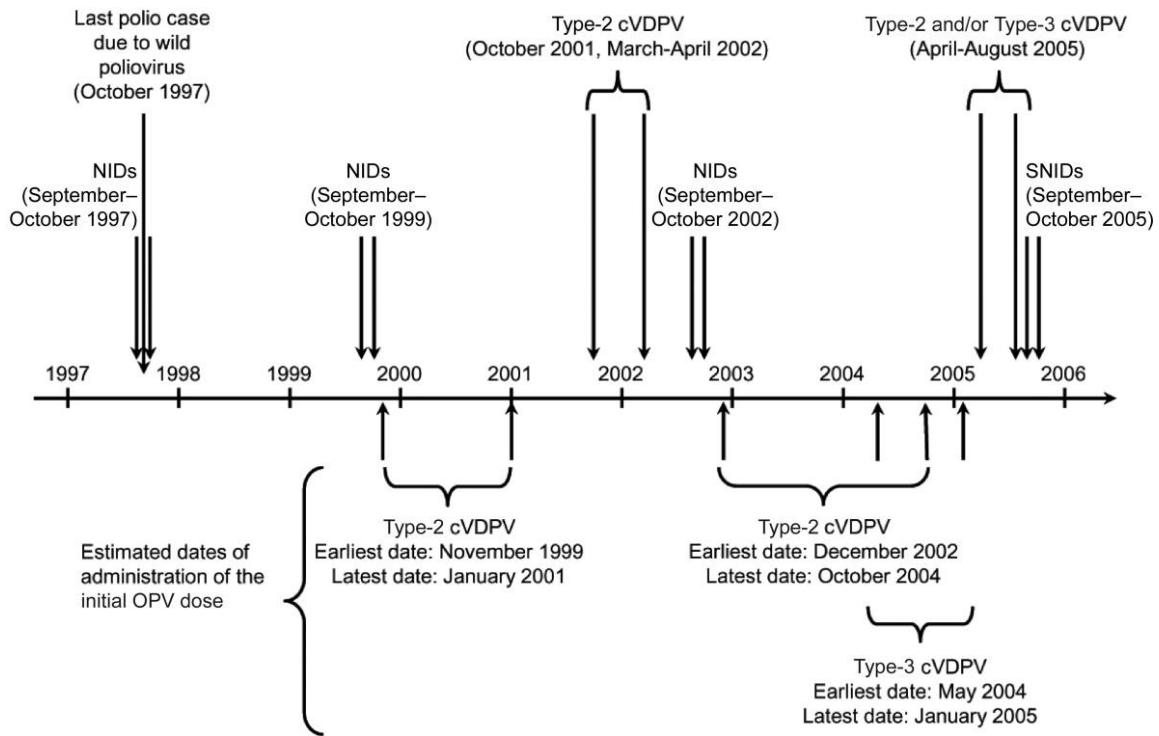
**Capsid VP1 sequences.** Among the 11 type-3 isolates—all from Toliara II district—8 were closely related (lineage, TOL/041), and their sequences differed from that of Sabin 3 by 9–16 nt (1.0%–1.8% difference) (figure 2A). They shared common nucleotide substitutions at 4 positions, which suggests a common OPV ancestor strain (data not shown). Assuming an evolution rate of  $3.4 \times 10^{-2}$  nt substitutions at synonymous third-base codon positions per year, as described elsewhere [22], type-3 VDPVs would have been circulating for ~5 to 13 months (i.e., since January 2005, at the earliest, or since May 2004, at the latest) before the dates of stool sample collection (figure 3). The VP1 sequences of the 9 type-2 isolates originating from Tsihombe, Toliara I, Beloha, and Sakaraha districts differed from that of Sabin 2 by 10–24 nt (1.1%–2.7% difference). They would have been circulating for ~11 to 32 months (since October 2004, at the earliest, or since December 2002, at the latest), assuming an evolution rate of  $2.5 \times 10^{-2}$  nt substitutions per synony-



**Figure 2.** Phylogenetic analysis of the VP1 region of the circulating vaccine-derived poliovirus (VDPV) isolates. These neighbor-joining trees were based on nucleotide sequence alignments of the VP1 coding regions of type-3 VDPVs (A) and type-2 VDPVs (B), as described in Methods. Numbers at nodes correspond to the percentages of 1000 puzzling steps supporting the distal cluster; bars, genetic distances. Isolate names in bold are those recovered from acute flaccid paralysis case patients. The dates of specimen collection are indicated in parentheses, and the names of the lineages are shown, as in figure 4. The nucleotide sequences used were from the polio vaccine strains Sabin 2 and Sabin 3 (GenBank accession number, X00595 and X00925, respectively) and from VDPVs isolated in 2001 and 2002 in Madagascar, MAD29 and MAD04 (GenBank accession number, AM084225 and AM084223, respectively).

mous site and per year, based on a study of the Egyptian polio type-2 isolates [13]. Their VP1 sequences differed by 15–42 nt (1.7%–4.6% difference) from that of the 2001–2002 isolates [11] and segregated into 3 clusters: V2-Tsi and their contacts (Tsihombe); V2-Sak (Sakaraha) and V2-Tol.1 (Toliara I); and V2-Bel (Beloha) and V2-Tol (Toliara I) (figure 2B). They shared common nucleotide substitutions at 4 positions (data not





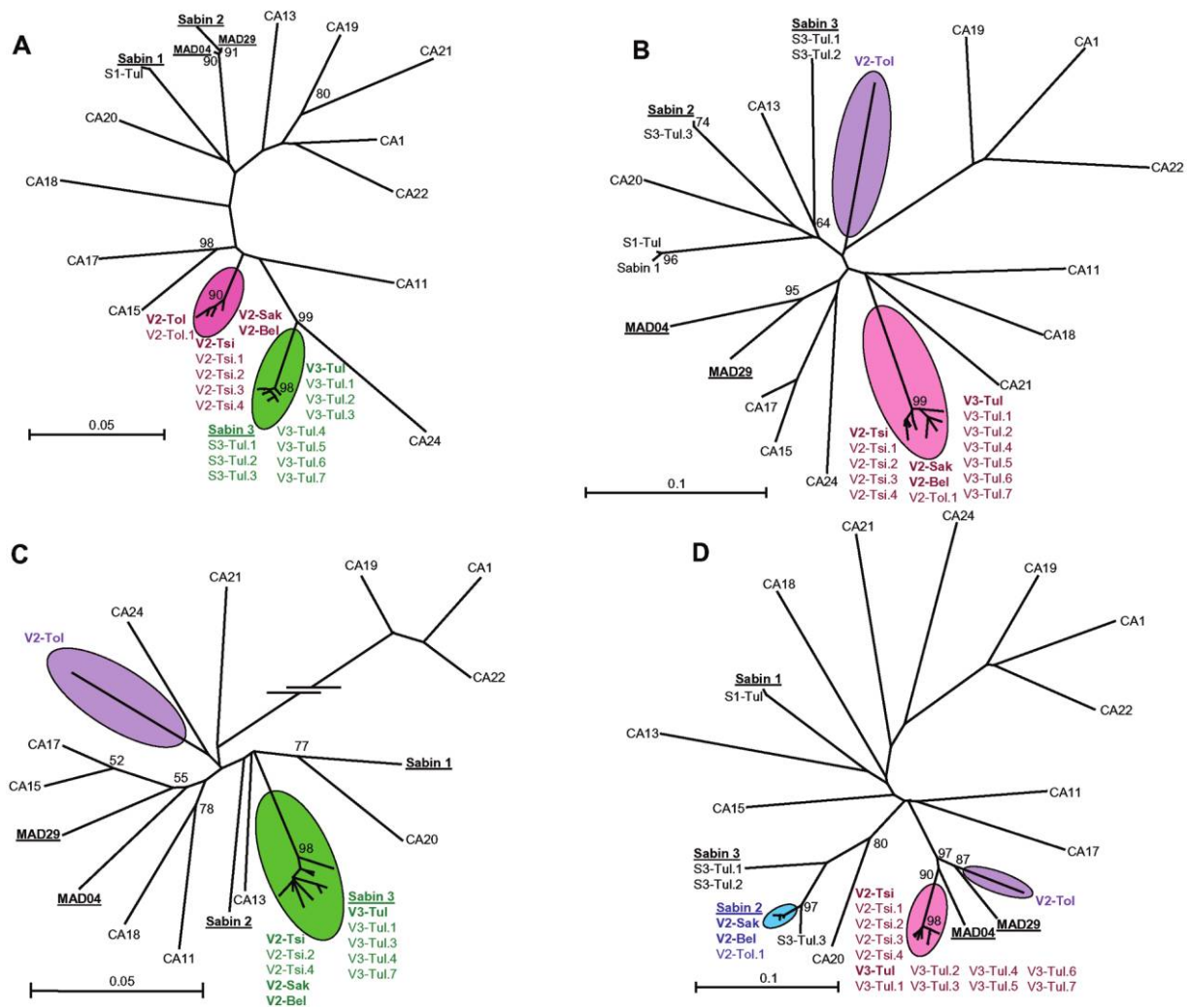
**Figure 3.** Time line of vaccination dates and detection of wild and circulating vaccine derived poliovirus (cVDPV) in Toliara province, Madagascar, 1997–2005. NIDs, national vaccination days (vaccination with oral polio vaccine [OPV]); SNIDs, subnational immunization days (vaccination with OPV) in Toliara province. The dates of administration for the initial OPV dose were estimated on the basis of the evolution rates of type-2 [13] and type-3 [22] poliovirus strains, as described elsewhere.

shown) and had the nucleotide change T2909>C, which is associated with the reversion of attenuated type-2 strain to neurovirulence.

**Recombinant features of VDPVs.** The 5'-NC sequences of the 8 type-3 VDPVs were closely related to those of Sabin 3 (figure 4A) but exhibited the T472>C nucleotide substitution, which is associated with the reversion to neurovirulence. In contrast, the 5'-NC sequences of the type-2 VDPVs were apparently derived from non-OPV viruses (8.7%–11.3% difference from the Sabin strains). The sequences of the 5' part of the 3D-coding region (figure 4B) for the type-2 and type-3 VDPV isolates were gathered in 2 distinct genetic clusters, which were related to HEV-C sequences but different from OPV sequences. In contrast, the sequences of the 3' part of the 3D-coding region and the 3'-NC region of most VDPVs were clearly derived from Sabin 3 (97%–99% nucleotide identities) (figure 4C). The analysis of the VDPV -nonstructural 2C regions showed a diversity of fragments derived from OPV and non-OPV strains (figure 4D). Non-OPV sequences present in these VDPVs presented 81%–87% nucleotide identities with HEV-C sequences available in GenBank and were probably derived from cocirculating enteroviruses of these species. Four other isolates recovered from contacts of the case patients showed sequences exclusively derived from OPV in all sequenced parts (figure 4), suggesting that they

were Sabin 3 (S3-Tul.1 and S3-Tul.2) isolates, recombinant Sabin 3/Sabin 2 (S3-Tul.3) isolates, and Sabin 1 (S1-Tul) isolates.

**Multipartite recombinant structure of VDPVs.** From the sequencing data presented in figure 4 and those of 2 other fragments localized in the 2B (nt 3832–4122) and 3D (nt 6574–7079) regions (data not shown), the VDPV isolates could be classified into 4 distinct lineages (figure 5). Lineage TOL/041 included the Toliara II isolates and was characterized by a tripartite recombinant genome that included 2 fragments derived from Sabin 3 flanking a fragment derived from HEV-C. Recombination sites were located in the 2C and 3D regions. Lineage TSI/073 included Tsihombe isolates and was characterized by a quadripartite recombinant genome derived from Sabin 2, Sabin 3, and 2 fragments of HEV-C sequences. Recombination sites were localized in the 2C and 3D regions. Lineage BEL/091 included the isolates from Beloha, Sakaraha, and Toliara I districts. This lineage showed the same quadripartite structure but differed from TSI/073 lineage because of differences in recombination sites between the Sabin 2-derived and the HEV-C sequences. Interestingly, the 3' end of the genome of the type-2 lineages TSI/073 and BEL/091 was identical to that of type-3 lineage TOL/041 with the same HEV-C–derived and Sabin 3–derived sequences, including an identical recombination site. Lineage TOL/082 included isolates from Toliara I district. This



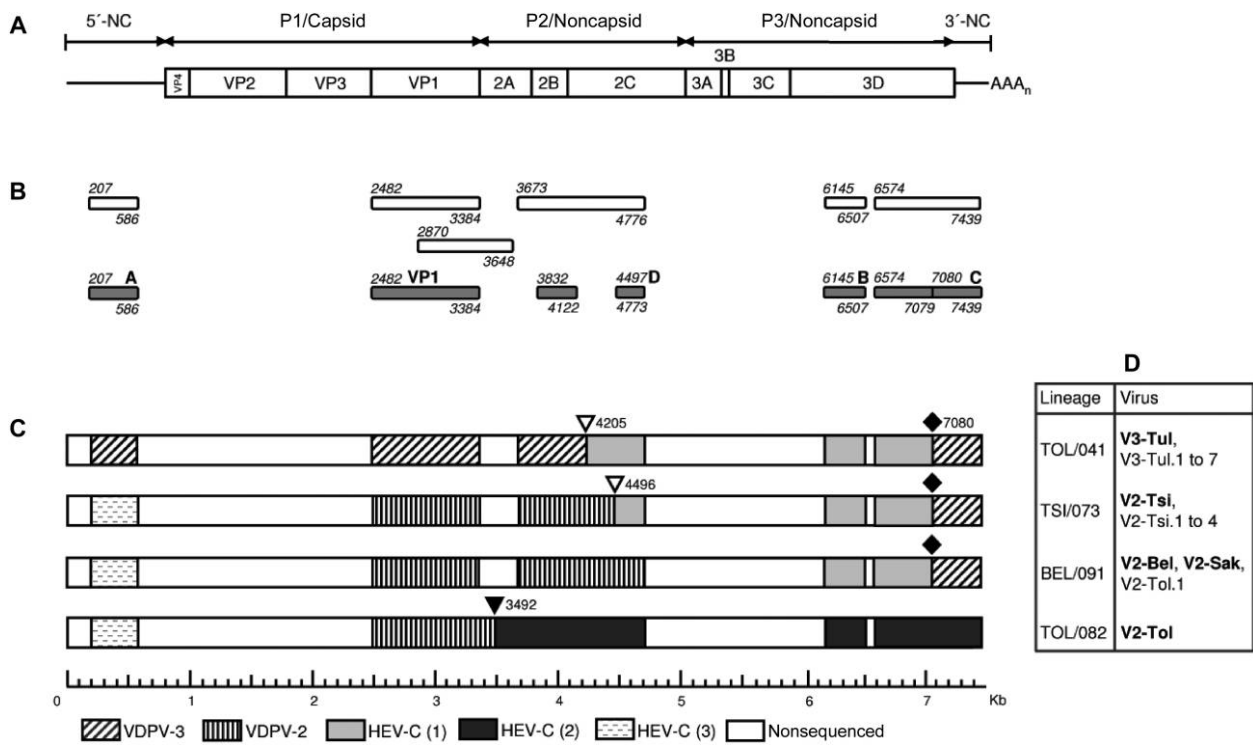
**Figure 4.** Genetic relationships between circulating vaccine-derived poliovirus (VDPV) isolates. These unrooted neighbor-joining trees were based on nucleotide sequence alignments of different subgenomic regions, as described in Methods. *A*, Tree built from 380-bp fragments of the 5' noncoding (NC) region (nt 207–586, with reference to Sabin 2). *B*, Tree built from 363-bp fragments of the 5' part of the 3D coding region (nt 6145–6507). *C*, Tree built from 367-bp fragments including the 3' part of the 3D coding region and the 3'-NC region (3D polymerase-3'-NC; nt 7080–7439). *D*, Tree built from 277-bp fragments of the 2C coding region (nt 4497–4773). Names in bold and underlined are VDPVs isolates recovered in 2001 (MAD29; GenBank accession number, AM084225) and 2002 (MAD04; AM084223) in Madagascar, and Sabin strains. Clusters of isolates with sequences related to Sabin 2 and Sabin 3 are colored blue and green, respectively; those related to unknown human enterovirus C species (HEV-C) are colored pink or purple. Sequences of HEV-C prototype strains in these trees had GenBank accession numbers AF499635 (CA1), AF499636 (CA11), AF499637 (CA13), AF499638 (CA15), AF499639 (CA17), AF499640 (CA18), AF499641 (CA19), AF499642 (CA20), AF546702 (CA21), AF499643 (CA22), D90457 (CA24), V01150 (Sabin 1), X00595 (Sabin 2), and X00925 (Sabin 3).

lineage showed a tripartite recombinant genome structure and a recombination site located in the 2A region. Its 5'-NC region is closely related to those of TSI/073 and BEL/091 (99%–100% nucleotide identities) (figure 4A). However, TOL/082 non-OPV HEV-C sequences present in the P2, P3, and 3'-NC regions showed no relationship with sequences in the other 3 lineages.

## DISCUSSION

In this article we report a new outbreak due to cVDPV, which was associated with 2 serotypes (2 and 3) and an intense circu-

lation of these viruses in the population. Outbreaks due to type-2 cVDPVs have been reported in Egypt in 1988–1993 [13] and in Madagascar in 2001–2002 [11]; the outbreak reported in the present article is the third description of this sort of outbreak. It is known that the type-2 OPV strain spreads to unvaccinated children more easily than the other 2 serotypes [23]. This characteristic would certainly favor its divergence and emergence as an outbreak-causing organism among nonvaccinated children. Type-3 VDPV strains have also been recovered from a healthy child [24], from sewage [25], and from chronically infected patients with hypogammaglobulinemia [8, 22], and they have been



**Figure 5.** Schematics of the genomes for the 4 recombinant circulating vaccine-derived poliovirus (VDPV) lineages. *A*, Schematic genetic organization of the enteroviral genome. The single open reading frame is flanked by the 5' and 3' noncoding (NC) regions, with different subgenomic regions and viral proteins. *B*, Different subgenomic regions that were sequenced to determine the overall structure of the genomes of the 5 strains isolated from the acute flaccid paralysis case patients and their contacts (*open boxes*). The nucleotide sequences of these subgenomic regions are used to construct the unrooted phylogenetic trees shown in figures 2 and 4 (*closed boxes*). Nucleotide numbering is indicated in accordance with Sabin 2 sequences. *C*, Structures of the 4 lineages, as inferred from partial nucleotide sequencing. The type-2 and type-3 VDPV sequences are indicated (VDPV-2 and VDPV-3, respectively), as well as the unknown human enterovirus C species (HEV-C) sequences, HEV-C (1) to (3). *Open triangles*, localization of the recombination junctions between the vaccine-derived and the nonpolio enterovirus parts in the 2C region (TOL/041 and TSI/073); *closed triangles*, localization of the recombination junctions between the vaccine-derived and the nonpolio enterovirus parts in the 2A region (TOL/082). *Rhombuses*, localization of the recombination junctions between the nonpolio enterovirus and the VDPV-3 genomic parts of TOL/041, TSI/073, and BEL/091 in the 3D coding region. *D*, Names of the 4 lineages. The viral isolates are classified by lineages, according to the partial nucleotide sequences indicated in *B*.

identified as the predominant cause of vaccine-associated paralytic poliomyelitis [26, 27]. Type-3 VDPV strains have been isolated in Cambodia from a patient with AFP in 2002 [28] and during an outbreak in 2005–2006 [8]. In the present study, the type-3 VDPV lineage was implicated in a single AFP case but had spread widely in the population.

Almost all of the outbreak-associated cVDPVs described thus far have been viruses that have recombined with HEV-C [8]. The 2 serotypes reported here also had recombinant genomes, confirming that, in most cases, cVDPVs are associated with the co-circulation of HEV-C and with genetic exchanges between this species and OPV strains. We recently reported that HEV-C might be a dominant cluster among the circulating enteroviruses in Madagascar [19]. Moreover, HEV-C was frequently found during the last 2001–2002 outbreaks [29] and during the present outbreak. However, the implications of the genetic exchange between poliovirus and HEV-C in terms of evolution and pathogenicity require further investigation.

One particular genomic feature of the cVDPVs described here is the complex mosaic recombinant structures of the genomes. At least 4 lineages had been circulating in different districts of the Toliara province in 2005. When we consider the VP1 sequences, the type-2 lineages had diverged from a common OPV ancestor and had been multiplying and circulating along multiple chains of transmission, whereas the type-3 lineage spread along a unique and independent chain. The similar HEV-C sequences of the 5'-NC region of the type-2 lineages suggest that this part of the genome also derived from a common ancestor. Moreover, a common ancestor can explain the presence of similar HEV-C/Sabin 3 recombinant sequences at the 3' end of the genome in 2 type-2 lineages and the type-3 lineages. These results indicate a relationship among the 4 lineages. Because no genetic relationship was found among the 2001–2002 and the 2005 type-2 isolates, we conclude that the last cVDPVs emerged from an OPV strain and then evolved and spread in distant districts during a short period (~3 years). Because the type-3 lineage appears to

have emerged after the emergence of the type-2 lineages, it is likely that the type-3 lineage acquired its 3' end from the type-2 ones. However, we cannot exclude the possibility that a widely circulating HEV-C with a recombinant 3' end served as an intermediate virus. In all cases, the relationship between these lineages indicates an intense cocirculation and a rapid coevolution between OPV strains and indigenous HEV-C. Rapid evolution by recombination has also been described for HEV-B species [30, 31], for wild polioviruses [5], and for OPV strains [3]. Here we report a rapid evolution between polioviruses and HEV-C involving multiple rounds of intratypic and intertypic recombination events in a short period of time and in a restricted geographic area. This finding emphasizes the fact that this mechanism of evolution is common for enteroviruses.

At the time of this writing, no case due to VDPV had been reported in Toliara province after the 2 rounds of supplemental vaccination activities with OPV conducted in August and September 2005. Nationwide OPV campaigns were conducted in September and October 1999 as part of the polio eradication strategy and in September and October 2002, in response to the 2002 VDPV outbreak [11]. The first VDPV outbreak in December 2001 and March–April 2002 and the second outbreak in April–August 2005 were probably associated with 2–3 years of low routine OPV coverage after each of these campaigns. The finding that 72% of healthy children considered in this study had never been vaccinated in 2005 provides further evidence of a serious immunity gap that would facilitate the emergence, rapid spread, and evolution of VDPVs. The observation of low population immunity as an important risk factor for outbreaks due to VDPV is consistent with findings of other studies [7, 32].

All cases due to VDPV, even those in the urban city of Toliara I, occurred in a context of poor hygiene and sanitation, as well as in a subtropical climate that could favor poliovirus transmission [7]. Some persons with humoral immunodeficiency are known to excrete modified diverse VDPV strains for prolonged periods (5–12 years) [33, 34], compared with immunocompetent individuals. In this study, no evidence of global humoral immunodeficiency disorder was found among the blood samples obtained from 2 case patients infected with VDPV and from 103 selected contacts (data not shown). Moreover, none of the modified diverse VDPV strains described thus far have been recombinant with NPEV. It is thus unlikely that strains from immunodeficient individuals were the source of this outbreak. Other possible epidemiological and clinical factors may have contributed to the development of VDPVs in Toliara province. The 2 patients infected with VDPV for whom detailed clinical histories were obtained had received multiple intramuscular injections and administrations of corticoid and had a history of chronic illness during the weeks preceding the onset of paralysis. However, the contribution of these or other factors to VDPV emergence requires more thorough study.

In our study, all polioviruses were found to be similar to OPV strains by RT-PCR and ELISA the usual intratypic differentiation methods [15]. Their mutated and recombinant characters were strongly suggested by the results of a multiple RFLP assay [17]. This finding emphasizes the need to develop efficient and convenient methods for more sensitive detection of VDPVs.

In conclusion, 2 outbreaks occurred successively in 2001–2002 and in 2005 in Toliara province. Our findings have important implications for the future of the polio eradication program and associated viral surveillance. In both cases, these outbreaks emerged after efficient mass vaccination campaigns with OPV and subsequent low vaccine coverage during a period of 2–3 years. Therefore, priority should be given to (1) achieving and maintaining high population immunity to prevent the emergence of VDPV; (2) continuing research to refine the risk management strategies for possible emergence of VDPVs during the posteradication and OPV cessation era; (3) improving surveillance and diagnostic tools for VDPVs; and (4) conducting detailed epidemiological, clinical, and laboratory studies to clarify the risk factors for VDPV and help prevent its reemergence.

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