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RESEARCH ARTICLE

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Reversion of neurovirulent mutations, recombination and high intra-host diversity in vaccine-derived poliovirus excreted by patients with primary immune deficiency

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Abstract

Patients with Primary immunodeficiency (PIDs) may be infected by Polioviruses (PVs), especially when vaccinated with live Oral Polio Vaccine before diagnosis. They may establish long-term shedding of divergent strains and may act as reservoirs of PV transmission. This study delved into the effect of the genetic evolution of complete PV genomes, from MHC class II-deficient patients, on the excretion duration and clinical outcomes. Stool samples from three PID patients underwent analysis for PV detection through inoculation on cell culture and real-time PCR, followed by VP1 partial sequencing and full genome sequencing using the Illumina technology. Our findings revealed a low number of mutations for one patient who cleared the virus, while two exhibited a high intra-host diversity favoring the establishment of severe outcomes. Neurovirulence-reverse mutations were detected in two patients, possibly leading to paralysis

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development. Furthermore, a recombination event, between type 3 Vaccine-Derived Poliovirus and Sabin-like1 (VDPV3/SL1), occurred in one patient. Our findings have suggested an association between intra-host diversity, recombination, prolonged excretion of the virus, and emergence of highly pathogenic strains. Further studies on intra-host diversity are crucial for a better understanding of the virus evolution as well as for the success of the Global Polio Eradication Initiative.

KEYWORDS
intra-host diversity, neurovirulent-reverse mutations, recombination, vaccine-derived poliovirus

1 | INTRODUCTION

The WHO Global Polio Eradication Initiative (GPEI), launched in 1988, has led to the cessation of circulation of type 2 and type 3 wild polioviruses (WPVs). Only type 1 WPV continues to circulate in Pakistan and Afghanistan.[1] Due to the use of Oral Polio Vaccine (OPV), Vaccine-Derived Polioviruses (VDPVs) also pose a major concern. Thus, meticulous investigation of any suspected PV circulation is of great interest for the success of this program.[2] VDPVs are identified according to the degree of divergence observed in the Viral Protein 1 (VP1) coding region in comparison to the original vaccine strain, they have a 1% or more nucleotide sequence divergence from the original Sabin strains for type 1 VDPV and type 3 VDPV, and 0.6% divergence rate for type 2 VDPV.[3,4] As a result of this genetic evolution, strains may exhibit neurovirulence and/or transmissibility properties similar to those of wild-type viruses. VDPVs were classified into 3 categories: circulating VDPVs (cVDPVs) showing evidence of person-to-person transmission, immunodeficient VDPVs (iVDPVs) isolated from Primary Immunodeficient Patients (PIDs) and ambiguous VDPVs (aVDPVs), for which the clinical, epidemiologic and virologic data are insufficient for definitive assignment to an iVDPV or cVDPV.[5,6]

Genetic recombination and accumulation of mutations play a key role in the evolution of Polioviruses. Mutations are generated as a result of error-prone polymerase that lacks corrective 3'-5' exonuclease activity. They can affect the nucleotide sites associated to neurovirulence which cause reverse mutations. Those sites are serotype-specific and distributed throughout the viral genome.[7] RNA recombination is common in RNA viruses.[8] It induces insertion of new genomic fragments into the parental strains and can occur between PV strains and other enteroviruses. Recombination may restore the virulence and transmissibility potential leading to outbreaks.[9] It has been recorded in many circulating strains such as the type 2 cVDPV which caused epidemic between 2001 and 2002 in Madagascar and type 1 cVDPV that caused epidemics between 2000 and 2001 in the Dominican Republic, the Philippines and Haiti.[10]

Genetic evolution of PVs in PID patients is also an issue of concern. Indeed, PIDs can serve as long-term carriers of the virus, replicating and shedding PV for extended periods ranging from several months to decades, even in the absence of clinical symptoms.[11] During the long replication period, neurovirulent strains exhibiting multiple mutations and recombination events can be generated.[4] Such strains can induce paralysis for the PID patient or lead to outbreaks in areas with low vaccination coverage. This was the case of the Philippines, in 2019.[12,13] The factors contributing to chronic PV infections are not known. One hypothesis is that occurrence of specific mutations in the excreted PV genome may enhance prolonged PV excretion. The establishment of a particular PV intra-host population in the patient's gut may also help PVs to establish chronic infection.

In Tunisia, surveillance of Acute Flaccid Paralysis (AFP) has been conducted since 1991. Last WPV detection was recorded in 1994 and since then, the majority of detected strains were Sabin-like with low recombination rate and limited replication period.[14–16] An earlier investigation on 82 Tunisian PIDs, conducted between 2006 and 2010, detected six Sabin-like PV excretors. Five of them successfully cleared the virus rapidly, whereas one patient excreted a type 1 PV for up to 134 days, accumulating 14 mutations in the VP1 region.[17,18] Two other PID patients excreting PVs and presenting with paralysis were detected in 2016 and 2019. The aim of this work is to study the genetic evolution of the complete PV genome of 13 isolates obtained from the three patients. Specific mutations, recombination and intra-host diversity are assessed to shed more light about their possible effects on the duration of excretion and the case outcome.

2 | MATERIAL AND METHODS

2.1 | Patients and specimens

A total of 29 stool samples were collected from three PID cases with MHC class II primary immunodeficiency at regular intervals (one or two samples per month approximately). The initial samples were received in October 2009, December 2016 and March 2019, from Patient A, B and C, respectively. Patient A was asymptomatic, while patients B and C presented Acute Flaccid Paralysis (AFP). Patient A received OPV according to the national vaccination calendar during the first 2 years of life and again at the age of four, before being diagnosed with immunodeficiency. Patient B received OPV 44 days before developing clinical signs including coma, dyspnea and tetraplegia. The three patients were from consanguineous marriages.

2.2 | Virus isolation in cell culture and viral RNA quantification

PV detection was conducted through cell culture isolation according to the WHO standard protocols for PV surveillance.[19] Stool suspensions were inoculated into two cell lines: RD cells (derived from a human rhabdomyosarcoma) and L20B cells (transgenic mouse cell line expressing the gene for human cellular receptor for PV).[20] The inoculated cells were followed daily to detect a cytopathic effect (CPE). The presence of a CPE on L20B and RD cell lines indicated the presence of PVs, which were then identified using intratypic real-time RT-PCR (ITD), a multiplex real-time PCR enabling the identification of the serotypes of PV strains as well as their vaccine or wild origin.[19]

For viral RNA quantification in the original stool samples obtained from the patients, serotype-specific real-time PCR was applied directly to treated stool samples after RNA extraction with the QIAamp viral RNA mini kit (Qiagen).[19]

2.3 | Sequencing of the VP1 region and sequence analysis

The complete VP1 region (positions 2480- 3385) of all isolates was amplified using Reverse transcription PCR (RT-PCR), as previously described.[16,18] Amplification and sequencing used forward primer Y7R (nucleotides 2399 – 2421: 5'-GGTTTTGTGTCAGGITGYAAYGA-3') and reverse primer Q8 (nucleotides 3504–3485:5'-AAGAGGTCTCTRTTC CACAT-3').[21] PCR products were purified with the QIAquick PCR kit (Qiagen) and sequenced bidirectionally using an ABI 3130 DNA sequencer (ABI 3130, Applied Biosystems) and ABI PRISM BigDye Terminator Cycle Sequencing Ready Process kit. Sequence data were then analyzed using Sequencher software v5.4.1 and compared to Sabin 1 and Sabin 3 PV reference strains, listed in the GenBank database under accession number AY184219.1 and AY184221.1, respectively.

2.4 | Whole genome sequencing and consensus sequence generation

Full genome sequencing was carried out at the Robert Koch Institute, Genome Competence Center (MF1), Berlin, Germany, using Illumina technology. cDNA synthesis was performed using random hexamer or oligo-dT primer, followed by library preparation using NexteraXT protocol.[22] An Illumina MiSeq device was used to read 2 × 300 bp paired end reads. To generate a full genome consensus sequence, the sequencing data underwent quality control and reads trimming using fastQC and fastp.[23] The Burrows-Wheeler Aligner (BWA) was employed for mapping to the Sabin 1 (AY184219.1) or Sabin 3 (AY184221.1) reference genomes.[24] The consensus sequence was then generated using Ivar tool.[25] The obtained consensus sequences were submitted to NCBI; accession numbers are available in Supporting Information S1: Table S1.

2.5 | Recombination analysis

The full-genome sequences were analyzed using Bootscan in SimPlot v.3.5.1[26] to identify recombination break points. Default settings were applied (Step: 20 bp; GapString: On, Reps: 100, Kimura [2-parameter]; T/t: 2.0, Neighbor-Joining). Breakpoints were confirmed by sequence alignment using Molecular Evolutionary Genetics Analysis (MEGA) software v7.0.26.[27]

2.6 | Evaluation of the evolution rate

To explore the evolution rate of PVs, a smart selection model with SMS[28] was performed before a Bayesian phylogeographic analysis using BEAST v2.6.3 and applying the HKY model. The strict clock model and the skyline tree model were inferred in our Bayesian analysis (500 million iterations and sampling every 10,000 states). The evolution rate was estimated using tracer tool v1.7.0. The phylodynamic tree was generated using tree annotator v2.6.3 and visualized using figtree v1.4.3.

2.7 | Intra-host diversity investigation

Mutations and amino-acid changes, along with their frequencies, were identified using Ivar[29] to generate tab-separated values (tsv) and variant call format (vcf) files. Data and heatmap visualization of mutation rates were performed using the R library ggplot2 v2.0.0.[30] For data visualization, the ggplot function was used to bind plots to specific data frames using the data argument. Heatmap representation of the matrix data was achieved using the heatmap function. The "BioRender" web server (https://www.biorender.com/) was utilized to map nucleotide and amino-acid mutations in the VP1 region. The distribution of SNV presented in Figure 8 was based on the calculation of Major and minor SNVs (M-SNVs and m-SNVs) rates. M-SNVs corresponds to the Major mutations presenting 90-100% Alternative frequency (ALT-FREQ) obtained by IVAR tool in the tsv files and m-SNVs corresponds to minor mutations that presented less than 90% ALT-FREQ. m-SNVs with different ALT-FREQ intervals were indicated in different shades of green color.

2.8 | Neurovirulence-Reverse mutations identification

The identification of neurovirulence mutations relied on comparing observed mutations with previously described neurovirulence mutations present in the P3/LEON/37[31] for PV3 sequences and the Mahoney strains[32] for PV1 sequences.

2.9 | Mutation mapping

The "Swiss Model" bioinformatics web server (www.swissmodel.expasy.org) was utilized to generate a three-dimensional

representation of PV structural proteins (VP1, VP2 and VP3). Protein structures and the localization of amino-acid changes were visualized using PyMOL software v2.3.3.[33]

3 | RESULTS

3.1 | Poliovirus detection and identification

Among the 29 stool specimens, 24 tested positives for PV, using isolation on cell culture. Only the first isolate from patient A was identified as Sabin-like1, while the remaining isolates were identified as VDPVs through ITD testing: 5 type 1 VDPVs obtained from Patient A, 5 type 3 VDPVs from Patient B and 13 type 3 VDPVs from Patient C. Viral RNA quantification by serotype-specific real-time PCR of the original stool samples obtained from Patient A, B and C showed Ct values ranging from 19 to 26, 25–26 and 16–30, respectively. Detailed Ct values are summarized in Table 1.

3.2 | Excretion kinetics

Patient A excreted type 1 VDPV for at least 134 days (approximately 4 months) (Figure 1A). The four samples obtained during the last two successive months were negative, indicating the cessation of excretion. Patient B excreted type 3 VDPV for at least 44 days (2 months) before dying (Figure 1B). For Patient C, prolonged type 3 VDPV shedding was observed during a period of minimum 271 days (9 months) before passing away (Figure 1C).

TABLE 1 Cycle threshold values of the stool samples obtained by real-time RT-PCR.

Patient	Sample ID	Ct Value
Patient A	D1	19
	D32	26
	D89	20
	D134	20
Patient B	D1	26
	D22	26
	D44	25
Patient C	D1	30
	D15	18
	D62	17
	D92	17
	D119	16
	D226	22
	D271	21

Abbreviation: RT-PCR, Reverse transcription PCR.

3.3 | Genetic variability in the VP1 region

Compared to the VP1 sequences of the parental Sabin1 (Supporting Information S1: Figure S1A) and Sabin3 strains (Supporting Information S1: Figure S1B), Patient A accumulated 7 to 14 nucleotide changes (Figure 2A), patient B had 10 to 18 (Figure 2B) and patient C accumulated 15 to 36 nucleotide changes (Figure 2C). Several mutations induced changes in the amino acid sequence in comparison to Sabin1 (Supporting Information S1: Figure S2A) and Sabin3 strains (Supporting Information S1: Figure S2B). Patient A accumulated 3 to 6 amino-acid changes including 1 to 2 mixed amino-acids, mainly into or near the antigenic site1 (Figure 3A). Patient B presented 5 to 9 amino-acid changes (Figure 3B). For Patient C, the number of amino-acid changes varied between 8 and 13 (Figure 3C). As shown in Supporting Information S1: Figure 2S2B, six of them were located in antigenic sites 1, 2 and 3.

3.4 | Recombination event investigation

Whole genome sequencing was performed for 13 isolates; with one isolate per month considered for each patient. Only one VDPV strain per month underwent complete genome sequencing.

All obtained sequences were examined for possible occurrence of recombination events. No recombination was detected in sequences from patients A and C. However, sequences from patient B showed recombination between type 3 VDPV and SL1, as indicated by SIMPLOT analysis and nucleotide alignment (Figure 4). The analysis revealed nearly identical regions from nucleotides 4905 to 4915, in reference to the Sabin 3 (AY184221.1) and Sabin 1 (AY184219.1) sequences which can be considered as the recombination site (Figure 5).

3.5 | Evolution of the nucleotide and amino-acid changes

Patient A exhibited the highest diversity rate in the VP1 and 2 A regions, with mutation rates of 1% to 1.9% for VP1 and 0% to 1.9% for 2 A (Figure 6A). Meanwhile, the highest rate of amino-acid changes was observed only in the VP1 region, with a frequency ranging from 1.5% to 1.9% (Figure 6B). In contrast, for Patients B and C sequences, the highest rate of diversity was seen in the VP1 and VP3 regions, as well as the 2B region for Patient B (Figure 6A). In term of the amino-acid changes, the most variable region was the VP1 protein for both patients, as well as the 2 A and 2B regions for Patient C (Figure 6B).

3.6 | Divergence rate

The divergence rates of the sequences from Patient C were calculated given the significant number of obtained samples. It was

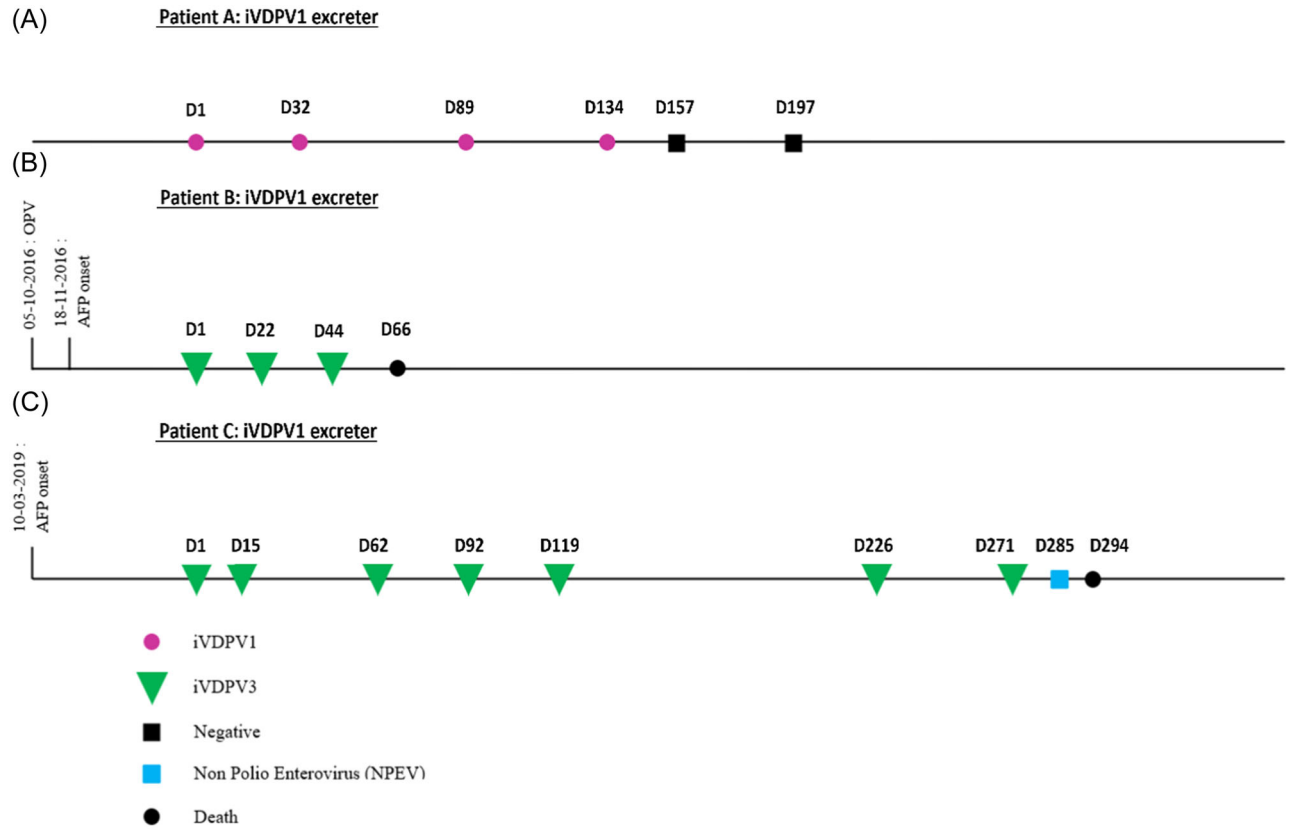

FIGURE 1 (A) Timeline of the excretion kinetics of PV strains for patient A; (B) Timeline of the excretion kinetics of PV strains for patient B; (C) Timeline of the excretions kinetics of PV strains for patient C. PV, Polioviruse.

estimated at 1.429×10^{-2} mutations per site per year. The phylodynamic tree (Figure 7) revealed three distinct lineages: 1, 2 and 3. One branch from lineage 1 evolved into lineage 2, which was detected up to Day 92, and then another branch from lineage 2 evolved into lineage 3, who continued evolution and excretion up to Day 271.

3.7 | Intra-host diversity evolution

In Patient A, the number of Single Nucleotide Variants (SNVs) increased from 47 SNVs at Day 32 to 62 SNVs by the end of excretion (Supporting Information S1: Figure S3A), including both Major SNVs (M-SNVs) (90%–100% frequency) and minor SNVs (m-SNVs) (less than 90% frequency). Interestingly, the m-SNVs rate, reflecting the viral population diversity, decreased from 52% in the first sample to 0% in the last one before virus clearance, showing evidence of viral diversity decline (Figure 8A).

In Patients B and C, SNV numbers remained important during the excretion period, varying from 155 to 119 SNVs from Day 1 to Day 44 (Supporting Information S1: Figure S3B) and from 50 to 160 SNVs from Day 1 to Day 271 for Patients B (Supporting Information S1: Figure S3C) (Figure 8B) and C (Figure 8C), respectively. Similarly, m-SNVs rates were high all along the shedding period, indicating a high level of intra-host diversity: 21% to 36% and 43% to 80% for Patients B and C, respectively. In the last samples, m-SNV rates were about 36% and 43% for Patients B and C, respectively.

3.8 | Mutations of interest

The neurovirulence-associated reverse mutations were assessed based on the 10 and 57 neurovirulence mutations documented in the literature for P3/LEON/37 strains and Mahoney strains respectively.[32,34] Among these, two neurovirulence-associated reverse mutations were identified for the type 3 VDPV excreted by Patients B and C, and 4 mutations for the type 1 VDPV excreted by Patient A. In Patient A, these mutations were located in the 5'UTR region at position 480, near to the loop V, and in the VP1 region at positions 2775, 2749 and 2795 (Table 2). Patients B and C shared the same neurovirulence-associated reverse mutations in the 5'UTR at position 472 near to the loop V, and in the VP3 region at position 2034 (Table 2).

3.9 | Mapping of mutations in VP1, VP2 and VP3 proteins

In antigenic site1, the three type 1 VDPV strains from Patient A exhibited 3 amino-acid changes in the VP1 protein at positions 90, 99

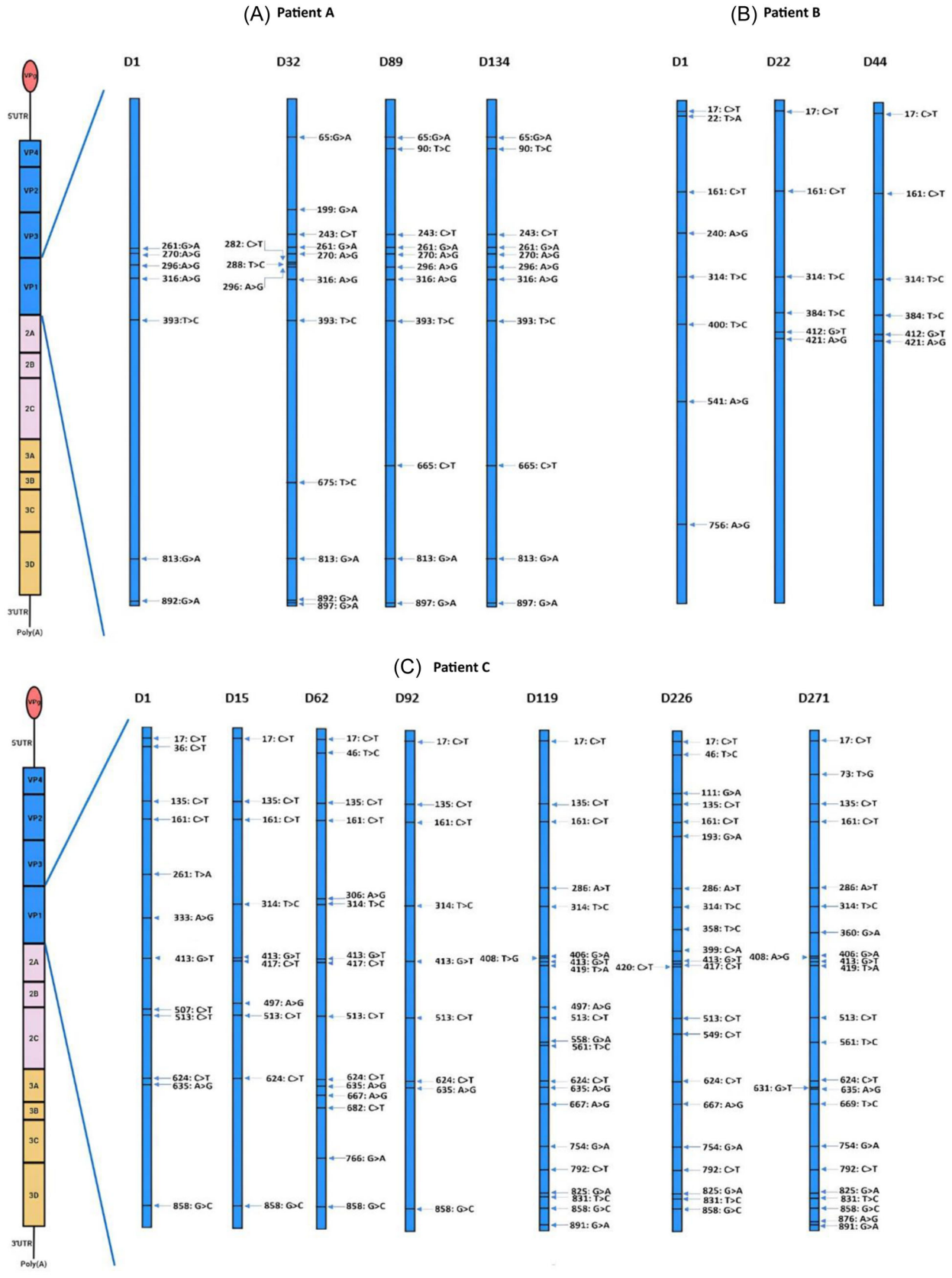

FIGURE 2 Nucleotide mutations of the VP1 region for patients (A–C). The colours blue, pink and yellow correspond to polypeptide1, polypeptide 2 and polypeptide 3 respectively D = Day.

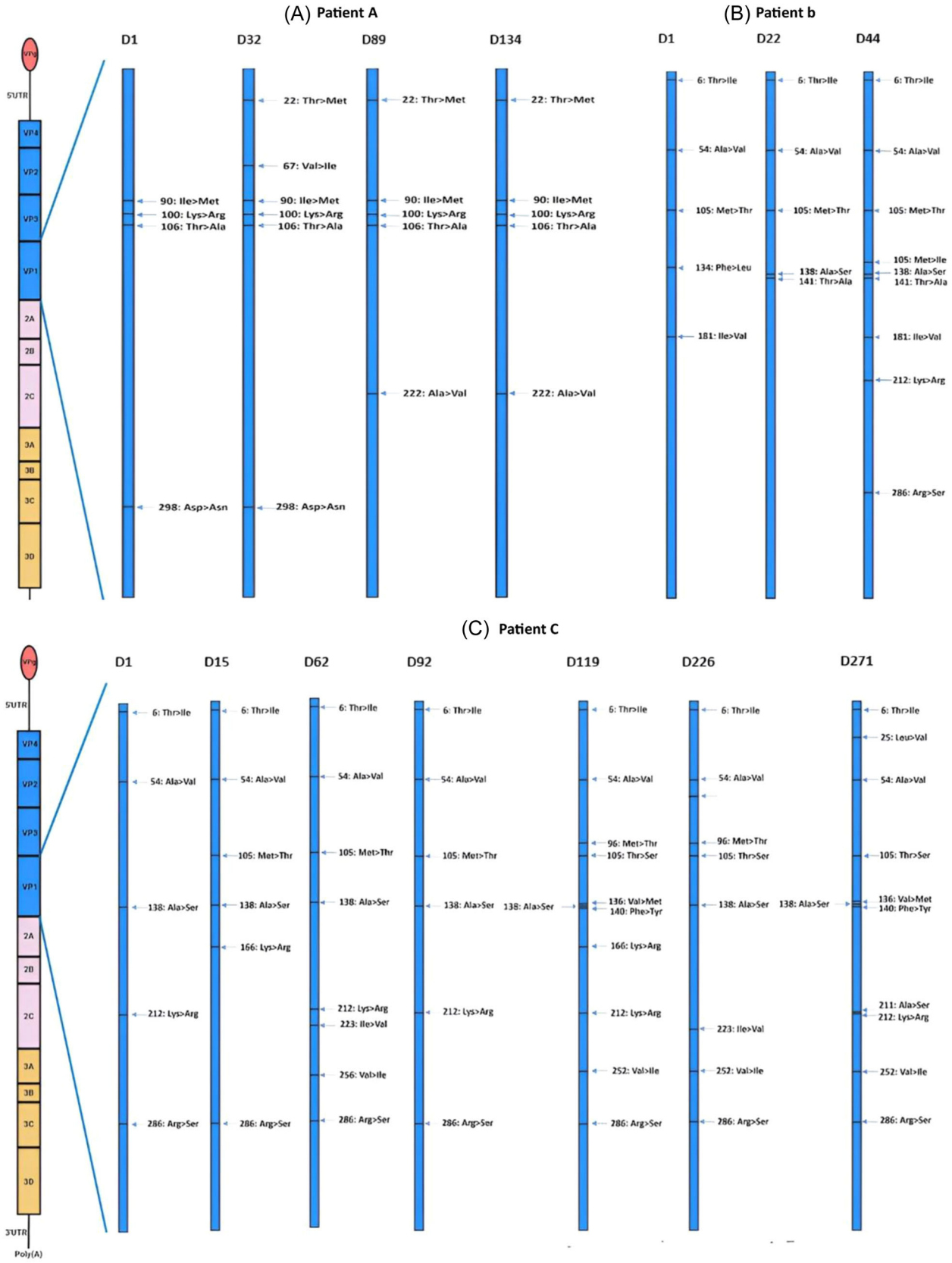

FIGURE 3 Nucleotide mutations of the VP1 region for patients (A–C). The colours blue, pink and yellow correspond to polypeptide1, polypeptide 2 and polypeptide 3 respectively D = Day.

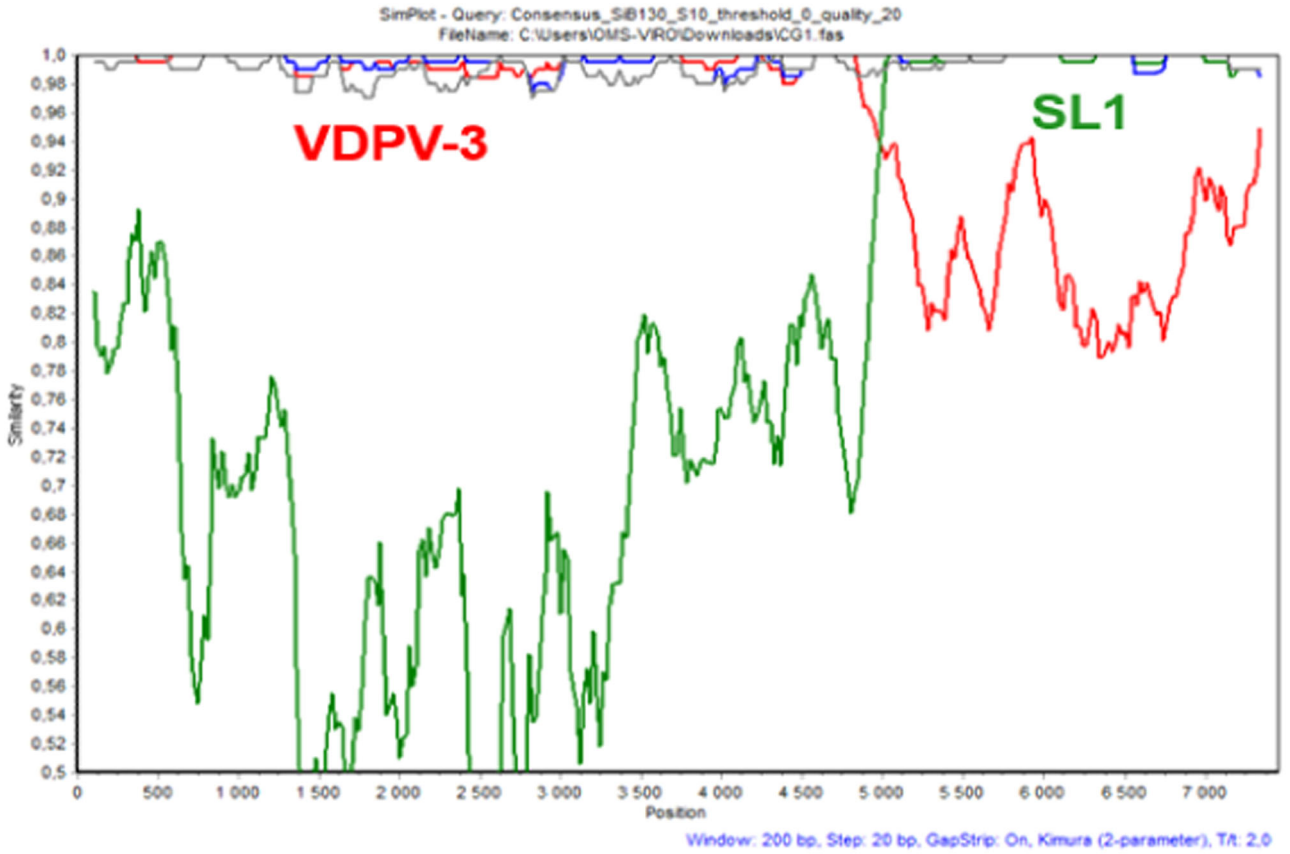

FIGURE 4 Recombination event between type 3 VDPV and SL1 sequences using SimPlot software Red = Sabin 3 sequence; Green = Sabin 1 sequence; VDPV = Vaccine-Derived Poliovirus; SL = Sabin-like. VDPV, Vaccine-Derived Polioviruses.

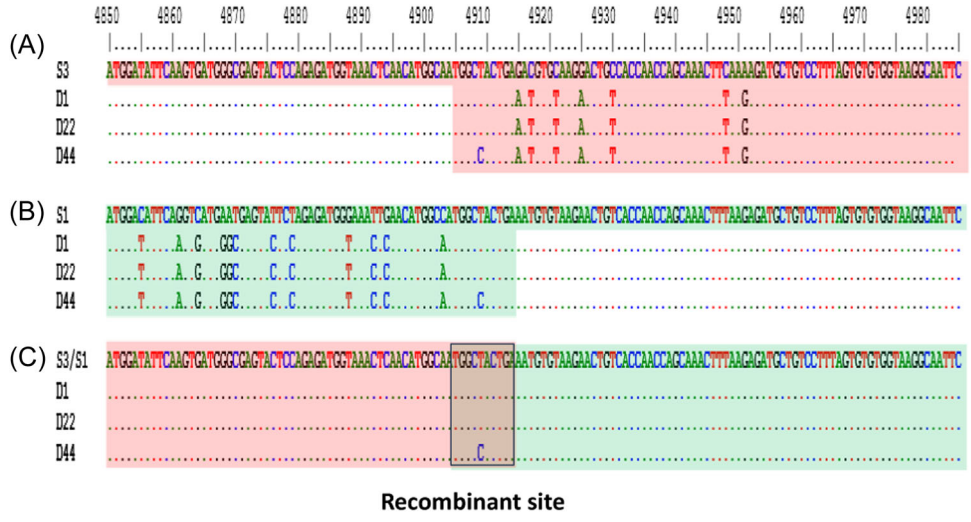

FIGURE 5 Alignment of patient 2 sequences: (A) with Sabin 3 reference sequence; (B) with Sabin 1 reference sequence; (C) with the recombinant Sabin3/Sabin1 S = Sabin; D = Day Red = Sabin 3 sequence; Green = Sabin 1 sequence.

and 106 within the 3 excreted strains (Figure 9A). Type 3 VDPV strains excreted by Patient B showed 2 amino-acid changes (VP1 protein): modification in position 105 within the 3 excreted strains, while the one at position 141 was detected in only 2 out of the 3 strains (Figure 9B). For type 3 VDPV strains excreted by Patient C, only one amino-acid change was detected at position 96 (VP1 protein), present in only 3 out of the 7 strains (Figure 9C) (Supporting Information S1: Table S2).

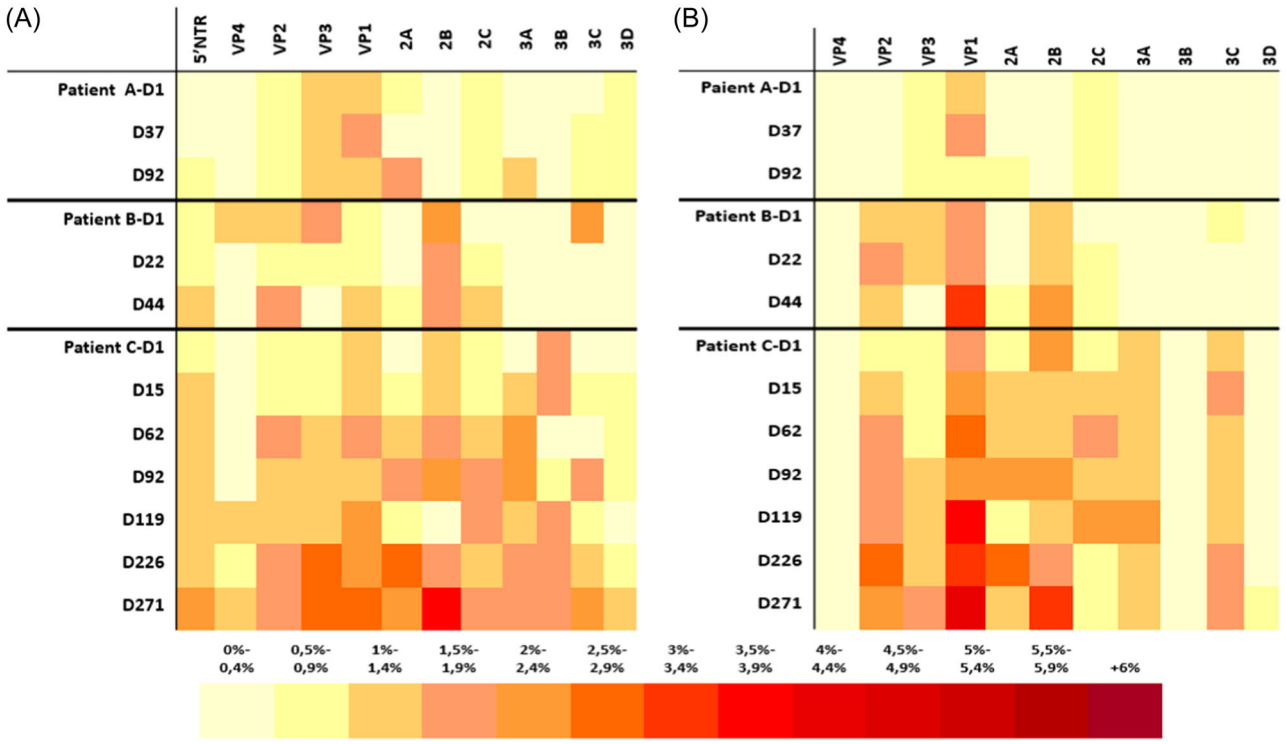

FIGURE 6 Heatmap of the evolution rate of nucleotide and amino-acid changes D: Day; 5'NTR: 5' Non-Translated Region; VP1, VP2, VP3, VP4: Structural proteins; 2 A, 2B, 2 C, 3 A, 3B, 3 C, 3D: Nonstructural proteins.

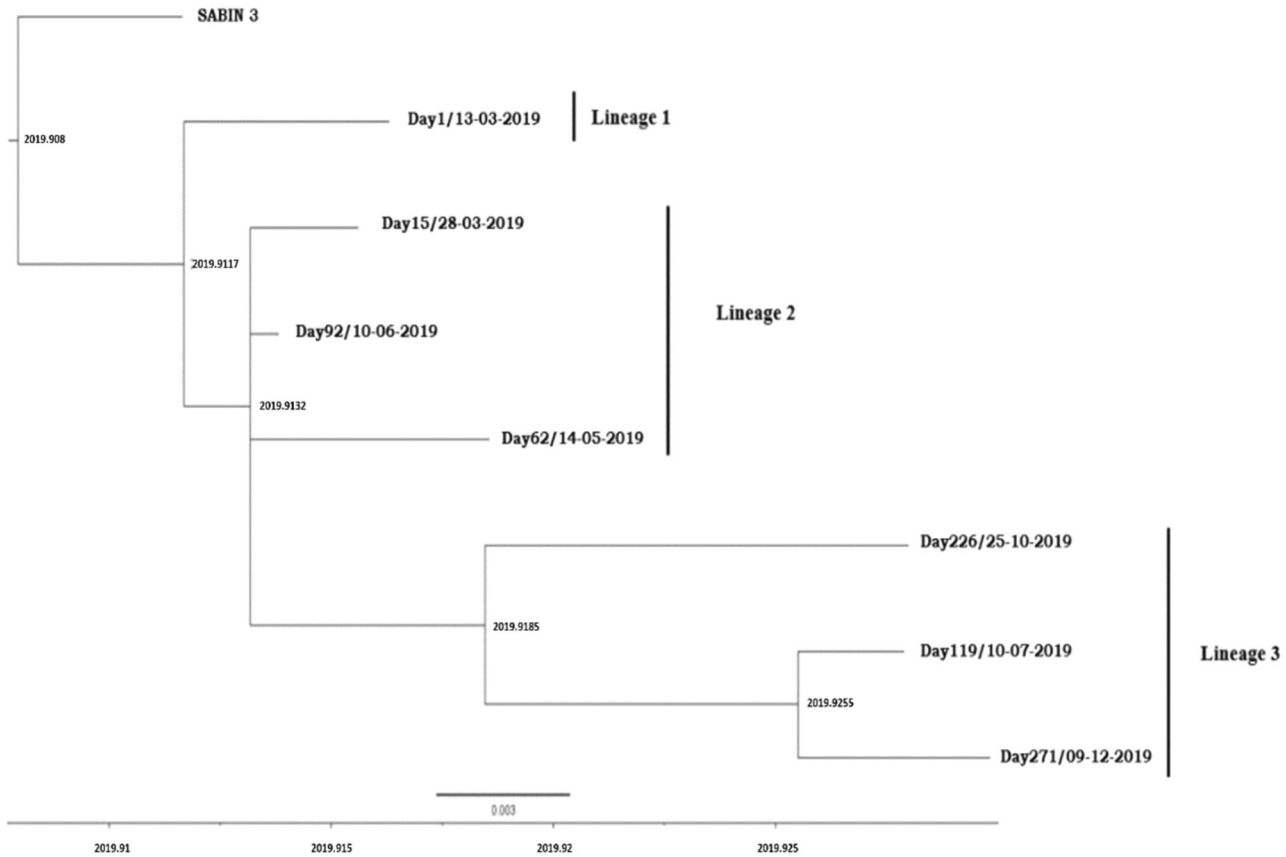

FIGURE 7 The phylodynamic tree of patient C strains.

FIGURE 8 Distribution of M-SNVs (blue) and m-SNVs (shades of green) for the three patients according to their frequency. (A) Distribution of M-SNVs and m-SNVs for Patient A; (B) Distribution of M-SNVs and m-SNVs for Patient B; (C) Distribution of M-SNVs and m-SNVs for Patient C. M-SNVs corresponds to the Major mutations presenting 90%–100% Alternative frequency (ALT-FREQ) and m-SNVs corresponds to minor mutations that presented less than 90% ALT-FREQ. m-SNVs with different ALT-FREQ intervals were indicated in different shade of green colors. D corresponds to Day.

TABLE 2 Neurovirulence reverse mutations.

Patient	Region	Serotype	Function	Position	Amino acid change and positions
Patient A	5'UTR	Type 1 VDPV	Affect the RNA secondary structure[35]	G480A*	No change
	VP1		Temperature resistance[36]	A2775C*	Lys99Thr*
				A2749G*	Ile90Met*
				A2795G*	Thr106Ala*
Patient B and Patient C	5′UTR	Type 3 VDPV	Affect the RNA secondary structure[31,34]	T472C*	No change
	VP3		*ts** phenotype[31,34]	T2034C*	Ser91Phe*

Note: (*) Thermosensitive phenotype.
Abbreviations: VDPV, Vaccine-Derived Polioviruses.

In the antigenic site 2, no amino-acid changes were detected for type 1 VDPV strains from Patient A (Figure 9A). Regarding type 3 VDPV strains from Patient B, they exhibited one amino-acid change at position 169 (VP2 protein) within 2 out of the 3 strains (Figure 9B). type 3 VDPV strains obtained from Patient C showed one amino-acid at position 223 (VP1 protein) in 2 out of the 7 strains and 2 amino-acid changes at positions 164 and 166 (VP2 protein) within 4 out of the 7 excreted strains (Figure 9C) (Supporting Information S1: Table S2).

In the antigenic site 3, type 1 VDPV strains from Patient A exhibited one amino-acid at position 60 (VP3 protein) within the three excreted strains (Figure 9A). For type 3 VDPV strains from Patient B, one amino-acid change was detected at position 286 (VP1 protein) within 1 out of the 3 excreted strains (Figure 9B). For type 3 VDPV strains from Patient C, two amino-acid changes were identified at position 286 (VP1 protein) within the 7 excreted strains and at position 59 (VP3 protein) within 5 out of the 7 strains (Figure 9C) (Supporting Information S1: Table S2).

4 | DISCUSSION

The prolonged excretion and evolution of PVs in patients with Primary Immunodeficiency (PID) pose major concerns for the Global Polio Eradication Initiative (GPEI).[37] In Tunisia, a country from the Eastern Mediterranean Region, previous studies[17,18] on 82 PID patients, between 2006 and 2010 identified six Sabin-like PV excretors, including one patient who excreted type 1 PV for up to 134 days, accumulating 14 mutations in the VP1 region. In continuity, the current study describes PV shedding in three PID patients with MHC class II deficiency. It provides an in-depth genetic characterization of complete genomes of the excreted PV strains, to highlight its possible effects on the duration of excretion and patient's outcomes. The accumulation of mutations and amino-acid changes, the occurrence of recombination events and the intra-host diversity were explored. The three investigated patients include Patient A detected in 2009 as prolonged excretor of type 1 PV for up to 134 days[17] and Patients B and C, detected later in 2016 and 2019, through the AFP surveillance program as AFP cases and then revealed PID after immunological investigation.

Limited knowledge exists about PV excretion and outcomes of such types of patients notably prevalent in the Eastern Mediterranean region.[38] A previous preliminary study, carried out in our laboratory, identified asymptomatic prolonged iVDPV excretion for at least 134 days in a patient with MHC class II deficiency.[17,18] In the present study, two of the three patients experienced severe outcomes. Another study conducted in Egypt reported the occurrence of paralysis and encephalopathy in three out of nine patients with MHC class II deficiency, who accidentally received OPV doses. Two of them experienced fatal outcomes.[39] Combined Immunodeficiencies such as severe Combined Immunodeficiency (SCID), pose a higher risk of eliciting severe outcomes and long-term iVDPV shedding.[37,40–42] Our findings give another testimony of the possible association of MHC class II immunodeficiency, as a combined immunodeficiency, to prolonged excretion and severe outcomes of PV excretors.

The screening for recombinant strains revealed a recombination event in the type 3 VDPV isolates from Patient B, involving recombination between a type 3 VDPV strain and SL1, at positions 4905 to 4915. These recombinants, often generated into the patient's gut after vaccination with OPV, may be associated with possible increase in the replicative potential of strains, particularly in the competitive context of the presence of 3 serotypes.[16] Virulent recombinant strains linked to epidemics were mainly poliovirus/non-polio enterovirus recombinant ones, specifically with type A Coxsackieviruses (CVA).[43] Nevertheless, recombination might indirectly increase virus virulence by enhancing its replicative potential, thereby increasing the intra-host diversity of the viral population.

The accumulation of mutations and intra-host diversity were also key focuses of our study. Indeed, mutations within RNA viruses play a crucial role in their evolution and virulence potential, particularly during prolonged replication in immunocompromised patients.[44]

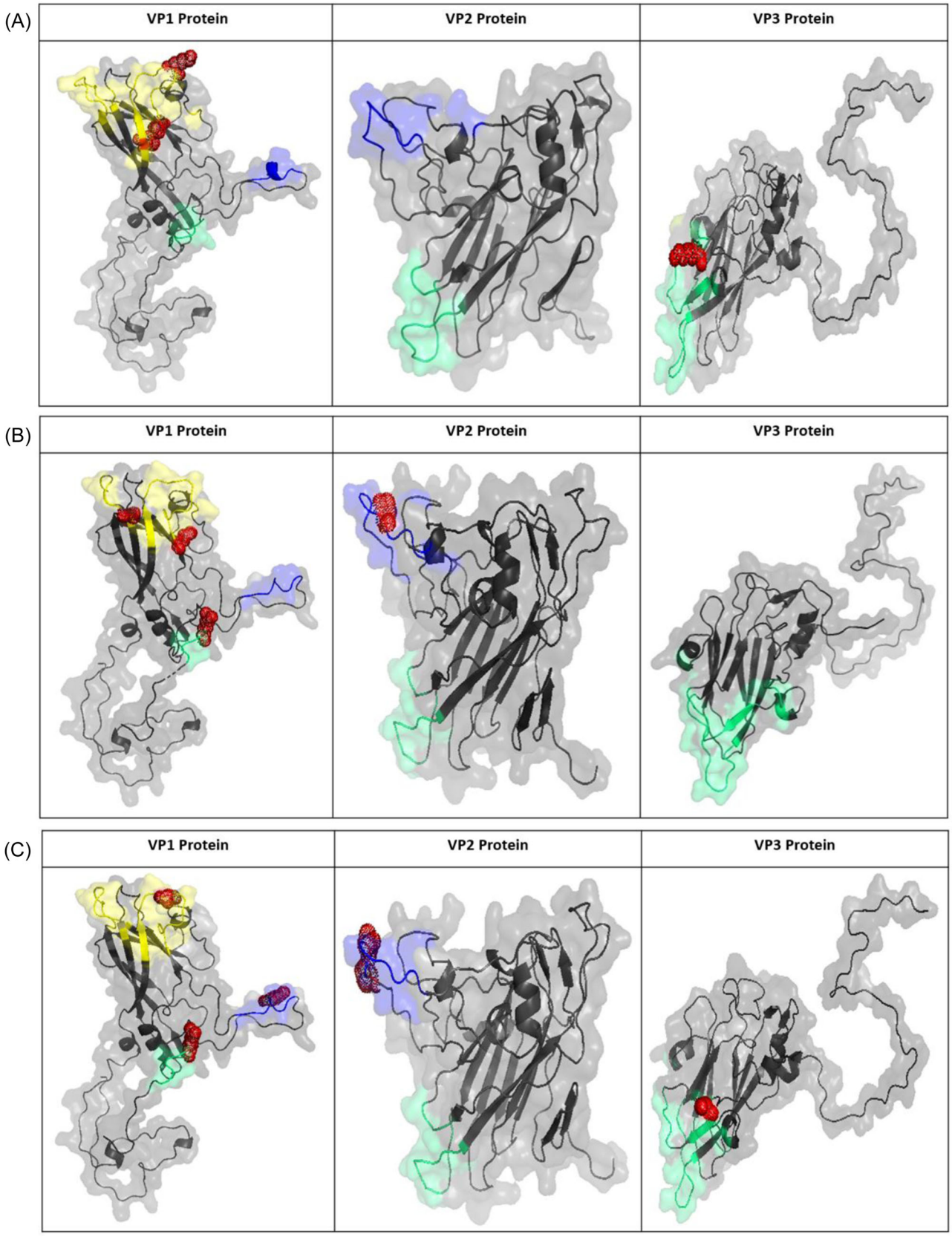

FIGURE 9 Three-dimensional representation of amino-acid changes in the antigenic sites of the structural proteins (VP1, VP2 and VP3) for the 3 patients: (A): Patient A, (B): Patient B, (C): Patient C Yellow: Antigenic Site1, Blue: Antigenic Site2, Green: Antigenic Site3, Red: Positions of amino-acid changes into antigenic sites.

Strains from Patients B and C, who experienced fatal outcomes, exhibited an increasing number of mutations in both the VP1 region and the entire genome compared to patient A, who presented favorable outcomes. Furthermore, amino-acid changes in the VP1 sequences were more important for patient B and C compared to patient A and presented different positions, considering the antigenic site in the VP1. Additionally, they exhibited an important intra-host diversity throughout the excretion period. In contrast, strains from Patient A, who cleared the virus, displayed fewer mutations and a rapid decrease in intra-host diversity before clearance. It was suggested that the occurrence of minor-SNV (m-SNV) could be the result of replication errors that are not strongly deleterious for the virus[45] indicating the existence of significant viral intra-host diversity.[46] Our phylodynamic tree of the type 3 iVDPV strains excreted by Patient C confirmed the coexistence of multiple lineages. While some lineages vanished quickly, new ones emerged, continued to multiply and were excreted. This funding aligns with studies by Martin et al.[47] an Yang et al. [44] on VDPV excretion within a chronic excretor, where a major genotypic lineage was identified together with minor branches that diverted from the main lineage suggesting the coexistence of multiple iVDPV strains in the gastrointestinal tract. The authors suggest the genesis of different lineages as a consequence of the virus replication within distinct locations into the digestive system.[44,47] These reported results indicate that as the shedding period prolongs, a greater diversity of lineages is observed.

Interestingly, the depletion of intra-host diversity in Patient A before virus clearance, compared to the important diversity observed in Patients B and C who experienced fatal outcomes, may suggest an impact on disease prognosis. These findings align with previous experiences in other RNA viruses, such as Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV2). Indeed, studies involving a substantial number of Coronavirus Disease 2019 (COVID-19) cases have reported notable intra-host diversity in samples from severe cases compared to mild cases.[48] Likewise, other studies have suggested that intra-host diversity contributes to the persistence of COVID-19 infections and the emergence of new lineages resistant to the host immune response, particularly in immunocompromised patients.[49,50] This emphasizes the importance of intra-host diversity in viral disease progression, indicating a possible impact of iVDPV intra-host diversity on the outcome of polio infection in PID patients. Therefore, continuous genetic surveillance of PVs excreted by PIDs should be prioritized for a better management of patients and limitation of virus spread.

In the present work, we also examined amino-acid changes in PV type 1 and type 3 strains, in relation to clinical presentation. Remarkebly, the two patients who presented paralysis and excreted type 3 iVDPV strains displayed two mutations associated with neurovirulence potential, along with four to six amino-acid changes located within the antigenic sites 1, 2 and 3. These neurovirulence mutations were found in the 5'UTR region at position 472 and in the VP3 protein at position 2034. Previous studies have described these mutations as acting in concert and being associated with a high degree of neurovirulence.[31] The fatal outcomes of Patients "B" and "C" are consistent with this finding. Moreover, amino-acid changes in the antigenic sites may facilitate the evasion from neutralizing antibodies, thereby increasing neurovirulence potential.[36] In contrast, Patient A who was asymptomatic and succeeded to clear the virus, neurovirulence reverse mutations were also detected in the 5'UTR at position 480 and in the VP1 region at positions 2775, 2749, 2795 but seems not affect deeply the attenuated phenotype of Sabin1 strains. The mutation at the 5'UTR was previously reported to be rapidly selected in the human gut after OPV administration[16] while mutations in VP1 have been associated with temperature resistance.[36]

In conclusion, our study underscores the possible role of genetic and intra-host diversity in the prolonged virus shedding as well as the genesis of virulent strains. Such findings highlight the critical importance of ongoing genetic surveillance of excreted samples, particularly from PID, to enhance patient management, mitigate virus spread, and prevent the emergence of epidemic strains, especially in communities with inadequate vaccination coverage.

AUTHOR CONTRIBUTIONS

Imene Ben Salem: Data curation; investigation; supervision; visualization and original draft preparation; writing; writing—review and editing. **Haifa Khemiri:** Data curation; investigation; visualization and original draft preparation. **Oliver Drechsel:** Data curation; investigation; funding acquisition; supervision; validation; visualization and original draft preparation. **Marwa Arbi:** Data curation; investigation; visualization and original draft preparation. **Sindy Böttcher:** Data curation; investigation. **Najla Mekki:** Data curation; investigation. **Ilhem Ben Fraj:** Resources. **Oussama Souiai:** Data curation; investigation; supervision; validation; visualization and original draft preparation. **Mahrez Yahyaoui:** Resources. **Essia Ben Farhat:** Resources. **Zina Meddeb:** Investigation. **Henda Touzi:** Investigation. **Imene Ben Mustapha:** Data curation. **Alia BenKahla:** Data curation; investigation; supervision; validation; visualization and original draft preparation. **Monia Ouederni:** Resources. **Mohamed-R. Barbouche:** Resources; validation. **Sabine Diedrich:** Conceptualization; funding acquisition; supervision; validation. **Henda Triki:** Conceptualization; resources; funding acquisition; writing—review and editing. **Sondes Haddad-Boubaker:** Conceptualization; data curation; investigation; resources; funding acquisition; supervision; validation; writing; writing—review and editing. All authors reviewed the manuscript and agreed to its submission to this journal.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

ETHICS STATEMENT

This study was approved by the Bio-Medical Ethics Committee of Pasteur Institute of Tunis, Tunisia (2019/20/I/LR161PT/V1). Written informed consent was provided by the parents or legal guardians of participants. The samples were gathered as part of PV surveillance activities and investigated after de-identification with regard to patient anonymity.

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