CHAPTER 5

PREVALENCE OF VACCINE-DERIVED POLIOVIRUSES IN SEWAGE AND RIVER WATER IN SOUTH AFRICA

The editorial style of Water Research was followed in this chapter

5.1 Abstract

Research has shown that poliovirus (PV) isolates in the environment are genetically and epidemiologically related to those circulating in the community. The properties of PV isolates from sewage and river water would reflect those of PVs excreted by humans after oral poliovirus vaccination (OPV). Polioviruses are not associated with waterborne transmission to the same extent as many other enteric viruses. However, they are typically transmitted by the faecal-oral route, which implies that the risk of infection by exposure to the viruses in water, cannot be underestimated. The risk appears particularly high for rural communities, which use sewage-polluted river water for domestic purposes. Thus, the presence in the environment of highly evolved, neurovirulent VDPV strains in the absence of polio cases would have important implications for strategies to terminate immunisation with OPV following global polio eradication. The aim of the current study was to determine the prevalence of VDPVs in selected sewage and river water samples collected from 2001 to 2003, and to construct phylogenetic trees of the partially sequenced 5'untranslated region (5'UTR) and the VP1 region of the genomes to deduce the genetic relatedness between these PV strains. Using the monolayer plaque assay, 703 plaques from sewage and 157 plaques from river water samples were analysed. Application with a RT-multiplex PCR revealed that 176 of these plaques were non-polio enteroviruses, and 49 were PV isolates. The Sabin-specific RT-triplex PCR revealed the presence of 29 Sabin PV type 1 (59.2%), 8 Sabin PV type 2 (16.3%) and 12 Sabin PV type 3 (24.5%) isolates. The 5'UTR and the VP1 region of 13 PV type 1, 7 PV type 3 and 6 PV type 2 isolates were partially sequenced. The majority of the OPV isolates (24 out of 26) displayed close sequence relationships (>99% VP1 sequence identity) to the parental Sabin PV vaccine strains and were classified as "OPV-like viruses". Two isolates (D1 08/28 and OF1 05/21) were found to be highly divergent and were

classified as "suspected" vaccine-derived polioviruses (VDPVs). Isolate OF1 05/21 (a "suspected" VDPV type 1) showed more than 0.9% divergence in VP1 nucleotides, whereas isolate D1 08/28 (a "suspected" VDPV type 2) showed the highest percentage divergence (at 1.4%) from the parental Sabin PV vaccine strains. As with most of the other OPV-like isolates, these "suspected" VDPVs were carrying mutations at specific positions in their partially sequenced regions, which have previously been associated with reversion of the attenuated Sabin PV vaccine strains to increased neurovirulence. It was estimated that the total period of replication for these two "suspected" VDPVs was between 12 to 16 months. In conclusion, this study provided new and relevant information on the prevalence of "suspected" VDPVs in sewage and river water, and opened the way to assess the possible broader significance of the findings reported here.

Keywords: Divergence, Oral poliovirus vaccine strains, Prevalence, River water, Sewage, Vaccine-derived polioviruses

Abbreviations: AFP, Acute flaccid paralysis; ATCC, American Type Culture Collection; BGM, Buffalo green monkey kidney; cVDPV, Circulating vaccine-derived poliovirus; CPE, Cytopathogenic effect; DNA, Deoxyribonucleic acid; EVs, Enteroviruses; ECACC, European Collection of Cell Culture; HEp-2, Human epidermoid carcinoma; NPEVs, Non-polio enteroviruses; OPV, Oral poliovirus vaccine; PBS, Phosphate-buffered saline; PEG, Polyethylene glycol; PV, Poliovirus; PLC/PRF/5, Primary liver carcinoma; RT-PCR, Reverse transcription polymerase chain reaction; RNA, Ribonucleic acid; UTR, Untranslated region; USA, United States of America; VAPP, Vaccine-associated paralytic poliomyelitis; VDPV, Vaccine-derived poliovirus; VP, Virus protein; WHO, World Health Organization.

5.2 Introduction

The polio eradication program is close to the final stage of replacing wild-type poliovirus (PV) in the population with vaccine-type by mass live attenuated oral poliovirus vaccine (OPV) immunisation (Yoshida *et al.*, 2002). Mutations of the PV vaccine strains during genomic replication in the gastrointestinal tract may result in loss of attenuation and an increase in neurovirulence (Wood and Thorley, 2003). If the mutations lead to poliomyelitis

in a vaccine recipient or close contact it is defined as vaccine-associated paralytic poliomyelitis (VAPP) (Wood and Thorley, 2003).

All clinical and environmental PV isolates that are related to OPV strains are vaccine-derived polioviruses (VDPVs) (World Health Organization [WHO], 2004). The extensive sequence divergence from the respective OPV strain is a distinguishing feature of VDPVs (Kew *et al.*, 1998; Bellmunt *et al.*, 1999; Martin *et al.*, 2000; Kew *et al.*, 2004). Derivatives of the Sabin live attenuated vaccine strains present in OPV have been classified into two broad categories for programmatic reasons (WHO, 2004). A vaccine-related isolate is considered a VDPV if it has diverged by ≥1% of VP1 nucleotides from the reference OPV strain (WHO, 2002; Kew *et al.*, 2004; WHO, 2004). The demarcation of 1% VP1 divergence implies that replication of vaccine virus had occurred for approximately 1 year (Kew *et al.*, 2004; WHO, 2004). The vast majority of vaccine related isolates are "OPV-like viruses" and have close sequence relationships (<1% VP1 sequence identity) to the original OPV strains (WHO, 2004). Isolates having <1% divergence would not necessarily lack the capacity for person-to-person transmission in poorly immunised populations, as it is likely that the critical attenuating mutations of the Sabin PV strains generally revert before nucleotide substitutions accumulate to the level of 1% (Kew *et al.*, 2004; WHO, 2004). By this definition, nearly all minimally diverged "OPV-like" isolates would be excluded and VDPVs that had replicated for at least one year would be included (Kew *et al.*, 2004; 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; Kew *et al.*, 2004).

A study conducted by Divizia and colleagues (1999), confirmed the environmental circulation in Albania of recombinant PV strains (Sabin-like PV type 2/wild PV type 1), sustained by a massive immunisation effort and by the presence in the environment of a PV type 1, isolated from a river 2 months before the first case of symptomatic acute flaccid paralysis (AFP). Shulman and colleagues (2000) have isolated an unusual, highly diverged derivative of the Sabin PV type 2 strain from environmental samples during routine screening for wild-type PV in Israel. The extensive genetic divergence of the isolate from its parental Sabin PV type 2 strain suggested that the virus had replicated in one or more individuals for approximately 6 years (Shulman *et al.*, 2000). According to other studies, VDPVs (with 1.4% nucleotide

divergence from the vaccine strain) were isolated from sewage and river water in Japan within 3 months following OPV vaccination, and several of these VDPV type 1 and type 3 isolates showed increased neurovirulence (Horie *et al.*, 2002; Yoshida *et al.*, 2002). More recently, a highly evolved VDPV type 3 strain harbouring a 13% sequence drift from Sabin PV type 3 vaccine strain has been isolated from sewage in Estonia (Blomqvist *et al.*, 2004).

Vaccine strains of PV isolated from the environment have been found to be genetically and epidemiologically related to those circulating in the community (Divizia *et al.*, 1999; Shulman *et al.*, 2000). The properties of vaccine PV isolates from sewage and river water would reflect those of strains excreted by humans after OPV immunisation (Yoshida *et al.*, 2002). These VDPVs may potentially cause poliomyelitis or related illness in susceptible individuals (Yoshida *et al.*, 2002; Buttinelli *et al.*, 2003). Thus, the presence in the environment of highly evolved, neurovirulent VDPV strains in the absence of polio cases would have important implications for strategies to terminate immunisation with OPV following global polio eradication.

This study addressed some of the issues regarding the evolution and nucleotide divergence of OPV strains isolated from selected sewage and river water samples in South Africa. Genotyping of the 5'UTR and VP1 regions of these OPV isolates using various molecular techniques demonstrated the prevalence of "suspected" VDPVs in the environment and the potential health risk they might constitute after termination of OPV immunisation in the near future.

5.3 Materials and methods

5.3.1 Virus stock and cell cultures

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 BGM (buffalo green monkey kidney) (Code ECACC 90092601, passage 80 - 95), HEp-2 (human epidermoid carcinoma) (Code ATCC CCL-23, passage 350 - 365) and PLC/PRF/5 (primary liver carcinoma) (Code ATCC 8024, passage 90 - 105) cell lines. 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.

5.3.2 Isolation of polioviruses from sewage and river water

During 2001 and 2003, 213 sewage samples were obtained from the Daspoort Sewage Treatment Plant, Pretoria and from the East Rand Water Care Company, Johannesburg, South Africa. Both of these plants treat domestic and industrial sewage for approximately 3 500 000 people (Table 5.1). During the same period of time, a total of 138 river water samples (occasionally used by the rural community for domestic purposes) were obtained from selected rural areas in South Africa. The sewage and river water samples were decontaminated using chloroform (Merck, Darmstadt, Germany). Ten millilitres of chloroform were added to 50 ml of sewage/river water samples and mixed for 30 min in a shaking incubator (Labcon, Labotec, South Africa) at 200 rpm (±25°C). The samples were centrifuged at 4 500 x *g* (Sorvall Super T 21, Wilmington, USA) at 4°C for 10 min in order to separate the supernatant from the chloroform (Merck). Viruses were recovered from these samples using the polyethylene glycol (PEG)/NaCl precipitation method (PEG 6000, Merck) (Minor, 1985). The resulting pellet was re-suspended in 10 ml phosphate-buffered saline (PBS) (Sigma Chemical Co., Louis, United States of America [USA]) and inoculated onto BGM, HEp-2 and PLC/PRF/5 cell monolayers. Plaque assays were carried out for the detection of PVs using 92 mm Nunclon tissue culture plates (Nalge Nunc, Denmark) containing pre-formed monolayers of BGM, HEp-2 and PLC/PRF/5 cells at a concentration of 2.0 x 10^5 cells.ml^{-1} (Manor *et al.*, 1999). The viruses from 10 well-separated plaques were picked for further propagation on BGM, HEp-2 and PLC/PRF/5 cell cultures.

5.3.3 Ribonucleic acid extraction and typing of poliovirus isolates

Ribonucleic acid (RNA) was extracted from infected cell culture fluid by means of a commercial RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The RNase-Free DNase kit (Qiagen) provided efficient on-column digestion of deoxyribonucleic acid (DNA) during RNA purification from cells. A reverse transcription multiplex PCR (RT-multiplex PCR) as described by Egger *et al.* (1995) was used for the

rapid detection of PVs and for their distinction from non-polio enteroviruses (NPEVs). Primers specific for either enterovirus (EV) or PV were combined in a RT-multiplex PCR (Promega Access RT-PCR system, Promega Corp., Madison, USA) and gave rise to amplicons of different sizes (Table 5.2). Optimised final concentrations in a total volume of 50 µl were as follows: AMV/*Tfl* Reaction Buffer (1x), 2.0 mM $MgSO_4$, dNTP Mix (final concentration of 0.2 mM), 25 pmol each of primers E1, E2, Po1, Po2, Po3 and Po4 (Sigma-Genosys Ltd., Pampisford, Cambridgeshire, United Kingdom), and 5 U each of AMV Reverse Transcriptase and *Tfl* DNA Polymerase (Promega Corp.). The PCR conditions included: reverse transcription for 45 min at 48°C, {DNA denaturation for 1 min at 94°C, primer annealing for 1.5 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, United Kingdom). After 30 cycles, 20 µl of each PCR product were subjected to agarose (2%) (Seakem LE agarose, Bioproducts, USA) gel electrophoresis (Midicell Primo Gel Apparatus, Holbrook, New York, USA).

5.3.4 Sabin specific RT-triplex PCR

Three sets of primers specific for Sabin PV type 1, 2 and 3 were combined in a RT-triplex PCR to confirm the isolated PVs as Sabin vaccine strains and gave rise to amplicons of different sizes (Table 5.3) (Yang *et al.*, 1991; Yang *et al.*, 1992; Chezzi, 1996). 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 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 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, Hercules, California, USA) gel electrophoresis using a Hoefer electrophoresis unit at 120 Volts (Hoefer, San Francisco, USA).

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

The 5'untranslated region (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 5.4). 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 Primo Gel Apparatus).

5.3.6 Partial genomic sequencing of the VP1 capsid protein of polioviruses

The virus protein (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 5.4). 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). After 30 cycles, 20 µl of each PCR product were subjected to agarose (2%) (Seakem) gel electrophoresis (Midicell Primo Gel Apparatus).

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 5.4). 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 each nested PCR product were subjected to agarose (2%) (Seakem) gel electrophoresis (Midicell Primo Gel Apparatus).

5.3.7 Nucleotide sequencing and phylogenetic analysis

Before initiating sequencing, the exconuclease I/Shrimp alkaline phosphatase (Fermentas, Vilnus, 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. Nucleotide sequence comparisons were made with previously published sequences (GenBank accession numbers: AY177685; AY184219; AY184220; AY184221; PI3L37; POL430385; V01149).

5.3.8 Nucleotide sequence accession numbers

The sequences of the 5'UTR and the VP1 of PVs isolated from sewage and river water 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 5.5).

5.4 Results and discussion

Using the monolayer plaque assay, 703 plaques from sewage and 157 plaques from river water samples were analysed. Application of the RT-multiplex PCR revealed that 176 of these plaques were NPEVs, and 49 were PV isolates. The remaining 634 plaques might possibly have been reoviruses or adenoviruses, since the HEp-2 cell line was applied in this assay. However, these plaques were not subjected to further analysis. The Sabin specific RT-triplex PCR showed that all 49 PV isolates were vaccine strains consisting of 29 Sabin PV type 1 (59.2%), 8 Sabin PV type 2 (16.3%) and 12 Sabin PV type 3 (24.5%) isolates. No wild-type PVs were detected in the sewage and river water samples, which was in agreement with epidemiological data indicating that poliomyelitis has been eradicated in South Africa (Centers for Disease Control and Prevention [CDC], 2003).

A representative number of PVs (26 from the initial 49 PVs) isolated from sewage and river water samples were selected for sequencing analysis of the 5'UTR and VP1 regions. The total number of PVs sequenced included: 13 PVs type 1, 7 PVs type 3 and 6 PVs type 2.

Phylogenetic analysis of the 5'UTR of the poliovirus genome

The unrooted phylogenetic tree, resulting from comparison of the 5'UTR nucleotide sequences of the PVs isolated from selected sewage and river water samples in this study, consisted of three main groups (Figure 5.1). In Group 1, five PV type 1 isolates (AJ783725, AJ783727, AJ783728, AJ783730 and AJ783731) displayed 100% nucleotide sequence identity to the attenuated Sabin PV type 1 (AY184219) reference strain (Table 5.5). Seven PV type 1 isolates (AJ635236, AJ783722, AJ783723, AJ783724, AJ783726, AJ783729, AJ783732) shared 100% nucleotide sequence identity with the neurovirulent reference strain PV type 1 Mahoney (V01149) and 99.7% similarity to the attenuated (AY184219) reference strain. Isolate AJ783721 showed 99.3% identity to the AY184219 reference strain. In addition, isolate AJ783721 and the subgroup of V01149 (AJ635236, AJ783722, AJ783723,

AJ783724, AJ783726, AJ783729 and AJ783732) had a mutation at position 480 in the 5'UTR, which involved a direct reversion of a G base (found in the attenuated Sabin strain) to A base (found in the virulent strain). This mutation is typically associated with reversion of the attenuated Sabin PV type 1 vaccine strain to increased neurovirulence (Minor, 1992; Minor, 1999; 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 and PV type 2 strain (AY177685) (Figure 5.1). The reference PV type 2 strain (AY177685) was isolated by Buttinelli and colleagues (2003) from an immunodeficient patient soon after the onset of paralysis. Even though the patient had permanent paralysis and 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), the virus did not cause paralysis when tested in transgenic mouse strains (Buttinelli *et al.*, 2003). Nonetheless, AY177685 was used as a reference PV type 2 strain, because most of the PV type 2 isolates in this study were to a certain extent genetically related to this virus. Five of the PV type 2 isolates in this study (AJ783720, AJ783733, AJ783734, AJ783736, AJ783737) differed at 0.5% in 5'UTR sequences from the attenuated AY184220 reference strain (Table 5.5). Isolate AJ783735 showed a higher nucleotide sequence divergence (0.9%) from the attenuated AY184220 reference strain. All PV type 2 isolates in this study had the key mutation at position 481 in the 5'UTR (a conversion from A to a G base).

Group 3 was comprised of PV type 3 strains (Figure 5.1). All PV type 3 isolates (AJ783738, AJ783739, AJ783770, AJ783771, AJ783772, AJ783773 and AJ783774) displayed 100% nucleotide sequence identity with the neurovirulent reference PV strain P3/Leon/37 (PI3L37) (Table 5.5). In addition, these PV isolates diverged at 0.5% in 5'UTR sequences from the attenuated reference strain Sabin PV type 3 (AY184221). All PV type 3 isolates (AJ783738, AJ783739, AJ783770, AJ783771, AJ783772, AJ783773, AJ783774) displayed a mutation at position 472 in the 5'UTR, which involved reversion of an U base (found in the attenuated vaccine strain) to a C base (found in the virulent strain). This mutation is typically associated with 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

Three main groups were evident from the unrooted phylogenetic tree of the VP1 capsid-encoding region of the PVs isolated in this study (Figure 5.2). In Group 1, five of the PV type 1 isolates (AJ783806, AJ783808, AJ783809, AJ783847, AJ783848), showed 100% nucleotide sequence similarity with the attenuated Sabin PV type 1 (AY184219) reference strain (Table 5.5). However, these isolates differed from the Cox type 1 live-attenuated PV (POL430385) at 1.2% and the virulent PV type 1 Mahoney (V01149) at 2.2% of VP1 nucleotides. 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. Isolate AJ783855 and isolates (AJ783805, AJ783853, AJ783856) differed from the attenuated AY184219 reference strain at 0.5% and 0.6% of VP1 nucleotides, respectively. High nucleotide divergences from the AY184219 strain (0.8% in VP1 sequences) were observed for isolates (AJ783810, AJ783857, AJ783858). Isolate AJ635237 displayed the highest nucleotide divergence (>0.9%) from the AY184219 reference strain. Although different mutations can be 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 (Figure 5.2). Isolate AJ783811 showed 100% nucleotide sequence similarity to the attenuated Sabin PV type 2 (AY184220) reference strain (Table 5.5). Three PV type 2 isolates (AJ783804, AJ783807 and AJ783852) differed at 0.2% and isolate (AJ783813) at 0.7% of VP1 sequences from the attenuated AY184220 strain. One of the isolates (AJ783852) showed 100% nucleotide sequence similarity to the AY177685 reference strain and had the highest divergence in VP1 sequences (>1.4%) from the attenuated AY184220 reference strain.

Group 3 consisted of PV type 3 isolates (Figure 5.2). All PV type 3 isolates (AJ783812, AJ783814, AJ783815, AJ783849, AJ783850, AJ783851, AJ783854) showed 100% nucleotide sequence identity with the neurovirulent PV reference strain P3/Leon/37 (PI3L37) and diverged at 0.2% from the attenuated Sabin PV type 3 (AY184221) reference strain (Table 5.5). All isolates (AJ783812, AJ783814, AJ783815, AJ783849, AJ783850, AJ783851 and AJ783854) had in their sequenced VP1 regions a mutation at position 2 493, which involved the reversion of a C base (found in the attenuated vaccine strain) to a T base (found in the neurovirulent strain). This mutation involved the substitution of threonine (Thr) to isoleucine (Ise) at residue 6 of capsid protein VP1 (VP1-6). According to scientific reports,

the presence of this mutation may be involved with reversion to increased neurovirulence of Sabin PV type 3 strains (Tatem *et al.*, 1992; Macadam *et al.*, 1993; Georgescu *et al.*, 1997; Minor, 1999; Martin and Minor, 2002).

In this study, the majority of the OPV isolates (24 out of 26 PVs) displayed close sequence relationships (>99% VP1 sequence identity) to the parental Sabin PV vaccine strains (Table 5.5). Based on a recent WHO classification (2004), these isolates were classified as "OPV-like viruses". In addition, four Sabin-like type 1 PVs (Ts1, Sbnd2, Lv1 and Mb1) showed 100% nucleotide sequence identity to the attenuated Sabin PV type 1 vaccine strain in both the VP1 region as well as the 5'UTR.

Seven Sabin-like type 1 PVs (D2, DP1 09/13, KspntDR, Mcc1, Mcc4, MF3 and Sbnd1) and one Sabin-like type 2 PV (OF2 05/21) showed approximately 0.5% to 0.8% divergence in VP1 nucleotides from the attenuated Sabin PV vaccine strains. By assuming that the rate of VP1 evolution (approximately 1-2% change.year^{-1}) was constant over the entire period of replication and similar to the rates observed for the other types of PVs, and without correcting for the small effects of multiple substitutions at site, it can be estimated that the total period of replication for these PV isolates was between 5 to 8 months.

Two isolates (D1 08/28 and OF1 05/21) were classified as "suspected" vaccine-derived polioviruses (VDPVs), since these isolates showed ≤99% VP1 sequence identity to the parental Sabin PV vaccine strains. Isolate OF2 05/21 (a "suspected" VDPV type 1) showed >0.9% divergence in VP1 nucleotides, whereas isolate D1 08/28 (a "suspected" VDPV type 2) showed the highest percentage divergence (at 1.4%). As with most of the other OPV-like isolates, these "suspected" VDPVs carried mutations at specific positions in their partially sequenced regions (5'UTR and VP1), which have been associated with reversion of the attenuated Sabin PV vaccine strains to increased neurovirulence. The extent of sequence divergence of OF1 05/21 and D1 08/28 suggested that these "suspected" VDPVs had replicated in one or more people from 12 to 16 months since the administration of the initiating OPV dose. These results were in agreement with findings from other studies, in which VDPVs (with 1.4% nucleotide divergence from the vaccine strain) were isolated from sewage and river waters within 3 months following OPV vaccination (Horie *et al.*, 2002;

Yoshida *et al.*, 2002). Several of these reported VDPV type 1 and type 3 isolates showed increased neurovirulence (Horie *et al.*, 2002; Yoshida *et al.*, 2002).

5.5 Conclusions

The risk of infection with VDPVs from river water can be considerably high, taking into account, that access to river water is easy for many individuals living in rural communities, which generally use this water for domestic purposes such as washing and drinking. However, the risk of infection with VDPVs from sewage is very low, but not zero, since in some instances raw sewage is used for irrigation purposes and in this way individuals may come into direct contact with VDPVs present in the sewage (Yoshida *et al.*, 2002). The survival in the environment of VDPVs excreted by humans is of concern, because these PVs might be transmitted and continue to circulate in a non-immune population after the cessation of polio vaccination (Friedrich, 2000; Buttinelli *et al.*, 2003).

Two conclusions could be drawn from this study. Sequencing confirmed the typing results and more importantly, sequencing gave no indication of an intertypic recombination event between the 5'UTR and the VP1 part of the PV genome. Nonetheless, the identification of OPV-like viruses and "suspected" VDPVs in this study emphasised that there is a potential environmental risk of VAPP as long as the attenuated live OPV is not replaced by the inactivated poliovirus vaccine in the near future.

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5.6 References

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Table 5.1: Types of sewage samples used in the isolation of polioviruses from selected water treatment plants in South Africa

System	Design	Waste Type	Population size
Daspoort (DP)	3-stage Bardenpho	100% Domestic	200 000
Daveyton (D)	3-stage Bardenpho	100% Domestic	13 000
Grundlingh (GR)	3-stage Bardenpho	60% Domestic and 40% Industrial	5 000
Heidelberg (Hb)	3-stage Bardenpho	70% Domestic and 30% Industrial	10 000
Mccomb (Mcc)	3-stage Bardenpho	100% Industrial	5 000
Modderfontein (MF)	3-stage Bardenpho	40% Domestic and 60% Industrial	3 000
Olifantsfontein (OF)	3-stage Bardenpho	60% Domestic and 40% Industrial	100 000
Tsakane (Ts)	3-stage Bardenpho	100% Domestic	20 000
Rynfield (RnF)	5-stage Phoredox	100% Domestic	20 000
Vlakplaats (VP)	3-stage Bardenpho	70% Domestic and 30% Industrial	130 000

Table 5.2: Enterovirus and poliovirus specific primers[a] used in the RT-multiplex PCR (Egger *et al.*, 1995)

Primer region and map position[b]	Primer	Sequence[c]	Amplicon length (bp)	Specificity
5'UTR 163-178 443-460	 E1 E2	 5'-AAG CAC TTC TGT TTC C-3' 5'-CAT TCA GGG GCC GGA GGA-3'	297	 EV EV
P2 region 4460-4478 4634-4653	 Po1 Po2	 5'-CAG TTC AAG AGC AA (A/G) CAC C-3' 5'-TC (A/G) TCC AT (A/G) AT (A/C) AC (T/C) AC (T/A) CC-3'	193	 PV PV
P2-P3 region 4922-4941 5467-5487	 Po3 Po4	 5'-GAA ATG TGT AAG AAC TGT CA-3' 5'-GTA ACA ATG TTT CTT TTA GCC-3'	565	 PV PV

***a* = Primers E1 and E2 were described previously (Hyypiä *et al.*, 1989).**
***b* = Map position and sequence of PV type 1 (Sabin) are given.**
***c* = For the degenerate primers Po1 and Po2, the sequence of the Sabin strain is underlined.**

Table 5.3: 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 5.4: 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 5.5: 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
0.3	AJ783729	DP1 (Daspoort sewage) (13/09/2001)	PV1	AJ783857	0.8
0.3	AJ783732	Kspnt DR (river water) (15/04/2002)	PV1	AJ783810	0.8
0.3	AJ635236	OF1 (Olifantsfontein sewage) (21/05/2002)	PV1	AJ635237	0.9
0	AJ783727	Ts1 (Tsakane sewage) (11/09/2002)	PV1	AJ783847	0
0.3	AJ783726	RnF2 (Rynfield sewage) (17/09/2002)	PV1	AJ783848	0
0.3	AJ783724	MF3 (Modderfontein sewage) (08/10/2002)	PV1	AJ783853	0.6
0.3	AJ783722	D2 (Daveyton sewage) (08/10/2002)	PV1	AJ783858	0.8
0.3	AJ783723	Mcc4 (Mccomb sewage) (08/10/2002)	PV1	AJ783856	0.6
0	AJ783725	Mcc1 (Mccomb sewage) (22/10/2002)	PV1	AJ783855	0.5
0	AJ783728	Sbnd2 (river water) (09/01/2003)	PV1	AJ783806	0
0	AJ783730	Lv1 (river water) (09/01/2003)	PV1	AJ783808	0
0	AJ783731	Mb1 (river water) (15/04/2003)	PV1	AJ783809	0
0.7	AJ783721	Sbnd1 (river water) (15/04/2003)	PV1	AJ783805	0.6
0.5	AJ783737	OF2 (Olifantsfontein sewage) (21/05/2002)	PV2	AJ783813	0.7
0.5	AJ783720	OF2 (Olifantsfontein sewage) (28/05/2002)	PV2	AJ783804	0.2
0.9	AJ783735	OF2 (Olifantsfontein sewage) (02/07/2002)	PV2	AJ783859	0.2
0.5	AJ783736	TCspntDR (river water) (16/07/2002)	PV2	AJ783811	0
0.5	AJ783733	D1 (Daveyton sewage) (28/08/2002)	PV2	AJ783852	1.4
0.5	AJ783734	Mt2 (river water) (15/04/2003)	PV2	AJ783807	0.2
0.5	AJ783774	DP1 (Daspoort sewage) (13/08/2001)	PV3	AJ783812	0.2
0.5	AJ783770	VP1 (Vlaakplats sewage) (09/07/2002)	PV3	AJ783854	0.2
0.5	AJ783739	MF1 (Modderfontein sewage) (23/07/2002)	PV3	AJ783815	0.2
0.5	AJ783772	GR2 (Grundlingh sewage) (23/07/2002)	PV3	AJ783850	0.2
0.5	AJ783773	OF5 (Olifantsfontein sewage) (23/07/2002)	PV3	AJ783849	0.2
0.5	AJ783738	Hb1 (Heidelberg sewage) (11/09/2002)	PV3	AJ783814	0.2
0.5	AJ783771	D9 (Daveyton sewage) (08/10/2002)	PV3	AJ783851	0.2

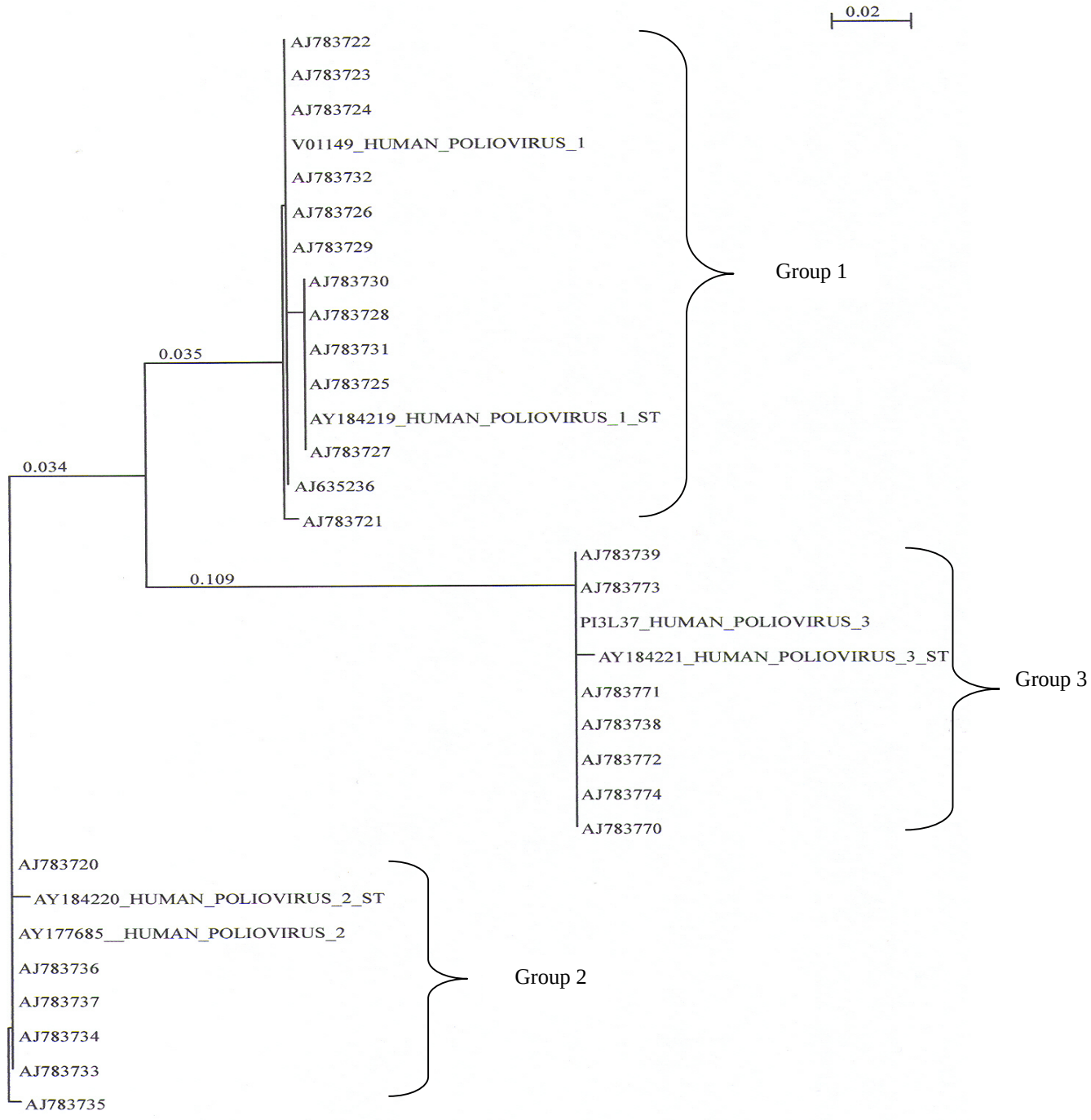

Figure 5.1: **Unrooted phylogenetic tree re-constructed with the neighbour-joining method from the comparative 5'untranslated region sequence analysis of the sewage isolated oral poliovirus vaccine strains and the poliovirus reference strains. Branch lengths are proportional to the phylogenetic distances, while the vertical branches are non-informative. The scale bar shows 2% nucleotide sequence difference**

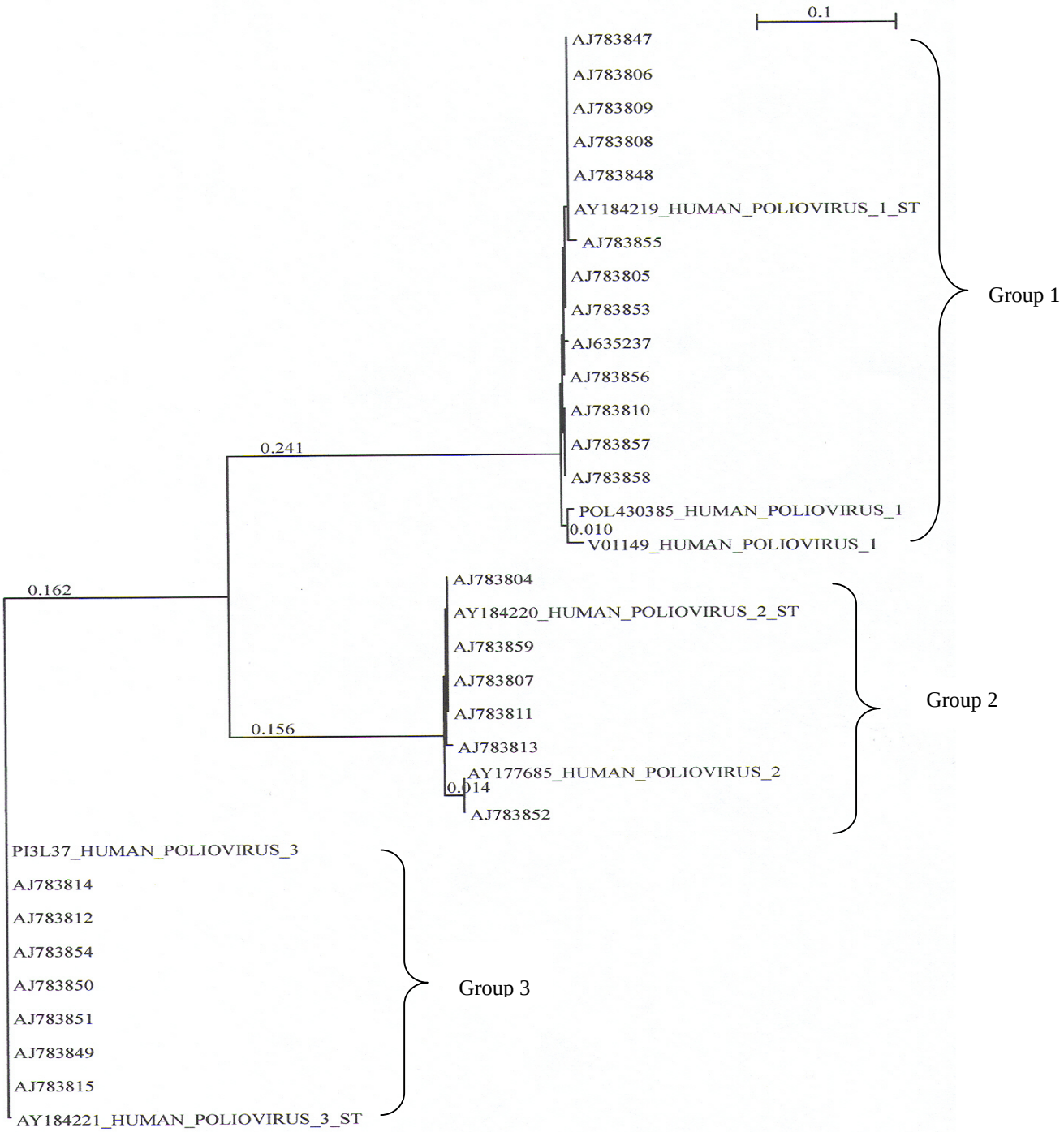

Figure 5.2: **Unrooted phylogenetic tree re-constructed with the neighbour-joining method from the comparative VP1 region sequence analysis of the sewage isolated oral poliovirus vaccine strains and the poliovirus reference strains. Branch lengths are proportional to the phylogenetic distances, while the vertical branches are non-informative. The scale bar shows 10% nucleotide sequence difference**