GENOMIC MUTATIONS IN ORAL POLIOVIRUS VACCINE STRAINS: IMPLICATIONS FOR THE ERADICATION OF POLIOVIRUS

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GENOMIC MUTATIONS IN ORAL POLIOVIRUS VACCINE STRAINS: IMPLICATIONS FOR THE ERADICATION OF POLIOVIRUS

by

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I, the undersigned, declare that the thesis hereby submitted to the University of Pretoria for the degree PhD (Medical Virology) and the work contained therein is my own original work and has not previously, in its entirely or in part, been submitted to any university for a degree.

Signed:_____ this_____day of_____2004

"In all human affairs… there is a single dominant factor- time. To make sense of the present state of science, we need to know how it got like that: we cannot avoid an historical account… To extrapolate into the future we must look backwards a little into the past."

J.M. Ziman

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TABLE OF CONTENTS

GENOMIC MUTATIONS IN ORAL POLIOVIRUS VACCINE STRAINS: IMPLICATIONS FOR THE ERADICATION OF POLIOVIRUS

by

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SUMMARY

Large epidemics of poliomyelitis spread across the world in the first half of the 20th century. However, polio incidence fell rapidly across the world following the introduction of the oral poliovirus vaccine (OPV). Since the introduction of immunisation with OPV, the vaccine had a remarkable track record of success, because the number of wild-type polio cases decreased from 350 000 to 500 and the number of polio endemic countries declined from 125 to 10. Thus, the global eradication of wild-type poliovirus (PV) seems a realistic goal for the foreseeable future.

Despite its many advantages, one disadvantage of the OPV is the potential risk of revertants of the OPV strains, which may cause neurological complications in vaccine recipients and susceptible contacts. Immunocompetent persons excrete OPV strains for a limited period of time. In contrast, immunodeficient people may become chronically infected and excretion times as long as 10 years have been reported. As a consequence, in the last phase of polio eradication this group of people may serve as potential reservoirs for vaccine-derived polioviruses (VDPVs). Two cases of vaccine-associated paralytic poliomyelitis have been reported in human immunodeficiency virus (HIV)-positive children, although, presently

there is no evidence for prolonged excretion of PV from patients with HIV and acquired immunodeficiency syndrome (AIDS). Highly evolved VDPVs have been isolated from sewage and river water even in the absence of cases of paralytic poliomyelitis.

This study aimed to investigate the prevalence of PVs in sewage and river water as well as in stool specimens of HIV-positive children (including those with an AIDS indicator condition according to the Centers for Disease Control and Prevention classification). Secondly, the study investigated the occurrence of genomic mutations in these OPV isolates.

A total of 49 PV vaccine strains were isolated from the sewage and river water, and 13 PV vaccine strains were detected in the stools of immunodeficient children. Two of the immunodeficient patients (vaccinated 15 months ago) tested positive for Sabin PVs type 1 and 3. Another immunodeficient patient (vaccinated 42 months ago) tested positive for Sabin PV type 1.

The 5'untranslated and the VP1 regions in the genomes of the OPV isolates were partially sequenced. The majority of the OPV strains detected in the sewage and river water displayed >99% VP1 sequence identity to the original PV vaccine strains and were classified as "OPV-like viruses". Two OPV isolates were identified as "suspected" VDPVs, since these isolates showed ≤99% VP1 sequence identity to the PV vaccine strains and had probably replicated in one or more people for 12 to 16 months since the administration of the initiating OPV dose. In contrast, three "suspected" immunodeficient VDPVs were identified in the stools of the immunodeficient children. All of the OPV-like and "suspected" VDPV isolates carried genomic mutations, which had been associated with reversion of the attenuated PV phenotypes to increased neurovirulence.

The identification of OPV-like and "suspected" VDPVs in this study emphasised the fundamental importance regarding the control of health risks constituted by OPV vaccination, particularly with regard to immunodeficient individuals such as HIV-positive children, and the possible role of water in the transmission of potentially hazardous VDPVs. These research findings provided valuable data, concerning prolonged excretion of OPV strains by individuals with secondary immunodeficiency and this could have major implications for strategies aimed for the global post-polio eradication era.

GENOMIESE MUTASIES IN ORALE POLIOVIRUS VAKSIEN STAMME: IMPLIKASIES VIR DIE UITWISSING VAN DIE POLIOVIRUS

deur

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OPSOMMING

Groot epidemies van poliomiëlitis het wêreldwyd gedurende die eerste helfte van die 20ste eeu voorgekom. Die voorkoms van polio het skerp afgeneem met die ingebruikneming van die orale poliovaksien (OPV). Sedert die begin van immunisering met OPV, het die vaksien merkwaardige sukses getoon, aangesien die aantal wilde-tipe poliogevalle afgeneem het vanaf 350 000 tot 500 terwyl die aantal lande waar polio endemies voorgekom het, vanaf 125 tot 10 gedaal het. Gevolglik blyk die toekomstige wêreldwye uitwissing van wilde-tipe poliovirus (PV) 'n realistiese doelwit te wees.

Ten spyte van die vele voordele van OPV, blyk die potensiële risiko van terugmutering van die OPV stamme ‘n belangrike nadeel te wees, aangesien dit tot neurologiese komplikasies in gevaksineerdes en vatbare kontakte kan lei. Immuunkompetente persone skei OPV stamme vir 'n beperkte tydperk uit. In teenstelling hiermee kan immuunonderdrukte persone kronies geïnfekteer word en gevalle waar OPV vir so lank as 10 jaar uitgeskei is is al gerapporteer. As gevolg hiervan kan hierdie groep persone as ‘n potensiële reservoir van vaksien-afkomstige poliovirusse (VAPV) optree gedurende die laaste fase van polio-uitwissing. Twee gevalle van vaksien-geassosieerde paralitiese poliomiëlitis is al gerapporteer in menslike immuniteitsgebrek virus (MIV)-positiewe kinders, nogtans is daar

tans geen bewyse van verlengde uitskeiding van PV in pasiënte met MIV en verworwe immuniteitsgebrek sindroom (VIGS) nie. Hoogs gemuteerde VAPV is al geïsoleer vanuit riool- en rivierwater selfs in die afwesigheid van gevalle van paralitiese poliomiëlitis.

Hierdie studie het gepoog om die voorkoms van poliovirusse in riool- en rivierwater asook in stoelgange van MIV-positiewe kinders (ingesluit die met ‘n VIGS indikator kondisie volgens die “Centers for Disease Control and Prevention” klassifikasie) te ondersoek. Tweedens is die voorkoms van genomiese mutasies in hierdie OPV isolate ondersoek.

'n Totaal van 49 PV vaksienstamme is geïsoleer vanuit riool- en rivierwater, en 13 PV vaksienstamme is opgespoor in die stoelgange van imuunonderdrukte kinders. Twee van die imuunonderdrukte pasïente (15 maande tevore gevaksineer) het positief vir Sabin PV tipe 1 en 3 getoets. 'n Volgende imuunonderdrukte pasïent (42 maande tevore gevaksineer) het positief vir Sabin PV tipe 1 getoets.

Die nukleotied volgorde van die 5’ongetransleerde en die VP1 gebiede in die genome van die OPV isolate is gedeeltelik bepaal. Die meeste van die OPV stamme wat in die riool- en rivierwater gevind is, het >99% ooreenstemmig getoon tussen die VP1 nukleotied volgordes en die van die oorspronklike PV vaksienstamme en is daarvolgens as "OPV-soortgelyke virusse" geklassifiseer. Twee OPV isolate is geïdentifiseer as “verdagte” VAPV, aangesien hierdie isolate ≤99% ooreenstemmigheid getoon tussen hulle VP1 nukleotied volgordes en die volgordes van die oorspronklike PV vaksienstamme wat aantoon dat hierdie isolate moontlik in een of meer persone vir 12 tot 16 maande vanaf die aanvanklike OPV toediening gerepliseer het. Daarteenoor, is drie “vedagte” imuunonderdrukte VAPV vanuit die stoelgange van imuunonderdrukte kinders geïdentifiseer. Al die OPV-soortgelyke en “verdagte” VAPV isolate het genomiese mutasies bevat wat geassosieer word met terugmutering van die verswakte PV fenotipes wat kan lei tot toenemende neurovirulensie.

Die identifisering van OPV-soortgelyke en “verdagte” VAPV in hierdie studie het die fundamentele belangrikheid van die kontrolering van gesondheidsrisikos wat gepaard gaan met OPV vaksinering beklemtoon, veral in die geval waar imuunonderdrukte individue soos MIV-positiewe kinders betrokke is, asook die moontlike rol wat water in die oordrag van potensieel gevaarlike VAPV kan speel. Hierdie navorsingsbevindings het waardevolle inligting gelewer, met betrekking tot die verlengde uitskeiding van OPV stamme deur

individue met sekondêre imuunonderdrukking. Dit kan groot implikasies inhou vir strategieë gemik op die wêreldwye post-polio uitwissingsera.

LIST OF ABBREVIATIONS

AFP	Acute flaccid paralysis
AIDS	Acquired immunodeficiency syndrome
AMPS	Ammonium per sulphate
ATCC	American Type Culture Collection
BCG	Bacille Calmette-Guérin vaccine
BGM	Buffalo green monkey kidney
bp	Base pair
C	Concentration
CAV	Coxsackievirus A
CBV	Coxsackievirus B
CDC	Centers for Disease Control and Prevention
cm	Centimetre
CNS	Central nervous system
CO_2	Carbon dioxide
CPE	Cytopathogenic effect
CSF	Cerebrospinal fluid
cVDPV	Circulating vaccine-derived polioviruses
CVID	Common variable immunodeficiency
DNA	Deoxyribonucleic acid
dNTP	DiNucleotide triphosphate
DT	Diphtheria, tetanus vaccine
DTP	Diphtheria, tetanus, pertussis vaccine
ECACC	European Collection of Cell Culture
ECV	Echovirus
EDTA	Ethylenediaminetetraacetate
ELISA	Enzyme linked immunosorbent assay
EMBL	European Bioinformatics Institute
EV	Enteroviruses
FCS	Foetal calf serum
Fig	Figure
g	Gram
g	Gravitational force
h	Hour
HCl	Hydrochloric acid
HEp-2	Human epidermoid carcinoma
HIV	Human immunodeficiency virus
H_2O	Water

Ile	Isoleucine
ITD	Intratypic differentiation
IPV	Inactivated poliovirus vaccine
iVDPV	Immunodeficient vaccine-derived polioviruses
kb	Kilobytes
KCl	Potassium chloride
L	Litre
L20B	Mouse L cells
M	Molar
mA	Milliampere
MEM	Minimum Essential Medium
µg	Microgram
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulphate
µl	Microlitre
ml	Millilitre
min	Minute
mm	Millimetre
mM	Millimolar
NaCl	Sodium chloride
ng	Nanogram
NIDs	National immunisation days
NIV	National Institute for Virology
nm	Nanometre
NPEVs	Non-polio enteroviruses
OPV	Oral poliovirus vaccine
ORF	Open reading frame
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PEI	Poliomyelitis Eradication Initiative
Pen/strep	Penicillin/streptomycin
PFU	Plaque forming units
Phe	Phenylalanine
PLC/PRF/5	Primary liver carcinoma
pmol	Picomole
ppm	Parts per million
PV	Poliovirus
PVR	Poliovirus receptor
RD	Rhabdomyosarcoma
RE	Restriction enzyme

RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT-PCR	Reverse transcription polymerase chain reaction
s	Second
Ser	Serine
Thr	Threonine
U	Unified atomic mass unit
USA	United States of America
UTR	Untranslated region
UV	Ultraviolet light
V	Volume
Val	Valine
VAPP	Vaccine-associated paralytic poliomyelitis
VDPV	Vaccine-derived poliovirus
VP	Virus protein
V/W	Vaccine/wild
WHO	World Health Organization

LIST OF TABLES

LIST OF FIGURES

LIST OF PUBLICATIONS AND CONFERENCE CONTRIBUTIONS

SUBMITTED PUBLICATIONS:

Ehlers, M.M., Grabow, W.O.K., Pavlov, D.N. (2004a) Detection of enteroviruses in untreated and treated drinking water supplies in South Africa. Submitted for publication in *Water Research.*

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Pavlov, D.N., Van Zyl, W.B., Kruger, M., Blignaut, L., Grabow, W.O.K., Ehlers, M.M (2004) Isolation of poliovirus vaccine strains from stool specimens of immunodeficient children in South Africa. To be submitted for publication in the *Journal of Clinical Virology*.

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CONFERENCE CONTRIBUTIONS:

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Ehlers, M.M., Grabow, W.O.K., Pavlov, D.N. (2003) Detection of enteroviruses in raw and treated drinking water supplies in South Africa. Poster presentation (CT-54) at the IWA Health Related Microbiology, 14 -19 September 2003, Cape Town, South Africa.

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CHAPTER 1

INTRODUCTION

In 1988, the World Health Assembly resolved to eradicate poliomyelitis globally by the year 2000 and this term was later postponed until 2005 (Centers for Disease Control and Prevention [CDC], 2003; World Health Organization [WHO], 2003). Substantial progress has been achieved towards this goal (CDC, 2003; WHO, 2003) and with the eliminated circulation of wild-type poliovirus (PV) in most parts of the world, attention has focussed on examining the potential for vaccine-derived polioviruses (VDPVs) to circulate where wild-type PV has disappeared.

The Sabin live attenuated oral poliovirus vaccine (OPV) has been effectively used in the reduction and control of poliomyelitis. The OPV has had a remarkable track record of success since the number of wild-type polio cases decreased from 350 000 to less than 500 and the number of polio endemic countries declined from more than 125 to 10 (Wood and Thorley, 2003). The last case of polio, caused by a wild-type PV in South Africa occurred in 1989 (CDC, 2003). Despite the advantages in using the attenuated OPV, one disadvantage is the potential risk of revertants of the PV vaccine strains, which may cause neurological complications in vaccine recipients and their susceptible contacts (Minor, 1992). During prolonged replication of the attenuated PV vaccine strains in humans different genomic modifications such as mutations, deletions, insertions and recombinations may occur, thus, leading to the almost invariable reversion of the OPV strains to increased neurovirulence (Bellmunt *et al.,* 1999; Hovi *et al.,* 2004).

As OPV-derived strains are excreted in nasopharyngeal secretions and stool after vaccination, this vaccine could become a source of dissemination of PVs and the potential cause of poliomyelitis (Bellmunt *et al.,* 1999). The choice of strategies for the termination of immunisation depends, among other things, on the circulation of VDPVs and their potential health implications (Dowdle *et al.,* 1999). Although the transmission of vaccine strains of PVs from person-to-person in family situations is well established, little is known about

circulation in the community, especially in settings of susceptible populations such as immunodeficient individuals (Dowdle *et al.*, 1999).

Rapid evolution is characteristic of both wild and vaccine-derived polioviruses. Nucleotide substitutions accumulate at a rate of approximately 1% per year and consist primarily of changes at synonymous codon positions, in other words, do not result in amino acid changes at those loci (Kew *et al.*, 1995; Martin *et al.*, 2000). The mutations initially appearing and fixed into the genomes of the Sabin PV vaccine strains upon administration of OPV are frequently associated with reversion of the attenuated phenotype and alteration of the neutralising antigenic sites of the OPV strains (Minor, 1992). Reversion of the OPV strains to increased neurovirulence is one key factor for the occurrence of vaccine-associated paralytic poliomyelitis (VAPP), which occurs at a rate of approximately 1 per 500 000 first doses of OPV in immunocompetent individuals, and at a 3 000 fold higher rate in immunodeficient patients (Sutter and Prevots, 1994).

Poliovirus excretion in immunocompetent hosts is usually short lived, seldom exceeding 2 months (Wood *et al.*, 2000). Hovi and colleagues (2004) have shown that excretion of wild-type PV by healthy children may continue for at least 6 months and is associated with the accumulation of single nucleotide substitutions during replication within an individual host. In the early 1960s and 1970s, several instances of prolonged excretion of VDPV for up to 2 years were reported, all among individuals suffering from B-cell deficiencies (Wood *et al.*, 2000). Bellmunt *et al.* (1999) reported the complete sequence of two PV isolates obtained from a patient with common variable immunodeficiency syndrome (CVID). The isolates were taken after the onset of paralysis and after 5.5 years of continuous virus excretion demonstrating evolutionary changes toward a “wild-type-like” genotype (Bellmunt *et al.*, 1999). Combined anamnestic and evolutionary data indicated about 10 years of persistent enteral PV replication (Bellmunt *et al.*, 1999). Similar molecular analyses from other examples of long-term polio excretion among individuals with primary immunodeficiencies have been recently reported (Kew *et al.*, 1998; Martin *et al.*, 2000; Minor, 2001; Buttinelli *et al.*, 2003).

The spectrum of possibilities for behaviour of PVs in immunodeficient individuals was illustrated by the accidental discovery in Europe of an immunodeficient man who was

carrying highly evolved VDPV type 2 strain (Minor, 2001; MacLennan *et al.,* 2004). This individual is known to have been excreting VDPV type 2 for an estimated 20 years and is still excreting at present without showing any clinical symptoms (MacLennan *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). "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 Sabin PV vaccine strains (WHO, 2004). "Vaccine-derived polioviruses" are those strains showing ≤99% VP1 sequence identity to the parental Sabin PV vaccine strains and are uncommon (WHO, 2004). The sequence drift shown in VDPVs is indicative of prolonged replication of the vaccine strain either in one individual or in the community (circulating VDPVs) (WHO, 2004).

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 vaccine strain suggested that the virus had replicated in one or more individuals for approximately 6 years (Shulman *et al.,* 2000). More recently, a highly evolved VDPV type 3 harbouring a 13% sequence drift from Sabin PV type 3 vaccine strain has been isolated from sewage in Estonia (Blomqvist *et al.,* 2004). The presence in the environment of highly evolved, neurovirulent VDPV strains in the absence of polio cases would have important implications for strategies to interrupt immunisation with OPV following global polio eradication.

Concerns about the potential risks constituted by VDPV strains are supported by recent outbreaks of acute flaccid paralysis (AFP) caused by circulating VDPVs that have been reported in four different regions of the world, namely the Middle East (Egypt), the Americas (Hispaniola: Dominican Republic and Haiti), the Western Pacific (Philippines), and Africa (Madagascar) (Kew *et al.,* 2004). In all four cases, the outbreaks occurred in pockets of unvaccinated or incompletely vaccinated individuals (Kew *et al.,* 2004). During the Hispaniola outbreak, VDPV type 1 was spread in the poorly vaccinated population and was able to cause more than 20 paralytic cases (Kew *et al.,* 2004). In Madagascar, five cases of AFP associated with VDPV 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).

These findings suggest, that the final step of PV eradication will require details on the possibility of persistent infections and excretion of VDPVs for long periods by immunodeficient patients, and the survival in the environment of these strains to the extent that they may infect non-immune individuals after termination of PV vaccination (Fine and Carneiro, 1999). Research is required to provide information in the following priority areas: the extent and duration of circulation of VDPVs in populations; risk factors for prolonged replication of PV among immunodeficient individuals; assessment of the prevalence and behaviour of VDPV strains in the environment (notably water resources) and in different population settings (Wood *et al.,* 2000).

There is little information on secondary immunodeficiency as a risk factor for VAPP or prolonged PV excretion (Wood *et al.,* 2000). The likelihood of prolonged PV excretion in cohorts of infected with human immunodeficiency virus (HIV) children is being investigated in several developing countries (Wood *et al.,* 2000; Haisey *et al.,* 2004). The current situation in South Africa offers opportunities well suited for research along these lines, since OPV immunisation is compulsory in the country. The purpose of this study was to isolate OPV strains from stool specimens of immunodeficient children as well as from environmental water samples (sewage and river water), and to investigate the genetic features of these PV isolates using advanced molecular techniques.

The objectives of this study were as follows:

1. To investigate the circulation of OPV strains in the environment (sewage and river water).
2. To isolate OPV strains from stool specimens of immunodeficient patients at Kalafong Hospital, such as HIV-positive children (including those with an acquired immunodeficiency syndrome [AIDS] indicator condition, according to the CDC classification).
3. To determine the occurrence of genomic mutations in the OPV isolates.
4. To determine the prevalence of VDPV strains in the environment and in stool specimens of immunodeficient patients.

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CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Poliomyelitis, or polio, is a life-threatening acute paralytic disease caused by poliovirus (PV), a member of the genus *Enterovirus* in the family Picornaviridae (Melnick, 1996a; Hovi *et al.*, 2004). Like other ribonucleic acid (RNA) viruses, PVs exist as mixtures of microvariants, called quasispecies (Mulders *et al.*, 1999). This is caused by the error-prone, virus-encoded RNA polymerase, which lacks proof-reading activity, resulting in a rapid accumulation of mutations upon replication (Mulders *et al.*, 1999; Hovi *et al.*, 2004). An additional mode of generating divergence among PVs and other enteroviruses (EVs) is their ability to recombine with other serotypes (intertypic recombinants) or with another genome of the same serotype (intratypic recombinants) (Mulders *et al.*, 1999; Hovi *et al.*, 2004). During replication in humans and upon transmission between hosts, some of the mutations are enriched, which has resulted in numerous genetic lineages within each serotype of PV that co-circulate worldwide (Mulders *et al.*, 1999).

To date, there are three PV serotypes, designated type 1, type 2 and type 3, that were originally distinguished from the other EVs by neutralisation with serotype-specific antisera and the propensity to cause paralytic illness (Bodian *et al.*, 1949; Georgopoulou *et al.*, 2000). Furthermore, PVs have been associated with seasonal undifferentiated febrile illness, particularly during summer outbreaks and enteroviral meningitis (Melnick, 1996a; Georgopoulou *et al.*, 2000).

Protective immunity against poliomyelitis is conferred through immunisation or natural PV infection (Ghendon and Robertson, 1994; Wood *et al.*, 2000; Centers for Disease Control and Prevention [CDC], 2002a). The use of highly efficacious PV vaccines, the oral live attenuated vaccine made from the Sabin strains (oral poliovirus vaccine [OPV]) and the inactivated Salk vaccine (inactivated poliovirus vaccine [IPV]), has resulted in a dramatic global decrease in the circulation of wild-type PVs (Wood *et al.*, 2000; Cherkasova *et al.*,

2002). Due to the ability of OPV to induce a higher level of intestinal immunity (providing long-term protection against polio through durable humoral immunity), the ability to spread and immunise unvaccinated contacts of vaccine recipients (increasing the impact of OPV), plus the advantages of oral administration and lower costs, made OPV the vaccine of choice for the poliomyelitis eradication initiative (PEI) (Wood *et al.*, 2000; Kew *et al.*, 2004).

Immunisation with OPV has been so effective that the global eradication of wild-type PV seems a realistic goal for the foreseeable future (Cherkasova *et al.*, 2002). Since the PEI was launched in 1988, extraordinary progress has been made to stop transmission of wild-type PV and to achieve global certification of eradication by 2005 (World Health Organization [WHO], 2004). By the end of 2002, the number of wild-type polio cases decreased from 350 000 to less than 500 and the number of polio endemic countries declined from more than 125 to 7 (Wood and Thorley, 2003; WHO, 2004). Poliomyelitis transmission has been interrupted in the American, European and Western Pacific Regions, and by the end of 2002 more than 180 countries and territories were declared as polio-free (Wood and Thorley, 2003; WHO, 2004). The last case of polio, caused by a wild-type PV in South Africa occurred in 1989 (CDC, 2003). However, this global initiative may be jeopardised due to recent outbreaks of polio in several African countries such as Botswana, Guinea, Mali and Sudan (ProMED-mail, 2004a). The current eradication strategies recommended by the WHO include: (1) high, routine infant immunisation coverage with at least three doses of OPV plus a dose at birth in polio-endemic countries; (2) national immunisation days (NIDs) targeting all children <5 years; acute flaccid paralysis (AFP) surveillance and laboratory investigations; and (4) mop-up immunisation campaigns with OPV to interrupt final chains of transmission (WHO, 2004).

However, the success of the OPV was tempered by its genetic lability, because mutations at critical sites of the live attenuated PV during genomic replication have resulted in loss of attenuation and concomitant increase in neurovirulence (Wood and Thorley, 2003; Kew *et al.*, 2004). If these mutations lead to poliomyelitis in a vaccine recipient or a close contact, it is defined as vaccine-associated paralytic poliomyelitis (VAPP) (Wood and Thorley, 2003). Long-term persistence (in some instances up to several years) of vaccine-derived polioviruses (VDPVs) in immunodeficient individuals and the ability of the evolved variants to cause paralytic disease are well-established phenomena (Kew *et al.*, 1998; Bellmunt *et al.*, 1999; Martin *et al.*, 2000; Cherkasova *et al.*, 2002). Outbreaks of poliomyelitis in Belarus

(1965-1966), Egypt (1988-1993), Hispaniola (2000-2001), the Philippines (2001) and Madagascar (2001-2002) associated with circulating VDPVs (cVDPVs), support the notion that there is a significant risk of prolonged circulation of the PV vaccine strains in populations with low immunity level, as well as their conversion into epidemic strains (Cherkasova *et al.,* 2002; Kew *et al.,* 2004). Highly evolved VDPVs have been isolated from environmental samples (such as sewage and river water) even in the absence of apparent cases of paralytic poliomyelitis (Shulman *et al.,* 2000; Cherkasova *et al.,* 2002; Horie *et al.,* 2002; Yoshida *et al.,* 2002; Blomqvist *et al.,* 2004).

The purpose of this study was, firstly, to isolate OPV strains from the environment (such as selected sewage and river water samples) and from stool specimens of children infected with human immunodeficiency virus (HIV) (including those with acquired immunodeficiency syndrome [AIDS] indicator condition according to the CDC classification) at Kalafong Hospital, South Africa. Secondly, this study aimed to determine the presence of mutations in the OPV genomes (associated with reversion of attenuation to increased neurovirulence) and to determine the prevalence of VDPVs in the stool specimens of the immunodeficient children studied as well as the environment.

2.2 History of poliomyelitis

Sporadic cases of paralytic poliomyelitis have been occurring for at least as long as human history has been recorded (Melnick, 1996a). In 1920, the former United States president F.D. Roosevelt developed a febrile illness during his summer vacation that was followed by paralysis (Zaoutis and Klein, 1998). However, the first evidence of any human disease being attributed to a PV infection was a funerary stele from Middle Kingdom Egypt, dated at ~1300 BC, which depicted the priest Rom with the classical withered limb and down-flexed foot that is a well-known characteristic of poliomyelitis (Minor, 1999). Since ancient times and into the late 1800s, PVs were widely distributed in most of the world's populations, surviving in an endemic fashion by continuously infecting susceptible infants newly born into the community (Melnick, 1996a).

Although records from antiquity mention crippling diseases compatible with poliomyelitis, it was Michael Underwood from Britain who, in 1789, first described debility of the lower extremities in children that was recognisable as poliomyelitis (CDC, 2002a). The first

outbreaks of paralytic poliomyelitis were reported in Europe (initially in Sweden) and North America in the early 19th century (CDC, 2002a). These epidemics became increasingly severe, more frequent, more widespread and the average age of persons affected rose (CDC, 2002a). Cases of infantile paralysis began to be observed in adolescents and in young adults (Melnick, 1996a; Minor, 1999). This was primarily due to improved sanitation, so that children were older when they were first exposed to PV infection and therefore, no longer protected by the antibodies that they had passively acquired from their mothers (Minor, 1999).

Large epidemics of poliomyelitis spread across the world in the first half of the 20th century (Melnick, 1996a). In the United States in the summer of 1916, over 27 000 persons were reported to have been paralysed, with 6 000 deaths (Melnick, 1996a). In New York alone, more than 9 000 cases and more than 2 000 deaths were recorded (Melnick, 1996a). In 1952, over 21 000 paralytic cases were reported in the United States (Melnick, 1996a; CDC, 2002a).

Polio incidence, however, fell rapidly across the world following the introduction of effective vaccines and the global WHO-sponsored PEI (Muir *et al.*, 1998; CDC, 2002a). The last case of wild-type PV acquired in the United States was reported in the year 1979, whereas the last case of polio associated with a wild-type PV in South Africa occurred in 1989 (CDC, 2002a; CDC, 2003).

2.3 Clinical manifestations of poliovirus infections

A specific protein receptor on susceptible human cells allows the attachment and entry of PV in the human body (Melnick, 1996a). As the virus travels from the portal of entry (the mouth), implantation and multiplication take place in the oropharynx from where the PV enters the blood stream and infects other susceptible tissues, such as lymph nodes and the central nervous system (CNS) (Melnick, 1996a). The incubation period is between 7 and 14 days but may range from 2 to 35 days (Melnick, 1996a). Infected persons without symptoms shed PVs in faeces and are able to transmit PVs to other people (Melnick, 1996a; CDC, 2002a).

The response to PV infection is variable and has been categorised based on the severity of clinical presentation (CDC, 2002a). Ninety percent or more of wild-type PV infections are asymptomatic or unapparent (Rotbart, 1997). Three clinical syndromes are attributed to PV infection, namely abortive poliomyelitis, aseptic meningitis and paralytic poliomyelitis (Melnick, 1996a; Zaoutis and Klein, 1998). As the infection progresses, a minor illness may be followed by a major, severe illness (Melnick, 1996a; Rotbart, 1997). However, such biphasic course is more common in young children and infants than in adults (Melnick, 1996a; Rotbart, 1997).

Approximately 4% - 8% of PV infections are characterised by a minor, non-specific illness without clinical or laboratory evidence of CNS invasion (CDC, 2002a). This syndrome is known as abortive poliomyelitis and results in upper respiratory infection (sore throat and fever), gastrointestinal disturbances (nausea, vomiting, abdominal pain, constipation or diarrhoea) as well as influenza-like illness (CDC, 2002a). Nearly 10% of patients with abortive poliomyelitis will develop aseptic meningitis (non-paralytic poliomyelitis) indistinguishable from the minor illness due to non-polio enteroviruses (NPEVs) (Rotbart, 1997). Typically, symptoms (stiffness of the neck, back and legs) will last from 2 to 10 days, followed by a complete recovery (Rotbart, 1997; CDC, 2002a).

Less than 2% of all polio infections result in flaccid paralysis, which begins with a minor febrile illness followed by a short asymptomatic period of 2 to 3 days (Zaoutis and Klein, 1998). A sudden onset of asymmetric flaccid paralysis with no significant sensory loss is the characteristic finding of paralytic disease (Zaoutis and Klein, 1998). Paralysis is presented by severe myalgia in the involved limb resulting from involvement of single or multiple muscle groups (Rotbart, 1997). Motor and sensory disturbances may be observed in the same affected muscle groups (Rotbart, 1997).

Paralytic polio is classified into three types (spinal, bulbar and bulbospinal polio) depending on the level of involvement (CDC, 2002a). Spinal polio is characterised by asymmetric paralysis mostly of the legs (CDC, 2002a). Cranial nerve involvement may result in bulbar paralysis, which leads to difficulties in breathing, speech, swallowing, eye movement and facial muscle involvement (Rotbart, 1997). Bulbospinal polio accounted for 19% of cases in the United States during the period of 1969 to 1979 and was due to a combination of bulbar and spinal paralysis (Melnick, 1996a; Rotbart, 1997; CDC, 2002a).

2.4 Genomic characterisation of poliovirus

Polioviruses are small (27 - 30 nm in diameter; 1.34 g.ml^{-1} buoyant density), non-enveloped viruses, consisting of a simple protein capsid and a single strand of positive sense RNA, which constitutes approximately 30% of the virion mass (Rotbart, 1997). The capsid contains four proteins, VP1 to VP4, arranged in 60 repeating protomeric units of an icosahedron (Figure 2.1) (Rotbart, 1997; Verlinden *et al.*, 2002). Each protomer carries a single attachment site for the PV receptor (PVR) molecule, termed a canyon; five protomers for each of the 12 pentamers corresponding to 60 PVR binding-site canyons per virion (Arita *et al.*, 1999). Neutralisation sites are densely clustered on the VP1 protein, whereas VP4 is in close association with the RNA core, functioning as an anchor to the capsid (Rotbart, 1997). Destabilisation of the VP4 protein would result in virus uncoating (Rotbart, 1997).

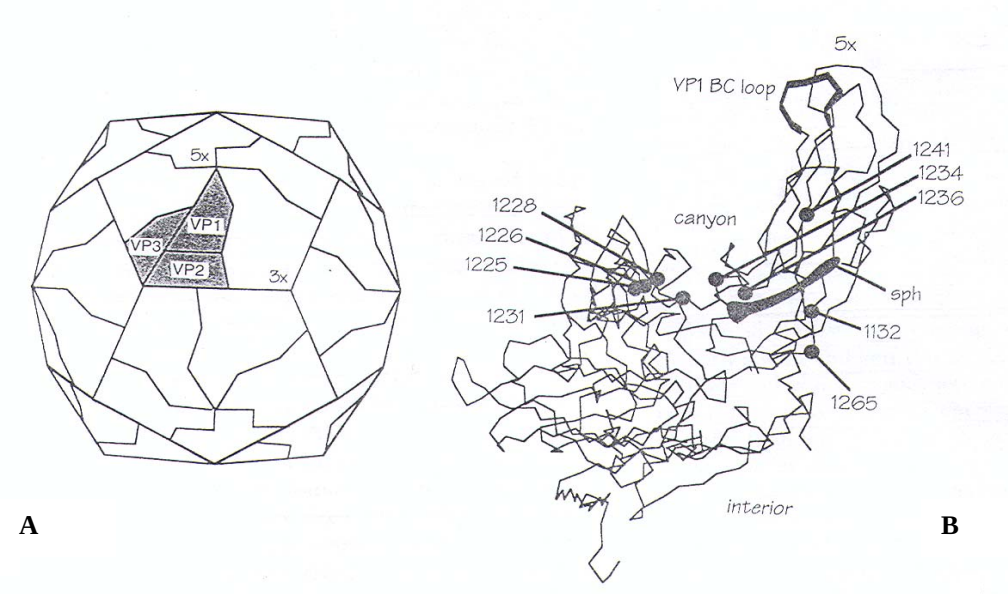

Figure 2.1: (A) Schematic representation of the icosahedral viral capsid structure of polioviruses. The fivefold (5x) and three fold (3x) axes of symmetry are indicated, as is the position of one of the 60 repeating protomeric units, each comprised of VP1, VP2 and VP3 surface proteins. (B) Line drawing of the VP1 and VP2 proteins in their tertiary configuration (Rotbart, 1997)

The genomic RNA of PV (approximately 7.4 kb in length) is infectious when introduced into host cells and serves as a template for both viral protein translation and RNA replication (equivalent to messenger RNA) (Rotbart, 1997; Muir *et al.*, 1998; Verlinden *et al.*, 2002).

At the 5'end of the genome there is a virally coded, covalently linked polypeptide (Vpg) (Figure 2.2) (Muir *et al.*, 1998; Minor, 1999). A 5'untranslated region (5'UTR or also known as 5'non-coding region [5'NCR]) consisting of approximately 750-nucleotides is followed by a long open reading frame (ORF) coding for an approximately 2 100 amino-acid polyprotein, which is succeeded by a short 3'untranslated region (3'UTR) and a polyadenylate (A) tail (Figure 2.2) (Muir *et al.*, 1998; Martin and Minor, 2002).

The polyprotein is cleaved as it is translated by virus-encoded proteases (P2A, P3C and uncleaved P3CD), producing the active proteins that are involved in virus replication (Minor, 1999). The structural proteins (VP1, VP2, VP3 and VP4) that form the shell of the virus particle are encoded before the non-structural proteins (P2A, P2B, P2C, P3A, P3B, P3C and P3D), which are involved in the replication of the PV genome and alter the host cell's synthetic machinery to produce viral proteins (Minor, 1999).

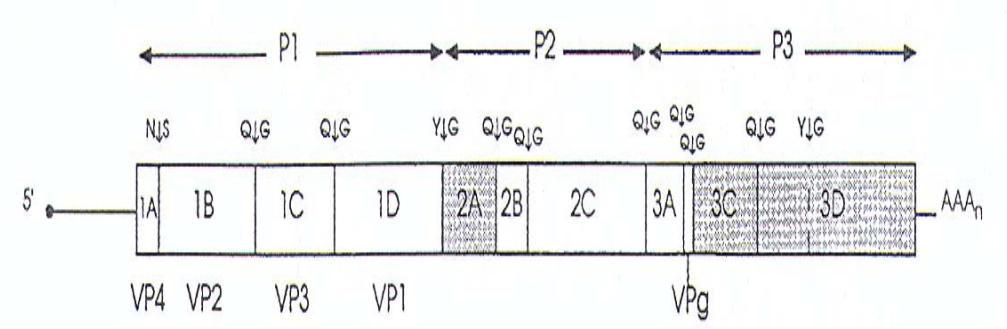

Figure 2.2: **Genomic organisation of poliovirus type 1 (Mahoney). The polyprotein encoded by the single open reading frame is shown as an elongated rectangle, the 5' and 3'untranslated regions are shown as lines and the genome-linked protein (VPg) is indicated by a black arrow. Cleavage sites between individual viral proteins are shown above the genome at appropriate locations; these proteins are described within the rectangle according to the L434 nomenclature (Rueckert and Wimmer, 1984); the capsid proteins 1AB, 1A, 1B, 1C and 1D are commonly referred to as VP0, VP4, VP2, VP3 and VP1, respectively. The proteinases 2A^{pro}, 3C^{pro} and 3CD^{pro} are represented by shaded boxes. The structural protein precursor P1 and the non-structural protein precursors P2 and P3 are indicated above the polyprotein (Muir *et al.*, 1998)**

Several important functions of the 5'UTR, the ORF and the 3'UTR of PV have been identified (Muir *et al.*, 1998; Martin and Minor, 2002). The first 500 nucleotides of the 5'UTR play an important role in viral replication (internal initiation of translation rather than the ribosome-scanning model proposed for cellular mRNAs) (Muir *et al.*, 1998). Point mutations in this region are known to affect virulence, temperature sensitivity and plaque morphology (Minor, 1992; Muir *et al.*, 1998).

The ORF following the 5'UTR is translated into a polyprotein, which is co- and post-translationally cleaved to give four structural proteins (VP4, VP3, VP2 and VP1) and seven non-structural proteins (P2A, P2B, P2C, P3A, P3B, P3C and P3D) (Muir *et al.*, 1998). Four neutralisation determinants have been identified and have been mapped to VP1, VP2 and VP3 (Minor, 1992; Muir *et al.*, 1998). Determinants of attenuation of virulence, virion thermostability, altered host range, *in vitro* cell tropism, persistent infection and plaque morphology have been mapped to the capsid-encoding region (Muir *et al.*, 1998).

The coding region in the PV genome is followed by a 70 to 100 nucleotide 3'UTR (Muir *et al.*, 1998; Martin and Minor, 2002). This region is important in the initiation of the minus-strand RNA synthesis, but no specific sequences have been identified for polymerase binding (Muir *et al.*, 1998). A genomic polyadenylate (A) tail with an average length of 75 nucleotides follows the 3'UTR (Muir *et al.*, 1998; Martin and Minor, 2002).

Development of a molecular typing system for EVs requires an understanding of the structure and function of the EV genome and a knowledge of the variability of genome sequences among EVs (Muir *et al.*, 1998). A number of PV genomes have been sequenced completely and the extensive knowledge concerning the molecular biology of PV, suggests that it may be possible to understand virulence and attenuation in molecular terms (Minor, 1992).

2.5 Mode of transmission of poliovirus

There are several routes of PV transmission but in most developing countries such as South Africa the faecal-oral route remains the most common mode of transmission (Melnick, 1996b; Zaoutis and Klein, 1998). Poliovirus replicates efficiently in the human intestinal tract and is shed in stools for several weeks (typically for 2 to 4 weeks) depending on the

immune status and immune competence of the individual (Fogarty *et al.*, 1995; Zaoutis and Klein, 1998).

Factors such as the extent of crowding, levels of hygiene, water quality and sewage handling facilities may affect the transmission of PV (Benenson, 1995; Zaoutis and Klein, 1998). In areas of good sanitary conditions and uncontaminated drinking water, other routes of transmission such as secretions from the upper respiratory tract are more important (Fogarty *et al.*, 1995; Zaoutis and Klein, 1998). Respiratory tract secretions are infectious (since the PV replicates in the upper respiratory tract) and may provide a source of virus for close contact spread through direct person-to-person contact, large-particle aerosols, or vomitus (Fogarty *et al.*, 1995; Zaoutis and Klein, 1998). Thus, although person-to-person spread of PV via the faecal-oral route seems as the most important route of transmission, the oral-oral route may account for some of the cases for PV infection (CDC, 2002a).

2.6 Survival of poliovirus in nature

Humans are the only known natural reservoirs of PV (Dowdle and Birmingham, 1997; WHO, 1999; CDC, 2002b). Higher non-human primates (chimpanzees and gorillas) are susceptible to infection and disease but their populations are too small to sustain PV transmission in the absence of human infection (Dowdle and Birmingham, 1997; WHO, 1999; CDC, 2002b).

Poliovirus in the environment is the direct result of recent PV infections in the human community (WHO, 1999; CDC, 2002b). Soil may become contaminated as a result of human defecation near dwellings, crop fertilisation with untreated or inadequately treated sewage and the recycling of wastewater for irrigation (WHO, 1999; CDC, 2002b). Surface water may become contaminated through the discharge of untreated or inadequately treated sewage or through run-off from contaminated soil (WHO, 1999; CDC, 2002b).

In general, wild-type PVs have a distinct seasonal pattern of circulation that may vary in geographic areas (WHO, 1999; CDC, 2002b). In tropical and semitropical areas circulation tends to be year round (or associated with the rainy season), whereas in temperate areas PVs are most prevalent in summer and fall although outbreaks may continue into the winter

(WHO, 1999; CDC, 2002b). Vaccine-like PVs can be found constantly in countries, which routinely use OPV specifically around the time of the NIDs (WHO, 1999; CDC, 2002b).

Polioviruses are distinguished from the other picornaviruses on the basis of physical properties such as buoyant density in caesium chloride and stability in weak acids (WHO, 1999; CDC, 2002b). Polioviruses are relatively heat resistant, resistant to acid pH (pH 3 to 5 for one to three hours) and resistant to many common detergents and disinfectants, including common soap, non-ionic detergents, alcohol, cresol, chloroform, deoxycholate, ether, phenol, quaternary ammonium compounds and other lipid solvents (Melnick, 1996b; WHO, 1999; CDC, 2002b). The virus is readily inactivated by dilute solutions of formaldehyde or free residual chlorine (bleach), ultraviolet light, heat (50°C or more) and drying (Melnick, 1996b; WHO, 1999; CDC, 2002b). Treatment with 0.3% formaldehyde, 0.1 M HCl, or free residual chlorine at a level of 0.3-0.5 parts per million (ppm) causes rapid inactivation, but the presence of extraneous organic matter may protect the PV from such inactivation (Melnick, 1996b; WHO, 1999; CDC, 2002b). Chlorine bleach (0.5%) is the recommended disinfectant for laboratories working with PVs (Melnick, 1996b; WHO, 1999; CDC, 2002b).

Rates of PV inactivation in nature are influenced by the immediate environment (WHO, 1999; CDC, 2002b). According to scientific data, PV infectivity in soil decreases by 90% every 20 days in winter and every 1.5 days in summer, at temperatures (±25°C) a 90% decrease in infectivity occurs in sewage every 26 days, in freshwater every 5.5 days and in seawater every 2.5 days (WHO, 1999; CDC, 2002b).

Although it is known that PV exists widely in nature, in soil, sewage, drinking water and food such as shellfish, there is little evidence to connect it directly with an outbreak of poliomyelitis (Goyal *et al.*, 1979; Metcalf *et al.*, 1995; Jaykus, 1997; Yoshida *et al.*, 2002). In most cases infection with PV are not apparent, because the infection is only recognised when secondary, person-to-person transmission leads to the onset of poliomyelitis (Yoshida *et al.*, 2002). Therefore, it would be difficult to address the risk of infection by PV from the environment (Metcalf *et al.*, 1995; Yoshida *et al.*, 2002).

2.7 Poliovirus vaccines

Protective immunity against poliomyelitis is conferred through immunisation or natural PV infection (Ghendon and Robertson, 1994; Wood *et al.*, 2000; CDC, 2002a). Immunity is PV serotype-specific (Ghendon and Robertson, 1994). Protection against infection is associated with both circulating antibodies in the blood and secretory antibodies in the gut and upper respiratory tract, which prevent the spread of PV to the CNS (Ghendon and Robertson, 1994; Wood *et al.*, 2000; CDC, 2002a).

Poliovirus is the only EV for which a vaccine is available (Zaoutis and Klein, 1998). The first clinical trials of vaccines against poliomyelitis were carried out in 1935 (Minor, 1999). These vaccines were made from the ground-up spinal cord of infected monkeys and were of limited success, because of their association with poliomyelitis at a high frequency in recipients (Minor, 1999). In 1955, the first successful vaccine against poliomyelitis was developed by Jonas Salk and contained the formaldehyde-inactivated poliovirus (known as inactivated poliovirus vaccine or IPV) (Zaoutis and Klein, 1998; Minor, 1999). In 1963, Albert Sabin developed an oral, live attenuated poliovirus vaccine (OPV) that contained all three PV serotypes (Zaoutis and Klein, 1998; Wood *et al.*, 2000). After its introduction, the OPV was adopted rapidly world-wide as the vaccine of choice (Zaoutis and Klein, 1998). Virologists were of the opinion that Salk's vaccine (IPV) could not provide long-lasting protection and that this could only be achieved with the Sabin's live attenuated version, which had sufficient immunogenicity to provide protection (Blume and Geesink, 2000). However, none of these vaccines (IPV and OPV) can provide 100% immunity against infection or re-infection with PV (Zaoutis and Klein, 1998; Blume and Geesink, 2000; Wood *et al.*, 2000).

2.7.1 Inactivated poliovirus vaccine

Two enhanced forms of inactivated poliovirus vaccine (IPV) are currently available in the United States, but only one vaccine (IPOL, Pasteur Merieux Connaught) is distributed (CDC, 2002a). This vaccine is prepared by growing the virus in a type of monkey kidney tissue culture (Vero cell line) and inactivation is achieved with formaldehyde (CDC, 2002a). The vaccine contains 2-phenoxyethanol, neomycin, streptomycin, polymyxin B (used to prevent bacterial and fungal growth) and all three serotypes of PV (CDC, 2002a).

The IPV is very effective in inducing circulating antibodies in the blood, thus preventing PV in the gut from entering and replicating in the CNS (Wood *et al.*, 2000). The use of IPV in several Northern European countries (Denmark, Finland, Sweden, The Netherlands) succeeded in effectively eliminating wild-type PV circulation (Lapinleimu, 1984; WHO, 2003a). The IPV provides protection for many years after a complete series, however, this duration of immunity is not certain (Wood *et al.*, 2000; CDC, 2002a).

Although IPV is very effective in inducing circulating antibodies against PV for individual protection, it is less effective than OPV in inducing mucosal immunity at replication sites in the gastrointestinal tract (Wood *et al.*, 2000). The IPV stimulates production of serum antibodies in the bloodstream, which cannot prevent the PV from initially multiplying in the intestine (Pelczar *et al.*, 1993). However, these serum antibodies do prevent PV in the bloodstream from reaching the spinal cord and causing paralysis (Pelczar *et al.*, 1993). The OPV produces long-lasting mucosal immunity by stimulating the formation of secretory IgA antibodies in the intestine and also serum antibodies in the bloodstream (Pelczar *et al.*, 1993). Thus, mucosal immunity restricts viral replication following exposure to PV and is important for community protection (Wood *et al.*, 2000). The intestinal secretory antibodies can prevent the primary intestinal infection by neutralising the infectivity of virulent PV strains that a person may encounter later (Pelczar *et al.*, 1993).

2.7.2 Oral poliovirus vaccine

Different strategies were used to produce early versions of live attenuated PV strains, starting from either virulent or naturally attenuated PV isolates obtained from humans (Sabin and Boulger, 1973; Martin and Minor, 2002). Sabin 1, CHAT and Cox are all attenuated strains derived from Mahoney strain PV type 1 and selected on the basis of their lack of neurovirulence in monkeys to be used as vaccines (Sabin and Boulger, 1973; Martin and Minor, 2002). Sabin 1 was developed from passage in monkey tissues, whereas the CHAT and Cox strains involved passages in monkey, mouse and chicken embryo cell substrates (Martin and Minor, 2002).

The trivalent OPV contains live attenuated strains of all three serotypes of PV in a ratio of 10:1:6, because of the higher immunogenicity to PV types 2 and 3 compared to PV type 1 (CDC, 2002a; Kew *et al.*, 2004). These attenuated PV strains replicate in the human gut,

inducing mucosal immunity that inhibits replication of the virus in the gastrointestinal tract (CDC, 2002a; Wood and Thorley, 2003; Kew *et al.*, 2004). A single dose of OPV produces immunity to all three PV vaccine strains in about 50% of the recipients and three doses of OPV will produce immunity in 95% of the recipients (CDC, 2002a). According to scientific reports, PV is excreted from healthy children for not more than 2-3 months following vaccine administration (Marker Test Subcommittee and the Japan Live Poliovaccine Research Commission, 1967; Alexander *et al.*, 1997).

The OPV has been found to confer longer-lasting immunity, so that repeated boosters are not necessary and acts quickly, immunity being achieved in a matter of days (Blume and Geesink, 2000; Wood and Thorley, 2003; Kew *et al.*, 2004). Most importantly, the OPV offers the prospect of passive vaccination, because it causes an active infection of the human gastrointestinal tract that results in the excretion of live attenuated virus (Blume and Geesink, 2000; Wood and Thorley, 2003; Kew *et al.*, 2004). Thus, through faecal-oral transmission OPV is capable of protecting those who have not been vaccinated (herd immunity) (Blume and Geesink, 2000; Hovi *et al.*, 2004).

The effective use of the OPV by many countries involved with the global PEI, has nearly achieved elimination of wild-type PV circulation (Kew *et al.*, 2004). However, maintenance of high immunisation coverage is crucial to protect against imported wild-type PVs and to prevent person-to-person transmission of OPV-derived viruses (Buttinelli *et al.*, 2003). It is important that all countries maintain a high quality acute flaccid paralysis (AFP) surveillance system and that a global strategy is developed for the cessation of OPV immunisation after global certification of polio eradication (Buttinelli *et al.*, 2003).

2.8 Genetic basis for the attenuation of Sabin vaccine strains of live attenuated poliovirus

The attenuation of neurovirulence of a PV strain can be defined as the inability of the virus to replicate in neural cells (Equestre *et al.*, 1991). Such PV strains have lost their neurovirulence, but not the capacity to multiply in the gut and to induce a type specific protection against subsequent infection with neurovirulent PVs (Friedrich, 2000). The basis of attenuation or reversion of the vaccine strains (whereby a PV recovers its ability to cause disease) has been studied by comparing the vaccine strain of each serotype with a closely

related strain, either a precursor of the vaccine strain or an isolate from a VAPP case (Minor, 1999).

The genome of Sabin PV type 1 differs from the parental virulent Mahoney PV by 54 point mutations, 20 of which are coding changes (Georgescu *et al.*, 1997; Martin and Minor, 2002). The large number of mutations have been used to explain the relatively lower rate of VAPP cases with the Sabin PV type 1 (Martin and Minor, 2002). However, the main mutation that is responsible for the attenuation of the Sabin PV type 1 includes this at residue 480 in the 5'UTR (Figure 2.3) (Minor, 1999; Martin and Minor, 2002). Mutations in the structural proteins at residues 65 of VP4, 225 of VP3, 106 and 134 of VP1 all have an attenuating effect (Minor, 1999). However, none of the mutations in any of these non-structural proteins have been implicated as a major factor in changes of PV virulence for any of the serotypes (Minor, 1999).

Several differences have been found to exist between the Sabin PV type 2 vaccine strain and the wild PV type 2 (Figure 2.3) (Minor, 1999). Mutations, at residue 481 (in which a G is converted to an A) in the 5'UTR and at base 2 908 or 2 909, which produces an amino acid change from an isoleucine (IIe) to a valine (Val) at residue 143 of the virus protein coat VP1 (VP1-143), are responsible for attenuating the wild PV type 2 (Macadam *et al.*, 1993; Minor, 1999; Martin and Minor, 2002). All derivatives of the Sabin PV type 2 associated with disease in humans are reported to have lost these attenuating mutations and both mutations are selected against in the human gut (Equestre *et al.*, 1991; Macadam *et al.*, 1993).

The genome of the Sabin PV type 3 differs from that of the virulent wild-type progenitor at 12 nucleotide positions, three of which determine the attenuated phenotype (Georgescu *et al.*, 1997). Two mutations, at 472 bp in the 5'UTR and at 2 034 bp in the capsid protein VP3 (VP3-91) (which produces an amino acid change at residue 91 of the virus protein coat VP3 from a serine [Ser] to a phenylalanine [Phe]), are responsible for the attenuation of the PV type 3 (Figure 2.3) (Macadam *et al.*, 1993; Georgescu *et al.*, 1997; Minor, 1999; Martin and Minor, 2002). Recent evidence suggested that a third mutation at 2 493 bp in the capsid protein VP1 may be involved, leading to an amino acid change from isoleucine (Ile) to threonine (Thr) at residue 6 of capsid protein VP1 (VP1-6) (Tatem *et al.*, 1992; Macadam *et al.*, 1993; Georgescu *et al.*, 1997; Martin and Minor, 2002).

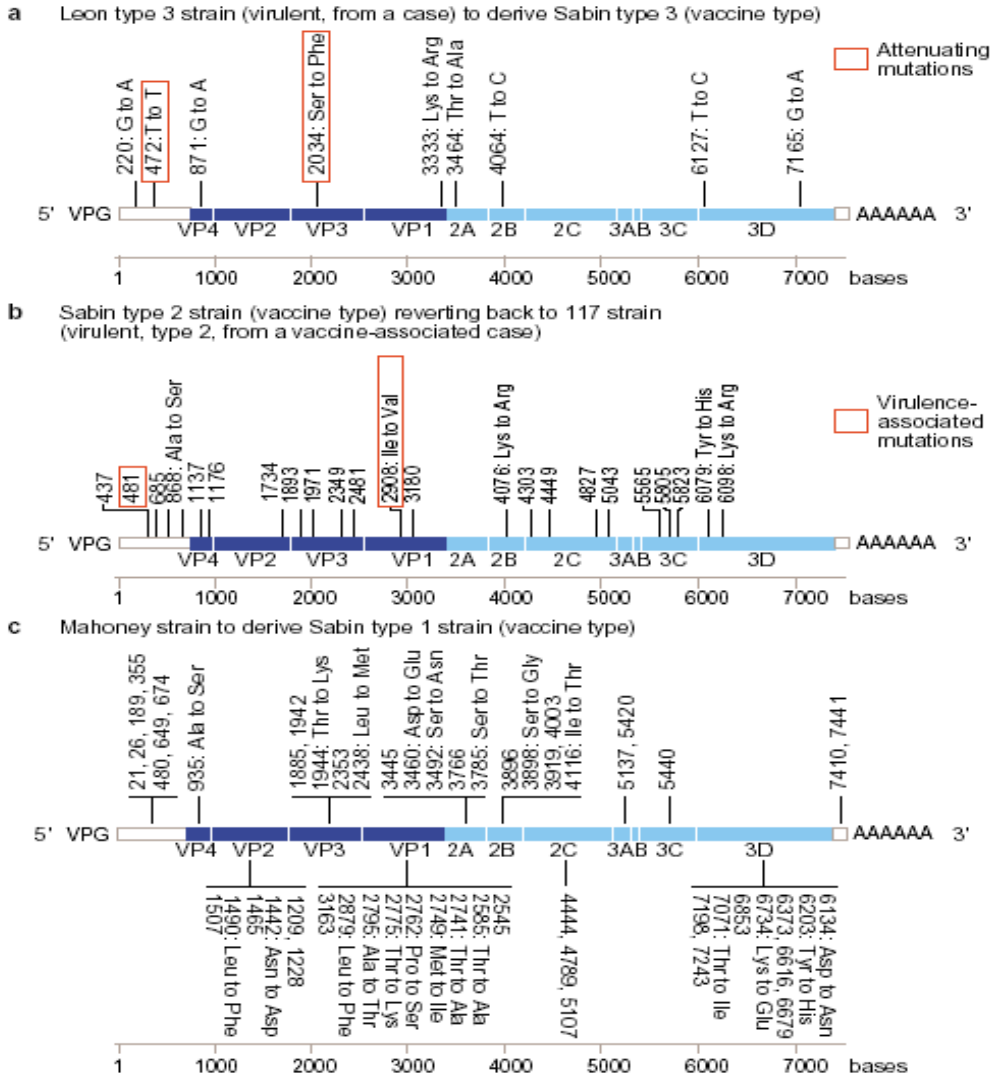

Figure 2.3: Comparisons of the sequences of polioviruses used to study the basis of attenuation and reversion of the Sabin vaccine strains of poliovirus. (A) Mutations that are involved in deriving the Sabin type 3 vaccine strain from the Leon strain. (B) Attenuating mutations in the Sabin PV type 2. (C) Mutations that are involved in deriving the Sabin PV type 1 strain from the Mahoney strain. The most common base changes and the amino acid differences produced in the encoded protein are shown (Minor, 1999)

Thus, the molecular biology of the live attenuated Sabin PV vaccine strains has been studied extensively and interestingly a few mutations are required to account for the greater part of the attenuated phenotype (Minor, 1992). The attenuating mutations of the OPV strains are strongly selected against when the vaccine replicates in the intestinal tract of OPV recipients

(Kew *et al.*, 2004). However, the OPV strains proved capable of rapid, extensive and precise variation in the vaccine recipient to adapt from the attenuated form to a form able to grow successfully in the host, causing almost no disease (Minor, 1992).

2.9 Complications resulting from the use of oral poliovirus vaccine

In the global PEI, the WHO included OPV in the packet of subsidised vaccines for poor countries because of its lower cost and long-term efficacy (Blume and Geesink, 2000). However, there were growing suspicions that in a small number of cases, mostly adults, the live attenuated PV vaccine could lead to paralytic poliomyelitis or the so-called VAPP (Blume and Geesink, 2000; Wood *et al.*, 2000; Wood and Thorley, 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 virus leads to a more neurotropic form of the PV vaccine strain, known as vaccine-derived poliovirus (VDPV) (Minor, 1992; Bellmunt *et al.*, 1999; Wood and Thorley, 2003; Hovi *et al.*, 2004). Although mutations, associated with reversion of attenuation to increased neurovirulence in the PV vaccine strains, occur generally in immunodeficient patients, the same mutations have been shown to occur in healthy immunocompetent vaccine recipients (Minor, 1999). In fact, VDPVs type 2 and type 3 isolated from VAPP cases and those isolated from healthy recipients are indistinguishable in their properties (Minor, 1992; Minor, 1999). Since VDPVs are excreted in nasopharyngeal secretions and stools after prolonged replication in the human body, OPV could become a source for the dissemination of these potentially neurovirulent PV strains in the environment as well as in the community and the potential cause of VAPP (Friedrich, 1998; Bellmunt *et al.*, 1999).

Cases of VAPP usually occur within 70 days following vaccination, however, VAPP has not been connected with an outbreak of poliomyelitis due to circulation of the virus within a population (Wood and Thorley, 2003). Vaccine-derived polioviruses type 2 and 3 have frequently been associated with VAPP, and rarely VDPV type 1 (only 10% of all VAPP cases) (Guillot *et al.*, 2000; Wood and Thorley, 2003). The large number of mutations (the attenuating phenotype of Sabin PVs type 2 and 3 has been ascribed to a few mutations, compared to 54 point mutations in Sabin PV type 1) has usually been thought to explain the relatively lower rate of VAPP cases with the Sabin PV type 1 (Martin and Minor, 2002). The vaccine-associated paralysis is identical to that caused by the wild-type PV and may be

permanent (Guillot *et al.*, 2000).

The overall incidence of VAPP in the United States is estimated to be 1 in 530 000 for first-time vaccine recipients or 1 in 200 000 for vaccine recipients overall (Minor, 1999). Since 1980 to 1999, a total of 144 cases of VAPP in the United States have been caused by the live OPV (American Academy of Paediatrics Committee on Infectious Diseases, 1999). The risk of VAPP has increased by a factor of 10 to 14 over the last three decades in some European countries such as Romania (Guillot *et al.*, 2000). The last VAPP case in Italy occurred in the year 2000 in an immunodeficient child (with agammaglobulinaemia) who received three doses of OPV (Buttinelli *et al.*, 2003). The child developed paralysis several months after vaccination and a Sabin-like type 2 PV was isolated (Buttinelli *et al.*, 2003).

Thus, genetic differences distinguish OPV strains from their parental strains and characteristic mutations are associated with attenuation of neurovirulence of parental strains (WHO, 2004). Typically, OPV strains will mutate during their replication in the human intestine and some mutations may result in recovery of the capacity for higher neurovirulence and lead to VAPP (WHO, 2004).

2.10 Molecular changes of poliovirus vaccine strains in vaccine recipients

Poliovirus vaccine strains are reputed to grow less efficiently in the human intestine than wild-type epidemic PVs (Guillot *et al.*, 2000). A large number of genetic variations are likely to arise during multiplication of these Sabin PV strains in the human digestive tract (Guillot *et al.*, 2000). Two mechanisms may be responsible for these variations: mutation and recombination (Guillot *et al.*, 2000). Theoretically, recombination is a more powerful mechanism than mutation, because it may transfer a number of properties to the original virus in a single event (Guillot *et al.*, 2000).

Vaccine-derived polioviruses, with mutations and recombinations known to result in increased neurovirulence, have been isolated from stool and the CNS of patients with VAPP cases, from patients with other OPV-associated diseases and from healthy vaccinees (Lipskaya *et al.*, 1991; Macadam *et al.*, 1993; Georgescu *et al.*, 1994; Li *et al.*, 1996; Friedrich, 2000). Recent studies have isolated VDPVs with genomic modifications from immunodeficient patients and from environmental samples as well (Divizia *et al.*, 1999;

Muscillo *et al.,* 1999; Friedrich, 2000; Buttinelli *et al.,* 2003).

Given the high frequency of recombinant genomes in the OPV strains excreted by healthy vaccinees, their contacts in the community and VAPP patients, it seems that genetic recombination is possibly involved in the natural evolution of Sabin PV vaccine strains (Guillot *et al.,* 2000). Recombination alone or in combination with reverse mutations at the attenuating sites of Sabin PV vaccine strains is not sufficient to render the original vaccine virus highly neurovirulent (Guillot *et al.,* 2000). However, this moderate increase in neurovirulence is sufficient to allow VDPVs to cause poliomyelitis in people, as demonstrated by the identification of such strains as the etiological agents in certain VAPP cases (Georgescu *et al.,* 1997; Guillot *et al.,* 2000).

2.10.1 Mutations in Sabin poliovirus vaccine strains

Each time the three serotypes of PV present in OPV replicate in the gastrointestinal tract, random mutations are incorporated into the viral genomes (Wood and Thorley, 2003). Mutations, which attenuate the vaccine strains of PV, have been shown to be reverted or suppressed in the isolates taken from normal healthy vaccine recipients and VAPP cases so that the particular nucleic acid bases are the same as those found in the virulent strain (Minor, 1999). Loss of the original attenuated phenotype of Sabin PV vaccine strains has been attributed to single- or multiple-nucleotide substitutions at precise sites of the genome (Guillot *et al.,* 2000).

Molecular characterisation of VDPV type 1 from VAPP cases confirmed that several of the Sabin PV type 1 mutations reverted to wild-type Mahoney PV 1 sequences following replication in the human intestine but indicated that reversion at only few of those mutations, which in all cases included the mutation at position 480 in the 5'UTR, had a more pronounced effect in increasing the neurovirulence of Sabin PV type 1 (Li *et al.,* 1996; Georgescu *et al.,* 1997; Martin and Minor, 2002). In Sabin PV type 1 vaccine strains, the attenuating mutation at position 480 is lost in approximately 50% of the vaccine recipients possibly due to a second-site suppression (Minor, 1999; Martin and Minor, 2002). Analysis of four isolates from cases of VAPP related to the CHAT vaccine (attenuated PV type 1 derived from the wild-type Mahoney PV 1) revealed genetic and phenotypic properties of the CHAT strain following replication in the human gut (Martin and Minor, 2002). The CHAT-

VAPP strain 134 contained a viral genome highly evolved from CHAT showing 1.1% nucleotide changes in the VP1 region (Martin and Minor, 2002). This difference corresponded to approximately 1 year of replication and circulation in humans, based on the "molecular clock" of PV evolution (nucleotide substitutions accumulate at a rate of approximately 1% per year) (Kew *et al.*, 1998; Martin and Minor, 2002). The CHAT-VAPP case 134 was classified as a community case, no contact with a vaccinee or any of his/her contacts was identified and therefore, it may have involved at least two steps of human-to-human transmission (Martin and Minor, 2002). Similarly, a Sabin type 1-VAPP isolate from Romania (1-IIs strain) has been reported to have contained 0.95% nucleotide changes in the VP1 region with respect to the Sabin PV type 1 genome (Georgescu *et al.*, 1997; Martin and Minor, 2002).

Among the Sabin PV type 1 strains analysed by Georgescu and colleagues (1997), the most neurovirulent strain (1-IIs) was highly divergent from the Sabin PV vaccine strain, with 71 nucleotides differences, including 13 missense mutations. The localisation of these mutations indicated that this strain was not a Mahoney-like wild-type PV, but a strain derived from Sabin PV type 1, since the 44 nucleotides which differentiate the Sabin PV type 1 from the Mahoney PV were maintained (Georgescu *et al.*, 1997). Thus, the large divergence of strain 1-IIs from the Sabin PV type 1 and even more from the Mahoney strain reflected *in vivo* genetic variation, suggesting the circulation of this vaccine strain for a long period of time in the community (Georgescu *et al.*, 1997).

Mutations at base 481 in the 5'UTR and at nucleotide 2 908 or 2 909 in the codon of amino acid (aa) 143 of VP1 are known as the two mutations important for the reversion of Sabin PV type 2 to an increased neurovirulence, although other mutations such as the mutation at nucleotide 398 might have some effect in increasing the neurovirulence as well (Friedrich, 2000). The attenuating mutations of Sabin PV type 2 at base 481 in the 5'UTR is lost 7 days following immunisation and at residue 143 of VP1 is lost in some of the PVs isolated from healthy vaccinees (Minor, 1999). According to reports, VDPV type 2 (exhibiting 93% - 96% nucleotide sequence identity to the Sabin type 2 OPV strain) remained in circulation for 10 years originating from populations with low immunity levels in Egypt (CDC, 2002a). Other incidents of prolonged persistence of VDPV type 2 occurred in China and Israel (Zhang *et al.*, 1998; Shulman *et al.*, 2000).

According to Minor (1999), in vaccine recipients who excrete PV type 3 vaccine strains, the base at position 472 in the 5'UTR of type 3 PV always reverts to the base that is found in the virulent strain by day 3 and within 6 days after vaccination. By day 11 following vaccination, Sabin PV type 3 loses the effects of both attenuating mutations (at base 472 in the 5'UTR and at 2 034 bp in the VP3) and may recombine its genome with the genome of either type 1 or type 2 VDPVs (Minor, 1999). Individuals can continue to excrete these PVs for 5 to 6 weeks following vaccination and 1% can continue for 10 weeks (Minor, 1999). Thus, a VDPV type 3 strain (carrying an U to C reversion in position 472 in the 5'UTR) was isolated from a 3.5 month-old child in Albania, who had received one dose of OPV 10 days before the onset of paralysis and was classified as having VAPP (Fiore *et al.*, 1998).

The identification of highly drifted VDPV isolates such as the CHAT-VAPP strain 134 and the strain 1-IIs (Georgescu *et al.*, 1997) from the Sabin PV type 1 vaccine strain represents clear evidence that these PV strains can survive in the human population for long periods, possibly even within well-immunised communities (Martin and Minor, 2002). These PV strains are a potential source of poliomyelitis epidemics, particularly in populations with low polio immunity and in the absence of wild-type PV competitors (Martin and Minor, 2002).

2.10.2 Recombination in Sabin poliovirus vaccine strains

Frequently occurring homologous recombination provides an additional mode of generating divergence among PVs and EVs (Hovi *et al.*, 2004). It has been suggested that recombination could increase the neurovirulence or has some replication advantage for VDPVs (Friedrich, 2000). Vaccine-derived poliovirus strains with recombinant intertypic genomes have been found to occur naturally and to be selected frequently in the intestine of VAPP patients (Lipskaya *et al.*, 1991; Georgescu *et al.*, 1994; Guillot *et al.*, 2000). Vaccine/wild (V/W) PV recombinants have been found, in which vaccine-specific segments of the Sabin PV genome have been replaced by non-vaccine sequences derived from wild-type PV or from NPEVs (Lipskaya *et al.*, 1991; Guillot *et al.*, 2000). According to a study conducted by Guillot and colleagues (2000), non-vaccine sequences were found to be present in 6% of VDPV strains (V/W PV recombinants) and OPV-derived sequences were detected in 9% of the genomes of wild strains (Guillot *et al.*, 2000).

According to a study conducted by Friedrich (2000), nucleotide sequence analysis of isolates from VAPP cases and their healthy contacts confirmed the transmission of mutant PV type 2 with recombinant genomes (Friedrich, 2000). In this study, isolate P2/1400 from a patient with VAPP and isolate P2/1402 from one of his healthy contacts had the same nucleotide sequences in the regions analysed (such as the 5'UTR and VP1) confirming the transmission of a VDPV type 2 (Friedrich, 2000). Characterisation of VDPV type 3 isolates from healthy vaccinees identified recombinant genomes as well (Friedrich, 2000).

Recombination alone or in combination with reverse mutations at the attenuating sites of Sabin PVs is not sufficient to render the original vaccine virus highly neurovirulent (Guillot *et al.*, 2000). However, this moderate increase in neurovirulence is sufficient to allow Sabin PVs to cause poliomyelitis in people, as demonstrated by the identification of such strains as the etiological agents in certain VAPP cases (Georgescu *et al.*, 1997; Guillot *et al.*, 2000).

2.11 Vaccine-associated paralytic poliomyelitis and immunodeficiency

Although immunodeficiencies are listed as contraindication for receiving OPV, patients with these clinical conditions may occasionally receive OPV before their immunodeficiency is diagnosed and/or may be infected with OPV strains excreted by a vaccinee or circulating in the community (Triki *et al.*, 2003). Patients with primary immunodeficiency disorders affecting the B-cell system (such as persons with X-linked or sporadic agammaglobulinaemia and common variable immunodeficiency [CVID]) appear to be at highest risk for prolonged PV replication and excretion (Wood *et al.*, 2000; Khetsuriani *et al.*, 2003). According to recent reports, protracted PV replication can take place and may last as long as 10 years, when infection with VDPVs occurs in patients with primary immunodeficiencies (Kew *et al.*, 1998; Bellmunt *et al.*, 1999; Triki *et al.*, 2003; WHO, 2004). As a consequence, chronically infected individuals may present with paralytic disease some years after OPV administration and may transmit VDPVs to their close contacts such as newly born children who are no longer being vaccinated following the global polio eradication campaign (Kew *et al.*, 1998; Hovi *et al.*, 2004). In a study conducted by Khetsuriani and colleagues (2003), 3 out of 6 long-term immunodeficient PV excretors identified, developed paralysis after a lengthy period of asymptomatic VDPV persistence.

Limited information exists on secondary immunodeficiency as a risk factor for VAPP or prolonged PV excretion (Wood *et al.*, 2000). According to current scientific data, HIV infection is not a risk factor for paralytic poliomyelitis caused by wild-type PV or VDPV (Wood *et al.*, 2000). The short life expectancy and the predominant T cell deficiency of severely immunodeficient HIV-positive patients argues against the risk of prolonged PV excretion (Dowdle and Birmingham, 1997). However, two case reports (one from Romania and one from Zimbabwe) have linked HIV infection and VAPP (Ion-Nedelscu *et al.*, 1994; Chitsike and van Furth, 1999; Wood *et al.*, 2000). The likelihood of prolonged PV excretion in cohorts of HIV-infected children is currently being investigated in several developing countries (Wood *et al.*, 2000; Haisey *et al.*, 2004).

Immunodeficient persons with asymptomatic VDPV persistence should be considered at high risk of VAPP (Khetsuriani *et al.*, 2003). The overall risk of VAPP from OPV in a highly immune community is approximately 1 in 2.5 million doses administered, however, the risk is higher in a population with low vaccine coverage or inadequately vaccinated individuals such as persons older than 18 years of age and immunodeficient children (Guillot *et al.*, 2000; Martin *et al.*, 2000; 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), which reduce the synthesis of immunoglobulins (Sutter and Prevots, 1994; Guillot *et al.*, 2000; Triki *et al.*, 2003; Wood and Thorley, 2003). It is likely that the absence of specific antibodies to PVs in these patients is responsible for the lack of viral neutralisation during the viremic phase, which precedes neurological localisation (Triki *et al.*, 2003). Thus, OPV should never be administered to immunodeficient recipients or to those living in households that contain immunodeficient individuals, such as HIV-positive patients or patients with lymphoma, leukemia, solid tumours, abnormal immunoglobulin synthesis and chemotherapy (Zaoutis and Klein, 1998; Hovi *et al.*, 2004).

In a move to eliminate the rare cases of polio that result from OPV, the Advisory Committee on Immunisation Practices of the Federal CDC in the United States has recommended a change in the polio vaccination schedule from the current practice of administering OPV only at 2, 4 and 6 months of age to a sequential schedule of injection of IPV at 2 and 4 months followed by the administration of two doses of OPV at 12 to 18 months and 4 to 6

years of age (Buonagurio *et al.,* 1999). It is believed that the immunity acquired from the first two doses of IPV, which is unlikely to cause paralytic poliomyelitis, should be sufficient to protect the small number of children who contract disease from the OPV (Buonagurio *et al.,* 1999).

2.12 Vaccine-derived polioviruses

A variety of OPV-derived viruses can be isolated from OPV recipients and their contacts (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 per year have been proposed (Kew *et al.,* 1998; Kew *et al.,* 2002). All clinical and environmental PV isolates that are related to OPV strains are VDPVs (WHO, 2004). Derivatives of Sabin OPV strains have been classified into two broad categories for programmatic reasons: "OPV-like viruses" and "Vaccine-derived polioviruses" (WHO, 2004).

The vast majority of vaccine related isolates are "OPV-like" and have close sequence relationships (>99% VP1 sequence identity) to the original OPV strains (WHO, 2004). Immunologically normal OPV recipients are known to excrete PVs for an average of three to four weeks (WHO, 2004). Short excretion periods and high population immunity normally limit the person-to-person spread of these OPV-like viruses (WHO, 2004).

Rare "vaccine-derived poliovirus" (VDPV) isolates show ≤ 99% VP1 sequence identity to the parental Sabin PV vaccine strains and the extent of their genetic changes indicates prolonged replication (WHO, 2004). Up to date, two categories of VDPV isolates have been identified: immunodeficient VDPVs (iVDPVs) and circulating VDPVs (cVDPVs) (WHO, 2004).

2.12.1 Immunodeficient vaccine-derived polioviruses

The potential for prolonged replication of PV vaccine strains in patients with B-cell immunodeficiencies has been recognised for many years (WHO, 2004). The first iVDPV

isolates to be characterised with modern molecular techniques were from patients with defects in antibody production (generally CVID or X-linked agammaglobulinemia) (Yoneyama *et al.*, 1982; Sutter and Prevots, 1994; Bellmunt *et al.*, 1999; Kew *et al.*, 1998; Wood *et al.*, 2000; Yang *et al.*, 2003). Some iVDPV isolates are highly divergent (~90% VP1 sequence identity to the parental Sabin PV vaccine strain), suggesting that the chronic PV infections had persisted for 10 years or more (Kew *et al.*, 1998; Wood *et al.*, 2000; Yang *et al.*, 2003). Eighteen chronic iVDPV excretors were detected world-wide through the end of 2002, although this number may be an underestimate in the absence of systematic screening of immunodeficient patients (WHO, 2004). So far, all reports of persistent iVDPV infections have been from countries with high or intermediate levels of development, where the rates of OPV coverage are high and where the survival times of immunodeficient patients may be extended by their access to appropriate clinical management (Yang *et al.*, 2003). 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).

2.12.2 Circulating vaccine-derived polioviruses

In regions of low OPV coverage, a VDPV may result from transmission of Sabin PV vaccine strains from one immunised individual to another and accumulation of sufficient mutations to adopt wild-type PV characteristics of neurovirulence and transmissibility (Wood and Thorley, 2003). 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).

According to scientific reports, any PV that is circulating will eventually recombine with another related EV and that recombination is an indicator of circulation rather than a step in the increased ability to transmit from person-to-person (WHO, 2004). All cVDPVs, but none of the iVDPVs described in scientific reports thus far appear to be recombinants with EVs closely related to PVs (Kew *et al.*, 2002; Rousset *et al.*, 2003; Yang *et al.*, 2003; Kew *et al.*, 2004). The possible role of recombination in the phenotypic reversion of OPV is unclear (Kew *et al.*, 2004). Recombination with EVs appears to be an indicator of circulation, as the cVDPVs in Hispaniola and Egypt had participated in successive rounds of recombination during the outbreaks (Kew *et al.*, 2002; Yang *et al.*, 2003; Kew *et al.*, 2004).

Several outbreaks of poliomyelitis due to cVDPV have been documented (Wood and Thorley, 2003). A type 2 vaccine-related PV circulated in Belarus following local cessation of OPV use from 1963 to 1966 (Korotkova *et al.*, 2003; Kew *et al.*, 2004). An outbreak of type 3 poliomyelitis in Poland in 1968 was associated with PV strains derived from the USOL-D-bac vaccine (Martin *et al.*, 2000; Martin and Minor, 2002). In Egypt between 1983 and 1993, 32 cases of paralytic disease from a cVDPV type 2 were reported, including many retrospective cases (CDC, 2001; Martin and Minor, 2002; Wood and Thorley, 2003; Kew *et al.*, 2004). Polio cases attributed to cVDPV type 1 have been found in Haiti, Philippines and the Dominican Republic during 2000 and 2001 (WHO, 2000; Kew *et al.*, 2002; Wood and Thorley, 2003; Kew *et al.*, 2004). The small cluster of cases marked the first polio outbreak in the Western Hemisphere in more than 9 years (Greensfelder, 2000).

There have been 19 reports of AFP in the Dominican Republic and one in Haiti (WHO, 2000; Dove, 2001; Kew *et al.*, 2002). Though AFP can be caused by conditions other than polio, laboratory tests confirmed that a cVDPV type 1 was involved in recent outbreaks (Dove, 2001). In Hispaniola (Dominican Republic and Haiti) and Philippines, the cVDPV had undergone recombination with NPEVs (Wood and Thorley, 2003; Kew *et al.*, 2004). This has been the first reliable report that a VDPV strain reverted to a virulent form and spread contagiously (Greensfelder, 2000). The virus in these episodes showed more than 2% genetic sequence difference from the parent Sabin PV vaccine strain (VP1 region of the genome) and probably circulated for more than 2 years before being detected (Kew *et al.*, 2004). The outbreaks began when a VDPV infected inadequately vaccinated individuals, leading to the spread of the pathogenic virus (Dove, 2001; Kew *et al.*, 2004).

Outbreaks of a similar kind have occurred more recently in the Philippines and Madagascar (WHO, 2002; Rousset *et al.*, 2003; Kew *et al.*, 2004). In Madagascar, five cases of 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). Mass vaccination campaigns with OPV interrupted circulation of cVDPVs in Hispaniola and have been underway in the Philippines and Madagascar (CDC, 2002a; Kew *et al.*, 2004).

A common factor to all cVDPV outbreaks has been low population immunity, consistent with low OPV coverage and the apparent absence of circulating indigenous wild-type PV of

the same serotype (Kew *et al.*, 2004; WHO, 2004). Other risk factors are typical for wild-type PV circulation and include crowding, high birth rates, poor hygiene as well as sanitation and tropical climate (Kew *et al.*, 2004; WHO, 2004).

2.13 Environmental surveillance of poliovirus circulation

Environmental surveillance has been used successfully in monitoring enteric virus circulation and assessing the extent or duration of epidemic PV circulation in specific populations (WHO, 2002; WHO, 2004). The rationale for environmental surveillance is based on the characteristic PV excretion pattern (WHO, 2002; WHO, 2004). Infected individuals excrete PVs in faeces for periods up to several weeks, whether or not they are symptomatic and therefore, large numbers of PVs may remain infectious in the environment for varying lengths of time depending on the immediate conditions (WHO, 2002; WHO, 2004).

Wild-type PVs and cVDPVs have been detected in the environment even in the absence of reported cases of AFP, which is of major concern, since 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; WHO, 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 AFP. An unusual highly diverged derivative of the Sabin PV type 2 strain was isolated from environmental samples during routine screening for wild-type PV in Israel (Shulman *et al.*, 2000). The extensive genetic divergence of the isolate from its parental Sabin PV type 2 vaccine 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 3 isolates showed increased neurovirulence (Horie *et al.*, 2002; Yoshida *et al.*, 2002*).*

More recently, two Sabin-like PVs were found by environmental surveillance 8 and 11 months after any OPV vaccine was used in New Zealand and showed 99.8% as well as

99.9% homology with Sabin PV type 2 vaccine strain in the VP1 region (WHO, 2003b). This suggested that these PVs could have been excreted by recently vaccinated children (one or two months) visiting from a country using OPV (WHO, 2003b). Furthermore, 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).

Research has shown that PV isolates in the environment are genetically and epidemiologically related to those circulating in the community (Divizia *et al.*, 1999; Shulman *et al.*, 2000). Thus, the properties of PV isolates from sewage and river water would reflect those of PVs excreted from humans after OPV immunisation and for susceptible individuals, VDPVs have the potential to be the causative agents of poliomyelitis (Yoshida *et al.*, 2002; Buttinelli *et al.*, 2003). However, it is difficult to address the risk of infection from the environment, since there is little chance that individuals come into direct contact with raw sewage (Yoshida *et al.*, 2002). In contrast, access to river water or any other water source (used by the community for domestic purposes) is easy and therefore, susceptible individuals should be regarded as at greater risk of infection from such water sources (Yoshida *et al.*, 2002). Nonetheless, although it is possible to eliminate wild-type PV from the human community and environment, it will be difficult to eradicate poliomyelitis completely as long as OPV is not replaced by IPV (Yoshida *et al.*, 2002).

It is evident that environmental surveillance is still epidemiologically important, because the results of virus surveillance retrospectively reflect the properties of virus circulating in the community and it assesses the potential risk of infection from the environment as well as food (Divizia *et al.*, 1999; Shulman *et al.*, 2000; Yoshida *et al.*, 2002). The examination of composite human faecal samples through environmental surveillance links PV isolates from unknown individuals to populations served by the wastewater system (WHO, 2004). In addition, environmental surveillance provides valuable information, particularly in urban populations where AFP surveillance is absent and where persistent PV circulation or re-introduction is suspected (WHO, 2004).

2.14 Isolation and identification of polioviruses

In the natural course of PV infection, excretion of PV in stools continues for a limited period of time (usually 2 to 3 months) (Hovi *et al.*, 2001). The question of whether VDPVs can

circulate indefinitely in the environment remains unanswered (Manor *et al.,* 1999a; Manor *et al.,* 1999b). Search for PVs in wastewater has been used for assessment of the extent of the epidemic spread of PVs and to approximate the proportion of infected individuals in the source population (Hovi *et al.,* 2001). It has been suggested that routine environmental surveillance of wastewater for PVs has been a powerful method to detect wild-type PVs as well as VDPVs and could be used to assess elimination of PV circulation in communities where OPV is still in use and where no poliomyelitis cases have been reported (Manor *et al.,* 1999b; Hovi *et al.,* 2001; Horie *et al.,* 2002).

2.14.1 Recommended cell lines for the isolation of polioviruses

Polioviruses grow readily in a wide variety of continuous human and primate cell lines (Wood and Hull, 1999). Two continuous cell culture lines of human origin were previously used by the laboratories of the WHO global poliovirus network in their initiative to eradicate wild-type PV (Wood and Hull, 1999). Human rhabdomyosarcoma (RD cells) and human epidermoid carcinoma (HEp-2 cells) cells were mainly used for virus isolation and characterisation because of their availability, ease of maintenance and proven sensitivity to infection by EVs (Manor *et al.,* 1999a; Wood and Hull, 1999).

The development of mouse cell lines (L20B cells) that express the gene for the human cellular receptor for PVs allows selective PV culture, because very few NPEVs grow in these murine cells (Wood and Hull, 1999; WHO, 2004). Scientific data have indicated that L20B cells can provide greater sensitivity and specificity for detection of PVs and can provide a quicker diagnosis of PV infection especially in samples that contain another EV (Wood and Hull, 1999; WHO, 2004). However, confirmatory serotyping subsequent to isolation in L20B cells should still be attempted, because some NPEVs may produce a cytopathogenic effect (CPE) on these cell lines (Chezzi, 1996; WHO, 2004).

2.14.2 Serological diagnosis of poliovirus infection

The demonstration of poliovirus-specific IgM antibody in a single serum specimen, indicating a current antigenic stimulus, provides evidence for a recent PV infection (Muir *et al.,* 1998). Detecting virus-specific IgM in cerebrospinal fluid (CSF) is more likely to indicate a causal relationship to CNS symptoms and is more sensitive than isolation of PV

from stool for confirming a diagnosis of paralytic poliomyelitis (Nibbeling *et al.,* 1994; Muir *et al.,* 1998). Thus, determination of virus-specific IgM in serum has proved useful in monitoring a recent PV outbreak in the Netherlands and in defining the epidemiology of NPEV outbreaks (Oostvogel *et al.,* 1994; Goldwater, 1995; Muir *et al.,* 1998).

Following the isolation of PV, the serotypic identity can be determined by neutralisation of infectivity with serotype-specific antisera (Muir *et al.,* 1998; WHO, 2002; WHO, 2004). This neutralisation test uses a selected set of monospecific polyclonal antisera to PV types 1, 2 and 3 combined as antiserum pools (Van der Avoort *et al.,* 1995; WHO, 2002; WHO, 2004). These antisera have been developed by the National Institute of Public Health and the Environment (RIVM), Bilthoven, The Netherlands and are supplied to the WHO Polio Laboratory Network laboratories (WHO, 2002; WHO, 2004). Using the micro-neutralisation technique, the serum/virus mixtures are incubated to allow the antibodies to bind to the virus (WHO, 2002; WHO, 2004). The antiserum that prevents the development of CPE indicates the identity of the PV (WHO, 2002; WHO, 2004).

2.14.3 Molecular techniques for the detection of polioviruses

Recent developments in molecular detection technology made it possible to diagnose PVs by non-culture-based methods such as the reverse transcription polymerase chain reaction (RT-PCR) (Muir *et al.,* 1998; Donaldson *et al.,* 2002). Compared to PCR, cell culture techniques are more costly, laborious and less sensitive (Donaldson *et al.,* 2002). The CPE produced by a PV strain on cell culture cannot readily be distinguished from the CPE caused by a non-poliovirus pathogen (Abraham *et al.,* 1993). Nonetheless, one important advantage of the cell culture method is its ability to distinguish between viable and non-viable PVs (because PVs would infect susceptible host cells and replicate their RNA), which molecular techniques cannot do and this is most important in environmental surveillance (Grabow *et al.,* 1999). However, it is well known that some PCR tests are capable of rapid differentiation between PV vaccine strains, wild-type PVs and NPEVs due to the sensitivity and specificity of the techniques, which in contrast cell cultures cannot do (Zoll *et al.,* 1992; Casas *et al.,* 2001).

Reverse transcription PCR assays have been developed to detect poliovirus RNA directly from clinical and environmental samples because of the high sensitivity for small amounts of

RNA (Casas *et al.*, 2001). In addition, scientific reports have shown that a RT-PCR followed by a nested PCR is a more sensitive and time-saving technique than those employing PCR only or a combination of PCR and hybridisation (Severini *et al.*, 1993; Kuan, 1997). Most of these methods use non-degenerated primers designed to target conserved sequences within the 5'UTR or the capsid protein-coding regions of the PV genome (Kuan, 1997; Casas *et al.*, 2001). Recently, the region of the genome encoding VP1 has been used to investigate the molecular evolution of PVs, to determine PV genotypes and to develop PV serotype-specific PCR primers (Caro *et al.*, 2001).

2.14.3.1 Reverse transcription multiplex PCR

A reverse transcription multiplex PCR (RT-multiplex PCR) has been developed for the rapid and sensitive detection of PVs and for their discrimination from NPEVs, which is an important factor in the PV surveillance program (Egger *et al.*, 1995). The RT-multiplex PCR combines EV-specific primers (E1 and E2) and PV-specific primers Po1 to Po4 (Table 2.1), thus giving rise to amplicons of different sizes (Egger *et al.*, 1995).

Table 2.1: Enterovirus and poliovirus specific primers[a] (Egger *et al.*, 1995)

Primer region and map position[b]	Sequence[c]	Primer designation	Amplicon length (bp)	Specificity
5'UTR 163-178 443-460	 5'-AAG CAC TTC TGT TTC C-3' 5'-CAT TCA GGG GCC GGA GGA-3'	 E1 E2	 297	 EV EV
P2 region 4460-4478 4634-4653	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'	Po1 Po2	193	PV PV
P2-P3 region 4922-4941 5467-5487	 5'-GAA ATG TGT AAG AAC TGT CA-3' 5'-GTA ACA ATG TTT CTT TTA GCC-3'	 Po3 Po4	 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.**

The RT-multiplex PCR is straightforward and in addition obviates the use of a series of different hybridisation probes, which would be necessary to compensate for the genetic diversity of PVs (Egger *et al.*, 1995). Reports have shown that the sensitivity of the RT-multiplex PCR, as measured with RNA extracted from a virus suspension, was found to be

100 plaque forming units (PFU) and is up to 2 orders of magnitude more sensitive than the RT-PCR performed directly on clinical specimens (Egger *et al.*, 1995).

2.14.3.2 Sabin specific RT-triplex PCR

A Sabin specific RT-triplex PCR method that allows the simultaneous identification of Sabin PV types 1, 2 and 3 in a single reaction has been reported by Yang *et al.* (1991). Contemporary wild-type PV strains are not detected (Yang *et al.*, 1991; Yang *et al.*, 1992; Buonagurio *et al.*, 1999). The PCR primers map to the region of the PV genome encoding the amino terminus of the VP1 capsid protein just upstream of the major antigenic site (Table 2.2) (Buonagurio *et al.*, 1999). Nucleotide sequence heterogeneity in this region among the three Sabin PV serotypes allows discrimination (Buonagurio *et al.*, 1999).

Table 2.2: Sequences of the oligonucleotides used for the detection of Sabin poliovirus types 1, 2 and 3 (Yang *et al.*, 1991; Yang *et al.*, 1992)

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

The PCR assay was reported to be highly sensitive for the detection of purified Sabin PV RNA in stools (Buonagurio *et al.*, 1999). This Sabin specific RT-triplex PCR could be used to monitor the impact of the change in vaccination schedule (change from OPV to IPV vaccination) on virus shedding (Buonagurio *et al.*, 1999). In addition, this RT-PCR could be implemented to evaluate how new OPV formulations affect PV excretion in the stools of vaccinees (Buonagurio *et al.*, 1999).

2.14.3.3 Restriction fragment length polymorphism

Restriction fragment length polymorphism (RFLP) is used as an alternative means of differentiating various serotypes of prototypical EVs (Kuan, 1997). Restriction enzymes (REs) recognise short deoxyribonucleic acid (DNA) sequences and cleave double-stranded DNA at specific sites within or adjacent to the recognition sequences (Fuchs and Blakesley,

1983). Thus, by combining three REs (*Sty* I, *Bgl* I and *Xmn*I), each one having their own specific six-base recognition site (Table 2.3), a digestion pattern can be obtained that is easily interpreted (Kuan, 1997).

Table 2.3: Restriction enzymes for the genotyping of enteroviruses (Kuan, 1997)

Restriction enzyme	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 restriction enzymes.**

Although the 5'UTR is the most conserved region among EVs, utilisation of differential restriction patterns to distinguish various serotypes may yield inconclusive results (Kuan, 1997). Some of the prototypes possess recognition sequences identical to those of the current REs, so that additional kinds of REs should be adopted to achieve further classification (Kuan, 1997).

2.14.3.4 Nucleotide sequencing of the enteroviral genomes

Phylogenetic analyses have been used to study the epidemiology of different EV serotypes (Muir *et al.*, 1998). Using this approach, it has been possible to estimate the date of emergence of newly arising EV strains, to study their subsequent molecular evolution and global spread, to confirm the common source of isolates from a single EV outbreak and to study their genetic relationship to strains from previous outbreaks (Muir *et al.*, 1998).

The sequencing method uses primers that target sequences, which are relatively conserved among isolates of a given serotype such as the 5'UTR and the VP1-2A junction region, thus allowing the amplification of most isolates and provides maximal discrimination potential between unrelated strains (Muir *et al.*, 1998). Sequence variation is the highest in the VP1 region, which codes for the major antigenic sites and the most type-specific neutralisation determinants (Oberste *et al.*, 1999). Research has confirmed the presence of sequence domains in the VP1 region that are conserved among all members of the EV genus, as well as intervening domains that vary in sequences between strains of different serotypes and in some cases within a serotype (Oberste *et al.*, 1999). Molecular assays directed to specific

sequences in VP1 have been applied to serotyping, genotyping and group identification of PVs (Kilpatrick *et al.*, 1998). The success of PV molecular diagnostics targeting the VP1 region suggested that future molecular development efforts should be directed at the genomic region encoding VP1 (Oberste *et al.*, 1999).

Studies on the three serotypes of PV have shown that a partial VP1 sequence correlates well with PV serotype (Kilpatrick *et al.*, 1998). Sequences of the VP1 region correlate better with the serotype than do sequences of either the 5'UTR or the VP4-VP2 junction (Oberste *et al.*, 1999). A 100% correlation exists between the nucleotide sequence of the 3'half of VP1 and antigenic typing by the standard neutralisation test for clinical isolates of various serotypes (Oberste *et al.*, 1999). Oberste and colleagues (1999) have developed a molecular typing system based on RT-PCR and nucleotide sequencing of the 3'half of the genomic region encoding VP1, which can be used to type isolates that are difficult or impossible to type with standard immunological reagents (Oberste *et al.*, 1999). The technique is useful for the rapid determination of whether viruses isolated during an outbreak are epidemiologically related (Oberste *et al.*, 1999).

2.14.4 Intratypic differentiation methods recommended by the WHO

In the PEI, AFP is the gold standard for surveillance, although under certain circumstances valuable supplementary information can be obtained by environmental surveillance (WHO, 2002; WHO, 2004). There is a strong school of thought that under circumstances environmental surveillance may be the more reliable and practical approach to monitor the circulation of enteric viruses in communities (Grabow *et al.*, 1999). However, environmental surveillance should be restricted to selected populations where deficiencies in AFP surveillance are suspected and where conditions (such as unvaccinated or incompletely vaccinated individuals in developing countries, the extent of crowding, levels of hygiene, poor water quality and sewage treatment facilities) exist that render the population at risk for PV circulation (WHO, 2002; WHO, 2004). Thus, isolation and identification of PVs from AFP cases is the first step in detecting the circulation of wild-type PV and VDPV in the community (WHO, 2002; WHO, 2004). Vaccine PV strains may circulate during and after immunisation campaigns and may be found in healthy and symptomatic children (WHO, 2002; WHO, 2004). Five methods for intratypic differentiation (ITD) are recommended for

use in Regional Reference laboratories of the Network for determining whether PV isolates are wild or vaccine-derived (WHO, 2002; WHO, 2004).

These ITD methods include:

1) Enzyme linked immunosorbent assay (ELISA) using cross-adsorbed antisera developed by National Institute of Public Health and the Environment (RIVM)
2) The probe hybridisation method developed by CDC
3) The diagnostic PCR method developed by CDC (WHO, 2002; WHO, 2004).

The other two, the PCR-RFLP developed by the Pasteur Institute, Paris and NIID Tokyo and the monoclonal antibody assay developed by the Pasteur Institute, Paris and NIBSC, Potters Bar, are accepted methods but are not currently supported by the Network (WHO, 2002; WHO, 2004).

The methods of intratypic differentiation that have been selected for routine use by the Network are based on different approaches (WHO, 2004). The enzyme linked immunosorbent assay with polyclonal cross-adsorbed antisera detects antigenic differences between wild and Sabin-derived strains (WHO, 2004). On the other hand, the nucleic acid probe hybridisation and diagnostic PCR are molecular methods, detecting differences in the viral RNA (WHO, 2004).

2.15 Eradication of poliomyelitis: Progress and Challenges

Despite substantial progress towards polio eradication since 1988, polio still remains endemic in several countries around the world (World Health Assembly, 1988; Sever, 2001; WHO, 2003b). Therefore, the WHO in coalition with various international organisations as well as governments set a new target for global polio eradication and certification by the year 2005 (Sever, 2001). In 2001, the number of countries where polio was endemic decreased from 125 to 10 and the number of reported polio cases decreased by more than 99% from an estimated 350 000 to less than 500 (CDC, 2002a; ProMED-mail, 2004b). In 2003, 784 cases were reported, a major drop from 1 918 cases in 2002 (ProMED-mail, 2004c). In 2004, wild-type PV remains endemic in only 6 countries, such as Nigeria, India, Pakistan, Niger, Afghanistan and Egypt (ProMED-mail, 2004c). The last reported human infection with indigenous wild-type PV type 2 was in 1999 in Aligarh, Uttar Pradesh, India (WHO, 2001a;

Yang *et al.,* 2003). Since 1999, all PV type 2 isolates from clinical specimens or from environmental samples, have been derived from OPV (WHO, 2001b; WHO, 2003a).

The American and Western Pacific Regions of the WHO have been certified free of indigenous wild-type PV (CDC, 2002a; Wood and Thorley, 2003). In the American region, no wild-type PV or VDPV positive cases have been detected in 2003 (WHO, 2003c). In the Western Pacific, nine cases of virologically confirmed poliomyelitis have been reported in 1997 mainly from China, Mekong River area of Cambodia and Vietnam (Hull *et al.,* 1997; Dowdle *et al.,* 1999). However, this region has been free from endemic wild-type PV circulation since March 1997 (WHO, 2003c). No VDPV has been detected in this region since early 2001 (WHO, 2003c).

Densely populated countries such as India, Pakistan and Nigeria represent major PV reservoirs and pose the risk of re-seeding neighbouring countries (Dowdle *et al.,* 1999; CDC, 2003; WHO, 2003c). India remains the only country with wild-type PVs detected in the South-East Asian Region, with 73 wild-type PV cases detected in 2003 (WHO, 2003c). In the first quarter of 2003, wild-type PVs were detected from AFP cases in the countries of Nigeria (31 PVs), Ghana (1 PV) and Niger (1 PV) (WHO, 2003c). Sequence and epidemiological data suggested that the PVs detected in Zambia in 2002 and Ghana in 2003 were imported from Angola and Nigeria, respectively (WHO, 2003c). In 2003, Nigeria accounted for 45% of the world's polio cases and in 2004, Nigeria had 48 of the world's 72 reported infections (ProMED-mail, 2004c). Wild-type PVs genetically linked to northern Nigeria have caused new polio cases in the previously polio-free countries of Benin, Burkina Faso, Cameroon, Central African Republic, Chad, Cote d'Ivoire, Ghana and Togo (ProMED-mail, 2004c). The importations have been associated with an extensive outbreak of polio in Nigeria, which followed after the suspension of polio immunisation campaigns in some states of the country (ProMED-mail, 2004c). In 2004, the ongoing polio outbreak which originated in northern Nigeria continues to infect new countries, underscoring the threat of a major epidemic across west and central Africa (Fleck, 2004; ProMED-mail, 2004a). Epidemiologists from the global PEI confirmed new polio cases in Botswana, Guinea, Mali, as well as 3 new cases in the Darfur region of Sudan (Fleck, 2004; ProMED-mail, 2004a). In Sudan, these were the first confirmed cases of polio, which mainly affected children under the age of five, in the country for three years (Fleck, 2004). Thus, it is evident that wild-type PVs are spreading at an alarming pace in African countries following suspension of

immunisation in Nigeria and the low OPV coverage in the previously polio free countries (Fleck, 2004). The tragedy is that many of these countries are becoming re-infected and this is why the number of polio cases has nearly doubled globally from 183 during 2002 to 333 in 2004 (Fleck, 2004).

Wild-type PVs have been detected in 20 AFP cases from Pakistan and one case from Lebanon in 2003 (WHO, 2003c). Sequence and epidemiological data suggested that the PV detected in Lebanon was imported from India (WHO, 2003c). Testing of sewage samples as part of a supplementary surveillance project in Egypt has revealed two wild PV type 1 isolates from two settlements in 2003 (WHO, 2003c).

During 2001, two polio-free European countries detected importations of wild-type PV: Bulgaria and Georgia (CDC, 2002a). In 2002, the WHO officially declared Europe free from endemic wild-type PV circulation (Wood and Thorley, 2003; WHO, 2003c). In 2003, a VDPV type 2 was isolated from a single AFP case in Kazakhstan (WHO, 2003c).

In South Africa, the poliovirus vaccination schedule starts with the administration of OPV at birth, 6 weeks, 10 weeks, 14 weeks, 18 months and 5 years (Table 2.4) (Department of Health, 1995). Vaccination with OPV is compulsory in South Africa and immunodeficient patients such as HIV-positive patients receive the OPV although it is a contra-indication to be vaccinated (WHO, 2004). The high number of immunodeficient individuals living in the country, therefore, represents a major concern, because these individuals could serve as potential reservoirs for the dissemination of VDPVs after cessation of PV immunisation in the near future.

Table 2.4: South African immunisation schedule (Department of Health, 1995)

Age	Vaccines
At birth	*Oral polio vaccine*, BCG
6 weeks	*Oral polio vaccine*, DTP, Hepatitis B vaccine
10 weeks	*Oral polio vaccine*, DTP, Hepatitis B vaccine
14 weeks	*Oral polio vaccine*, DTP, Hepatitis B vaccine
9 months	Measles vaccine
18 months	*Oral polio vaccine*, DTP, Measles vaccine
5 years	*Oral polio vaccine*, DT

BCG = Vaccine against tuberculosis.
DT = Diphtheria, tetanus vaccine.
DTP = Diphtheria, tetanus, pertussis vaccine.
OPV = Trivalent oral poliovirus vaccine (Sabin PV type 1, type 2 and type 3).

Fortunately, there have not been currently any reports of an increased incidence of VAPP in countries with a high prevalence of HIV infections (Bellmunt *et al.*, 1999). In HIV patients, the production of neutralising antibodies is less impaired than in patients with CVID and neutralising antibodies have been detected in HIV-infected children after the application of OPV, although titres were found to be lower than in HIV-negative children (Ryder *et al.*, 1993; Bellmunt *et al.*, 1999). However, production of neutralising antibodies is impaired in advanced stages of AIDS and future studies on prolonged PV excretion from HIV-infected patients are required (Bellmunt *et al.*, 1999).

The current situation in South Africa (the high number of immunodeficient individuals and the compulsory polio vaccination) offers opportunities well suited for research along these lines. The purpose of this study was therefore to isolate OPV strains from selected sewage and river water samples, and from immunodeficient patients (such as HIV-positive children including those with an AIDS indicator condition according to the CDC classification), and to type the OPV isolates using advanced molecular techniques. Secondly, two distant regions of the OPV genomes (the 5'UTR and VP1 region) were partially sequenced in order to determine the prevalence of VDPVs in immunodeficient children and the environmental samples. These features and implications of OPV vaccination have not previously been investigated in South Africa.

2.16 Summary

The effort to eradicate polio world-wide by the year 2005 received a major set-back, as a case of paralytic poliomyelitis due to an imported wild PV type 1 was reported in Botswana in 2004, a country which had been polio-free since 1991 (ProMED-mail, 2004b). The virus was closely linked genetically to a PV endemic in northern Nigeria (ProMED-mail, 2004b). The polio case in Botswana underlined the magnitude of the risk posed to polio-free areas by ongoing polio outbreaks in west and central Africa (ProMED-mail, 2004b). Children across Africa will continue to be at risk of polio from such importations until the disease is eradicated world-wide (ProMED-mail, 2004b).

There is no long-term carrier state of PV in immunocompetent persons (Dowdle and Birmingham, 1997). After exposure to OPV, immunocompetent persons excrete PV vaccine strains for a limited period of time (usually 2 to 3 months) (Khetsuriani *et al.*, 2003; Hovi *et*

al., 2004). However, Hovi and colleagues (2004) have shown that excretion of wild-type PV by healthy children may continue for at least 6 months and is associated with the accumulation of single nucleotide substitutions during replication within an individual host. It is important to consider the possibility of persistent infections and excretion of VDPVs for long periods by immunodeficient patients (Buttinelli *et al.,* 2003). In patients with deficiencies in antibody production, PV replication and excretion may differ (Kew *et al.,* 1998; Buttinelli *et al.,* 2003). The spectrum of possibilities for behaviour of PVs in immunodeficient individuals was illustrated by the accidental discovery in Europe of an immunodeficient man who was carrying a highly evolved VDPV type 2 strain (Minor, 2001; MacLennan *et al.,* 2004). This individual is known to have been excreting VDPV type 2 for an estimated 20 years and is still excreting at present without showing any clinical symptoms (MacLennan *et al.,* 2004). Furthermore, in a study conducted in the United Kingdom, two of 30 immunodeficient individuals excreted VDPVs for more than 6 months (WHO, 1998; Dowdle *et al.,* 1999). Several reports described the excretion of VDPV for periods ranging from 2 to 8 years (Yoneyama *et al.,* 1982; Kew *et al.,* 1998; Dowdle *et al.,* 1999).

The circulation of the VDPVs in the environment is of major 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). Shulman and colleagues (2000) have isolated an unusual, highly diverged derivative of the Sabin 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 vaccine strain suggested that the virus had replicated in one or more individuals for approximately 6 years (Shulman *et al.,* 2000). More recently, a highly evolved VDPV type 3 harbouring a 13% sequence drift from the Sabin PV type 3 vaccine strain has been isolated from sewage in Estonia (Blomqvist *et al.,* 2004).

This study will address some of the issues regarding the evolution and nucleotide divergence of OPV strains replicating in carrier communities, as well as the prevalence of these strains in the environment. Genotyping of the VDPV strains will cast light on their potential health risk. This information is essential for strategies aimed at the protection of immunodeficient patients against complications of vaccination with OPV. Thus, data on the excretion of VDPV strains by carrier communities (notably immunodeficient individuals) will give an indication of the quantitative release of these strains into the environment. Data on the

prevalence of VDPV strains in the environment will reveal details on the possible role of water in the transmission of potentially hazardous mutants of OPV strains. These features and implications of OPV vaccination have not previously been investigated in South Africa. Furthermore, no research has previously been conducted in South Africa to determine the prevalence of VDPV strains as well as the prolonged excretion of OPV strains specifically by immunodeficient children. In conjunction with this study, an additional study will be conducted by medical professionals from Kalafong Hospital (South Africa), which will investigate the clinical status of the HIV-positive children taking part in this project. This study will provide new and relevant information on the circulation of VDPVs, which will be of importance not only for South Africa but the global polio eradication program.

The WHO is considering a sequential world-wide vaccination schedule in which OPV is followed by the IPV in order to reduce the risk of VAPP and to facilitate the transition to the exclusive use of IPV before termination of PV vaccination. The results obtained in this study will be of fundamental importance regarding the control of health risks constituted by OPV vaccination, particularly with regard to immunodeficient individuals such as HIV-positive patients, the planning of how and when to terminate PV vaccination, and the possible role of water in the transmission of potentially hazardous VDPVs.

2.17 References

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CHAPTER 3

POLIOVIRUS VACCINE STRAINS IN SEWAGE AND RIVER WATER IN SOUTH AFRICA

The editorial style of Water Research was followed in this chapter

3.1 Abstract

Since the initiation of the global poliomyelitis eradication program in 1988, the number of wild-type polio cases decreased from 350 000 to less than 500 and the number of polio endemic countries declined from more than 125 to 10. The last case of polio, caused by a wild-type poliovirus (PV) in South Africa occurred in 1989. The live attenuated oral poliovirus vaccine (OPV) has been effectively used in the reduction and control of poliomyelitis. However, as OPV strains are excreted in stools after vaccination, this vaccine could become a source of dissemination of PVs in the environment and the potential cause of poliomyelitis. The aim of the study was, therefore, to determine the occurrence of OPV strains in selected sewage and river water samples. During the period between 2001 and 2003, 138 samples of river water and 213 samples of settled sewage were collected from selected areas of South Africa. A total of 860 plaques were analysed, which consisted of 703 plaques from the sewage and 157 plaques from the river water samples. Using a RT-multiplex PCR, 49 PVs were successfully distinguished from 176 non-polio enteroviruses (NPEVs). The 176 NPEVs consisted of 50 coxsackieviruses B2 (CBV2) (28.4%), followed by 39 echoviruses 11 (ECV11) (22.2%), 25 CBV5 (14.2%), 21 CBV3 (11.9%), 15 CBV4 (8.5%), 14 coxsackieviruses A6 (CAV6) (8.0%), 7 CBV6 (4.0%), 2 CAV5 (1.1%), 2 CBV1 (1.1%) and 1 ECV19 (0.6%), which was in agreement with the prevalence of these EVs in other parts of the world. 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. Buffalo green monkey kidney (BGM) and primary liver carcinoma (PLC/PRF/5) cell cultures allowed the amplification of a broad spectrum of EVs, whereas human epidermoid carcinoma (HEp-2) cells were more selective for PVs. This study addressed some of the issues regarding the prevalence of OPV strains in the environment. The

identification of 49 viable OPV isolates in this study confirmed the presence and circulation of PV vaccine strains in sewage and river water. The extent of the potential health risk constituted by these OPV isolates remains to be investigated.

Keywords: Poliovirus vaccine strains, Enteroviruses, Circulation, Sewage, River water, Molecular techniques

Abbreviations: ATCC, American Type Culture Collection; BGM, Buffalo green monkey kidney; CAV, Coxsackievirus A; CBV, Coxsackievirus B; CDC, Centers for Disease Control and Prevention; CPE, Cytopathogenic effect; DNA, Deoxyribonucleic acid; ECV, Echovirus; EVs, Enteroviruses; ECACC, European Collection of Cell Culture; HEp-2, Human epidermoid carcinoma; IPV, Inactivated poliovirus vaccine; L20B, Mouse L cells; MEM, Minimum Essential Medium; NPEVs, Non-polio enteroviruses; OPV, Oral poliovirus vaccine; PBS, Phosphate-buffered saline; PCR, Polymerase chain reaction; PEG, Polyethylene glycol; PFU, Plaque forming units; PV, Poliovirus; PLC/PRF/5, Primary liver carcinoma; RE, Restriction enzyme; RFLP, Restriction fragment length polymorphism; RNA, Ribonucleic acid; RT-PCR, Reverse transcription polymerase chain reaction; USA, United States of America; UTR, Untranslated region; VAPP, Vaccine-associated paralytic poliomyelitis; VDPV, Vaccine-derived poliovirus; VP, Virus protein; WHO, World Health Organization.

3.2 Introduction

Human enteroviruses (EVs) belong to the family Picornaviridae and consist of more than 60 serotypes that include polioviruses (PVs), coxsackieviruses A (CAVs), coxsackieviruses B (CBVs), echoviruses (ECVs) and the more recently identified enteroviruses (EVs) 68 to 71 (Zaoutis and Klein, 1998; Caro *et al.*, 2001). Enteroviruses are among the most important viral pathogens of humans and cause an estimated 30 million infections in the United States of America (USA) each year (Caro *et al.*, 2001; Donaldson *et al.*, 2002). Enteroviruses are transmitted by the faecal-oral route and infection can be acquired through contaminated water, food or vomitus (Fogarty *et al.*, 1995; Zaoutis and Klein, 1998). The spectrum of diseases ranges from a mild febrile illness to aseptic meningitis, hand, foot and mouth

disease, myocarditis, meningo-encephalitis, poliomyelitis and neonatal multi-organ failure (Muir *et al.*, 1998; Caro *et al.*, 2001).

Although PV is known to exist widely in nature such as soil, sewage, wastewater, drinking water and shellfish, there is little evidence to connect it directly with an outbreak of poliomyelitis from the environment (Goyal *et al.*, 1979; Jaykus, 1997; Yoshida *et al.*, 2002). It is difficult to address the risk of infection by PV from the environment, because most cases of infection by PV are not apparent until person-to-person spread leads to the onset of poliomyelitis (Metcalf *et al.*, 1995). Poliovirus strains isolated from the environment were found to be genetically and epidemiologically related to those circulating in the community (Divizia *et al.*, 1999; Shulman *et al.*, 2000). The properties of PV isolates from sewage and river water would reflect those of PVs excreted from humans after OPV immunisation, and vaccine-derived polioviruses (VDPVs) may potentially cause poliomyelitis or related symptoms in susceptible individuals (Yoshida *et al.*, 2002; Buttinelli *et al.*, 2003). Therefore, environmental surveillance is epidemiologically important, because the results of PV surveillance retrospectively reflect the properties of PV circulating in the community and can be used to assess the potential risk of infection from the environment and food (Jaykus, 1997; Divizia *et al.*, 1999; Richards, 1999; Shulman *et al.*, 2000; Yoshida *et al.*, 2002).

Polioviruses include three distinct serotypes (designated PV type 1, type 2 and type 3) that were originally defined by their patterns of reactivity with neutralising antibodies (Zaoutis and Klein, 1998; Georgopoulou *et al.*, 2000). Three clinical syndromes are attributed to PV infection, namely: minor, non-specific illness (abortive poliomyelitis), aseptic meningitis (non-paralytic poliomyelitis) and paralytic poliomyelitis (Melnick, 1996a; Zaoutis and Klein, 1998). Poliovirus type 1 is the most paralytogenic and type 2 the least (Dowdle and Birmingham, 1997). Ninety percent or more of wild-type PV infections are asymptomatic or unapparent and only 0.1% to 1% of PV infections result in paralysis (Dowdle and Birmingham, 1997).

Poliomyelitis is being effectively controlled by the inactivated poliovirus vaccine (IPV) and the oral poliovirus vaccine (OPV) (Georgopoulou *et al.*, 2000; Wood *et al.*, 2000; Wood and Thorley, 2003). Although IPV is effective in inducing circulating antibodies against PV for individual protection, it is less effective than OPV in inducing mucosal immunity at

replication sites in the gastrointestinal tract (Wood *et al.*, 2000; Wood and Thorley, 2003). Vaccination with live attenuated OPV strains (Sabin PV types 1, 2 and 3) mounts a long-lasting immune response that protects the human host from future viral infections with wild-type PV strains (Georgopoulou *et al.*, 2000; Wood *et al.*, 2000; Wood and Thorley, 2003). The ability of OPV to induce a higher level of mucosal immunity, plus the advantages of oral administration and lower costs, made OPV the vaccine of choice for the World Health Organization (WHO) in their polio eradication initiative (Wood *et al.*, 2000; Yoshida *et al.*, 2002; Wood and Thorley, 2003). The OPV has had a remarkable track record of success since the number of wild-type polio cases decreased from 350 000 to less than 500 and the number of polio endemic countries declined from more than 125 to 10 (Wood and Thorley, 2003). The last case of polio, caused by a wild-type PV in South Africa occurred in 1989 (Centers for Disease Control and Prevention [CDC], 2003).

After termination of OPV in the near future (2005), the possibility of an outbreak caused by VDPVs must be considered, since several studies have revealed that nucleotide substitution in the virus genome occurs gradually during replication in the human gut after OPV administration and the phenotype of excreted viruses changes from attenuated to virulent (Wood and Macadam, 1997; Yoshida *et al.*, 2002; Wood and Thorley, 2003). If the mutations lead to poliomyelitis in a vaccine recipient or a close contact, it is defined as vaccine-associated paralytic poliomyelitis (VAPP) (Wood and Macadam, 1997; Wood and Thorley, 2003). Vaccine-associated paralytic poliomyelitis is most frequently associated with OPV serotypes 2 and 3, which contain fewer differences to the wild-type PV they were derived from, in comparison to serotype 1 (Wood and Macadam, 1997; Wood and Thorley, 2003).

In the past PV isolates were typed by neutralisation tests with pools of type-specific antisera or enzyme-linked immunosorbent assays with antisera specific for individual serotypes (Van der Avoort *et al.*, 1995; Melnick, 1996b; Kilpatrick *et al.*, 1998). Recent advances in molecular virology by polymerase chain reaction (PCR) amplification methods have provided new alternatives to PV detection and typing (Georgopoulou *et al.*, 2000). Polymerase chain reaction genotyping of PVs includes serotype-specific PCR primers (Kilpatrick *et al.*, 1998), genotype Sabin-specific PCR primers (Yang *et al.*, 1991) and restriction fragment length polymorphism (RFLP) analysis (Furione *et al.*, 1993), which allow inter- and intratypic

differentiation (Georgopoulou *et al.,* 2000). Nucleotide sequence determination of PCR products provides the most detailed discrimination between strains (Mulders *et al.,* 1995). The sequencing method uses primers that target sequences, which are relatively conserved among isolates of a given serotype such as the 5'untranslated region (5'UTR) and the VP1-2A junction region, thus allowing the amplification of most isolates and providing maximal discrimination potential between unrelated strains (Muir *et al.,* 1998; Wood and Thorley, 2003).

Data on the prevalence of OPV strains in the environment will reveal details on the potential role of water in the transmission of potentially hazardous mutants of OPV strains. The presence in the environment of a highly evolved, neurovirulent VDPV in the absence of polio cases would have important implications for strategies regarding the cessation of immunisation with OPV following global polio eradication. Therefore, the occurrence of OPV strains in sewage and river water in South Africa was studied, as a first step towards future research on the prevalence and potential health impact of VDPVs.

3.3 Materials and methods

3.3.1 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.

3.3.2 Recovery of viruses from sewage and river water samples

During 2001 and 2003, a total of 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 3.1). During the same period of time, a total of 138 river water samples (occasionally used by the rural community for drinking and washing 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 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). A mixture consisting of 14.0 g PEG (PEG 6000, Merck) and 1.17 g NaCl (Sigma Chemical Co., Louis, USA) was prepared and this mixture was dissolved in 100 ml of sewage/river water sample. After settling down at 4°C for 24 h, each sample was centrifuged at 2 500 x *g* (Sorvall Super T 21, Wilmington, USA) for 30 min at 4°C. The resulting pellet was re-suspended in 10 ml phosphate-buffered saline (PBS) (Sigma) and sonicated for 20 s (Soniprep 150, MSE) in order to break up any pre-formed virus clumps. After centrifugation at 600 x *g* (BHG Roto-Uni II, Separation Scientific, South Africa) for 10 min at 4°C, the concentrates were treated with a nystatin and penicillin/streptomycin neomycin mix (150 µl of each antibiotic) (Whittaker M.A. Bioproducts, Maryland, USA) and inoculated onto BGM, HEp-2 and PLC/PRF/5 cell monolayers.

3.3.3 Cell culture techniques for assaying plaque forming polioviruses

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.*, 1999a). The tissue culture plates were washed with 2 ml of 0.01 M PBS at pH 7.4 and incubated in a 5% CO_2 incubator (Galaxy CO_2 Incubator- Biotech, Northants, England) at 37°C for 1 h in order to starve the cells, after which the PBS was decanted. Each of these cell monolayers was

inoculated with a volume of test sample concentrate (1 ml) appropriate to cover the cell surface area of the cell culture plate used. Inoculated cell cultures were incubated at 37°C for 90 min to permit viruses to adsorb onto and infect cells. Overlay medium stock was prepared using filter sterilised double-strength Eagle's Minimum Essential Medium (96% of MEM) (Highveld Biological, Lyndhurst, South Africa), 3% foetal bovine serum (Delta Bioproducts, Kempton Park, South Africa) and 1% penicillin/streptomycin fungizone mix. A 2% Sea Kem ME Agarose solution (FMC Bioproducts, ME, USA), consisting of 2 g agarose in 100 ml of PBS at pH 7.2 (Sigma), was prepared separately and autoclaved at 121°C for 15 min. Equal volumes of the double-strength MEM and the agarose were mixed at 50°C to give a final concentration of 1% agarose. To each cell culture plate, 20 ml of warm (42°C) agarose overlay medium was added and allowed to set. Plaques appeared between 5 to 7 days. The viruses from 10 well-separated plaques were picked for further propagation onto BGM, HEp-2 and PLC/PRF/5 cell cultures.

3.3.4 Extraction of the ribonucleic acid from viral isolates

Ribonucleic acid (RNA) was extracted from infected tissue culture fluid by means of a commercial RNeasy Mini Kit (Qiagen, Hilden, Germany) and the extractions were performed according to the manufacturer's instructions. A total of 1.5 ml of virus infected cells were centrifuged at 300 x *g* (Eppendorf Centrifuge 5402D) for 5 min at ± 25°C and the pellet was dissolved in the appropriate volume of buffer RLT containing β-mercaptoethanol (Sigma). Cell cultures were homogenised using a biopolymer shredding system (QIAshredder™, Hilden, Germany) prior to RNA extraction. A DNase step was included in the extraction to remove contaminating deoxyribonucleic acid (DNA) from RNA preparations. Generally, DNase digestion is not required for RNA purified with the RNeasy Mini Kit since the silica-membrane, spin-column technology efficiently removes the majority of the DNA without DNase treatment. The additional use of RNase-Free DNase kit provides efficient on-column digestion of DNA during RNA purification from cells and tissues (Qiagen).

3.3.5 Reverse transcription multiplex PCR to distinguish polioviruses from non-polio enteroviruses

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 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 3.2). 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).

3.3.6 Sabin specific RT-triplex PCR to distinguish between Sabin PV types 1 to 3

Three sets of primers specific for Sabin PV types 1 to 3 were combined in a RT-triplex PCR to confirm the isolated PVs as Sabin PV vaccine strains and gave rise to amplicons of different sizes (Table 3.3) (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).

3.3.7 Restriction enzymes used in the typing of non-polio enteroviruses

Non-polio enteroviruses were typed with restriction enzymes (REs) such as *Sty* I, *Bgl* I and *Xmn* I (Promega Corp.) (Table 3.4) (Kämmerer *et al.*, 1994; Kuan, 1997). Aliquots of 10 µl of the RT-multiplex 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) gel electrophoresis (Hoefer electrophoresis unit). The RE patterns of some of the EVs were evaluated, although there has been a lack of sequence data for most of the EVs (Table 3.5) (Kämmerer *et al.*, 1994; Kuan, 1997).

3.3.8 Quality control of the amplification method

Standard precautions were applied in all the manipulations to reduce the possibility of sample contamination by amplified DNA molecules. Separate laboratories were used for reagents, treatment of samples and manipulation of amplified fragments. Negative controls for cell culture, RNA extraction and RT-PCRs were included in each assay.

3.4 Results and discussion

In the natural course of PV infection, excretion of PV in stools continues for a period of several weeks (Hovi *et al.*, 2001). However, in immunodeficient patients it may be prolonged up to several years and subsequently, these PVs may end up in the environment (Hovi *et al.*, 2001). Search for PVs in sewage or in other wastewater has been used for assessment of the extent of the epidemic spread of PV and to approximate the proportion of infected individuals in the source population (Manor *et al.*, 1999a; Hovi *et al.*, 2001). Therefore, environmental surveillance could be used as one approach to assess elimination of PV circulation in a given human population and to demonstrate the final success of PV eradication (Hovi *et al.*, 2001).

In this study, a total of 213 sewage and 138 river water samples were analysed during a two-year period. Previous research has shown that the PEG precipitation method could be highly effective for the concentration of environmental samples such as sewage and river water

(Hovi *et al.*, 2001). In agreement with these findings, the PEG/NaCl precipitation method and the monolayer plaque assay using HEp-2 cells (in the absence of mouse L [L20B] cells) proved sensitive for the isolation and purification of PVs from the environment. The fact that L20B cells are transfected with the gene for the human cellular receptor for PV, renders them susceptible to infection with PV (Wood and Hull, 1999). However, L20B cells are not absolutely specific for PVs since some other human enteric viruses, such as wild-type reoviruses, will grow in these cells (Wood and Hull, 1999). Although generally recommended for the isolation of PVs, L20B cells are not readily available and therefore, could not be applied in the current study.

The HEp-2 cells on the other hand were found to be highly permissive for PVs but much less so for other EVs and have been previously applied in the detection of PVs by researchers such as Fiore *et al.* (1998), Manor *et al.* (1999a), Manor *et al.* (1999b) and Buttinelli *et al.* (2003). Furthermore, this study revealed that BGM and PLC/PRF/5 cell cultures allowed the amplification of a broad spectrum of EVs. The ability of the isolated EVs to infect susceptible host cells and to form plaques (Figure 3.1), confirmed that they were viable and therefore, potentially infectious. Earlier studies carried out on similar water environments revealed that plaque-forming EVs actually represented a small minority of the EVs present, because the majority of the EVs infected the cell monolayers and replicated their RNA but failed to complete the replication cycle to produce plaques. However, it was possible to detect the RNA of these EVs by means of molecular techniques (Vivier *et al.*, 2001; Vivier *et al.*, 2004).

Using the monolayer plaque assay, 703 plaques from the sewage and 157 plaques from the river water samples were analysed. The RT-multiplex PCR proved useful for the rapid, specific and sensitive detection of PVs and for their distinction from NPEVs. This distinction is important in PV surveillance programs, specifically in the WHO polio eradication campaign. This methodology can be used to detect circulating wild-type PVs still circulating around the world. A total of 49 PV isolates were successfully distinguished from 176 NPEVs (Table 3.6). The remaining 634 plaques picked from the tissue culture plates were probably enteric viruses such as adenoviruses or reoviruses since HEp-2 cells were used, however, these plaques were not subjected to further analysis. A higher number of PVs (37 isolates or 76%) were detected in the sewage than in the river water samples. Furthermore,

approximately 70% of the PV isolates were detected on HEp-2 cells compared to 18% on BGM and 12% on PLC/PRF/5 cells (Table 3.6).

In the current study, the RNAs of 24 PV isolates (48%) showed the PV-specific bands of 193 bp, 297 bp and 565 bp (Figure 3.2; Table 3.2). The RNAs of 3 PV isolates (6%) displayed an additional 1 000 bp product resulting from a read through between primers Po1 and Po4, and the RNAs of 2 PV isolates (4%) displayed a 193 bp, 297 bp and 1 000 bp product (Table 3.6). The RNAs of 21 PV isolates (42%) showed only the PV-specific band at 193 bp and the 297 bp (Table 3.6). The RNA of PV type 3 showed a 193 bp (in addition to the 297 bp), the 565 bp band of the PV-specific Po3-Po4 primers was missing possibly due to mismatches of primer Po3 with type 3 sequences, which favours read through between the perfectly matching Po1 and Po4 primers (Figure 3.2) (Egger *et al.,* 1995). These results were in agreement with findings reported previously by other researchers such as Egger *et al.* (1995).

Non-polio enteroviruses were positive only with the EV-specific primers that generated a 297 bp product (Table 3.2). However, an exception was observed with CBV5 and CAV19, which displayed an additional band (193 bp) with a PV-specific primer pair (Figure 3.2). Similarly, Abraham *et al.* (1993) described primers that recognised all but certain PV type 2 strains and which still cross-reacted with echovirus strains. This cross-reactivity can be explained with the close relatedness of certain EVs such as coxsackieviruses and echoviruses to the PVs rather than to the other NPEVs. Egger and colleagues (1995) observed cross-reactivity with CAV21, because in the P2 genomic region (in which the sequences of the PV-specific primers are located), CAV21 showed 90% similarity to PV types 1 to 3.

The sensitivity of the RT-multiplex PCR, as measured with RNA extracted from a virus suspension, was found to be 10^2 plaque forming units (pfu) of Sabin PV type 1 in a 140 µl volume of sample. Since it has been estimated, that the ratio of virus particles to infectious units is between 100 and 1 000 for the EVs, it was concluded that the RT-multiplex PCR allowed the detection of 10^4 and 10^5 copies of enteroviral RNA per 140 µl volume of sample (Rotbart, 1990; Egger *et al.*, 1995; Melnick, 1996b; Vivier *et al*, 2001).

Polioviruses detected with the RT-multiplex PCR were further typed with a Sabin RT-triplex PCR, which combined three sets of primers specific for Sabin PV types 1 to 3 and resulted in

DNA bands of the expected sizes (Figure 3.3; Table 3.3). Results showed, that all 49 PV isolates were vaccine strains with Sabin PV type 1 being the most prevalent (29 isolates or 59.2%), followed by Sabin PV type 3 (12 isolates or 24.5%) and Sabin PV type 2 (8 isolates or 16.3%). The sensitivity of the Sabin RT-triplex PCR was found to be similar to that of the RT-multiplex PCR (10^2 pfu of Sabin PV type 1). 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 (CDC, 2003). None of the other EV prototype strains amplified with the Sabin specific primers. All negative controls were negative.

A total of 176 NPEVs were isolated in this study and the RNAs of all of these viruses were successfully typed with REs (*Sty* I, *Bgl* I *and Xmn* I each having their own specific six-base recognition site, Table 3.4). A digestive pattern was obtained that was easily visible and thus not too complicated to interpret (Figure 3.4; Table 3.5). In most of the cases the restriction patterns corresponded to those described by Kämmerer *et al.* (1994) and Kuan (1997). Thus, the NPEVs consisted of 50 CBV2 (28.4%), followed by 39 ECV11 (22.2%), 25 CBV5 (14.2%), 21 CBV3 (11.9%), 15 CBV4 (8.5%), 14 (CAV6) (8.0%), 7 CBV6 (4.0%), 2 CAV5 (1.1%), 2 CBV1 (1.1%) and 1 ECV19 (0.6%) (Figure 3.5), which was in agreement with the prevalence of these EVs in other parts of the world such as USA and Canada (Payment *et al.*, 1985; Rose *et al.*, 1986). In a study conducted by Hovi *et al.* (1996), EVs such as CBV2, CBV3, CBV4, CBV5, ECV6 and ECV11 were the most common serotypes in sewage. According to Vivier and colleagues (2001), CBV2, CBV3 and CBV5 were the most abundant of the EVs isolated from the environment in selected areas of South Africa.

The RT-multiplex PCR, followed by RE analysis and the Sabin specific RT-triplex PCR might prove some of the most efficient aids currently available for the rapid detection and typing of EVs in water environments. The availability of efficient methods to detect PVs and the potential presence of VDPVs might open the way to assess the possible broader significance of the findings reported in the current study.

3.5 Conclusions

In conclusion, this study addressed some of the issues regarding the prevalence of OPV strains in the environment. The identification of 49 viable OPV isolates in the sewage and river water sources (used in some cases for human consumption by the rural community) warrants further investigation into the presence and circulation of VDPVs as well as the potential health risk they might constitute. Therefore, the OPV isolates detected in this study will be subjected to nucleotide sequencing in order to determine the presence of possible mutations leading to the reversion of these OPV isolates.

This notion is supported by recent outbreaks of acute flaccid paralysis associated with circulating vaccine-derived polioviruses (cVDPVs) in four different regions of the world, namely the Middle East (Egypt), the Americas (Hispaniola: Dominican Republic and Haiti), the Western Pacific (Philippines), and in Africa (Madagascar) (Kew *et al.*, 2004). Since OPV vaccination is compulsory in South Africa and the country has a high number of immunodeficient people, it would appear that the risk of circulation of OPV strains and revertants of these strains might be relatively high. Thus, the final step of PV eradication will require details on the possibility of persistent infections and excretion of VDPVs for long periods by immunodeficient patients and the survival in the environment of these strains to the extent that they may infect non-immune individuals after termination of PV vaccination. Therefore, strategies aimed at the protection of immunodeficient patients against complications of OPV-vaccination are essential.

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3.6 References

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Table 3.1: Sewage samples used in the isolation of polioviruses from selected water treatment plants in South Africa

System	Design	Waste Type	Population size
Ancor (AN)	3-stage Bardenpho	80% Domestic and 20% Industrial	5 000
Benoni (Bn)	3-stage Bardenpho	60% Domestic and 40% Industrial	25 000
Boksburg (B)	3-stage Bardenpho	70% Domestic and 30% Industrial	30 000
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
JP Marais (JP)	3-stage Bardenpho	100% Domestic	29 000
Jan Smuts (Js)	3-stage Bardenpho	90% Domestic and 10% 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
Rynfield (RnF)	5-stage Phoredox	100% Domestic	20 000
Tsakane (Ts)	3-stage Bardenpho	100% Domestic	20 000
Vlakplaats (VP)	3-stage Bardenpho	70% Domestic and 30% Industrial	130 000

Table 3.2: 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	E1	5'-AAG CAC TTC TGT TTC C-3'	297	EV
443-460	E2	5'-CAT TCA GGG GCC GGA GGA-3'		EV
P2 region				
4460-4478	Po1	5'-CAG TTC AAG AGC AA (A/G) CAC C-3'	193	PV
4634-4653	Po2	5'-TC (A/G) TCC AT (A/G) AT (A/C) AC (T/C) AC (T/A) CC-3'		PV
P2-P3 region				
4922-4941	Po3	5'-GAA ATG TGT AAG AAC TGT CA-3'	565	PV
5467-5487	Po4	5'-GTA ACA ATG TTT CTT TTA GCC-3'		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 3.3: 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 3.4: 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 3.5: Fragments resulting from digestion by *Sty* I, *Bgl* I and *Xmn* I REs of 297 bp amplified EVs (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 3.6: Detection of poliovirus vaccine strains in sewage and river water samples in South Africa between 2001 and 2003

Date	Sample isolate	Patterns with amplicons obtained by RT-multiplex PCR	Sabin specific RT-PCR	Cell culture
13/08/2001	DP1 (Daspoort)	PV (193bp, 297bp)	PV3	BGM
13/08/2001	DP2 (Daspoort)	PV (193bp, 297bp)	PV3	BGM
13/09/2001	DP1 (Daspoort)	PV (193bp, 297bp, 565bp, 1000bp)	PV1	BGM
15/04/2002	Kspnt DR (River water)	PV (193bp, 297bp)	PV1	PLC/PRF/5
15/04/2002	Kppt DR (River water)	PV (193bp, 297bp)	PV1	PLC/PRF/5
15/04/2002	Ksppt DR (River water)	PV (193bp, 297bp)	PV1	PLC/PRF/5
15/04/2002	Kssppnt DR (River water)	PV (193bp, 297bp)	PV1	PLC/PRF/5
21/05/2002	OF1 (Olifantsfontein)	PV (193bp, 297bp, 565bp, 1000bp)	PV1	BGM
21/05/2002	OF2 (Olifantsfontein)	PV (193bp, 297bp, 565bp)	PV2	BGM
28/05/2002	OF2 (Olifantsfontein)	PV (193bp, 297bp, 565bp, 1000bp)	PV2	BGM
02/07/2002	OF2 (Olifantsfontein)	PV (193bp, 297bp, 565bp)	PV2	BGM
09/07/2002	VP1 (Vlakplaats)	PV (193bp, 297bp)	PV3	BGM
16/07/2002	TCspnt DR (River water)	PV (193bp, 297bp)	PV2	PLC/PRF/5
16/07/2002	TCppt DR (River water)	PV (193bp, 297bp)	PV2	PLC/PRF/5
23/07/2002	MF1 (Modderfontein)	PV (193bp, 297bp)	PV3	HEp-2 cells
23/07/2002	GR2 (Grundlingh)	PV (193bp, 297bp)	PV3	HEp-2 cells
23/07/2002	GR3 (Grundlingh)	PV (193bp, 297bp)	PV3	HEp-2 cells
23/07/2002	OF5 (Olifantsfontein)	PV (193bp, 297bp)	PV3	HEp-2 cells
23/07/2002	Bn4 (Benoni)	PV (193bp, 297bp)	PV3	HEp-2 cells
30/07/2002	AN2 (Ancor)	PV (193bp, 297bp)	PV3	HEp-2 cells
13/08/2002	MF5 (Modderfontein)	PV (193bp, 297bp, 565bp)	PV1	HEp-2 cells
13/08/2002	MF6 (Modderfontein)	PV (193bp, 297bp, 565bp)	PV1	HEp-2 cells
28/08/2002	D1 (Daveyton)	PV (193bp, 297bp, 1000bp)	PV2	HEp-2 cells
28/08/2002	D2 (Daveyton)	PV (193bp, 297bp, 1000bp)	PV1	HEp-2 cells
11/09/2002	Hb1 (Heidelberg)	PV (193bp, 297bp)	PV3	HEp-2 cells
11/09/2002	Ts1 (Tsakane)	PV (193bp, 297bp, 565bp)	PV1	HEp-2 cells

Table 3.6: Detection of poliovirus vaccine strains in sewage and river water samples in South Africa between 2001 and 2003 (continued)

Date	Sample isolate	Patterns with amplicons obtained by RT-multiplex PCR	Sabin specific RT-PCR	Cell culture
17/09/2002	B3 (Boksburg)	PV (193bp, 297bp, 565bp)	PV1	HEp-2 cells
17/09/2002	RnF1 (Rynfield)	PV (193bp, 297bp)	PV1	HEp-2 cells
17/09/2002	RnF2 (Rynfield)	PV (193bp, 297bp)	PV1	HEp-2 cells
08/10/2002	MF3 (Modderfontein)	PV (193bp, 297bp, 565bp)	PV1	HEp-2 cells
08/10/2002	D2 (Daveyton)	PV (193bp, 297bp, 565bp)	PV1	HEp-2 cells
08/10/2002	D3 (Daveyton)	PV (193bp, 297bp, 565bp)	PV1	HEp-2 cells
08/10/2002	D5 (Daveyton)	PV (193bp, 297bp, 565bp)	PV1	HEp-2 cells
08/10/2002	D8 (Daveyton)	PV (193bp, 297bp, 565bp)	PV1	HEp-2 cells
08/10/2002	D9 (Daveyton)	PV (193bp, 297bp)	PV3	HEp-2 cells
08/10/2002	D10 (Daveyton)	PV (193bp, 297bp, 565bp)	PV1	HEp-2 cells
08/10/2002	D11 (Daveyton)	PV (193bp, 297bp)	PV3	HEp-2 cells
08/10/2002	D21 (Daveyton)	PV (193bp, 297bp, 565bp)	PV1	HEp-2 cells
08/10/2002	JS4 (Jan Smuts)	PV (193bp, 297bp, 565bp)	PV1	HEp-2 cells
08/10/2002	Mcc4 (Mccomb)	PV (193bp, 297bp, 565bp)	PV1	HEp-2 cells
22/10/2002	JP1 (JP Marais)	PV (193bp, 297bp, 565bp)	PV1	HEp-2 cells
22/10/2002	Mcc1 (Mccomb)	PV (193bp, 297bp, 565bp)	PV1	HEp-2 cells
22/10/2002	D2 (Daveyton)	PV (193bp, 297bp, 565bp)	PV1	HEp-2 cells
09/01/2003	Sbnd2 (River water)	PV (193bp, 297bp, 565bp)	PV1	HEp-2 cells
09/01/2003	Lv1 (River water)	PV (193bp, 297bp, 565bp)	PV1	HEp-2 cells
15/04/2003	Mt1 (River water)	PV (193bp, 297bp, 565bp)	PV2	HEp-2 cells
15/04/2003	Mt2 (River water)	PV (193bp, 297bp, 565bp)	PV2	HEp-2 cells
15/04/2003	Mb1 (River water)	PV (193bp, 297bp, 565bp)	PV1	HEp-2 cells
15/04/2003	Sbnd1 (River water)	PV (193bp, 297bp, 565bp)	PV1	HEp-2 cells

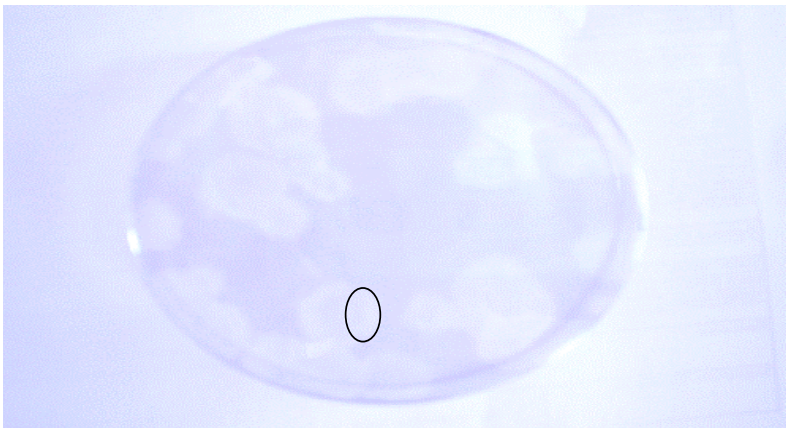

Figure 3.1: **Plaques formed on HEp-2 cell monolayers by poliovirus isolates**

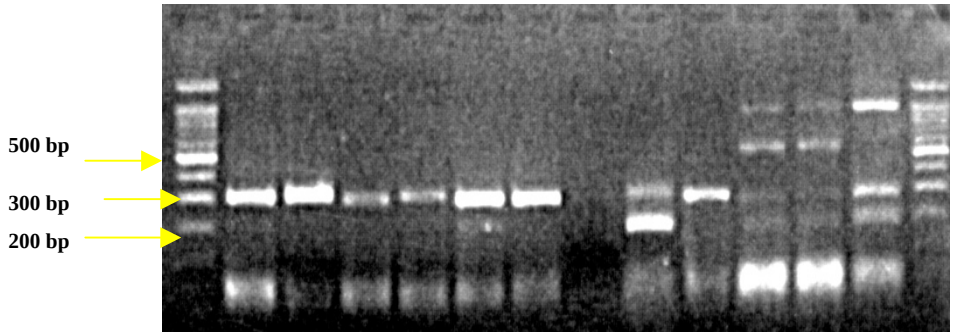

Figure 3.2: **Band patterns observed with the RT-multiplex PCR of the Sabin PV types 1 to 3 and the non-polio enteroviruses. Lane 1: 100 bp Marker; Lane 2: CBV1 (297 bp); Lane 3: CBV2 (297 bp); Lane 4: CBV3 (297 bp); Lane 5: CBV4 (297 bp); Lane 6: CBV5 (193 bp and 297 bp); Lane 7: CBV6 (297 bp); Lane 8: CAV9 (297 bp); Lane 9: CAV19 (193 bp and 297 bp); Lane 10: ECV1 (297 bp); Lane 11: Sabin PV type 1 (193 bp, 297 bp, 565 bp and 1 000 bp); Lane 12: Sabin PV type 2 (193 bp, 297 bp, 565 bp and 1 000 bp); Lane 13: Sabin PV type 3 (193 bp and 297bp); Lane 14: 100 bp Marker. In the RT-multiplex PCR, the Sabin PV type 3 strain did not show the Po3-Po4 band (565 bp)**

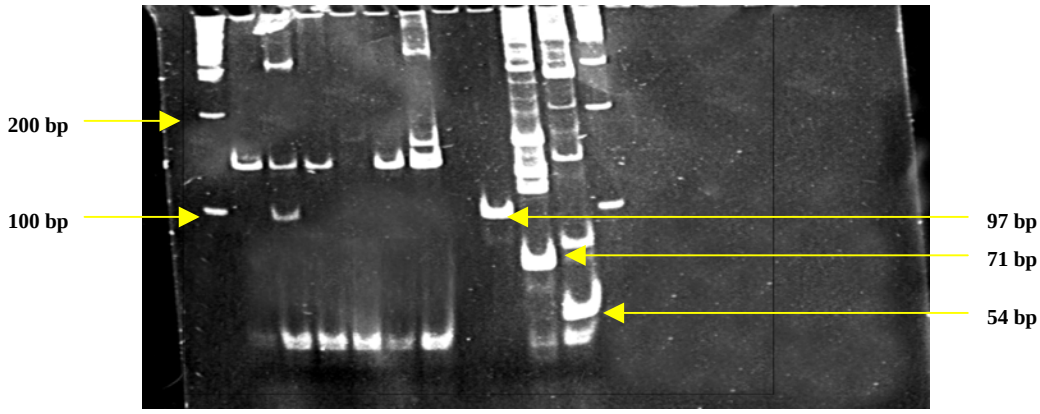

Figure 3.3: **Sabin specific RT-triplex PCR of the PV isolates and the positive controls. Lane 1: Marker 100 bp; Lane 2: negative sample; Lane 3: Sabin PV type 1 (positive isolate 97 bp); Lanes 4-8: Negative isolates; Lane 9: Sabin PV type 1 (positive control 97 bp); Lane 10: Sabin PV type 2 (positive control 71 bp); Lane 11: Sabin PV type 3 (positive control 54bp); Lane 12: Marker 100 bp**

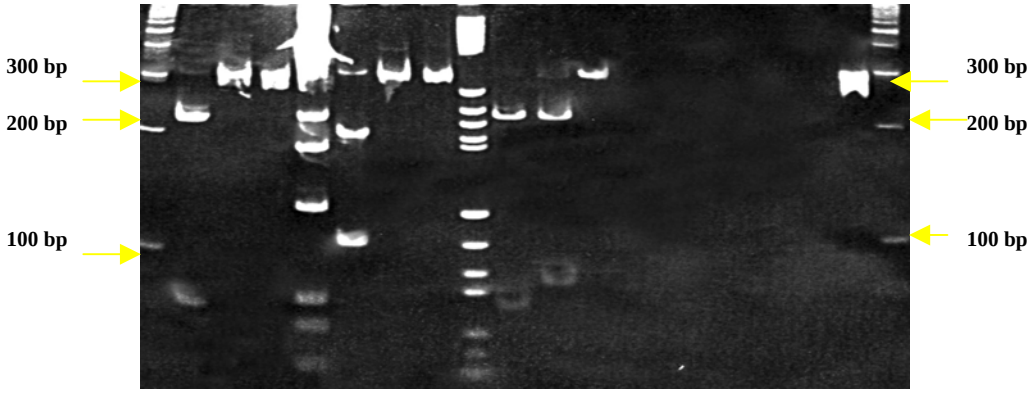

Figure 3.4: **Restriction enzyme digestion of 297 bp products from prototype strains of enteroviruses with three restriction enzymes (*Sty* I, *Bgl* I and *Xmn* I). Lane 1: Marker 100 bp; Lane 2: *Sty* I (226 bp and 71 bp), Lane 3: *Bgl* I (297 bp), Lane 4: *Xmn* I (297bp) - CBV3; Lane 5: PGem Marker; Lane 6: *Sty* I (197 bp and 100 bp), Lane 7: *Bgl* I (297 bp), Lane 8: *Xmn* I (297 bp) - Sabin PV type 2; Lane 9: Marker V; Lane 10: *Sty* I (226 bp and 71 bp), Lane 11: *Bgl* I (217 bp and 80 bp), Lane 12: *Xmn* I (297 bp) - ECV19; Lane 13-18: empty; Lane 19: EV uncut (297 bp); Lane 20: Marker 100 bp**

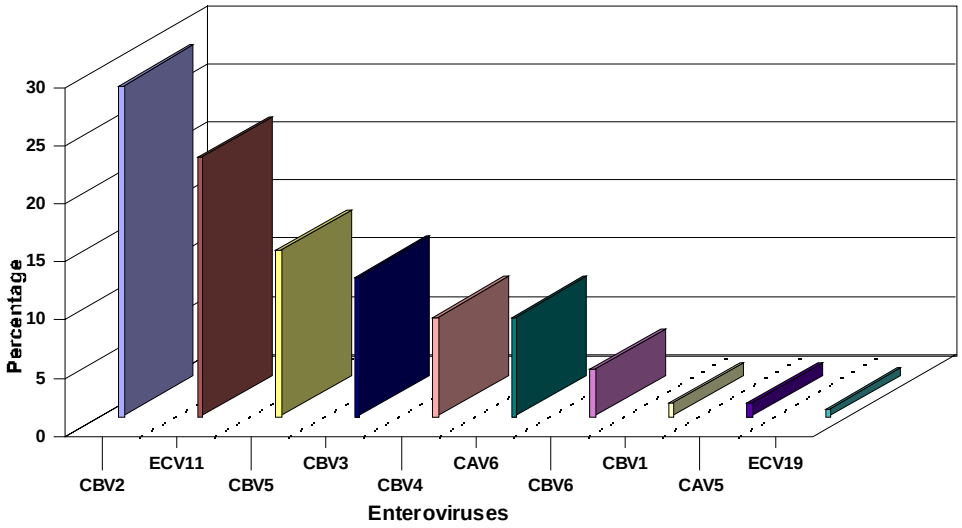

Figure 3.5: Detection of enteroviruses in sewage and river water samples collected from selected areas in South Africa from 2001 to 2003

CHAPTER 4

ISOLATION OF POLIOVIRUS VACCINE STRAINS FROM STOOL SPECIMENS OF IMMUNODEFICIENT CHILDREN IN SOUTH AFRICA

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

4.1 Abstract

After exposure to the oral poliovirus vaccine (OPV), immunocompetent persons excrete poliovirus (PV) vaccine strains for a limited period of time (usually 2 to 3 months). In contrast, immunodeficient individuals remain sometimes chronically infected and in some cases PV excretion times as long as 10 years have been reported. During prolonged replication in the human intestine, the PV vaccine strain almost invariably reverts its attenuated character and acquires neurovirulent properties (vaccine-derived polioviruses), which resemble wild-type PV strains. Concerns about potential health risks constituted by these vaccine-derived polioviruses (VDPVs) are supported by recent outbreaks of acute flaccid paralysis associated with circulating VDPVs among unvaccinated or incompletely vaccinated individuals in four different regions of the world. The aim of this study was to determine the occurrence of OPV strains in stools of immunodeficient children from a selected area in South Africa, as a first step towards future research on the prevalence and potential health impact of VDPVs. In a period of one year (2003-2004), a total of 164 stool samples of HIV-positive children aged 4 months to 8 years (including those with an AIDS indicator condition according to the CDC classification) were studied for the excretion of OPV strains. In addition, 23 stool samples from healthy immunocompetent children were analysed after receiving their OPV immunisation. 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. Using restriction enzyme analysis, 13 PVs were distinguished from 41 non-polio enteroviruses (NPEVs). A Sabin specific RT-triplex PCR confirmed the presence of 7 Sabin PV type 1 (53.8%), 4 Sabin PV type 3 (30.8%) and 2 Sabin PV type 2 (15.4%) isolates. The majority of the NPEV group was made up of 7 coxsackievirus B3 (CBV3) (17.1%), 6 echovirus 11 (ECV11) (14.6%), 5 ECV9 (12.2%) and 3 coxsackievirus

A6 (CAV6) (7.3%) isolates. According to the results, two of the immunodeficient patients (P023 and P140) who had received their last OPV immunisation more than 15 months before (vaccinated at 14 weeks of age) tested positive for Sabin PVs type 3 and type 1, respectively. A five year old immunodeficient patient (P052) who had received her last OPV immunisation more than 42 months before (vaccinated at 18 months of age) tested positive for Sabin PV type 1. These results suggested that immunodeficient patients vaccinated with OPV may excrete potentially pathogenic VDPVs for a prolonged period of time. These VDPVs may circulate in the community resulting in possible infections in the unvaccinated population. Therefore, the information obtained in this study would be essential for strategies aimed at the protection of both immunodeficient as well as immunocompetent individuals against complications of vaccination with OPV.

Keywords: Immunocompetent, Immunodeficient children, OPV strains, Prolonged excretion, Stool specimens, Vaccine-derived polioviruses

4.2 Introduction

Poliovirus (PV) is the only enterovirus (EV) for which a vaccine is available (Zaoutis and Klein, 1998). Two vaccines were developed to control poliomyelitis: the inactivated poliovirus vaccine (IPV) and the oral poliovirus vaccine (OPV) (Wood *et al.*, 2000). Although the merits of the two different types of polio vaccines have been the subject of heated debates, both of them were highly effective in eradicating polio from the Western Hemisphere and in decreasing the incidence of poliomyelitis world-wide (Minor, 1999; Wood and Thorley, 2003). The introduction of the IPV reduced the number of cases by 90% and this decline continued after the introduction of the OPV during the early 1960s (Minor, 1999; Wood *et al.*, 2000). The recent declaration of three major regions of the world, the Americas, Europe and the Western Pacific as being free of circulating wild-type PV constitutes a major achievement in public health (Wood and Thorley, 2003).

Following certification of PV eradication by the year 2005 or shortly thereafter, the public health community and policy makers will be faced with the decision of how and when to stop polio vaccination (Dowdle *et al.*, 2001). The risks are obvious, considering the fact that if PV is re-introduced into a susceptible population, a catastrophic epidemic of paralytic disease,

disability and death could ensue (Dowdle *et al.,* 2001). It is believed that PV could emerge through re-introduction of PV from a laboratory, prolonged replication in immunodeficient patients and persistent transmission of vaccine-derived polioviruses (VDPVs) (which are highly divergent PVs with at least 1% divergence in nucleotide sequence from the prototype Sabin PV strain) in susceptible populations (Dowdle *et al.*, 2001; Khetsuriani *et al.,* 2003).

Previously, no long-term carrier stage in humans has been reported following wild-type PV infection (Wood *et al.,* 2000; Hovi *et al.,* 2004). In paralytic cases caused by the wild-type PV, virus titres in faeces rapidly decrease during the few weeks following onset of disease (Alexander *et al.,* 1997; Hovi *et al.,* 2004). However, Hovi and colleagues (2004) have reported prolonged excretion of wild-type PV for 7 months by two immigrant siblings in Finland (Hovi *et al.,* 2004). In immunocompetent OPV recipients, the excretion of PVs is usually short-lived, seldom exceeding 2 months (Alexander *et al.,* 1997; Kew *et al.,* 1998; Wood *et al.,* 2000; Hovi *et al.,* 2004). In contrast, several OPV recipients with severe deficiencies in humoral immunity tend to remain chronically infected and have been reported to excrete PVs for long periods of time (in some cases as long as 10 years) (Kew *et al.,* 1998; Bellmunt *et al.,* 1999; Shulman *et al.,* 2000).

During prolonged replication in the human intestine, the OPV strain invariably reverts its attenuated character and acquires neurovirulent properties as well as transmissibility characteristics typical of wild-type PV strains (Cherkasova *et al.,* 2002; Buttinelli *et al.,* 2003; Hovi *et al.,* 2004). Reversion of the OPV strains to increased neurovirulence is one key factor for the occurrence of vaccine-associated paralytic poliomyelitis (VAPP), which occurs at a rate of approximately 1 per 500 000 first doses of OPV in immunocompetent individuals and at an approximately 3 000 fold higher rate for immunodeficient patients (Strebel *et al.,* 1992; Sutter and Prevots, 1994; Bellmunt *et al.,* 1999; Shulman *et al.,* 2000; Hovi *et al.,* 2004). As a consequence chronically infected individuals may present with paralytic disease some years after OPV administration and may also transmit VDPVs to their close contacts (Kew *et al.,* 1998; Bellmunt *et al.,* 1999; Hovi *et al.,* 2004). This raises concerns for the desired future global cessation of OPV immunisation, which will be considered after the eradication program has been completed (Hovi *et al.,* 2004).

Patients with primary immunodeficiency disorders affecting the B-cell system appear to be at highest risk for prolonged PV replication and excretion (World Health Organization [WHO] Scientific Group, 1997; Wood *et al.*, 2000). This group includes people with either X-linked or sporadic agammaglobulinaemia XLA and those with common variable immunodeficiency (CVID) (WHO Scientific Group, 1997; Wood *et al.*, 2000). There are very limited data on secondary immunodeficiency as a risk factor for VAPP or prolonged VDPV excretion (Centers for Disease Control and Prevention [CDC], 1997; Wood *et al.*, 2000). According to current scientific data, human immunodeficiency virus (HIV) infection is not a risk factor for paralytic poliomyelitis caused by wild-type PV or VDPV (Wood *et al.*, 2000). However, two case reports, one from Romania and one from Zimbabwe, have linked HIV infection and VAPP (Ion-Nedelscu *et al.*, 1994; Chitsike and van Furth, 1999; Wood *et al.*, 2000). The OPV is therefore not advisable for immunodeficient people (Minor, 2001; Buttinelli *et al.*, 2003). Although immunodeficiencies are listed as a contraindication for receiving OPV, patients with these clinical conditions may receive the OPV before their immunodeficiency is diagnosed or may be infected with OPV strains excreted by other vaccinees or due to circulating OPV strains within the community (person-to-person transmission) (Triki *et al.*, 2003).

Currently, the identification and characterisation of PVs in stool specimens collected from OPV-vaccinees rely on virus isolation in susceptible tissue culture cells (Melnick, 1996; Buonagurio *et al.*, 1999). A second culture step, in which the PV is neutralised in the presence of serotype-specific antiserum pools is usually required to identify the serotypes of the PV isolates (Buonagurio *et al.*, 1999). Recent developments in molecular detection technology made it possible to diagnose PVs by non-culture-based methods such as the polymerase chain reaction (PCR) (Muir *et al.*, 1998). Most of these methods use non-degenerated primers designed to target conserved sequences within the 5'untranslated region (5'UTR) or VP1 capsid protein-coding regions of the PV genome (Kuan, 1997; Caro *et al.*, 2001; Casas *et al.*, 2001). A reverse transcription-triplex PCR (RT-triplex PCR) method that allows the simultaneous identification of Sabin PV type 1, 2 and 3 vaccine strains in a single reaction has been reported by Yang *et al.* (1991). Wild-type PV strains are not detected by this PCR method (Yang *et al.*, 1991; Buonagurio *et al.*, 1999).

In the current study, various molecular techniques were applied in order to determine the presence of OPV strains in stool specimens of immunodeficient patients (such as HIV-positive children including those with an acquired immunodeficiency syndrome [AIDS] indicator condition according to the CDC classification) from a selected area in South Africa. In a follow-up study, the genomes of these OPV strains will be sequenced in order to find mutations leading to the reversion of the OPV strains to increased neurovirulence. In conjunction with this study, medical professionals from the Department of Paediatrics, Kalafong Hospital/University of Pretoria, South Africa have been investigating the clinical status of the immunodeficient children involved and the results of this project will be published in a separate article.

4.3 Materials and methods

4.3.1 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) (Fiore *et al.*, 1998; Manor *et al.*, 1999a; Manor *et al.*, 1999b; Buttinelli *et al.*, 2003). 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.

4.3.2 Sample size

In order to estimate the prevalence of OPV strains (possibly VDPVs) in the stool specimens of immunodeficient children, a maximum sample size associated with an expected prevalence of 50% was analysed. Sample sizes for different levels of confidence and accuracy of estimation are shown in Table 4.1 (Biostatistics Unit, Medical Research Council, Pretoria, South Africa). 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%. Lowering of the sample size to 95 stool specimens would give a 95% confidence level and an accuracy of estimation of 10%.

4.3.3 Patient specimens

In a period of one year (2003-2004), one stool specimen was collected from each of the 164 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. The research protocol was approved by the Faculty of Health Sciences Research Ethics Committee, University of Pretoria and the parents of the research participants gave informed consent for the inclusion of their children in the study.

During the same period of time, 23 stool samples from 3 healthy immunocompetent babies were collected after receiving their scheduled OPV immunisations. This group of children served as a control group in order to demonstrate the type of PVs being excreted and possibly the duration of excretion of OPV strains by immunocompetent children. The stool samples were collected regularly from the immunocompetent children during their OPV immunisation schedule (Table 4.2): one stool specimen 48 h after each vaccination (at birth, 6 weeks, 10 weeks, 14 weeks and 18 months) and then one stool sample on a weekly basis until no PV was detected in the stools.

4.3.4 Extraction of the ribonucleic acid

Stool specimens were homogenised and clarified by mixing 300 µl of 10-50% faecal suspension with an equal volume of freon (Sigma Chemical Co., Louis, United States of America [USA]), and the mixture was centrifuged at 12 000 x *g* (Eppendorf Centrifuge 5402D, Hamburg, Germany) for 5 min at room temperature (±25°C). A total of 140 µl of the supernatant was mixed with 500 µl of TRIzol (Invitrogen Life Techno, Paisley, Scotland), and incubated at room temperature (±25°C) for 5 min to permit complete dissociation of the nucleoprotein complex for the extraction of viral ribonucleic acid (RNA) according to the manufacturer's instructions. Following the addition of pure chloroform (100 µl) (Sigma), each mixture was centrifuged at 12 000 x *g* (Eppendorf Centrifuge 5402D) for 15 min at 4°C. The aqueous phase (300 µl) was transferred to sterile Eppendorf tubes containing 30 µl of

sodium acetate (pH 5.2) (Merck, Darmstadt, Germany) and 600 µl of 100% ethanol (Merck). After 24 h at -20°C, the samples were centrifuged at 12 000 x *g* (Eppendorf Centrifuge 5402D) for 15 minutes at 4°C. Each RNA pellet was washed with 300 µl of 70% ethanol (Merck) and centrifuged at 12 000 x *g* (Eppendorf Centrifuge 5402D) for 5 min at 4°C. The pellets were briefly air-dried and dissolved in 35 µl of RNase free water (DEPC-water, Promega Corp., Madison, USA). The dissolved pellets were incubated for 10 min at 42°C in a hybridisation oven (Techne Hybridiser HB-1D, Techne, Cambridge, United Kingdom). The extracted RNA was frozen at -70°C for further analysis.

4.3.5 Reverse transcription polymerase chain reaction

The reverse transcription polymerase chain reaction (RT-PCR) for the amplification of RNA was carried out using a Promega Access RT-PCR system (Promega Corp.) 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 4.3), 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 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). 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).

4.3.6 Nested polymerase chain reaction

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 (Promega Corp.). 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 4.3), 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) gel electrophoresis (Midicell Primo Gel Apparatus).

4.3.7 Reverse transcription multiplex PCR to distinguish polioviruses from non-polio enteroviruses

A reverse transcription multiplex PCR (RT-multiplex PCR) as described by Egger *et al.* (1995) was used for the rapid detection and distinction of PVs from non-polio enteroviruses (NPEVs). Primers specific for either EV or PV were combined in a RT-multiplex PCR (Promega Corp.) to obtain amplicons of different sizes (Table 4.4). 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), 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). After 30 cycles, 20 µl of each PCR product were subjected to agarose (2%) (Seakem LE agarose) gel electrophoresis (Midicell Primo Gel Apparatus).

4.3.8 Restriction enzyme analysis

Enteroviruses were partially typed with restriction enzymes (REs) such as *Sty* I, *Bgl* I and *Xmn* I (Promega Corp.) (Table 4.5) (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 for 3 h at 37°C and were analysed using 7% polyacrylamide (BioRad, Hercules, CA, USA) gel-electrophoresis (Hoefer, San Francisco, USA). The restriction patterns of the EVs were evaluated based on previously published RE patterns (Kämmerer *et al.*, 1994; Kuan, 1997) (Table 4.6).

4.3.9 Sabin specific RT-triplex PCR

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 4.7) (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).

4.3.10 Quality control of the amplification methods

Standard precautions were applied in all the manipulations to reduce the possibility of sample contamination by amplified DNA molecules. Separate laboratories were used for reagents, treatment of samples and manipulation of amplified fragments. Negative controls for RNA extraction and RT-PCRs were included in each assay.

4.4 Results and discussion

Nearly four decades of experience have shown that OPV is very safe and effective in preventing poliomyelitis (Buttinelli *et al.*, 2003). Despite the advantages in using the attenuated OPV strains, one disadvantage of OPV is the potential risk of VDPVs, which may cause neurological complications in vaccine recipients and their susceptible contacts. In comparison to immunocompetent people, immunodeficient individuals are known to remain chronically infected and may act as potential reservoirs for PV resulting in the re-introduction of PVs after the polio eradication initiative (Hovi *et al.*, 2004). Oral poliovirus vaccination is, therefore, not advisable for people with severe deficiencies in humoral immunity, because of

the possible chronic excretion of PV vaccine strains (notably VDPVs) by these individuals (Buttinelli *et al.*, 2003).

A RT-multiplex PCR has been developed for the rapid and sensitive detection of PVs (Egger *et al.*, 1995). This RT-multiplex PCR has been applied in the discrimination of PVs from NPEVs, which is an important factor in the PV surveillance program (Egger *et al.*, 1995). This is achieved by combining EV-specific primers (E1 and E2) and PV-specific primers (Po1 to Po4), thus giving rise to amplicons of specific sizes (Table 4.4) (Egger *et al.*, 1995). In this study, the sensitivity of the RT-multiplex PCR, as measured with RNA extracted from a virus suspension, was found to be 10^2 plaque forming units (pfu) of PV type 1 in 140 µl volume of sample and these results were in agreement with reports by other researchers such as Egger *et al.* (1995), Melnick (1996) and Vivier *et al.* (2001). However, in this study the RT-multiplex PCR generally failed to detect the majority of PVs and EVs being excreted by the immunodeficient as well as immunocompetent children (the control group). This outcome could be attributed in part to the components of the stool specimens that inhibit the PCR reaction. Since the RT-multiplex PCR includes one-step RT-PCR without a nested PCR, this method may not be sensitive enough to detect the presence of a low number of PVs and EVs in stool specimens. This is in agreement with findings by Buonagurio and colleagues (1999), that 21% of culture-positive PV samples produced a negative PCR result.

In order to avoid false negative results, a RT-PCR in combination with a nested PCR was applied in the current study. Since, this method is very sensitive, wild-type PV sequences could still be picked up if they circulated anywhere in the world. In this study, 54 EVs were detected in the stool specimens of the 164 HIV-positive children. The detection of EVs in the faeces of the immunodeficient children did not completely confirm an EV diagnosis, because the excretion of EVs may persist for several weeks after an EV infection in some patients (Zaoutis and Klein, 1998). 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, septicaemia and upper respiratory tract infections. Some of the children were hospitalised for anaemia, chronic diarrhoea,

dehydration and malnutrition. In total, 17 of these immunodeficient children died during the course of the study, therefore, the excretion of EVs by these patients could not be followed.

Enteroviruses isolated in the current study were successfully amplified with both sets of primers for the RT-PCR and nested PCR, resulting in deoxyribonucleic acid (DNA) bands of the expected sizes (Table 4.3). Sensitivities of both PCRs were determined by using enteroviral RNA extracted from virus culture supernatant of known titre. The RT-PCR detected 10 pfu of PV type 1 in 140 µl volume sample, whereas the nested PCR allowed the detection of between 1 and 10 copies of enteroviral RNA. Thus, a higher level of sensitivity was obtained with the nested PCR. These levels of sensitivity were in agreement with findings reported previously by other researchers such as Kämmerer *et al.* (1994), Kuan (1997) and Vivier *et al.* (2001).

Nested PCR products (Figure 4.1) obtained from stool specimens of the immunodeficient children were analysed with the REs *Sty* I, *Bgl* I and *Xmn* I. Most of the EVs isolated from the immunodeficient children exhibited RE patterns (Table 4.6) identical to those described by Kuan (1997). Based on the RE analysis, 13 PVs were successfully distinguished from 41 NPEVs (Figure 4.2). 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 4.8). Using the Sabin specific RT-triplex PCR (Figure 4.3), all of these PV isolates were typed as Sabin PV vaccine strains. The sensitivity of the Sabin RT-triplex PCR was found to be similar to that of the RT-multiplex PCR (10^2 pfu of PV type 1). 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). None of the other EV prototype strains amplified with the Sabin specific primers.

The majority of the NPEV group (41 isolates) detected in the stool specimens of the immunodeficient children consisted of 7 coxsackievirus B3 (CBV3) (17.1%), 6 echovirus 11 (ECV11) (14.6%), 5 ECV9 (12.2%) and 3 coxsackievirus A6 (CAV6) (7.3%) isolates (Figure 4.4). Other NPEVs detected included CAV3, CAV4, CAV5, CAV7, CAV17, CAV22, CBV2, CBV5, CBV6, ECV13 and ECV30 (Figure 4.4). Some of the NPEVs (4 isolates) could not be typed and were, therefore, unidentifiable with the techniques applied (Figure

4.4). These results were in agreement with findings reported previously by other researchers such as Druyts-Voets (1997), Nairn and Clements (1999) as well as Vivier *et al.* (2001).

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 (Table 4.9). All EVs were typed as PVs using the RE analysis, and the Sabin specific RT-triplex PCR identified them as Sabin PV vaccine strains. Six of the PV isolates were typed as Sabin PVs type 1 (Table 4.9). One of the seven PV isolates was typed as Sabin PV type 2 and was isolated from one of the healthy babies 48 h after receiving its OPV immunisation at 10 weeks of age (Table 4.9). According to the results, PV excretion generally stopped by the end of the second week following each vaccination (at birth, 6 weeks and 10 weeks) or the number of PVs in the stool specimens was too low to be detected by the molecular techniques applied (Table 4.9). Furthermore, in this study no PVs could be detected in the stool samples collected from one of the immunocompetent children after the 14th week vaccination, even 48 h following vaccination (Table 4.9). In two of the immunocompetent children, who had received the 18 month polio vaccination, PVs were detected in the stool samples 48 h following vaccination, but not in the samples collected on a weekly basis following the last polio vaccination, thus indicating cessation of PV excretion by these children (Table 4.9). These results indicated that the immunocompetent children involved in this study did not excrete PVs for more than a month following each polio vaccination. These findings were in agreement with the results of studies in other parts of the world, in which PV was found to be excreted by healthy children for not more than 2-3 months following vaccine administration (Marker Test Subcommittee and the Japan Live Poliovaccine Research Commission, 1967; Alexander *et al.*, 1997). It was shown that the period over which PV was excreted by individuals previously immunised with OPV or infected naturally was shorter than the excretion period of susceptible individuals (Marker Test Subcommittee and the Japan Live Poliovaccine Research Commission, 1967; Alexander *et al.*, 1997).

In eight of the immunodeficient children (P020, P025, P031, P039, P045, P069, P114 and P126), PVs were detected in stool specimens collected less than 3 months following their last polio immunisation (Table 4.8). Patient P114 (6 months old baby) was immunised at the age of 14 weeks (02/09/2003) and Sabin PV type 1 was detected in a stool specimen collected three months after the last recorded vaccination (01/12/2003) (Table 4.8). Patients P025 (18

months old baby) and P126 (20 months old baby) were immunised at 18 months on the 16/07/2003 and 08/10/2003, respectively. Polioviruses type 3 were detected in stool samples collected from both patients two weeks (29/07/2003 for P025) and two months (17/12/2003 for P126) following the last polio vaccination (Table 4.8). In comparison to these immunodeficient patients, OPV strains could not be detected in the current study in stool samples of the immunocompetent children collected following the 14th week vaccination and a week following the 18th month vaccination. Patient P045 (4 months old baby) was immunised lastly at 6 weeks of age (22/07/2003) and tested positive for PV type 3 one month following vaccination (22/08/2003) (Table 4.8). A second follow-up stool sample, collected from patient P045 (21/10/2003), tested negative for PV three months after the last polio vaccination (at the age of 6 weeks) indicating a possible cessation of PV excretion by this patient (Table 4.8). Patient P045 was admitted to the hospital for pneumonia and had CD4^{+} counts between 200-499 cells.mm^{-3} (Table 4.8). In general, there was a lack of a complete record in the polio immunisation schedule for several of the immunodeficient patients, because most of them could not visit the hospital for routine immunisation or have omitted immunisation dates for various unknown reasons and therefore, the excretion of OPV strains by these patients could not be monitored throughout the whole study. Thus, it is not certain whether patients P020, P031, P039, P045 and P069 have received a 10th and a 14th week polio vaccination due to the lack of a complete vaccination record (Table 4.8).

In this study, stool specimens collected from two of the immunodeficient children (P085 and P095) tested positive for OPV strains approximately seven months following the last recorded polio vaccination (Table 4.8). Patient P085 (one-year-old baby) was immunised at the age of 14 weeks (12/03/2003) and Sabin PV type 1 was detected in a stool specimen collected seven months (23/10/2003) following the last polio vaccination. This baby was hospitalised for pneumonia and marasmus, and presented with CD4^{+} counts of less than 200 cells.mm^{-3} (Table 4.8). Patient P095 (2 years old child) was lastly immunised at the age of 18 months (18/02/2003) and Sabin PV type 1 was detected in a stool specimen collected seven months after this last polio vaccination (01/10/2003). The patient was hospitalised for chronic diarrhoea and also had CD4^{+} counts of less than 200 cells.mm^{-3} (Table 4.8). Since, immunocompetent children are known to excrete OPV strains for up to three months following vaccination (Alexander *et al.*, 1997), the excretion of OPV strains by the immunodeficient children (P085 and P095) in this study could be considered as prolonged.

Prolonged excretion of OPV strains for more than 15 months was observed in two other immunodeficient children (P023 and P140) involved in this study (Table 4.8). Patient P023 (18 months old baby) had his last recorded OPV immunisation at the age of 14 weeks (04/2002) and a stool specimen taken 15 months later (29/07/2003) tested positive for Sabin PV type 3. The other patient (P140, a 19 month old baby) had his last recorded OPV immunisation at 14 weeks (28/08/2002) and a stool specimen taken 16 months later (02/01/2004) tested positive for Sabin PV type 1. Both of these children had $CD4^+$ counts between 200-499 cells.mm^{-3} and were hospitalised for various diseases such as encephalopathy, gastroenteritis, pulmonary tuberculosis and pneumonia (Table 4.8). A five year old child (patient P052) who had received her last polio immunization at the age of 18 months (27/03/2000) tested positive for Sabin PV type 1, which was more than 42 months after the stool specimen was collected (04/09/2003) (Table 4.8). Patient P052 had $CD4^+$ counts of less than 200 cells.mm^{-3} and was hospitalised with miliary tuberculosis (Table 4.8). Since patient P052 was five years old the last polio vaccination date according to the immunisation schedule (Table 4.2) should have been on the 26/09/2003, however, the last stool sample was collected on the 04/09/2003 before the child was due to be vaccinated. Thus, based on the immunisation records this case represented the most prolonged period of OPV excretion by an immunodeficient patient in this study, which may have important implications regarding the control of health risks constituted by OPV vaccination, particularly with regard to immunodeficient patients.

Based on these results, it can be concluded that immunodeficient patients have the potential to excrete PVs for a prolonged period of time and therefore, these patients may serve as potential reservoirs for the re-introduction of PVs in the post eradication era. However, this prolonged excretion cannot definitely be attributed to the vaccine alone since there is the possibility of person-to-person transmission as well as acquiring PVs from the environment. Live vaccines should never be given to immunodeficient patients, in their own interest and in the interest of the community. But even if these patients have received OPV before the deficiency is diagnosed, strict monitoring will not prevent person to person contact and/or re-infection.

4.5 Conclusions

Since the PV eradication campaign has relied almost entirely on the live, attenuated OPV, every person who is vaccinated (even immunocompetent individuals) would excrete live OPV strains into the environment for at least a limited period of time (usually 2 to 3 months) (Wood and Thorley, 2003). The attenuated Sabin PV vaccine strains are known to revert their neurovirulent characteristics and numerous potentially virulent PVs can be released into sewage systems and aquifers of countries, which are considered "polio-free" (Wood and Thorley, 2003).

Although this study could not present a definitive hard evidence for long-term excretion of PVs in HIV-positive children, one important conclusion that can be made from the results is that HIV-positive children seem to be more susceptible to viral infections than other healthy children. According to the results, two of the immunodeficient children (P023 and P140) who had received their last OPV immunisation more than 15 months ago (vaccinated at 14 weeks of age) tested positive for Sabin PV type 3 and Sabin PV type 1, respectively. A five year old immunodeficient child (P052) who had lastly received OPV immunisation more than 42 months ago (vaccinated at 18 months of age) tested positive for Sabin PV type 1. As a next step in this study, the genomes of the OPV strains isolated from the immunodeficient as well as from the immunocompetent children will be sequenced in order to find any possible mutations leading to increased neurovirulence of these vaccine strains (VDPVs). Data on the excretion of VDPVs by carrier communities (notably immunodeficient individuals) will give an indication of the quantitative release of these strains into the environment and the potential health risk they might constitute. This information would be essential for strategies aimed at the protection of newly born children who are no longer being vaccinated during the post-eradication era as well as protecting immunodeficient patients against complications of OPV-vaccination such as acute flaccid paralysis (AFP).

In a move to eliminate the rare cases of polio that result from OPV, the Advisory Committee on Immunisation Practices of the Federal CDC in the USA recommended a change in the polio vaccination schedule from the current practice of administering OPV only at 2, 4 and 6 months of age to a sequential schedule of injection of IPV at 2 and 4 months followed by the administration of two doses of OPV at 12 to 18 months and 4 to 6 years of age (Buonagurio *et*

al., 1999). It is believed that the immunity acquired from the first two doses of inactivated vaccine, which is unlikely to cause paralytic poliomyelitis, should be sufficient to protect the small number of children who contract disease from the oral poliovirus vaccine (Buonagurio *et al.*, 1999; Yang *et al.*, 2003). In non-endemic developing countries, such as South Africa, the conversion to exclusive use of IPV in the routine immunisation schedule, although more costly than OPV, will avoid VAPP and maintain immunity to wild-type PV importations (Wood and Thorley, 2003).

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Table 4.1: Sample sizes for different levels of confidence and accuracy of estimation for the prevalence of OPV strains in stool specimens of immunodeficient children (Biostatistics Unit, Medical Research Council, South Africa)

		Accuracy of estimation			
		10%	**5%**	**3%**	**2%**
Level of confidence	**80%**	41	162	436	931
	90%	67	263	699	1446
	95%	95	370	964	1936

Table 4.2: South African childhood immunisation schedule (Department of Health, 1995)

Age	Vaccines
At birth	*Oral polio vaccine*, BCG
6 weeks	*Oral polio vaccine*, DTP, Hepatitis B vaccine
10 weeks	*Oral polio vaccine*, DTP, Hepatitis B vaccine
14 weeks	*Oral polio vaccine*, DTP, Hepatitis B vaccine
9 months	Measles vaccine
18 months	*Oral polio vaccine*, DTP, Measles vaccine
5 years	*Oral polio vaccine*, DT

BCG = Vaccine against tuberculosis.
DT = Diphtheria, tetanus vaccine.
DTP = Diphtheria, tetanus, pertussis vaccine.
OPV = Trivalent oral poliovirus vaccine (Sabin PV type 1, type 2 and type 3).

Table 4.3: 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	EP1	5'-CGG TAC CTT TGT GCG CCT GT-3'	408
454-473	EP4	5'-TTA GGA TTA GCC GCA TTC AG-3'	
163-178	E1	5'-AAG CAC TTC TGT TTC C-3'	297
443-460	E2	5'-CAT TCA GGG GCC GGA GGA-3'	

Table 4.4: 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 4.5: 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 4.6: 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 4.7: 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 4.8: Poliovirus vaccine strains isolated from stool specimens of immunodeficient children from a selected area in South Africa

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

Table 4.9: Isolation of poliovirus vaccine strains from stool specimens of immunocompetent children (the control group)

Sample number	Gender	Date of birth	Polio immunisations	Stool specimens collected	Type of virus
Nat 05/24	Female	22/05/2003	22/05/2003 (at birth)	24/05/2003 (after 48 h)	PV1
				02/06/2003 (after one week)	PV1
				10/06/2003 (after two weeks)	-
				18/06/2003 (after three weeks)	-
Nat 07/03			03/07/2003 (at 6 weeks)	05/07/2003 (after 48 h)	PV1
				12/07/2003 (after one week)	PV1
				19/07/2003 (after two weeks)	-
				01/08/2003 (after three weeks)	-
Nat 08/02			02/08/2003 (at 10 weeks)	04/08/2003 (after 48 h)	PV2
				12/08/2003 (after one week)	-
				20/08/2003 (after two weeks)	-
				27/08/2003 (after three weeks)	-
Nat 09/04			04/09/2003 (at 14 weeks)	06/09/2003 (after 48 h)	-
				13/09/2003 (after one week)	-
Mrsa 06/03	Female	18/11/2002	02/03/2003 (at 14 weeks)	03/06/2003 (after three months)	-
Mrsa 06/01			01/06/2004 (at 18 months)	03/06/2004 (after 48 h)	PV1
				10/06/2004 (after one week)	-
				17/06/2004 (after two weeks)	-
				24/06/2004 (after three weeks)	-
Ln 06/09	Male	10/12/2001	24/03/2002 (at 14 weeks)	09/06/2003 (after 15 months)	-
Ln 06/17			17/06/2003 (at 18 months)	19/06/2003 (after 48 h)	PV1
				22/06/2003 (after one week)	-
				30/06/2003 (after two weeks)	-

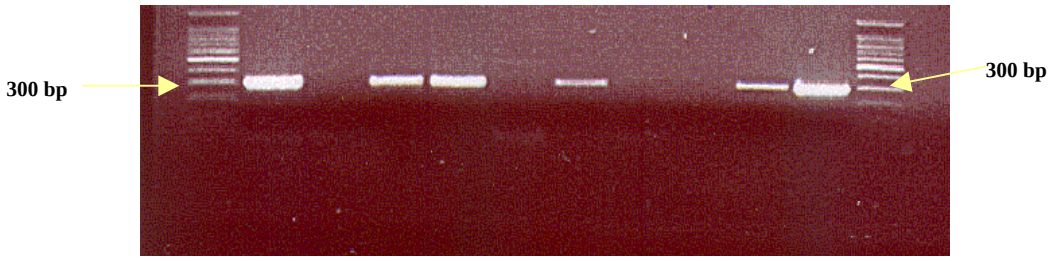

Figure 4.1: **Band patterns observed with the nested PCR in the detection of enteroviruses (EVs) in selected stool samples. Lane 1: Marker 100 bp; Lane 2: EV (297 bp); Lane 3: Negative; Lane 4: EV (297 bp); Lane 5: EV (297 bp); Lane 6: Negative; Lane 7: EV (297 bp); Lanes 8-9: Negative; Lane 10: EV (297 bp); Lane 11: Positive control (297 bp); Lane 12: Marker 100 bp**

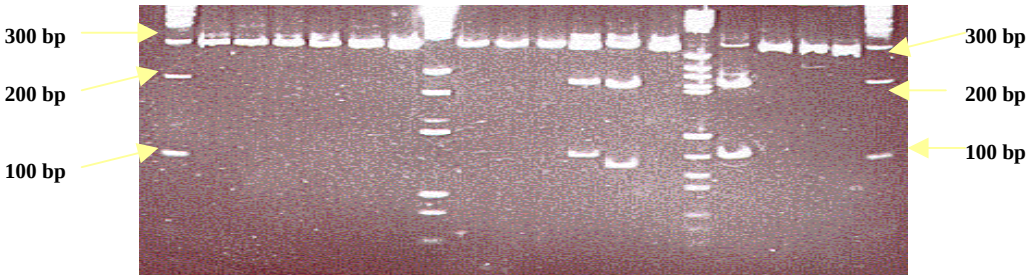

Figure 4.2: **Restriction enzyme digestion of 297 bp products from prototype strains of enteroviruses (EVs) with three restriction enzymes (*Sty* I, *Bgl* I and *Xmn* I). Lane 1: Marker 100 bp; Lane 2: *Sty* I (297 bp), Lane 3: *Bgl* I (297 bp), Lane 4: *Xmn* I (297bp) - PV type 3; Lane 5: *Sty* I (297 bp); Lane 6: *Bgl* I (297 bp), Lane 7: *Xmn* I (297 bp) – PV type 3; Lane 8: PGem marker; Lane 9: *Sty* I (297 bp), Lane 10: *Bgl* I (297 bp), Lane 11: *Xmn* I (297 bp) – PV type 3; Lane 12: *Sty* I (197 bp + 100 bp), Lane 13: *Bgl* I (196 bp + 80 bp + 21 bp), Lane 14: *Xmn* I (297 bp) – CAV17; Lane 15: Marker V; Lane 16: *Sty* I (197 bp + 100 bp), Lane 17: *Bgl* I (297 bp), Lane 18: *Xmn* I (297 bp) – PV type 2; Lane 19: uncut 297 bp product; Lane 20: Marker 100 bp**

100 bp

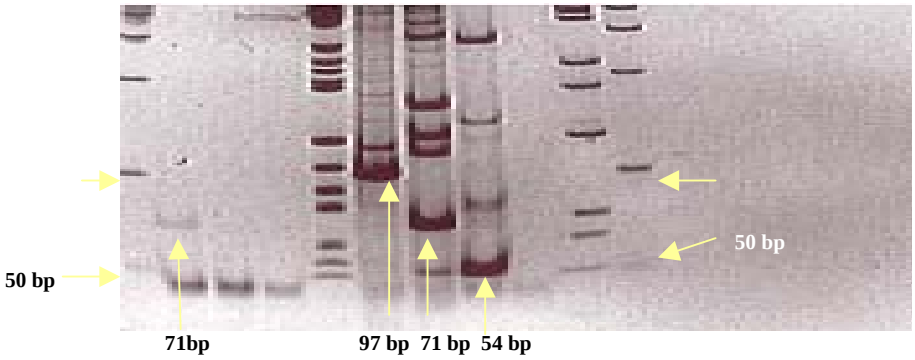

Figure 4.3: **Sabin RT-triplex PCR of the PV isolates and the positive controls. Lane 1: Marker 100 bp; Lane 2: Sabin PV type 2 (positive isolate 71 bp); Lanes 3-4: Negative isolates; Lane 5: PGem Marker; Lane 6: Sabin PV type 1 (97 bp); Lane 7: Sabin PV type 2 (71 bp); Lane 8: Sabin PV type 3 (54bp); Lane 9: Negative control; Lane 10: Marker V; Lane 11: Marker 100 bp**

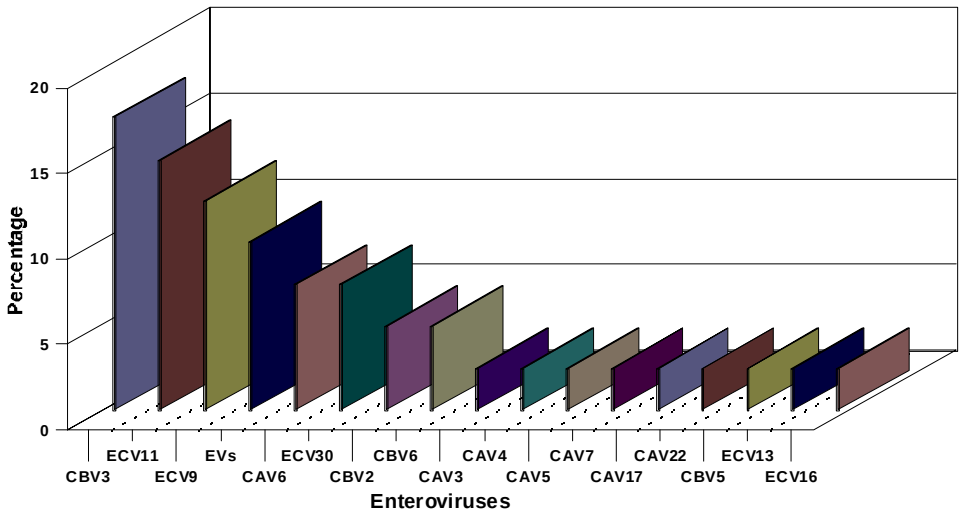

Figure 4.4: **Detection of non-polio enteroviruses in stool specimens of immunodeficient children from a selected area in South Africa during a period of one year**

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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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

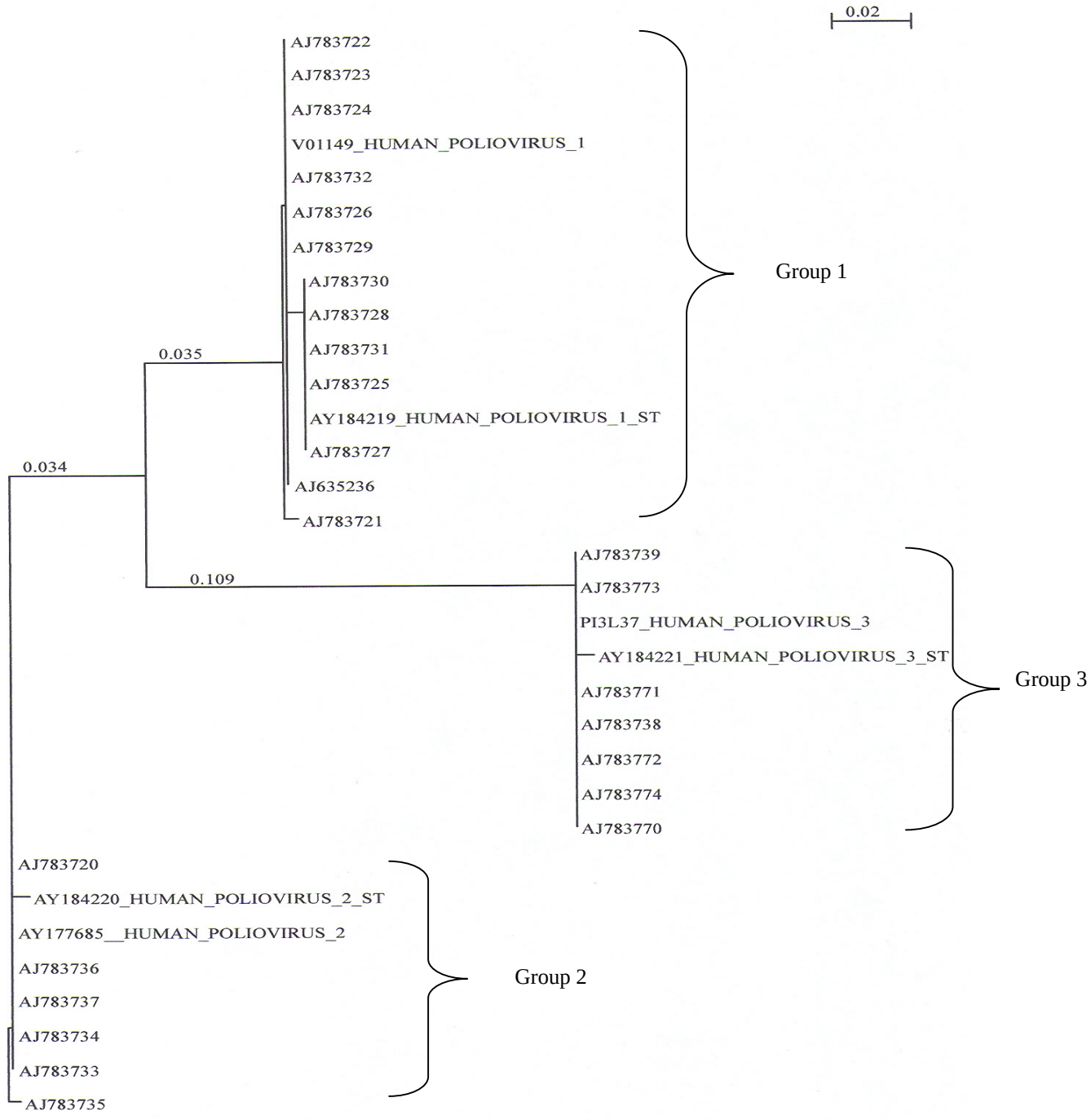

Figure 5.1: **Unrooted phylogenetic tree re-constructed with the neighbour-joining method from the comparative 5'untranslated region sequence analysis of the sewage isolated oral poliovirus vaccine strains and the poliovirus reference strains. Branch lengths are proportional to the phylogenetic distances,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

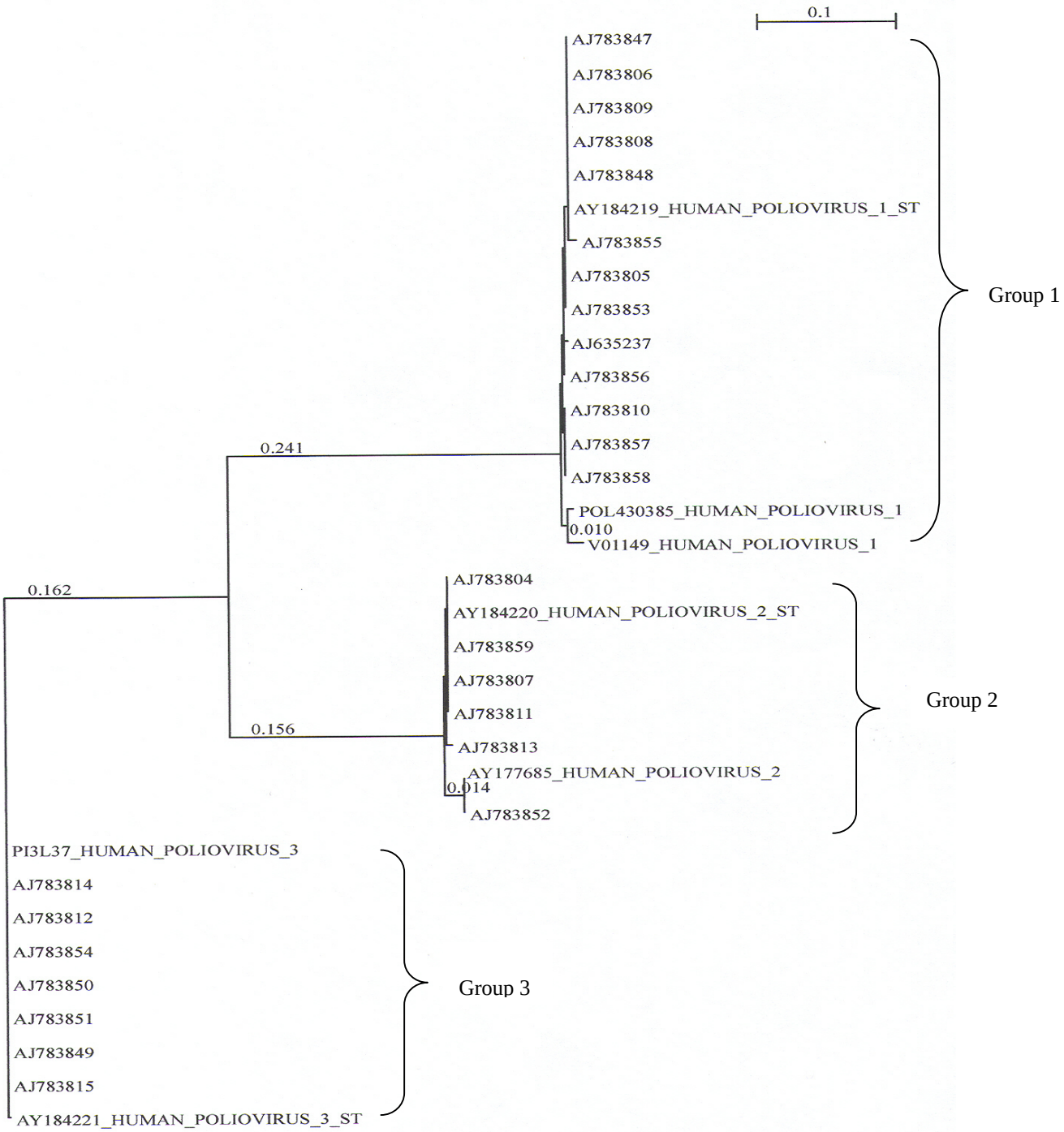

Figure 5.2: **Unrooted phylogenetic tree re-constructed with the neighbour-joining method from the comparative VP1 region sequence analysis of the sewage isolated oral poliovirus vaccine strains and the poliovirus reference strains. Branch lengths are proportional to the phylogenetic distances, while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

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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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.

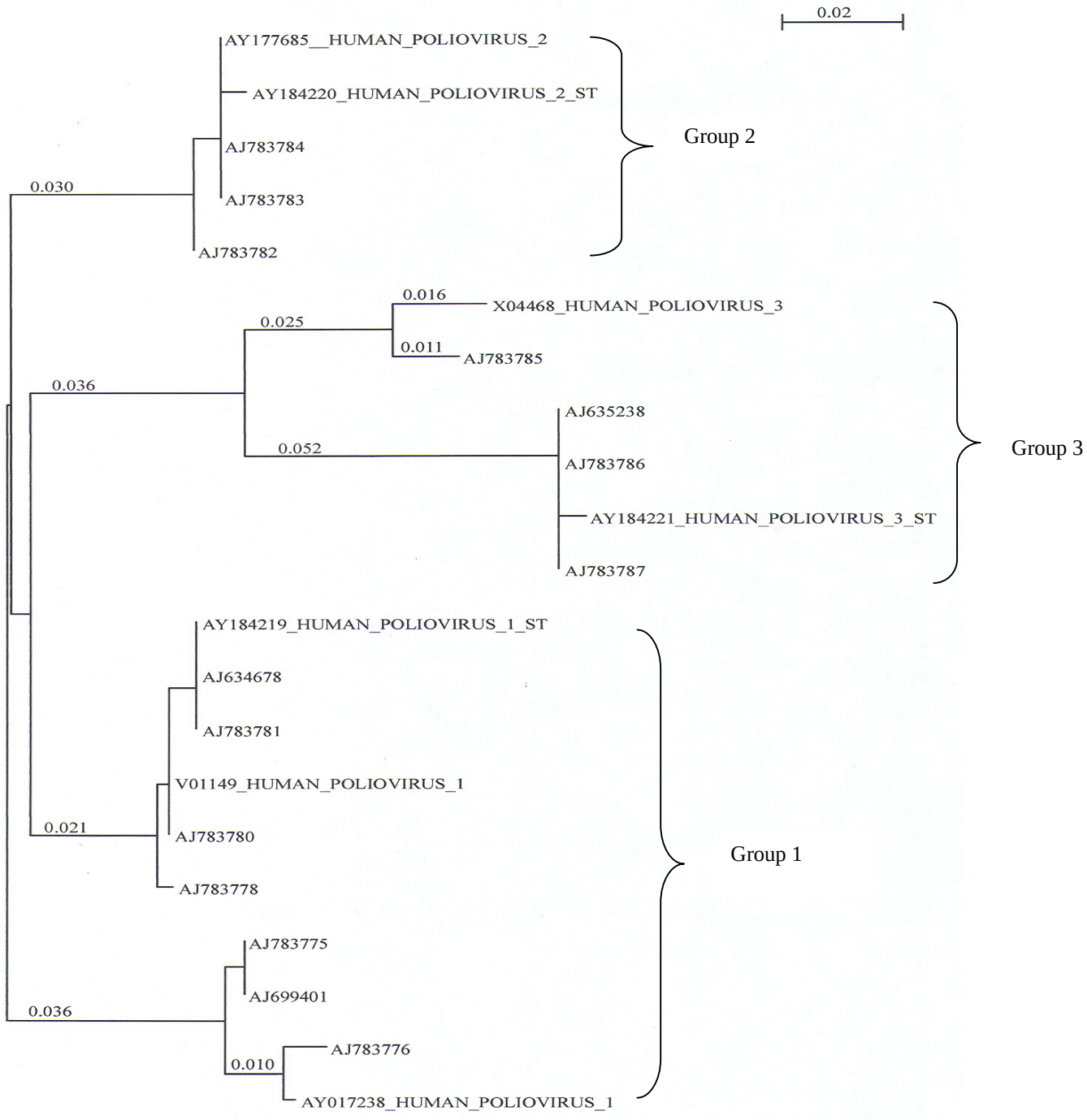

Figure 6.1: **Unrooted phylogenetic tree re-constructed with the neighbour-joining method from the comparative 5'untranslated region sequence analysis of the isolated oral poliovirus vaccine strains from immunodeficient children and the poliovirus reference strains. Branch lengths are proportional to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

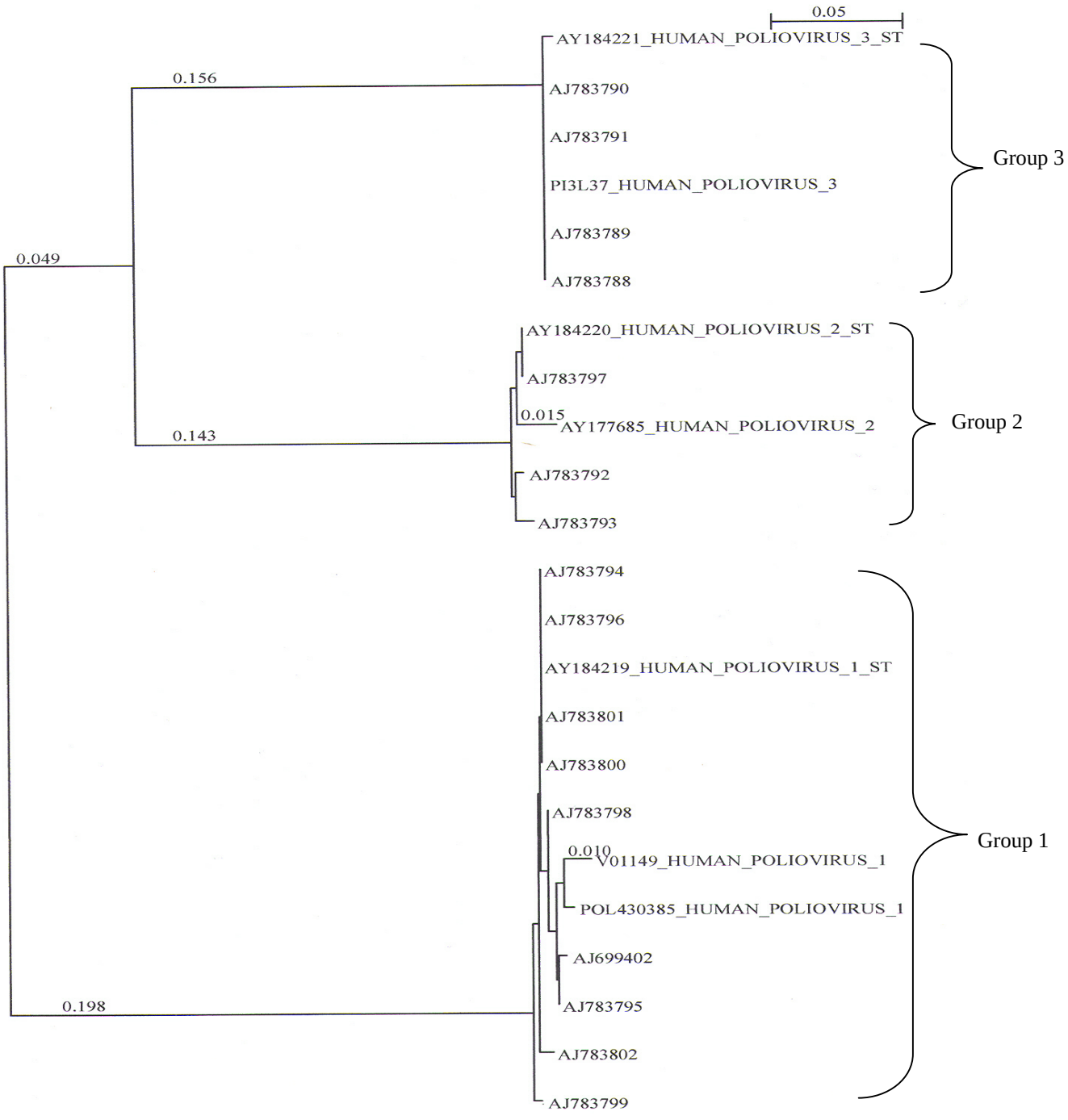

Figure 6.2: **Unrooted phylogenetic tree re-constructed with the neighbour-joining method from the comparative VP1 region sequence analysis of the isolated oral poliovirus vaccine strains from immunodeficient children and the poliovirus reference strains. Branch lengths are proportional to the phylogenetic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

CHAPTER 7

GENERAL DISCUSSION

At the present advanced stage of the Global Polio Eradication Initiative, when 3 regions of the world namely the Americas, Western Pacific and Europe, have been certified as polio-free, developing the "endgame" strategies has become a high priority (Centers for Disease Control and Prevention [CDC], 2002a; Khetsuriani *et al.*, 2003). The major threat to world-wide polio eradication is still represented by wild-type poliovirus (PV), localised to a few reservoirs in Africa and Asia (CDC, 2002a). The maintenance of polio-free status in certified regions until global certification is jeopardised by importations of wild-type PV from endemic countries (ProMED-mail, 2004a). In 2004, outbreaks of paralytic poliomyelitis due to imported wild-type PVs were reported from several African countries such as Botswana, Guinea, Mali and Sudan (ProMED-mail, 2004a). These importations were associated with an extensive outbreak of polio in Nigeria, which resulted following the suspension of polio immunisation campaigns in some states of the country (ProMED-mail, 2004b). In other parts of the world, threat comes from the continued use of oral poliovirus vaccine (OPV) and the associated risk of circulating vaccine-derived polioviruses (cVDPVs) (WHO, 2004a). In recent years, at least 4 episodes of poliomyelitis outbreaks or endemic transmission associated with cVDPVs have been recorded such as Egypt (1982-1993), Hispaniola (2000-2001), Philippines (2001) and Madagascar (2002) (CDC, 2001a; CDC, 2001b; CDC, 2002b; Kew *et al.*, 2002; Khetsuriani *et al.*, 2003). These findings suggest that a better understanding of VDPV persistence and circulation is critical for making the decision about when and how to stop immunisation with OPV after the global eradication of poliomyelitis (Fine and Carneiro, 1999; Wood *et al.*, 2000; Technical Consultative Group to the World Health Organization on the Global Eradication of Poliomyelitis, 2002; Khetsuriani *et al.*, 2003).

Long-term excretors of PV, like immunodeficient patients (with primary or secondary immunodeficiency) represent an additional concern, since these individuals may act as potential reservoirs for PV re-introduction after polio eradication (Buttinelli *et al.*, 2003). Following exposure to OPV, immunocompetent persons are known to excrete PV vaccine strains for a limited period of time (from 2 - 3 months) (Alexander *et al.*, 1997). However,

cases of prolonged excretion of VDPVs by immunodeficient persons, including those with vaccine-associated paralytic poliomyelitis (VAPP), have been previously reported (Kew *et al.*, 1998; Bellmunt *et al.*, 1999; Martin *et al.*, 2000; Khetsuriani *et al.*, 2003). Polioviruses shed in stools of vaccinated individuals typically revert their attenuated phenotype to increased neurovirulence (Haisey *et al.*, 2004). As a consequence, immunodeficient individuals receiving OPV during the last campaign of immunisation may transmit neurovirulent VDPVs to close contacts such as newly born children who are no longer being vaccinated, thus representing a potential health risk (Haisey *et al.*, 2004; Hovi *et al.*, 2004).

Vaccine-derived polioviruses from immunodeficient people with long-term excretion have been classified as immunodeficient VDPVs (iVDPVs) (Kew *et al.*, 1998; Gavrilin *et al.*, 2000; Wood *et al.*, 2000; Wood and Thorley, 2003). Such cases have not been associated with cVDPVs, which have the increased capacity for sustained person-to-person transmission (Wood and Thorley, 2003; Kew *et al.*, 2004). Two cases of VAPP have been reported in children infected with human immunodeficiency virus (HIV) (Ion-Nedelscu *et al.*, 1994; Chitsike and Furth, 1999), although, at present there is no evidence for prolonged excretion of PV from patients with HIV and acquired immunodeficiency syndrome (AIDS) (Wood and Thorley, 2003).

Although, acute flaccid paralysis (AFP) is the gold standard for surveillance of PV circulation, under certain circumstances valuable supplementary information can be obtained by environmental surveillance (WHO, 2004b). A number of researchers in various parts of the world have submitted strong and convincing evidence that routine screening of sewage is a powerful and practical tool for monitoring enteric viruses (including PVs) circulating in communities under a diverse spectrum of conditions (Grabow *et al.*, 1999). Thus, highly evolved VDPVs have been isolated from sewage and river water even in the absence of apparent cases of paralytic poliomyelitis (Shulman *et al.*, 2000; Horie *et al.*, 2002; Yoshida *et al.*, 2002; Blomqvist *et al.*, 2004). Shulman and colleagues (2000) have isolated a highly diverged derivative of the Sabin PV type 2 strain from environmental samples during routine screening for wild-type PV in Israel. 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). Vaccine-derived polioviruses, with increased neurovirulence (showing 1.4% nucleotide divergence from the vaccine strain), were isolated from sewage and river water in Japan (Horie *et al.*, 2002; Yoshida *et al.*, 2002). More recently, two Sabin-like

PVs were found by environmental surveillance 8 and 11 months after any OPV vaccine was used in New Zealand and showed 99.8% as well as 99.9% homology with Sabin PV type 2 vaccine strain in the VP1 region (WHO, 2003b). Furthermore, a VDPV type 3 strain harbouring a 13% sequence drift from the parental PV vaccine strain has been isolated from sewage in Estonia (Blomqvist *et al.,* 2004). Thus, it is evident from these findings that the presence of VDPVs in the environment is of major concern, because these viruses might be transmitted and continue to circulate in a non-immune population after cessation of polio vaccination (Buttinelli *et al.,* 2003).

The development of "endgame" strategies will require details on the possibility of persistent infections and excretion of VDPVs for long periods by immunodeficient patients, and the survival in the environment of these strains to the extent that they may infect non-immune individuals after termination of PV vaccination in the near future (Fine and Carneiro, 1999). The aim of the current study was, firstly, to investigate the occurrence of OPV strains in selected sewage and river water samples as well as to isolate OPV strains from stool specimens of immunodeficient patients (such as HIV-positive children including those with an AIDS indicator condition according to the CDC classification) at Kalafong Hospital, South Africa. Secondly, the study aimed to determine the occurrence of genomic mutations in these OPV isolates and to determine the prevalence of VDPVs in the sewage and river water as well as in stool specimens of the immunodeficient children studied.

In this study during the period between 2001 and 2003, a total of 213 sewage samples (domestic and industrial sewage of approximately 3 500 000 people) and a total of 138 river water samples (occasionally used by the rural community for drinking and washing purposes) were obtained from selected areas in South Africa. Using the monolayer plaque assay, 703 plaques from the sewage and 157 plaques from the river water samples were analysed. This study revealed that the buffalo green monkey kidney (BGM) and the primary liver carcinoma (PLC/PRF/5) cell cultures allowed the amplification of a broad spectrum of enteroviruses (EVs). The mouse L (L20B) cell line recommended for the selective recovery of PVs was not used in this study. However, human epidermoid carcinoma (HEp-2) cells proved suitable to isolate large numbers of PVs and smaller numbers of other non-polio enteroviruses (NPEVs) from water environments. The HEp-2 cells have previously been applied in the detection of PVs in water environments by other researchers such as Fiore *et al.* (1998), Manor *et al.* (1999a), Manor *et al.* (1999b) and Buttinelli *et al.* (2003).

Using a reverse transcription multiplex PCR (RT-multiplex PCR), 49 PVs isolated from the various cell lines (BGM, HEp-2 and PLC/PRF/5) were successfully distinguished from 176 NPEVs. Plaques, other than those of EVs, might have been reoviruses or adenoviruses, however, they were not subjected to further analysis. The RT-multiplex PCR proved useful for the rapid, specific and sensitive detection of PVs and for their distinction from NPEVs. A higher number of PVs (37 isolates or 76%) were detected in the sewage than in the river water samples. Furthermore, approximately 70% of the PV isolates were detected on HEp-2 cells compared to 18% on BGM and 12% on PLC/PRF/5 cells. All of the 49 PV isolates were typed as vaccine strains using the Sabin specific RT-triplex PCR, which showed that Sabin PV type 1 isolates were the most prevalent (29 isolates), followed by Sabin PV type 3 (12 isolates) and Sabin PV type 2 (8 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 (CDC, 2003). The ability of the isolated OPV strains to infect susceptible host cells and to form plaques, confirmed that they were viable and therefore, potentially infectious. Thus based on these results, it was concluded that the identification of 49 viable OPV strains in the sewage and the river water sources (which are used in some cases for human consumption by the rural community) warrants further investigation into the presence and circulation of VDPVs as well as the potential health risk they might constitute.

In order to estimate the prevalence of OPV strains (possibly VDPVs) in the stool specimens of immunodeficient children, a maximum sample size associated with an expected prevalence of 50% was analysed. Thus, stool specimens were collected from 164 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. During the same period of time (2003-2004), 23 stool samples from 3 healthy immunocompetent babies (the control group) were collected after receiving their scheduled OPV immunisations. By applying a RT-PCR in combination with a nested PCR, a total of 54 EVs were detected in the stool specimens of the immunodeficient children. These immunodeficient children were between the ages of 4 months to 8 years and were hospitalised for various diseases such as bronchopneumonia, encephalopathy, gastroenteritis, herpes stomatitis, meningitis, miliary tuberculosis, pneumocystis carinii pneumonia, pulmonary tuberculosis, pneumonia and etc. In total, 17 of the immunodeficient children died during the course of the study. Using restriction enzyme (RE) analysis, 13 PVs were distinguished from 41 NPEVs.

The Sabin specific RT-triplex PCR confirmed the presence of Sabin PV type 1 (7 isolates), Sabin PV type 3 (4 isolates) and Sabin PV type 2 (2 isolates). In total, 7 of the 23 stool samples taken from the healthy immunocompetent children tested positive for EVs after receiving their polio immunisation. Six of the PV isolates were typed as Sabin PVs type 1 and one as Sabin PV type 2. It was concluded from the results that the immunocompetent children involved in this study did not excrete PVs for more than a month following each polio vaccination, which was in agreement with other international research studies (Alexander *et al.*, 1997). In comparison to these findings, two of the immunodeficient children (P023 and P140) who had received their last OPV immunisation more than 15 months before (vaccinated at 14 weeks of age) tested positive for Sabin PV type 3 and type 1, respectively. Furthermore, a five year old immunodeficient patient (P052) who had lastly received OPV immunisation more than 42 months before (vaccinated at 18 months of age) tested positive for Sabin PV type 1. Based on these results, it was concluded that immunodeficient patients vaccinated with OPV could indeed be considered as prolonged excretors (at least for a longer period of time than the immunocompetent persons) of potentially pathogenic VDPVs. These VDPVs might circulate in the community resulting in possible infections in the unvaccinated population in the near future.

As a next step, the 5'untranslated region (5'UTR) and the VP1 capsid-encoding region in the genomes of the OPV strains isolated from the selected sewage and river water samples were partially sequenced in order to determine the presence of mutations that may lead to the increased neurovirulence of the OPV isolates. The evolutionary relationships between the studied OPV isolates were deduced from unrooted phylogenetic trees of the sequenced genomic regions. A representative number of the PV isolates (26 from the initial 49 PVs) were selected for sequencing analysis. The total number of PVs sequenced included: PV type 1 (13 isolates), PV type 3 (7 isolates) and PV type 2 (6 isolates). The majority of the OPV strains (24 out of 26) displayed close sequence relationships (>99% VP1 sequence identity) to the original Sabin PV vaccine strains and were classified according to the WHO classification as "OPV-like viruses". Only two from the sequenced 26 OPV isolates (D1 08/28 and OF1 05/21) were classified as "suspected" VDPVs, since these isolates showed ≤99% VP1 sequence identity to the reference Sabin PV vaccine strains. It was evident from the sequencing results that isolate OF1 05/21 (a "suspected" VDPV type 1) displayed 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 reference Sabin PV vaccine

strains. 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 previously 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 for 12 to 16 months since the administration of the initiating OPV dose. However, the estimate of the duration of replication was only approximate and was based upon the assumption that the rate of VP1 evolution for PV type 1 and type 2 was essentially constant over the period of replication and similar to the rate observed for PV type 3 (approximately 1-2% change.year^{-1}) (Kew *et al.*, 2002; WHO, 2004b).

Similarly, the 5'UTR and the VP1 regions in the genomes of the OPV strains isolated from stool specimens of the immunodeficient and the immunocompetent children in this study were partially sequenced. In the control group, two Sabin-like type 1 PVs (isolated from Nat 05/24 and Ln 06/17) showed 100% nucleotide sequence similarity in both regions to the parental Sabin PV vaccine strain. In addition, these isolates had a mutation at position 480 in the 5'UTR, which had previously been identified as a determinant of attenuation (Minor, 1999; Martin and Minor, 2002). However, one isolate (a Sabin-like type 2 PV) showed a very high divergence (0.9%) in VP1 nucleotides from the Sabin PV vaccine strain and had the key mutation at position 481 (5'UTR) associated with reversion of the attenuated phenotype to increased neurovirulence. This Sabin-like type 2 PV was isolated from a stool sample collected from one of the immunocompetent children (Nat 08/02) at the 10th week vaccination. According to the results, excretion of this PV strain stopped by the end of the second week following vaccination and no Sabin-like type 2 PV was detected in the stool samples collected following the 14th week vaccination. In contrast to the control group, the majority of the OPV isolates (10 out of 13) 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. Furthermore, three of the 13 OPV isolates from the immunodeficient children (P069, P085 and P114), showed ≤99% VP1 sequence identity to the Sabin PV vaccine strains and were classified as "suspected" immunodeficient VDPVs (iVDPVs). All of the OPV-like isolates and the "suspected" iVDPVs carried mutations at specific positions in their partially sequenced 5'UTR and VP1 regions, which had been associated with reversion of the attenuated Sabin PV vaccine strains to increased

neurovirulence. Thus, it was concluded that immunodeficient patients might excrete OPV strains with potential neurovirulent phenotypes and these OPV strains could circulate in the environment posing a potential health risk after termination of the OPV vaccination program.

In this study, one of the immunodeficient patients (P052) had received her last OPV immunisation more than 42 months before (vaccinated at 18 months of age) and a stool sample, collected three weeks before the due date for vaccination at five years of age, tested positive for Sabin PV type 1. The time between vaccine assumption and isolation of Sabin-like type 1 PV from patient P052, however, was not compatible with the mutation rate observed (>0.6% divergence in VP1 from the attenuated Sabin PV vaccine strain). The partially sequenced 5'UTR of the Sabin-like type 1 PV isolate revealed a very high percentage divergence (>8.5%) in the 5'UTR from the parental Sabin PV vaccine strain. Similarly, the time between vaccine assumption and PV isolation from two other immunodeficient patients (P023 and P140) was not compatible with the mutation rate observed (>0.3% and >0.6% nucleotide sequence divergence in the VP1 region from the Sabin PV vaccine strains, respectively). These immunodeficient patients (P023 and P140) had received their last OPV immunisations more than 15 months before (vaccinated at 14 weeks of age) and tested positive for Sabin PV type 3 and type 1, respectively. The partially sequenced 5'UTR of the Sabin-like type 1 PV isolate (patient P140), revealed a very high percentage divergence (>7%) in the 5'UTR from the Sabin PV vaccine strain. One explanation for this low divergence in the partially sequenced VP1 regions of the two type 1 isolates and the one type 3 isolate with respect to their corresponding parental vaccine strains could have been that these isolates were acquired through a close contact such as another vaccine recipient. However, generation of such isolates can not simply be explained by long-term replication of VDPVs in a single individual but rather by recombination of vaccine-like strains with other polio or non-polio enteroviruses. Furthermore, the evolution rate measurements used to estimate the duration of infection in this study generally had several limitations to precision and therefore, deviation from the molecular clock was possible. Firstly, only a portion of the sequence information available was used for the evolution rate estimates, because the 5'UTR and VP1 regions of the genomes of the OPV isolates were partially sequenced. Nevertheless, these regions carried mutations at specific positions associated with reversion of the attenuated phenotype of PV to increased neurovirulence. 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 is unproven (Kew *et al.*, 1998).

In conclusion, this study addressed some of the issues regarding the evolution and nucleotide divergence of OPV strains replicating in carrier communities (notably immunodeficient patients), as well as the prevalence of these strains in the environment. Although this study could not show evidence for long-term excretion of vaccine-derived polioviruses in HIV-positive children, however, the results made an important suggestion that HIV-positive children seem to be more susceptible to viral infections than other healthy children. The data in the current study could not be extrapolated to the actual situation in South Africa because of the small size of the study group (only 164 immunodeficient children). The present study, however, illustrated that a number of potentially dangerous PVs could be found in a small group of patients to a fairly high frequency. At least two of the 26 PVs detected in the sewage and at least three of the 13 PV sequences found in the stools of the HIV-positive children could be classified as "suspected" VDPVs. Complete sequencing of the isolated VDPVs would have revealed a more detailed description of the genomic mutations present. These results were in agreement with findings reported from other studies. A survey of PVs in river and sewage water conducted in Japan, revealed the presence of 25 VDPVs type 2 (Yoshida *et al.*, 2002) and more recently a VDPV type 3 strain was isolated from sewage in Estonia (Blomqvist *et al.*, 2004). Similarly, VDPVs have been isolated from stools of immunodeficient patients and in the past 40 years since the beginning of the polio vaccination, nineteen chronic iVDPV excretors were detected world-wide, although this number may be an underestimate in the absence of systematic screening of immunodeficient patients (Haisey *et al.*, 2004; WHO, 2004a).

It is evident from this study that environmental surveillance is still epidemiologically important, because the results of virus surveillance retrospectively reflect the properties of virus circulating in the community and it assesses the potential risk of infection from the environment (Divizia *et al.*, 1999; Grabow *et al.*, 1999; Shulman *et al.*, 2000; Yoshida *et al.*, 2002). The examination of human faecal samples through environmental surveillance links PV isolates from unknown individuals to populations served by the wastewater system (WHO, 2004b). Thus, the identification of 49 viable OPV strains in the sewage and river water in this study, supports the view that the screening of sewage and river water is a

practical and valuable tool for monitoring the circulation of PV strains in communities and the possible role of water in the transmission of potentially hazardous mutants of OPV strains.

Future research

A decision to stop OPV vaccination could potentially have serious public health consequences, because uncoordinated discontinuation by countries is likely to create unacceptable risks for emergence of circulating VDPVs (Wood *et al.*, 2000; Kew *et al.*, 2004). Transition to inactivated poliovirus vaccine (IPV) should be encouraged at the present time in developed countries, where IPV efficacy is known to be high and where high rates of IPV coverage can be maintained through routine immunisation (Kew *et al.*, 2004). The use of IPV in tropical developing countries presents special challenges, because the rates of routine immunisation are often inadequate and IPV efficacy is uncertain due to logistical and financial problems (Kew *et al.*, 2004). The Advisory Committee on Immunisation Practices of the Federal CDC in the United States of America has recommended a change in the polio vaccination schedule from the current practice of administering OPV at 2, 4 and 6 months of age to a sequential schedule of injection of IPV at 2 and 4 months followed by the administration of two doses of OPV at 12 to 18 months and 4 to 6 years of age (Buonagurio *et al.*, 1999). Stockpiles of polio vaccine must be established to enable a rapid response to the detection of any PV infection in the post-OPV era (Kew *et al.*, 2004). Sensitive field and laboratory surveillance must be maintained until there is evidence that the risk of any PV re-emergence is negligible (Kew *et al.*, 2004). Other challenges include: verification of laboratory containment; development of an appropriate mechanism to confirm the absence of circulating VDPVs in the future; the maintenance of polio-free status in certified regions until global certification (WHO, 2004a).

There is little information on secondary immunodeficiency as a risk factor for VAPP or prolonged PV excretion (Wood *et al.*, 2000). The likelihood of prolonged PV excretion in cohorts of HIV-infected children is being investigated in several developing countries (Wood *et al.*, 2000; Haisey *et al.*, 2004). Research is, therefore, required to provide information in the following priority areas: the extent and duration of circulation of VDPVs in populations; risk factors for prolonged replication of VDPVs among immunodeficient individuals such as HIV-positive children; assessment of the prevalence and behaviour of VDPV strains in the environment (notably water resources) and in different population settings (Wood *et al.*, 2000). Furthermore, future studies should focus on determining the period of excretion of PV

strains by HIV-positive children (preferably a larger study group) after vaccination with OPV and by following-up the excretion of these OPV strains. Secondly, the effect of anti-retroviral treatment on HIV-positive patients receiving OPV may result in interesting findings. These research findings will be of extreme importance and will provide new valuable data to help devise the most appropriate immunisation policies for the post-eradication era.

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APPENDIX I

CULTURE MEDIA, REAGENTS AND MOLECULAR TECHNIQUES

I. CELL CULTURE TECHNIQUES AND REAGENTS

A. Buffalo green monkey kidney, human epidermoid carcinoma and primary liver carcinoma cell line cultivation (Whitaker, 1972):

1. Five percent of Eagle's Minimum Essential Medium (MEM) (Highveld Biological, Lyndhurst, South Africa) was used as the growth medium for the cultivation of buffalo green monkey kidney (BGM), human epidermoid carcinoma (HEp-2) and primary liver carcinoma (PLC/PRF/5) cell lines (American Type Culture Collection, Virginia, United States of America [USA]).
2. The medium was replaced twice weekly. An yellow colour indicated the growth of cells and the production of metabolic substances due to acid excretion.
3. As soon as a monolayer of BGM, HEp-2 or PLC/PRF/5 cell lines was formed on the base of the 250 ml tissue culture flask (Cellstar, Greiner Labortechnik), the cells were passaged in a ratio of 1:2 to 1:6, depending on the thickness of the cell monolayer.
4. The medium in the flask was discarded and the walls of the flask were washed with 5 ml of phosphate-buffered saline (PBS) (Sigma Chemical Co., Louis, USA).
5. Three millilitres of trypsin EDTA (National Institute for Virology, South Africa) were added to the flask and incubated at 37°C for 1 min in a 5% CO_2 incubator (Galaxy CO_2 Incubator- Biotech, Northants, England).
6. The cells were detached from the bottom of the flask by lightly tapping the flask firmly against the hand.
7. An amount of 3 ml of a 10% MEM (Highveld Biological) was added to the flask and swirled to neutralise the effect of trypsin EDTA (National Institute for Virology, South Africa).
8. The whole suspension was transferred from the flask to a centrifuge tube (Corning Costar Corporation, Cambridge, Canada), which was centrifuged at 500 x *g* (BHG Roto-Uni II, Separation Scientific, South Africa) for 2 min in order to pellet the cells.

9. The supernatant was discarded and the pellet of cells was re-dissolved in 10% MEM (Highveld Biological).
10. After thorough mixing, 1 ml of the cell suspension was transferred into each flask containing 14 ml of the 5% MEM (Highveld Biological) that has been pre-heated to 37°C. Flasks were incubated at 37°C in a 5% CO_2 incubator (Galaxy CO_2 Incubator).

B. Preparation of 5% MEM (Whitaker, 1972):

1. Foetal calf serum (FCS) (Delta Bioproducts, Kempton Park, South Africa) was de-complemented in a water bath (Gallenkamp, England) at 56°C for 30 min.
2. Thirty millilitres of serum-free medium were abstracted from a 500 ml MEM bottle (Highveld Biological).
3. Twenty-five millilitres of filter sterilised foetal calf serum and 5 ml of sterile penicillin/streptomycin (pen/strep) fungizone mix (Whittaker M.A. Bioproducts, Maryland, USA) were added aseptically to the medium.

C. Preparation of 10% MEM (Whitaker, 1972):

1. Foetal calf serum (FCS) (Delta Bioproducts) was de-complemented in a water bath (Gallenkamp, England) at 56°C for 30 min.
2. Fifty-five millilitres of serum-free medium were abstracted from a 500 ml MEM bottle (Highveld Biological).
3. Fifty millilitres of filter sterilised foetal calf serum and 5 ml of sterile pen/strep fungizone mix (Whittaker) were added aseptically to the medium.

D. Determination of total BGM, HEp-2 and PLC/PRF/5 cell counts (Bird and Forrester, 1981):

1. The total number of viable cells of a monolayer of BGM (HEp-2 and PLC/PRF/5 cells) formed on the base of the flask (250 ml) was determined.
2. The medium in the flask was discarded and the walls of the flask were washed with 5 ml phosphate-buffered saline (PBS) (Sigma).
3. Trypsin EDTA (National Institute for Virology, South Africa) (3 ml) was added to the flask. The flask was incubated at 37°C for 1 min.
4. The cells were detached from the bottom of the flask by lightly tapping the flask firmly against the hand.

5. Three millilitres of a 10% MEM (Highveld Biological) were added to the flask and swirled to neutralise the effect of trypsin EDTA.
6. The suspension was transferred from the flask to a centrifuge tube (Corning Costar), which was centrifuged at 500 x *g* (BHG Roto-Uni II) for 2 min to pellet the cells.
7. The supernatant was discarded and the pellet of cells was re-dissolved by mixing thoroughly in a serum-free media, with a volume (generally, $V_{initial}$= 10 ml) dependent on the thickness of the monolayer.
8. In a separate centrifuge tube, 500 µl of 0.4% trypan blue stain (Sigma) were added. This cell counting method was based on the fact that viable cells (with intact membranes) do not take up the trypan blue stain, whereas non-viable cells do.
9. An equal amount of the cell suspension (500 µl) (dilution factor of 2) was added to the trypan blue and mixed thoroughly to receive an even suspension without excessive clumping. The trypan blue-cell suspension was stained for 5 min.
10. A cover-slip was placed on one of the two counting chambers of a Neubauer hemacytometer (Superior, Germany). This counting chamber had four identical ruled squares, each measuring 1 by 1 mm. The space between the cover-slip and the ruled squares (with surface areas of 1 mm^2) was 0.1 mm. Therefore, the volume of one ruled square was 0.1 mm^3, or 10^{-4} cm^3. Using a Pasteur pipette, a small amount of the trypan blue-cell suspension (10 µl) was transferred to the counting chamber by carefully touching the edge of the cover-slip with the pipette tip and allowing the chamber to fill by capillary action. The chamber was not allowed to overfill or underfill.
11. The counting chamber was placed and examined under a light microscope. The low-power objective was focused on the ruled squares and all of the cells in the four 1 mm^2 corner squares were counted. Cells lying outside the borders of the squares were not counted.
12. The total number of viable cells in the 4 squares was divided by 4 to determine the mean count per square. This represented the number of cells per 0.1 mm^3. This number is multiplied by 10 000 to determine the number of cells per cubic centimetre. Since 1 cm^3 is equivalent to 1 ml, the cell number can be expressed per millilitre. The final number is adjusted by the appropriate dilution factor.

Determination of the total number of viable cells (Bird and Forrester, 1981):

Cells per ml = the average count per square x 10^4

Total cells = cells per ml x the dilution factor x the original volume of fluid from which cell sample was removed.

Example: $C_{initial} = 24 \times 10^4$ cells.ml^{-1}

$C_{final} = 2 \times 10^5$ cells.ml^{-1}

$V_{initial} = 10$ ml

$2 \times C_{initial} \times V_{initial} = C_{final} \times V_{final}$

$2 \times (24 \times 10^4 \text{ cells.ml}^{-1}) \times 10 \text{ ml} = (2 \times 10^5 \text{ cells.ml}^{-1}) \times V_{final}$

$V_{final} = (4.8 \times 10^6 \text{ cells.ml}^{-1}) / (2 \times 10^5 \text{ cells.ml}^{-1})$

$V_{final} = 24$ ml

13. The pellet of cells was dissolved in serum-free media and centrifuged at 500 x *g* (BHG Roto-Uni II) for 2 min to pellet the cells.
14. The supernatant was discarded and the cell pellet was re-dissolved in the necessary volume (V_{final}) of 5% MEM growth medium.

E. Freezing of BGM, HEp-2 and PLC/PRF/5 cell cultures for storage (Whitaker, 1972):

Add the following to prepare the freeze medium:

1. Twenty-five millilitres of filter sterilised double-strength Eagle's Minimum Essential Medium (Highveld Biological).
2. Twenty millilitres of filter sterilised foetal bovine serum (Delta Bioproducts).
3. Five millilitres of filter sterilised glycerol (Sigma).
4. Sterile pen/strep fungizone mix (0.5 ml) (Whittaker).
5. Mix together.

Procedure:

1. Cells were trypsinised and neutralised with 10% MEM (Highveld Biological) medium.
2. The cell suspension was centrifuged at 500 x *g* (BHG Roto-Uni II) to pellet cells.
3. The supernatant was removed and cells were re-suspended in 1 ml of freeze-medium.
4. The cell suspension was carried over to a sterile cryogenic vial (Corning Costar) and frozen at 4°C for 2 h.

5. After 2 h, the cell suspension was frozen at -20°C for 24 h and finally the cell suspension was stored at -70°C for future analysis.

II. RECOVERY OF VIRUSES FROM SEWAGE AND RIVER WATER SAMPLES

A. Decontamination of the sewage and river water samples (Minor, 1985):

1. 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).
2. The samples were centrifuged at 4 500 x *g* (Sorvall Super T 21, Wilmington, USA) at 4°C in order to separate the supernatant from the chloroform.

B. Polyethylene glycol/sodium chloride precipitation method (Minor, 1985):

1. A mixture consisting of 14.0 g polyethylene glycol (PEG) (PEG 6000, Merck) and 1.17 g sodium chloride (NaCl) (Sigma) was prepared and this mixture was dissolved into 100 ml of sewage/river water sample.
2. After settling down at 4°C for 24 h, each sample was divided into two 50 ml centrifuge tubes (Corning Costar) and each sample tube was centrifuged at 2 500 x *g* (Sorvall Super T 21) for 30 min at 4°C.
3. The supernatant was discarded and 10 ml of PBS (Sigma) were added into each centrifuge tube. The two tubes of each sample were added together and mixed well.
4. The sonicator (Soniprep 150, MSE) was cleaned with 70% ethanol (Merck) before use and the samples were sonicated for 20 s in order to break loose any pre-formed virus clumps.
5. The samples were centrifuged at 600 x *g* (BHG Roto-Uni II) for 10 min at 4°C before being added to bottles containing nystatin and penicillin/streptomycin neomycin mix (150 µl of each antibiotic) (Whittaker).
6. Finally, the sewage and river water samples were stored at -20°C for further analysis.

III. ENTEROVIRUS DETECTION

A. Plaque assay for the detection of plaque forming enteroviruses (Manor *et al.*, 1999):

1. The BGM, HEp-2 and PLC/PRF/5 cells were seeded in 92 mm Nunclon tissue culture plates (Nalge Nunc, Denmark) at a concentration of 2 x 10^5 cells.ml^{-1} and incubated at 37°C in a 5% CO_2 incubator (Galaxy CO_2 Incubator).
2. After 48 h of incubation, the medium was discarded aseptically and a confluent BGM, HEp-2 or PLC/PRF/5 cell monolayer was observed at the bottom of each tissue culture plate.
3. The cells were starved by adding 2 ml of a medium consisting of MEM and 1% pen/strep fungizone mix on the side of each tissue culture plate.
4. The infectious plates were incubated for 1 h at 37°C. After 1 h, the MEM + 1% pen/strep fungizone medium was pulled off.
5. The cells were infected with 1 ml of the PEG sample (sewage and river water) and incubated for 1 h 30 min at 37°C with rotation of the flask every 10-15 min.
6. Overlay medium stock was prepared using filter sterilised double-strength Eagle's MEM, consisting of 96% MEM and 4% foetal bovine serum. A 2% Sea Kem ME Agarose solution (FMC Bioproducts, ME, USA), consisting of 2 g agar in 100 ml of PBS (pH 7.2) (Sigma), was prepared separately and autoclaved at 121°C for 15 min. Equal amounts of the double-strength MEM and the agar were mixed at 50°C to give a final concentration of 1% agar.
7. Each tissue culture plate received 20 ml of the overlay medium, which was poured aseptically on the side of each plate without disrupting the pre-formed BGM, HEp-2 or PLC/PRF/5 cell monolayers.
8. The agarose in the tissue culture plates was allowed to solidify for 10-20 min at 22°C.
9. The tissue culture plates were incubated at 37°C and plaques appeared within 5 - 7 days.
10. Plaques were picked up from the plates and the virus was allowed to replicate in 50 ml tissue culture flasks (Cellstar, Greiner Labortechnik) containing pre-formed BGM, HEp-2 and PLC/PRF/5 cell monolayers.
11. The concentrated virus was extracted from each tissue culture flask and stored at -20°C for further analysis.

B. Extraction of the ribonucleic acid from sewage and river water samples (RNeasy, Qiagen, Hilden, Germany):

1. The ribonucleic acid (RNA) was extracted by means of a RNeasy Viral RNA extraction kit (RNeasy, Qiagen, Hilden, Germany).
2. A total of 1.5 ml of virus infected cells were centrifuged at 300 x *g* (Eppendorf Centrifuge 5402D, Hamburg, Germany) for 5 min (±25°C).
3. The supernatant was removed carefully by aspiration.
4. The cells were disrupted by the addition of buffer RLT (350 µl per reaction). A ß-mercaptoethanol (10 µl) was mixed with 1 ml of buffer RLT before use. The cell pellets were dissolved thoroughly by inverting each centrifuge tube.
5. Each lysate was pipetted directly onto QIAshredder spin columns placed in 2 ml collection tubes and the lysates were centrifuged for 2 min at a maximum speed (Eppendorf Centrifuge 5402D) at ±25°C.
6. A volume of 350 µl of 70% ethanol per reaction was added to each homogenised lysate and mixed well by pipetting.
7. Up to 700 µl of each sample were added to RNeasy mini columns placed in 2 ml collection tubes (supplied) and each sample was centrifuged for 15 s at 8 000 x *g* (Eppendorf Centrifuge 5402D) at ±25°C. The flow-through was discarded.
8. A buffer RW1 (350 µl per reaction) was pipetted into each spin column and the spin columns were centrifuged for 15 s at 8 000 x *g* (Eppendorf Centrifuge 5402D) at ±25°C. The flow-through was discarded and the collection tubes were re-used.
9. DNase I (10 µl) stock solution was added to 70 µl of Buffer RDD and this solution was mixed gently by inverting each tube.
10. The DNase I incubation mix (80 µl per reaction) was pipetted directly onto each spin-column membrane and the columns were placed on the benchtop (20°C to 30°C) for 15 min.
11. After 15 min, buffer RW1 (350 µl per reaction) was pipetted into each spin column and the spin columns were centrifuged for 15 s at 8 000 x *g* (Eppendorf Centrifuge 5402D) at ±25°C. The flow-through and collection tubes were discarded.
12. Each spin column was placed in a new 2 ml collection tube (supplied). Buffer RPE (500 µl per reaction) was pipetted into each spin column and the spin columns were

centrifuged for 15 s at 8 000 x *g* (Eppendorf Centrifuge 5402D) at ±25°C. The flow-through was discarded and each collection tube was re-used.

13. Buffer RPE (500 µl per reaction) was pipetted into each spin column and the columns were centrifuged for 2 min at a maximum speed (Eppendorf Centrifuge 5402D) at ±25°C to dry the silica-gel membranes, thus ensuring that no ethanol is carried over during elution.
14. Each spin column was transferred to new 1.5 ml collection tubes (supplied) and 55 µl of RNase-free water were pipetted directly onto the silica-gel membranes. The columns were centrifuged for 1 min at 8 000 x *g* (Eppendorf Centrifuge 5402D) at ±25°C in order to elute the RNA.
15. The extracted RNA was stored at -70°C for further analysis.

C. TRIzol method for the extraction of viral RNA from stool samples (Center for Pediatric Research, EVMS/CHKD, Virginia, USA):

1. In the homogenisation and clarification of the stool specimens, 300 µl of 10 - 50% faecal suspension was mixed with an equal volume of freon (Sigma).
2. The samples were vortexed for 30 s at ±25°C.
3. The samples were centrifuged at 12 000 x *g* (Eppendorf Centrifuge 5402D) for 5 min at room temperature (±25°C) and 140 µl of each supernatant were withdrawn for RNA extraction.
4. The clarified stool suspensions (140 µl per reaction) were mixed with 500 µl of TRIzol (Invitrogen Life Techno, Paisley, Scotland). The mixtures were incubated at room temperature (±25°C) for 5 min to permit complete dissociation of the nucleoprotein complex.
5. Pure chloroform (100 µl per reaction) (Sigma) was added to 500 µl of TRIzol and mixed vigorously by hand for 15 s. The mixtures were incubated for 3 min at room temperature (±25°C). The samples were centrifuged at 12 000 x *g* (Eppendorf Centrifuge 5402D) for 15 min at 4°C.
6. In separate 1.5 ml eppendorf tubes (Eppendorf), 30 µl of 3 M sodium acetate (pH 5.2) (Merck) per reaction were added to 600 µl of 100% ethanol (Merck). After 15 min of centrifugation, the aqueous phase (300 µl) was transferred to each of the 1.5 ml eppendorf tubes.

7. The samples were stored at -20°C overnight.
8. After 24 h, the samples were centrifuged at 12 000 x *g* (Eppendorf Centrifuge 5402D) for 15 min at 4°C.
9. Each supernatant was discarded and the RNA pellets were washed with 300 µl of 70% ethanol (Merck) per reaction.
10. The samples were centrifuged at 12 000 x *g* (Eppendorf Centrifuge 5402D) for 5 min at 4°C. Each supernatant was discarded and the RNA pellets were air-dried from 5 to 10 min.
11. Each RNA pellet was dissolved in 35 µl of RNase free water (DEPC-water, Promega Corp., Madison, USA) and the samples were incubated for 10 min at 42°C in the hybridisation oven (Techne Hybridiser HB-1D, Techne, Cambridge, United Kingdom). After 10 min, the samples were centrifuged briefly at 8 000 x *g* (Eppendorf Centrifuge 5402D) at ±25°C and stored at -70°C for further analysis.

D. Reverse transcription polymerase chain reaction for the detection of enteroviruses (Gow *et al.*, 1991):

1. A reverse transcription polymerase chain reaction (RT-PCR) was carried out using a Promega Access RT-PCR kit.
2. 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), 5 U each of AMV Reverse Transcriptase and *Tfl* DNA Polymerase.

PCR conditions applied:

48°C for 45 min, reverse transcription
94°C for 1 min, DNA denaturation
56°C for 1 min, primer annealing } 30 cycles
72°C for 1 min, primer extension
72°C for 10 min, final extension

Preparation of master mix cocktails:

Reagents	Master mix per reaction
Nuclease-Free H_2O	22.00 µl
AMV/*Tfl* 1x Reaction Buffer	10.00 µl
DNTP Mix, 10 mM (10 mM each of dATP, dCTP, dGTP, dTTP)	1.00 µl
EP1 (50 pmol)	0.50 µl
EP4 (50 pmol)	0.50 µl
$MgSO_4$, 25 mM	4.00 µl
AMV Reverse Transcriptase, 5 units.ul^{-1} (0.1 M Potassium phosphate pH 7.2, 0.2% Triton-X-100, 2 mM DTT, 50% Glycerol)	1.00 µl
Tfl DNA Polymerase, 5 units.ul^{-1} (50% Glycerol, 20 mM Tris HCl pH 8.0, 1 mM DTT, 100 mM KCl, 0.1 mM EDTA, 0.5% Nonidet P40, 0.5% Tween 20)	1.00 µl
Total	**40.00 µl**

3. In total, 40 µl of master mix cocktails were dispensed into 0.5 ml eppendorf tubes (Eppendorf).
4. Two drops of mineral oil (Promega Corp.) were added into each eppendorf tube to avoid evaporation of the PCR product.
5. Ten microlitres of extracted RNA were added into each eppendorf tube containing the PCR cocktails in an ultraviolet (UV) cabinet, to give a final volume of 50 µl per reaction.
6. The amplification (30 cycles) was performed in a Hybaid Thermocycler (Hybaid OmniGene Thermocycler, United Kingdom).
7. Enteroviruses displayed a 408 bp product.

E. Nested PCR amplification (Kuan, 1997):

1. A second PCR run was undertaken, in which 1 µl of the amplified RT-PCR product was added to 49 µl of previously prepared PCR mixture (Promega Corp.).
2. 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), and 1.5 U of *Taq* DNA polymerase (Promega Corp.).

Nested PCR conditions applied:

94°C for 3 min, DNA denaturation

94°C for 1 min, DNA denaturation
45°C for 1 min, primer annealing
72°C for 1 min, primer extension
} 30 cycles

72°C for 10 min, final extension

Preparation of master mix cocktails:

Reagents	Master mix per reaction
Nuclease-Free H_2O	37.50 µl
1x PCR buffer	5.00 µl
$MgCl_2$	4.00 µl
dNTP Mix, 10 mM (10 mM each of dATP, dCTP, dGTP, dTTP)	1.00 µl
E1 (50 pmol)	0.50 µl
E2 (10 pmol)	0.50 µl
Taq DNA Polymerase, 5 units.µl^{-1} (50% Glycerol, 20 mM Tris HCl pH 8.0, 1 mM DTT, 100 mM KCl, 0.1 mM EDTA, 0.5% Nonidet P40, 0.5% Tween 20)	0.50 µl
Total	**49.00 µl**

3. In total, 49 µl of master mix cocktails were dispensed into 0.5 ml eppendorf tubes (Eppendorf).
4. One microlitre of each PCR product was added into each eppendorf tube containing the pre-formed master mix cocktails in an UV cabinet, to give a final volume of 50 µl per reaction.
5. After 30 cycles, 20 µl of each nested PCR product were subjected to agarose (2%) (Seakem LE agarose) gel electrophoresis (Midicell Primo Gel Apparatus).
6. Enteroviruses displayed a 297 bp product.

F. Reverse transcription multiplex PCR to distinguish poliovirus from non-polio enteroviruses (Egger *et al.*, 1995):

1. Primers specific for either enterovirus or poliovirus were combined in a reverse transcription multiplex PCR (RT-multiplex PCR) (Promega Access RT-PCR system).
2. 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), 25 pmol each of primers E1, E2, Po1, Po2, Po3 and Po4 (Sigma-Genosys), 5 U each of AMV Reverse Transcriptase and *Tfl* DNA Polymerase (Promega Corp.).

PCR conditions applied:

48°C for 45 min, reverse transcription

94°C for 1 min, DNA denaturation
45°C for 1.5 min, primer annealing } 30 cycles
72°C for 1 min, primer extension

72°C for 10 min, final extension

Preparation of master mix cocktails:

Reagents	Master mix per reaction
Nuclease-Free H_2O	22.50 µl
AMV/*Tfl* 1x Reaction Buffer	10.00 µl
dNTP Mix, 10 mM (10 mM each of dATP, dCTP, dGTP, dTTP)	1.00 µl
E1 (25 pmol)	0.25 µl
E2 (25 pmol)	0.25 µl
Po1 (25 pmol)	0.25 µl
Po2 (25 pmol)	0.25 µl
Po3 (25 pmol)	0.25 µl
Po4 (25 pmol)	0.25 µl
$MgSO_4$, 25 mM	3.00 µl
AMV Reverse Transcriptase, 5 units.ul^{-1} (0.1 M Potassium phosphate pH 7.2, 0.2% Triton-X-100, 2 mM DTT, 50% Glycerol)	1.00 µl
Tfl DNA Polymerase, 5 units.ul^{-1} (50% Glycerol, 20 mM Tris HCl pH 8.0, 1 mM DTT, 100 mM KCl, 0.1 mM EDTA, 0.5% Nonidet P40, 0.5% Tween 20)	1.00 µl
Total	**40.00 µl**

3. In total, 40 µl of master mix cocktails were dispensed into 0.5 ml eppendorf tubes (Eppendorf).
4. Two drops of mineral oil (Promega Corp.) were added into each eppendorf tube to avoid evaporation of the PCR product.
5. Ten microlitres of extracted RNA were added into each eppendorf tube containing the PCR cocktails in an UV cabinet, to give a final volume of 50 µl per reaction.
6. The amplification was performed in a Hybaid Thermocycler (Hybaid OmniGene Thermocycler).
7. After 30 cycles, 20 µl of each PCR product were subjected to agarose (2%) (Seakem LE agarose) gel electrophoresis (Midicell Primo Gel Apparatus).
8. Poliovirus vaccine strains displayed base pair bands at 193 bp, 297 bp, 565 bp and in several cases at 1 000 bp, whereas non-polio enteroviruses showed the enterovirus-specific 297 bp band.

G. Sabin specific RT-triplex PCR to distinguish between the Sabin poliovirus types 1 to 3 (Yang *et al.*, 1991; Yang *et al.*, 1992):

1. Three sets of primers specific for Sabin poliovirus vaccine strains were combined in a RT-triplex PCR to confirm the isolated polioviruses as OPV strains (Promega Corp.).
2. A 50 µl reaction volume containing the following was prepared: 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 $MgSO_4$, 5 U each of AMV Reverse Transcriptase and *Tfl* DNA Polymerase (Promega Corp.).
3. The amplification was performed in 30 cycles in a Mini Thermocycler (MJ Research, Inc., Watertown, USA) or in a Hybaid Thermocycler (Hybaid OmniGene Thermocycler).

PCR conditions applied:

42°C for 45 min, reverse transcription

95°C for 30 s, DNA denaturation
56°C for 45 s, primer annealing } 30 cycles
72°C for 1 min, primer extension

72°C for 10 min, final extension

Preparation of master mix cocktails:

Reagents	Master mix per reaction
Nuclease-Free H_2O	22.50 µl
AMV/*Tfl* 1x Reaction Buffer	10.00 µl
dNTP Mix, 10 mM (10 mM each of dATP, dCTP, dGTP, dTTP)	1.00 µl
S1-1 (25 pmol)	0.25 µl
S1-2 (25 pmol)	0.25 µl
S2-1 (25 pmol)	0.25 µl
S2-2 (25 pmol)	0.25 µl
S3-1a (25 pmol)	0.25 µl
S3-2 (25 pmol)	0.25 µl
$MgSO_4$, 25 mM	3.00 µl
AMV Reverse Transcriptase, 5 units.ul^{-1} (0.1 M Potassium phosphate pH 7.2, 0.2% Triton-X-100, 2 mM DTT, 50% Glycerol)	1.00 µl
Tfl DNA Polymerase, 5 units.ul^{-1} (50% Glycerol, 20 mM Tris HCl pH 8.0, 1 mM DTT, 100 mM KCl, 0.1 mM EDTA, 0.5% Nonidet P40, 0.5% Tween 20)	1.00 µl
Total	**40.00 µl**

4. In total, 40 µl of master mix cocktails were dispensed into 0.5 ml eppendorf tubes (Eppendorf).
5. Two drops of mineral oil (Promega Corp.) were added into each eppendorf tube to avoid evaporation of the PCR product.
6. Ten microlitres of extracted RNA were added into each eppendorf tube containing the PCR cocktails in an UV cabinet, to give a final volume of 50 µl per reaction.
7. The amplification was performed in 30 cycles in a Mini Thermocycler (MJ Research) or in a Hybaid Thermocycler (Hybaid OmniGene Thermocycler).
8. The amplified products (20 µl) were separated with 7% polyacrylamide (BioRad, Hercules, California, USA) gel electrophoresis using a Hoefer electrophoresis unit (SE 600 Electrophoresis Unit, Hoefer, Scientific Instruments, San Francisco, California, USA).
9. Sabin poliovirus type 1 showed 97 bp band, Sabin poliovirus type 2 showed 71 bp band and Sabin poliovirus type 3 displayed 54 bp band.

H. Restriction enzyme analysis (Kämmerer *et al.*, 1994; Kuan, 1997):

1. Non-polio enteroviruses were typed using three restriction enzymes (*Sty* I, *Bgl* I and *Xmn* I) (Promega Corp.).
2. Aliquots of 10 µl of the RT-multiplex PCR products were incubated with 10 U of each of the restriction enzymes in 30 µl reaction volumes with the buffers recommended by the manufacturer (Promega Corp.).

Preparation of master mix cocktails:

Reagents	Master mix per reaction
Sty I (10 u.µl^{-1}), *Bgl* I (10 u.µl^{-1}), *Xmn* I (10 u.µl^{-1})	1.0 µl
Restriction enzyme 10xBuffer F, RE 10xBuffer D, RE 10xBuffer	3.0 µl
Acetylated BSA, 10 µg.µl^{-1}	0.3 µl
sterile, de-ionised H_2O	15.7 µl
Total	**20.0 µl**

3. In total, 20 µl of master mix cocktails were dispensed into 0.5 ml eppendorf tubes (Eppendorf).
4. Ten microlitres of each RT-multiplex PCR product (obtained from method F) were added into each eppendorf tube containing the pre-formed master mix cocktails in an UV cabinet, to give a final volume of 30 µl per reaction.

5. The samples were incubated at 37°C for 3 h in a Hybaid Thermocycler (Hybaid OmniGene Thermocycler) and were analysed using 7% polyacrylamide (BioRad) gel electrophoresis (Hoefer, SE 600 Electrophoresis Unit).

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

Restriction enzyme	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, CA7, CBV1, CBV2	236 + 61

I. Preparation of 7% polyacrylamide gel

1. In total, 7 ml of acrylamide (40%) (BioRad) were mixed with 29 ml of sterile distilled water.
2. Four millilitres of 10 x TAE (Amresco, Solon, Ohio, USA), 250 µl of ammonium persulphate (AMPS) (Amresco) and 50 µl of temed (Sigma) were added to the above solution.
3. A spacer mate (Hoefer) and two spacers were placed on a glass plate (16 cm x 18 cm x 0.3 cm). A second glass plate was placed on top and the glass plates were tightened with clamps.
4. The spacer mate was removed and the gel was poured between the two glass plates.
5. After the gel solidified, the glass plates were placed in a Hoefer electrophoresis unit. The gel was run at 120 Volts at 6°C using a BioRad power supply (BioRad, 500/200 Power Supply).

IV. NUCLEOTIDE SEQUENCE ANALYSIS

A. A RT-PCR for the 5'untranslated region of poliovirus vaccine strains (Divizia *et al.*, 1999; Guillot *et al.*, 2000):

1. The RT-PCR was performed as described by Divizia *et al.* (1999) and Guillot *et al.* (2000) with a few modifications.
2. The primers used for the 5'untranslated region (5'UTR) were as follows: UG52 (nt 160 to 180) and UC53 (nt 599 to 580) (Sigma-Genosys).
3. 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 UG52 and UC53 (Sigma-Genosys), 5 U each of AMV Reverse Transcriptase and *Tfl* DNA Polymerase (Promega Corp.).
4. The amplification was performed in 30 cycles in a Mini Thermocycler (MJ Research).

PCR conditions applied:

42°C for 45 min, reverse transcription

95°C for 30 s, DNA denaturation
45°C for 45 s, primer annealing } 30 cycles
72°C for 1 min, primer extension

72°C for 10 min, final extension

Preparation of master mix cocktails:

Reagents	Master mix per reaction
Nuclease-Free H_2O	28.80 µl
AMV/*Tfl* 1x Reaction Buffer	10.00 µl
dNTP Mix, 10 mM (10 mM each of dATP, dCTP, dGTP, dTTP)	1.00 µl
UG52 (10 pmol)	0.10 µl
UC53 (10 pmol)	0.10 µl
$MgSO_4$, 25 mM	3.00 µl
AMV Reverse Transcriptase, 5 units.ul^{-1} (0.1 M Potassium phosphate pH 7.2, 0.2% Triton-X-100, 2 mM DTT, 50% Glycerol)	1.00 µl
Tfl DNA Polymerase, 5 units.ul^{-1} (50% Glycerol, 20 mM Tris HCl pH 8.0, 1 mM DTT, 100 mM KCl, 0.1 mM EDTA, 0.5% Nonidet P40, 0.5% Tween 20)	1.00 µl
Total	**45.00 µl**

5. In total, 45 µl of master mix cocktails were dispensed into 0.5 ml eppendorf tubes (Eppendorf).
6. Five microlitres of each poliovirus RNA were added into each eppendorf tube containing the pre-formed master mix cocktails in an UV cabinet, to give a final volume of 50 µl per reaction.
7. After 30 cycles, 20 µl of each PCR product were subjected to agarose (2%) (Seakem) gel electrophoresis (Midicell Primo Gel Apparatus).
8. Poliovirus vaccine strains displayed base pair bands at 440 bp and this region was sequenced using the automated sequencer (Inqaba Biotechnical Industries Pty [Ltd], Pretoria, South Africa).

B. A RT-PCR for the VP1 capsid-encoding region of poliovirus vaccine strains (Divizia *et al.*, 1999; Guillot *et al.*, 2000):

1. The RT-PCR was performed as described by Divizia *et al.* (1999) and Guillot *et al.* (2000) with a few modifications.
2. The primers used for the VP1 region were as follows: UG1 (nt 2402 to 2422) and UC1 (nt 2881 to 2862) (Sigma-Genosys).
3. 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 (Sigma-Genosys), 5 U each of AMV Reverse Transcriptase and *Tfl* DNA Polymerase (Promega Corp.).
4. The amplification was performed in 30 cycles in a Mini Thermocycler (MJ Research, USA).

PCR conditions applied:

42°C for 45 min, reverse transcription

95°C for 30 s, DNA denaturation
50°C for 45 s, primer annealing } 30 cycles
72°C for 1 min, primer extension

72°C for 10 min, final extension

Preparation of master mix cocktails:

Reagents	Master mix per reaction
Nuclease-Free H_2O	28.80 µl
AMV/*Tfl* 1x Reaction Buffer	10.00 µl
dNTP Mix, 10 mM (10 mM each of dATP, dCTP, dGTP, dTTP)	1.00 µl
UG1 (10 pmol)	0.10 µl
UC1 (10 pmol)	0.10 µl
$MgSO_4$, 25 mM	3.00 µl
AMV Reverse Transcriptase, 5 units.ul^{-1} (0.1 M Potassium phosphate pH 7.2, 0.2% Triton-X-100, 2 mM DTT, 50% Glycerol)	1.00 µl
Tfl DNA Polymerase, 5 units.ul^{-1} (50% Glycerol, 20 mM Tris HCl pH 8.0, 1 mM DTT, 100 mM KCl, 0.1 mM EDTA, 0.5% Nonidet P40, 0.5% Tween 20)	1.00 µl
Total	**45.00 µl**

5. In total, 45 µl of master mix cocktails were dispensed into 0.5 ml eppendorf tubes (Eppendorf).
6. Five microlitres of each poliovirus RNA were added into each eppendorf tube containing the pre-formed master mix cocktails in an UV cabinet, to give a final volume of 50 µl per reaction.
7. After 30 cycles, 20 µl of each PCR product were subjected to agarose (2%) (Seakem) gel electrophoresis (Midicell Primo Gel Apparatus).
8. Poliovirus vaccine strains displayed a 480 bp product.
9. A nested PCR was performed as described by Divizia *et al.* (1999) with a few modifications immediately after completion of the RT-PCR step.
10. The primers used for the nested PCR were as follows: N2426 (nt 2426 to 2446) and N2812 (nt 2812 to 2792) (Sigma-Genosys).
11. 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) (Sigma-Genosys) and 1.5 U of *Taq* DNA Polymerase (Promega Corp.).
12. Cycling was carried out 30 times in a Mini Thermocycler (MJ Research).

Nested PCR conditions applied:

94°C for 2 min, DNA denaturation

94°C for 1 min, DNA denaturation
45°C for 1 min, primer annealing
72°C for 1 min, primer extension
} 30 cycles

72°C for 10 min, final extension

Preparation of master mix cocktails:

Reagents	Master mix per reaction
Nuclease-Free H_2O	37.80 µl
1x PCR buffer	5.00 µl
$MgCl_2$	4.00 µl
dNTP Mix, 10 mM (10 mM each of dATP, dCTP, dGTP, dTTP)	1.00 µl
N2426 (10 pmol)	0.10 µl
N2812 (10 pmol)	0.10 µl
Taq DNA Polymerase, 5 units.µl^{-1} (50% Glycerol, 20 mM Tris HCl pH 8.0, 1 mM DTT, 100 mM KCl, 0.1 mM EDTA, 0.5% Nonidet P40, 0.5% Tween 20)	1.00 µl
Total	**49.00 µl**

13. In total, 49 µl of master mix cocktails were dispensed into 0.5 ml eppendorf tubes (Eppendorf).
14. One microlitre of each PCR product was added into each eppendorf tube containing the pre-formed master mix cocktails in an UV cabinet, to give a final volume of 50 µl per reaction.
15. After 30 cycles, 20 µl of each nested PCR product was subjected to agarose (2%) (Seakem) gel electrophoresis (Midicell Primo Gel Apparatus).
16. Poliovirus vaccine strains displayed base pair bands at 387 bp and this region was sequenced using the automated sequencer (Inqaba Biotechnical Industries).

C. Cloning PCR products with pGEM®-T Easy vectors (Promega Corp.). Ligation using 2x rapid ligation buffer

1. The pGEM®-T Easy Vector and Control Insert DNA (Promega Corp.) were briefly centrifuged in order to collect the contents at the bottom of each tube.
2. The ligation reactions were prepared by mixing vigorously the 2x rapid ligation buffer (LB) (Promega Corp.). A 0.5 ml tube (Eppendorf) known to have low DNA-binding capacity was used in each ligation step.

3. The reactions were mixed by pipetting and incubated for 1 h at room temperature (±25°C). Alternatively, the reactions can be incubated overnight at 4°C in order to obtain the maximum number of transformants.

Reagents	Standard reaction	Positive control	Negative control
2x rapid ligation buffer	5 µl	5 µl	5 µl
pGEM®-T Easy vector (50 ng)	1 µl	1 µl	1 µl
PCR product	X µl	-	-
Control insert DNA	-	2 µl	-
T4 DNA ligase (3 units per L)	1 µl	1 µl	1 µl
Deionised water to a final volume of:	10 µl	10 µl	10 µl

Preparation of LB medium

1. The following reagents were mixed with 1 L of distilled water in a 1 L Erlenmeyer flask: 10 $g.L^{-1}$ Bacto tryptone (Difco Laboratories, Detroit, USA), 10 $g.L^{-1}$ Bacto yeast extract (Difco) and 5 $g.L^{-1}$ NaCl (Difco).
2. The pH was adjusted to pH 7.

Preparation of LB/ampicillin/ITPG/X-Gal plates

1. LB medium was prepared by mixing LB broth (Difco) and 15 g of agar (Difco), the top of the flask was wrapped with aluminium foil and autoclaved.
2. The LB medium was incubated at 50°C in order to prevent solidification of the medium.
3. The medium was heated to 50°C at which the following substances were added: 400 µl ampicillin (Mast Diagnostics, Mast group Ltd, Merseyside, United Kingdom), 400 µl ITPG and 400 µl X-Gal (Promega Corp.). The medium was gently mixed by swirling to avoid formation of air bubbles.
4. Approximately, 20 ml of the medium was poured in each Petri dish near a burner in a sterile area. The plates were allowed to cool to room temperature (25°C) before use.
5. The plates were stored at 4°C till further analysis.

Modified transformation of JM109 (Promega Corp.) high efficiency competent cells

1. LB/ampicillin/ITPG/X-Gal plates were prepared.
2. The ligation reaction was briefly vortexed.

3. Each ligation reaction (10 µl) was pippeted into a sterile 1.5 ml tube (Eppendorf) on ice. The JM109 (Promega Corp.) high efficiency competent cells were incubated on ice until thawed (5 min).
4. The cells were gently mixed by flicking the tube. Cells (100 µl) were carefully pipetted to the ligation reaction tubes.
5. The tubes were gently mixed and incubated on ice for 20 min.
6. The cells were heat-shocked for 45-50 s in a heat block (QBT2, Grant Instruments, Cambridge, United Kingdom) at exactly 42°C and tubes were not shaken.
7. The tubes were immediately put back on ice for 2 min. LB broth (900 µl) was added to the ligation reaction transformations at room temperature (±25°C).
8. The tubes were incubated for 1 h at 37°C in a shaking incubator (Labcon, Labotec, South Africa) at 200 rpm.
9. Each transformation culture (100 µl) was plated onto duplicate LB/ampicillin/IPTG/X-Gal plates. The plates were incubated overnight at 37°C.
10. The white colonies were selected and each colony represented one transformation.

Screening of transformed (white) colonies with PCR for inclusion of DNA fragments

1. The white colonies were diluted in 20 µl of nuclease-free water (Promega Corp.).
2. A 45 µl reaction volume for the PCR containing the following was prepared: 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.), and finally 5 µl of transformed (white) colonies suspension.
3. The amplification was performed in 30 cycles in a Mini Thermocycler (MJ Research). The PCR conditions were as follows: reverse transcription for 45 min at 42°C, denaturation for 30 s at 95°C, annealing for 45 s at 45°C, extension for 1 min at 72°C and after 30 cycles final extension for 10 min at 72°C.

Preparation for use of QIAprep spin miniprep kit protocol (Qiagen)

a) Growing culture

1. LB broth (10 ml) was mixed with 10 µl ampicillin and with 5 µl of the colony suspensions (found positive when screening with UG52 and UC53 primers).

2. The suspension was incubated overnight (18-24 h) at 37°C with agitation. Cells were pelleted by centrifugation at 1 000 x *g* for 10 min (Eppendorf Centrifuge 5402D) at ±25°C.

b) Preparation of reagents

1. The RNase A solution was added to buffer P1, mixed and stored at 2-8°C.
2. Absolute ethanol (Merck) was added to buffer PE.
3. Buffers P2 and N3 were investigated for salt precipitation before use. The precipitate was dissolved by warming solution to 37°C.
4. Buffer P2 should not be shaken vigorously. Buffer P2 was closed immediately after use to avoid acidification from CO_2 in the air.

c) QIAprep spin miniprep kit protocol

1. The pelleted bacterial cells were re-suspended in 250 µl of buffer P1 and transferred to a microcentrifuge tube (Eppendorf).
2. Buffer P2 (250 µl) was added and the tube was gently inverted 4-6 times to mix the suspension. The suspension was not vortexed as this would result in sharing of genomic DNA.
3. Buffer N3 (350 µl) was added and the tube was inverted gently 4-6 times. To avoid localized precipitation, the solution was mixed gently but thoroughly, immediately after addition of buffer N3. The solution should become cloudy.
4. The suspension was centrifuged for 10 min at 10 000 x *g* (Eppendorf Centrifuge 5402D) at ±25°C.
5. The supernatants were pipetted into the QIAprep column. The suspension was centrifuged for 60 s at 10 000 x *g* (Eppendorf Centrifuge 5402D) at ±25°C. The flow-through was discarded.
6. The QIAprep spin column was washed by adding 0.5 ml buffer PB and centrifuged for 60 s at 10 000 x *g* (Eppendorf Centrifuge 5402D) at ±25°C. The flow-through was discarded.
7. The QIAprep spin column was washed by adding 0.75 ml buffer PE and centrifuged for 60 s at 10 000 x *g* (Eppendorf Centrifuge 5402D) at ±25°C.
8. The flow-through was discarded and centrifuged for an additional 1 min at 10 000 x *g* (Eppendorf Centrifuge 5402D) at ±25°C to remove residual wash buffer.

9. The QIAprep spin column was placed in a clean 1.5 ml microcentrifuge tube (Eppendorf). To elute, 50 µl diluted buffer EB (1 µl EB: 7 µl water; 80°C) was added to the center of each QIAprep spin column, the tubes were incubated for 1 min at room temperature (±25°C) and centrifuged for 1 min at 10 000 x *g* (Eppendorf Centrifuge 5402D).
10. The elute was used for sequencing of DNA samples.

D. Sequencing using the automated sequencer (Inqaba Biotechnical Industries)

1. The exconuclease I/Shrimp alkaline phosphatase (Fermentas, Vilnus, Lithuania) was used to clean the PCR samples from primers and nucleotides.
2. The ABI BigDye Terminator cycle sequencing kit version 3.1 was used to sequence the PCR products.
3. 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. Both strands of the amplified fragments were sequenced to confirm the nature of the product obtained.
4. The data was analysed on a Spectrumedix SCE2410 genetic analysis system.

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