CHAPTER 6

PREVALENCE OF VACCINE-DERIVED POLIOVIRUSES IN STOOLS OF IMMUNODEFICIENT CHILDREN IN SOUTH AFRICA

The editorial style of the Journal of Clinical Virology was followed in this chapter

6.1 Abstract

The discovery of prolonged excretion of polioviruses (PVs) by immunodeficient individuals has raised concern. When exposed to oral poliovirus vaccine (OPV), these patients may become chronically infected, spreading potentially neurovirulent vaccine-derived polioviruses (VDPVs) for many months or years to close contacts and children who are no longer being vaccinated. Cases of VDPVs from immunodeficient people with long-term excretion of PV have been recorded and classified as immunodeficient VDPVs (iVDPVs). Two cases of vaccine-associated paralytic poliomyelitis (VAPP) have been reported in children infected with the human immunodeficiency virus (HIV). However, at present there is no evidence for prolonged excretion of PV by patients with the acquired immunodeficiency syndrome (AIDS). The current situation in South Africa offers opportunities well suited for research along these lines, since vaccination with OPV is compulsory and the incidence of immunodeficient individuals is high. The aim of the study was to determine the prevalence of VDPVs in stool specimens of immunodeficient patients such as HIV-positive children (including those with an AIDS indicator condition, according to the Centers for Disease Control and Prevention classification) by applying various molecular techniques. A total of 164 stool samples from HIV-positive children and 23 stool samples from healthy immunocompetent children (the control group) were analysed during 2003 and 2004. By applying a RT-PCR in combination with a nested PCR, a total of 54 enteroviruses were detected in the stool specimens of the immunodeficient children. The use of restriction enzymes and a Sabin specific RT-triplex PCR confirmed the presence of 13 PVs, such as 7 Sabin PV type 1 (53.8%), 4 Sabin PV type 3 (30.8%) and 2 Sabin PV type 2 (15.4%) isolates. The 5'untranslated region and the VP1 capsid-encoding protein of the 13 PVs and the 3 PVs from the stools of the immunocompetent children were partially sequenced and their genetic

relatedness was deduced from the constructed phylogenetic trees. The majority of the PVs isolated from the stools of the immunodeficient children (10 out of 13 isolates) were classified as "OPV-like viruses", since these isolates had close sequence relationships (>99% in VP1 nucleotide sequences) to the original Sabin PV vaccine strains. Three PVs showed ≤99% VP1 sequence identity to the Sabin PV vaccine strains and were classified as "suspected" iVDPVs. All of the OPV-like isolates and the "suspected" iVDPVs carried mutations at specific positions in their partially sequenced regions, which have been associated with reversion of the attenuated Sabin PV vaccine strains to increased neurovirulence. Thus, this study confirmed the notion that immunodeficient patients may indeed excrete OPV strains with potential neurovirulent phenotypes for a prolonged period of time. These OPV strains can circulate in the environment posing a potential health risk after termination of vaccination with OPV in the near future. Additional studies on prolonged PV excretion from HIV-infected patients are, therefore, required.

Keywords: Immunocompetent, Immunodeficient children, Oral poliovirus vaccine, Sequencing, 5'untranslated region, Vaccine-derived polioviruses, VP1 region

6.2 Introduction

The oral poliovirus vaccine (OPV) of Albert Sabin is nearly ideal for the global polio eradication (Nathanson and Fine, 2002; Dowdle *et al.,* 2003; Sutter *et al.,* 2003; Kew *et al.,* 2004). The vaccine is easily administered by mouth and provides long-term protection against polio through durable humoral immunity (Kew *et al.,* 2004). The poliovirus (PV) vaccine strains can spread to and immunise unvaccinated contacts of vaccine recipients, thereby, increasing the impact of OPV beyond those immunised (Kew *et al.,* 2004). However, despite its many advantages, the genetic stability of OPV (including attenuation of neurovirulence and immunogenicity in humans) was a prime concern during its development (Sabin, 1957; Fine and Carneiro, 1999; Dowdle *et al.,* 2003; Kew *et al.,* 2004).

The first evidence of the clinical consequences of the genetic lability of OPV was the appearance of cases of vaccine-associated paralytic poliomyelitis (VAPP), which have become an increasingly significant proportion of the global polio burden (Sutter *et al.,* 2003; Kew *et al.,* 2004). Vaccine-associated paralytic poliomyelitis is a rare adverse event

following vaccination with OPV, in which a mutation or reversion of the vaccine PV leads to a more neurotropic form of the vaccine strain (in some instances as neurovirulent as wild-type PV) (Minor, 1992; Wood and Thorley, 2003; Hovi *et al.*, 2004). The overall risk of VAPP from OPV in a highly immune community is approximately 1 in 2.5 million doses administered (Guillot *et al.*, 2000; Martin *et al.*, 2000a; Wood and Thorley, 2003). Compared with immunocompetent children, the risk of VAPP is approximately 7 000 times higher for persons with certain types of immunodeficiencies, such as B lymphocyte disorders (agammaglobulinemia and hypogammaglobulinemia) (Sutter and Prevots, 1994; Triki *et al.*, 2003; Haisey *et al.*, 2004).

A variety of OPV-derived viruses can be isolated from OPV recipients and their contacts (World Health Organization [WHO], 2004). All clinical and environmental PV isolates that are related to OPV strains are vaccine-derived polioviruses (VDPVs) (WHO, 2004). The extent of sequence divergence of the VP1 capsid gene from Sabin PV strains can be used as a "molecular clock" to estimate the duration of PV replication (WHO, 2004). A constant rate of accumulation of synonymous nucleotide substitutions is assumed to exist and for the PV genome rates of approximately 1-2% change.year^{-1} have been proposed (Kew *et al.*, 1998; Kew *et al.*, 2002). Based on this "molecular clock" derivatives of Sabin OPV strains have been classified into two broad categories for programmatic reasons: "OPV-like viruses" and "Vaccine-derived polioviruses" (Yang *et al.*, 2003; WHO, 2004). Oral poliovirus vaccine-like viruses represent the vast majority of vaccine related isolates and have close sequence relationships (>99% VP1 sequence identity) to the original OPV strains (Wood and Thorley, 2003; Yang *et al.*, 2003; WHO, 2004). Vaccine-derived polioviruses are those strains showing ≤99% VP1 sequence identity (from 1-15% genetic sequence difference) to the parental Sabin strains and those with greater than 15% genetic sequence difference are classified as wild-type PVs (Wood and Thorley, 2003; Yang *et al.*, 2003; WHO, 2004).

Up to date, two categories of VDPV isolates have been identified: immunodeficient VDPVs (iVDPVs) and circulating VDPVs (cVDPVs) (WHO, 2004). Some iVDPVs isolated from patients with defects in antibody production (generally common variable immunodeficiency or X-linked agammaglobulinemia) are highly divergent (~90% VP1 sequence identity to the parental OPV strain), suggesting that the chronic PV infections had persisted for 10 years or more (Kew *et al.*, 1998; Bellmunt *et al.*, 1999; Wood *et al.*, 2000; Yang *et al.*, 2003).

Nineteen chronic iVDPV excretors were detected world-wide in the past 40 years since the beginning of polio vaccination, although this number may be an underestimate in the absence of systematic screening of immunodeficient patients (Haisey *et al.*, 2004; WHO, 2004). Currently, there is no clear evidence of spread of iVDPV from immunodeficient patients to the wider community (Wood *et al.*, 2000; Yang *et al.*, 2003; WHO, 2004).

A VDPV may cause an outbreak of poliomyelitis and if there is evidence of person-to-person transmission, based on epidemiological and phylogenetic studies, it is defined as a circulating VDPV (cVDPV) (Wood and Thorley, 2003; Yang *et al.*, 2003; Kew *et al.*, 2004). The immediate public health importance of cVDPVs was underscored by the occurrence of several outbreaks of poliomyelitis due to cVDPV (Yang *et al.*, 2003; Kew *et al.*, 2004). An outbreak of PV type 3 poliomyelitis in Poland in 1968 was associated with PV strains derived from the USOL-D-bac vaccine (Martin *et al.*, 2000b; Kew *et al.*, 2004). In Egypt between 1983 and 1993, 32 cases of paralytic disease from a cVDPV type 2 were reported, including many retrospective cases (Centers for Disease Control and Prevention [CDC], 2001; Kew *et al.*, 2004). Polio cases attributed to cVDPV type 1 (which had undergone recombination with non-polio enteroviruses) have been found in Haiti, Philippines and the Dominican Republic during 2000 and 2001 (Kew *et al.*, 2002; Wood and Thorley, 2003; Kew *et al.*, 2004). In Madagascar, five cases of acute flaccid paralysis (AFP) associated with cVDPV type 2 were reported and partial genomic sequencing indicated that two of the PV strains had been circulating for approximately 1 and 2.5 years, respectively (Rousset *et al.*, 2003; Kew *et al.*, 2004). A common factor to all these cVDPV outbreaks has been the low population immunity, consistent with low OPV coverage and the previous eradication of the corresponding serotype of indigenous wild-type PV (Kew *et al.*, 2004; WHO, 2004).

Immunodeficient people exposed to OPV may become chronically infected and excrete VDPVs for many months or years (Kew *et al.*, 1998; Bellmunt *et al.*, 1999; Martin *et al.*, 2000a; Kew *et al.*, 2004). As a consequence, in the last phase of polio eradication this group of people could serve as potential reservoirs for VDPVs, spreading these potentially neurovirulent viruses to close contacts and children who are no longer being vaccinated (Martin *et al.*, 2002; Buttinelli *et al.*, 2003).

In the current study, various molecular techniques were applied to determine the prevalence of VDPV strains in stool specimens from immunodeficient patients such as HIV-positive children (including those with an AIDS indicator conditon, according to the CDC classification) from a selected area in South Africa. The applied molecular techniques included partial genomic sequencing of the 5'untranslated region (5'UTR) and VP1 capsid-encoding protein of PVs excreted by the immunodeficient children studied.

6.3 Materials and methods

6.3.1 Poliovirus stock

Poliovirus controls included: PV type 1, 2 and 3 vaccine strains, which were clinical isolates obtained from the National Institute of Virology (NIV), Johannesburg, South Africa. These PVs were recovered in HEp-2 cells (human epidermoid carcinoma) (Code ATCC CCL-23, passage 350 - 365). The cell cultures were frozen and thawed three times, after demonstrating a cytopathogenic effect (CPE). The debris was removed by centrifugation at 600 x *g* (Eppendorf Centrifuge 5402D, Hamburg, Germany) for 10 min at room temperature (±25°C). The PV suspensions were stored at -70°C for further analysis.

6.3.2 Patient specimens

In order to estimate the prevalence of VDPV in the stool specimens of immunodeficient children, a maximum sample size associated with an expected prevalence of 50% was analysed. Statistically, a sample size of 162 stool specimens of immunodeficient children was studied to achieve a confidence level of 80% and the prevalence was estimated to an accuracy of 5% (Biostatistics Unit, Medical Research Council, Pretoria, South Africa).

During a period of one year (2003-2004), one stool specimen was collected from each of the 164 immunodeficient patients such as HIV-positive children (including those with an AIDS indicator condition according to the CDC classification) from the Department of Paediatrics, Kalafong Hospital/University of Pretoria, South Africa. In addition, 23 stool samples from 3 healthy immunocompetent babies were collected regularly during their OPV immunisation schedule: one stool specimen 48 h after each vaccination (at birth, 6 weeks, 10 weeks, 14

weeks and 18 months) and one stool sample on a weekly basis until no PV was detected in the stools. These children served as a control group to demonstrate the type of PVs being excreted and possibly the duration of excretion of PVs by immunocompetent children.

6.3.3 Ribonucleic acid extraction and isolation of polioviruses

In the homogenisation and clarification of the stool specimens, 300 µl of 10-15% faecal suspension was mixed with an equal volume of freon (Sigma Chemical Co., Louis, United States of America [USA]) and centrifuged at 12 000 x *g* (Eppendorf Centrifuge 5402D, Hamburg, Germany) for 5 min at room temperature (±25°C). The ribonucleic acid (RNA) was extracted from the stool specimens by means of a TRIzol reagent (Invitrogen Life Techno, Paisley, Scotland) as per manufacturer's instructions.

A reverse transcription polymerase chain reaction (RT-PCR) was performed using a Promega Access RT-PCR system (Promega Corp., Madison, USA) as described by Gow *et al.* (1991). Optimised final concentrations in a total volume of 50 µl included: AMV/*Tfl* reaction buffer (1x), 1.5 mM $MgSO_4$, dNTP Mix (final concentration of 0.2 mM), 50 pmol each of primers EP1 and EP4 (Sigma-Genosys Ltd., Pampisford, Cambridgeshire, United Kingdom) (Table 6.1), 5 U each of AMV Reverse Transcriptase and *Tfl* DNA Polymerase. The PCR conditions included: reverse transcription for 45 min at 48°C, {DNA denaturation for 1 min at 94°C, primer annealing for 1 min at 56°C and primer extension for 1 min at 72°C} 30 cycles and final extension for 10 min at 72°C (Hybaid OmniGene Thermocycler, United Kingdom). A second PCR (nested PCR) run was undertaken as described by Kuan (1997), in which 1 µl of the amplified RT-PCR product was added to 49 µl of previously prepared PCR mixture. The PCR mixture contained the following: 1x PCR buffer (10 mM Tris-HCl, pH 9; 50 mM KCl; 0.1% Triton X-100), $MgCl_2$ (final concentration of 1.5 mM), dNTP mix (final concentration of 0.2 mM), 50 pmol each of primers E1 and E2 (Sigma-Genosys) (Table 6.1), and 1.5 U of *Taq* DNA polymerase. The PCR conditions included: DNA denaturation for 3 min at 94°C, {DNA denaturation for 1 min at 94°C, primer annealing for 1 min at 45°C and primer extension for 1 min at 72°C} 30 cycles and final extension for 10 min at 72°C (Hybaid OmniGene Thermocycler). After 30 cycles, 20 µl of each PCR product were subjected to 2% agarose (Seakem LE agarose, Bioproducts, USA) gel electrophoresis (Midicell Primo Gel Apparatus, Holbrook, New York, USA).

6.3.4 Typing of the poliovirus isolates

The RNAs of PVs were partially typed with restriction enzymes (REs) such as *Sty* I, *Bgl* I and *Xmn* I (Promega Corp.) (Table 6.2) (Kämmerer *et al.*, 1994; Kuan, 1997). Aliquots of 10 µl of the nested PCR products were incubated with 10 U of the REs in a 30 µl reaction volume with the buffer recommended by the manufacturer. Samples were incubated at 37°C for 3 h and were analysed using 7% polyacrylamide (BioRad, Hercules, California, USA) gel electrophoresis (Hoefer, San Francisco, USA). The RE patterns of the PVs were evaluated based on previously published RE patterns (Kämmerer *et al.*, 1994; Kuan, 1997) (Table 6.3).

Three sets of primers specific for Sabin PV types 1 to 3 were combined in a Sabin specific RT-triplex PCR to confirm the isolated PVs as OPV strains based on the production of amplicons of specific sizes (Table 6.4) (Chezzi, 1996; Yang *et al.*, 1991; Yang *et al.*, 1992). A 50 µl reaction volume was prepared using: AMV/*Tfl* Reaction Buffer (1x), dNTP Mix (final concentration of 0.2 mM), 25 pmol each of primers S1-1, S1-2, S2-1, S2-2, S3-1a, S3-2 (Sigma-Genosys), 1.5 mM of $MgSO_4$, and 5 U of AMV Reverse Transcriptase as well as *Tfl* DNA Polymerase (Promega Corp.). The PCR conditions included: reverse transcription for 45 min at 42°C, {DNA denaturation for 30 s at 95°C, primer annealing for 45 s at 56°C and primer extension for 1 min at 72°C} 30 cycles and final extension for 10 min at 72°C (Hybaid OmniGene Thermocycler). The amplified products (20 µl) were separated using 7% polyacrylamide (BioRad) gel electrophoresis using a Hoefer electrophoresis unit at 120 Volts (Hoefer).

6.3.5 Partial genomic sequencing of the 5'untranslated region of polioviruses

The 5'UTR of the PV genome was subjected to RT-PCR amplification as described by Divizia *et al.* (1999) and Guillot *et al.* (2000) with a few modifications. The primers used for the 5'UTR were as follows: UG52 (nt 160 to 180) and UC53 (nt 599 to 580) (Sigma-Genosys) (Table 6.5). Optimised final concentrations in a total volume of 50 µl included: AMV/*Tfl* Reaction Buffer (1x), 2.0 mM $MgSO_4$, dNTP Mix (final concentration of 0.2 mM), 10 pmol each of primers UG52 and UC53, 5 U each of AMV Reverse Transcriptase and *Tfl* DNA Polymerase (Promega Corp.). The PCR conditions included: reverse transcription for 45 min at 42°C, {DNA denaturation for 30 s at 95°C, primer annealing for 45 s at 45°C and primer

extension for 1 min at 72°C} 30 cycles and final extension for 10 min at 72°C (Hybaid OmniGene Thermocycler). After 30 cycles, 20 µl of each PCR product were subjected to agarose (2%) (Seakem) gel electrophoresis (Midicell).

6.3.6 Partial genomic sequencing of the VP1 capsid protein of polioviruses

The VP1 region of the PV genome was subjected to RT-PCR amplification as described by Divizia *et al.* (1999) and Guillot *et al.* (2000) with a few modifications. The primers used for the VP1 region were as follows: UG1 (nt 2402 to 2422) and UC1 (nt 2881 to 2861) (Sigma-Genosys) (Table 6.5). Optimised final concentrations in a total volume of 50 µl were: AMV/*Tfl* Reaction Buffer (1x), 2.0 mM $MgSO_4$, dNTP Mix (final concentration of 0.2 mM), 10 pmol each of primers UG1 and UC1, 5 U each of AMV Reverse Transcriptase and *Tfl* DNA Polymerase (Promega Corp.). The PCR conditions were as follows: reverse transcription for 45 min at 42°C, {DNA denaturation for 30 s at 95°C, primer annealing for 45 s at 50°C and primer extension for 1 min at 72°C} 30 cycles and final extension for 10 min at 72°C (Hybaid OmniGene Thermocycler). A nested PCR was performed as described by Divizia *et al.* (1999) immediately after completion of the RT-PCR step. The primers used for the nested PCR were as follows: N2426 (nt 2426 to 2446) and N2812 (nt 2812 to 2792) (Sigma-Genosys) (Table 6.5). The nested PCR mixture contained the following: 1x PCR buffer (10 mM Tris-HCl, pH9; 50 mM KCl; 0.1% Triton X-100), $MgCl_2$ (final concentration of 1.5 mM), dNTP mix (final concentration of 0.2 mM), primers N2426 and N2812 (10 pmol each) and 1.5 U of *Taq* DNA Polymerase (Promega Corp.). The nested PCR conditions were as follows: DNA denaturation for 2 min at 94°C, {DNA denaturation for 1 min at 94°C, primer annealing for 1 min at 45°C and primer extension for 1 min at 72°C} 30 cycles and final extension for 10 min at 72°C (Hybaid OmniGene Thermocycler). After 30 cycles, 20 µl of PCR products were subjected to agarose (2%) (Seakem) gel electrophoresis (Midicell).

6.3.7 Nucleotide sequencing and phylogenetic analysis

Several of the PCR products (those with faint bands) were cloned prior to sequencing so that high numbers of that sequence were produced. The deoxyribonucleic acids (DNA) were cloned into a pGEM®-T Easy Vector (Promega Corp.) and transformed to JM109 high efficiency competent cells (Promega Corp.). Plasmids from positive recombinants (white

colonies) were purified by the QIAprep spin miniprep kit protocol (Qiagen, Hilden, Germany). Before initiating sequencing, the exconuclease I/Shrimp alkaline phosphatase (Fermentas, Vilnius, Lithuania) was used to clean the PCR samples from primers and nucleotides (Inqaba Biotechnical Industries Pty (Ltd), Pretoria, South Africa). The ABI BigDye Terminator cycle sequencing kit version 3.1 was used to sequence the PCR products (Inqaba Biotechnical Industries). In the latter procedure, approximately 500 ng of PCR product and 0.8 pmol of each of the same primers used in the amplification reaction were employed in each sequencing reaction (Inqaba Biotechnical Industries). Both strands of the amplified fragments were sequenced to confirm the nature of the product obtained (Inqaba Biotechnical Industries). The data was analysed on a Spectrumedix SCE2410 genetic analysis system (Inqaba Biotechnical Industries).

The sequences of the PV genomes described in this study were compared with all sequences in the GenBank database by using the PubMed National Centre for Biotechnology Information BLAST program as well as the European Bioinformatics Institute (EMBL) databases and submitted via the Internet to these databases. Multiple sequence alignments were performed by the ClustalX program (Higgins and Sharp, 1988). Phylogenetic trees were constructed using the maximum-likelihood analysis programs PHYLIP (version 3.57c) and PUZZLE (version 4.0), and were visualised using NJPLOT or TREEVIEW (version 1.5.3) (Felsenstein, 1981). Bootstrap analysis of the 5'UTR and VP1 nucleotide sequences were performed with the SEQBOOT program of the PHYLIP package with 1 000 replicates. Comparisons were made with previously published sequences (GenBank accession numbers: AY017238; AY177685; AY184219; AY184220; AY184221; PI3L37; POL430385; V01149; X04468).

6.3.8 Nucleotide sequence accession numbers

The sequences of the 5'UTR and the VP1 of PVs isolated from stool specimens of the immunocompetent (serving as the control group) and the immunodeficient children in South Africa described in this study have been deposited in the GenBank data library. Accession numbers have been assigned to all of the PV isolates (Table 6.6).

6.4 Results and discussion

Using a RT-PCR in combination with a nested PCR, 54 enteroviruses (EVs) were isolated from the stool specimens of the 164 immunodeficient children studied. These immunodeficient children were between the ages of 4 months to 8 years and were hospitalised for various diseases such as bronchopneumonia, cardiomyopathy, encephalopathy, gastroenteritis, herpes stomatitis, lymphocytic intestinal pneumonia, meningitis, miliary tuberculosis, pneumocystis carinii pneumonia (*Pneumocystis jereveci*), pulmonary tuberculosis, pneumonia, septicemia and upper respiratory tract infections. Some of the children were hospitalised for anaemia, chronic diarrhoea, dehydration and malnutrition. In total, 17 of these immunodeficient patients died during the course of the study, therefore, the excretion of EVs by these patients could not be followed.

Based on the RE analysis, 13 PVs from the immunodeficient children were successfully distinguished from 41 non-polio enteroviruses (NPEVs). These 13 PVs were identified as 7 PV type 1 (53.8%), 4 PV type 3 (30.8%) and 2 PV type 2 (15.4%) isolates (Table 6.7). Using the Sabin specific RT-triplex PCR, all of these PV isolates were typed as Sabin PV vaccine strains. No wild-type PVs were detected in the stool samples, which was in agreement with epidemiological data indicating that the last case of polio associated with wild-type PV in South Africa was in 1989 (CDC, 2003).

In total, 7 of the 23 stool samples taken from healthy immunocompetent children (the control group) tested positive for EVs after receiving their polio immunisation. All these EVs were typed as PVs using the RE analysis and the Sabin specific RT-triplex PCR identified them as Sabin PV vaccine strains (six PV type 1 and one PV type 2 isolates).

A total of 13 PVs isolated from the stool specimens of the immunodeficient patients and 3 PVs from the stools of the immunocompetent children (the control group) were analysed by sequencing the 5'UTR and VP1 regions of their genomes. Two of the PV type 1 isolates (P020, Accession No: AJ783777 and P069, Accession No: AJ783779) could not be incorporated into the phylogenetic tree of the 5'UTR. The nucleotide sequences in the 5'UTR for these isolates were too short to be compared by aligning them against the other PV type 1 isolates as well as the reference strains.

Phylogenetic analysis of the 5'UTR of the poliovirus genome

Three main groups were evident from the unrooted phylogenetic tree constructed from nucleotide sequences of the 5'UTR of PVs isolated in this study (Figure 6.1). Group 1 consisted of PV type 1 isolates and three reference strains: the attenuated Sabin PV type 1 (AY184219), the PV type 1 Mahoney (V01149) and wild-vaccine recombinant PV type 1 (AY017238), isolated in China by Liu and colleagues (2003) (Figure 6.1). Isolates (AJ634678, AJ783781) showed 100% nucleotide sequence similarity to the attenuated AY184219 reference strain (Table 6.6). Isolate AJ783778 showed 0.9% divergence in 5'UTR nucleotides from the attenuated AY184219 reference strain and lesser divergence (0.4%) from the virulent Mahoney V01149 strain. Isolates AJ783775 and AJ699401 had 100% nucleotide sequence similarity between each other and as a subgroup differed from the attenuated AY184219 strain at 7% in 5'UTR nucleotides. The most divergent isolate in this group from the attenuated reference strain AY184219 was isolate AJ783776 with 8.5%. Isolates (AJ699401, AJ783775, AJ783776, AJ783777, AJ783778, AJ783779, AJ783780) had a mutation at position 480 in the 5'UTR, which involved a direct reversion of a G base (found in the vaccine strain) to an A base (found in the virulent strain). This mutation is the key mutation responsible for reversion of the attenuated Sabin PV type 1 to increased neurovirulence (Li *et al.*, 1996; Georgescu *et al.*, 1997; Martin and Minor, 2002).

Group 2 consisted of PV type 2 isolates and two reference strains: the attenuated Sabin PV type 2 (AY184220) and a Sabin-like type 2 PV (AY177685), which was isolated by Buttinelli and colleagues (2003) from an immunodeficient patient soon after the onset of paralysis. Comparing the genome of AY177685 with its parental Sabin vaccine strain, it was determined that two mutations were present in positions 481 of the 5'UTR and 2 908 of VP1 (amino acid 143), known to be correlated with the neurovirulent phenotype (Minor and Dunn, 1988; Equestre *et al.*, 1991; Minor, 1999; Martin and Minor, 2002; Buttinelli *et al.*, 2003). Since, the PV type 2 isolates in this study were to a certain extent genetically related to this PV, therefore, AY177685 was used as a reference PV type 2 strain. Two of the PV type 2 isolates (AJ783783, AJ783784) showed 100% nucleotide sequence identity to the AY177685 reference strain and differed at 0.4% in 5'UTR nucleotides from the attenuated AY184220 reference strain (Table 6.6). The PV type 2 isolate (AJ783782) differed at 0.9% from the AY184220 reference strain. All PV type 2 isolates in this study had the key mutation at position 481 in the 5'UTR (conversion of A base to a G base).

Group 3 was constituted by PV type 3 isolates and two reference strains: the attenuated reference strain Sabin PV type 3 (AY184221) and a PV type 3 strain 23127 (X04468), isolated during an outbreak of poliomyelitis in Finland by Hughes and colleagues (1986) (Figure 6.1). Isolates (AJ635238, AJ783786 and AJ783787) differed at 0.4% of nucleotides in the 5'UTR from the attenuated AY184221 reference strain (Table 6.6). The sequence divergence in the 5'UTR of isolate AJ783785 from the attenuated AY184221 reference strain was higher at 9.2%. All PV type 3 isolates in this study had a mutation at position 472 in the 5'UTR, which involved the reversion of an U base (found in the vaccine strain) to a C base (found in the virulent strain). This mutation at position 472 in the 5'UTR is reported responsible for the reversion to neurovirulence in Sabin PV type 3 vaccine strains (Minor, 1992; Minor, 1999; Martin and Minor, 2002).

Phylogenetic analysis of the VP1 region of the poliovirus genome

The unrooted phylogenetic tree, of the VP1 region of the PV genomes isolated in this study, consisted of three main groups (Figure 6.2). Group 1 consisted of PV type 1 isolates and three reference strains: the attenuated Sabin PV type 1 (AY184219), the virulent PV type 1 Mahoney (V01149) and the Cox type 1 live-attenuated PV (POL430385). According to Martin and Minor (2002), the Cox type 1 strain (POL430385) was closely related to the Mahoney PV type 1, which was evident in this study as well (1.4% divergence between the two). Two of the PV type 1 isolates (AJ783794, AJ783796) showed 100% nucleotide sequence similarity to the attenuated AY184219 reference strain (Table 6.6). Isolates (AJ783799, AJ783800, AJ783801, AJ783802) differed at >0.6% in VP1 sequences from the attenuated AY184219 strain. High sequence divergence (>0.9%) from the attenuated AY184219 strain was recorded for isolate AJ783798. However, only isolates AJ699402 and AJ783795 diverged at 1.3% and 1.1%, respectively from the attenuated AY184219 strain. Although different mutations (at nucleotides 2502, 2581, 2586, 2707, 2741, 2745, 2749, 2775, 2795 and etc.) were found in the VP1 genomes of PV type 1 isolates, none of these mutations have ever been implicated as major factors in changes of PV virulence.

Group 2 consisted of PV type 2 isolates and two reference strains: the attenuated Sabin PV type 2 (AY184220) and the Sabin-like type 2 PV (AY177685) (Figure 6.2). Isolate AJ783797 showed 100% nucleotide sequence similarity to the attenuated AY184220 reference strain (Table 6.6). Isolate AJ783792 differed at 0.6% in VP1 sequences from the attenuated reference strain AY184220. Isolate AJ783793 showed the highest nucleotide divergence in

VP1 sequences (>0.9%) from the attenuated AY184220 strain. Some of the mutations found in the partially sequenced VP1 region of the PV2 isolates, although not known to be associated with increased neurovirulence, included those at nucleotides 2543, 2580, 2655, 2661 and etc.

Group 3 was comprised of PV type 3 isolates and two reference strains: the attenuated Sabin PV type 3 (AY184221) and the neurovirulent reference PV strain P3/Leon/37 (PI3L37). All PV type 3 isolates (AJ783788, AJ783789, AJ783790, AJ783791) in this study showed 100% nucleotide sequence similarity to the PI3L37 reference strain and 0.3% divergence in VP1 sequences from the attenuated AY184221 reference strain (Table 6.6). All of the PV type 3 isolates had a mutation at position 2 493, which involved the reversion of a C base (found in the vaccine strain) to a T base (found in the neurovirulent strain). This mutation involved the substitution of the amino acid threonine (Thr) to isoleucine (Ise) at residue 6 of capsid protein VP1 (VP1-6). The presence of this mutation has been associated with reversion to increased neurovirulence of Sabin PV type 3 strains (Tatem *et al.,* 1992; Macadam *et al.,* 1993; Minor, 1999; Martin and Minor, 2002).

The majority of the PVs (10 out of 13 isolates) detected in stool specimens of the immunodeficient children in this study were classified as “OPV-like viruses”, since these isolates had close sequence relationships (>99% in VP1 nucleotide sequences) to the original Sabin PV vaccine strains (WHO, 2004). The Sabin-like type 3 PVs, isolated from patients P023, P025, P045 and P126, had approximately 0.3% nucleotide sequence divergence in the VP1 from the respective parental Sabin PV type 3 vaccine strains (Table 6.6). By assuming that the rate of VP1 evolution (approximately 1-2% change.year^{-1}) was constant over the period of replication, the time between vaccine assumption and PV isolation from patients P025, P045 and P126 was compatible with the mutation rate observed (possibly less than 3 months). Patient P023 (18 months old baby) had his last OPV immunisation at the age of 14 weeks (the last recorded date 04/2002) and a stool specimen taken 15 months later (29/07/2003) tested positive for Sabin-like type 3 PV (Table 6.7). Thus, the time between vaccine assumption and PV isolation from P023 was not compatible with the mutation rate observed. However, patient P023 might have received an additional polio vaccination at 18 months of age (on the 01/07/2003, although not recorded in the immunisation schedule) or could have acquired the OPV strain through a close contact with another vaccine recipient.

Another explanation is possible recombination of the OPV-like strain with other polio or non-polio enteroviruses.

Another group of OPV-like viruses, showing >0.6% divergence in VP1 from the parental vaccine strains, were isolated from patients P020, P031, P052, P095 and P140 (Table 6.6). For patient P020, a Sabin-like type 1 PV was isolated from a stool sample collected 3 months (29/07/2003) following the last recorded polio vaccination (23/04/2003) and for P031, a Sabin-like type 2 PV was isolated from a stool sample collected one month following vaccination (Table 6.7). A Sabin-like type 1 PV was isolated from a stool sample taken from P095 approximately 8 months (01/10/2003) following vaccination (18/02/2003) (Table 6.7). The time between vaccine assumption and PV isolation from patients P020, P031 and P095 was thus compatible with the mutation rate observed (>0.6% divergence in VP1).

For patient P140 the time between vaccine assumption and PV isolation was not compatible with the mutation rate observed. Patient P140 (a 19 month old baby) had his last recorded OPV immunisation at 14 weeks of age (28/08/2002) and a stool specimen taken 16 months later (02/01/2004) tested positive for Sabin-like type 1 PV. The 5'UTR sequences found in samples taken from patients P095 and P140 were identical (7%, about 24-25 mutations from the original OPV strains) and the chance that so many identical mutations have occurred independently is very low. However, since both patients were hospitalised, a person-to-person transmission in the hospital would be a common epidemiological source explaining the identity for the presence of these two sequences in the two samples.

The time between vaccine assumption and isolation of Sabin-like type 1 PV from patient P052 was not compatible with the mutation rate observed (>0.6% divergence in VP1 from the Sabin PV vaccine strain). The partially sequenced 5'UTR of the Sabin-like type 1 PV isolate, showed a very high percentage divergence (8.5%) from the parental Sabin PV vaccine strain (Table 6.6). Patient P052 (a five-year-old child) had received polio immunisation at the age of 18 months (27/03/2000) and a stool specimen collected 42 months later (04/09/2003) tested positive for Sabin-like PV type 1 (Table 6.7). Since patient P052 was five years old, the last polio vaccination date according to the immunisation schedule should have been on the 26/09/2003 (five-year vaccination). However, the last stool sample was collected on the 04/09/2003, 3 weeks before the child was due for vaccination. One explanation for this low divergence in the partially sequenced VP1 region from the vaccine strain might be that the

Sabin-like type 1 PV isolate was acquired through a close contact such as another vaccine recipient (person-to-person transmission). However, generation of such an isolate cannot simply be explained by long-term replication of VDPV in a single individual but rather by recombination of vaccine-like strains with other polio or non-polio enteroviruses.

In this study three of the PVs isolated from stool specimens of immunodeficient children (P069, P085 and P114), showed ≤99% VP1 sequence identity to the parental Sabin PV vaccine strains (Table 6.6). Based on the WHO classification (2004), these PVs were identified as "suspected" immunodeficient VDPVs (iVDPVs). These "suspected" iVDPVs (as well as the OPV-like isolates) carried mutations at specific positions in their partially sequenced regions, which have been associated with reversion to increased neurovirulence. Patient P085 (one-year-old baby) was immunised at the age of 14 weeks (12/03/2003) and "suspected" iVDPV type 1 was detected in a stool specimen collected seven months (23/10/2003) following the last polio vaccination (Table 6.7). The time between vaccine assumption and PV isolation from P085 was compatible with the mutation rate observed (>0.9% divergence in VP1 sequences). However, the time between vaccine assumption and isolation of "suspected" iVDPVs type 1 from patients P069 and P114 was not compatible with the mutation rate observed (>1.1% divergence in VP1 sequences). A "suspected" iVDPV type 1 was isolated from a stool specimen collected from P069 approximately one month (10/10/2003) following last polio immunisation (18/09/2003) (Table 6.7). Patient P114 (6 months old baby) was immunised previously at the age of 14 weeks (02/09/2003) and "suspected" iVDPV type 1 was detected in a stool specimen collected three months following last polio vaccination (01/12/2003) (Table 6.7). Thus, it seems that deviation from the molecular clock was possibly due to mutations, which appeared to accumulate non-linearly with time.

In the control group two Sabin-like type 1 PVs, isolated from the immunocompetent children Nat 05/24 and Ln 06/17, showed 100% nucleotide sequence similarity in both regions (the 5'UTR and the VP1) to the parental Sabin PV vaccine strain (Table 6.6). A Sabin-like type 2 PV, isolated from Nat 08/02, showed a very high divergence (0.9%) in VP1 nucleotides from the parental Sabin PV vaccine strain (Table 6.6) and had the key mutation at position 481 (5'UTR) associated with reversion of the attenuated phenotype to increased neurovirulence. The Sabin-like type 2 PV was isolated from stool samples at the 10th week vaccination and

according to the results, excretion of PV stopped by the end of the second week following vaccination and no PVs were detected in the stools collected after the 14th week vaccination.

Two conclusions were drawn from the sequencing analysis of the selected regions in the genomes of the PV isolates studied. Firstly, sequencing confirmed the typing results and secondly, sequencing gave no indication of an intertypic recombination event between the 5'UTR and the VP1 part of the genome. However, the data obtained in this study (the various mutations observed in the sequenced regions) were consistent with the view that PV is able to escape the immune pressure in the gut to some extent by improving its general fitness rather than evasion of immunity (Minor *et al.*, 2005).

6.5 Conclusions

Currently, the most urgent priority is to eliminate the remaining reservoirs of wild-type PV endemicity (Kew *et al.*, 2004). The Technical Consultative Group to the WHO on the Global Eradication of Poliomyelitis (2002) suggested that when poliomyelitis is eradicated globally and OPV administration is ceased, that vaccine-derived strains would continue to circulate for 2-3 months. During this time, the potential risk of infection would be greatest for the 0- to 5-year-old age group, who may be unvaccinated and who could transmit PV via the faecal-oral route and thus, perpetuate circulation of the PV (Wood and Thorley, 2003). Excretion of PV is prolonged in immunodeficient people leading to another reservoir for continued circulation of PV following vaccination cessation (Wood and Thorley, 2003). Cases of VDPV from immunodeficient people (iVDPV) with long-term excretion of PV have been recorded, but such cases have not been associated with cVDPV (no person-to-person transmission of these iVDPVs) (Kew *et al.*, 1998; Gavrilin *et al.*, 2000; Wood *et al.*, 2000; Wood and Thorley, 2003). Two cases of VAPP have been reported in HIV-infected children (Ion-Nedelscu *et al.*, 1994; Chitsike and Furth, 1999). However, at present there is no evidence for prolonged excretion of PV from patients with HIV and AIDS (Technical Consultative Group to the WHO on the Global Eradication of Poliomyelitis, 2002; Wood and Thorley, 2003).

The evolution rate measurements used to estimate the duration of infection in this study have several limitations to precision (Kew *et al.*, 1998). Firstly, only a portion of the sequence information available was used for the evolution rate estimates, because only the 5'UTR and VP1 regions of the genomes of the OPV isolates were partially sequenced. Sequencing the

whole genomes of the OPV isolates or other parts of these genomes (such as the 3'UTR) would have given more information on the evolution rates of the PVs isolated in this study. Finally, the underlying assumption that the rate of VP1 sequence evolution in immunodeficient persons is effectively constant over a prolonged period of time (several months or years) is unproven (Kew *et al.*, 1998). Future studies on prolonged VDPV excretion from HIV-infected patients are required in order to make decisions about when and how to stop immunisation with OPV after the global eradication of poliomyelitis.

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6.6 References

Bellmunt A, May G, Zell R, Pring-Akerblom P, Verhagen W, Heim A. Evolution of poliovirus Type I during 5.5 years of prolonged enteral replication in an immunodeficient patient. Virol 1999; 265, 178-184.

Buttinelli G, Donati V, Fiore S, Marturano J, Plebani A, Balestri P, Soresina AR, Vivarelli R, Delpeyroux F, Martin J, Fiore L. Nucleotide variation in Sabin type 2 poliovirus from an immunodeficient patient with poliomyelitis. J Gen Virol 2003; 84, 1215-1221.

Centers for Disease Control and Prevention. Circulation of a type 2 vaccine-derived poliovirus- Egypt, 1982-1993. Morb Mortal Wkly Rep 2001; 50, 41-51.

Centers for Disease Control and Prevention. Progress toward poliomyelitis eradication- Southern Africa, 2001 – March 2003. Morb Mortal Wkly Rep 2003; 52, 521-524.

Chezzi C. Rapid diagnosis of poliovirus infection by PCR amplification. J Clin Microbiol 1996; 34, 1722-1725.

Chitsike I, van Furth R. Paralytic poliomyelitis associated with live oral poliomyelitis vaccine in a child with HIV infection in Zimbabwe: case report. Brit Med J 1999; 318, 841-843.

Divizia M, Palombi L, Buonomo E, Donia D, Ruscio V, Equestre M, Leno L, Pana A, Degener AM. Genomic characterisation of human and environmental polioviruses isolated in Albania. Appl Environ Microbiol 1999; 65, 3534-3539.

Dowdle WR, De Gourville E, Kew OM, Pallansch MA, Wood DJ. Polio eradication: the OPV paradox. Rev Med Virol 2003; 13, 277-291.

Equestre M, Genovese D, Cavalieri F, Fiore L, Santoro R, Bercoff RP. Identification of a consistent pattern of mutations in neurovirulent variants derived from the Sabin vaccine strain of poliovirus type 2. J Virol 1991; 65, 2707-2710.

Felsenstein J. Evolution trees from DNA sequences: a maximum likelihood approach. J Mol Evol 1981; 17, 368-376.

Fine PE, Carneiro IAM. Transmissibility and persistence of oral polio vaccine viruses: implications for the global poliomyelitis eradication initiative. Am J Epidemiol 1999; 150, 1001-1021.

Gavrilin GV, Cherkasova EA, Lipskaya GY, Kew OM, Agol VI. Evolution of circulating wild poliovirus and of vaccine-derived poliovirus in an immunodeficient patient: a unifying model. J Virol 2000; 74: 7381-7390.

Georgescu MM, Balanant J, Macadam A, Otelea D, Combiescu M, Combiescu AA, Crainic R, Delpeyroux F. Evolution of the Sabin type 1 poliovirus in humans: characterization of strains isolated from patients with vaccine-associated paralytic poliomyelitis. J Virol 1997; 71, 7758-7768.

Gow JW, Behan WMH, Clements GB, Woodall C, Riding M, Behan PO. Enteroviral RNA sequences detected by polymerase chain reaction in muscle of patients with postviral fatigue syndrome. Br Med J 1991; 302, 692-696.

Guillot S, Caro V, Cuervo N, Korotkova E, Combiescu M, Persu A, Aubert-Combiescu A, Delpeyroux F, Crainic R. Natural genetic exchanges between vaccine and wild poliovirus strains in humans. J Virol 2000; 74, 8434-8443.

Haisey NA, Pinto J, Espinosa-Rosaies F, Faure-Fontenla MA, da Silva E, Khan AJ, Webster ADB, Minor P, Dunn G, Asturias E, Hussain H, Pallansch MA, Kew OM, Winkelstein J, Sutter R, the Polio Project Team. Search for poliovirus carriers among people with primary immune deficiency diseases in the United States, Mexico, Brazil and the United Kingdom. Bull WHO 2004; 82, 16-23.

Higgins DG, Sharp PM. Clustal: a package for performing multiple sequence alignment on a microcomputer. Gen 1988; 73, 237-244.

Hovi T, Lindholm N, Savolainen C, Stenvik M, Burns C. Evolution of wild-type 1 poliovirus in two healthy siblings excreting the virus over a period of 6 months. J Gen Virol 2004; 85, 369-377.

Hughes PJ, Evans DM, Minor PD, Schild GC, Almond JW, Stanway G. The nucleotide sequence of a type 3 poliovirus isolated during a recent outbreak of poliomyelitis in Finland. J Gen Virol 1986; 67, 2093-2102.

Ion-Neldescu N, Dobrescu A, Strebel PM, Sutter RW. Vaccine-associated paralytic poliomyelitis and HIV infection. Lancet 1994; 343, 51-52.

Kämmerer U, Kunkel B, Korn K. Nested polymerase chain reaction for specific detection of and rapid identification of human picornaviruses. J Clin Microbiol 1994; 32, 285-291.

Kew OM, Sutter RW, Nottay BK, MacDonough MJ, Prevots DR, Quick L, Pallansch MA. Prolonged replication of a type 1 vaccine-derived poliovirus in an immunodeficient patient. J Clin Microbiol 1998; 36, 2893-2899.

Kew OM, Morris-Glasgow V, Landaverde M, & 21 other authors. Outbreak of poliomyelitis in Hispaniola associated with circulating type 1 vaccine-derived poliovirus. Sci 2002; 296, 356-359.

Kew OM, Wright PF, Agol VI, Delpeyroux F, Shimizu H, Nathanson N, Pallansch MA. Circulating vaccine-derived polioviruses: current state of knowledge. Bull WHO 2004; 82, 16-23.

Kuan MM. Detection and rapid differentiation of human enteroviruses following genomic amplification. J Clin Microbiol 1997; 35, 2598-2601.

Li J, Zhang LB, Yoneyama T, Yoshida H, Shimizu H, Yoshii K, Hara M, Nomura T, Yoshikura H, Miyamura T, Hagiwara A. Genetic basis of the neurovirulence of type 1 polioviruses isolated from vaccine-associated paralytic patients. Arch Virol 1996; 141, 1047-1054.

Liu HM, Zheng DP, Zhang LB, Oberste MS, Kew OM, Pallansch MA. Serial recombination during circulation of type 1 wild-vaccine recombinant polioviruses in China. J Virol 2003; 77, 10994-11005.

Macadam AJ, Pollard SR, Ferguson G, Skuce R, Wood D, Almond JW, Minor PD. Genetic basis of attenuation of the Sabin type 2 vaccine strain of poliovirus in primates. Virol 1993; 192, 18-26.

Martin J, Dunn G, Hull R, Patel V, Minor PD. Evolution of the Sabin strain of type 3 poliovirus in an immunodeficient patient during the entire 637-day period of virus excretion. J Virol 2000a; 74, 3001-3010.

Martin J, Ferguson GL, Wood DJ, Minor PD. The vaccine origin of the 1968 epidemic of type 3 poliomyelitis in Poland. Virol 2000b; 278, 42-49.

Martin J, Minor PD. Characterisation of CHAT and Cox type 1 live-attenuated poliovirus vaccine strains. J Virol 2002; 76, 5339-5349.

Martin J, Samoilovich E, Dunn G & 7 other authors. Isolation of an intertypic poliovirus capsid recombinant from a child with vaccine-associated paralytic poliomyelitis. J Virol 2002; 76, 10921-10928.

Minor PD, Dunn G. The effect of sequences in the 5'non-coding region on the replication of polioviruses in the human gut. J Gen Virol 1988; 69, 1091-1096.

Minor PD. The molecular biology of poliovaccines. J Gen Virol 1992; 73, 3065-3077.

Minor PD. Poliovirus vaccination: current understanding of poliovirus interactions in humans and implications for the eradication of poliomyelitis. Exp Rev Mol Med 1999. http://www-ermm.cbcu.cam.ac.uk

Minor PD, Dunn G, Ramsay ME, Brown D. Effect of different immunisation schedules on the excretion and reversion of oral poliovaccine strains. J Med Virol 2005; 75, 153-160.

Nathanson N, Fine P. Poliomyelitis eradication- a dangerous endgame. Sci 2002; 296, 269-270.

Rousset D, Rakoto-Andrianarivelo M, Razafindratsimandresy R, Randriamanalina B, Guillot S, Balanant J, Mauclère P, Delpeyroux F. Recombinant vaccine-derived poliovirus in Madagascar. Emerg Infect Dis 2003; 9, 885-887.

Sabin AB. Properties and behaviour of orally administered attenuated poliovirus vaccine. J Am Microbiol Assoc 1957; 164, 1216-1223.

Sutter RW, Prevots DR. Vaccine-associated paralytic poliomyelitis among immunodeficient persons. Infect Med 1994; 11, 426-438.

Sutter RW, Kew OM, Cochi SL. Poliovirus vaccine- live. In: Plotkin SA, Orenstein WA, editors. Vaccines, 4th edition. Philadelphia (PA): WB Saunders, 2003; 651-705.

Tatem JM, Weeks-Levy C, Georgiu A, DiMichele SJ, Gorgacz EJ, Racaniello VR, Cano FR, Mento SJ. A mutation present in the amino terminus of Sabin 3 poliovirus VP1 protein is attenuating. J Virol 1992; 66, 3194-3197.

Technical Consultative Group to the World Health Organization on the Global Eradication of Poliomyelitis. "Endgame" issues for the global polio eradication initiative. Clin Infect Dis

2002; 34, 72-77.

Triki H, Barbouche MR, Bahri O, Bejaoui M, Dellagi K. Community-acquired poliovirus infection in children with primary immunodeficiencies in Tunisia. J Clin Microbiol 2003; 41, 1203-1211.

Wood DJ, Sutter RW, Dowdle WR. Stopping poliovirus vaccination after eradication: issues and challenges. Bull WHO 2000; 78, 347-357.

Wood N, Thorley B. Viewpoint Towards global poliomyelitis eradication: The successes and challenges for a developed country. J Paediatr Child Health 2003; 39, 647-650.

World Health Organization. Manual for the virological investigation of poliomyelitis. Geneva, Switzerland: World Health Organization 2004. (WHO/EPI/GEN/04).

Yang C-F, De L, Holloway BP, Pallansch MA, Kew OM. Detection and identification of vaccine-related polioviruses by the polymerase chain reaction. Virus Res 1991; 20, 159-179.

Yang C-F, De L, Yang S-J, Gòmez JR, Cruz JR, Holloway BP, Pallansch MA, Kew OM. Genotype-specific in vitro amplification of sequences of the wild type 3 polioviruses from Mexico and Guatemala. Virus Res 1992; 24, 277-296.

Yang C-F, Naguib T, Yang S-J, Nasr E, Jorba J, Ahmed N, Campagnoli R, van der Avoort H, Shimizu H, Yoneyama T, Miyamura T, Pallansch M, Kew O. Circulation of endemic type 2 vaccine-derived poliovirus in Egypt from 1983-1993. J Virol 2003; 77, 8366-8377.

Table 6.1: Primers used in the detection of enteroviruses in stool specimens using RT-PCR and nested PCR methods (Gow *et al.*, 1991; Kuan, 1997)

Primer region	Primer	Sequence	Amplicon length (bp)
65-84 454-473	EP1 EP4	5'-CGG TAC CTT TGT GCG CCT GT-3' 5'-TTA GGA TTA GCC GCA TTC AG-3'	408
163-178 443-460	E1 E2	5'-AAG CAC TTC TGT TTC C-3' 5'-CAT TCA GGG GCC GGA GGA-3'	297

Table 6.2: Restriction enzymes (REs) used for the genotyping of enteroviruses (Kämmerer *et al.*, 1994; Kuan, 1997)

REs	Recognition site
Sty I	C*C(A/T)(A/T)GG
Bgl I	GCCNNNN*NGGC
Xmn I	GAANN*NNTTC

N = A, G, C or T.
*** = Recognition site for REs.**

Table 6.3: Fragments resulting from digestion by *Sty* I, *Bgl* I and *Xmn* I REs of 297 bp amplified enteroviruses (Kämmerer *et al.*, 1994; Kuan, 1997)

REs	Prototype enteroviruses	DNA fragment size (bp)
Sty I	ECV4, ECV9, ECV11, ECV20, PV3, CAV2, CAV3, CAV5, CAV7, CBV4	297
	ECV6, ECV19, PV1, CAV6, CBV2, CBV3	226+71
	ECV7, PV2, CBV1	197+100
	CBV2, CBV3, CBV6	212+75+10
	CBV5	112+102+83
Bgl I	ECV7, ECV9, ECV11, ECV20, PV2, PV3, CBV1, CBV3, CAV1, CAV5, CAV6, CAV7	297
	ECV4, ECV6, ECV14, ECV19, PV1, CAV2, CAV3, CBV2, CBV4, CBV5	217+80
	CBV6	196+80+21
Xmn I	ECV7, ECV11, PV2, PV3, CAV1, CBV3	297
	ECV4, ECV6, ECV9, ECV14, ECV20, PV1, CAV2, CAV3, CAV5, CAV6, CAV7, CBV1, CBV2	236+61

Table 6.4: Sabin specific RT-PCR primers used in the detection and differentiation of Sabin PV types 1, 2 and 3 (Yang *et al.*, 1991; Yang *et al.*, 1992)

Primer region	Primer	Sequence	Amplicon length (bp)	Specificity
2584-2601 2505-2523	S1-1 S1-2	5'-TCC ACT GGC TTC AGT GTT-3' 5'-AGG TCA GAT GCT TGA AAG C-3'	97	Sabin PV type 1
2580-2595 2525-2544	S2-1 S2-2	5'-CGG CTT GTG TCC AGG C-3' 5'-CCG TTG AAG GGA TTA CTA AA-3'	71	Sabin PV type 2
 2537-2553	S3-1a S3-2	5'-AGT ATC AGG TAA GCT ATC C-3' 5'-AGG GCG CCC TAA CTT TG-3'	54	Sabin PV type 3

Table 6.5: Primers used in the RT-PCRs for the amplification of the 5'UTR and VP1 region of the poliovirus genome (Divizia *et al.*, 1999; Guillot *et al.*, 2000)

Primer region	Primer	Sequence	Amplicon length (bp)
5'UTR 160-180 580-599	 UG52 UC53	 5'-AAC AAG CAC TTC TGT TTC CCC-3' 5'-GTG ATT GTC ACC ATA AGC AG-3'	440
VP1 2402-2422 2862-2881	 UG1 UC1	 5'-TTT GTG TCA GCG TGT AAT GAC-3' 5'-AAA[1] TTC CAT ATC AAA TCT AG-3'	480
VP1 2426-2446 2792-2812	 N2426 N2812	 5'-AGC GTG CGC TTG ATG CGA GAT-3' 5'-AGT GAT CTT CCA CAC TGT[2] AAA-3'	387

[1]= For Mahoney poliovirus type 1 there is a G, instead of an A in the VP1 genome.
[2]= For Mahoney poliovirus type 1 there is a C, instead of a T in the VP1 genome.

Table 6.6: Extent of nucleotide divergence between the characterised 5'UTR and VP1 regions of polioviruses isolated in this study from their attenuated parental Sabin poliovirus vaccine strains

% Difference in the 5'UTR	Accession number for the 5'UTR region	Sample	Type of virus	Accession number for the VP1 region	% Difference in the VP1
Not done**	AJ783777	P020	PV1	AJ783799	0.7
8.5	AJ783776	P052	PV1	AJ783800	0.6
Not done**	AJ783779	P069	PV1	AJ699402	1.3
0.9	AJ783778	P085	PV1	AJ783798	0.9
7.0	AJ699401	P095	PV1	AJ783802	0.6
0.4	AJ783780	P114	PV1	AJ783795	1.1
7.0	AJ783775	P140	PV1	AJ783801	0.6
0	AJ634678	Nat 05/24*	PV1	AJ783794	0
0	AJ783781	Ln 06/17*	PV1	AJ783796	0
0.4	AJ783784	P031	PV2	AJ783792	0.6
0.9	AJ783782	P039	PV2	AJ783797	0
0.4	AJ783783	Nat 08/02*	PV2	AJ783793	0.9
0.4	AJ635238	P023	PV3	AJ783790	0.3
0.4	AJ783787	P025	PV3	AJ783788	0.3
0.4	AJ783786	P045	PV3	AJ783789	0.3
9.2	AJ783785	P126	PV3	AJ783791	0.3

*** = control group of immunocompetent children.**
**** = nucleotide sequences in the 5'UTR were too short to be incorporated into the phylogenetic tree.**

Table 6.7: Poliovirus vaccine strains isolated from stool specimens of immunodeficient children from a selected area in South Africa

Sample	Type of virus	Clinical condition	Date of birth	Polio immunisation	Stool specimens collected	CDC classification
P020 2385048	PV1	PCP Died	10/03/2003	10/03/2003 23/04/2003	29/07/2003	C3 $CD4^+$ count of < 200 cells.mm^{-3}
P052 2391424	PV1	Miliary TB	26/09/1998	26/09/1998 07/11/1998 05/12/1998 02/01/1999 27/03/2000	04/09/2003	C3 $CD4^+$ count of < 200 cells.mm^{-3}
P069 2386771	PV1	Pneumonia	07/08/2003	07/08/2003 18/09/2003	10/10/2003	B
P085 2400161	PV1	Marasmic pneumonia	23/10/2002	24/10/2002 03/12/2002 23/01/2003 12/03/2003	23/10/2003	B3 $CD4^+$ count of < 200 cells.mm^{-3}
P095 2353775	PV1	Chronic diarrhoea	13/08/2001	15/08/2001 27/09/2001 15/11/2001 29/01/2002 18/02/2003	01/10/2003	C3 $CD4^+$ count of < 200 cells.mm^{-3}
P114 2404862	PV1	G/E Dehydration	25/05/2003	26/05/2003 07/07/2003 05/08/2003 02/09/2003	01/12/2003	B3 $CD4^+$ count of < 200 cells.mm^{-3}
P140 2332025	PV1	Pneumonia	06/05/2002	06/05/2002 19/06/2002 31/07/2002 28/08/2002	02/01/2004	B2 $CD4^+$ count of 200–499 cells.mm^{-3}
P031 2374125	PV2	Meningitis	02/05/2003	02/05/2003 13/06/2003	29/07/2003	B
P039 2388823	PV2	BPN	15/05/2003	16/05/2003 27/06/2003	19/08/2003	B1 $CD4^+$ count of >500 cells.mm^{-3}
P023 2379238	PV3	Encephalitis G/E PTB	01/2002	01/2002 02/2002 03/2002 04/2002	29/07/2003	C2 $CD4^+$ count of 200–499 cells.mm^{-3}
P025 2330375	PV3	Herpes stomatitis Pneumonia	14/01/2002	14/01/2002 26/02/2002 25/03/2002 24/04/2002 16/07/2003	29/07/2003	B2 $CD4^+$ count of 200–499 cells.mm^{-3}
P045 2389052	PV3	Pneumonia	10/06/2003	10/06/2003 22/07/2003	22/08/2003 21/10/2003	B2 $CD4^+$ count of 200–499 cells.mm^{-3}
P126 2369107	PV3	G/E Dehydration	12/04/2002	12/04/2002 22/05/2002 19/06/2002 17/07/2002 08/10/2003	17/12/2003	A3 $CD4^+$ count of < 200 cells.mm^{-3}

Abbreviations: A, documented asymptomatic HIV infection; B, symptomatic HIV infection; C, symptomatic HIV infection with an AIDS indicator condition; BPN, bronchopneumonia; G/E, gastroenteritis; PCP, pneumocystis carinii pneumonia; PTB, pulmonary tuberculosis; TB, tuberculosis.

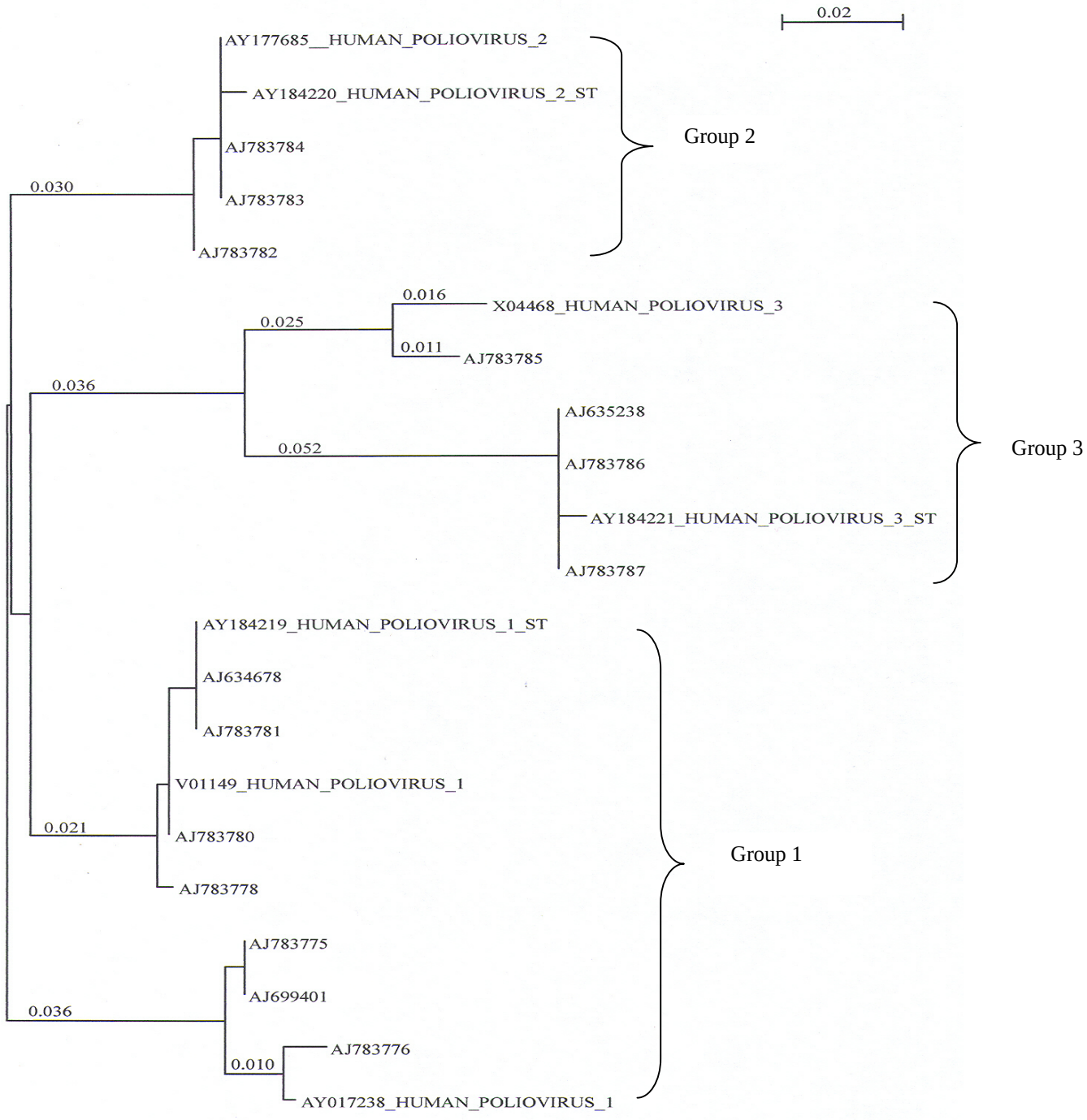

Figure 6.1: **Unrooted phylogenetic tree re-constructed with the neighbour-joining method from the comparative 5'untranslated region sequence analysis of the isolated oral poliovirus vaccine strains from immunodeficient children and the poliovirus reference strains. Branch lengths are proportional to the phylogenetic distances, while the vertical branches are non-informative. The scale bar shows 2% nucleotide sequence difference**

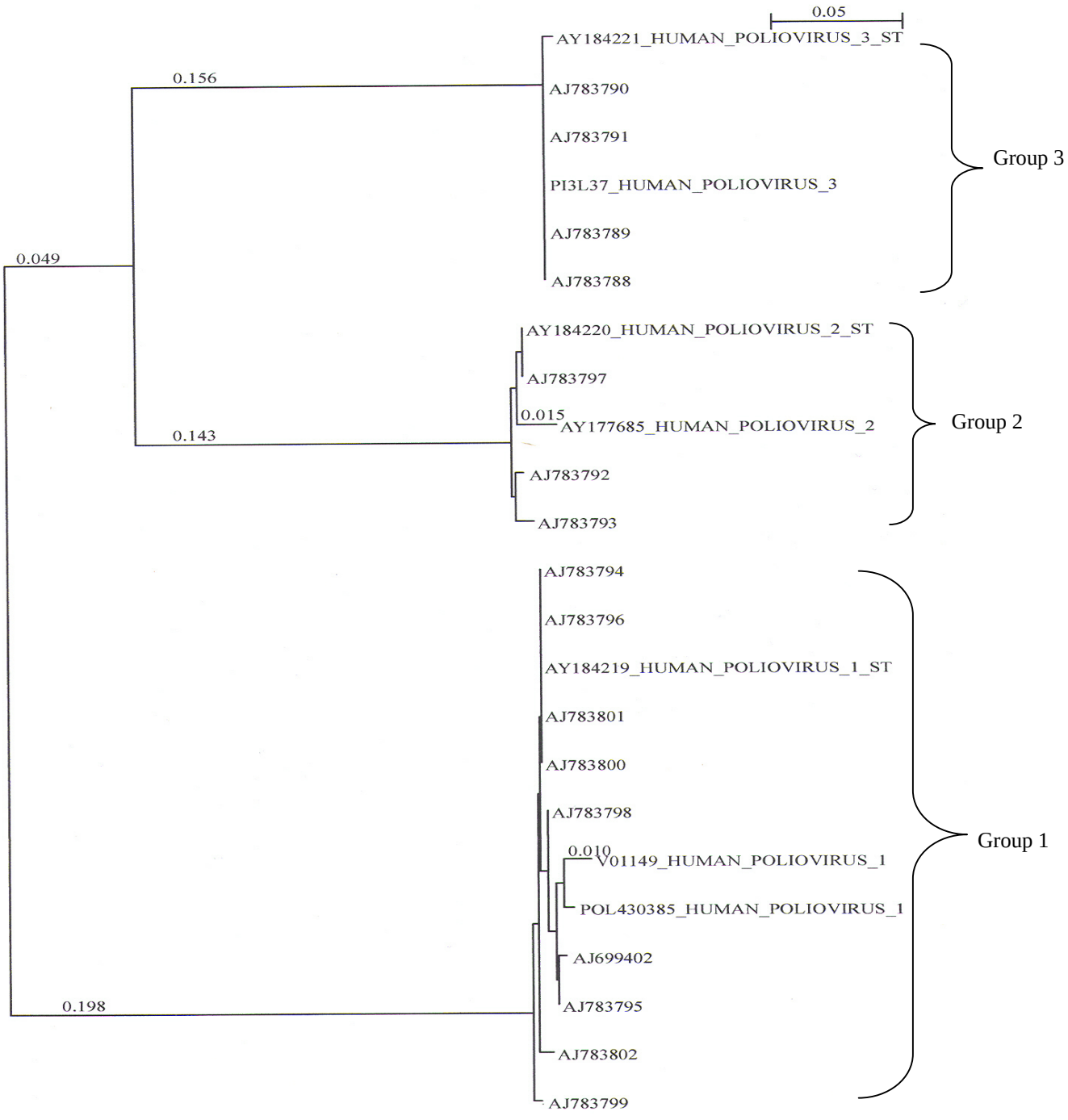

Figure 6.2: **Unrooted phylogenetic tree re-constructed with the neighbour-joining method from the comparative VP1 region sequence analysis of the isolated oral poliovirus vaccine strains from immunodeficient children and the poliovirus reference strains. Branch lengths are proportional to the phylogenetic distances, while the vertical branches are non-informative. The scale bar shows 5% nucleotide sequence difference**