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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