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)	-

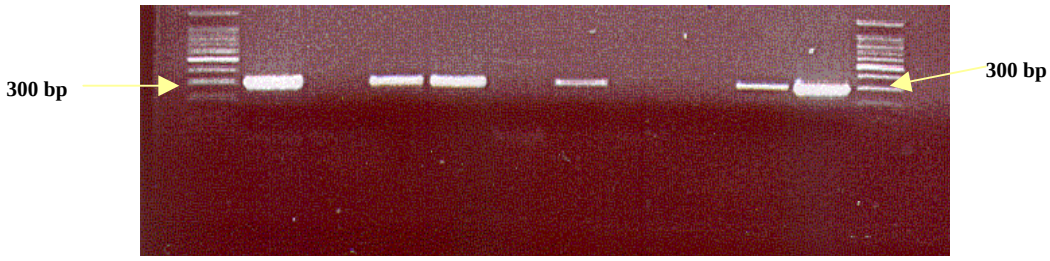

Figure 4.1: **Band patterns observed with the nested PCR in the detection of enteroviruses (EVs) in selected stool samples. Lane 1: Marker 100 bp; Lane 2: EV (297 bp); Lane 3: Negative; Lane 4: EV (297 bp); Lane 5: EV (297 bp); Lane 6: Negative; Lane 7: EV (297 bp); Lanes 8-9: Negative; Lane 10: EV (297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

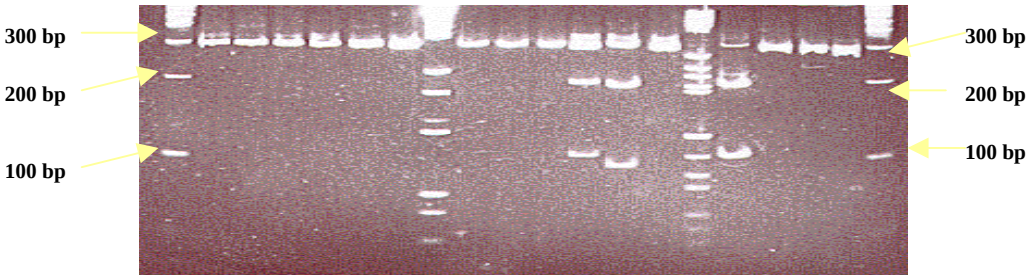

Figure 4.2: **Restriction enzyme digestion of 297 bp products from prototype strains of enteroviruses (EVs) with three restriction enzymes (*Sty* I, *Bgl* I and *Xmn* I). Lane 1: Marker 100 bp; Lane 2: *Sty* I (297 bp), Lane 3: *Bgl* I (297 bp), Lane 4: *Xmn* I (297bp) - PV type 3; Lane 5: *Sty* I (297 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

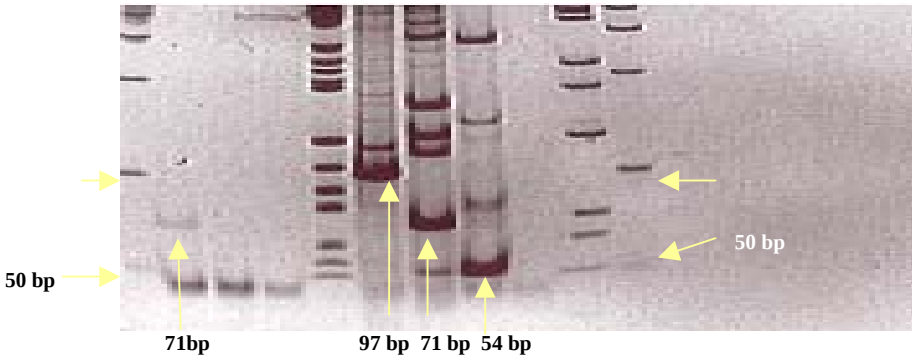

Figure 4.3: **Sabin RT-triplex PCR of the PV isolates and the positive controls. Lane 1: Marker 100 bp; Lane 2: Sabin PV type 2 (positive isolate 71 bp); Lanes 3-4: Negative isolates; Lane 5: PGem Marker; Lane 6: Sabin PV type 1 (97 bp); Lane 7: Sabin PV type 2 (71 bp); Lane 8: Sabin PV type 3 (54bp);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

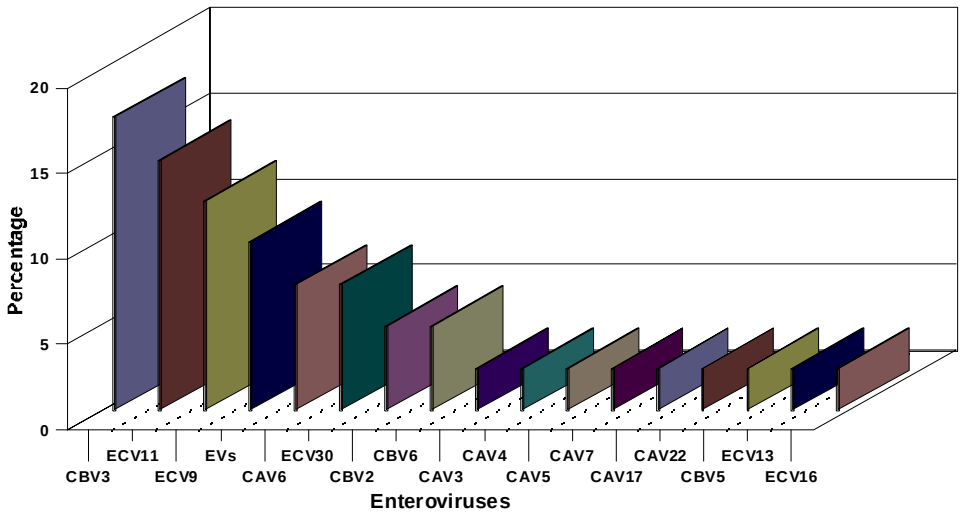

Figure 4.4: **Detection of non-polio enteroviruses in stool specimens of immunodeficient children from a selected area in South Africa during a period of one year**