CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

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

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

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

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

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

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

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

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

2.2 History of poliomyelitis

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

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

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

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

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

2.3 Clinical manifestations of poliovirus infections

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

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

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

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

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

2.4 Genomic characterisation of poliovirus

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

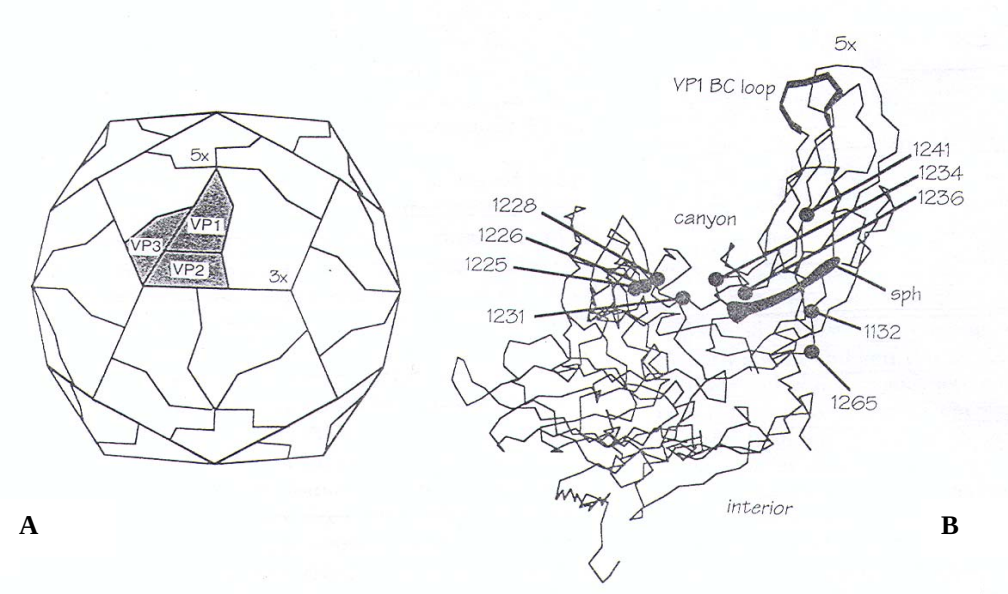

Figure 2.1: (A) Schematic representation of the icosahedral viral capsid structure of polioviruses. The fivefold (5x) and three fold (3x) axes of symmetry are indicated, as is the position of one of the 60 repeating protomeric units, each comprised of VP1, VP2 and VP3 surface proteins. (B) Line drawing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

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

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

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

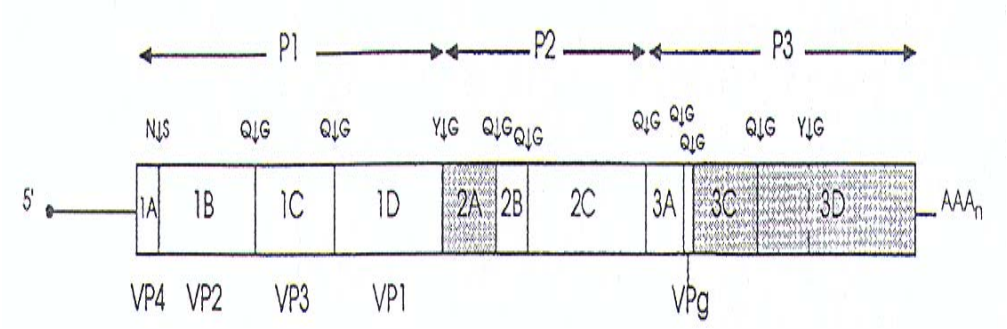

Figure 2.2: **Genomic organisation of poliovirus type 1 (Mahoney). The polyprotein encoded by the single open reading frame is shown as an elongated rectangle, the 5' and 3'untranslated regions are shown as lines and the genome-linked protein (VPg) is indicated by a black arrow. Cleavage sites between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

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

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

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

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

2.5 Mode of transmission of poliovirus

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

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

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

2.6 Survival of poliovirus in nature

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

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

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

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

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

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

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

2.7 Poliovirus vaccines

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

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

2.7.1 Inactivated poliovirus vaccine

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

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

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

2.7.2 Oral poliovirus vaccine

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

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

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

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

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

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

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

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

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

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

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

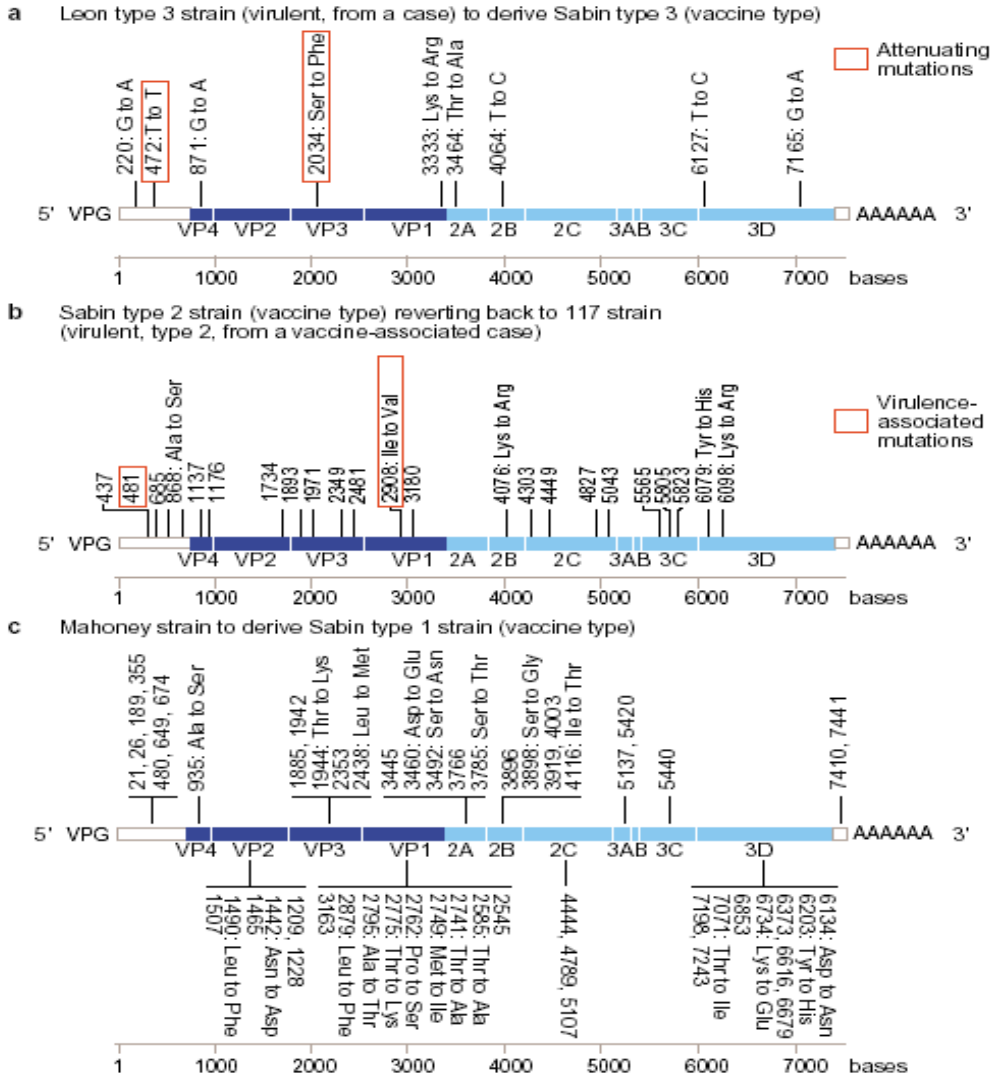

Figure 2.3: Comparisons of the sequences of polioviruses used to study the basis of attenuation and reversion of the Sabin vaccine strains of poliovirus. (A) Mutations that are involved in deriving the Sabin type 3 vaccine strain from the Leon strain. (B) Attenuating mutations in the Sabin PV type 2.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

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

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

2.9 Complications resulting from the use of oral poliovirus vaccine

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

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

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

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

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

2.10 Molecular changes of poliovirus vaccine strains in vaccine recipients

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

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

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

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

2.10.1 Mutations in Sabin poliovirus vaccine strains

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

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

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

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

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

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

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

2.10.2 Recombination in Sabin poliovirus vaccine strains

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

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

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

2.11 Vaccine-associated paralytic poliomyelitis and immunodeficiency

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

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

Immunodeficient persons with asymptomatic VDPV persistence should be considered at high risk of VAPP (Khetsuriani *et al.*, 2003). The overall risk of VAPP from OPV in a highly immune community is approximately 1 in 2.5 million doses administered, however, the risk is higher in a population with low vaccine coverage or inadequately vaccinated individuals such as persons older than 18 years of age and immunodeficient children (Guillot *et al.*, 2000; Martin *et al.*, 2000; Wood and Thorley, 2003). Compared with immunocompetent children, the risk of VAPP is approximately 7 000 times higher for persons with certain types of immunodeficiencies, such as B-lymphocyte disorders (agammaglobulinemia and hypogammaglobulinemia), which reduce the synthesis of immunoglobulins (Sutter and Prevots, 1994; Guillot *et al.*, 2000; Triki *et al.*, 2003; Wood and Thorley, 2003). It is likely that the absence of specific antibodies to PVs in these patients is responsible for the lack of viral neutralisation during the viremic phase, which precedes neurological localisation (Triki *et al.*, 2003). Thus, OPV should never be administered to immunodeficient recipients or to those living in households that contain immunodeficient individuals, such as HIV-positive patients or patients with lymphoma, leukemia, solid tumours, abnormal immunoglobulin synthesis and chemotherapy (Zaoutis and Klein, 1998; Hovi *et al.*, 2004).

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

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

2.12 Vaccine-derived polioviruses

A variety of OPV-derived viruses can be isolated from OPV recipients and their contacts (WHO, 2004). The extent of sequence divergence of the VP1 capsid gene from Sabin PV strains can be used as a "molecular clock" to estimate the duration of PV replication (WHO, 2004). A constant rate of accumulation of synonymous nucleotide substitutions is assumed to exist and for the PV genome rates of approximately 1-2% change per year have been proposed (Kew *et al.,* 1998; Kew *et al.,* 2002). All clinical and environmental PV isolates that are related to OPV strains are VDPVs (WHO, 2004). Derivatives of Sabin OPV strains have been classified into two broad categories for programmatic reasons: "OPV-like viruses" and "Vaccine-derived polioviruses" (WHO, 2004).

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

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

2.12.1 Immunodeficient vaccine-derived polioviruses

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

isolates to be characterised with modern molecular techniques were from patients with defects in antibody production (generally CVID or X-linked agammaglobulinemia) (Yoneyama *et al.*, 1982; Sutter and Prevots, 1994; Bellmunt *et al.*, 1999; Kew *et al.*, 1998; Wood *et al.*, 2000; Yang *et al.*, 2003). Some iVDPV isolates are highly divergent (~90% VP1 sequence identity to the parental Sabin PV vaccine strain), suggesting that the chronic PV infections had persisted for 10 years or more (Kew *et al.*, 1998; Wood *et al.*, 2000; Yang *et al.*, 2003). Eighteen chronic iVDPV excretors were detected world-wide through the end of 2002, although this number may be an underestimate in the absence of systematic screening of immunodeficient patients (WHO, 2004). So far, all reports of persistent iVDPV infections have been from countries with high or intermediate levels of development, where the rates of OPV coverage are high and where the survival times of immunodeficient patients may be extended by their access to appropriate clinical management (Yang *et al.*, 2003). Currently, there is no clear evidence of spread of iVDPV from immunodeficient patients to the wider community (Wood *et al.*, 2000; Yang *et al.*, 2003; WHO, 2004).

2.12.2 Circulating vaccine-derived polioviruses

In regions of low OPV coverage, a VDPV may result from transmission of Sabin PV vaccine strains from one immunised individual to another and accumulation of sufficient mutations to adopt wild-type PV characteristics of neurovirulence and transmissibility (Wood and Thorley, 2003). A VDPV may cause an outbreak of poliomyelitis and if there is evidence of person-to-person transmission, based on epidemiological and phylogenetic studies, it is defined as a circulating VDPV (cVDPV) (Wood and Thorley, 2003; Yang *et al.*, 2003).

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

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

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

Outbreaks of a similar kind have occurred more recently in the Philippines and Madagascar (WHO, 2002; Rousset *et al.*, 2003; Kew *et al.*, 2004). In Madagascar, five cases of AFP associated with cVDPV type 2 were reported and partial genomic sequencing indicated that two of the PV strains had been circulating for approximately 1 and 2.5 years, respectively (Rousset *et al.*, 2003). Mass vaccination campaigns with OPV interrupted circulation of cVDPVs in Hispaniola and have been underway in the Philippines and Madagascar (CDC, 2002a; Kew *et al.*, 2004).

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

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

2.13 Environmental surveillance of poliovirus circulation

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

Wild-type PVs and cVDPVs have been detected in the environment even in the absence of reported cases of AFP, which is of major concern, since these PVs might be transmitted and continue to circulate in a non-immune population after the cessation of polio vaccination (Friedrich, 2000; Buttinelli *et al.*, 2003; WHO, 2004). A study conducted by Divizia and colleagues (1999), confirmed the environmental circulation in Albania of recombinant PV strains (Sabin-like PV type 2/wild PV type 1), sustained by a massive immunisation effort and by the presence in the environment of a PV type 1, isolated from a river 2 months before the first case of symptomatic AFP. An unusual highly diverged derivative of the Sabin PV type 2 strain was isolated from environmental samples during routine screening for wild-type PV in Israel (Shulman *et al.*, 2000). The extensive genetic divergence of the isolate from its parental Sabin PV type 2 vaccine strain suggested that the virus had replicated in one or more individuals for approximately 6 years (Shulman *et al.*, 2000). According to other studies, VDPVs (with 1.4% nucleotide divergence from the vaccine strain) were isolated from sewage and river water in Japan within 3 months following OPV vaccination, and several of these VDPV type 1 and 3 isolates showed increased neurovirulence (Horie *et al.*, 2002; Yoshida *et al.*, 2002*).*

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

99.9% homology with Sabin PV type 2 vaccine strain in the VP1 region (WHO, 2003b). This suggested that these PVs could have been excreted by recently vaccinated children (one or two months) visiting from a country using OPV (WHO, 2003b). Furthermore, a highly evolved VDPV type 3 strain harbouring a 13% sequence drift from Sabin PV type 3 vaccine strain has been isolated from sewage in Estonia (Blomqvist *et al.*, 2004).

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

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

2.14 Isolation and identification of polioviruses

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

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

2.14.1 Recommended cell lines for the isolation of polioviruses

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

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

2.14.2 Serological diagnosis of poliovirus infection

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

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

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

2.14.3 Molecular techniques for the detection of polioviruses

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

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

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

2.14.3.1 Reverse transcription multiplex PCR

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

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

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

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

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

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

2.14.3.2 Sabin specific RT-triplex PCR

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

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

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

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

2.14.3.3 Restriction fragment length polymorphism

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

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

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

Restriction enzyme	Recognition site
Sty I	C*C(A/T)(A/T)GG
Bgl I	GCCNNNN*NGGC
Xmn I	GAANN*NNTTC

N = A, G, C or T.
*** = Recognition site for restriction enzymes.**

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

2.14.3.4 Nucleotide sequencing of the enteroviral genomes

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

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

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

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

2.14.4 Intratypic differentiation methods recommended by the WHO

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

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

These ITD methods include:

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

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

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

2.15 Eradication of poliomyelitis: Progress and Challenges

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

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

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

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

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

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

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

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

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

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

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

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

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

2.16 Summary

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

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

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

The circulation of the VDPVs in the environment is of major concern, because these PVs might be transmitted and continue to circulate in a non-immune population after the cessation of polio vaccination (Friedrich, 2000; Buttinelli *et al.,* 2003). Shulman and colleagues (2000) have isolated an unusual, highly diverged derivative of the Sabin type 2 strain from environmental samples during routine screening for wild-type PV in Israel. The extensive genetic divergence of the isolate from its parental Sabin PV type 2 vaccine strain suggested that the virus had replicated in one or more individuals for approximately 6 years (Shulman *et al.,* 2000). More recently, a highly evolved VDPV type 3 harbouring a 13% sequence drift from the Sabin PV type 3 vaccine strain has been isolated from sewage in Estonia (Blomqvist *et al.,* 2004).

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

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

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

2.17 References

Abraham, R., Chonmaitree, T., McCombs, J., Prabhakar, B., Lo Verde, P.T., Ogra, P.L. (1993) Rapid detection of poliovirus by reverse transcription and polymerase chain amplification: application for differentiation between poliovirus and non-poliovirus enteroviruses. *Journal of Clinical Microbiology* **31:** 395-399.

Alexander, J.P.Jr., Gary, H.E.Jr., Pallansch, M.A. (1997) Duration of poliovirus excretion and its implications for acute flaccid paralysis surveillance: a review of the literature. *Journal of Infectious Diseases* **175:** S176-S182.

American Academy of Paediatrics Committee on Infectious Diseases (1999) Poliomyelitis prevention: revised recommendations for use of inactivated and live oral poliovirus vaccines. *Paediatrics* **103:** 171-172.

Arita, M., Horie, H., Arita, M., Nomoto, A. (1999) Interaction of poliovirus with its receptor affords a high level of infectivity to the virion in poliovirus infections mediated by the Fc receptor. *Journal of Virology* **73:** 1066-1074.

Bellmunt, A., May, G., Zell, R., Pring-Akerblom, P., Verhagen, W., Heim, A. (1999) Evolution of poliovirus Type I during 5.5 years of prolonged enteral replication in an immunodeficient patient. *Virology* **265:** 178-184.

Benenson, A.S. (1995) *Control of communicable diseases manual.* 16th edition. American Public Health Association, Washington D.C., pp. 370.

Blomqvist, S., Savolainen, C., Laine, P., Hirttiö, P., Lamminsalo, E., Pentillä, E., Jöks, S., Roivainen, M., Hovi, T. (2004) Characterisation of a highly evolved vaccine-derived poliovirus type 3 isolated from sewage in Estonia. *Journal of Virology* **78:** 4876-4883.

Blume, S., Geesink, I. (2000) A brief history of polio vaccines. *Science* **288:** 1593-1594.

Bodian, D., Morgan, I.M., Howe, H.A. (1949) Differentiation of types of poliomyelitis viruses. *American Journal of Hygiene* **49:** 234-245.

Buonagurio, D.A., Coleman, J.W., Patibandla, S.A., Prabhakar, B.S., Tatem, J.M. (1999) Direct detection of Sabin poliovirus vaccine strains in stool specimens of first-dose vaccinees by a sensitive reverse transcription-PCR method. *Journal of Clinical Microbiology* **37:** 283-289.

Buttinelli, G., Donati, V., Fiore, S., Marturano, J., Plebani, A., Balestri, P., Soresina, A.R., Vivarelli, R., Delpeyroux, F., Martin, J., Fiore, L. (2003) Nucleotide variation in Sabin type 2 poliovirus from an immunodeficient patient with poliomyelitis. *Journal of General Virology* **84:** 1215-1221.

Caro, V., Guillot, S., Delpeyroux, F., Crainic, R. (2001) Molecular strategy for "serotyping" of human enteroviruses. *Journal of General Virology* **82:** 79-91.

Casas, I., Palacios, G.F., Trallero, G., Cisterna, D., Freire, M.C., Tenorio, A. (2001) Molecular characterisation of human enteroviruses in clinical samples: comparison between VP2, VP1 and RNA polymerase regions using RT nested PCR assays and direct sequencing of products. *Journal of Medical Virology* **65:** 138-148.

Centers for Disease Control and Prevention (2001) Circulation of a type 2 vaccine-derived poliovirus- Egypt, 1982-1993. *Morbidity and Mortality Weekly Report* **50:** 41, 42, 51.

Centers for Disease Control and Prevention (2002a) Progress toward global eradication of poliomyelitis, 2001. *Morbidity and Mortality Weekly Report* **51:** 253-256.

Centers for Disease Control and Prevention (2002b) Global progress toward laboratory containment of wild polioviruses. *Morbidity and Mortality Weekly Report* **51:** 993-996.

Centers for Disease Control and Prevention (2003) Progress toward poliomyelitis eradication- Southern Africa, 2001 – March 2003. *Morbidity and Mortality Weekly Report* **52:** 521-524.

Cherkasova, E.A., Korotkova, E.A., Yakovenko, M.L., Ivanova, O.E., Eremeeva, T.P., Chumakov, K.M., Agol, V.I. (2002) Long-term circulation of vaccine-derived poliovirus that causes paralytic disease. *Journal of Virology* **76:** 6791-6799.

Chezzi, C. (1996) Rapid diagnosis of poliovirus infection by PCR amplification. *Journal of Clinical Microbiology* **34:** 1722-1725.

Chitsike, I., van Furth, R. (1999) Paralytic poliomyelitis associated with live oral poliomyelitis vaccine in a child with HIV infection in Zimbabwe: case report. *British Medical Journal* **318:** 841-843.

Department of Health. (1995) The new childhood immunisation schedule. *Epidemiological Comments* **22:** 39.

Divizia, M., Palombi, L., Buonomo, E., Donia, D., Ruscio, V., Equestre, M., Leno, L., Pana, A., Degener, A.M. (1999) Genomic characterisation of human and environmental

polioviruses isolated in Albania. *Applied and Environmental Microbiology* **65:** 3534-3539.

Donaldson, K.A., Griffin, D.W., Paul, J.H. (2002) Detection, quantitation and identification of enteroviruses from surface waters and sponge tissue from the Florida Keys using real-time RT-PCR. *Water Research* **36:** 2505-2514.

Dove, A. (2001) Paralytic poliomyelitis returns to the Western Hemisphere. *PICO News: Caribbean Outbreak.*

Dowdle, W.R., Birmingham, M.E. (1997) The biologic principles of poliovirus eradication. *The Journal of Infectious Diseases* **175:** S286-S292.

Dowdle, W.R., Featherstone, D.A., Birmingham, M.E., Hull, H.F., Aylward, R.B. (1999) Poliomyelitis eradication. *Virus Research* **62:** 185-192.

Egger, D., Pasamontes, L., Ostermayer, M., Bienz, K. (1995) Reverse transcription multiplex PCR for differentiation between polio- and enteroviruses from clinical and environmental samples. *Journal of Clinical Microbiology* **33:** 1442-1447.

Equestre, M., Genovese, D., Cavalieri, F., Fiore, L., Santoro, R., Bercoff, R.P. (1991) Identification of a consistent pattern of mutations in neurovirulent variants derived from the Sabin vaccine strain of poliovirus type 2. *Journal of Virology* **65:** 2707-2710.

Fiore, L., Genovese, D., Diamanti, E., Catone, S., Ridolfi, B., Ibrahimi, B., Konomi, R., van der Avoort, H.G.A.M., Hovi, T., Crainic, R., Simeoni, P., Amato, C. (1998) Antigenic and molecular characterisation of wild-type 1 poliovirus causing outbreaks of poliomyelitis in Albania and neighbouring countries in 1996. *Journal of Clinical Microbiology* **36:** 1912-1918.

Fleck, F. (2004) WHO warns of a polio epidemic in Africa. *British Medical Journal* **328:** 1513.

Fogarty, J., Thornton, L., Hayes, C., Laffoy, M., O'Flanagan, D., Devlin, J., Corcoran, R. (1995) Illness in a community associated with an episode of water contaminated with

sewage. *Epidemiology and Infection* **114:** 289-295.

Friedrich, F. (1998) Neurologic complications associated with oral poliovirus vaccine and genomic variability of the vaccine strains after multiplication in humans. *Acta Virologica* **42:** 187-195.

Friedrich, F. (2000) Genomic modifications in oral poliovirus vaccine strains after multiplication in humans and implications for the eradication of poliovirus. *Acta Virologica* **44:** 109-117.

Fuchs, R, Blakesley, R. (1983) Guide to the use of type II restriction endonucleases. *Methods of Enzymology* **100:** 3-38.

Georgescu, M.M., Delpeyroux, F., Tardy-Panit, M., Balanant, J., Combiescu, M., Combiescu, A.A., Guillot, S., Crainic, R. (1994) High diversity of poliovirus strains isolated from the central nervous system from patients with vaccine-associated paralytic poliomyelitis. *Journal of Virology* **68:** 8089-8101.

Georgescu, M.M., Balanant, J., Macadam, A., Otelea, D., Combiescu, M., Combiescu, A.A., Crainic, R., Delpeyroux, F. (1997) Evolution of the Sabin type 1 poliovirus in humans: characterization of strains isolated from patients with vaccine-associated paralytic poliomyelitis. *Journal of Virology* **71:** 7758-7768.

Georgopoulou, A., Markoulatos, P., Spyrou, N., Vamvakopoulos, NC. (2000) Improved genotyping vaccine and wild-type poliovirus strains by restriction fragment length polymorphism analysis: clinical diagnostic implications. *Journal of Clinical Microbiology* **38:** 4337-4342.

Ghendon, Y., Robertson, S.E. (1994) Interrupting the transmission of wild polioviruses with vaccines: immunological considerations. *Bulletin of the World Health Organization* **72:** 973-983.

Goldwater, P.N. (1995) Immunoglobulin M capture immunoassay in investigation of coxsackievirus B5 and B6 outbreaks in South Australia. *Journal of Clinical Microbiology* **33:** 1628-1631.

Goyal, S.M., Gerba, C.P., Melnick, J.L. (1979) Human enteroviruses in oysters and their overlying waters. *Applied and Environmental Microbiolology* **37:** 572-581.

Grabow, W.O.K., Botma, K.L., de Villiers, J.C., Clay, C.G., Erasmus, B. (1999) An assessment of cell culture and PCR procedures for the detection of polioviruses in waste water. *Bulletin of the World Health Organization* **77:** 973-978.

Greensfelder, L. (2000) Polio outbreak raises questions about vaccine. *Science* **290:** 1867-1869.

Guillot, S., Caro, V., Cuervo, N., Korotkova, E., Combiescu, M., Persu, A., Aubert-Combiescu, A., Delpeyroux, F., Crainic, R. (2000) Natural genetic exchanges between vaccine and wild poliovirus strains in humans. *Journal of Virology* **74:** 8434-8443.

Haisey, N.A., Pinto, J., Espinosa-Rosaies, F., Faure-Fontenla, M.A., da Silva, E., Khan, A.J., Webster, A.D.B., Minor, P., Dunn, G., Asturias, E., Hussain, H., Pallansch, M.A., Kew, O.M., Winkelstein, J., Sutter, R., the Polio Project Team (2004) Search for poliovirus carriers among people with primary immune deficiency diseases in the United States, Mexico, Brazil and the United Kingdom. *Bulletin of the World Health Organization* **82:** 16-23.

Horie, H., Yoshida, H., Matsuura, K., Miyazawa, M., Wakabayashi, K., Nomoto, A., Hashizume, S. (2002) Isolation of vaccine-derived type 1 polioviruses displaying similar properties to virulent wild strain Mahoney from sewage in Japan. *Journal of Medical Virology* **68:** 445-451.

Hovi, T., Stenvik, M., Partanen, H., Kangas, A. (2001) Poliovirus surveillance by examining sewage specimens. Quantitative recovery of virus after introduction into sewerage at remote upstream location. *Epidemiology of Infection* **127:** 101-106.

Hovi, T., Lindholm, N., Savolainen, C., Stenvik, M., Burns, C. (2004) Evolution of wild-type 1 poliovirus in two healthy siblings excreting the virus over a period of 6 months. *Journal of General Virology* **85:** 369-377.

Hyypiä, T., Auvinen, P., Maaronen, M. (1989) Polymerase chain reaction for human picornaviruses. *Journal of General Virology* **70:** 3261-3268.

Hull, H.F., Birmingham, M.E., Melgaard, B. (1997) Progress toward global polio eradication. *Journal of Infectious Diseases* **175:** S4-S9.

Ion-Neldescu, N., Dobrescu, A., Strebel, P.M., Sutter, R.W. (1994) Vaccine-associated paralytic poliomyelitis and HIV infection. *Lancet* **343:** 51-52.

Jaykus, L.A. (1997) Epidemiology and detection as options for control of viral and parasitic food-borne disease. *Emerging Infectious Diseases* **3:** 529-539.

Kew, O.M., Sutter, R.W., Nottay, B.K., MacDonough, M.J., Prevots, D.R., Quick, L., Pallansch, M.A. (1998) Prolonged replication of a type 1 vaccine-derived poliovirus in an immunodeficient patient. *Journal of Clinical Microbiology* **36:** 2893-2899.

Kew, O.M., Morris-Glasgow, V., Landaverde, M., & 21 other authors. (2002) Outbreak of poliomyelitis in Hispaniola associated with circulating type 1 vaccine-derived poliovirus. *Science* **296:** 356-359.

Kew, O.M., Wright, P.F., Agol, V.I., Delpeyroux, F., Shimizu, H., Nathanson, N., Pallansch, M.A. (2004) Circulating vaccine-derived polioviruses: current state of knowledge. *Bulletin of the World Health Organization* **82:** 16-23.

Khetsuriani, N., Prevots, D.R., Quick, L., Elder, M.E., Pallansch, M., Kew, O., Sutter, R.W. (2003) Persistence of vaccine-derived polioviruses among immunodeficient persons with vaccine-associated paralytic poliomyelitis. *The Journal of Infectious Diseases* **188:** 1845-1852.

Kilpatrick, D.R., Nottay, B., Yang, C.F., Yang, S.J., da Silva, E., Penaranda, S., Pallansch, M., Kew, O.M. (1998) Serotype-specific identification of poliovirus by PCR using primers containing mixed-base or deoxyinosine residues at positions of codon degeneracy. *Journal of Clinical Microbiology* **36:** 352-357.

Korotkova, E.A., Park, R., Cherkasova, E.A., Lipskaya, G.Y., Chumakov, K.M., Feldman, E.V. (2003) Retrospective analysis of a local cessation vaccination against poliomyelitis: a possible scenario for the future. *Journal of Virology* **77:** 12460-12465.

Kuan, M.M. (1997) Detection and rapid differentiation of human enteroviruses following genomic amplification. *Journal of Clinical Microbiology* **35:** 2598-2601.

Lapinleimu, K. (1984) Elimination of poliomyelitis in Finland. *Reviews of Infectious Diseases* **6:** S457-S460.

Li, J., Zhang, L.B., Yoneyama, T., Yoshida, H., Shimizu, H., Yoshii, K., Hara, M., Nomura, T., Yoshikura, H., Miyamura, T., Hagiwara, A. (1996) Genetic basis of the neurovirulence of type 1 polioviruses isolated from vaccine-associated paralytic patients. *Archives of Virology* **141:** 1047-1054.

Lipskaya, G.Y., Muzychenko, A.R., Kutitova, O.K., Maslova, S.V., Equestre, M., Drozdov, S.G., Bercoff, R.P., Agol, V.I. (1991) Frequent isolation of intertypic poliovirus recombinants with serotype 2 specificity from vaccine-associated polio cases. *Journal of Medical Virology* **35:** 290-296.

Macadam, A.J., Pollard, S.R., Ferguson, G., Skuce, R., Wood, D., Almond, J.W., Minor, P.D. (1993) Genetic basis of attenuation of the Sabin type 2 vaccine strain of poliovirus in primates. *Virology* **192:** 18-26.

MacLennan, C.A., Arnoud, G.D., Huissoon, P., Kumararatne, D.S., Martin, J., O'Leary, P., Thompson, R.A., Osman, H., Wood, P., Minor, P., Wood, D.J., Pillay, D. (2004) Failure to clear persistent vaccine-derived neurovirulent poliovirus infection in an immunodeficient man. *Lancet* **363:** 1509-1513.

Manor, Y., Handsher, R., Halmut, T., Neuman, M., Abramovitz, B., Mates, A., Mendelson, E. (1999a) A double-selective tissue culture system for isolation of wild-type poliovirus from sewage applied in a long term environmental surveillance. *Applied and Environmental Microbiology* **65:** 1794-1797.

Manor, Y., Handsher, R., Halmut, T., Neuman, M., Bobrov, A., Rudich, H., Vonsover, A., Shulman, L., Kew, O.M., Mendelson, E. (1999b) Detection of poliovirus circulation by environmental surveillance in the absence of clinical cases in Israel and the Palestinian Authority. *Journal of Clinical Microbiology* **37:** 1670-1675.

Marker Test Subcommittee, the Japan Live Poliovaccine Research Commission. (1967) Evaluation of Sabin live poliovirus vaccine in Japan, IV- Marker tests on poliovirus strains recovered from vaccinees and their contacts. *Japanese Journal of Medical Sciences and Biology* **20:** 167-173.

Martin, J., Ferguson, G.L., Wood, D.J., Minor, P.D. (2000) The vaccine origin of the 1968 epidemic of type 3 poliomyelitis in Poland. *Virology* **278:** 42-49.

Martin, J., Minor, P.D. (2002) Characterisation of CHAT and Cox type 1 live-attenuated poliovirus vaccine strains. *Journal of Virology* **76:** 5339-5349.

Melnick, J.L. (1996a) Current status of poliovirus infections. *Clinical Microbiology Review* **9:** 293-300.

Melnick, J.L. (1996b) Enteroviruses: polioviruses, coxsackieviruses, echoviruses and newer enteroviruses. In: Fields BN, Knipe DM, Howley PM, Chanock RM, Melnick JL, Monath TP, Roizman B, Straus SE, editors. Fields Virology, 3rd ed. Lippincott-Raven, Philadelphia, PA, pp: 655-712.

Metcalf, T.G., Melnick, J.L., Estes, M.K. (1995) Environmental virology: from detection of virus in sewage and water by isolation to identification by molecular biology - a trip of over 50 years. *Annual Reviews of Microbiology* **49:** 461-487.

Minor, P.D. (1992) The molecular biology of poliovaccines. *Journal of General Virology* **73:** 3065-3077.

Minor, P.D. (1999) Poliovirus vaccination: current understanding of poliovirus interactions in humans and implications for the eradication of poliomyelitis. *Expert Reviews in Molecular Medicine.* http://www-ermm.cbcu.cam.ac.uk

Minor, P.D. (2001) Characteristics of poliovirus strains from long-term excretors with primary immunodeficiencies. *Developments Biologicals (Basel)* **105:** 75-80.

Muir, P., Kämmerer, U., Korn, K., Mulders, M.N., Pöyry, T., Weissbrich, B., Kandolf, R., Cleator, G.M., van Loon, A.M. (1998) Molecular typing of enteroviruses: current status and future requirements. *Clinical Microbiology Reviews* **11:** 202-227.

Mulders, M.N., Reimerink, J.H.J., Stenvik, M., Alaeddinoglu, I., van der Avoort, H.G.A.M., Hovi, T., Koopmans, M.P.G. (1999) A Sabin vaccine-derived field isolate of poliovirus type 1 displaying aberrant phenotypic and genetic features, including a deletion in antigenic site 1. *Journal of General Virology* **80:** 907-916.

Muscillo, M., La Rosa, G., Carducci, A., Cantiani, L., Marianelli, C. (1999) Molecular and biological characterisation of poliovirus 3 strains isolated in Adriatic seawater samples. *Water Research* **33:** 3204-3212.

Nibbeling, R., Reimerink, J.H.J., Agboatwala, M., Naquib, T., Ras, A., Poelstra, P., van der Avoort, H.G.A.M., van Loon, A.M. (1994) A poliovirus type-specific IgM antibody-capture enzyme-linked immunosorbent assay for the rapid diagnosis of poliomyelitis. *Clinical and Diagnostic Virology* **2:** 113-126.

Oberste, M.S., Maher, K., Kilpatrick, D.R., Flemister, M.R., Brown, B.A., Pallansh, M.A. (1999) Typing of human enteroviruses by partial sequencing of VP1. *Journal of Clinical Microbiology* **37:** 1288-1293.

Oostvogel, P.M., van Wijngaarden, J.K., van der Avoort, H.G., Mulders, M.N., Conyn-van Spaendonck, M.A., Rumke, H.C., van Steenis, G., van Loon, A.M. (1994) Poliomyelitis

outbreak in an unvaccinated community in The Netherlands, 1992-1993. *Lancet* **344:** 665-670.

Pelczar, M.J.Jr., Chan, E.C.S., Krieg, N.R. (1993) Microbiology: Concepts and Applications. McGraw-Hill, Inc. pp. 698.

ProMED-mail (2004a) Poliomyelitis- Africa: Guinea, Mali. Source: *WHO Press Release*, Tue 24 Apr 2004 [edited]. From: ProMED-mail promed@promedmail.org

ProMED-mail (2004b) Poliomyelitis- Nigeria. Source: *Yahoo News Online*, Sat 20 Mar 2004 [edited]. From: ProMED-mail promed@promedmail.org

ProMED-mail (2004c) Poliomyelitis- Botswana ex Nigeria. Source: *Boston Globe Online*, Wed 14 Apr 2004 [edited]. From: ProMED-mail promed@promedmail.org

Rotbart, H.A. (1997) Enteroviruses. In: *Clinical Virology.* Ed by Richman, D.D., Whitley, R.J., Hayden, F.G. Churchill Livingstone Inc, New York, USA, pp.997-1008.

Rousset, D., Rakoto-Andrianarivelo, M., Razafindratsimandresy, R., Randriamanalina, B., Guillot, S., Balanant, J., Mauclère, P., Delpeyroux, F. (2003) Recombinant vaccine-derived poliovirus in Madagascar. *Emerging Infectious Diseases* **9:** 885-887.

Rueckert, R.R., Wimmer, E. (1984) Systematic nomenclature of picornavirus proteins. *Journal of Virology* **50:** 957-959.

Ryder, R.W., Oxtoby, M.J., Mvula, M., Batter, V., Baende, E., Nsa, W., Davachi, F., Hassig, S., Onorato, I., Deforest, A. (1993) Safety and immunogenicity of bacille Calmette-Guerin, diphtheria-tetanus-pertussis, and oral polio vaccines in newborn children in Zaire infected with human immunodeficiency virus type 1. *Journal of Paediatrics* **122:** 697-702.

Sabin, A.B., Boulger, L.R. (1973) History of Sabin attenuated poliovirus oral live vaccines. *Journal of Biological Standards* **1:** 115-118.

Sever, J.L. (2001) Rotary International: a partner in polio eradication. *Developments Biologicals* **105:** 105-108.

Severini, G.M., Mestroni, L., Falaschi, A., Camerini, F., Giacca, M. (1993) Nested polymerase chain reaction for high-sensitivity detection of enteroviral RNA in biological samples. *Journal of Clinical Microbiology* **31:** 1345-1349.

Shulman, L.M., Manor, J., Handsher, R., Delpeyroux, F., MacDonough, M.J., Halmut, T., Silberstein, I., Alfandari, J., Quay, J., Fisher, T., Robinov, J., Kew, O.M., Crainic, R., Mendelson, E. (2000) Molecular and antigenic characterisation of a highly evolved derivative of the type 2 oral poliovaccine strain isolated from sewage in Israel. *Journal of Clinical Microbiology* **38:** 3729-3734.

Sutter, R.W., Prevots, D.R. (1994) Vaccine-associated paralytic poliomyelitis among immunodeficient persons. *Infection and Medicine* **11:** 426-438.

Tatem, J.M., Weeks-Levy, C., Georgiu, A., DiMichele, S.J., Gorgacz, E.J., Racaniello, V.R., Cano, F.R., Mento, S.J. (1992) A mutation present in the amino terminus of Sabin 3 poliovirus VP1 protein is attenuating. *Journal of Virology* **66:** 3194-3197.

Triki, H., Barbouche, M.R., Bahri, O., Bejaoui, M., Dellagi, K. (2003) Community-acquired poliovirus infection in children with primary immunodeficiencies in Tunisia. *Journal of Clinical Microbiology* **41:** 1203-1211.

Van der Avoort, H.G.A.M., Hull, B.P., Hovi, T., Pallansch, M.A., Kew, O.M., Crainic, R., Wood, D.J., Mulders, M.N., van Loon, A.M. (1995) Comparative study of five methods for intratypic differentiation of polioviruses. *Journal of Clinical Microbiology* **33:** 2562-2566.

Verlinden, Y., Cuconati, A., Wimmer, E., Rombaut, B. (2002) The viral protein 3CD induces an equilibrium between the viral protein and RNA synthesis in a cell-free system for poliovirus replication. *Archives of Virology* **147:** 731-744.

Wood, D.J., Hull, B. (1999) L20B cells simplify culture of polioviruses from clinical samples. *Journal of Medical Virology* **58:** 188-192.

Wood, D.J., Sutter, R.W., Dowdle, W.R. (2000) Stopping poliovirus vaccination after eradication: issues and challenges. *Bulletin of the World Health Organization* **78:** 347-357.

Wood, N., Thorley, B. (2003) Viewpoint Towards global poliomyelitis eradication: The successes and challenges for a developed country. *Journal of Paediatrics and Child Health* **39:** 647-650.

World Health Assembly (1988) Global eradication of poliomyelitis by the year 2000. Resolution of the 41st World Health Assembly. Geneva, Switzerland: World Health Organization (resolution no. 41.28).

World Health Organization (1998) Report of the meeting on the scientific basis for stopping polio immunisation, Geneva, 23-25 March 1998. Geneva, Switzerland: World Health Organization (WHO/EPI/GEN/98.12).

World Health Organization (1999) WHO global action plan for laboratory containment of wild polioviruses. Geneva, Switzerland: World Health Organization (WHO/V&B/99.32).

World Health Organization (2000) Outbreak news. *Weekly Epidemiological Record* **75:** 397-399.

World Health Organization (2001a) Transmission of wild poliovirus type 2 – apparent global interruption. *Weekly Epidemiological Record* **76:** 95-97.

World Health Organization (2001b) Network strategy for detecting cVDPV is defined. *Polio Laboratory Network Quarterly Update* **7:** 1-2.

World Health Organization (2002) Manual for the virological investigation of poliomyelitis. Geneva, Switzerland: World Health Organization (WHO/EPI/GEN/02.1).

World Health Organization (2003a) Progress towards the global eradication of poliomyelitis, 2002. *Weekly Epidemiological Record* **78:** 138-144.

World Health Organization (2003b) OPV virus circulation and evolution investigated in New Zealand. *Polio Laboratory Network Quarterly Update* **9:** 2-3.

World Health Organization (2003c) Poliovirus surveillance report, January - March 2003. *Polio Laboratory Network Quarterly Update* **9:** 4.

World Health Organization (2004) Manual for the virological investigation of poliomyelitis. Geneva, Switzerland: World Health Organization (WHO/EPI/GEN/04).

Yang, C.-F., De, L., Holloway, B.P., Pallansch, M.A., Kew, O.M. (1991) Detection and identification of vaccine-related polioviruses by the polymerase chain reaction. *Virus Research* **20:** 159-179.

Yang, C.F., De, L., Yang, S.J., Gòmez, J.R., Cruz, J.R., Holloway, B.P., Pallansch, M.A., Kew, O.M. (1992) Genotype-specific in vitro amplification of sequences of the wild type 3 polioviruses from Mexico and Guatemala. *Virus Research* **24:** 277-296.

Yang, C.-F., Naguib, T., Yang, S.-J., Nasr, E., Jorba, J., Ahmed, N., Campagnoli, R., van der Avoort, H., Shimizu, H., Yoneyama, T., Miyamura, T., Pallansch, M., Kew, O. (2003) Circulation of endemic type 2 vaccine-derived poliovirus in Egypt from 1983-1993. *Journal of Virology* **77:** 8366-8377.

Yoneyama, T., Hagiwara, A., Hara, M., Shimojo, H. (1982) Alteration in oligonucleotide fingerprint pattern of the viral genome in poliovirus type 2 isolated from paralytic patients. *Infection and Immunology* **37:** 46-53.

Yoshida, H., Horie, H., Matsuura, K., Kitamura, T., Hashizume, S., Miyamura, T. (2002) Prevalence of vaccine-derived polioviruses in the environment. *Journal of General Virology* **83:** 1107-1111.

Zaoutis, T., Klein, J.D. (1998) Enterovirus infections. *Pediatrics in Review* **19:** 183-191.

Zhang, L., Li, J., Hou, X., Zheng, D. (1998) Analysis of the characteristics of polioviruses isolated from AFP cases in China. *Chinese Journal of Vaccination and Immunology* **4:** 247-254.

Zoll, G.J., Melchers, W.J.G., Kopecka, H., Jambroes, G., van der Poel, H.J.A., Galama, J.M.D. (1992) General primer-mediated polymerase chain reaction for detection of enteroviruses: application for diagnostic routine and persistent infections. *Journal of Clinical Microbiology* **30:** 160-165.