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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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

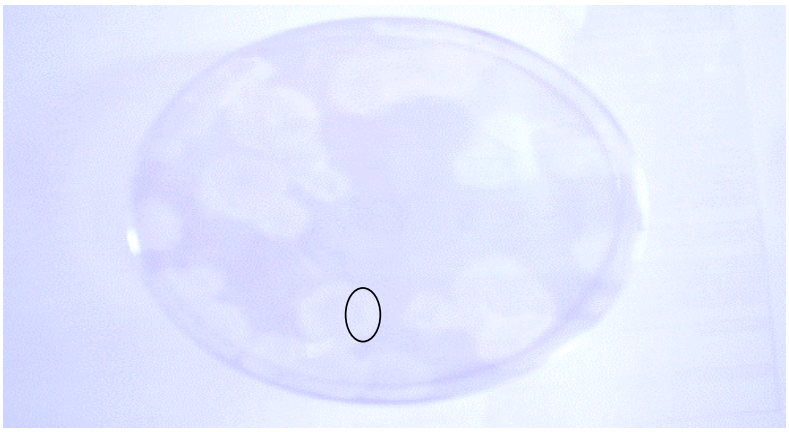

Figure 3.1: **Plaques formed on HEp-2 cell monolayers by poliovirus isolates**

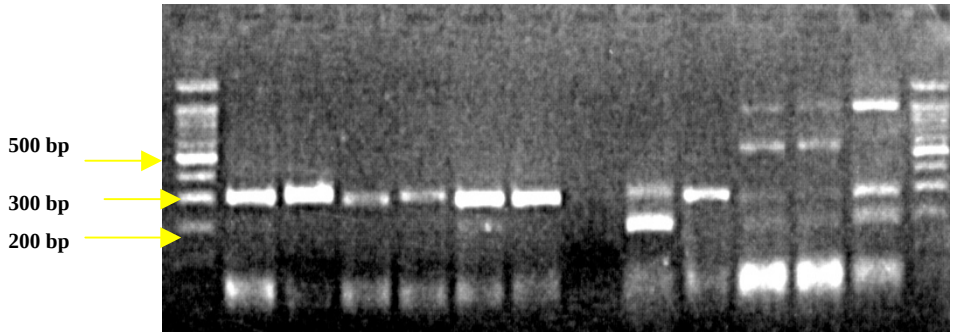

Figure 3.2: **Band patterns observed with the RT-multiplex PCR of the Sabin PV types 1 to 3 and the non-polio enteroviruses. Lane 1: 100 bp Marker; Lane 2: CBV1 (297 bp); Lane 3: CBV2 (297 bp); Lane 4: CBV3 (297 bp); Lane 5: CBV4 (297 bp); Lane 6: CBV5 (193 bp and 297 bp); Lane 7: CBV6 (297 bp); Lane 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)**

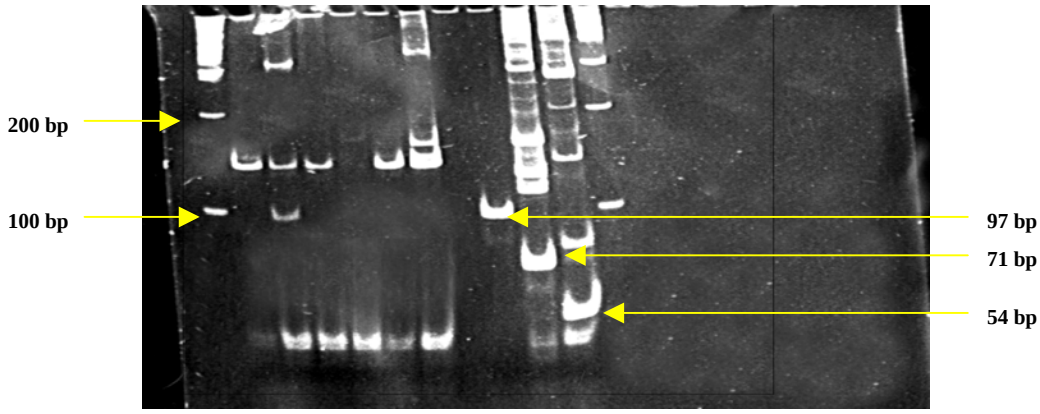

Figure 3.3: **Sabin specific RT-triplex PCR of the PV isolates and the positive controls. Lane 1: Marker 100 bp; Lane 2: negative sample; Lane 3: Sabin PV type 1 (positive isolate 97 bp); Lanes 4-8: Negative isolates; Lane 9: Sabin PV type 1 (positive control 97 bp); Lane 10: Sabin PV type 2 (positive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

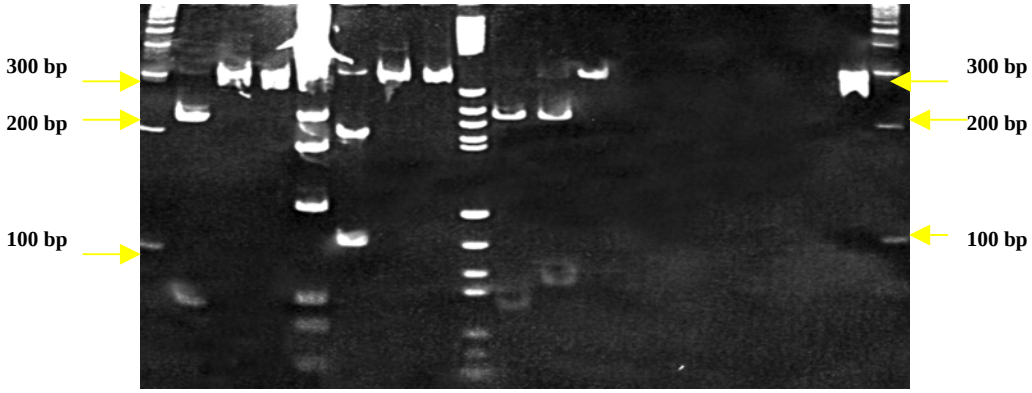

Figure 3.4: **Restriction enzyme digestion of 297 bp products from prototype strains of enteroviruses with three restriction enzymes (*Sty* I, *Bgl* I and *Xmn* I). Lane 1: Marker 100 bp; Lane 2: *Sty* I (226 bp and 71 bp), Lane 3: *Bgl* I (297 bp), Lane 4: *Xmn* I (297bp) - CBV3; Lane 5: PGem Marker; 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**

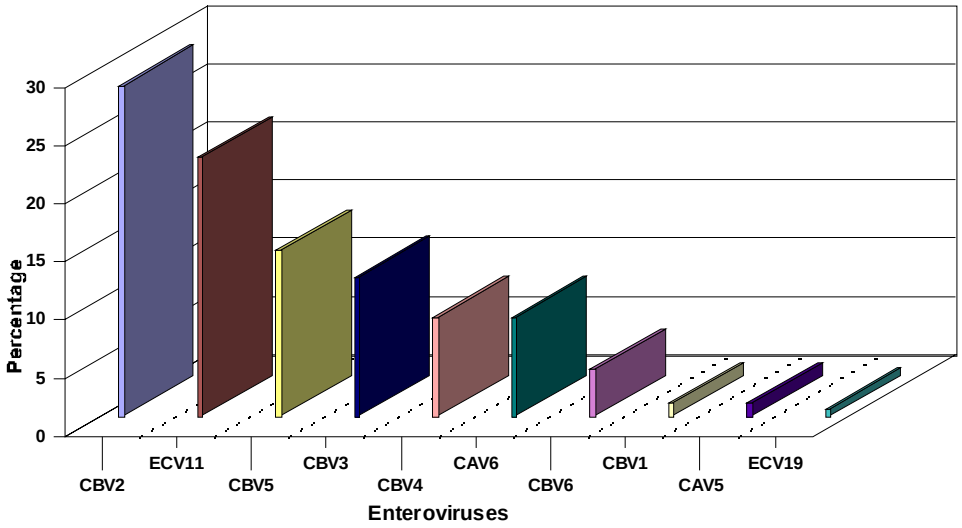

Figure 3.5: Detection of enteroviruses in sewage and river water samples collected from selected areas in South Africa from 2001 to 2003