DYNAMICS OF GENOMIC VARIATION IN POLIOVIRUS IN AFRICA

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DECLARATION

I, Claudia Chezzi, declare that this thesis is my own unaided work. It is being submitted for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg, South Africa. It has not been submitted before for any degree or examination at this or any other University.

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25 day of May, 1998

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LIST OF ABBREVIATIONS USED IN THE TEXT

1. STANDARD ABBREVIATIONS:

AFP	Acute flaccid paralysis
ATP	Adenosine triphosphate
bp	Base pair
cDNA	Complementary DNA
CNS	Central nervous system
CSF	Cerebrospinal fluid
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
ds DNA	Double stranded DNA
ELISA	Enzyme-linked immunosorbent assay
eIPV	Enhanced potency IPV
EPI	Expanded Programme on Immunisation
FCS	Foetal calf serum
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HMA	Heteroduplex mobility assay
IPV	Inactivated polio vaccine
IRES	Internal ribosome entry site
kD	Kilodalton
Ig	Immunoglobulin
IgA	Immunoglobulin A
IPV	Inactivated polio vaccine
IRES	Internal ribosome entry site
L (l)	Litre
M	Molar
ml	Millilitre
mm	Millimetre
mM	Millimolar
mRNA	Messenger RNA
NCR	Non coding region
NID	National Immunization Day
NIV	National Institute for Virology
nm	Nanometre

nt	Nucleotide
OPV	Oral polio vaccine
ORF	Open reading frame
P1; P2; P3	Poliovirus type 1; poliovirus type 2; poliovirus type 3
PCR	Polymerase chain reaction
PEI	Poliomyelitis Eradication Initiative
PFU	Plaque forming unit
ppm	Parts per million
PVR	Poliovirus receptor
RFLP	Restriction fragment polymorphism assay
RI	Replicative intermediate
RNA	Ribonucleic acid
RNase	Ribonuclease
RT	Reverse transcription
RT-PCR	Reverse transcription-polymerase chain reaction
S1; S2; S3	Sabin 1; Sabin 2; Sabin 3
ss DNA	Single stranded DNA
TCID	Tissue culture infective dose
TOPV	Trivalent OPV
μ	Micro
UV	Ultraviolet
VAPP	Vaccine associated paralytic poliomyelitis
VK	Vervet kidney
WHO	World Health Organization

2. COUNTRY ABBREVIATIONS:

ANG	Angola
BFA	Burkina Faso
CAE	Cameroun
CAF / CAR	Central African Republic
CIV / IVC	Cote D'Ivoire
D.R.Congo	Democratic Republic of Congo (former Zaire)
EGY	Egypt
ETH	Ethiopia
GAM	Gambia
GHA	Ghana
IND	India
ISR	Israel
JOR	Jordan
KEN	Kenya
KUW	Kuwait
LIB	Liberia
NAM	Namibia
NIE	Nigeria
NIG	Niger
PAK	Pakistan
SEN	Senegal
SOA	South Africa
SUD	Sudan
TAN	Tanzania
TOG	Togo
UGA	Uganda
ZAI	Democratic Republic of Congo (former Zaire)
ZAM	Zambia
ZIM	Zimbabwe

1. INTRODUCTION

In May 1988, the 41st World Health Assembly of the World Health Organization (WHO) accepted a resolution for the worldwide eradication of poliomyelitis and its causative agent, poliovirus, by the year 2000 (WHO, 1988). Specific operational strategies have been devised for the achievement of this goal, as follows: (1) reaching and maintaining the highest possible routine immunisation coverage (over 80%) with at least 3 doses of oral polio vaccine (OPV); (2) annual National Immunisation Days (NID's) to deliver 2 supplemental doses of OPV to all children less than 5 years of age in countries and regions where polio is still endemic or where there is a risk of re-introduction from other areas; (3) laboratory-based surveillance to detect and investigate every case of acute flaccid paralysis (AFP) in children less than 15 years of age, and all cases of suspected poliomyelitis of any age; and (4) house-to-house "mopping up" immunisation campaigns to deliver OPV to children in areas where poliovirus transmission persists.

Laboratory-based surveillance is a critical component of the WHO's strategy for global polio eradication. The underlying objective of wild poliovirus surveillance is the development and implementation of effective strategies for poliomyelitis control, and in this context, the most important surveillance questions centre on (1) the identification of the local, regional and global reservoirs sustaining poliovirus circulation; (2) the identification of links between poliomyelitis cases; and (3) the identification of local, regional and global pathways of poliovirus transmission. Because 99% or more of poliovirus infections are subclinical, answers to these questions can often only be obtained by analysis of the poliovirus strains associated with cases and outbreaks. Techniques for wild-type poliovirus strain characterisation have, until recently, been based on the antigenic properties of the viruses and have been serological in nature (Nakano *et al.*, 1978; van Wezel and Hazendonk, 1979; Humphrey *et al.*, Minor *et al.*, 1982; Crainic *et al.*, 1983; Osterhaus *et al.*, 1983). However, the information obtained using serological techniques may be limited due to the limited antigenic variability between poliovirus strains. Polioviruses, being RNA viruses, mutate rapidly at a fixed rate during replication in humans (Nottay *et al.*, 1981), and thus the potential resolving power of molecular epidemiological studies based on genomic, rather than antigenic, characteristics of the viruses is very high. Of the molecular techniques available for genomic characterisation of polioviruses (discussed in Chapter 3), sequence analysis is by far the most powerful (Rico-Hesse *et al.*, 1987), and comparison of sequence data from different poliovirus strains can provide epidemiological information that can be of considerable programmatic value.

Although remarkable progress in the control of poliomyelitis worldwide has been made since the implementation of the WHO control strategies (Hull *et al.,* 1994), elimination of poliomyelitis from Africa remains one of the major challenges to achieving global eradication (WHO, 1997b). Vaccination coverages in Africa are among the lowest in the world (WHO, 1998c); lack of infrastructure and political instability in many African countries make the delivery and administration of vaccine, both for routine purposes and during NID's, very difficult. Extreme poverty and poor sanitation in many countries, and large movements of refugees escaping war-torn regions provide the ideal conditions for both continuous endemic wild-type poliovirus transmission, and the introduction of wild-type viruses into polio-free areas. If the goal of elimination of poliomyelitis from Africa is to be achieved, the regions of continued endemic poliovirus circulation need to be identified, and the patterns of transmission of wild-type viruses determined, so that improved strategies for the interruption of transmission can be designed and implemented.

One of the original aims of this study was to identify the molecular epidemiological characteristics of wild-type polioviruses associated with cases and outbreaks in South Africa. However, the extent of genomic diversity of polioviruses circulating not only in South Africa, but throughout southern and sub-Saharan Africa, was unknown. Increased surveillance during the past few years, the occurrence of outbreaks in Namibia in 1993 (Van Niekerk *et al.,* 1994) and the former Zaire in 1995 (Lambert *et al.,* 1995). and the designation of the National Institute for Virology (NIV) in Johannesburg as a WHO Regional Reference Centre for Poliomyelitis resulted in the availability of poliovirus isolates from many African countries. This provided an ideal opportunity to investigate the molecular epidemiology of wild-type polioviruses circulating not only in South Africa, but throughout sub-Saharan Africa, with the view to identifying areas of endemic circulation and patterns of transmission throughout the continent. In addition, characterisation of the viruses circulating in Africa could permit the design of reagents for the rapid and sensitive detection of wild-type viruses in clinical and possibly environmental specimens.

Routine and mass vaccination with OPV is also a major component of the WHO's eradication campaign. OPV, despite its excellent safety record, has the potential for reversion to neurovirulence, and may cause paralysis in a very small proportion of vaccinees (Joce *et al.,* 1992; CDC 1997a). OPV is produced in cultures of monkey kidney cells, the availability of which is strictly dependent on a regular supply of healthy monkeys. Because of problems with regular supplies of monkeys, and the increasing presence adventitious monkey viruses in monkey tissue, vaccine manufacturers have recently switched to continuous cell lines such as Vero cells for the production of OPV (Montagnon, 1989). Whether the genetic stability of OPV produced on the Vero cell substrates was in

any way altered during passage in cell culture and in the intestinal tract of vaccinees had not yet been determined; differences in the stability of the vaccines might have direct bearing on the safety of the vaccine and on the number of cases of vaccine-associated paralysis cases occurring both in South Africa as well as in other African countries where Vero-cell produced OPV is used for routine and mass immunisations. Thus a second component of this study was to compare the genetic stability of OPV produced on primary monkey kidney or Vero cell substrates, when passaged in cell culture and the gastrointestinal tract of vaccinees, with respect to mutations at the sites considered most important for attenuation.

The specific objectives of this study are thus:

(1) To characterise, at the genomic level using partial sequence analysis, the polioviruses associated with poliomyelitis outbreaks in South Africa between 1980 and 1989, and isolated during the pre- and post-epidemic years.

(2) To characterise, also using partial sequence analysis, recent wild-type polioviruses responsible for cases and outbreaks of poliomyelitis in sub-Saharan Africa, with the view to identifying reservoirs of endemic wild-type circulation, patterns of transmission, and epidemiological links between cases.

(3) To develop sensitive and rapid techniques and reagents for intratypic differentiation and identification of wild-type polioviruses circulating in sub-Saharan Africa.

(4) To compare the excretion rates and genetic stability of OPV produced on two different cell substrates, primary monkey kidney or Vero cells, when passaged in cell culture and the gastrointestinal tract of vaccinees, with respect to mutations at the nucleotide positions considered most important for attenuation.

2. LITERATURE REVIEW

Poliovirus is taxonomically classified as a member of the family Picornaviridae, and subfamilial genus Enterovirus (Rueckert, 1996). Picornaviruses are among the smallest RNA viruses known, and their genetic material is a single strand of RNA contained within a small capsid (pico = small; rna = ribonucleic acid). Three serotypes of poliovirus exist, termed type 1(PV1), type 2 (PV2) and type 3 (PV3). Polioviruses are the causative agents of poliomyelitis, a severe paralytic affliction of the central nervous system (polio = grey; myelos = marrow, spinal cord; Koch and Koch, 1985). The only natural hosts of polioviruses are humans and monkeys. Polioviruses are transmitted primarily through the faecal-oral route, and until the beginning of this century, poliomyelitis was primarily a disease of infants (hence the German name "kinderlahmung" = infantile paralysis; Koch and Koch, 1985). This pattern is still seen today in communities with substandard sanitation, where the disease is endemic. Prior to widespread immunisation, improvement in sanitation was accompanied by an increasing prominence of poliomyelitis epidemics, concurrent with a drift in the age distribution of the disease to include older persons, in whom an increase in disease severity was observed. Since the introduction and wide scale application of inactivated vaccines in the early 1950's, followed soon afterwards by that of attenuated live vaccines, the incidence of poliomyelitis has declined drastically throughout the world. In 1988, spurred by the success of the smallpox eradication campaign, the WHO set a goal for the global eradication of poliomyelitis by the year 2000 (WHO, 1988).

2.1 History

The earliest documented record of poliomyelitis can be found on an Egyptian stele from the 18th dynasty (1580 - 1350 BC), which shows a young man with a withered leg characteristic of paralytic poliomyelitis (Fanconi *et al.*, 1945). Characteristic descriptions and examples of poliomyelitis-like disease can be found in ancient literature and archeological specimens, suggesting that occasional cases of poliomyelitis have occurred throughout the history of man (Koch and Koch, 1985); Hippocrates described paralysis that afflicted patients primarily in summer and autumn, the seasons most commonly associated with an increased incidence of poliomyelitis (Armstrong, 1950); biblical reports of persons with paralysed or crippled extremities may also reflect affliction by poliomyelitis; 15th century skeletons, excavated in southern Greenland, showed bone deformities reminiscent of those typically associated with severe poliomyelitis. It was not until the 19th century, however, that, as a result of severe epidemics in Europe and North America, poliomyelitis became recognised

as a distinct clinical entity (Rissler, 1888). Research on this devastating but relatively rare disease progressed relatively slowly until the early 20th century - in 1909, the viral aetiology of poliomyelitis was established by Landsteiner and Popper (1909), who successfully transmitted the disease to monkeys by intracerebral inoculation of spinal cord filtrate obtained from a child who had suffered from poliomyelitis; in 1910 Flexner and Lewis (1910) passed the viral agent from monkey to monkey. The agent for poliomyelitis did not come to be called poliovirus, however, until the 1950's (von Magnus *et al.,* 1955). In 1916, the worst polio epidemic known in history spread throughout the USA, afflicting more than 27 000 people in New York city alone. This epidemic gave great impetus to polio research, as did the contracting of the disease by Franklin D. Roosevelt, elected to American presidency in 1932; in 1938, a private research and welfare programme, the National Foundation for Infantile Paralysis, was founded in his name (Koch and Koch, 1985). During the 1930's, Paul and Trask demonstrated that virus could be recovered repeatedly over a period of several weeks from the faeces of both patients and healthy carriers, and the concept of poliomyelitis as an enteric infection became established (Melnick, 1996). A landmark in polio research occurred in 1949 when Enders, Weller and Robbins (1949) demonstrated that poliovirus could be isolated and readily propagated in cells of non-neuronal human or monkey tissue - this discovery earned them the Nobel prize in 1954. Within 3 years, a formalin-inactivated vaccine was developed by Salk (1954), and large vaccination programmes with inactivated virus were launched in many countries. The difficulties of producing sufficient quantities of safe and potent vaccines led to the development of tissue culture-prepared live attenuated vaccines by Sabin in the 1950's (Sabin, 1955). Such vaccines began to be administered on a large scale in 1959 in the former Soviet Union (Chumakov *et al.,* 1961).

The successful cultivation of poliovirus in tissue culture also paved the way for detailed studies on the molecular biology of polioviruses. Other crucial advances were the development of a plaque assay for infectivity (Dulbecco and Vogt, 1954), and the development of methods for purification and crystallisation of poliovirus (Schaffer and Schwerdt, 1955), thus opening the way for structural analysis by X-ray crystallography (Hogle *et al.,* 1985). The determination of the nutritional requirements of cultured cells (Eagle, 1955) provided defined nutrient media so that the poliovirus proteins and replication cycle could be studied (Darnell, 1958; Darnell and Levintow, 1960; Darnell et al., 1961). The finding that isolated polioviral RNA was infectious (Alexander *et al.,*1958) facilitated demonstration that susceptibility of cells correlates with the presence of specific receptors (Holland and McLaren, 1959; Darnell and Sawyer, 1960). Important advances with implications for molecular biology as a whole included the characterisation of replicative form of dsRNA (Montagnier and Sanders, 1963) and replicative intermediate (RI) (Girard,

1969), the demonstration of the first RNA-dependent RNA polymerase (Baltimore and Franklin, 1963) and the description of the kinetics of RNA synthesis (Baltimore, 1968), and the discovery that poliovirus synthesises its gene products by proteolytic cleavage of a single polyprotein precursor (Summers and Maizel, 1968).

Recently, refinements in biochemical and immunological methods, and the development of recombinant DNA technology, have led to the elucidation of the entire nucleotide sequence of the poliovirus genome (Kitamura *et al.*, 1981; Racaniello and Baltimore, 1981; Stanway *et al.*, 1984; Toyoda *et al.*, 1984), the discovery of the genome-associated protein VPg (Wimmer, 1979), and the elucidation of important principles of virus structure and assembly (Rueckert, 1976). The adaptation of molecular techniques such as oligonucleotide fingerprinting (Nottay *et al.*, 1981; Kew and Nottay, 1984a) and RNA sequencing to study poliovirus genetics (Rico-Hesse *et al.*, 1987), and the development of monoclonal antibodies against several polioviral proteins (Icenogle *et al.*, 1981; Emini *et al.*, 1982; Minor *et al.*, 1982), has led to the elucidation of the antigenic structure of polioviruses (Minor *et al.*, 1986a), and has enabled monitoring of the antigenic and genetic stability of the viruses. The genetic basis for the attenuation of the poliovirus vaccines has been elucidated (reviewed in Minor *et al.*, 1993), and molecular techniques have been developed to replace the monkey neurovirulence tests for assessing the safety of attenuated polio vaccines (Chumakov et al., 1991). The discovery of the human cellular receptor for polioviruses (Mendelsohn *et al.*, 1989) has led to the development of transgenic mice (Ren *et al.*, 1990; Koike *et al.*, 1994) to replace monkeys as animal models for studies of infectivity and neurovirulence.

2.2 Virus structure and antigenicity

The poliovirus virion is roughly spherical, with no lipid envelope. It contains a positive sense single-stranded RNA core of approximately 7500 nucleotides that is polyadenylated at its 3' end and linked at its 5' end to a viral polypeptide, VPg. The RNA is tightly packed within the central cavity of a thin protein shell. Some of the physical properties of the virion are listed in Table 2.1. Electron micrographs suggest that the diameter of the particle ranges between 24 and 30 nm - the wide range in size is due to the flattening of particles or variable penetration of heavy metal stains during the drying and staining procedures required for preparation of samples for electron microscopy (Rueckert, 1996). Methods which measure the diameter of wet particles, such as sedimentation equilibrium and small angle x-ray scattering and x-ray diffraction analysis indicate diameters in the range of 29.8 to 30.7 nm (Rueckert, 1996). Polioviruses are insensitive to ether, deoxycholate, and

various detergents that destroy other viruses (Melnick, 1996). Treatment with 0.3% formaldehyde, 0.1 N HCl, or free residual chlorine at a level of 0.3-0.5 ppm causes rapid inactivation, but the presence of extraneous organic matter protects the virus from inactivation (Trask *et al.*, 1945). Polioviruses are thermolabile, and exposure at 50 °C rapidly destroys the virus. However, in the presence of molar magnesium chloride, only partial inactivation occurs after 1 hour at 50 °C (Wallis and Melnick, 1961). Polioviruses are stable at freezing temperatures for many years, remain viable for weeks at 4 - 8 °C, and for days at room temperature. Their inactivation at all environmental temperatures is inhibited by magnesium chloride, and this property has led to the widespread use of $MgCl_2$ as a stabilizer of oral polio vaccines (OPV's) (Melnick *et al.*, 1961). Polioviruses are rapidly inactivated by ultraviolet light and usually by drying (Le Bouvier, 1955); dyes such as neutral red, acridine orange and proflavine, when incorporated into the viral structure, render the viruses readily susceptible to visible light (Wallis and Melnick, 1965).

Table 2.1 [a] Physical properties of the poliovirion

Diameter (hydrated)		About 30.5 nm
Symmetry		5:3:2: (icosahedral)
Capsomers (EM; 32; 42 or 60)		Indistinct
Sedimentation coefficient $_{20,w}$		156S
D $_{20,w}$		1.40 X 10^{-7} cm^2/sec
Partial specific volume (v)		0.685 ml/g
Virion mass		8.43 X 10^6
% RNA (as K-salt)		31.6
% Protein		68.4
Virions/mg		7.07 X10^{13}
Virions/OD_{260}-unit		9.4 X 10^{12}
pH stability		3 - 8.5
Stable		To lipid solvents
		1%SDS, EDTA at pH 7
		4M Urea
		Up to 45 °C in isotonic salt
		Up to 56 °C in hypertonic salt, 1 M $MgCl_2$
Copies per particle	VP0	1 - 2
	VP1	60
	VP2	58 - 59
	VP3	60
	VP4	58 - 59
	VPg-RNA	1
Ions	K^+	4900
	Na^+	900
	Mg^{2+}	110
Polycations		54
Lipid		Sphingosine?
Carbohydrate		Not detectable

[a] compiled from Mirzayan and Wimmer (1994), and Rueckert, (1996).

Poliovirus has icosahedral symmetry and consists of 60 identical asymmetrical protomers arranged around fivefold, threefold and twofold axes (Rueckert, 1996). Each protomer is composed of a single copy of each of the 3 capsid proteins VP1, VP2 and VP3. The smallest capsid protein, VP4, is located on the inner surface of the virion and may be considered an extension of VP2 (Hogle *et al.,* 1985). Of the 4 proteins, VP1 exhibits the greatest sequence variability, and VP4 the least. VP1 is also the dominant protein, playing key roles in surface topography and in several viral functions, including antigenicity and receptor attachment (Rueckert, 1996). Although VP1, VP2 and VP3 differ in size and amino acid sequence, they have similar tertiary structures (Hogle *et al.,* 1985). Each capsid protein presents a common structural motif, an 8-stranded antiparallel β-barrel core (Figure 2.1). The capsids differ in their N– and C- terminal extensions, and in the size and structure of the loops that connect the outer strands of the β-barrels. The loop extensions protrude from the surface of the virion where they may become well exposed and thus represent the major antigenic sites of the virus. The folding of the β-strands gives the barrel the shape of a triangular wedge where the thin end of the VP1 wedge is directed toward the fivefold axis, and the equivalent ends of the VP2 and VP3 alternate around the threefold axis. The N-terminal extensions of the capsid proteins (and VP4) form an intertwined network of connections in the interior of the capsid shell which contributes largely to its stability (Hogle *et al.,* 1985).

As a result of the C-terminal extensions and surface loops, the exterior of the virion is marked by protrusions, 'broad plateaus' and 'deep crevices' (Hogle *et al.,* 1985). One notable surface feature is a depression or 'canyon' formed at the junction of VP1 and VP3, encircling the fivefold axis (Figure 2.1 B), which is thought to be the receptor binding site on the virion, at least certainly for human rhinovirus 14 and by analogy for polioviruses (Rossmann *et al.,* 1985). A number of hydrophobic drugs known as 'WIN compounds', a third generation of neutralising antivirals derived first from rhodamine (Eggers, 1977) and then from arildone (McSharry *et al.,* 1979) have been reported to inhibit attachment of picornaviruses to the cellular receptor (Andries *et al.,* 1988, 1992; Pevear *et al.,* 1989), or uncoating (Fox *et al.,* 1986; McSharry *et al.,* 1979; Mosser and Rueckert, 1993), the effect depending on the type of virus. These compounds insert into a hydrophobic pocket which lies just beneath the floor of the canyon (Zhang *et al.,* 1992). Drug binding induces a conformational change in this pocket, which inhibits virus binding to the cellular receptor.

Crystallographic analysis of poliovirus has shown the presence in the drug-binding pocket of types 1 and 3 of a long sphingosine-like molecule, and it has been suggested that the neutralising antiviral compounds described above are actually analogs of viral pocket

molecules involved in assembly or uncoating of the virus (Hogle *et al.*, 1987).

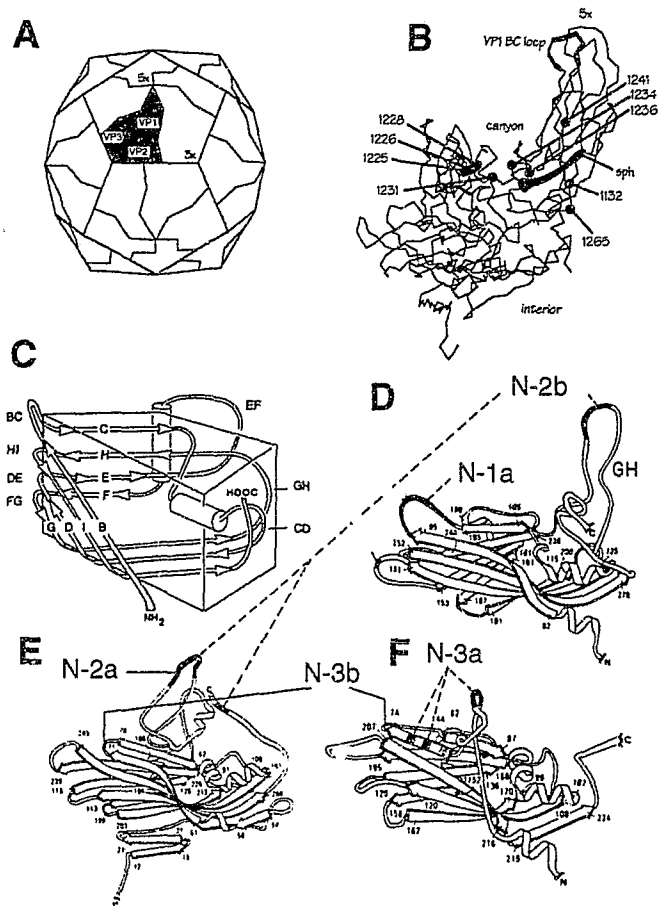

Figure 2.1 (A) Schematic representation of the icosahedral capsid structure of poliovirus, and (B) line drawing of the VP1 and VP2 proteins in their tertiary configuration, illustrating the canyon structure, with its sphingosine (sph) hydrocarbon-binding pocket into which the cellular receptor for poliovirus fits (reproduced from Rotbart, 1997). (C) depicts the common structural motif, an eight-stranded anti-parallel β-barrel core which is shared among each capsid protein, and (D) (E) and (F) represent VP1, VP2 and VP3 respectively, with the neutralization antigenic sites (N-Ags) mapped to surface loop extensions coloured black (reproduced from Melnick, 1996).

Two distinct antigenic forms of poliovirus exist, designated D and C antigen (also referred to as N - native -, and H - heated -; Mayer *et al.*, 1957). D (N) antigen is expressed on native infectious virus, and C (H) on non-infectious empty particles. Partial denaturation of the virus may occur by relatively mild treatments, such as heating at 56 °C for 10 minutes or UV irradiation, and results in a change in antigenic properties from the D (N) form found in the infectious virus to a C (H) form (Le Bouvier, 1955). This change is also associated with the first stages of virus uncoating.

The poliovirus neutralising antigenic sites have been characterised by the use of neutralising monoclonal antibodies to select for mutant viruses resistant to neutralisation. These neutralisation escape mutations have been localised to surface loop structures or adjacent β-strands on the exterior of the virion (Hogle *et al.*, 1985; see Figure 2.1 D, E, and F). Three distinct sites have been identified, designated site 1, site 2, and site 3, summarised in Table 2.2 (Minor *et al.*, 1986a). Site 1 includes a region of 12 amino acids of VP1, from residues 89 to 100. This site is strongly immunodominant in type 2 and 3, but has not been detected for type 1. This site has been shown to be sensitive to trypsin (Icenogle *et al.*, 1986), rendering the site antigenically inactive in its natural site of replication in the human gut. Site 2 is a complex site including residues 220 to 222 from VP1 (site 2a) with residues including 169 and 170 and others of VP2 (site 2b). Both site 2a and 2b have been detected in type 1 poliovirus, while only site 2b has been detected in type 3 poliovirus. Site 3 is a complex site including residues 286 to 290 from VP1 (site 3a) with residues 58 and 59 and others of VP3 (site 3b). Both sites 3a and 3b have been detected in type 3 poliovirus, while only site 3b has been detected in type 1 poliovirus.

Table 2.2 Location and occurrence of antigenic sites in poliovirus of serotypes 1, 2 and 3.

Site	Location	Serotype
1	VP1: 89-100	2, 3
2a	VP1: 220-222	1
2b	VP2: 164-172	1, 3
3a	VP1: 286-290	3
3b	VP3: 58-60, 70, 71, 77, 79	1, 3

2.3 Genome organization and proteolytic processing

The poliovirus genome consists of a single positive-sense strand of RNA that is polyadenylated at the 3' terminus and carries a small protein, VPg, covalently attached to its 5' end (Rueckert, 1996). The VPg protein is attached to the 5' terminal pUpUp of the RNA through a phosphodiester linkage to the phenolic (O^4) hydroxyl group of a tyrosine residue (Wimmer, 1982). The poliovirus genome is monocistronic, containing a single long open reading frame (ORF) which encodes a 247 kD polyprotein (Figure 2.2).The N-terminal half of the genome encodes, in the order VP4, VP2, VP3, VP1, the 4 non-identical capsid polypeptides that are products of proteolytic processing of the precursor, P1. VP4 and its precursors VP0 and P1 are myristoylated at the N–terminus. The downstream P2 and P3 precursors encode the nonstructural proteins. Proteolytic processing of the P2 region yield polypeptides $2A^{pro}$, 2B, and 2C, and that of the P3 region yields polypeptides 3A, $3B^{VPg}$, $3C^{pro}$ and $3D^{pol}$.

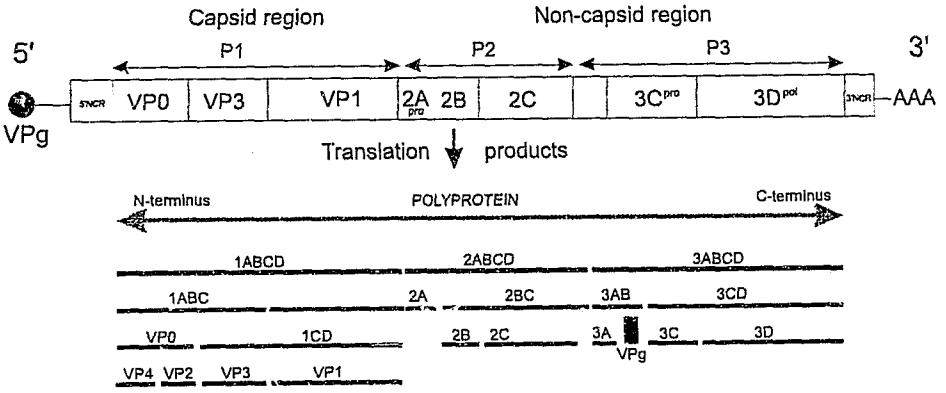

Figure 2.2 Organization and expression of the poliovirus genome (Modified from Rueckert, 1996)

Proteolysis of the polyprotein can be divided into 3 steps (Mirzayan and Wimmer, 1994). The first step is the cleavage of the P1 capsid protein precursor from the nascent polypeptide. This primary cleavage, which occurs at the junction of VP1 and 2A, is catalysed in *cis* by the viral protease $2A^{pro}$, and serves to separate replicative enzymes from structural proteins (Toyoda *et al.*, 1986). In the second step, the capsid and non-capsid

precursors are processed, catalysed by $3C^{pro}$ and $3CD^{pro}$. Product 3B represents VPg, which is thought to be derived from a precursor 3AB thought to be involved in initiation of RNA synthesis (Pallansch *et al.*, 1980; Semler *et al.*, 1981). Product 3D is an enzyme capable of elongating nascent RNA chains from an RNA template (Flanegan *et al.*, 1977). Proteins 2B (Bernstein *et al.*, 1986) and 2C (Li and Baltimore, 1988) are both involved in RNA synthesis. The third step is the processing of VP0 into VP4 and VP2 (the 'maturation cleavage') which takes place at the time of encapsidation of the viral RNA. The VP0 cleavage site lies buried inside the shell near the RNA, and the proteinase responsible for this cleavage is not yet precisely known (Rueckert, 1996). Substrate recognition by the poliovirus proteinases is highly restricted and few polypeptides other than viral gene products are cleaved. It has been shown that concomitant with poliovirus infection is rapid shut-off of host cell protein synthesis (Franklin and Baltimore, 1962), an event accompanied by the proteolytic cleavage of a polypeptide of 220 kD, termed p220. This protein is part of the cap-binding complex eIF-4F (which consists, in addition to p220 of the cap-binding protein, the initiation factor eIF-4A) that recognises the capped 5' end of eukaryotic mRNA's in initiation of translation. Proteinase 2A has been found to be directly involved in abolishing cap-dependent translation (Bernstein *et al.*, 1985). In addition, it has been found to be directly involved in the process of cap-independent translation (Macadam *et al.*, 1994). Proteinase 3C has been implicated in the inhibition of the polymerase III transcription system, specifically in the cleavage of a TFIIIC-containing complex (Mirzayan and Wimmer, 1994), although whether the $3C^{pro}$ cleaves TFIIIC, or a component of this complex, directly or indirectly, is unclear. Proteinase 3C has also been linked to the cleavage of microtubule associated protein 4 (MAP-4), leading to the collapse of cytoskeletal structure in infected cells (Joachims *et al.*, 1995).

The protein coding region is flanked on each end by non-translated regions, whose sequences are strongly conserved and carry signals for initiation of translation near the 5' end, and for initiation of RNA synthesis at the 3' end of the positive and negative sense strands respectively (Rueckert, 1996). The 5' untranslated region (5' non-coding region, 5' NCR) is approximately 740 nucleotides in length, and lacks the m^7GpppNp cap structure that is present at the extreme 5' end of most eukaryotic mRNA's (Kitamura *et al.*, 1981; Racaniello and Baltimore, 1981). Within the first 620 bases there are regions in which the sequence is totally conserved between all polioviruses and enteroviruses, whereas the 100 bases immediately preceding the start codon are hypervariable. The 5' NCR contains stable secondary stem-loop structures (Rivera *et al.*, 1988; Skinner *et al.*, 1989; Pilipenko *et al.*, 1989; Figure 2.3) and an unusually large number - 8 - of AUG's preceding the initiation codon at nucleotide 743.

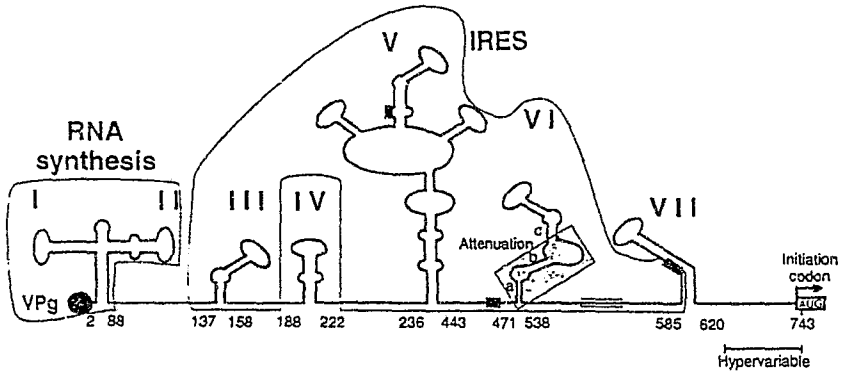

Figure 2.3 Schematic representation of the RNA secondary structure of the poliovirus 5' noncoding region (reproduced from Macadam *et al.*, 1994b). Domain VI may be referred to as domain V in other publications.

The first 90 nucleotides of the genome form cloverleaf structure that interacts with a cellular protein of 36 kD and with the viral protein 3CD to form a complex that is involved in the synthesis of RNA of the same (positive) sense (Skinner *et al.*, 1989; Andino *et al.*, 1990; Harris *et al.*, 1994; Xiang *et al.*, 1995). Because the significant structure can only form after synthesis of the first 100 or so nucleotides of the positive strand, the cloverleaf-protein complex is unlikely to act in *cis*, and is thought to be involved in the initiation of a new positive strand in *trans* (Andino *et al.*, 1993).

The 5'NCR has been found to act as an internal ribosome entry site (IRES) for cap-independent initiation of translation (Pelletier and Sonnenberg, 1988). The important *cis*-acting elements in the 5' NCR appear to be a secondary (or tertiary) structure involving domains III, V, and VI (Percy *et al.*, 1992; Haller *et al.*, 1993), and an AUG triplet approximately 22 nucleotides downstream of an oligopyrimidine tract (Nicholson *et al.*, 1991; Pestova *et al.*, 1991; Pilipenko *et al.*, 1992) that has been shown to be complementary to conserved sequences in the 18S ribosomal RNA (Nicholson *et al.*, 1991; Le *et al.*, 1992). Ribosomes are thought to bind at or near this AUG, and then scan the downstream hypervariable region until they encounter the authentic initiation codon (Jackson *et al.*, 1990).

At least 3 of the *trans*-acting cellular factors required for internal ribosome entry have been identified: one is e-IF2, which interacts with nucleotides 502 to 636 of the 5'NCR (Del Angel *et al.*, 1989). The second is the nuclear 57 kD polypyrimidine-tract-binding protein (PTB), which binds specifically to the poliovirus 5'NCR upstream of the oligopyrimidine tract and

appears to be essential for internal ribosome entry (Pestova *et al.*, 1991; Hellen *et al.*, 1993; Toyoda *et al.*, 1994). The third is also a nuclear protein, known as the LA autoantigen, of molecular mass 52 kD, which binds specifically to a region that encompasses the oligopyrimidine tract and domain VII, and stimulates translation from the authentic initiation codon (Meerovitch *et al.*, 1993; Svitkin *et al.*, 1994; Toyoda *et al.*, 1994).The efficiency of translation has been shown to vary markedly between different cell types and cell-free lysates, suggesting that *trans*-acting factors that are of critical importance for translation may be limiting in some cell types, and that the difference in the spectrum of initiation factors may play a significant role in determining host range and neurovirulence (Svitkin *et al.*, 1988; Ehrenfeld and Gebhard, 1994; Gutierrez *et al.*, 1997).

All 3 serotypes of poliovirus have been shown to carry specific mutations in the 5' NCR that attenuate neurovirulence (see section 2.10). Major attenuating mutations are located at position 480 for type 1 (Kawamura *et al.*, 1989) 481 for type 2 (Equestre *et al.*, 1991; Macadam *et al.*, 1991b; Ren *et al.*, 1991), and 472 for type 3 (Cann *et al.*, 1984; Evans *et al.*, 1985; Westrop *et al.*, 1989). Evidence suggests that attenuation caused by mutations in the 5' NCR impairs the ability of the mutant RNA to initiate translation (Svitkin *et al.*, 1988; Ehrenfeld and Gebhard, 1994; Gutierrez *et al.*, 1997).

The 3' NCR of poliovirus is relatively short, 72 bases in length. Its function is unknown but may be important at some stage of replication because an 8-base insertion in this region produces a temperature-sensitive phenotype (Sarnow *et al.*, 1986).

2.4 The poliovirus receptor

The poliovirus infectious cycle is initiated by attachment and internalisation of the virus via a cellular receptor, followed by uncoating of the virus and release of the viral genome into the cytoplasm. The cellular receptor for poliovirus (PVR) has been mapped to chromosome 19 (Miller *et al.*, 1974; Couillin *et al.*, 1986), and has been identified as a new member of the immunoglobulin (Ig) supergene family, with 3 distinct Ig-like domains, arranged in the order V-C2-C2, where V is variable and C is constant (Mendelsohn *et al.* 1989). It has a transmembrane portion and a C-terminal cytoplasmic tail (Singer, 1990; Figure 2.4).

All 3 serotypes of poliovirus compete for the same receptor. The predicted size of the poliovirus receptor is a peptide of 46 kD (Mendelsohn *et al.*, 1989), but the predominant moiety observed by western blot analyses of Hela cell membranes and in recombinantly expressed PVR is a 67 kD protein (Zibert *et al.*, 1991), probably due to n-glycosylation of

8 potential sites on the extracellular portion (Mendelsohn *et al.*, 1989; Koike *et al.*, 1991). However, deglycosylation experiments have suggested that the 67 kD form is an intracellular high-mannose glycosylation intermediate, and that mature membrane-bound forms of the PVR possess complex branched-chain oligosaccharides and are 80 kD in size (Bernhardt *et al.*, 1994a). Although it was originally demonstrated that the V domain of the PVR is both necessary and sufficient for virus binding and infection (Koike *et al.*, 1991), it has subsequently been shown that all 3 domains are required for efficient receptor function (Bernhardt *et al.*, 1994b; Morrison *et al.*, 1994).

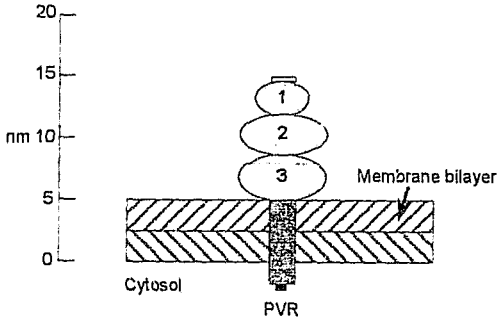

Figure 2.4 Schematic representation of the poliovirus receptor (reproduced with modifications from Rueckert, 1996).

It has been suggested that the depression or 'canyon' around the virion fivefold axis is the virus binding site for the cellular receptor (see Section 2.2 and Figure 2.1 B).

The poliovirus receptor mRNA has been shown to be ubiquitously expressed in human tissues (Mendelsohn *et al.*, 1989). Polioviruses, however, display restricted tissue tropism, infecting cells of the nasopharynx, Peyer's patches of the gut and the motor neurons of the spinal cord (Bodian, 1959, 1972). It thus appears that transcription of the PVR mRNA is not sufficient for the biosynthesis of a functional receptor molecule. Recent results obtained using transgenic mice expressing the human PVR (see following paragraph) suggest that tissue distribution of poliovirus occurs independently of the PVR transgene, and that polioviruses can permeate through the blood-brain barrier independently of receptor expression (Yang *et al.*, 1997). Receptor function may depend on glycosylation and/or post-translational modifications such as phosphorylation and splicing, or on the presence of ancillary proteins which may act as regulatory subunits and promote receptor-virus interactions (Mirzayan and Wimmer, 1994). The membrane-bound form of the PVR has

been identified as a serine phosphoprotein which is phophorylated by calcium/calmodulin kinase II (CaMKII) (Bibb *et al.*, 1994). This enzyme is particularly concentrated in areas of the spinal cord, hippocampus and motor cortex, and localises subcellularly to membrane fractions of synaptosomes (cited from Bibb *et al.*, 1994). It is interesting to note that the histopathology of poliomyelitis (Bodian, 1972) correlates well with this pattern. Furthermore, viral binding to neural tissue homogenates has been reported to be highest in synaptosomes (Brown *et al.*, 1987), suggesting that PVR expression and CaMKII activity co-localise with respect to their distribution in the central nervous system (Bibb *et al.*, 1994).

Mouse cells transfected with the human PVR gene have been shown to be susceptible to poliovirus infection (Mendelsohn *et al.*, 1986), and mouse cell lines transformed with the human PVR have been established (Pipkin *et al.*, 1993).These cells have proved to be useful for the selective isolation of polioviruses (Hovi and Stenvik, 1994). Transgenic mice expressing the human PVR gene have been generated (Ren *et al.*, 1990; Koike *et al.*, 1994); these mice are susceptible to all 3 serotypes of poliovirus and show similar clinical signs and histopathologies as those observed in infected humans and monkeys. These mice are useful for studying poliovirus pathogenesis (Racaniello and Ren, 1994), neurovirulence, attenuation, and tissue tropism (Ren and Racaniello, 1992; Yang *et al.*, 1997), and for development and testing of poliovirus vaccine strains (Abe *et al.*, 1995).

2.5 The poliovirus infection cycle

The poliovirus replication cycle, which occurs entirely in the cytoplasm of infected cells, can be divided into 3 phases (presented in Figure 2.5, and cited from Rueckert, 1996): (i) the early phase, which comprises attachment, penetration and uncoating, (ii) translation of the viral RNA and synthesis of progeny RNA, and (iii) intracellular assembly and release of progeny virions. The initial event in infection is attachment of the virion to specific receptor units embedded in the plasma membrane (step 1). The function of the receptor is twofold: to position the virion to within striking distance of the membrane (step 1), then to trigger a conformational change in the virion (step 2), which involves loss of the internally located protein VP4 (De Sena and Mandel, 1977) and extrusion of the hydrophobic N-termini of VP1 (Fricks and Hogle, 1990), and delivery of the viral RNA across the membrane and into the cytosol (step 3), where translation can begin (step 4). Although the individual steps in the process of internalization are obscure, it is believed to result from receptor-mediated endocytosis (Madshus *et al.*, 1984a, 1984b).The RNA is thought to be extruded into the cytoplasm through a pore which is generated by the contact of the hydrophobic N-terminus of VP1 and the VP4-myristate moiety with the endosomal membrane (Rueckert, 1996).

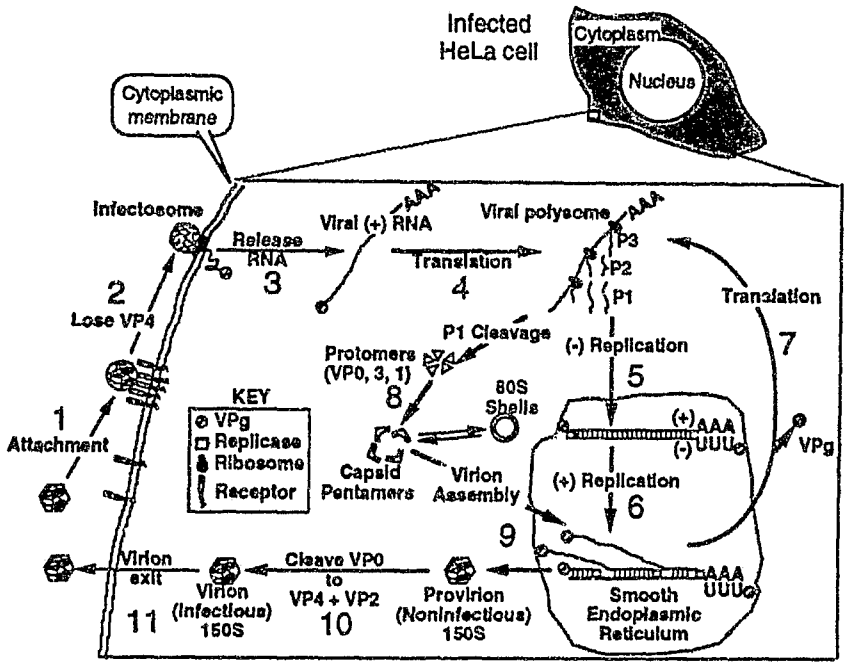

Figure 2.5 Overview of the poliovirus infection cycle (*reproduced from* Rueckert, 1996).

Translation is a crucial step because synthesis of new viral RNA cannot begin until the virus has successfully manufactured the virus-coded RNA-synthesizing machinery. By confiscating ribosomes and other protein-synthesizing machinery of the host cell, the incoming RNA strand directs synthesis of a polyprotein, which is then cleaved into segments while still in the process of synthesis. Translation of the viral message is not restricted to a single ribosome: polysomes carrying up to 40 ribosomes have been reported in virus-infected cells (Rueckert, 1996). The first fragment released from the nascent polyprotein is a coat precursor protein (P1); the next released is a mid-piece precursor protein (P2); and the last segment released is P3 (Rueckert, 1996). Each segment is released from the polyprotein by proteinases encoded in the polyprotein. Viral protein synthesis is accompanied by the shut-off of both protein and RNA synthesis in the host cell (Franklin and Baltimore, 1962).

The first step in synthesis of new viral RNA is to copy the incoming genomic RNA to form complementary minus-strand RNA (step 5, Figure 2.5), which then serves as a template for synthesis of new plus strands (step 6). Synthesis of plus-strand RNA occurs on the smooth endoplasmic reticulum (Caliguiri and Tamm, 1970), and is initiated so rapidly (20- to 50-fold

that of minus strands, Baltimore and Girard, 1966) that it generates multi-stranded replicative intermediates (RI's) consisting of 1 minus-stranded template and many plus-stranded copies (Baltimore, 1969). During the early steps of replication, newly synthesized plus-strand RNA molecules are recycled to form additional replication centres (step 7 → step 5 → step 6), until, with an ever-expanding pool of plus-stranded RNA, a greater and greater fraction of the plus-stranded RNA in the replication complex is packaged into virions (Baltimore, 1969).

Virion assembly (steps 8 and 9) is controlled by a number of events (Rueckert, 1996): one is that, before assembly can begin, coat precursor protein P1 must be cleaved to form immature protomers composed of 3 tightly aggregated proteins (VP0,3,1). Early in the infection cycle this cleavage is likely very slow because the concentrations of P1 and the necessary proteinase (3C or 3CD) are low. Later, with increasing proteinase activity, the rising concentration of immature (5S) protomers triggers assembly into pentamers (step 8), which then package the plus-stranded VPg-RNA to form provirions (step 9). The mechanism of RNA packaging has not yet been fully elucidated, but it has been proposed (Jacobson and Baltimore, 1968) that either (i) the RNA is threaded through a pore in the empty shell (threading model) or (ii) the RNA wraps around the procapsid, fitting into the appropriate channels and triggering reorientation of the subunits in such a way that the RNA is internalized (transfiguration model). Provirions are not infectious. Formation of infective 160S particles (step 10) requires a 'maturation cleavage', in which most of the VP0 chains are cleaved to form the mature four-chain subunits (VP4,2,3,1) characteristic of poliovirions. Complete virus particles, which often form crystals in infected cells, are ultimately released by infection-mediated disintegration of the host cell (step 11).

The time required for a complete multiplication cycle, from infection to completion of virus assembly, generally ranges from 5 to 10 hours. The precise timing depends on variables such as pH, temperature, the host cell, the nutritional vigour of the cell, and the number of particles that infect the cell (Baltimore *et al.*, 1966).

2.6 PATHOGENESIS, PATHOLOGY, AND CLINICAL FEATURES OF POLIOMYELITIS

The pathogenesis of poliovirus infection has been investigated extensively (Bodian, 1959; Bodian and Horstmann, 1965; Melnick, 1996). Poliovirus is transmitted primarily via the faecal-oral route, the portal of entry being the alimentary tract via the mouth, and less commonly by respiratory droplet. The incubation period, defined as the time from exposure

to onset of disease, is usually between 7 and 14 days. A schematic illustration of the pathogenesis of poliomyelitis is presented in Figure 2.6. Initial viral multiplication takes place in the tonsils, lymph nodes of the neck, Peyer's patches, and small intestine. A minor (primary) viraemia follows, during which poliovirus can be detected in the blood. More significant viral replication at the primary sites results in a major (secondary) viraemia, associated with the signs and symptoms of viral infection. If the central nervous system (CNS) has not been seeded with the initial viraemic episode, spread there may occur with the major viraemia. Invasion of the CNS may be by way of circulating blood, or alternatively by direct neural spread.

The mechanism by which poliovirus leaves the blood and enters the CNS is unknown, but recent evidence using transgenic mice (Ren and Racaniello, 1992) supports the importance of muscle infection: polioviruses may spread to skeletal muscle via the blood, reaching neuromuscular end plates from which the viruses ascend along nerves to the spinal cord, and from there may disseminate widely within the CNS. Neural spread may occur in children who have inapparent infections at the time of tonsillectomy; poliovirus present in the oropharynx may enter nerve fibres exposed during surgery and spread to the brain, resulting in bulbar paralysis. A similar spread along neural pathways may be responsible for cases of paralysis following injection with an irritating substance into a limb during periods of high poliovirus prevalence (*provocation paralysis*). Within the CNS, poliovirus spreads along nerve fibres and infects certain types of nerve cells, which may be damaged or destroyed during the process of viral multiplication. The anterior horn cells of the spinal cord are most prominently involved, but in severe cases the intermediate grey ganglia and even the posterior horn and dorsal root ganglia are often affected. Lesions are found as far forward as the hypothalamus and thalamus, and in the brain, the reticular formation, the vestibular nuclei, the cerebellar vermis, and the deep cerebellar nuclei are most often affected. The cortex is spared, with the exception of the motor cortex along the precentral gyrus. In nerve cells, rapid changes occur, from mild chromatolysis to neuronophagia and complete destruction. Inflammation occurs secondary to the attack on the nerve cells; the focal and perivascular infiltrations are chiefly lymphocytes, with some polymorphonuclear cells, plasma cells, and microglia.

In addition to pathological changes in the nervous system, hyperplasia and inflammatory lesions of lymph nodes and of Peyer's patches and other lymph follicles in the intestinal tract are also frequently observed.

Viruses may be shed for up to 2 weeks from the nasopharynx, and for several weeks to months from the faeces. Antibodies to poliovirus appear early in infection, and are usually

present by the time paralysis appears.

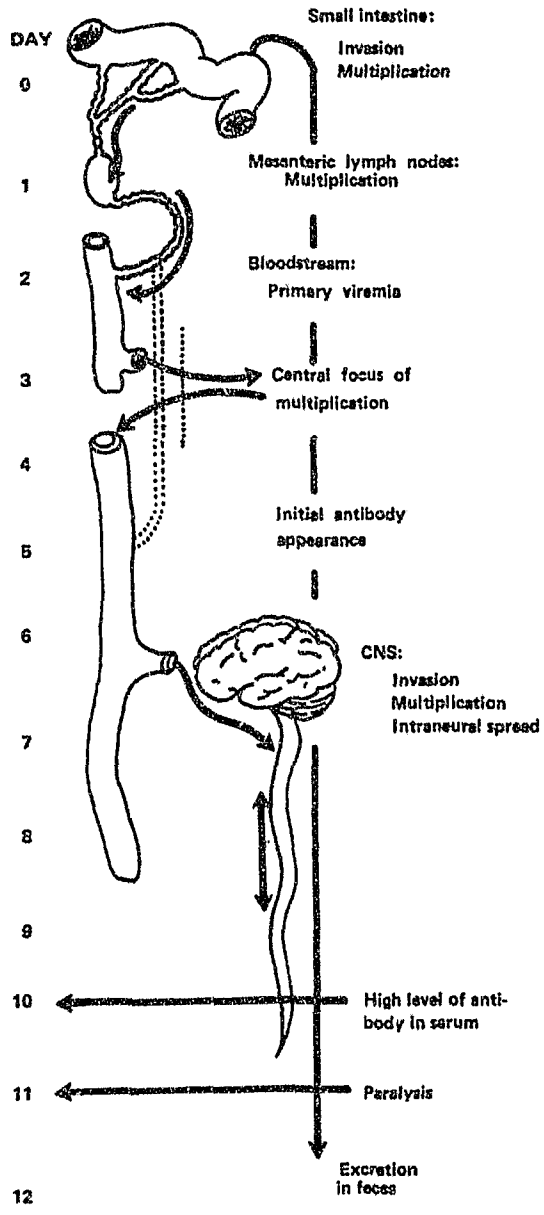

Figure 2.6 Schematic illustration of the pathogenesis of poliomyelitis (reproduced from Melnick, 1996).

Infection with poliovirus may result in one of the following responses: inapparent infection without symptoms, mild (minor) illness, aseptic meningitis, or paralytic poliomyelitis (Melnick, 1996). Ninety-nine percent or more of wild-type poliovirus infections are asymptomatic, and only 0.1% of poliovirus infections result in paralysis (Melnick, 1996; Rotbart, 1997). *Abortive poliomyelitis* or *minor illness* is the most common form of the disease, characterised by fever, malaise, drowsiness, headache, nausea, vomiting, or sore throat, lasting for 2-3 days and followed by complete recovery without neurologic sequelae; the symptoms are accompanied by viraemia. Approximately 10% of patients with abortive poliomyelitis (1% of patients with poliovirus infections) will develop concomitant aseptic meningitis (*non-paralytic poliomyelitis*) indistinguishable from that due to the non-polio enteroviruses (Melnick, 1996; Rotbart, 1997). In a small percentage of cases, the disease may advance to paralysis. The *major illness*, paralysis, when it does occur, may follow the minor illness, but it usually occurs without an antecedent first phase. The paralytic manifestations of poliovirus infections reflect the regions of the CNS most severely affected, with the predominating sign being flaccid paralysis resulting from lower motor neuron damage (Rotbart, 1997). The distribution of paralysis is characteristically asymmetric, with proximal muscles more affected than distal, and legs more than arms. Cranial nerve involvement may result in bulbar paralysis, with resultant difficulties in any or all of speech, swallowing, breathing, eye movement, and facial muscle movements (Rotbart, 1997). Medullary centres controlling respiration and vasomotor function can become involved, with potentially fatal outcome, and paralysis of the muscles of the diaphragm may also result in respiratory failure.

A fairly high proportion (25%) of individuals who recover from paralytic disease may develop the syndrome of *progressive post-poliomyelitis muscular atrophy* (post-polio syndrome; Dalakas *et al.*, 1984). This syndrome is characterised by recurrent weakness, pain and atrophy 25 to 30 years after the initial acute infection, and seldom results in total disability of the affected areas. Although persistent viral infection or reactivation has been postulated due to the presence of intrathecal antibodies (Sharief *et al.*, 1991) or polioviral RNA (Muir *et al.*, 1995; Leparc-Goffart *et al.*, 1996) in the CNS of patients with the post-polio syndrome, this association has not conclusively been established (Melchers *et al.*, 1992; Muir *et al.*, 1996); rather the post-polio syndrome appears to be the result of aging and neurological drop-out in already compromised neuromuscular connections (Dalakas *et al.*, 1995).

2.7 Poliovirus strain variation

Polioviruses exist as 3 serotypes (PV1, PV2 and PV3), classified according to the ability of immune sera or monoclonal antibodies to neutralize viral infectivity (McBride, 1959; Nakano and Gelfand, 1962; van Wezel and Hazendonk, 1979). The "Brunhilde", "Lansing" and "Leon" poliovirus strains are the prototype strains for type 1, type 2, and type 3 poliovirus serotypes respectively (Melnick, 1996). Immunity to one serotype does not confer significant immunity to the other two. P1 Mahoney was the first picornaviral genome to be sequenced in its entirety (Kitamura *et al.,* 1981). Representative strains of the 3 serotypes have subsequently been sequenced and found to be highly homologous in both nucleotide and amino acid sequence (Toyoda *et al.,* 1984). The 5' and 3' termini of the genomes of the 3 poliovirus serotypes are highly homologous: the 3 poliovirus serotypes exhibit approximately 70% homology at the nucleotide level, and 88% homology at the amino acid level. More than 80% of the nucleotide differences in the coding region occur in the third letter position of in-phase codons, resulting in a low frequency of amino acid differences; the observed constrained amino acid variability may be due to requirements for conservation of polypeptide structure. These observations confirm the notion that the 3 poliovirus serotypes are all derived from a common prototype poliovirus ancestor by evolutionary divergence.

Polioviruses within each serotype exhibit limited antigenic variation, and although point mutations in known antigenic sites are common during infections (Minor *et al.,* 1982; Crainic *et al.,* 1983), significant antigenic drift is not observed, and the viruses remain neutralisable by polyclonal type-specific sera. This phenomenon is best illustrated by the fact that the vaccine strains of virus in use since 1955 have remained able to induce protective immunity against wild strains. One notable exception is the poliovirus type 3 strain responsible for the outbreak in Finland in 1984, which was found to differ in both its immunological and molecular properties from the type 3 strain contained in the inactivated vaccine used in Finland (Hovi *et al.,* 1986).

Within each poliovirus serotype, vaccine-like and wild-type strains can be differentiated by neutralisation with strain specific (McBride, 1959; Nakano and Gelfand, 1962) or cross-adsorbed (van Wezel and Hazendonk, 1979) polyclonal antisera, or type-specific monoclonal antibodies (Osterhaus *et al.,* 1981; Ferguson *et al.,* 1982; Humphrey et al, 1982; Crainic *et al.,* 1993), as well as by nucleotide sequence (Nottay *et al.,* 1981; Kew and Nottay, 1984a; Rico-Hesse *et al.,* 1987; Balanant *et al.,* 1991; Yang *et al.,* 1991; De *et al.,* 1995).

Polioviruses, being RNA viruses, mutate rapidly upon passage in humans, at a constant rate of approximately 1-2 nucleotides over the entire genome per week, or 1-2% per year (Nottay *et al.,* 1981; Kew *et al.,* 1995). The majority of the mutations are silent, and do not result in amino acid changes (Rico-Hesse *et al.,* 1987). Wild-type polioviruses thus exist as "quasispecies" or genetic variants, termed genotypes, a genotype being defined as a group of related strains differing by ≤ 15% at the nucleotide level (Rico-Hesse *et al.,* 1987; Kew *et al.,* 1990). The maximum extent of genomic divergence between wild-type strains within a serotype has been found to be similar to that observed between serotypes, approximately 30% (Rico-Hesse *et al.,* 1987; Kew *et al.,* 1990). Poliovirus genotypes have been found to cluster geographically, with specific genotypes circulating endemically in defined geographical regions (Rico-Hesse *et al.,* 1987; Kew *et al.,* 1990; Kew *et al.,* 1995). Because mutations are fixed within the poliovirus genome at a constant rate, identification of genotypes and measurement of the extent of nucleotide sequence divergence between strains associated with cases and outbreaks can be of great value to the eradication programme, as it can identify the source of epidemics and provide a measure of the extent of endemic transmission and its interruption by vaccination.

Recombination between polioviruses of different serotypes is common *in vitro* (Hirst, 1962; Ledinko, 1963) and in recipients of live vaccines (Kew and Nottay, 1984b; Minor *et al.,* 1986b; Cammack *et al.,* 1988; Macadam *et al.,* 1989). It has also been detected in cases of vaccine-associated paralytic poliomyelitis (VAPP; Lipskaya *et al.,* 1991; Furione *et al.,* 1993; Georgescu *et al.,* 1994), and in cases of wild-type infection (Rico-Hesse *et al.,* 1987; Zheng *et al.,* 1993). Intertypic recombination has been found to occur more frequently in type 2 and type 3 viruses. The crossover sites have been mapped to the junction between the capsid and non-capsid region, and to within the P3 non-structural region. The frequency with which recombination occurs suggests that recombinants are not at a disadvantage for growth, and indeed may possess some selective advantage for reproduction in the human intestinal tract.

2.8 Immune response to poliovirus

Natural infection or immunisation with poliovirus confers permanent immunity to the serotype causing the infection. Virus-neutralising antibody develops within a few days after exposure to the virus, usually before the onset of illness, and may persist for life (Paul *et al.,* 1951). Passive immunity can be transferred from mother to offspring; maternal antibodies gradually disappear during the first 6 months of life (Melnick, 1996). Passively administered antibody lasts only 3 to 5 weeks.

Individuals in the acute phase of poliomyelitis mount a humoral immune response predominantly directed against the C (H) antigenic form of poliovirus, while those in the convalescent phase have antibodies to the D (N) form (Minor, 1994). Immune serum with antibodies specific for the D (N) form is protective.

The predominant protective immune response is believed to be humoral. Individuals suffering from primary immune deficiencies associated with defects in the humoral but not cellular arms of the immune response are particularly susceptible to disease caused by poliovirus. The significance of cellular immunity is not clear; infection or immunisation with type 2 virus, however, is believed to be able to prime for a secondary response to type 1 and type 3, and this may be due to cross-reactive T helper cells.

The formation of neutralising antibody early in the infection is a result of virus multiplication in the intestinal tract and deep lymphatic structures before invasion of the nervous system. Because antibodies must be present in the blood to prevent the dissemination of virus to the brain and are not effective after this has already occurred, immunisation is of value only if it precedes the onset of symptoms referable to the nervous system. Local or secretory IgA is produced in the nasopharynx and the gastrointestinal tract, and is recognized as having an important role in defence against poliovirus infection (Ogra *et al.*, 1980; Ogra, 1984). The development of both serum and secretory antibody responses to orally administered live polio vaccine and to intramuscular inoculation of killed polio vaccine (Ogra and Karzon, 1971) is shown in Figure 2.7.

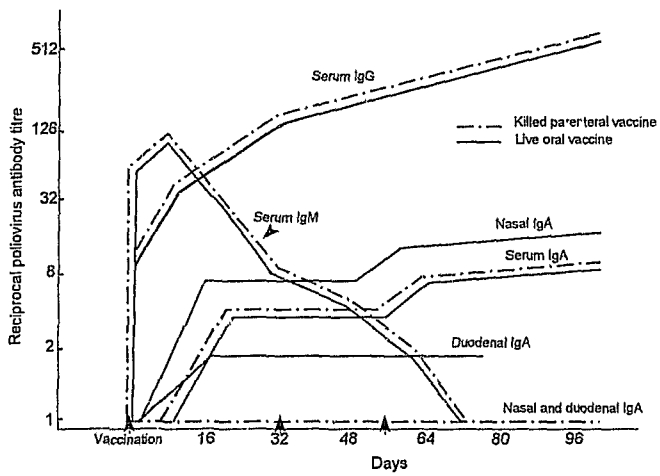

Figure 2.7 Serum and secretory antibody responses to oral administration of live attenuated polio vaccine and to intramuscular inoculation of killed polio vaccine (reproduced from Melnick, 1996)

2.9 Prevention and control of poliomyelitis

Strategies for the prevention and control of poliomyelitis depend on the use of vaccines either based on the formalin-inactivated preparations developed by Salk (Salk *et al.*, 1954) or the live attenuated strains developed by Sabin (Sabin, 1955; Sabin and Boulger, 1973).

2.9.1 Inactivated polio vaccine (IPV)

IPV was first developed in the 1950's by Salk and co-workers and was rapidly accepted for regular immunisation of children (Salk, 1960). The IPV preparations used today are of far greater quality and potency than those manufactured in the 1950's (Salk *et al.*, 1984; Onorato *et al.*, 1991), containing 40-8-32 D antigen units of poliovirus type 1, 2, and 3, respectively, compared to 20-2-4 D antigen units in the original vaccine; hence the designation "enhanced-potency IPV (eIPV). IPV is prepared by concentrating, purifying, and filtering cell-culture harvest to remove aggregates, then treating with 3mM formaldehyde at 37 °C for 2 weeks before a second filtration step. The prolonged and slow inactivation conserves the antigenic properties of the virus and the filtration steps remove aggregates which may protect virus from the formalin and may therefore contain live virus particles.

Excellent protective serum immunity is achieved with IPV. Mucosal immunity is also achieved, although not to the same degree as that induced by OPV (Ogra 1971; Sutter and Patriarca, 1993).

2.9.2 Live attenuated oral polio vaccine (OPV)

The 3 attenuated strains of poliovirus which constitute the Sabin vaccines, one from each serotype, were developed from a range of available candidates largely by the use of the monkey animal model. Different routes of inoculation were used to assess the ability of candidate strains to spread and cause histological damage, and their genetic stability was examined by isolating virus from various tissues of infected animals (Sabin and Boulger, 1973). These studies resulted in the isolation of: (1) the serotype 1 vaccine strain P1/LS-c,2ab, derived from the highly virulent wild-type strain P1/Mahoney by Li and Schaffer and subsequently passaged further by Sabin; (2) the type 2 vaccine strain P2/712Ch,2ab, derived from a relatively avirulent type 2 strain P2/712/56, isolated from a healthy child, which was further passaged including an oral passage in chimpanzees; and (3) the type 3 strain P3/Leon12a,b, derived from P3/Leon/37, an isolate from a fatal case of poliomyelitis in 1937 which was subjected to extensive passage *in vitro* by Sabin.

The Sabin 1, 2, and 3 polio vaccine strains differ from their neurovirulent progenitors at 57 (Nomoto *et al.*, 1982), 23 (Moss *et al.*, 1989; Pollard *et al.*, 1989) and 10 (Stanway *et al.*, 1984) nucleotide positions respectively.

Vaccine viruses are grown in susceptible cells - these were initially primary monkey kidney cells, but the presence of unacceptably high levels of adventitious monkey viruses in the cell preparations led to the use of continuous cell lines, predominantly Vero (Montagnon, 1989) or human diploid cells (Dunn *et al.*, 1990), for OPV preparation.

The vaccines are almost always administered as a standard trivalent formulation containing 10^6, 10^5, $10^{5.5}$ (300 000) $TCID_{50}$ of Sabin 1, 2 and 3 respectively ("10:1:3") formulation, or an enhanced formulation containing 10^6, 10^5, and $10^{5.8}$ (600 000) $TCID_{50}$ of Sabin1, 2, and 3 respectively ("10:1:6" formulation)(Patriarca *et al.*, 1993). The present EPI immunisation schedule with OPV in South Africa is a single trivalent OPV (TOPV) dose at birth, 6, 10 and 14 weeks, followed by booster doses at 18 months and 5 years (Department of National Health, 1995).

Live vaccines establish an asymptomatic infection in the gut which imitates the natural infection and thus stimulates a full range of immune responses including the production of both systemic and local secreted antibodies (Ogra, 1971). In rare instances, however, the vaccine strains have been implicated in paralytic poliomyelitis, as result of reversion to neurovirulence during passage in the gut of vaccinees. The estimated incidence of VAPP is approximately 1 per 500 000 primary vaccinees, and 1 to 2 cases per 2 500 000 doses overall (CDC, 1997a; Joce *et al.*, 1992).

2.9.3 Choice of poliovirus vaccine

The choice between OPV and eIPV remains a source of frequent ongoing and intense debate. The advantages and disadvantages of both vaccine types are listed in Tables 2.3 and 2.4 (adapted from Melnick, 1996).

When properly prepared and administered, IPV can induce adequate levels of serum antibodies, conferring humoral immunity. Because it contains no living virus, it cannot mutate towards increasing neurovirulence. It is also safe to administer to persons with immune-deficiency diseases and their contacts, and is suitable for combination with other injectable childhood vaccines. However, IPV does not induce a high degree of local immunity, and vaccinees can still act as reservoirs of wild-type virus, which is excreted and

can be a source of infection to others. IPV has been used for several decades in Finland, the Netherlands, and France. Despite the success of IPV in controlling poliomyelitis in these countries, outbreaks still occurred in Finland in 1984 (Hovi *et al.*, 1986), and in the Netherlands in 1978 (WHO, 1979) and again in 1992-93 (Oostvogel *et al.*, 1994). The Finnish outbreak was attributed to vaccine failure due a decline in the PV3 content of the vaccine, and a slight change in the immunologic and molecular characteristics of the epidemic virus compared to the vaccine virus (Hovi *et al.*, 1986). Both outbreaks in the Netherlands occurred as a result of importation of wild-type virus into religious communities that refused vaccination, and in both cases the epidemic virus subsequently spread to unvaccinated members of associated religious groups in Canada (Furesz *et al.*, 1978; Drebot *et al.*, 1997).

Table 2.3 Inactivated polio vaccine: advantages and disadvantages

Advantages:

- Confers excellent humoral immunity
- Confers some degree of mucosal immunity
- Can be incorporated into regular paediatric immunisation schedules with other vaccines
- No reversion to neurovirulence
- Greater stability than OPV
- No interference by other intestinal pathogens
- Can be administered to immunocompromised or immunodeficient individuals and their contacts

Disadvantages:

- Repeated boosters required to maintain detectable antibody levels
- Confers poor local intestinal immunity - vaccinees do not act as a block to wild-type virus transmission via the faecal-oral route
- More expensive than OPV, both in preparation and administration to vaccinees

OPV has been more widely used than IPV because of its greater ease of administration by the oral route, much lower cost for the developing world, its ability to induce not only serum antibodies but also intestinal resistance, and the rapidity with which vaccinees develop long-lasting resistance (Melnick, 1996). Vaccine viruses are abundantly excreted by the vaccinees and are able to infect and immunise unvaccinated contacts (Benyesh-Melnick *et al.*, 1967). Routine use of OPV has successfully eliminated wild-type poliovirus circulation

in the United States (Hull *et al.*, 1994), and in Japan (Miyamura *et al.*, 1992), and widespread mass vaccination with OPV has resulted in the elimination of polio from the Americas (PAHO, 1993), and to a dramatic reduction in cases throughout most of the world (WHO, 1997b).

Table 2.4 Live attenuated oral polio vaccine: advantages and disadvantages

Advantages:

- Mimics natural infection, conferring both humoral and intestinal immunity
- Immunity induced may be lifelong
- Induces antibodies very rapidly in a large proportion of vaccinees
- Oral administration is more acceptable than injection, and is easier to accomplish - does not require trained healthcare workers
- Under epidemic conditions, not only does it induce antibody quickly, but gastrointestinal immunity blocks spread of the epidemic virus
- Can spread to and induce immunity in un-immunised contacts of vaccinees (secondary spread)
- Relatively inexpensive to produce and to administer

Disadvantages:

- Vaccine viruses may revert to neurovirulence and cause VAPP
- Secondary spread of reverted progeny virus from vaccinees may cause VAPP in unvaccinated contacts
- Requires cold chain for maintenance of vaccine potency
- Contraindicated in some immunocompromised and immunodeficient individuals and their contacts
- Dosage of OPV as currently constituted may not induce sufficiently high levels of protective antibodies in developing countries

Although OPV is highly successful in temperate countries, in tropical and developing countries it often fails to induce adequate serum antibody responses. Several factors have been found to be responsible for the OPV failure in developing countries (Patriarca *et al.*, 1991; WHO, 1995a; Maldonado *et al.*, 1997; Triki *et al.*, 1997). These include vaccine instability resulting from incorrect storage, concurrent infections with other enteric pathogens which interfere with the response to OPV, the presence of passive maternal antibodies during the first few months of life, the presence of antibodies in breastmilk, and possibly too short an interval between vaccine doses.

An alternative approach to the exclusive use of either IPV or OPV has been the use of combined IPV-OPV schedules. Combined schedules have been used successfully in Denmark since 1966 (von Magnus and Petersen, 1984) and in Israel, Gaza and the West Bank since 1979 (Tulchinsky *et al.*, 1993). In many countries where wild-type virus has been eliminated and the only cases of poliomyelitis are the result of OPV which has reverted to neurovirulence, discussions have focussed on the introduction of a combined scheduled, with the goal to provide both humoral and intestinal immunity, and prevent the few cases of VAPP which occur every year (Melnick, 1988). With the goal of global elimination in sight, the focus of the WHO's Poliomyelitis Eradication Initiative is now shifting towards strategies for the eventual cessation of vaccination (WHO, 1998b).

2.10 Determinants of poliovirus neurovirulence

The Sabin polio vaccine strains are attenuated, with respect to neurovirulence, compared to their progenitor strains (Sabin, 1955). They are also temperature sensitive, exhibiting a temperature optimum of 34 °C and a reduced capacity to multiply at elevated temperatures (Lwoff and Lwoff, 1958; Sabin, 1961). Sequence analysis of the Sabin 1, 2, and 3 polio vaccine strains and their neurovirulent progenitors has revealed that the attenuated Sabin strains differ from their progenitors at very few nucleotide positions: Sabin 1, 2 and 3 exhibit respectively only 57 (Nomoto *et al.*, 1982), 23 (Moss *et al.*, 1989; Pollard *et al.*, 1989) and 10 (Stanway *et al.*, 1984) nucleotide changes from their neurovirulent progenitors. Using recombinant DNA technology and site-directed mutagenesis, the nucleotide polymorphisms between strains which directly influence neurovirulence have been defined.

For type 1, a significant attenuating mutation was identified as an A to G change at residue 480 of the 5'NCR (Kawamura *et al.*, 1989). Two additional mutations, one at position 6203, a C to U change which introduces a change in Sabin 1 from histidine to tyrosine at residue 73 of the polymerase 3D, and a G to A change at position 7441 at the extreme 3' end of the genome (Christodoulou *et al.* 1990), have also been found to affect attenuation by rendering the viruses less temperature sensitive (Christodoulou *et al.* 1990; Bouchard *et al.*, 1995; Georgescu *et al.*, 1995; McGoldrick *et al.*, 1995). The major determinants of attenuation and temperature sensitivity for type 1 are thus G-480, histidine-73, and G-7441.

For type 2, 2 mutations have been found to have the most significant effects on both reversion to neurovirulence and temperature sensitivity: an A to G change at position 481, and an A to G mutation at position 2903 of Sabin 1 which results in an amino acid change from isoleucine to valine at residue 143 of capsid protein VP1(Equestre *et al.*, 1991; Ren

et al., 1991; Macadam *et al.*, 1991b; Macadam *et al.*, 1993). Thus for type 2, A-481 and Isoleucine-143 are general determinants of attenuation.

Two nucleotide changes have been found to affect reversion to neurovirulence for type 3: a U to C change at nucleotide 472 in the 5'NCR (Cann *et al.*, 1984; Evans *et al.*, 1985; Westrop *et al.*, 1989), and a U to C change at nucleotide 2034 which causes an amino acid substitution at residue 91 of the capsid protein VP3 from phenylalanine in the attenuated strains to serine in neurovirulent strains (Minor *et al.*, 1989; Westrop *et al.*, 1989). A third Sabin-specific mutation, C at position 2493, has also been identified (Weeks-Levy *et al.*, 1991). A change from C to U at this position, which results in a threonine to isoleucine change at residue 6 of capsid VP1, was originally found to increase neurovirulence in monkeys (Tatem *et al.*, 1992), but has since been found not to be directly associated with neurovirulence, but to correlate with the source and the passage level of the seed virus used for vaccine production (Chumakov *et al.*, 1992). Thus the major determinants of attenuation for type 3 are U-472 and U-2304 (phenylalanine-91). The nucleotide at position 2034 has been found to determine temperature sensitivity (Minor *et al.*, 1989), which has been found to result from a defect early in the assembly process that inhibits the formation of 14S pentamers, empty capsids, and virions (Macadam *et al.*, 1991a).

The major attenuating mutations for all 3 Sabin polio vaccine serotypes are thus located within the 5' NCR. The 5' NCR contains stable secondary stem-loop structures (Rivera *et al.*, 1988; Skinner *et al.*, 1989; Pilipenko *et al.*, 1989), and the attenuating mutations at positions 472, 480 and 481 are found within domain VI of the stem-loop structure (see Figure 2.3). The primary effect of the attenuating mutations appears to be on secondary-structural stability of the base-paired stems in domain VI (Minor, 1992; Macadam *et al.*, 1994). Disruption of base pairs at each of 4 positions in stems VIa and VIb has been shown to result in attenuated phenotypes (Skinner *et al.*, 1989; Macadam *et al.*, 1992). Attenuation appears to result from the destabilisation of the secondary hairpin loop structure in this region, resulting in altered interactions with cellular factors mediating ribosome binding (Pelletier and Sonnenberg, 1989; Pestova *et al.*, 1991; Gebhard and Ehrenfeld, 1992; Gutierrez *et al.*, 1997) and a subsequent reduction in translation efficiency (Svitkin *et al.*, 1985; Svitkin *et al.*, 1988; Svitkin *et al.*, 1990).

The structural stability of domain VI therefore seems to be essential for neurovirulence. It is not clear whether destabilisation of domain VI is directly responsible for attenuation, perhaps by interfering with the correct presentation of a loop sequence, or whether it leads to the loss of important tertiary interactions in the 5' NCR (Macadam *et al.*, 1994). The

genetic instability of the attenuating mutations at positions 472, 480 and 481 is observed under certain conditions during passage in the gastrointestinal tract (Evans *et al.*, 1985; Minor and Dunn, 1988; Macadam *et al.*, 1989; Dunn *et al.*, 1990; Tatem *et al.*, 1991) and in tissue culture: selection pressure can be increased by raising the incubatio temperature (Chumakov *et al.*, 1994), which is consistent with the effects of the attenuating mutations on the temperature sensitivity of viral growth (Agol *et al.*, 1989). Selection pressures also depend on the cell line that is used for viral growth, with variation in reversion rates (Chumakov *et al.*, 1992; Chumakov *et al.*, 1994; Rezapkin *et al.*, 1994; Rezapkin *et al.*, 1995; Taffs *et al.*, 1995), temperature sensitivity (Macadam *et al.*, 1991b; Macadam *et al.*, 1992), and virus yield (Agol *et al.*, 1989; La Monica and Racaniello, 1989) between cell lines. These findings underlie the importance of interactions between domain VI and a cellular factor(s), the nature or abundance of which varies between cell lines; the attenuated phenotypes of the polio vaccine strains and the selection pressures in the gastrointestinal tract may be partly explained by deficiencies of these factors in neuronal tissues and relevant gut cells (Minor, 1992; Macadam *et al.*, 1994b).

2.11 Epidemiology

The circulation of the 3 poliovirus serotypes occurs at a similar rate, although infections with type 1 polioviruses are more likely to lead to disease than those with type 3, while infections with type 2 are the least likely to have clinical effects (Melnick, 1996). Poliovirus type 2, however, appears to be the most effective immunogen, and thus the highest rate of seroconversion following immunisation with TOPV is to this type (WHO, 1995b).

Poliomyelitis can be regarded as having 3 major epidemiological phases: endemic, epidemic, and "vaccine era" (Melnick, 1996). These phases have occurred sequentially in history, but at present they all co-exist in different regions of the world. From ancient times into the late 1800's polioviruses became established throughout most of the world's populations and survived for many centuries in an endemic fashion. Viral prevalence is favoured by warm wet conditions; in temperate climates, prevalence is thus highest in summer and autumn. Young children form a reservoir of infection and high frequencies of transmission are associated with poor living conditions and low socioeconomic status. Improvements in hygiene resulted in lesser exposure in infancy, with a consequent accumulation of susceptible older individuals, leading eventually to significant epidemics, rather than the original endemic pattern. In crowded developing countries, however, paralytic poliomyelitis continues to be a disease of infancy, circulating in the classic endemic pattern. The vaccine era began after 1955 when IPV was introduced, and became even

more firmly established after 1960 when live attenuated vaccines became available on a large scale. Since then, the progress made towards the control of poliomyelitis has been dramatic: in 1955, the Soviet Union, 23 other European countries, the United States, Canada, Australia and New Zealand experienced a total of more than 76 000 reported cases of poliomyelitis; in 1967, only 12 years later, only 1 013 cases were recorded in these same countries, a reduction of almost 99% (Melnick, 1996).

2.11.1 The Poliomyelitis Eradication Initiative (PEI)

Following the eradication of smallpox in 1978, the WHO Expanded Programme on Immunisation (EPI) was able to focus its attention on increasing global polio immunisation coverage. Despite impressive gains in routine immunisation coverages with OPV (85% worldwide coverage in 1990), wild-type poliovirus circulation continued in many regions (WHO, 1995a). It became clear that a more intense, targeted strategy was necessary to achieve poliomyelitis eradication; the Poliomyelitis Eradication Initiative (PEI) was thus established, and in May 1988, the 41st World Health Assembly accepted a resolution for the global eradication of poliomyelitis by the year 2000 (WHO, 1988). The WHO has developed specific operational strategies for the achievement of this goal, as follows: (1) reaching and maintaining the highest possible routine immunisation coverage (over 80%) with at least 3 doses of OPV; (2) annual National Immunisation Days (NID's) to deliver 2 supplemental doses of OPV to all children less than 5 years of age in countries and regions where polio is still endemic or where there is a risk of re-introduction from other areas; (3) laboratory-based surveillance to detect and investigate every case of acute flaccid paralysis (AFP) in children less than 15 years of age, and all cases of suspected poliomyelitis of any age; and (4) house-to-house "mopping up" immunisation campaigns to deliver OPV to children in areas where poliovirus transmission persists.

Remarkable progress has been made since the implementation of these strategies; Poliomyelitis has been successfully eliminated from the Western Hemisphere (Hull *et al.*, 1994), with the last indigenous case of poliomyelitis reported from Peru in 1991 (de Quadros *et al.*, 1992). Global routine OPV3 immunisation reached 81% in 1996, with >80% coverage in all WHO regions except the African and Eastern Mediterranean regions (WHO, 1997b). In Africa, coverage increased from 32% in 1988 to 58% in 1995, and 60% in 1996. Eighty two countries conducted NID's in 1996, with 419 million children under the age of 5, approximately two-thirds of the world's children under 5, immunised (WHO, 1997b). A total of 3 997 cases of poliomyelitis were reported globally in 1996, a decrease of 90% from the approximately 35 000 cases reported in 1988 (WHO, 1997b).

Elimination of poliomyelitis from Africa remains one of the major challenges to achieving global eradication, although progress achieved to date in the WHO African region suggests that the target of global eradication by the year 2000 remains feasible (WHO, 1998c). Reported routine OPV3 coverage in Africa is still low in the region overall, but has increased from 47% in 1993 to 54% in 1996. In 1996, 15 countries reported that less than 50% of their children were routinely immunised with OPV3; in 1996 the largest and epidemiologically most important countries of Angola, Democratic Republic of Congo (D.R.Congo, formerly Zaire), and Nigeria reported OPV3 coverages of 42%, 36% and 26% respectively (WHO, 1998c). During 1996, 1997 and the first quarter of 1998, NID's were conducted in all African countries except Liberia, the Congo and Sierra Leone. The D.R.Congo carried out "local immunisation days", which reached 25% of the total population. By 1997, AFP surveillance had been established in all but 6 countries (Burundi, Equatorial Guinea, Eritrea, Gabon, Liberia and Sierra Leone). However, the rate of AFP reporting for all epidemiological blocks in Africa is still low, less than 0.2 AFP cases per 100 000 children less than 15 years of age (target, 1 AFP case per 100 000 children less than 15). In 1996, 1 949 poliomyelitis cases were reported from the African Region, with 6 countries contributing the majority of cases: Angola (81), Chad (93), D.R.Congo (219), Ethiopia (264), Uganda (121) and Nigeria (942). In 1997, no wild poliovirus was recovered from countries in the East and Southern African epidemiological blocks. In West and Central African countries however, wild poliovirus was isolated widely, even after the first NID's, indicating that interruption of transmission has not yet been achieved in these areas. The 2 most important remaining reservoirs of wild poliovirus, which present major challenges to reaching the target of eradication in Africa by the year 2000, are Nigeria and D.R.Congo (WHO, 1998c).

2.11.2 Molecular epidemiology

Surveillance, including laboratory surveillance, is a critical component of the WHO's strategy for global polio eradication, and molecular epidemiology is an integral part of laboratory investigations to identify and characterise polioviruses associated with cases of AFP. The underlying objective of wild poliovirus surveillance is the development and implementation of effective strategies for poliomyelitis control, and in this context, the most important surveillance questions centre on (1) the identification of the local, regional and global reservoirs sustaining poliovirus circulation; (2) the identification of links between poliomyelitis cases; and (3) the identification of local, regional and global pathways of poliovirus transmission. Because 99% or more of poliovirus infections are subclinical (Melnick, 1996), standard epidemiological investigations cannot be employed to address these questions. Characterisation and comparison of the poliovirus strains associated with cases and

outbreaks, can, however, often provide information that cannot be obtained by any other means. Techniques for poliovirus strain characterisation have, until recently, been based on the antigenic properties of the viruses and have been serological in nature, using specific polyclonal antibodies (Nakano *et al.*, 1978; van Wezel and Hazendonk, 1979) or panels of monoclonal antibodies (Humphrey *et al.*, 1982; Minor *et al.*, 1982; Crainic *et al.*, 1983; Osterhaus *et al.*, 1983). The finding that a molecular clock exists for poliovirus evolution - poliovirus RNA genomes evolve rapidly during replication in humans, at a rate of approximately 1-2 nucleotide substitutions per week over the entire genome (10^{-2} substitutions per site per year) (Nottay *et al.*, 1981; Kew *et al.*, 1995; CDC, 1997b) - has meant that the potential resolving power of molecular epidemiological studies based on sequence comparisons is very high. As a result, poliovirus strain characterisation based on antigenic properties has now largely been replaced by molecular analyses based on the genomic characteristics of the viruses.

The most powerful approach to molecular epidemiological investigations is comparative genomic sequencing (Rico-Hesse *et al.*, 1987). Sequence comparisons of poliovirus strains has revealed the existence, for each poliovirus serotype, of numerous genotypes, defined as groups of polioviruses sharing ≥85% nucleotide similarity within a defined genomic interval (Rico-Hesse *et al.*, 1987; Kew *et al.*, 1990). Genotypes have been found to be distributed geographically and to be endemic to different regions of the world (Rico-Hesse *et al.*, 1987; Kew *et al.*, 1990, 1995). Sequence diversity within a genotype has been found to be reduced by epidemics (as 1 lineage predominates), as well as by intensive immunisation (as lineages are eliminated) (Kew *et al.*, 1995). Molecular epidemiological approaches have and are being used widely within the PEI to (1) identify reservoirs sustaining virus transmission; (2) follow the pathways of virus transmission; (3) determine the sources of imported viruses; (4) develop molecular reagents for rapid detection and characterisation of polioviruses in clinical and environmental samples, and (5) to monitor the success of the eradication programme, by monitoring the distribution and disappearance of genotypes, and finally provide critical evidence that poliovirus elimination and eradication has been achieved (Kew *et al.*, 1995).

3. MOLECULAR METHODS FOR THE DETECTION AND CHARACTERISATION OF POLIOVIRUSES

3.1 INTRODUCTION

The laboratory diagnosis of poliomyelitis is a critical component of the WHO 's initiative for poliomyelitis eradication by the year 2000 (WHO, 1988, 1990a), as it ultimately determines the specificity of AFP surveillance. The need for rapid and accurate identification of polioviruses in clinical or environmental specimens will become increasingly important as the goal of poliomyelitis eradication is approached and circulation of wild-type polioviruses decreases. Routine laboratory diagnosis of poliovirus infection still relies largely on cell culture enterovirus isolation (Lenette, 1992) followed by enterovirus subtyping by serum neutralisation techniques (Lim and Benyesh-Melnick, 1969). These techniques are laborious and time-consuming, as 5 to 7 days (on average) are typically required for the positive identification of poliovirus in a clinical specimen. Recently, mouse L-cells transfected with the human poliovirus receptor have been developed for selective isolation of polioviruses and exclusion of other enteroviruses (Pipkin *et al.*, 1993; Hovi and Stenvick, 1994). However, the sensitivity of the L-cells compared to the primate cell types used routinely has not yet been adequately assessed, and confirmatory serotyping following the isolation of a putative poliovirus is still necessary, thus precluding the routine use of these cells.

The determination of the vaccine or non-vaccine origins of poliovirus strains associated with cases is an important part of laboratory diagnosis. Techniques for intratypic differentiation between vaccine-like and wild-type isolates have included phenotypic markers e.g. rct markers (Lwoff and Lwoff, 1958), and neutralisation with strain-specific (McBride, 1959; Nakano and Gelfand, 1962) or cross-absorbed neutralising polyclonal antisera (van Wezel and Hazendonk, 1979). Recently, an enzyme-linked immunosorbent assay (ELISA), using the cross-adsorbed type-specific antisera, has been developed (Osterhaus *et al.*, 1983; van der Avoort *et al.*, 1995). The development of monoclonal antibodies (Osterhaus *et al.*, 1981; Crainic *et al.*, 1981, Ferguson *et al.*, 1982, Humphrey *et al.*, 1982; Crainic *et al.*, 1983) significantly improved the serodifferentiation methods based on antigenic characterisation of polioviruses. However, the differentiation of polioviruses based on antigenic characterisation is not always definitive and can lead to equivocal results, since the antigenic properties of the viruses are not always stable and wild-type variants may have antigenic properties indistinguishable from those of vaccine-derived strains (Nakano *et al.*, 1978; Crainic *et al.*, 1983).

Molecular methods based on genomic sequence differences between wild-type and vaccine-like strains have been developed; these include oligonucleotide fingerprinting (Nottay *et al.*, 1981), partial genomic sequencing (Rico-Hesse *et al.*, 1987), and probe hybridization (da Silva *et al.*, 1991; De *et al.*, 1995); while reliable results can be obtained using these techniques, with the exception of probe hybridization, they are laborious and time-consuming. Methods based on specific amplification of vaccine-like genomes (Yang *et al.*, 1991) or PCR followed by vaccine-specific probe hybridization (Takeda *et al.*, 1994) have also been developed for differentiation between vaccine-like and wild-type poliovirus strains. The Sabin-specific RT-PCR described by Yang *et al.* (1991) was selected as the method of choice for intratypic differentiation of poliovirus strains described in this thesis (discussed in section 3.3.1). However, procedures which positively detect only vaccine-like genomes are not ideally suited to routine monitoring purposes because isolates may contain mixed populations of vaccine-like and wild-type strains; the wild-type strains in mixed populations would be missed by the use of vaccine-specific PCR/hybridization. Genotype-specific probe hybridization (da Silva *et al.*, 1991, De *et al.*, 1997) and PCR (Yang *et al*, 1992) assays, while highly sensitive, are suitable only for the detection of strains for which sequence information is available and not ideal for use in areas where the distribution of circulating genotypes is unknown.

Restriction fragment length polymorphism (RFLP) assays have recently been developed for intratypic differentiation and genomic analysis. Balanant *et al.* (1991) describe PCR amplification followed by restriction enzyme digestion of a highly variable segment of the genomic region coding for VP1, including antigenic site 1. A possible drawback of this technique is that under immune pressure, the regions coding for the exposed surfaces of the virion are rapidly modified, resulting in the loss or generation of a restriction site. An RFLP assay based on the variable segment of the 5′ non-coding region (5′ NCR), which is not exposed to the selective pressure of neutralizing antibodies, was developed by Schweiger *et al.*, (1994). RFLP-based assays are, however, also not ideally suited for routine characterisation of strains; panels of restriction enzymes are required in order to obtain definitive results, and misleading results may still be obtained due to incomplete cleavage of PCR products or the presence of additional fragments after digestion.

The need thus exists for rapid, sensitive techniques which can complement or even replace the existing systems for poliovirus identification and intratypic differentiation. To this end, a rapid and sensitive one-tube poliovirus-specific RT-PCR assay was developed. The primers used in this assay span the VP1/2A region used for genotype determination (Rico-Hesse *et al.*, 1987), so that rapid genotype analysis can be performed by direct sequencing

of the amplified products. A novel method for poliovirus intratypic differentiation was also developed; this method, the heteroduplex mobility assay (HMA), is based on the formation of stable heteroduplexes, readily detectable by gel electrophoresis, between templates differing in genetic composition (Delwart *et al.*, 1993).

The underlying objective of wild poliovirus surveillance is the development and implementation of effective strategies for poliomyelitis control. In this context, the most important surveillance questions centre on (1) the identification of the local, regional and global reservoirs sustaining poliovirus circulation; (2) the identification of links between poliomyelitis cases; and (3) the identification of local, regional and global pathways of poliovirus transmission. Because as many as 99% or more of poliovirus infections are subclinical (Melnick, 1996), standard epidemiological techniques cannot be employed to provide answers to these questions. Thus characterisation of the poliovirus strains associated with cases and outbreaks is of critical importance to the eradication initiative, since this is often the sole means by which epidemiological links can be determined. Until recently, techniques for poliovirus strain characterisation have been based on the antigenic properties of the viruses and have been serological in nature, using specific polyclonal antibodies (Nakano *et al.*, 1978; van Wezel and Hazendonk, 1979) or panels of monoclonal antibodies (Humphrey *et al.*, 1982; Minor *et al.*, 1982; Crainic *et al.*, 1983; Osterhaus *et al.*, 1983). These techniques have now largely been replaced by molecular analyses based on genomic, rather than antigenic, characteristics of the viruses.

The most powerful approach to molecular epidemiological investigations is comparative genomic sequencing. Early sequencing studies demonstrated that sequence analysis of 150 nucleotides across the VP1/2A region of the poliovirus genome provided sufficient resolution to generate a broad overview of the global distribution and transmission of wild-type polioviruses (Rico-Hesse *et al.*, 1987; Kew *et al.*, 1990). Since these initial studies, comparative sequencing studies have been conducted to more thoroughly investigate the molecular epidemiology of wild-type viruses associated with cases and outbreaks in a number of geographic regions (discussed in section 3.3.4). Since the fundamental aim of the PEI is the systematic eradication of poliovirus genotypes, in addition to its role in the identification of areas of continued endemic wild-type circulation and pathways of transmission, sequence analysis and subsequent analysis of the distribution patterns of circulating poliovirus genotypes has also become a tool for monitoring the success of the eradication programme.

In this chapter, the molecular methods applied and developed for the characterisation of polioviruses circulating in sub-Saharan Africa will be described and discussed.

3.2 METHODS

3.2.1 Poliovirus isolation and typing

The polioviruses analysed for the purpose of this study are listed in the Materials and Methods section of Chapters 4, 5 and 6. Polioviruses were isolated from clinical specimens (stools, throat swabs, CSF) of patients presenting with poliomyelitis, or from healthy contacts of such cases. Stool suspensions were made up to 20% w/v in glycerol-buffered saline and inoculated onto primary vervet monkey kidney or Hep2-C cells. CSF and throat swabs were inoculated undiluted Cell cultures were monitored daily for appearance of cytopathic effects. Viruses isolated up until 1995 were typed by neutralisation using type-specific monkey antisera produced by the Vaccine Unit, NIV. From 1995, viruses were typed using a standard microplate neutralization assay (WHO, 1990b) with antiserum pools supplied by the National Institute for Public Health and Environmental Protection (RIVM), the Netherlands.

3.2.2 Viral RNA extraction

(a) RNA extraction from virus-containing cell-culture supernatants

Cell-culture supernatants were centrifuged briefly (3 minutes, 1000 rpm, Eppendorf microfuge) to remove cellular debris, and 160 μl of each clarified supernatant were transferred to microfuge tubes containing 40 μl 5X lysis buffer (250 mM Tris-HCl, pH 8.3; 350 mM KCl; 25 mM $MgCl_2$; 2.5% Nonidet-P40) Tubes were incubated on ice for 15 minutes. Nucleic acids were extracted once with an equal volume (200 μl) of Tris-buffered phenol (pH 8.0), once with phenol/chloroform (1:1) and once with chloroform/isoamylalcohol (24:1). The aqueous phase was transferred to a clean tube on ice and used directly for amplification reactions. Nucleic acid extracts were subsequently stored at -70 °C.

In order to avoid the continued use of potentially dangerous organic reagents, beginning in 1997 the HighPure Viral RNA kit (Boehringer Mannheim, GMBH, Germany) was used in preference to the phenol extraction method described above. RNA was extracted from 200 μl cell-culture supernatants according to the kit instructions, eluted in 50 μl RNase-free water, and stored at -70 °C.

(b) RNA extraction from clinical specimens

Commercial RNA extractions kits (Qiagen Viral RNA kit, Qiagen GMBH, Germany and HighPure Viral RNA kit, Boehringer Mannheim, GMBH, Germany) were used to extract viral RNA directly from 200 μl clarified stool or other clinical specimens. The kits were used according to the manufacturers' instructions, RNA was eluted in 50 μl RNAse-free water and stored at -70 °C.

3.2.3 *Intratypic differentiation by Sabin-specific RT-PCR*

The PCR assay described by Yang *et al.* (1991) was employed to differentiate between vaccine-like and wild-type poliovirus isolates. Single-tube, single step reverse-transcription PCR (RT-PCR) was performed in a final volume of 100 μl using the following conditions: 5 μl RNA, 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 50mM KCl, 200 μM of each deoxynucleotide triphosphate (dNTP), 10 units RNase inhibitor (Boehringer Mannheim), 5 units AMV-reverse transcriptase (Boehringer Mannheim), 2.5 units *Taq* polymerase (Boehringer Mannheim), and 20 pmol of each Sabin 1, Sabin 2 and Sabin 3 primer (see Table 3.1). Reactions were set up on ice in order to minimise the formation of non-specific amplification artifacts and primer dimers. Reaction tubes were overlaid with 2 drops of light mineral oil to prevent evaporation during cycling. Thermal cycling for RT-PCR was performed in a Biometra Trioblock using the following programme: 1 cycle of reverse transcription (42 °C, 45 minutes); 1 cycle of denaturation (95 °C, 3 minutes); 30 cycles of denaturation (95 °C, 30 seconds), annealing (56 °C, 45 seconds) and elongation (72 °C, 1 minute); and 1 final cycle of elongation (72 °C, 7 minutes). Reactions were held at 4 °C after completion of the programmed cycles. Amplified products were detected by electrophoresis through 10% polyacrylamide gels stained with ethidium bromide (0.1 μg/ml). The presence of primer pairs specific for each of the 3 poliovirus serotypes in the reactions permitted the concurrent detection of all 3 types in a single specimen.

3.2.4 VP1/2A poliovirus-specific RT-PCR for rapid poliovirus identification and generation of sequencing templates

In order to facilitate sequencing of the 150 bp VP1/2A junction region used for molecular epidemiological studies, a 293 bp DNA fragment which encompasses this target region was generated by RT-PCR. Primers were selected in order to permit selective amplification of the 3 serotypes of poliovirus, but not of the non-polio enteroviruses. Primer 2A, previously described by Rico-Hesse *et al.* 1987 was used as the reverse primer (Table 3.1). Forward

primer PVPCR (Table 3.1) was chosen from a conserved region of the poliovirus VP1 region after analysis of published Sabin and wild-type sequences (Genbank). The primer pair PVPCR/2A was checked for internal and 3' end complementarity, and the specificities of these primers for polioviruses were checked against the Genbank and EMBL databanks. No homology with other non-polio enteroviruses for which sequences were available was detected.

RT-PCR was performed in a single step in 100μl reaction volumes. A reverse transcription/PCR master mix containing 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 20 pmol of each primer, 200μM of each deoxynucleotide triphosphate, 10 units RNase inhibitor (Boehringer Mannheim), 5 units AMV-reverse transcriptase (Boehringer Mannheim) and 2.5 units *Taq* polymerase (Boehringer Mannheim) for each reaction was prepared and aliquoted in 95μl volumes into thin-walled 0.5ml reaction tubes held on ice. Reaction mixtures were overlaid with 2 drops of light mineral oil. RNA templates were heat-denatured (80°C, 2 minutes), and snap-cooled on ice for 5 minutes. Five microlitres of denatured template were added to each reaction tube.

Reverse transcription and amplification was performed using the following programme on a Biometra Trioblock thermal cycler: 1 cycle of reverse transcription (42 °C, 45 minutes); 1 cycle of denaturation (95 °C, 3 minutes); 30 cycles of denaturation (95 °C, 30 seconds), annealing (56 °C, 45 seconds) and elongation (72 °C, 1 minute); and 1 final cycle of elongation (72 °C, 7 minutes). Reactions were held at 4 °C after completion of the programmed cycles. Amplified products were detected by electrophoresis through 2% agarose gels containing ethidium bromide (0.1 μg/ml).

In order to check the integrity of the RNA used for amplification and the specificity of the poliovirus-specific primers, an enterovirus-specific RT-PCR was also established. Broadly reactive primers located in the conserved 5' NCR (Rotbart, 1990) were chosen to amplify a 154 bp target region. The reaction and cycling conditions were as described above for polio specific PCR, with the exception that a 50 °C annealing temperature was used for the general enterovirus amplification.

3.2.5 Sequencing of the VP1/2A region

Prior to sequencing, PCR products were purified using the Mermaid kit (Bio101). Forty microlitres of amplified product were electrophoresed on a 1.5% low gelling temperature Biogel (Bio101) in Mermaid buffer, and the target bands excised from the gel. DNA was

purified from the agarose slices according to the manufacturers' instructions, and eluted in a final volume of 20μl distilled water. Five microlitres of purified DNA were used for sequencing. Dideoxy sequencing was performed using the Sequenase PCR-product sequencing kit (United States Biochemicals) with ^{35}S-dATP and primer 2A as the sequencing primer. All sequence data obtained was confirmed by repeat sequencing with primer PVPCR. Sequencing products were resolved on 8% acrylamide gels containing 7M urea, and visualised by autoradiography. Sequence data was analysed using DNASIS 2.5 software (Hitachi).

3.2.6 Phylogenetic relationships between poliovirus strains

The VP1/2A sequences (150 bases from position 3296 to 2445) were aligned and a matrix of genetic distances between strains was generated by performing pairwise comparisons between all sequences using the DNADIST programme from the PHYLIP phylogenetic inference package (Felsenstein, 1993). Genetic relationships between poliovirus isolates were then determined by constructing, by the method of least squares using the KITSCH programme from PHYLIP, dendrograms representing graphically the extent of sequence divergence between strains. The reproducibility of the resulting branching patterns was assessed by carrying out bootstrap analysis on 100 replicates of the initial sequence datasets, and determining, using the CONSENSE programme in the PHYLIP package, the number of times out of 100 that a particular set of strains grouped together. Bootstrap values of ≥70% were considered significant, as bootstrap proportions of ≥70% usually correspond to a probability of ≥95% that the corresponding group is real (Hillis and Bull, 1993). In the dendrograms, the distance along the horizontal X-axis to the node connecting any two strains is a measure of the extent of sequence divergence between those strains. A genotype was defined as a group of viruses showing at least 85% nucleotide sequence similarity across the 150 bp VP1/2A interval (Rico-Hesse *et al.*,1987), and within genotypes, direct epidemiological links were defined by a minimum nucleotide similarity of 98% between strains (Rico-Hesse *et al.*, 1987).

3.2.7 The HMA as a tool for poliovirus intratypic differentiation and genotype analysis

For HMA analysis, a 480 bp fragment, encompassing the region coding for the N-terminal half of capsid protein VP1, was amplified using the VP3-1 and VP1-1 primers described by

Balanant *et al.* (1991). Reverse transcription was carried out for 45 minutes at 42°C in a 25μl reaction containing 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 1 mM DTT, 0.5 mM of each deoxynucleotide triphosphate, 20 units RNase inhibitor (Boehringer Mannheim), 100 units MuMLV reverse transcriptase (BRL), 10 pmol of the downstream primer VP1-1 and 5μl RNA. Upon completion of cDNA synthesis, reactions were heated to 95 °C for 3 minutes to denature the reverse transcriptase, and cooled on ice. PCR was performed using the whole 25 μl volume of cDNA mixture as template. Seventy-five microlitres of PCR master mix containing 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.01% gelatin, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 200 μM of each deoxynucleotide triphosphate, 20 pmol each of primers VP3-1 and VP1-1, and 2.5 units of *Taq* polymerase (Boehringer Mannheim) were added to the cDNA mixture. Amplification was performed using the following programme on a Stratagene Robocycler Gradient 40 automatic cycler: 1 cycle of 95 °C for 2 minutes; 40 cycles of 95 °C for 1 minute, 42 °C for 1 minute, and 72 °C for 2 minutes; 1 cycle of 72 °C for 8 minutes. Amplified products were detected by electrophoresis through 1.5% agarose gels containing ethidium bromide (0.1 μg/ml).

Heteroduplex formation between amplicons from different poliovirus strains was examined by using amplicons from the vaccine strains Sabin 1, 2 and 3 as reference reagents. Hybridization reactions were performed by combining, in a 500μl thin-walled microcentrifuge tube containing 1.1μl of 10X heteroduplex annealing buffer (1M NaCl, 0,1 M Tris pH 7.8, 20 mM EDTA), 5μl of the 480 bp VP1-1/VP3-1 amplicon from each poliovirus isolate with 5μl of the amplified fragment from the Sabin 1, 2 or 3 reference strains. Control reactions consisted of mixing 5μl of amplified product with 5μl of distilled water. Reactions were overlaid with one drop of light mineral oil, denatured at 95 °C for 4 minutes, and immediately cooled in an ice-water bath for 5 to 10 minutes. Electrophoresis loading dye (3.3μl of a 10X dye containing 0.25% bromophenol blue, 0.25% xylene cyanol and 25% Ficoll 400 in distilled H_2O) was added to each tube, and the entire reaction volume was electrophoresed through 7.5% acrylamide gels (acrylamide: bisacrylamide 37.5:1), 1.5 mm in thickness, at 150V in 0.6X TBE. Electrophoresis was stopped when the xylene cyanol dye front had migrated to within an inch from the bottom of the gel (approximately 5 hours depending on the apparatus used). Gels were stained in a solution of 0.1 μg/ml ethidium bromide in 0.6X TBE for 2 hours and visualised under UV light in order to detect the presence of heteroduplex bands.

Table 3.1 Primers for poliovirus amplification, sequencing and HMA

Primer	Position in genome*	Sequence (5'→ 3')	Amplicon size (bp)	Reference
Sabin-specific PCR				
Sab1-1 (reverse)	VP1 2584-2601	TCC ACT GGC TTC AGT GTT	97	Yang *et al.*, 1991
Sab1-2 (forward)	VP1 2505-2523	AGG TCA GAT GCT TGA AAG C		" "
Sab2-1 (reverse)	VP1 2580-2595	CGG CTT GTG TCC AGG C	71	" "
Sab2-2 (forward)	VP1 2525-2544	CCG TTG AAG GGA TTA CTA AA		" "
Sab3-1 (reverse)	VP1 2571-2589	AGT ATC AGG TAA GCT ATC C	53	Yang *et al.*, 1992
Sab3-2 (forward)	VP1 2537-2553	AGG GCG CCC TAA CTT TG		" "
Polio-specific PCR				
2A (reverse)	2A 3508-3527	AAG AGG TCT CTA TTC CAC AT	290-293	Rico-Hesse *et al.*, 1987
PVPCR (forward)	VP1 3235-3254	GTC AAT GAT CAC AAC CCA C		
General Enterovirus PCR				
Ent1 (reverse)	5'NCR 584-603	ATT GTC ACC ATA AGC AGC CA	154	Rotbart, 1990
Ent2 (forward)	5'NCR 449-474	CCT CCG GCC CCT GAA TGC GGC TAA T		" "
N-terminal region of VP1				
VP1-1 (reverse)	VP1 2880-2901	GAA TTC CAT GTC AAA TCT AGA	472-480	Balanant *et al.*, 1991
VP3-1 (forward)	VP3 2422-2441	TTT GTG TCA GCG TGT AAT GA		" "

* Position in the genome of Sabin 1. Numbering according to Toyoda *et al.*, 1984.

3.3 RESULTS AND DISCUSSION

3.3.1 Intratypic differentiation by Sabin-specific RT-PCR

The Sabin strain-specific PCR technique described by Yang *et al.*, (1991) was employed to determine the vaccine-like or wild-type identity of the polioviruses characterised in this study. The choice of this method in preference to methods based on antigenic differences between vaccine-related and wild-type strains was influenced by the high specificity and sensitivity of the technique, as well as its rapidity and ease of performance and interpretation. The principle of the Sabin-specific PCR is that vaccine-related strains are positively identified by a positive amplification reaction with the Sabin-specific primers, while wild-type polioviruses are identified by their failure to amplify with these primers (Figure 3.1). The primers are spaced along the genome in order to generate amplification products of 97 bp for Sabin 1, 72 bp for Sabin 2 and 54 bp for Sabin 3.

The N-terminal region of VP1 was selected for Sabin-specific amplification for the following reasons: nucleotide and amino acid sequences within this region vary widely among different poliovirus genotypes; this interval resides within the capsid region and is flanked by VP2, VP3 and VP1 sequences encoding type-specific antigenic sites, so that identification based on PCR correlates strongly with serotype; the structural domain encoded by this interval is internalised within the poliovirus virion, so that evolution of this domain does not appear to be driven by strong immune selection (Yang *et al.*, 1991).

The Sabin-specific primer pairs have been found to be highly specific for the Sabin vaccine strains and for vaccine-related clinical isolates. The difference in amplicon sizes for Sabin 1, 2 and 3 templates allows clear resolution of the individual amplicons by polyacrylamide gel electrophoresis, and thus by simultaneously including all three Sabin primer pairs in amplification reactions, the composition of vaccine strain mixtures can be rapidly determined. The Sabin-specific primers do, however, also efficiently amplify laboratory strains widely used as reference wild polioviruses: type 1 reference strains Mahoney and Brunhilde, and type 3 reference strains Leon and Saukett cross-amplify with the Sabin 1 and Sabin 3 primers respectively, yielding amplicons identical in size to those of the corresponding Sabin strains. This cross-reactivity is the result of the high degree of sequence similarity between the reference strains: Sabin 1 and Mahoney (>99% sequence similarity) and Sabin 3 and Leon (99.9% sequence similarity) exhibit identical primer binding sequences; Brunhilde exhibits only 1 mismatch within the Sabin 1 primer binding site; and

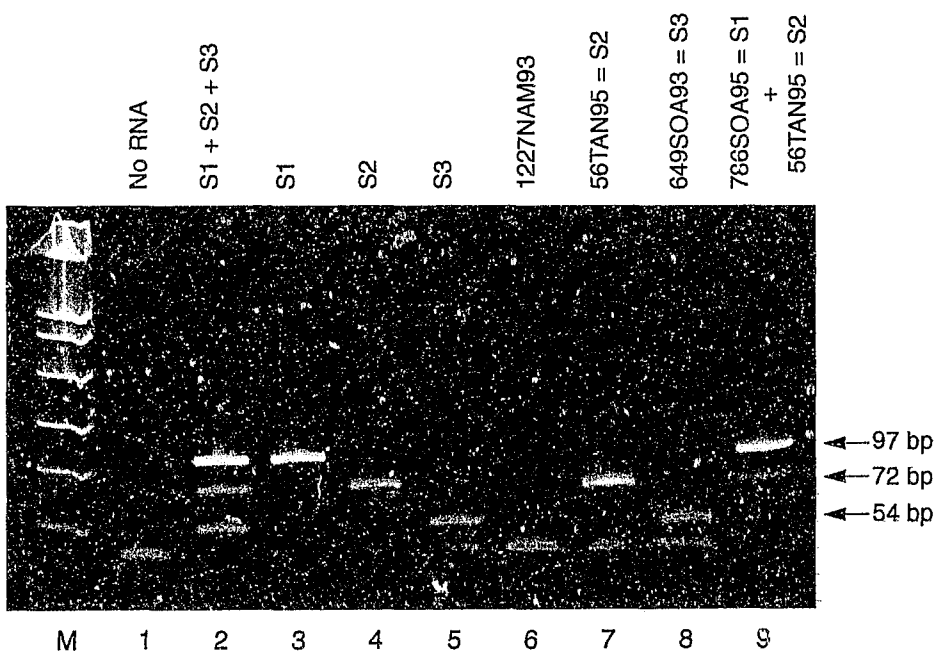

Figure 3.1 RT-PCR amplification with Sabin-specific primers. Sabin reference strains (lanes 2-5) and vaccine-like clinical isolates (lanes 7-9) yield amplicons of the expected sizes (Sabin 1 = 97 bp; Sabin 2 = 72 bp; Sabin 3 = 54 bp). The wild-type isolate 1277NAM93 fails to amplify with the Sabin-specific primers. M = *TaqI* digest of ΦX174.

Saukett exhibits 2 and 3 mismatches within the Sab3-1 and Sab3-2 sites respectively. The type 2 reference strains MEF-1 and Lansing do not amplify with the Sabin 2-specific primers, as both strains exhibit multiple mismatches within the Sabin 2 primer binding sites (Yang *et al.*, 1991). In this study, cross-amplification between Sabin 1 and Sabin 3 primers and the P1 Mahoney and P3 Saukett wild reference strains was also observed. However, in contrast to the findings of Yang *et al.* (1991) that MEF-1 did not cross-amplify with Sabin 2 primers, in our experience, cross-amplification was observed, although at a lower efficiency, (indicated by reduced product yield relative to the yield of the corresponding Sabin 2 template). These results may be explained by the fact that the slightly less stringent amplification conditions used in this study compared to those described by Yang *et al.* (1991) (optimization of the reaction conditions resulted in the choice of 56 °C for primer annealing during amplification, compared to 60 °C used by Yang *et al.*, (1991), since higher annealing temperatures resulted in a loss of amplification signal from the Sabin 2 and Sabin 3 templates) permitted sufficient annealing between the Sabin 2 primers and MEF-1, despite the multiple mismatches between their corresponding sequences.

The Sabin-specific primers have been shown to not cross-react with sequences of wild-type polioviruses most frequently obtained as clinical isolates, when tested against a panel of wild polioviruses representing different genotypes with widely separate geographic origins Despite the wide range of sequence variability among these wild isolates, the Sabin-specific primers did not support amplification of their sequences (Yang *et al.*, 1991). For the present study, several hundred clinical specimens were typed by Sabin-specific PCR. All wild-type isolates whose wild-type identity was confirmed by sequence analysis failed to amplify with the Sabin-specific primers, thus confirming their specificity for vaccine-like templates. To confirm the correct typing of vaccine-related poliovirus strains isolated from clinical specimens, 30 isolates (10 of each serotype) typed as vaccine-like by Sabin-specific PCR were randomly selected and sequenced. In all cases, sequence analysis confirmed the Sabin-specific PCR results.

A major drawback of the Sabin-specific PCR for intratypic differentiation is its inability to identify the wild-type component in isolates containing mixtures of vaccine-like and wild-type strains. Additional techniques which enable the identification of mixtures thus need to be used in conjunction with the Sabin-specific PCR. In a recent WHO-initiated collaborative study, several methods for poliovirus intratypic differentiation were evaluated independently in 4 different laboratories (van der Avoort *et al.*, 1995). Each method was based on a different principle: (i) ELISA with polyclonal cross-adsorbed antisera (PAb-E); (ii) neutralisation using type-specific monoclonal antibodies (MAb-N); (iii) RFLP; (iv) probe hybridisation with Sabin-specific probes (ProHyb) and (v) Sabin-specific PCR. The most

consistent results were obtained by the PAb-E assay, which was also the only assay able to correctly detect 2 mixtures of wild and Sabin-like type 2's. However, 2 polio type 1's clearly identified as wild type by sequence analysis were consistently characterised as Sabin-like by PAb-E. The most likely explanation for this phenomenon is that the 2 strains represent wild polio 1's in which the major antigenic determinant had mutated to Sabin-like by genetic drift. The MAb-N test, while making good use of existing resources, experience and skills in most laboratories where poliovirus isolates are serotyped by neutralisation, is time consuming, requiring a 5- to 7- day incubation period. In the collaborative evaluation, several vaccine strains were typed as wild-type using this technique. While this may be a fail-safe result in the context of screening for wild poliovirus in a community vaccinated with OPV, the incorrect identification of wild type viruses in communities which have been declared polio-free has major programmatic implications. In the RFLP test, isolates are typed by comparison of digestion profiles of amplified targets to those of reference strains. This technique is thus not only useful for intratypic differentiation, but also allows rapid characterisation of genetically related strains. A drawback of this technique is that rapid modification of the regions coding for the exposed surfaces of the virion may occur under conditions of immune pressure, which may result in the loss or addition of restriction sites. The ProHyb assay was found to be a very reliable method for intratypic differentiation, and its filter hybridization format offers a particular advantage in the analysis of large number of isolates. This feature, however, can be a disadvantage when few isolates require rapid typing. The PCR assay, while being very sensitive and correctly identifying single isolates, failed to detect the wild-type component in isolates containing mixtures of vaccine-like and wild type-strains. Since none of the methods evaluated exhibited a performance record of 100%, the WHO has recommended, on the basis of the study results, that intratypic differentiation should be performed by at least two WHO-approved methods, preferably based on different antigenic and genomic properties between vaccine-like and wild-type viruses. This recommendation minimises the risk of incorrect or incomplete results because of the emergence of antigenic or genetic variants of polioviruses (van der Avoort *et al.*, 1995). (In keeping with this recommendation, since 1997 the PAb-ELISA has been implemented in addition to Sabin-specific PCR for poliovirus intratypic differentiation at the NIV).

A second drawback of the Sabin-specific PCR is that the presence of inhibitors in vaccine-like isolates may inhibit amplification, and thus lead to the incorrect interpretation of results. To avoid this possibility, positive control PCR 's in which enterovirus- or poliovirus-specific primers must be included for each isolate. In the course of this study, 2 sets of amplification reactions were run for intratypic differentiation of isolates: a Sabin-specific PCR and in addition a poliovirus-specific amplification PCR (for method see section 3.2.4; discussed

in section 3.3.3). The Sabin-specific PCR results (positive = vaccine-like; negative = wild type) were only accepted if the result of the corresponding polio-specific PCR was positive.

3.3.2 The HMA as a tool for poliovirus intratypic differentiation and genotype analysis

As discussed in section 3.3.1, all of the existing methods for poliovirus intratypic differentiation have drawbacks such that rapid and efficient characterisation of poliovirus strains using one method alone is currently not possible. Recently, a novel technique, the HMA, based on the detection of mismatches between double stranded DNA containing one strand of known sequence composition and a complementary strand with an altered nucleotide sequence, has been described (Delwart *et al.*, 1993). The mismatched bases between the two strands cause structural distortions (bulges) in the resulting double-stranded molecule (heteroduplex). These structural distortions result in a decrease in the electrophoretic mobility of the heteroduplex compared to that of double- stranded DNA made up of perfectly complementary strands (homoduplexes). Thus sequence differences between strains can be detected simply by noting a shift in the electrophoretic mobility of heteroduplexes formed following the denaturation and reannealing of PCR-amplified fragments from a reference strain of known sequence and that of an unknown type. HMA is particularly suited to the analysis of rapidly evolving genetic systems such as RNA viruses, and has been used successfully for HIV subtype determination (Delwart *et al.*, 1993) for quasispecies analysis of HIV (Delwart *et al.*, 1994) and HCV (Wilson *et al.*, 1995), and recently for measles strain identification (Kreis and Whistler, 1997). Because the HMA is based on the difference in nucleotide composition between related strains, and sequence differences between poliovirus vaccine strains and wild-type viruses have been well documented (Nomoto *et al.*, 1982; Moss *et al.*, 1989; Pollard *et al.*, 1989; Stanway *et al.*, 1984; Rico-Hesse *et al.*, 1987; Kew *et al.*, 1990), an investigation into the suitability of the HMA as a reliable tool for poliovirus intratypic differentiation was thus carried out.

Forty seven poliovirus isolates (Table 3.2) were included in this study. Strains were selected to reflect the genomic variability between strains and represent a broad sample of laboratory reference strains, vaccine-related strains from clinical isolates, and contemporary wild-type isolates representing different genotypes. The Sabin 1, 2 and 3 polio vaccine strains were used as reference strains for HMA analysis of type 1, 2 and 3 strains isolates respectively.

Table 3.2 Poliovirus strains selected for the heteroduplex mobility assay

Poliovirus Strain		Genotype	Heteroduplex formation with reference strain[a]
Type 1	Sabin 1		-
	Mahoney		-
	1325SOA82	South African	+
	1531SOA82	(1982)	
	1677SOA82		+
	5918SOA82		+
	855SOA85		+
	2947SOA85	Southern African	+
	181SOA86	(1985-1989)	+
	307SOA88		+
	364SOA88		+
	441SOA89		+
	2SOA83	Middle Eastern	+
	1406SOA83		+
	73SOA83	West African	+
	1050SOA84		+
	1177NAM93		+
	1227NAM93		+
	1263NAM93		+
	1465NAM93		+
	6CAR94		+
	46CAR94		+
	72NAM95		+
	76NAM95		+
	266NAM95		+
	1199TAN95	East African	+
	50ZAM95		+
	59ZAM95		+
	564TAN95	P1 vaccine-like + wild-type	+
	432SOA92	P1 vaccine-like	-
	238NAM94	P1 vaccine-like	-
	786SOA95	P1 vaccine-like	-
Type 2	Sabin 2		-
	MEF-1		
	2307SOA81	Unknown wild-type	+
	isolate from Vietnam 1986	Unknown wild-type	+
	isolate from Cameroon 1986	Unknown wild-type	+
	52TAN95	P1 + P2 vaccine-like	+
	56TAN95	P2 vaccine-like	-
	1502SOA94	P2 vaccine-like	-
Type 3	Sabin 3		-
	Saukett		-
	isolate from Vietnam 1986	Unknown wild-type	+
	isolate from Spain 1986	Unknown wild-type	+
	735SOA95	P3 vaccine-like	-
	339NAM94	P3 vaccine-like	-
	649SOA93	P3 vaccine-like	-

[a] Sabin 1, 2 and 3 strains were used as reference strains for poliovirus types 1, 2 and 3 respectively.

The HMA was performed as described in section 3.2.7: equal quantities of the 480 bp VP1-1/VP3-1 amplicon from the reference vaccine and wild-type isolates were mixed, denatured, reannealed and electrophoresed through 5% polyacrylamide gels in order to detect heteroduplexes.

An important criterion for heteroduplex formation is both the number and the position of mutations within the region for analysis. Gaps formed by unpaired nucleotides have a stronger effect on heteroduplex mobility than mismatched nucleotides (Hsieh and Griffith, 1989). In addition, the exact sequence of base-paired nucleotides adjoining a gap, resulting in base stacking close to the helix distortion, also impacts on the final structure and resulting mobility (Wang and Griffith, 1991). The position of unpaired or mismatched nucleotides also markedly affects mobility - the more centrally located the mismatched/unpaired nucleotides are relative to the extremities of the fragment, the greater the mobility retardation (Bhattacharyya and Lilley, 1989). In this study we chose to use the 480 bp fragment described by Balanant *et al.* (1991) for RFLP analysis. This genome fragment encodes the amino terminus of VP1, including antigenic site 1, and is highly variable. This region has been shown to be effective as a polymorphic fragment for strain identification by RFLP (Balanant *et al.*, 1991), and we therefore thought that this target region could also serve as a potential target for heteroduplex shift analysis. The primer binding sites flanking the variable region are relatively well conserved between the 3 poliovirus serotypes, and by maintaining low stringency during PCR (annealing at 42°C), it was possible to amplify the genomes of all wild-type polioviruses tested. Sequence analysis of the amplified 480 bp products from wild-type isolates indicated that mutations were scattered uniformly throughout the target region (see Chapter 6). Sequence variation among isolates within the same genotype ranged between 0% and 12%. There was approximately 20% sequence divergence between isolates belonging to different genotypes, and all the wild-type sequences differed from the Sabin reference strains by approximately 20%. The degree of variation required for good discrimination of heteroduplexes has been shown to be within the range of 5% - 25% (Delwart *et al.*, 1995). Since the extent of sequence divergence between wild-type and reference templates was well within this range, it was thus expected that detectable heteroduplexes would be formed when wild-type polioviruses were allowed to reanneal with the reference vaccine strains. A semiquantitative relationship has been shown to exist between the electrophoretic mobility of heteroduplexes and the level of sequence divergence between the reannealed strands, with more closely related strains displaying a marked reduction in mobility compared to more distantly related strains (Delwart *et al.*, 1993, 1994). Because the extent of divergence from the Sabin strains was similar for all poliovirus genotypes used in this study, very large differences in mobility shifts were not expected between different genotypes.

The results of HMA analysis of poliovirus strains are presented in Figures 3.2 and 3.3. Heteroduplexes were detected in all reactions which contained DNA from isolates which differed in sequence from the Sabin reference strains. Three distinct banding patterns were observed:

(i) homoduplex bands, with indistinguishable or similar mobilities, produced by reannealing of completely complementary strands

(ii) two sharp heteroduplex bands, of reduced mobility compared to the homoduplexes, formed by annealing of non-identical single strands from two different sources to each other.

(iii) discrete multiple bands, migrating with a mobility of about 40% that of the homoduplexes, corresponding to collapsed single stranded fragments that failed to reanneal with a complementary strand. The sharp uniform positioning of these bands makes them useful for visual comparison of heteroduplex patterns.

The heteroduplexes, and to a lesser degree the single stranded DNA, were found to fluoresce less intensely than the homoduplexes when strained with ethidium bromide, and detection of the heteroduplexes was enhanced by leaving gels to stain for at least 2 hours. The decreased staining intensity of heteroduplex bands, compared to that of homoduplexes, is possibly a result of their low frequency of formation (about 50% of the denatured strands reanneal to form heteroduplexes, Nagamine *et al.*, 1989) and of the low affinity of ethidium bromide for both single stranded sequences and double stranded sequences with many unpaired bases. Staining of the gels with SybrGreen 1 (Molecular Probes Europe), which has been reported to exhibit a sensitivity of detection 25 times greater than that of ethidium bromide, did not increase the intensity of the heteroduplexes.

Initially, to determine whether HMA analysis could be employed to differentiate between the 3 Sabin serotypes, the 3 Sabin reference strains were reannealed with each other (S1 + S2; S1 + S3; S2 + S3). Unique, discrete heteroduplex bands, with shift patterns of reduced mobility compared to the single-stranded DNA, were produced (Figure 3.2, lanes 4-6); the patterns were highly reproducible and could be used to differentiate between the 3 Sabin serotypes. These highly reproducible patterns reflect the remarkable genetic stability of the Sabin vaccine strains. These 3 reactions were therefore subsequently included in all assays as positive controls for heteroduplex formation. The marked reduction in electrophoretic mobility of heteroduplexes formed between different poliovirus serotypes compared to those formed between different strains of the same serotype (Figure 3.2) is very likely due to the greater extent of sequence divergence between serotypes (approximately 30% between the 3 Sabin serotypes; Toyoda *et al.*, 1984) than that within individual serotypes, and to the presence, within the amplified targets, of small insertions and deletions characteristic of serotype.

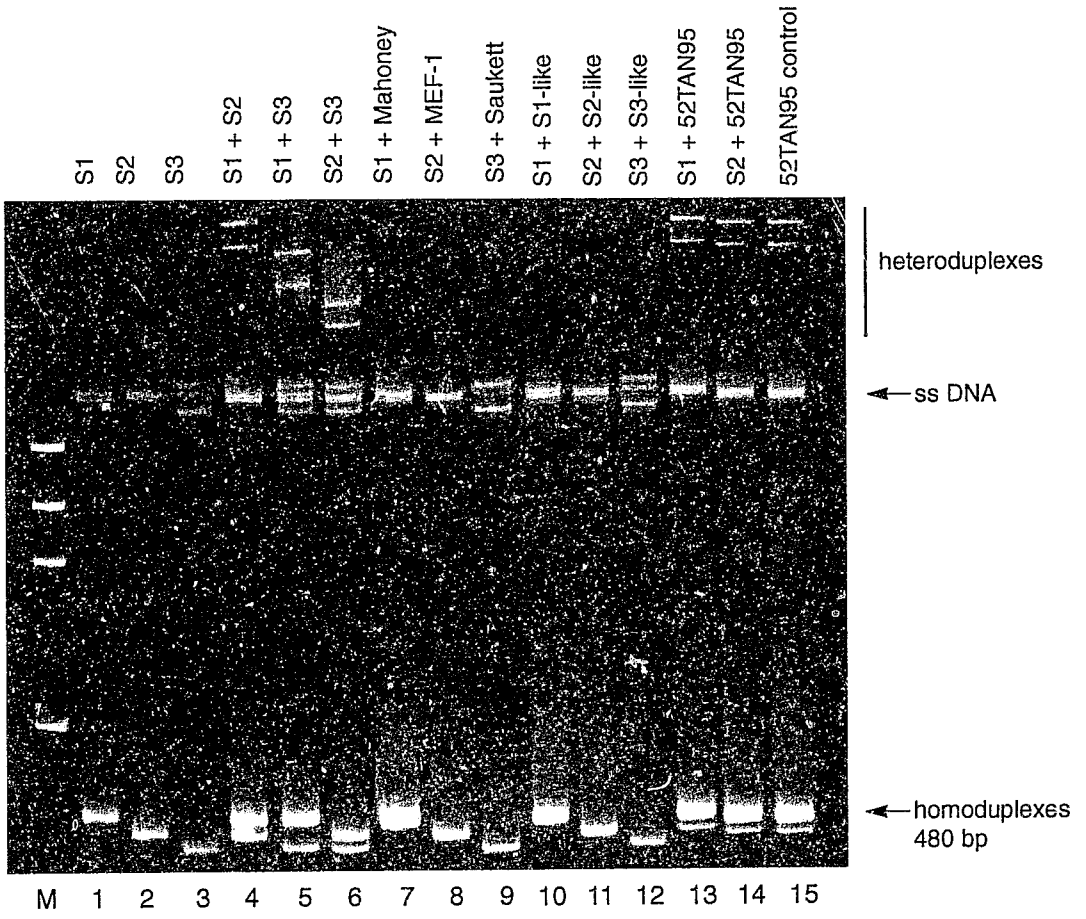

Figure 3.2 Heteroduplex mobility assay with poliovirus reference vaccine and wild-type laboratory strains, and with vaccine-like clinical isolates. Lanes 4-6 show the formation of heteroduplexes, of reduced mobility with respect to the ssDNA, between the 3 different Sabin poliovirus serotypes. Lanes 7-9 show the absence of heteroduplex formation between Mahoney, MEF-1 and Saukett strains and the respective Sabin 1, Sabin 2 and Sabin 3 reference strains. Lanes 10-12 show the absence of heteroduplex formation between vaccine-like clinical isolates and their respective Sabin reference strains (lane 10, 786SOA95 = Sabin 1; lane 11, 56TAN95 = Sabin2; lane 12, 649SOA93 = Sabin 3). The presence of the characteristic S1+S2 bands present in lanes 13, 14 and 15 show the presences of both Sabin 1 and Sabin 2 vaccine-like strains in isolate 52TAN95.
MWM; HaeIII digest of ΦX174.

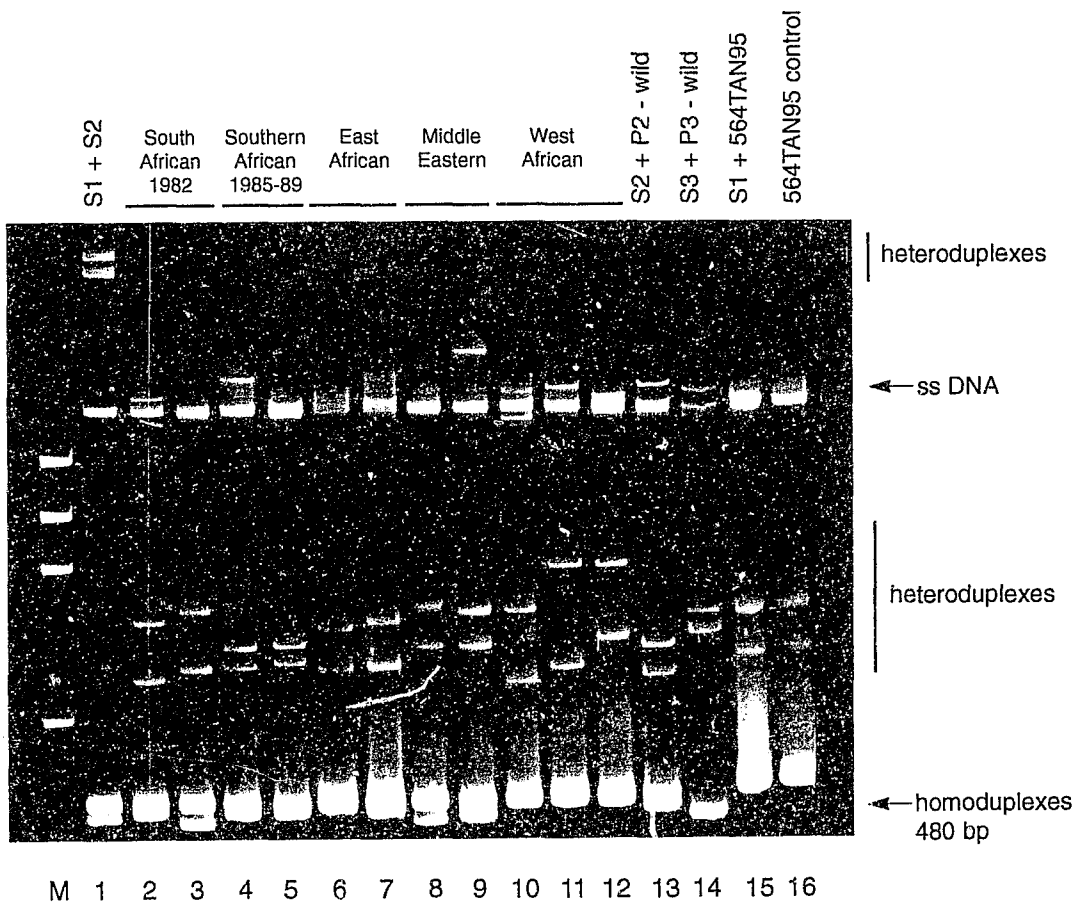

Figure 3.3 Heteroduplex formation between Sabin reference strains and wild-type poliovirus isolates. Lane 1, Sabin 1 + Sabin 2; lanes 2 - 3, Sabin 1 + 1531SOA82 and 885SOA85 respectively; lanes 4 - 5, Sabin 1 + 307SOA86 and 364SOA88 respectively; lanes 6 - 7; Sabin 1 + 50ZAM95 and 564TAN95 respectively; lanes 8 - 12; Sabin 1 + 2SOA83, 1406SOA83, 1227NAM93, 72NAM95 and 46CAR94 respectively. Lane 13, Sabin 2 + P2 wild Vietnam; lane 14, Sabin 3 + P3 wild Spain. The presence of both vaccine-like and wild-type strains in isolate 564TAN95 can be seen by the presence of heteroduplex bands when reannealed with the Sabin 1 reference template (lane 15) and in the control reaction containing only 564TAN95 (lane 16). MWM; HaeIII digest of ΦX174.

All clinical isolates that had been previously typed as vaccine-like were also characterised as vaccine-like with the HMA technique (Figure 3.2, lanes 10-12); the absence of heteroduplex formation when these templates were reannealed with the respective vaccine reference strains confirmed their vaccine-like identity. To additionally confirm both vaccine-like origins as well as the serotype of the vaccine-like isolates, these isolates were annealed, in separate reactions, to each of the 3 reference vaccine templates - in each case the characteristic S1/2, S1/3 and S2/3 band shift patterns were generated (results not shown). identity. The presence of more than one serotype of vaccine-related virus in specimen 52/TAN95 (S1 + S2) could be determined by the characteristic S1/2 band shift pattern observed in both the control reaction (no reference DNA) and the reactions containing the S1 and S2 reference templates (Figure 3.2, lanes 13-15). Isolates with mixtures of both wild-type and vaccine-like strains (564/TAN95) could also be identified; characteristic wild-type heteroduplex band shift patterns were present in both the control and reference template reactions (Figure 3.3, lanes 16 and 15 respectively), indicating the presence of both strains in the original isolate.

Mahoney, MEF-1 and Saukett could not be distinguished from their respective Sabin 1, 2 and 3 reference strains using the HMA technique (Figure 1, lanes 7-9). This was not unexpected since the Sabin vaccine strains exhibit limited divergence from their wild-type progenitors: Mahoney differs from Sabin 1 at only 8 (1.6%) positions within the 480 bp target region; MEF-1 differs from Sabin 2 at only 2 positions within the target, and none of the nucleotide differences between Saukett and Sabin 3 is located within the amplified region. The 480 bp amplicons of the wild-type vaccine progenitors and the Sabin reference target sequences do not differ sufficiently for detectable heteroduplexes to form. Differentiation between Mahoney and Sabin 1 and MEF-1 and Sabin 2 respectively may be possible using a more sensitive technique for heteroduplex detection, such as electrophoresis on MDE gels. This matrix significantly improves resolution of conformationally different DNA molecules, permitting the detection of single base substitutions, insertions and deletions. However, the inability to differentiate between these strains is not significant; wild-type polioviruses evolve at a rate of 1 to 2 base substitutions over the entire genome per week (Nottay *et al.*, 1981), and the Mahoney, MEF-1 and Saukett strains, if still in circulation, would have mutated substantially from their original progenitors.

All the wild-type isolates tested, when allowed to reanneal with the Sabin reference targets, produced detectable heteroduplexes clearly distinguishable from the homoduplex and single stranded DNA bands (Figure 3.3). Control reactions, consisting of both the reference and wild-type targets reannealed to themselves, were carried out for all templates, to ensure that any heteroduplex formation was indeed a result of annealing of wild-type and reference

targets, and not due to the presence of non-specific amplification products or inherent multiple sequence types within the individual PCR products. No heteroduplexes were formed when completely complementary targets were allowed to reanneal. The 480 bp targets from wild-type isolates were sequenced to determine the extent of sequence divergence between the different isolates and between wild-type and Sabin strains. Closely related (95 - 100% homology) wild-type isolates belonging to the same genotype displayed very similar heteroduplex shift patterns (Figure 3.3, lanes 3-4 and 7-8), whereas isolates belonging to different genotypes produced different, clearly distinguishable heteroduplex mobility shift patterns. Within the West African group of isolates, however, the mobility shift patterns between isolates were found to differ markedly - this was not unexpected as isolates were chosen to represent highly divergent strains within the same genotype. Isolates from Namibia 1993, Central African Republic 1993/4 and Namibia 1995 all belong to the West African genotype, but fall into 3 distinct groups differing from each other by as much as 12% across the target sequence. Three distinct heteroduplex patterns can be seen within this genotype, reflecting the genetic diversity between variants (Figure 3.3, lanes 10-12). Although within each serotype the heteroduplex patterns appear to be unique for each genotype, the reduction in electrophoretic mobility was similar for all genotypes, emphasising the much stronger positional, rather than numerical effect of mutations within the target region on heteroduplex mobility. The formation of unique patterns characteristic of genotype makes this technique useful not only for differentiation between vaccine-related and wild-type poliovirus isolates, but also for the rapid identification of genetically closely-related strains.

A particularly useful feature of the HMA technique for intratypic differentiation is the ability to detect mixtures of wild-type and vaccine-like strains in a single isolate. Simultaneous detection of both wild-type and vaccine-like strains is not possible with PCR- and probe hybridization- based techniques, unless both vaccine- and genotype-specific reagents are employed for each reaction. In the HMA, characteristic wild-type heteroduplex bands present in both the control and reference template reactions indicate the presence of both strains in the original isolate. As the poliovirus eradication campaign progresses, it will become increasingly important to be able to detect low levels of wild-type viruses from among the more abundant vaccine strains, and the HMA could thus be refined to allow the sensitive detection of very few copies of wild-type virus RNA within a large excess of vaccine virus.

In conclusion, excellent correspondence was obtained between intratypic differentiations based on PCR, sequence analysis and the HMA. Results of this study demonstrate that the HMA can be reliably employed to differentiate between wild-type and vaccine-like polioviruses, and may be a useful supplement to existing diagnostic virological procedures.

3.3.3 Poliovirus-specific amplification for the detection of poliovirus

As the poliomyelitis eradication campaign progresses and efforts are being directed towards the certification of polio-free status in many regions, the sensitive detection of wild-type polioviruses in clinical and environmental specimens will become of increasing importance, and molecular techniques, which offer the advantages of sensitivity and speed, can complement and enhance the existing conventional cell-culture based detection systems. PCR techniques for the detection of enteroviruses in clinical or environmental specimens have been well established (reviewed in Mulders *et al.*, 1995a). However, many of the reagents described cannot differentiate between poliovirus and other non-polio enteroviruses (Hypia *et al.*, 1989; Rotbart, 1990; Thoren *et al.*, 1992; Zoll *et al.*, 1992; Muir *et al.*, 1993) or the specific identification of poliovirus requires additional assays such as probe hybridisation (Chapman *et al.*, 1990) or RFLP analysis (Kammerer *et al.* 1994; Schweiger *et al.*, 1994) following amplification. A number of RT-PCR methods for the specific detection of polioviruses have also been described:

(i) Abraham *et al.* (1993) describe poliovirus primers in the 5' NCR which recognize all polioviruses but type 2 Lansing. However, these primers exhibited cross-reactivity with Echoviruses 11 and 32.

(ii) Two primer pairs, located in the VP2 region, for rapid detection of polioviruses, have been described by Egger *et al.* (1995); however, these two primer pairs, when used individually, did not recognise all poliovirus strains, thus necessitating two sets of reactions for each specimen tested, and the primers also exhibited cross-reactivity with coxsackievirus A21.

(iii) A sensitive RT-PCR assay for the independent detection of the 3 serotypes of the Sabin polio vaccine strains has been developed (Yang *et al.*, 1991). However, this assay allows the positive identification of vaccine-related strains only; identification of wild type strains, which are identified by their non-reactivity with Sabin-specific primers, requires the prior positive identification of poliovirus using conventional culture-based techniques.

(iv) Reagents for the amplification of specific wild-type polio genotypes circulating in Central America (Yang *et al.*, 1992), Colombia (Tambini *et al.*, 1993) the former Soviet Union (Lipskaya *et al.*, 1995) and the Netherlands (Mulders *et al.*, 1995c) have been described. However, these reagents cannot be employed for the identification of all poliovirus wild-types; the specificity of the primer sets allows the identification of only genetically similar wild-type strains, and as such these reagents are best used to confirm the circulation of defined genotypes rather than identify wild-type strains.

(v) Recently, poliovirus group-specific PCR primer sets targeting the VP1 region have been developed for the detection of both vaccine-related and wild-type poliovirus strains of all

three serotypes (Kilpatrick *et al.*, 1996). These primers, one of which binds codons of a poliovirus-specific 7-amino acid sequence within VP1, and the other to codons of a domain thought to interact with the poliovirus cell receptor, contain mixed-base and deoxyinosine residues to compensate for the high codon degeneracy within the target region.
(vi) Kilpatrick *et al.* (1998) have also developed poliovirus serotype-specific PCR primers. Three sets of serotype-specific antisense primers were designed to pair with codons of VP1 amino acid sequences that are conserved within but that differ across serotypes. The sense polarity primers were designed to match codons of more conserved capsid sequences. As for the poliovirus group-specific primers described in the preceding paragraph, the serotype-specific primers contain mixed-base and deoxyinosine residues to compensate for the high rate of degeneracy of the targeted codons.

One of the objectives of this thesis was to investigate, using sequence analysis, the molecular epidemiology of wild-type polioviruses circulating in sub-Saharan Africa (see Chapter 1, Introduction). In early poliovirus sequencing studies (Rico-Hesse *et al.*, 1987), poliovirus RNA templates were sequenced by primer extension sequencing with reverse transcriptase. This required the extraction of large amounts of high-purity viral RNA, a procedure which is both labour-intensive and time-consuming. Since then, PCR technology for the generation of large amounts of DNA templates, which are less difficult to sequence than RNA templates, has become available, and the poliovirus specific PCR developed in this study was originally designed for the purpose of generating poliovirus sequencing templates which encompassed the 150 bp VP1/2A junction region sequenced for molecular epidemiological studies (Rico-Hesse *et al.*, 1987). Primers which were located on either side of the region of interest were thus selected in order to permit selective amplification of the 3 serotypes of poliovirus, but not of the non-polio enteroviruses. Primer 2A, which binds to a conserved region of the poliovirus genome coding for protease 2A , has been shown to bind, albeit as a sequencing primer, to a large number of both vaccine-like and wild-type polioviruses (Rico-Hesse *et al.*, 1987; Kew *et al.*, 1990), and it was thus chosen as the reverse primer (Table 3.1). For the selection of the forward primer, all available poliovirus sequences encoding the VP1 capsid were aligned and scanned for regions of sequence conservation. A 20-base interval from position 3235 to 3254, which appeared to be conserved among all poliovirus strains examined, was identified. Primer PVPCR was designed to bind to this region (Table 3.1). The predicted size of the target region amplified using the PVPCR and 2A primer pair was 293 bp. The primer pair was checked for internal and 3' end complementarity, and the specificities of these primers for polioviruses were checked against the Genbank and EMBL databanks. No homology was observed with any available nucleic acid sequence other than those of polioviruses. Since no homology with available sequences of the non-polio enteroviruses was detected, it was thought that this

primer pair might be useful not only for the generation of DNA sequencing templates, but also for the specific detection of polioviruses. In order to test whether the primers were indeed specific for polioviruses, 125 enteroviral isolates identified as poliovirus by conventional typing techniques, and 39 non-polio enteroviruses were subjected to RT-PCR with primers PVPCR and 2A (Table 3.3). The poliovirus isolates were chosen to represent a range of both vaccine-like and genetically diverse wild-type strains of all 3 serotypes. Although the majority of wild-type strains available were from Africa, they were obtained from widely diverse geographical locations, and sequence analysis (see Chapters 4 and 5) indicated that within each serotype, strains exhibited as much as 25-30% genomic sequence divergence within the amplified region. RNA extracted from non-infected cells used for virus isolation was used as a negative control. All the 125 isolates (100%) typed as poliovirus by tissue culture neutralization were also positive by PCR (Table 3.3). An amplification product of the expected size (290-293 bp) was obtained with all the poliovirus-positive isolates but not with any of the non-polio enteroviruses (Figure 3.4). No cross-reactivity with any of the non-polio enteroviruses tested was observed. Echovirus 32 was not available for testing on our panel of enteroviruses. However, this enterovirus is very rarely isolated at the NIV laboratories (E. Maselesele, personal communication), and in the event of cross-reactivity PCR products can be sequenced to confirm identity. All of the PCR products were sequenced to confirm their identity, and sequence data confirmed that the amplicons were indeed from vaccine-like or wild-type poliovirus genomes (see Chapters 4 and 5).

In order to determine the end-point sensitivity of the poliovirus PCR, tenfold dilutions of polioviruses of known titre were performed (Sabin 1 and wild-type isolate 1277NAM93), RNA was extracted from the diluted specimens, and amplified using primers PVPCR and 2A. A detection limit of 0.4 PFU's per RT-PCR reaction was observed with ethidium bromide staining (Figure 3.5). Since the particle:infectivity ratio is approximately 100:1 for enteroviruses (Grandien *et al.*, 1989), a detection limit of 4 PFU's corresponds to the detection of approximately 40 virus particles per reaction.

To ensure that the negative PCR results obtained with non-polio enteroviruses using primers PVPCR/2A were due to the specificities of the primers and not due to RNA being of unsuitable quality for amplification, all of the non-polio enterovirus isolates were amplified with general enterovirus primers Ent1/Ent2 (Table 3.1). All isolates yielded a positive 154 bp product with the enterovirus-specific primers (Table 3.3), confirming the specificities of the poliovirus-specific primers.

Table 3.3 Specificities of primers for poliovirus amplification

Virus Type	Strain(s)	Number of PCR positive isolates Ent1/Ent2	PVPCR/2A
Polioviruses			
Poliovirus type 1	Mahoney		1
	Brunenders		1
	LSc2ab Sabin		1
	50 isolates from South Africa 1980-1989		50
	40 isolates from Namibia 1993-1995		40
	10 isolates from Central African Republic 1992-1993		10
	2 isolates from Angola 1994		2
	3 isolates from Tanzania 1995		3
	2 isolates from Zimbabwe 1995		2
	1 isolate from Curacao		1
	1 isolate from Brazil		1
	1 isolate from Greece		1
Poliovirus type 2	MEF-1		1
	P712ch2ab Sabin		1
	2 isolates from South Africa 1980-1995		2
	1 isolate from Cameroon		1
	1 isolate from Vietnam		1
	1 isolate from Brazil		1
Poliovirus type 3	Saukett		1
	Leon 12ab Sabin		1
	1 isolate from Spain		1
	1 isolate from Vietnam		1
	1 isolate from Israel		1
Total	**125 isolates**		**125**
Non-polio enteroviruses			
Coxsackievirus type A	1, 2, 3, 4, 6, 7, 8, 9, 10, 11, 12, 13, 14, 16, 17, 18, 19, 20, 21, 22, 23, 24	22	0
Coxsackievirus type B	1, 2, 3, 4, 5, 6	6	0
Echovirus	2, 6, 7, 11, 14, 16, 20, 25, 27	9	0
Enterovirus	70	1	0
Total	**38 isolates**	**38**	**0**

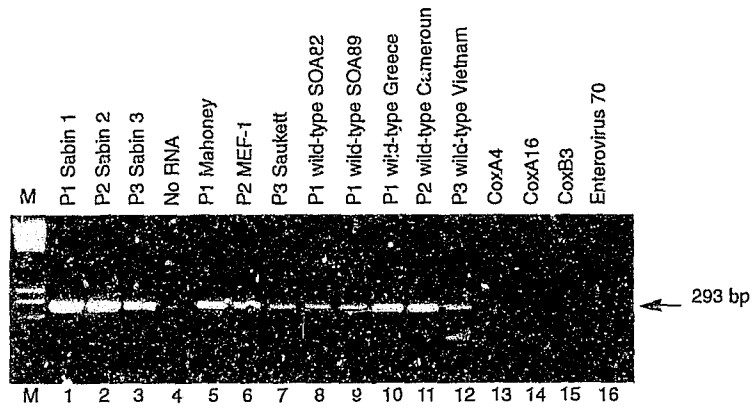

Figure 3.4 Poliovirus-specific RT-PCR amplification. All laboratory reference, vaccine-like and wild-type poliovirus strains amplify with the poliovirus-specific PVPCR/2A primer pair, whereas non-polio enteroviruses fail to amplify. M = *TaqI* digest of ΦX174.

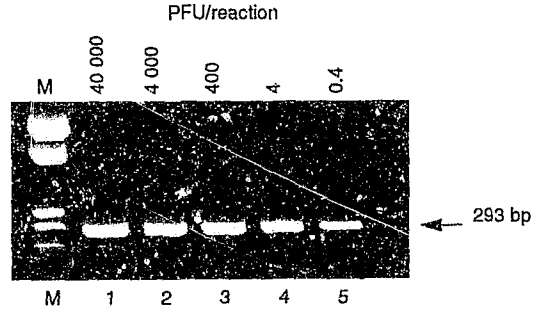

Figure 3.5 Sensitivity of poliovirus RNA detection by poliovirus-specific RT-PCR using the PVPCR/2A primer pair. Serial dilutions of RNA from strain 1277NAM93, representing 0.4 - 40 000 PFU's/reaction, were used as reaction templates. M = *TaqI* digest of ΦX174.

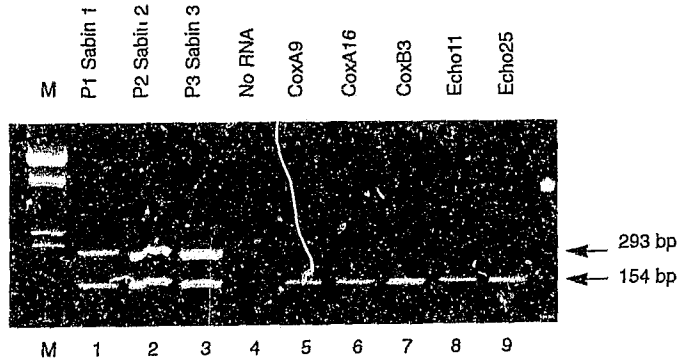

Figure 3.6 Duplex RT-PCR amplification of polioviruses and non-polio enteroviruses with primer pairs) /PCR/2A and Ent1/Ent2. M = *TaqI* digest of ΦX174.

In order to rapidly differentiate between polioviruses and other enteroviruses in a single reaction, the poliovirus-specific primers PVPCR and 2A (amplicon size 293 bp), and the general enterovirus-specific primers Ent1 and Ent2 (amplicon size 154 bp) were combined in a single duplex PCR. An annealing temperature of 50 °C was routinely used for the duplex amplification as higher annealing temperatures failed to amplify the enterovirus targets (results not shown). The use of 50 °C for primer annealing did not affect the specificity of primers PVPCR and 2A. Duplex RT-PCR's were carried out for several of the isolates, and in all cases the 290 bp poliovirus-specific amplicon was detected only in reactions containing poliovirus RNA, whereas the 154 bp enterovirus amplicon was detected in all cases (Figure 3.6). The addition of both enterovirus-specific and poliovirus specific primers in the same reaction can serve as an internal positive reaction control, as both the general enterovirus and poliovirus-specific amplicons will be expected if a specimen is positive for polioviruses. The sensitivity and specificity of the RT-PCR obviates the need for either restriction enzyme analysis or hybridization with poliovirus-specific probes, techniques which increase the time required for a positive diagnosis.

The ability of the PCR assay to detect polioviruses directly in clinical specimens was determined by amplifying RNA extracted from clarified stool specimens. RNA was extracted from 25 stool specimens from which polioviruses had been isolated; all 25 specimens were also positive for poliovirus by PCR (data not shown). Negative controls included 13 stool specimens from which no virus could be isolated, 3 stools from which non-polio enteroviruses were cultured (Coxsackievirus A, Coxsackievirus B2 and Echo 9), and 1 stool from which a reovirus was isolated. All culture-negative and poliovirus-negative controls were also negative for poliovirus by PCR. The amplicon bands obtained when RNA extracted from stool specimens was amplified, were, however, less intense than those obtained from tissue culture supernatants, suggesting either compromised amplification or a very low input RNA concentration. The latter explanation is likely to be correct, since the titre of virus in stool samples is considerably lower than that after amplification in cell culture. The number of virus particles in stool specimens are seldom higher than the minimum number required for the detection of a positive PCR result (approximately 0.4 PFU's per reaction). Egger *et al.* (1995) describe a method for circumventing both the problems of low input titre of virus, below the level detectable by R-T-PCR, and of the presence of PCR inhibitors in stool samples; a combination of short-term (overnight) cell culture followed by RT-PCR. This method could be applied for routine amplification from clinical and environmental samples, where low viral titres and PCR inhibitors could lead to false negative results.

In order to test the ability of the polio-specific PCR assay to complement or possibly replace cell culture for the detection of polioviruses, RNA was extracted and amplified from stool specimens obtained from AFP cases for which no tissue culture results were yet available (Table 3.4). Poliovirus RNA was detected in all 10 stool specimens tested. Four of the 10 specimens yielded

poliovirus upon isolation; no virus could be isolated from the remaining 6 specimens. It is interesting to note that all the above specimens were stored and transported to the laboratory under suboptimal conditions (specimens were stored at room temperature and remained in transit for several weeks), which may have led to viral deterioration below the levels detectable by conventional cell culture techniques.

Table 3.4 Poliovirus strains detected in clinical specimens by poliovirus-specific RT-PCR

Specimen lab number	Clinical specimen	Country of origin	Year specimen obtained	Cell culture isolate	Poliovirus-specific RT-PCR	Genotype *
072/95	stool	Namibia - Rundu	1995	Polio 1	+ ve	West African-C
077/95	stool	Namibia - Rundu	1995	negative	+ ve	" "
078/95	stool	Namibia - Rundu	1995	negative	+ ve	" "
079/95	stool	Namibia - Rundu	1995	negative	+ ve	" "
266/95	stool	Namibia - Rundu	1995	Polio 1	+ ve	" "
341/95	stool	Namibia - Rundu	1995	Polio 1	+ ve	" "
759/95	stool	D.R.Congo - Mbuji Mayi	1995	negative	+ ve	West African-B
762/95	stool	D.R.Congo - Mbuji Mayi	1995	negative	+ ve	" "
Bdd97004	stool	D.R.Congo Bandundu	1997	Polio 1	+ ve	" "
ZAI97-4	stool	D.R.Congo Kinshasa	1997	negative	+ ve	East African

* For detailed description of the genotypes, please see Chapter 5

RT-PCR using poliovirus-specific primers PVPCR and 2A can thus be used to rapidly identify polioviruses and differentiate them from non-polio enteroviruses. The primers PVPCR and 2A can be used in combination with Sabin-specific primers (Yang *et al.*, 1991) for intratypic differentiation between vaccine-like and wild-type polioviruses. In reactions with both sets of primers, the PVPCR/2A reaction acts as a positive control reaction for the positive identification of polioviruses: isolates that amplify as polio-positive, Sabin-negative can then by inference be typed as wild-type. Because the primers span the VP1/2A region commonly sequenced for molecular epidemiological studies, (Rico-Hesse *et al.*, 1987; Kew *et al.*, 1990), genotype analysis of wild-type isolates can rapidly be carried out by directly sequencing the amplicons generated with primers PVPCR and 2A. The identities of all poliovirus strains (vaccine-like and wild-type) analysed during the course of this study (see Chapters 4 and 5) were confirmed using primers PVPCR and 2A - no strain which was typed as poliovirus by conventional techniques failed to amplify with these primers. All VP1/2A sequence data was generated by direct sequencing of the PVPCR/2A amplicons.

3.3.4 Sequence analysis of the VP1/2A interval for determination of phylogenetic relationships between poliovirus strains

In recent years, recognition that genetic relatedness implies epidemiological linkage (Rico-Hesse *et al.*, 1987) has enhanced the precision and reliability of wild poliovirus surveillance. Molecular epidemiological studies have been able to provide answers to questions central to the development of effective strategies for the interruption of transmission, such as the identification of the reservoirs sustaining poliovirus circulation, and identification of transmission pathways and epidemiological links between cases.

The dynamics of the evolution of poliovirus genomes make it possible to utilize for epidemiological purposes information obtained from genomic comparisons. Polioviruses evolve rapidly during replication in humans, at a rate of approximately 1 -2 nucleotide substitutions per week (1 - 2% per year) over the entire genome (Nottay *et al.*, 1981; Kew *et al.*, 1995). Although the rate of evolution is not uniform across the entire genome (the 5' NCR is highly conserved among the enteroviruses, including poliovirus, whereas nucleotide sequence variability in the poliovirus coding region can reach approximately 25% within a serotype (Toyoda *et al.*, 1984; Rico-Hesse *et al.*, 1987), evidence suggests that different poliovirus genomes evolve at similar rates (Nottay *et al.*, 1981; Rico-Hesse *et al.*, 1987). Since most nucleotide mutations have been found to be fixed cumulative in the poliovirus genome, chains of transmission may be inferred by comparison of the number, location and types of mutations found in different poliovirus

genomes. Additionally, the extent of sequence divergence between related wild poliovirus strains can provide an approximate measure of the intervening infections separating cases (Kew *et al.*, 1990).

Several methods have been described for the determination of genetic relationships between poliovirus strains:

1. Oligonucleotide fingerprinting (T1 oligonucleotide mapping) has been used extensively in molecular studies of poliovirus epidemiology (Nottay *et al.*, 1981; Minor *et al.*, 1982; Kew and Nottay, 1984a). Fingerprints are produced by digestion of radiolabelled viral RNA with ribonuclease T1 and electrophoretically separating the resulting oligonucleotide fragments in two dimensions. Longer oligonucleotides (>12 nucleotides), which represent approximately 10 - 15% of the genome, resolve into patterns of spots which are highly characteristic for each RNA sequence. Fingerprinting is, however, highly sensitive to mutations (Aaronson *et al.*, 1982), such that quantitative estimates of relatedness are most reliable when RNA molecules exhibit no more than 5% nucleotide sequence divergence between them. Since, as discussed in the preceding paragraph, the average rate of mutation across the poliovirus genome has been found to be approximately 1-2% per year, fingerprinting is limited to recognising relationships between strains separated temporally by no more than 3 -5 years.

2. Genotype-specific PCR and probe hybridization are rapid methods for determining poliovirus sequence homology within a defined genomic interval. Hybridization probes have been prepared against each of the 3 Sabin strains (De *et al.*, 1995), as well as to each of the 4 major genotypes recently endemic to the America's (da Silva *et al.*, 1981; De *et al.*, 1997). Genotype-specific PCR primers have also been developed for the identification of genotypes from Mexico and Guatemala (Yang *et al.*, 1992), Colombia (Tambini *et al.*, 1993), the former Soviet Union (Lipskaya *et al.*, 1995), and the Netherlands (Mulders *et al.*, 1995c). These reagents, however, do not provide a quantitative measure of the extent of genetic similarity between strains, and are thus best used for rapid screening of genomic similarity to a defined reference strain.

3. An RFLP assay has also been developed for examining genomic variability between poliovirus strains (Balanant *et al.*, 1991). The assay is based on the comparison of restriction profiles produced after electrophoretic separation of digested PCR-amplified fragments which encode for the amino-terminal half of capsid protein V P1. The RFLP approach has been used successfully to characterize the natural genomic variability of poliovirus strains circulating throughout the world (Balanant *et al.*, 1991), and to

characterise strains associated with outbreaks in Israel (Vonsover *et al.*, 1993) and the Central African Republic (Morvan *et al.*, 1997). However, because the assay is highly sensitive to single mutations which may alter restriction enzyme sites, as for oligonucleotide fingerprinting RFLP is best suited for characterisation of closely related strains.

4. Genomic sequence analysis has proved to be the most definitive method for assessing genetic relatedness between strains. Initial surveys of wild poliovirus circulation (Rico-Hesse *et al.*, 1987; Kew *et al.*, 1990) compared a 150-nucleotide interval at the junction of the capsid and non-capsid domains (90 nucleotides encoding the carboxy-terminus residues of the major capsid protein VP1, and 60 nucleotides encoding the amino terminus residues of protease 2A). Since these initial studies, the same approach has been taken to investigate the molecular epidemiology of all 3 serotypes of wild-type polioviruses circulating in Europe, the Middle East and the Indian subcontinent (Mulders *et al.*, 1995b and 1995c); Pakistan (Huovilainen *et al.*, 1995), the former Soviet Union (Lipskaya *et al.*, 1995), China (Zheng *et al.*, 1993), Indochina (Yoshida *et al.*, 1997) and Africa (this study; Morvan *et al.*, 1997), and a large database of VP1/2A sequences of strains representing the major poliovirus genotypes circulating worldwide has been developed. The availability of comparative sequence data from viruses from diverse geographical areas can greatly facilitate molecular surveillance, particularly the identification of wild-type importation and pathways of transmission.

The VP1/2A junction was selected as the region of choice for molecular epidemiological studies for several reasons (Rico-Hesse *et al.*, 1987);
(i) the region spans an interval coding for parts of 2 functionally distinct proteins, capsid protein VP1 and protease 2A. The region encodes both internal domains and a surface polypeptide loop forming part of a potential binding site for neutralising antibodies; any differences in the evolution rates across the two intervals can thus be directly compared.
(ii) the region includes sequences characteristic of serotype, so that the serotype of an isolate can be determined or confirmed by sequence analysis.
(iii) a recombination crossover site has been found at the VP1/2A junction (Rico-Hesse *et al.*, 1987), so that recombinant strains comprising more than one genotype can be detected by sequencing across this region.
(iv) sequence analysis of the carboxy- terminus of VP1 opens the way for higher resolution studies sampling wider intervals, such as those coding for the entire VP1 protein, or complete capsid structures.

The initial sequence analysis surveys reinforced the view that sequence relatedness between poliovirus strains correlates better with the geographical origins of strains, rather than the time of isolation (Rico-Hesse *et al.*, 1987). The geographic clustering of genotypes suggests that local conditions, such as vaccine coverage rates and sanitation levels are more important determinants of transmission than specific properties, such as antigenic structure, of the viruses themselves (Kew *et al.*, 1990).

The extent of genomic divergence between poliovirus strains can be measured by aligning the sequences and determining the percentage of nucleotide sequence similarity between strains. However, direct comparison to only a single reference sequence may obscure relationships between strains, and thus the most practical way to assess the extent of genetic relatedness between any two strains is to perform all possible pairwise comparisons between all strains under investigation, and to generate a graphical representation of the extent of sequence relatedness between strains.

For the purpose of this study, a distance phylogenetic inference method was selected as the method of choice for determining genetic relatedness. Distance methods calculate the genetic distance between all strains based on analysis of all mutations across the entire interval of interest. The resultant genetic distances were then translated into graphical representations of sequence divergence between strains (dendrograms or sequence trees) using a programme which assumes similar mutation rates across the homologous interval of all strains compared, and the presence of a common ancestral strain (KITSCH, Felsenstein, 1993). The criteria for choosing the KITSCH method were satisfied by the previously established observations that different poliovirus genomes evolve at similar rates and that lineages diverge from a common ancestor (Rico-Hesse *et al.*, 1987). Using KITSCH, constraints are imposed on the lengths of branches in the resulting dendrograms, so that the total branch length from the root of the tree to the to any strain is the same (see Chapters 4 and 5 for dendrograms of sequence relatedness between polioviruses in Africa). This allows rapid determination of the genetic relationships between all strains included in each dendrogram, as the distance along the horizontal axis from the branch tip to the node connecting any two strains is a measure of the sequence divergence between those strains. However, because of the constrained branch lengths, the position of each node on the divergence scale represents the average sequence divergence between all strains connected by that node, and is not an absolute measure of the genetic distance between any two strains.

The previously defined criterion that a poliovirus genotype is defined as a group of polioviruses displaying no more than 15% divergence within the 150-nucleotide VP1/2A region (Rico-Hesse *et al.*, 1987) was also used to define genotypes throughout this study. While this single criterion

is subjective, it is sufficiently restrictive to exclude spurious associations and yet include linkages expected on epidemiological grounds and those confirmable by other means (Kew *et al.*, 1990). As the divergence between strains exceeds 15%, the reliability of the relationships established by the dendrograms diminish and must thus be interpreted with caution. Similarly, when mutational differences between strains are very small, sequence analysis of the 150-nucleotides interval alone may underestimate the extent of sequence divergence between closely related strains. Detailed determination of the evolutionary rates and transmission patterns of closely related strains, for example the detailed analysis of epidemics, may thus require the analysis of larger sequence intervals.

4. MOLECULAR EPIDEMIOLOGY OF TYPE 1 POLIOVIRUSES ASSOCIATED WITH EPIDEMICS IN SOUTH AFRICA, 1980 - 1989

4.1 INTRODUCTION

Two extensive epidemics of poliomyelitis occurred in South Africa during the 1980's. In 1982, an outbreak lasting from May to September, during which 260 cases of paralytic polio and 42 deaths were reported, took place in Gazankulu, which was a former self-governing National State in the north-eastern part of the country (Johnson *et al.*, 1984; Saayman *et al.*, 1984). The Gazankulu region now falls mostly within the borders of the Northern Province. A second, even more extensive outbreak, involving 412 paralytic cases and 42 deaths, occurred between December 1987 and November 1988 in Kwazulu-Natal along the south-eastern coast of the country (Schoub *et al.*, 1992; Van Middelkoop *et al.*, 1992). The Gazankulu outbreak was exclusively rural, and the Kwazulu-Natal outbreak was predominantly rural. Type 1 polioviruses were found to be responsible for both outbreaks, which, however, differed markedly from each other with respect to population immunity (Schoub *et al.*, 1992). The Gazankulu outbreak was characterised by a low level of population immunity, with 74% of patients in the epidemic exhibiting no detectable antibodies to polio types 2 and 3, while exhibiting high antibody titres to the causative type 1 virus (Johnson *et al.*, 1984). Surveillance carried out shortly after the epidemic revealed substantial underimmunisation, with vaccine coverage as low as 43% in some rural areas (Johnson *et al.*, 1984). Reduced vaccine potency, probably due to breaks in the cold chain, also contributed to the vaccine failure in the area.

In contrast, the Kwazulu-Natal epidemic occurred under conditions of high population immunity - a serological study of 2-year-old children carried out at the same time and in the same region as the epidemic revealed immunity levels of 84%, 95% and 90% to poliovirus types 1, 2 and 3 respectively, and immunity to all 3 polio serotypes in 76% of children (Schoub *et al.*, 1992). The Kwazulu-Natal epidemic thus appeared to be associated with the buildup of a high viral burden in the area, rather than being solely due to under-immunisation. The build-up of a massive viral burden was probably the result of severe flooding in the area during the previous year, which caused disruptions in basic health services and sanitation (Van Middelkoop *et al.*, 1992). The large amount of circulating virus was able to spread rapidly among the relatively small population of susceptibles in the

community, resulting in an extensive epidemic.

The molecular epidemiology of both outbreaks has been previously investigated by oligonucleotide fingerprint analysis. Using this technique it was found that the molecular epidemiological characteristics of the two outbreaks were different; during the Gazankulu epidemic, several different unrelated strains of viruses were found to be in circulation (Tsilimigras *et al.*, 1984), whereas a single strain of wild-type polio 1, also isolated from cases occurring in other areas of the country, was implicated in the Kwazulu-Natal outbreak (Tsilimigras *et al.*, 1991). Oligonucleotide fingerprinting has been used extensively in molecular epidemiology studies of poliovirus (Kew and Nottay, 1984); however, the technique is very sensitive to small mutational changes and reliable estimates of relatedness can be made only when viral strains show >95% sequence homology (Rico-Hesse *et al.*, 1987). Polioviruses evolve at a rate of approximately 1-2 base substitutions over the entire genome per week, or 1-2% per year (Nottay *et al.*, 1981), and fingerprinting is thus limited to recognising relationships between isolates separated by no more than 3-5 years. Comparative epidemiological investigations of the 2 epidemics thus required the use of a more appropriate technique. Partial genomic sequencing, which has proved to be by far the most definitive technique for determining genetic relationships between polioviruses (Rico-Hesse *et al.*, 1987), was thus selected as the method of choice to investigate in more detail the epidemiological interrelationships between poliovirus strains from the Gazankulu and Kwazulu-Natal epidemics, as well as strains isolated from different regions of South Africa both during the epidemic and inter-epidemic periods.

4.2 MATERIALS AND METHODS

4.2.1 Viruses

The type 1 poliovirus strains isolated in South Africa between 1980 and 1989 and selected for comparative sequence analysis are listed in Table 4.1. This table lists only wild-type strains that were selected for sequence analysis. Strains that were typed as vaccine-like were not analysed further and are thus not listed or discussed in this study. The type 1 reference strain used for alignments was the Sabin 1 poliovaccine strain (P1/Lsc2ab), obtained from Dr. C. Dommann, Vaccine Unit, NIV.

All viruses were isolated and typed as described in section 3.2.1, and intratypic differentiation between vaccine-like and wild-type strains was performed by Sabin-specific PCR as described in section 3.2.3.

4.2.2 Sequence analysis

DNA sequencing templates of the virus strains to be sequenced were generated by RT-PCR using polio-specific primers as described in section 3.2.4. Direct sequencing of the amplified products was performed using the Sequenase PCR-product sequencing kit as described in section 3.2.5.

Genetic relationships between poliovirus strains were established by carrying out pairwise comparisons between all strains, and generating graphical representations of the extent of sequence divergence between strains in the form of a dendrogram (section 3.2.6).
For comparative purposes, sequences of the following isolates, kindly made available to us by Dr. O.M. Kew, CDC, were included in the analyses: 1177/KUW77 (isolate from Kuwait 1977); 1197/JOR78 (isolate from Jordan 1978); 6224/ZIM85 (isolate from Zimbabwe 1985); and 6747/SEN86 (isolate from Senegal 1986).

Table 4.1 Wild poliovirus type 1 strains isolated in South Africa between 1980 and 1989 which were selected for comparative sequence analysis

Strain	Year of isolation	Region from which isolated	Associated with epidemic
D1072	1980	Gauteng	no
D1073	1980	Gauteng	no
D1881	1980	Kwazulu-Natal	no
D1897	1980	Kwazulu-Natal	no
D144	1982	Gauteng	no
D1321	1982	Gazankulu	yes
D1325	1982	Gazankulu	yes
D1326	1982	Gazankulu	yes
D1327	1982	Gazankulu	yes
D1343	1982	Gazankulu	yes
D1393	1982	Gazankulu	yes
D1429	1982	Gazankulu	yes
D1531	1982	Gazankulu	yes
D1532	1982	Gazankulu	yes
D1653	1982	Gazankulu	yes
D1663	1982	Gazankulu	yes
D1677	1982	Northern Province	yes
D1728	1982	Gazankulu	yes
D1902	1982	Gazankulu	yes
D2388	1982	Gazankulu	yes
D2552	1982	Gazankulu	yes
D4238	1982	Gazankulu	yes
D4926	1982	Namibia	no
D5818	1982	Kwazulu-Natal	no
D5859	1982	Kwazulu-Natal	no
D5917	1982	Kwazulu-Natal	no
D5918	1982	Kwazulu-Natal	no
D2	1983	Mpumalanga	no
D16	1983	Mpumalanga	no
D73	1983	Kwazulu-Natal	no
D106	1983	Kwazulu-Natal	no
D698	1983	Gauteng	no
D1170	1983	Northern Province	no
D1406	1983	Northern Province	no
D2661	1983	Kwazulu-Natal	no
D2889	1983	Kwazuku-Natal	no
D1050	1984	Free State	no
D1390	1984	Eastern Cape	no
D2716	1984	Northern Province	no
D2901	1984	Northern Province	no
D2972	1984	Northern Province	no
D3043	1984	Northern Province	no
D710	1984	Kwazulu-Natal	no
D912	1984	Free State	no
D1651	1985	Mpumalanga	no
D1669	1985	Northern Province	no
D1789	1985	Gauteng	no
D2177	1985	Gauteng	no
D2317	1985	Mpumalanga	no
D2497	1985	Kwazulu-Natal	no

Continued on following page...

Table 4.1 ..... continued from previous page

Strain	Year of isolation	Region from which isolated	Associated with epidemic
D2500	1985	Kwazulu-Natal	no
D2501	1985	Kwazulu-Natal	no
D2503	1985	Kwazulu-Natal	no
D2511	1985	Gauteng	no
D2596	1985	North-West Province	no
D2604	1985	Gauteng	no
D282	1985	Kwazulu-Natal	no
D427	1985	Mpumalanga	no
D442	1985	Mpumalanga	no
D477	1985	Northern Province	no
D553	1985	Northern Province	no
D576	1985	Gauteng	no
D635	1985	Mpumalanga	no
D662	1985	Free State	no
D795	1985	Northern Province	no
D850	1985	Kwazulu-Natal	no
D855	1985	Northern Province	no
D18/86	1985	Kwazulu-Natal	no
D1033	1986	Gauteng	no
D1115	1986	Gauteng	no
D206	1986	Gauteng	no
D307	1986	Gauteng	no
D501	1986	Gauteng	no
D516	1986	Gauteng	no
D541	1986	Gauteng	no
D571	1986	Kwazulu-Natal	no
D740	1986	Gauteng	no
D932	1986	Gauteng	no
D1305	1987	Northern Province	no
D1434	1987	Free State	no
D1495	1987	Kwazulu-Natal	no
D1596	1987	Gauteng	no
D1651	1987	Gauteng	no
D1701	1987	Kwazulu-Natal	no
D1711	1987	Gauteng	no
D190	1987	North West Province	no
D259	1987	Namibia	no
D857	1987	Kwazulu-Natal	no
D1048	1988	Eastern Cape	no
D1170	1988	Gauteng	no
D1422	1988	North West Province	no
D1522	1988	Gauteng	no
D1607	1988	Eastern Cape	no
D179	1988	Kwazulu-Natal	yes
D180	1988	Kwazulu-Natal	yes
D181	1988	Kwazulu-Natal	yes
D182	1988	Kwazulu-Natal	yes
D185	1988	Kwazulu-Natal	yes
D202	1988	Gauteng	no
D221	1988	Kwazulu-Natal	yes
D222	1988	Kwazulu-Natal	yes
D250	1988	Kwazulu-Natal	yes

Continued on following page...

Table 4.1 ..... continued from previous page

Strain	Year of isolation	Region from which isolated	Associated with epidemic
D349	1988	Northern Province	no
D359	1988	Kwazulu-Natal	yes
D362	1988	Kwazulu-Natal	yes
D364	1988	Kwazulu-Natal	yes
D366	1988	Kwazulu-Natal	yes
D400	1988	Gauteng	no
D402	1988	Gauteng	no
D420	1988	Gauteng	no
D427	1988	Gauteng	no
D522	1988	Gauteng	no
D699	1988	no details available	unknown
D441	1989	Gauteng	no
D93	1989	Free State	no

4.3 RESULTS

Wild-type polioviruses isolated during the 1982 and 1987- 88 epidemics in South Africa, as well as poliovirus strains isolated during the pre-and post epidemic years, were characterised by partial genomic sequencing of 150 bp across the VP1/2A junction. The geographical areas in which the epidemics occurred are shown in Figure 4.1. The genetic relationships between the South African type 1 poliovirus isolates are presented graphically in the form of a dendrogram in Figure 4.2. The sequences of representative isolates only have been included in the dendrogram and results for strains that are closely related are not presented.

All polioviruses investigated fell within 4 distinct genotypes, I to IV, defined by a shared nucleotide identity of 85% or more within the 150 bp region analysed. Three genotypes (I, II and IV) were co-circulating in South Africa between 1980 and 1985; the fourth genotype (III) was introduced into the country in 1985, and was the sole genotype in circulation until 1989. All poliovirus strains isolated in South Africa since 1989 have been vaccine-like.

Analysis of the relationships between wild-type polioviruses obtained during the two epidemics indicated that the outbreaks were caused by viruses belonging to different genotypes. All strains associated with the 1982 Gazankulu epidemic fell within genotype I. Epidemic isolates from the Gazankulu area (1325/SOA82; 1532/SOA82; 4238/SOA82) and from cases seen in neighbouring hospitals during the outbreak period (Pietersburg, 1677/SOA82) were very closely related (maximum divergence between them 3%) and formed a separate cluster within genotype I. In addition to the epidemic isolates, strains belonging to genotype I were isolated between 1980 and 1985 from the Gauteng area, (698/SOA83; 1897/SOA80) the Free State (1170/SOA83), Kwazulu/Natal (1897/SOA80; 5917/SOA82) and the Eastern Cape (282/SOA85). A high degree of diversity (8-11%) between strains belonging to the same genotype was evident. Sequence divergence between the epidemic strains and other strains within the same genotype was approximately 7%. Comparison of genotype I viruses to a databank of strains obtained worldwide revealed no relationship with known strains from other countries, making this genotype unique to South Africa.

The 1987-88 Kwazulu-Natal epidemic was associated with viruses belonging to genotype III. The majority of the epidemic isolates (359/SOA88; 221/SOA88; 364/SOA88) were very closely related (maximum sequence divergence 2%). Cases with definitive epidemiological links to the Kwazulu-Natal epidemic (less than 2% sequence divergence) were also seen in 1988 in Gauteng (1522/SOA88; 427/SOA88) and Port Elizabeth (Eastern Cape

Province) on the south-eastern coast of the country (1048/SOA88). Several epidemic strains (181/SOA88) and strains isolated in Gauteng during the same period (402/SOA88; 400/SOA88) formed a separate cluster diverging from the majority of the outbreak isolates by 4%, although within this group isolates from Kwazulu-Natal (181/SOA88) displayed only 2% divergence from the epidemic strains.

Polioviruses belonging to genotype III were first isolated in the former Transvaal province in the north of South Africa early in 1985 (427SOA85). (The former single province of Transvaal has now been replaced by 4 provinces, Gauteng in the centre, Northern Province in the north, Mpumalanga in the east and North West Province in the west). The closest relative to the genotype III strains was a 1985 strain from Zimbabwe (6224/ZIM85). By early 1986 strains belonging to this genotype were isolated throughout the former province of Transvaal (206/SOA86), and isolation of strain 571/SOA86 from a case in Durban in March 1986 indicated that genotype III had spread into Kwazulu-Natal as well. Viruses isolated throughout the country between 1985 and the end of 1987 displayed a relatively high degree of sequence divergence (5%-6%). The last circulating strain belonging to the genotype III, 441/SOA89, isolated in Gauteng in 1989, exhibited 97% sequence similarity to the Natal epidemic strains and 96% similarity to the 1988 isolates from Gauteng.

Two additional poliovirus genotypes, II and IV, were also in circulation in South Africa between 1982 and 1985. Strains belonging to genotype II were circulating in the former Transvaal province between 1983 and 1985. The minimum divergence between these strains was 3% (between 2/SOA83 and 140/SOA83): later isolates from 1984 (2972/SOA84) and 1985 (442/SOA85) exhibited 94% similarity to the 1983 isolates. The closest relatives to these strains (85 - 88% similarity) were older strains from Jordan (1197/JOR78) and Kuwait (1177/KUW77). Genotype IV comprised 5 isolates, obtained from geographically distant areas in the former Transvaal province, the Free State and Kwazulu-Natal. Sequence divergence between these isolates reached 11%. Comparison of these strains to the sequence databank showed them to be approximately 87% similar to strains from west Africa (6747/SEN86).

A time line presenting the temporal distribution of poliovirus type 1 genotypes in South Africa between 1980 and 1989 is presented in Figure 4.3.

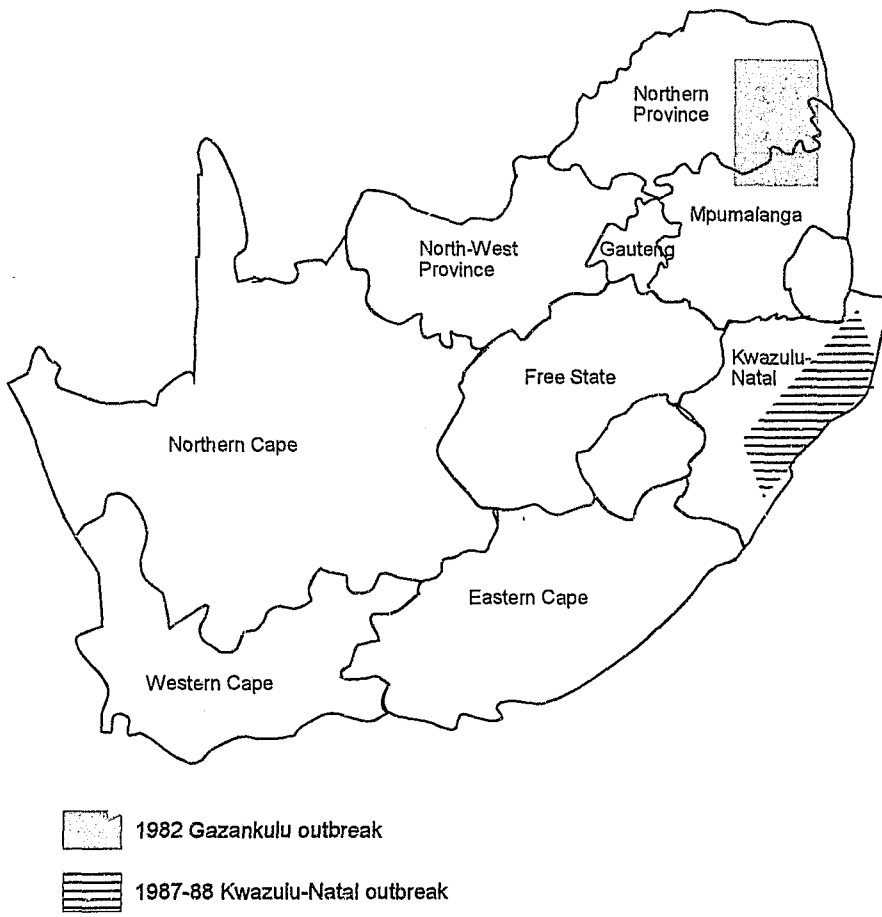

Figure 4.1 Map of South Africa indicating the regions where the 1982 Gazankulu and 1987-88 Kwazulu-Natal poliomyelitis epidemics took place. The map shows the newly-established provinces (1994).

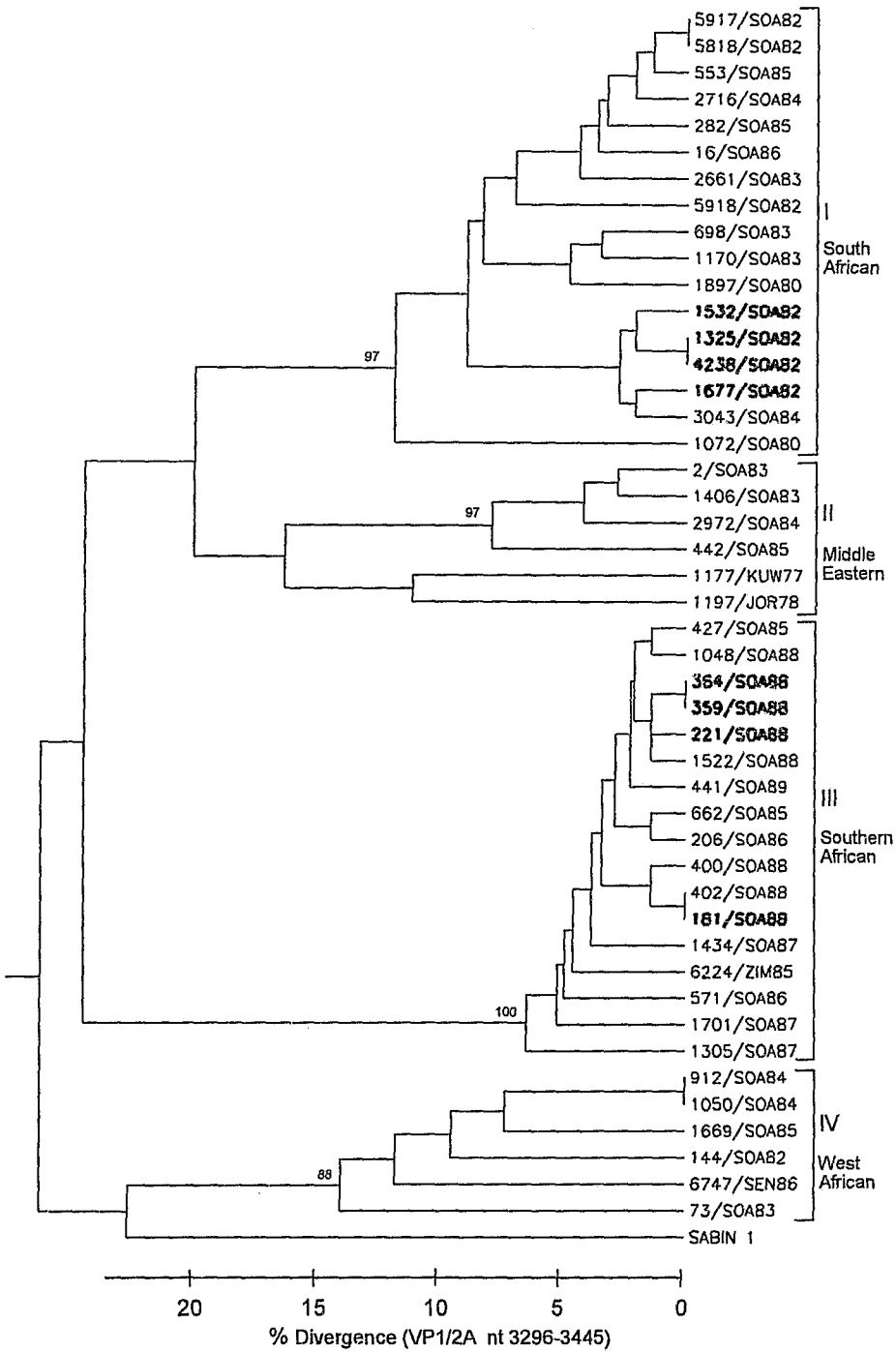

Figure 4.2 Dendrogram of sequence relationships between South African type 1 poliovirus strains. Genotypes are grouped with brackets and assigned numbers I - IV. Strains associated with epidemics are in bold print. The extent of sequence divergence between any 2 strains is determined by measuring the distance along the X-axis to the connecting node. The numbers at the external nodes represent bootstrap percentage values (100 replicates) for those nodes. Country abbreviations are listed in the Preface.

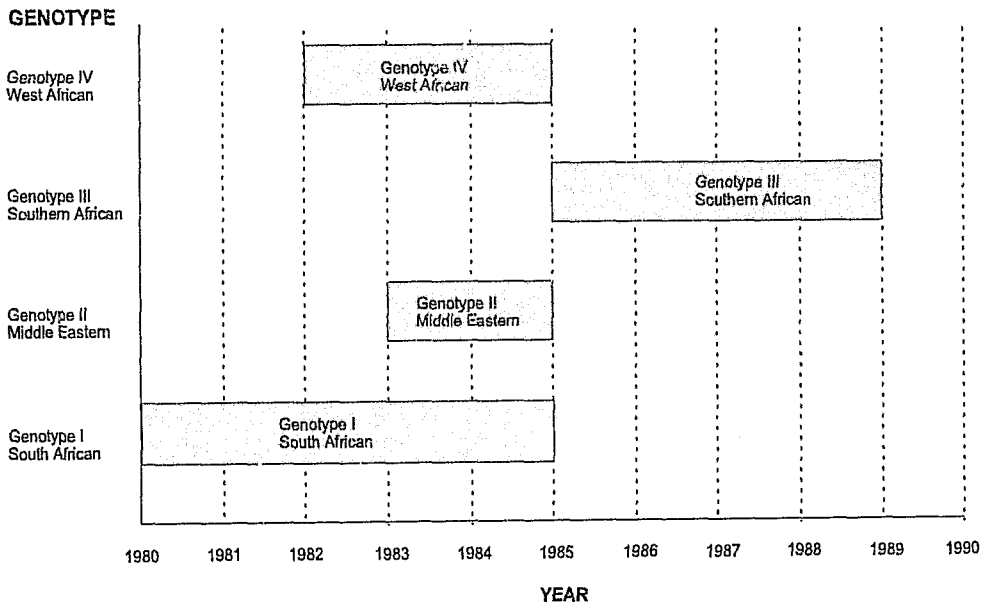

Figure 4.3 Graphical representation of the temporal distribution of poliovirus type 1 genotypes in South Africa between 1980 and 1989.

4.4 DISCUSSION

Partial genomic sequence analysis of 150 bp across the VP1/2A region was employed to characterise and study the molecular epidemiology of wild-type polioviruses associated with cases and outbreaks in South Africa in the past 2 decades. Four type 1 genotypes (I, II, III and IV) were found to have been present in South Africa during the 1980's. Between 1980 and 1985, genotypes I, II and IV co-circulated independently within the country. The majority of isolates obtained during this period fell into genotype I, which was unique to South Africa and circulated endemically throughout the country until its displacement in 1986. Genotype III first appeared in South Africa in 1985, displaced the locally circulating genotypes I, II and IV, and continued to circulate until 1989.

Genotype I was implicated in the 1982 Gazankulu outbreak (Saayman *et al.*, 1984). The outbreak was multifactorial in nature, with low population immunity and vaccine failure playing major roles (Johnson *et al.*, 1984). The high degree of sequence relatedness between outbreak isolates is consistent with rapid epidemic spread of a single strain. These results appear to contrast with those obtained during previous molecular epidemiological studies where oligonucleotide fingerprinting was used to determine relationships between strains. Tsilimigras *et al.* (1989) found that epidemic isolates displayed several different oligonucleotide patterns, indicating the existence of several different poliovirus strains associated with the outbreak, although one particular strain appeared to predominate. They concluded that the outbreak was multifocal, arising from several unrelated poliovirus strains present in the area at the same time. Sequence analysis results, however, indicate that the isolates from the Gazankulu area were highly homologous and diverged by a maximum of 2%, although isolates obtained from more outlying hospitals exhibited slightly less homology to the outbreak strains (3% divergence).The high degree of sequence relatedness between outbreak isolates is consistent with rapid epidemic spread of a single strain, and suggests that the epidemic arose from a single source. Oligonucleotide fingerprinting is highly sensitive to mutations and reliable estimates of relatedness can only be made when strains share greater than 95% sequence similarity. All strains showed less than 5% sequence divergence within the interval sequenced, and as such would have been expected to present similar oligonucleotide patterns. However, when the nucleotide differences between strains are very small, discrepancies between the fingerprinting and sequencing results may arise (Rico-Hesse *et al.*, 1987). Oligonucleotide fingerprinting samples a much greater portion of the genome (10-15%), thus increasing the genomic interval used for comparison, so that limited sequence data may underestimate the differences between very closely related viruses. The 2% divergence between epidemic strains isolated over a 6 month

period is suggestive of rapid virus transmission, and it is possible that viruses that were relatively closely related in sequence might have produced slightly different oligonucleotide patterns. More detailed sequence analysis of a larger portion of the genome would be necessary to reveal more complex relationships between the epidemic isolates. Nevertheless, the partial sequence data is strongly suggestive of a single source epidemic. The unavailability of strains isolated in the Gazankulu area before the epidemic does not allow us to unequivocally determine whether the epidemic strain was already present in the area before the outbreak or whether it was introduced from elsewhere. Genotype I strains were certainly in circulation in the former Transvaal province as early as 1980 (1072/SOA80), and the high degree of divergence between strains within genotype I (up to 11%) indicates that several pockets of susceptible individuals, each sustaining independent evolution of strains within the same genotype, existed in the country. Additionally, the absence of any close epidemiological links (<2% sequence divergence) between strains circulating in other areas of the country during (5917/SOA82, Kwazulu-Natal) or shortly after (698/SOA83, Gauteng) the epidemic would suggest that the epidemic strain was already present in the area prior to the outbreak.

Within genotype I, strains from the south-eastern coastal areas, the central and the north-eastern regions of the country tend to form separate clusters, representative of independent reservoirs of poliovirus in different geographical areas of the country. The presence of isolates from a distant geographical area in a specific cluster, however, may reflect population movements from rural to urban areas of the country (for example, strains 5917/SOA82 and 5818/SOA82 from rural Kwazulu-Natal cluster with isolate 553/SOA85 from the urban Gauteng region).

The extensive 1987-88 outbreak in Kwazulu-Natal was attributed to strains belonging to genotype III. The 95-97% sequence similarity between epidemic isolates and strains isolated in the area in the preceding years (571/SOA86; 1701/SOA87) indicates that the epidemic was caused by strains that were already in circulation in the area and not exclusively by the introduction of a novel genotype into a susceptible population. Sequence analysis of the isolates associated with the outbreak showed them to be very closely related, consistent with rapid epidemic spread of a single strain. Strains very closely related to the epidemic isolates were also isolated in the Gauteng area during the outbreak; travel between the rural and semi-rural areas of the country to the large cities is a common feature of life in South Africa, and the presence of epidemic strains in the former Transvaal province is most likely the result of patients from Kwazulu-Natal travelling to and seeking care in hospitals in the Gauteng area. Very good concordance was seen between the sequencing results reported in this study and earlier oligonucleotide fingerprint analysis of strains associated with the

outbreak, which implicated a single strain, which was also in circulation in other parts of the country during the epidemic (Tsilimigras *et al.*, 1991).

The absence of strains belonging to genotype III in South Africa prior to 1985, and evidence of genetic relatedness to a 1985 isolate from Zimbabwe strongly suggests that genotype III was introduced into South Africa from countries north of the border. The earliest isolates belonging to genotype III were made in the former Transvaal province; a likely route of importation into South Africa may have been across the border with Zimbabwe or Mozambique, via refugee movements due to political and social unrest. The isolation of strains related to those from the former Transvaal approximately one year later in Kwazulu-Natal suggests that the transmission route was from the former Transvaal into the other provinces. The relatively high degree of sequence divergence between strains isolated during the 3-year period between 1985 and the end of 1987 is suggestive of rapid transmission among susceptible individuals.

Genotype III displaced the locally circulating genotypes I, II and IV, and continued to circulate until 1989, when the last confirmed cases of poliomyelitis attributed to wild-type viruses were documented (Schoub *et al.*, 1995). Displacement of endemically circulating strains by an imported strain has been shown to be favoured by conditions of inadequate population immunity, thus allowing the introduction and sustained transmission of an imported strain amongst susceptible individuals (Rico-Hesse *et al.*, 1987). Vaccine coverage in South Africa dropped from 76-77% during 1984/85 to 70% in 1986 (Department of National Health, 1994), providing the conditions of low population immunity required for the establishment of endemicity by the imported genotype III. Displacement of endemic genotypes has also been reported from other developing countries including Venezuela and Honduras (Rico-Hesse *et al.*, 1987) and the Central African Republic (Morvan *et al.*, 1996).

Genotypes II and IV represent genotypes imported from countries in the Middle East and west Africa respectively. Although the number of isolates belonging to these 2 genotypes is small, the high degree of genetic diversity between isolates is suggestive of rapid establishment of endemicity, in separate pockets of susceptible populations, by progenitor infections in the late 1970's. Within genotype I too, the high degree of sequence heterogeneity between strains (up to 11%) is indicative of multifocal endemic transmission. In contrast, strains belonging to genotype III exhibited less sequence heterogeneity (maximum divergence 6%) and no apparent clustering other than that displayed by epidemic isolates. Independent circulation of several genotypes, as well as separate evolution of polioviruses within an individual genotype, has been found to occur when the population of susceptibles is sufficiently large to allow the establishment of endemicity by

imported genotypes and to sustain the local circulation of a specific genotype along different transmission pathways (Rico-Hesse *et al.*, 1987).

In conclusion, the molecular epidemiology of the wild-type 1 polioviruses associated with 2 major outbreaks of poliomyelitis in South Africa has been described. Sequence analysis suggests that both outbreaks originated from a single source, and both were caused by virus strains that were already circulating in the outbreak areas prior to the epidemics. Genotype I, responsible for the 1982 Gazankulu epidemic, was unique to South Africa and had been in circulation since at least 1980. In addition to this genotype, 2 imported genotypes circulated concurrently until 1985. The 1987-88 Kwazulu-Natal outbreak was caused by a different genotype, most probably introduced into South Africa several years earlier from countries north of the border. The displacement of the 3 locally circulating genotypes by the imported genotype reflects the transition from endemic to epidemic poliovirus circulation as vaccine coverage in South Africa increased. No wild-type viruses have been isolated in the country since 1989, and it thus appears that the transmission of wild-type polioviruses in South Africa has been effectively controlled.

Sequence relationships between type 1 poliovirus strains isolated in South Africa and those circulating in other countries in Africa will be discussed in Chapter 5.

5. MOLECULAR EPIDEMIOLOGY OF TYPE 1 POLIOVIRUSES IN SUB-SAHARAN AFRICA

5.1 INTRODUCTION

In recent years, remarkable progress has been made towards achieving the goal of global poliomyelitis eradication by the year 2000. In 1996, a total of 4074 cases of poliomyelitis were reported globally, a decrease of 43% from the 7032 cases reported in 1995 (WHO, 1998). Of these, 1949 cases (approximately 48%) were reported from Africa. Elimination of poliomyelitis from the African continent is thus one of the remaining major challenges to achieving global eradication by the target date. To this end, during 1996, 1997 and the first quarter of 1998, as part of the "Kick polio out of Africa" campaign, NID's were conducted in all African countries except Liberia, the Congo and Sierra Leone, targeting more than half of all children under 5 years - over 74 million children (WHO, 1997a; WHO, 1998c). In 1997, the African region reported only 219 confirmed cases of poliomyelitis (as of May 1998), a decrease of almost 90% from the number of cases reported in 1996. The global total of cases dropped from 4074 in 1996 to 3234 in 1997 (WHO, 1998a).

Reported overall vaccination coverage with 3 doses of OPV is still low in the African region as a whole, but has increased from 32% in 1988, to 47% in 1993, to 54% in 1996 (WHO, 1997b; WHO, 1998c). Many countries in southern Africa have for several years reported both high levels of OPV-3 coverage in children under 1 year of age, and very low or zero incidence of poliomyelitis, and WHO suggestions are that southern Africa may be emerging as a polio-free zone (WHO, 1994b). However, in 1996, 16 countries including 4 of the largest and epidemiologically most important countries - Angola, Ethiopia, Nigeria and the D.R.Congo - reported that less than 50% of children were routinely immunised with 3 doses of OPV (WHO, 1997a). Thus large areas of sub-optimal vaccine coverage exist in western and central Africa. These areas are also those from which the largest numbers of poliomyelitis cases are being reported (WHO, 1998c). Outbreaks of poliomyelitis have been reported in several African countries in recent years: an increase in the incidence of wild-type polio 1-associated AFP cases occurred during 1993-94 in Bangui in the Central African Republic (CAR) (Gouandjika *et al.*, 1995); D.R.Congo experienced an outbreak of poliomyelitis involving several hundred cases in 1995 (Lambert *et al.*, 1995); cases of paralytic poliomyelitis were reported from Tanzania and Zambia in 1995 (Mpabalwani *et al.*, 1996), and despite intensive national vaccination campaigns following the 1993 epidemic in Namibia (van Niekerk *et al.*, 1994), poliomyelitis cases continued to be reported

throughout 1995 from the north of the country and from southern Angola (Izurieta *et al.*, 1996).

Political instability and severe poverty in many countries in Africa results in large population movements across national borders, and the construction of large refugee camps in which sanitation and health services are virtually non-existent. The very real threat of reappearance of poliomyelitis in polio-free countries as a result of re-introduction from polio-endemic areas thus exists in many African countries.

If the goal of elimination of poliomyelitis from Africa is to be achieved, the regions where polioviruses continue to circulate endemically need to be identified, and the patterns of spread of wild-type viruses determined, so that improved strategies for the interruption of transmission may be designed and implemented. The continued surveillance for cases of AFP, and laboratory support in the form of identification and characterisation of wild-type virus infections are thus critical aspects of the WHO strategy for eradication.

With few exceptions, the majority of wild-type polioviruses circulating in Africa during the past 2 decades have been type 1. In this chapter, the molecular epidemiology, established by partial genomic sequence analysis, of wild-type 1 polioviruses circulating in sub-Saharan Africa during recent years will be discussed.

5.2 MATERIALS AND METHODS

5.2.1 Viruses

The type 1 poliovirus strains from Africa which were selected for molecular characterisation are listed in Table 5.1. Only wild-type strains that were selected for sequence analysis are included in the table, as strains that were typed as vaccine-like were not analysed further and are not discussed in this study. All polioviruses were isolated in the WHO national and regional reference laboratories in Africa, from clinical specimens of patients presenting with AFP or with a clinical diagnosis of poliomyelitis, or from contacts of such cases. Those strains which were not isolated at the NIV laboratories were submitted to the NIV for intratypic differentiation and/or sequence analysis. The Sabin 1 poliovaccine strain (P1/Lsc2ab), obtained from Dr. C. Dommann, Vaccine Unit, NIV, was used as the reference strain for alignments.

All polioviruses were isolated and typed as described in section 3.2.1, and intratypic differentiation between vaccine-like and wild-type strains was performed by Sabin-specific PCR as described in section 3.2.3.

5.2.2 Sequence analysis

DNA sequencing templates of the virus strains to be sequenced were generated by RT-PCR using polio-specific primers as described in section 3.2.4. Direct sequencing of the amplified products was performed using the Sequenase PCR-product sequencing kit as described in section 3.2.5.

Genetic relationships between poliovirus strains were established by carrying out pairwise comparisons between all strains, and generating graphical representations of the extent of sequence divergence between strains in the form of a dendrogram (section 3.2.6).

Table 5.1 Recent wild poliovirus type 1 strains from Africa which were selected for comparative sequence analysis

Strain	Country in which isolated	Year of isolation	Genotype	Included in dendrogram
1177NAM93	Namibia - Windhoek	1993	West African - B	✔
1178NAM93	Namibia - Windhoek	1993	West African - B	
1228NAM93	Namibia - Windhoek	1993	West African - B	
1229NAM93	Namibia - Keetmanshoop	1993	West African - B	
1232NAM93	Namibia - Windhoek	1993	West African - B	
1234NAM93	Namibia - Mariental	1993	West African - B	
1257NAM93	Namibia - Windhoek	1993	West African - B	
1258NAM93	Namibia - Windhoek	1993	West African - B	
1259NAM93	Namibia - Windhoek	1993	West African - B	
1263NAM93	Namibia - Windhoek	1993	West African - B	
1264NAM93	Namibia - Mariental	1993	West African - B	
1265NAM93	Namibia - Windhoek	1993	West African - B	
1266NAM93	Namibia - Keetmanshoop	1993	West African - B	
1281NAM93	Namibia - Windhoek	1993	West African - B	
1360NAM93	Namibia - Mariental	1993	West African - B	
1363NAM93	Namibia - Windhoek	1993	West African - B	
1376NAM93	Namibia - Windhoek	1993	West African - B	
1404NAM93	Namibia - Mariental	1993	West African - B	
1405NAM93	Namibia - Windhoek	1993	West African - B	✔
1407NAM93	Namibia - Windhoek	1993	West African - B	
1408NAM93	Namibia - Mariental	1993	West African - B	
1409NAM93	Namibia - Aranos	1993	West African - B	
1410NAM93	Namibia - Aranos	1993	West African - B	
1412NAM93	Namibia - Aranos	1993	West African - B	
1413NAM93	Namibia - Rehoboth	1993	West African - B	
1416NAM93	Namibia - Windhoek	1993	West African - B	
1417NAM93	Namibia - Mariental	1993	West African - B	
1441NAM93	Namibia - Aranos	1993	West African - B	
1455NAM93	Namibia - Windhoek	1993	West African - B	
1459NAM93	Namibia - Okahanya	1993	West African - B	
1460NAM93	Namibia - Aranos	1993	West African - B	
1462NAM93	Namibia - Windhoek	1993	West African - B	
1467NAM93	Namibia - Gobabis	1993	West African - B	
1497NAM93	Namibia - Windhoek	1993	West African - B	
1522NAM93	Namibia - Mariental	1993	West African - B	✔
1586NAM93	Namibia - Rehoboth	1993	West African - B	
1602NAM93	Namibia - Windhoek	1993	West African - B	
1607NAM93	Namibia - Gobabis	1993	West African - B	
1630NAM93	Namibia - Windhoek	1993	West African - B	
1CAR93	Central African Republic	1993	Indian	✔
3CAR93	Central African Republic	1993	Indian	✔
9CAR93	Central African Republic	1993	West African - F	
10CAR93	Central African Republic	1993	West African - F	
13CAR93	Central African Republic	1993	Middle Eastern	✔
16CAR93	Central African Republic	1993	Middle Eastern	✔
17CAR93	Central African Republic	1993	Indian	✔
1CAR94	Central African Republic	1994	West African - F	
2CAR94	Central African Republic	1994	West African - F	
6CAR94	Central African Republic	1994	West African - F	

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Table 5.1 ..... continued from previous page

Strain	Country in which isolated	Year of isolation	Genotype	Included in dendrogram
45CAR94	Central African Republic	1994	West African - F	
46CAR94	Central African Republic	1994	West African - F	
50CAR94	Central African Republic	1994	West African - F	✔
76CAR94	Central African Republic	1994	West African - F	✔
169CAR94	Central African Republic	1994	West African - F	
177CAR94	Central African Republic	1994	West African - F	
051NAM94	Namibia	1994	West African - B	
158NAM94	Namibia - Swakopmund	1994	West African - B	✔
1276ANG94	Angola - Luanda	1994	West African - B	✔
1277ANG94	Angola - Luanda	1994	West African - B	✔
1497ANG94	Angola - border with Namibia	1994	West African - C	✔
1498ANG94	Angola - border with Namibia	1994	West African - C	✔
072NAM95	Namibia - Rundu	1995	West African - C	✔
076NAM95	Namibia - Rundu	1995	West African - C	
077NAM95	Namibia - Rundu	1995	West African - C	
078NAM95	Namibia - Nankundu	1995	West African - C	
079NAM95	Namibia - Rundu	1995	West African - C	
109NAM95	Namibia - Rundu	1995	West African - C	
266NAM95	Namibia - Rundu	1995	West African - C	
284NAM95	Namibia - Rundu	1995	West African - C	
285NAM95	Namibia - Rundu	1995	West African - C	
341NAM95	Namibia - Rundu	1995	West African - C	✔
818NAM95	Namibia - Rundu	1995	West African - C	✔
4080ZAI95	D.R.Congo - Mbuji Mayi	1995	West African - B	
4097ZAI95	D.R.Congo - Mbuji Mayi	1995	West African - B	✔
4106ZAI95	D.R.Congo - Mbuji Mayi	1995	West African - B	
4131ZAI95	D.R.Congo - Mbuji Mayi	1995	West African - B	
4145ZAI95	D.R.Congo - Mbuji Mayi	1995	West African - B	
4156ZAI95	D.R.Congo - Mbuji Mayi	1995	West African - B	
4188ZAI95	D.R.Congo - Mbuji Mayi	1995	West African - B	
4779ZAI95	D.R.Congo - Mbuji Mayi	1995	West African - B	
757ZAI95	D.R.Congo - Mbuji Mayi	1995	West African - B	✔
759ZAI95	D.R.Congo - Mbuji Mayi	1995	West African - B	
761ZAI95	D.R.Congo - Mbuji Mayi	1995	West African - B	
762ZAI95	D.R.Congo - Mbuji Mayi	1995	West African - B	
764ZAI95	D.R.Congo - Mbuji Mayi	1995	West African - B	
765ZAI95	D.R.Congo - Mbuji Mayi	1995	West African - B	
766ZAI95	D.R.Congo - Mbuji Mayi	1995	West African - B	
767ZAI95	D.R.Congo - Mbuji Mayi	1995	West African - B	
768ZAI95	D.R.Congo - Mbuji Mayi	1995	West African - B	
769ZAI95	D.R.Congo - Mbuji Mayi	1995	West African - B	✔
564TAN95	Tanzania - Mbeya	1995	East African	✔
1199TAN95	Tanzania - Dodoma	1995	East African	✔
MpoTAN95	Tanzania - Mbeya	1995	East African	✔
ChansaZAM95	Zambia - Lusaka	1995	East African	✔
50ZAM95	Zambia - Lusaka	1995	East African	✔
HanyZAM95	Zambia - Lusaka	1995	East African	✔
63ZAM95	Zambia - Lusaka	1995	East African	✔
65ZAM95	Zambia - Lusaka	1995	East African	
042ZAM95	Zambia - Ikelenge	1995	West African - B	✔

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Table 5.1 ..... continued from previous page

Strain	Country in which isolated	Year of isolation	Genotype	Included in dendrogram
015CAE95	Cameroun	1995	West African - F	✔
012LIB95	Liberia	1995	West African - A	✔
001LIB96	Liberia	1996	West African - A	✔
001SEN96	Senegal	1996	West African - A	✔
002SEN96	Senegal	1996	West African - A	✔
010TOG96	Togo	1996	Nigeria-1	✔
K19GHA96	Ghana	1996	West African - A	
K21GHA96	Ghana	1996	West African - A	
K22GHA96	Ghana	1996	West African - A	✔
K24GHA96	Ghana	1996	West African - A	✔
K25GHA96	Ghana	1996	West African - A	
002NIE96	Nigeria	1996	Nigeria-1	✔
004NIE96	Nigeria	1996	Nigeria-1	✔
002CIV96	Cote D'Ivoire	1996	West African - A	✔
005CIV96	Cote D'Ivoire	1996	West African - E	✔
007CAF96	Central African Republic	1996	West African - B	✔
001ZAI96	D.R.Congo	1996	East African	✔
U19UGA96	Uganda	1996	East African	✔
73ZAM96	Zambia - Lusaka	1996	East African	✔
009TAN96	Tanzania - Hanang	1996	East African	✔
012TAN96	Tanzania - Mtwara	1996	East African	✔
015TAN96	Tanzania - Kigoma	1996	West African - B	✔
1579ANG96	Angola - Luena	1996	West African - B	✔
1587ANG96	Angola - Lumege	1996	West African - B	✔
001TOG97	Togo	1997	Nigeria-1	✔
001GHA97	Ghana	1997	West African - A	✔
002GHA97	Ghana	1997	West African - A	✔
001IVC97	Cote D'Ivoire	1997	West African - A	✔
001NIG97	Niger	1997	West African - F	✔
005NIG97	Niger	1997	Nigeria-1	✔
003NIG97	Niger	1997	West African - F	✔
005SEN97	Senegal	1997	West African - A	✔
001BFA97	Burkina Faso	1997	West African - F	✔
003BFA97	Burkina Faso	1997	West African - F	✔
005CAF97	Central African Republic	1997	West African - B	✔
006CAF97	Central African Republic	1997	West African - B	✔
008CAF97	Central African Republic	1997	West African - B	
009CAF97	Central African Republic	1997	West African - B	
010CAF97	Central African Republic	1997	West African - B	✔
97-4ZAI97	D.R.Congo - Kinshasa	1997	East African	✔
004ZAI97	D.R.Congo - Bandundu	1997	West African - B	✔
005ZAI97	D.R.Congo - Bandundu	1997	West African - B	✔
006ZAI97	D.R.Congo - Bandundu	1997	West African - B	✔

In order to obtain a more comprehensive picture of the distribution of polio type 1 genotypes throughout Africa, polioviruses from African countries, which were sequenced at the RIVM or CDC (Table 5.2) were also included in the dendrograms.

Table 5.2 Poliovirus type 1 strains which were not sequenced at the NIV, but which were included in the dendrograms for comparative purposes

Strain	Country in which isolated	Year of isolation	Genotype
Sequence data for the following strains kindly provided by Harrie G.A.M. van der Avoort, RIVM, the Netherlands:			
08659IND91	India	1991	Indian
11257EGY91	Egypt	1991	Middle Eastern
11300EGY91	Egypt	1991	Middle Eastern
21197PAK91	Pakistan	1991	Indian
24094SUD93	Sudan	1993	Indian
24115KEN93	Kenya	1993	Indian
24122EGY93	Egypt	1993	Indian
5135ETH93	Ethiopia	1993	Indian
5136ETH93	Ethiopia	1993	Indian
8423ISR88	Israel	1988	Middle Eastern
Sequence data for the following strains kindly provided by Olen M. Kew, CDC, Atlanta. USA:			
3862TOG92	Togo	1992	West African - B
55552NIE93	Nigeria	1993	Nigeria-1
6656CIV95	Cote D'Ivoire	1995	West African - E
6723NIE96	Nigeria	1996	Nigeria-2
6725NIE96	Nigeria	1996	Nigeria-2
6726NIE96	Nigeria	1996	West African - F
6727NIE96	Nigeria	1996	West African - F
6728SEN95	Senegal	1995	West African - D
6731TOG94	Togo	1994	Nigeria-2
6732TOG92	Togo	1992	West African - A
6735TOG92	Togo	1992	West African - A
6743GHA95	Ghana	1995	West African - A
6747GHA95	Ghana	1995	West African - A
6748GHA95	Ghana	1995	West African - A
6762UGA94	Uganda	1994	East African
6771UGA95	Uganda	1995	East African
6777UGA95	Uganda	1995	East African
6780UGA96	Uganda	1996	East African
6785UGA96	Uganda	1996	East African
6785UGA96	Uganda	1996	East African
6786UGA96	Uganda	1996	East African
6224ZIM85	Zimbabwe	1985	Southern African
9475ZAI	Zaire	not known	East African

5.3 RESULTS

5.3.1 Relationships between wild-type 1 polioviruses based on nucleotide sequence comparisons

Type 1 poliovirus strains recently isolated from countries in Africa were characterised by partial genomic sequencing of 150 bases across the VP1/2A junction region. The aligned sequences of representative strains are presented in Figures 5.3 and 5.4. All detectable mutations were base substitutions, with an observed transition/transversion ratio of 2. Within the major virus groups (genotypes), nearly all the substitutions between pairs of strains were transitions.

A graphical representation of the sequence relationships between strains, determined from the extent of genetic divergence between strains, is presented in the form of a dendrogram in Figures 5.1 and 5.2 Analysis of the dendrograms indicates that the polioviruses circulating in Africa during recent years (1990-1997) group into 6 major clusters, each representing a different genotype. The genotypes are defined as groups of viruses sharing ≥85% sequence similarity within the 150-base VP1/2A interval. Bootstrap analysis values for the nodes separating the genotypes are all > 75%, suggesting that the branching patterns defining the major genotypes are significant. The genotypes appear to be distributed geographically, and have thus been designated names (West African, Nigeria-1, Nigeria-2, East African, Middle Eastern and Indian) corresponding to their original geographical regions of endemicity. If the poliovirus type 1 strains circulating in South Africa between 1980 and 1989 are included in the analysis (see Chapter 4 and Figure 4.2), then the total number of identified type 1 genotypes circulating in Africa during the past 2 decades rises to 9.

The West African genotype (Figure 5.1) is the most widespread geographically, comprising isolates from countries in western, south-western, central, and southern Africa. Within this genotype are several smaller clusters, numbered A - F, which diverge from each other by 6% - 15 %. Cluster A comprises strains isolated from west African countries (Burkina Faso, Cote D'Ivoire, Ghana, Liberia, Senegal, Togo) between 1992 and 1997. Three separate branches are evident within cluster A, representing tighter geographical clustering of strains from i.) Ghana (1996), Cote D'Ivoire (1996), Liberia (1995-96), Senegal (1996-97) and Burkina Faso (1997); ii) Ghana (1995-96), Togo (1992-94) and Cote D'Ivoire (1997); and iii) recent isolates from Ghana (001GHA97; 002GHA97), obtained after the NID's in 1996, and which diverged from the earlier isolates by approximately 11%.

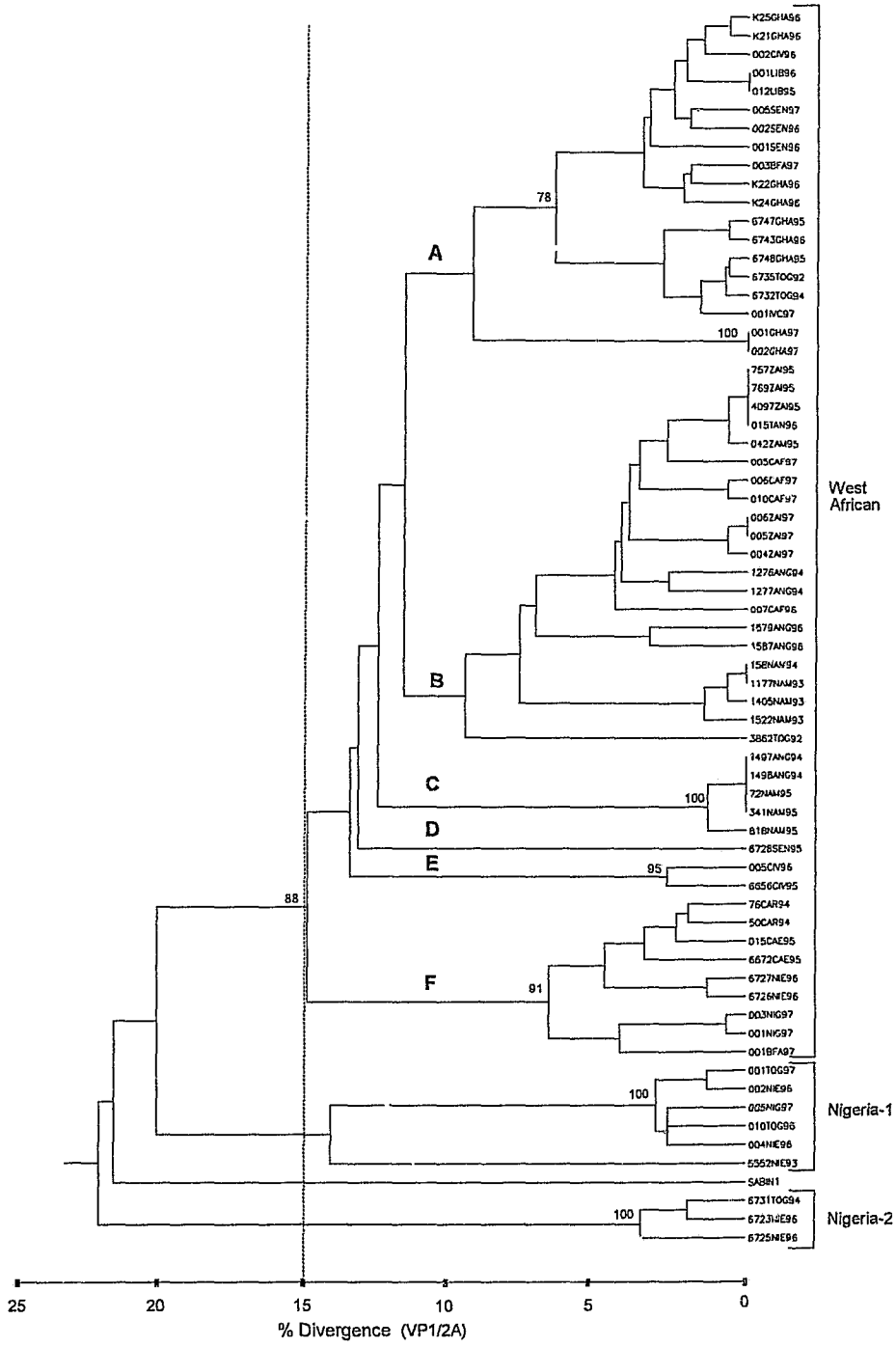

Figure 5.1 Dendrogram of sequence divergence (nt 3296-3445) between type 1 polioviruses from west, central and south-western Africa. The numbers at the external nodes represent bootstrap percentage values (100 replicates) for those nodes. Genotypes are grouped within brackets. Country abbreviations are listed in the Preface.

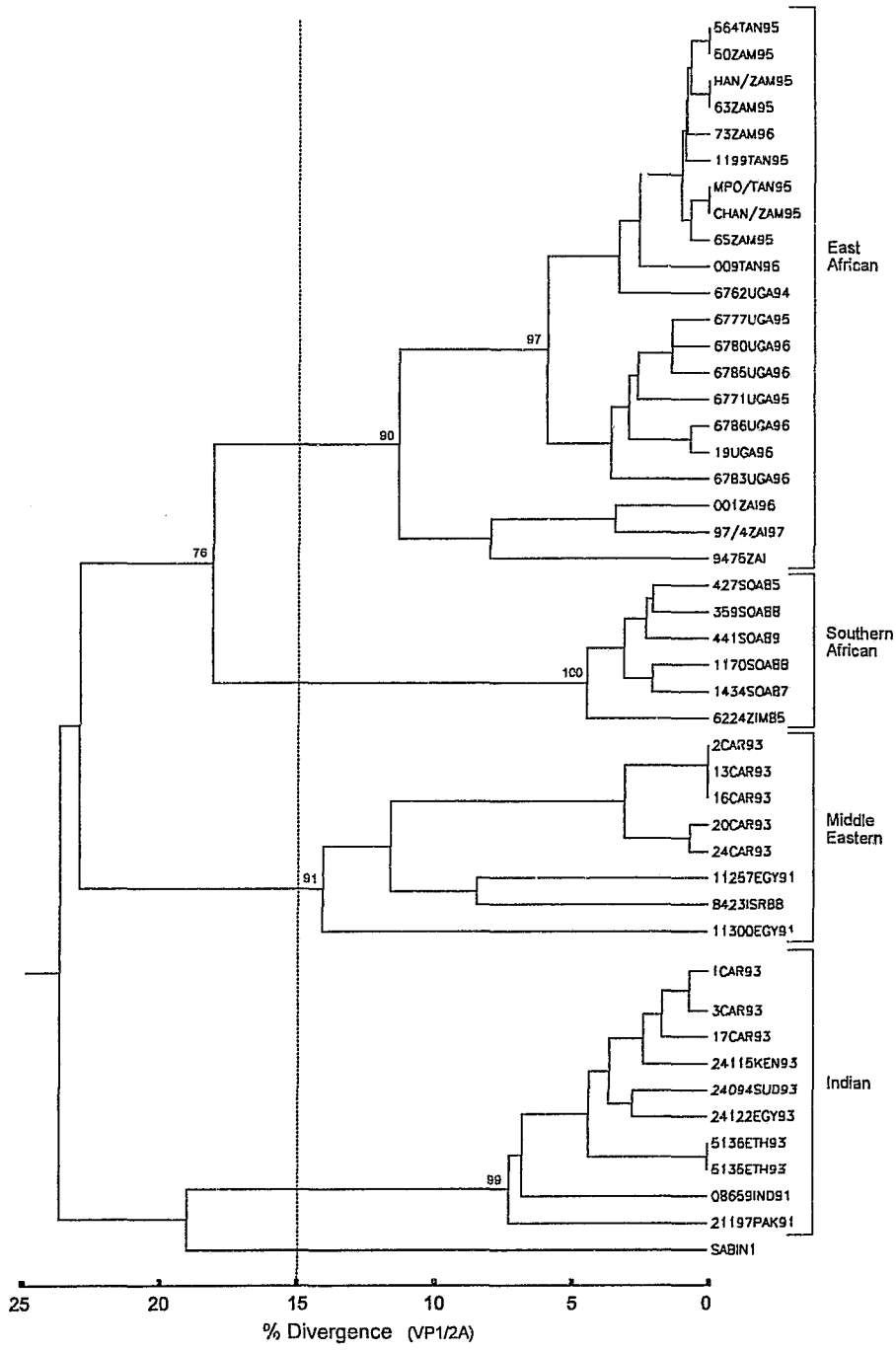

Figure 5.2 Dendrogram of sequence divergence (nt 3296-3445) between type 1 polioviruses from central, eastern, and southern Africa. The numbers at the external nodes represent bootstrap percentage values (100 replicates) for those nodes. Genotypes are grouped within brackets. Country abbreviations are listed in the Preface.

Cluster B comprises mostly strains from central and south-western Africa. Isolates obtained during the poliomyelitis outbreak in D.R.Congo in 1995 (757ZAI95; 4097ZAI95), from D.R.Congo and the CAR during 1997, and from central Angola in 1994 (5384ANG94 and 1276ANG94), exhibit approximately 96% nucleotide identity between them and tend to group together. All D.R.Congo 1995 epidemic isolates exhibited between 98% and 100% nucleotide similarity. Included in this group were strains 042ZAM95 and 015TAN96; these isolates exhibited 99% genetic similarity to the D.R.Congo outbreak strains, and were isolated respectively late in 1995 and early in 1996 from cases in Ikeienge in north-western Zambia and from Kigoma in western Tanzania (both towns close to the D.R.Congo border). Isolates obtained in Angola in 1996, from Togo in 1992, and from the 1993 outbreak in Namibia branch independently within cluster B. All the Namibia outbreak strains were closely related, displaying a maximum sequence divergence of 2%. The strains most genetically similar to those from the Namibian outbreak were from Angola - isolates from the Namibian epidemic (1405NAM93; 1177NAM93) and from central Angola (5384ANG94; 1276ANG94) displayed approximately 94% sequence identity.

Strains isolated from northern Namibia early in 1995 (72NAM95; 341NAM95; 818NAM95) belong to a third cluster, C, within genotype A. These strains diverged by 12% from isolates obtained during the 1993 outbreak in Namibia, but displayed a very high percentage sequence similarity (99%) with isolates made from cases in southern Angola late in 1994 (1497ANG94; 1498ANG94).

Clusters D and E comprise, respectively, a single strain obtained in Senegal during 1995 (6728SEN95), and strains from Cote D'Ivoire from 1995-6 (6656CIV95; 005CIV96).

Strains isolated from the CAR in 1994 (76CAR94; 50CAR94), Cameroun in 1995 (015CAE95; 6672CAE95), Nigeria in 1996 (6727NIE96; 6726NIE96), and Niger and Burkina Faso in 1997 (001NIG97; 003NIG97; 001BFA97) make up the sixth cluster, F, within the A genotype. Strains within cluster F displayed the highest degree of sequence divergence, approximately 12-14%, from other isolates within genotype A.

Two independent genotypes, termed Nigeria-1 and Nigeria-2, co-circulated with the West African genotype in west Africa. The Nigeria-1 genotype comprises recent strains from Nigeria (002NIE96; 004NIE96), Niger (005NIG97) and Togo (010TOG96; 001TOG97). The genomic sequence variation between these strains is limited to approximately 2.5%. Included in this genotype, but exhibiting far greater sequence divergence (13%) compared to the other isolates within the genotype, is a strain isolated in Nigeria in 1993 (5552NIE93).

The Nigeria-2 genotype also comprises strains isolated in Togo in 1994 (6731TOG94) and Nigeria in 1996 (6723NIE96; 6725NIE96). Theses isolates are closely related in sequence (maximum divergence approximately 3%), but diverge by more than 20% from strains falling within the Nigeria-1 genotype.

Recent poliovirus strains isolated in 1996-97 in D.R.Congo (001ZAI96; 97/4ZAI97) and in 1995-96 from Tanzania (564TAN95; 1199TAN95) and Zambia (50ZAM95) make up the East African genotype, which appears to be geographically restricted to central, eastern and southern Africa (Figure 5.2). Three clusters are evident within this genotype: the first comprises closely related isolates from Tanzania (maximum sequence divergence of 1.5%), and 6 strains isolated from cases in Lusaka, Zambia in 1995, which exhibited a maximum of 2% sequence divergence from the Tanzanian strains. Included in this cluster, and exhibiting at most 4 % sequence divergence from the Tanzania/Zambia strains, is a strain isolated in Uganda in 1994 (6762UGA94). The second cluster within the East African genotype comprises strains isolated in Uganda in 1995-6. Strains within this cluster are closely related, diverging by no more than 4%, and differ in sequence from the Tanzania/Zambia and the earlier Uganda strain by approximately 7%. The third distinct cluster comprises strains isolated in D.R.Congo in 1996-97 (001ZAI96; 97/4ZAI97) and a strain isolated in D.R.Congo before 1987 (9475ZAI; the exact date of isolation of this strain, except for the fact that it was definitely before 1987, is unknown); the D.R.Congo strains within this cluster exhibit a maximum sequence similarity of 87% with isolates from Tanzania, Zambia and Uganda.

Two additional genotypes, Middle Eastern and Indian, were also in circulation in central, eastern and north-eastern Africa between 1991 and 1993. The Indian genotype comprises strains from the CAR from 1993 (1CAR93; 3CAR93; 17CAR93), and includes strains from the same period from Kenya (24115KEN93), Sudan (24094SUD93), Ethiopia (5135ETH93; 5136ETH93) and Egypt (24122EGY93), and older strains from India (08659IND91) and Pakistan (21197PAK91). The Middle Eastern genotype co-circulated simultaneously with the Indian genotype in the CAR during 1993 (2CAR94; 13CAR93; 16CAR93; 20CAR93; 24CAR93); included in this group are older isolates from Egypt (11257EGY91; 11300EGY91) and the Middle East (8423ISR88).

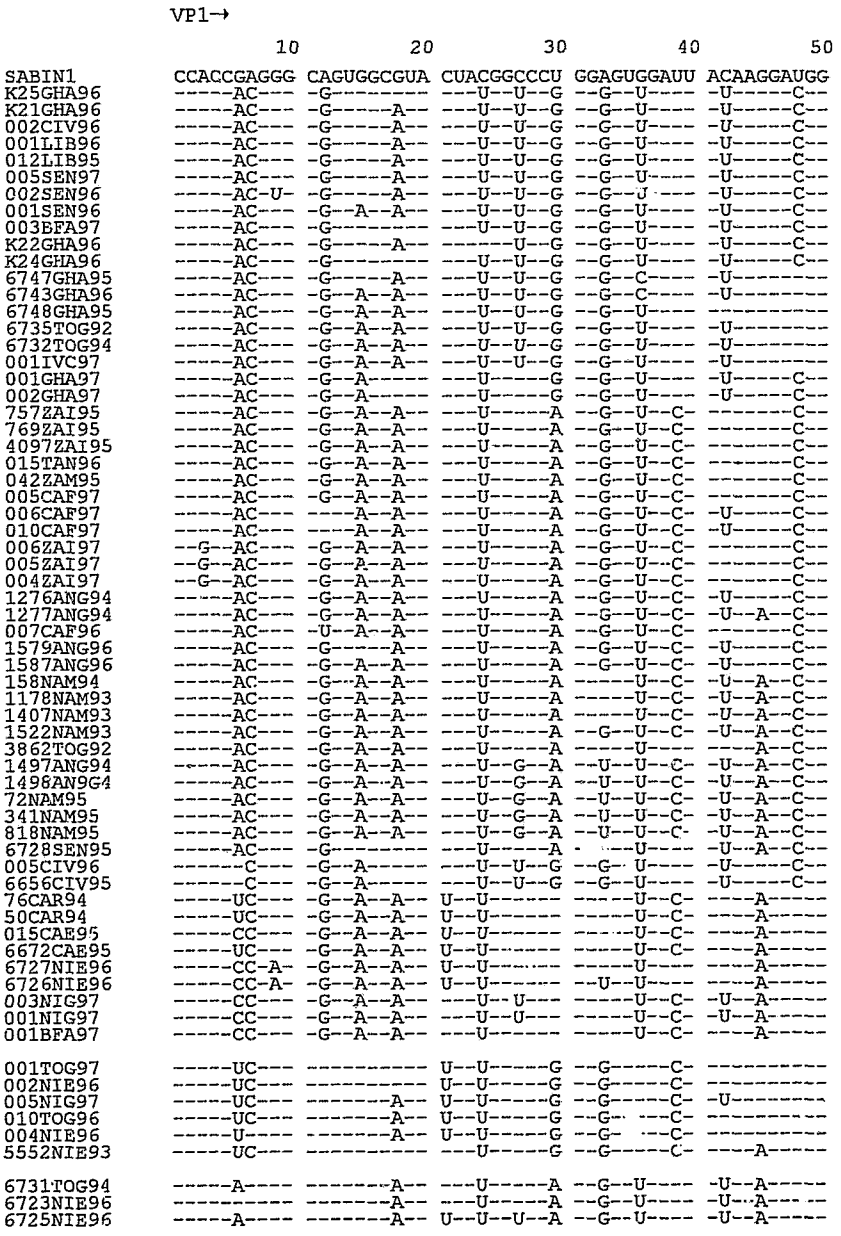

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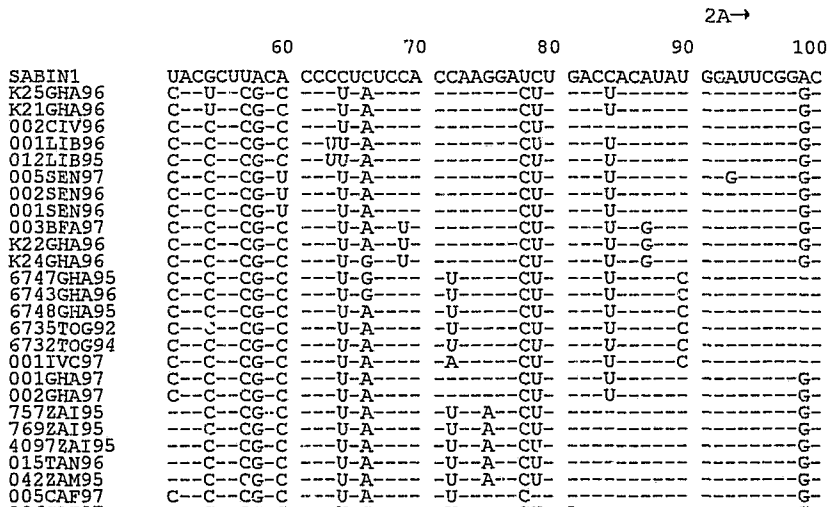
Figure 5.3 Nucleotide sequence comparison of 150 bases across the poliovirus VP1/2A junction (position 3296-3445) for wild-type 1 polioviruses belonging to the West African, Nigeria-1 and Nigeria-2 genotypes. Dashes represent nucleotides which are identical to those of Sabin 1.



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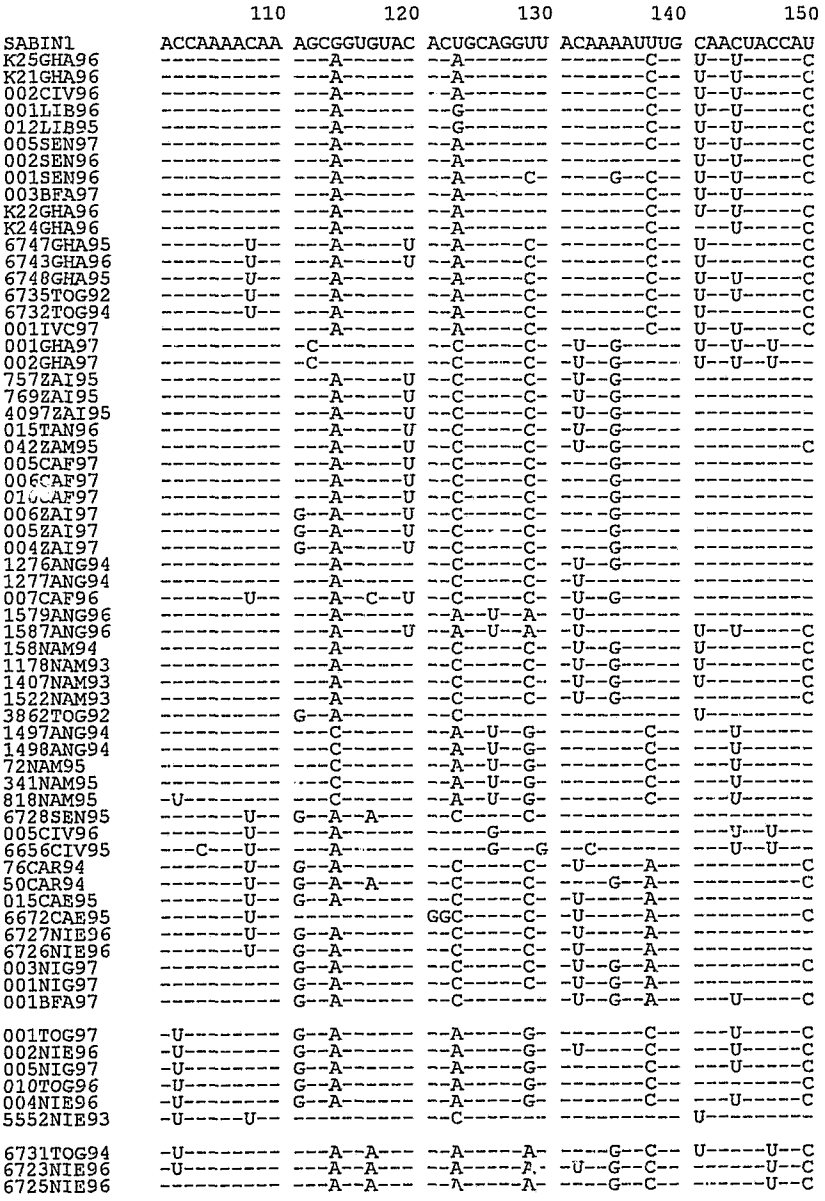

Figure 5.3 ..... *continued from previous page*

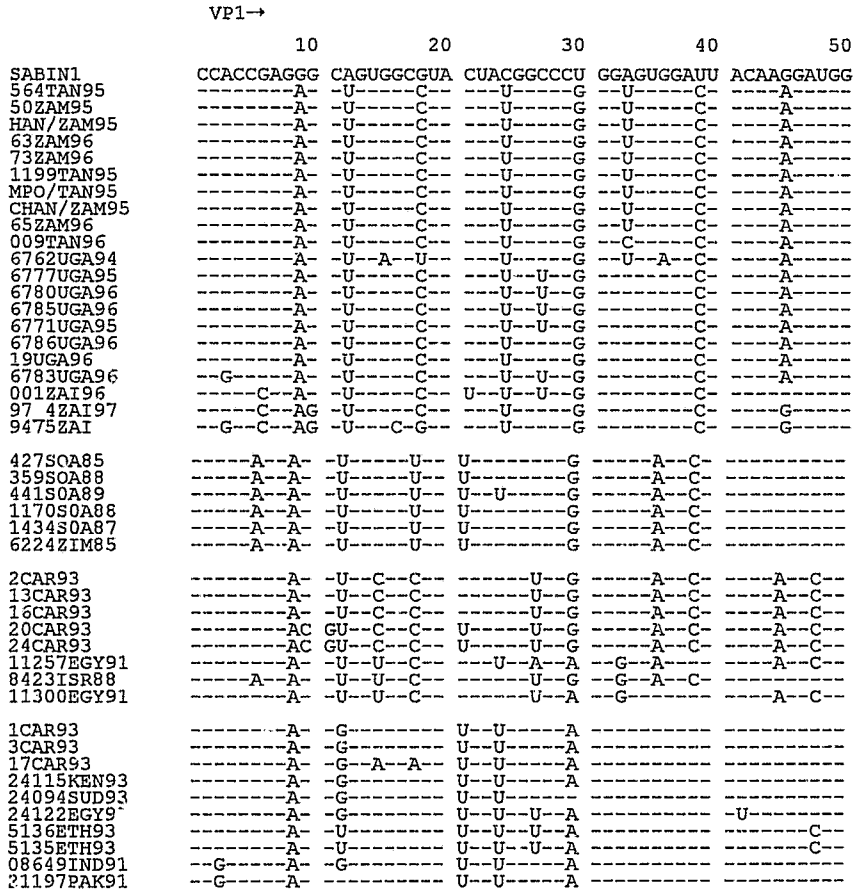

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Figure 5.4 Nucleotide sequence comparison of 150 bases across the poliovirus VP1/2A junction (position 3296-3445) for wild-type 1 polioviruses belonging to the East African, Southern African, Middle Eastern and Indian genotypes. Dashes represent nucleotides which are identical to those of Sabin 1.

```
                                                                2A→
                        60          70          80          90          100
SABIN1          UACGCUUACA CCCCUCUCCA CCAAGGAUCU GACCACAUAU GGAUUCGGAC
564TAN95        C--U---G-U ---U-G--U- -U-------- A--A--G--- --U--U--G-
50ZAM95         C--U---G-U ---U-G--U- -U-------- A--A--G--- --U--U--G-
HAN/ZAM95       C--U---G-U --UU-G--U- -U-------- A--A--G--- --U--U--G-
63ZAM96         C--U---G-U --UU-G--U- -U-------- A--A--G--- --U--U--G-
73ZAM96         C--U---G-U ---U-G--U- -U----G--- A--A--G--- --U--U--G-
1199TAN95       C--U---G-U ---U-G--U- -U-----A-- A--A--G--- --U--U--G-
MPO/TAN95       C--U-----U ---U-G--U- -U-------- A--A--G--- --U--U--G-
CHAN/ZAM95      C--U-----U ---U-G--U- -U-------- A--A--G--- --U--U--G-
65ZAM96         C--U-----U ---U-G--U- -G-------- A--A--G--- --U--U--G-
009TAN96        C--U---G-U ---U-G--U- -U-------- ---A--G--- --U--U--G-
6762UGA94       C--U--- G-U ---U-G--U- ---------- A--A--G--- --U--U--G-
6777UGA95       C--U---G-U ---U-G--U- ----A----- A--A--G--- --U-----G-
6780UGA96       C--U---G-U ---U-G--U- ----A----- A--A--G--- --U-----G-
6785UGA96       C--U---G-U ---U-G--U- ----A----- A--A--G--- --U-----G-
6771UGA95       C--U---G-U ---U-G--U- ------G--- A--A--G--- --U-----G-
6786UGA96       C--U---G-U ---U-G--U- ---------- A--A--G--- --U--U--G-
19UGA96         C--U---G-U ---U-G--U- ---------- A--A--G--- --U--U--G-
6783UGA96       C--U---G-U ---U-G--U- ---------- A--A--G--- --U-----G-
001ZAI96        ---U---G-U ---U-G--U- -U-------- A--A------ --C-----G-
97_4ZAI97       C--U---G-U ---U-G--U- -U-------- A--A------ --C-----G-
9475ZAI         C--U---G-U ---U-G--U- -U-------- ---A--G-C- --C-----G-

427S0A85        C--U--CG-U ---U-A--A- -A-----C-- A--A--G--- --UC-U----
359SOA88        C--U--CG-U ---U-A--A- -A--A--C-- A--A------ --UC-U----
441SOA89        C--U--CG-U ---U-A--A- -A--A----- A--A--G--- --UC-U----
1170SOA88       ---U--CG-U ---U-A--A- -A-----C-- A--U--G--- --UC-U----
1434SOA87       ---U--CG-U ---U-G--A- -A-----C-- A--A--G--- --UC-U----
6224ZIM85       C--U---G-U ---U-A--A- -------C-- A--A--G--- --UC-U--G-

2CAR93          C--A--CG-C --UU-GA--- ----A--C-- ---A--G--C --C-------
13CAR93         C--A--CG-C --UU-GA--- ----A--C-- ---A--G--C --C-------
16CAR93         C--A--CG-C --UU-GA--- ----A--C-- ---A--G--C --C-------
20CAR93         C--A--CG-C --UU-GA--- ----A--C-- ---A--G--C --C-------
24CAR93         C--A--CG-C --UU-GA--- ----A--C-- ---A--G--C --C-------
11257EGY91      C--C--CG-U ---U-GA--- ----A--C-- ---A-----C --U--U----
8423ISR88       C--C--CG-U ---U-AA--- ----A--C-- ---A--G--C --C--U----
11300EGY91      C--C--CG-- -----GA--- ----A--C-- ---A--C--C -----U--CU

1CAR93          G--A--C--C ---------- ----A--C-- ---------- --G-A---G-
3CAR93          G--A--C--C ---------- ----A--C-- ---------- --G-A---G-
17CAR93         G--A--CG-C ---------- ----A--C-- ---------- --G-A---G-
24115KEN93      G--A--C--C ---------- ----A--C-- ---------- --GCAU--G-
24094SUD93      ---A--C--C ---------- ----A--C-- ---------- --GCAU--G-
24122EGY93      ---A--C--C ---------- ----A--C-- ---------- --G-AU--G-
5136ETH93       G--A--C--C ----U----- ----A--C-- ---------- --G-AU--G-
5135ETH93       G--A--C--C -----U---- ----A--C-- ---------- --G-AU--G-
08649IND91      A--A--C--C ---------- ----A----- ---------- --G-AU--G-
21197PAK91      A--A--C--C ---------- ----A--C-- A--U------ --G-AU--G-
```

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Figure 5.4 ..... continued from previous page

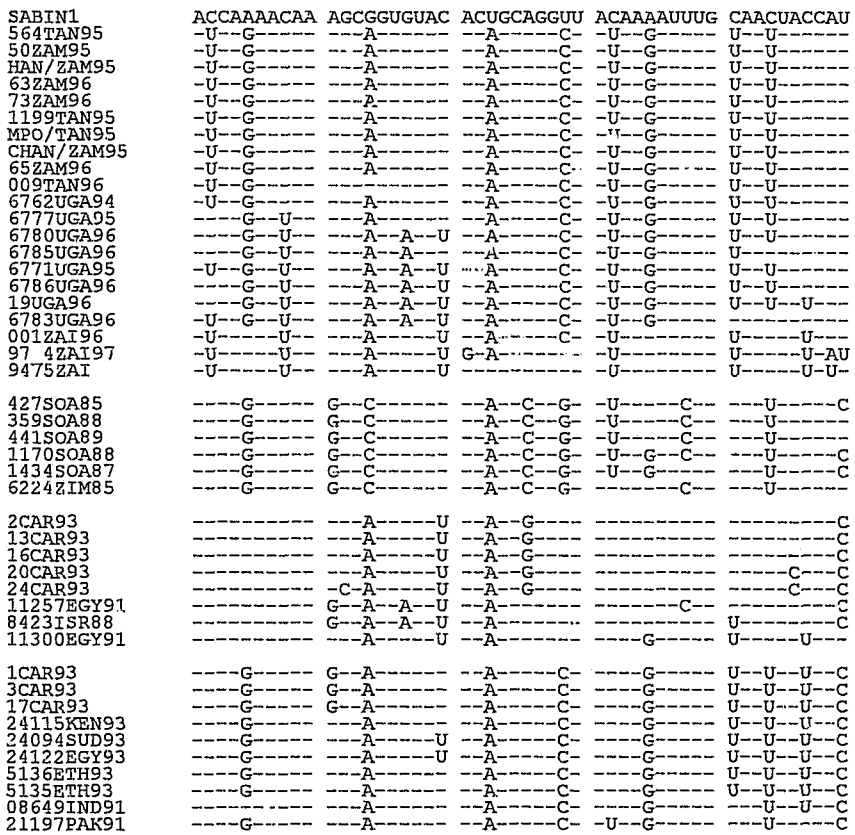

Figure 5. 4 ..... continued from previous page

5.3.2 Amino acid substitutions in the VP1/2A region

The predicted amino acid sequences encoded by the 150 bp VP1/2A sequences of representative strains belonging to each of the 9 genotypes found to be circulating in Africa (including the South African, Southern African and older Middle Eastern genotypes described in Chapter 4) are presented in Figure 5.5. The majority of the nucleotide differences between strains were silent, resulting in synonymous amino acid codons. Strains belonging to the Nigeria-2 genotype, and strains belonging to the South African genotype, (with the exception of isolates from 1984 and 1985, 710SOA84 and 855SOA85, which contained an alanine to arginine substitution at position 8 of protease 2A) had an amino acid composition identical to that of Sabin 1. Within the other genotypes, the most common substitution occurred at residue 20 of VP1; all strains within the West African, East African, Southern African and Middle Eastern (including the older Middle Eastern strains) genotypes substituted an alanine for the threonine at this position. Strains within the newer Middle Eastern genotype contained an additional substitution of a threonine for serine at position 295 (codon 23) of VP1. Two strains within this genotype, 20CAR93 and 24CAR93, contained additional substitutions of an arginine for alanine at residue 276 of VP1, and a tyrosine to serine substitution at residue 19 of protease 2A. With the exception of 17CAR93, which also exhibited the alanine for threonine VP1-20 substitution, all strains within the Indian genotype had a single substitution of a tyrosine or histidine for phenylalanine at residue 2 of protease 2A. A leucine for phenylalanine substitution at this residue was seen for isolates within the Southern African genotype. Two strains within the Nigeria-1 genotype, 004NIE96 and 5552NIE93, also contained the leucine for phenylalanine 2A-2 substitution, whereas strain 1276ANG94, in the West African genotype, contained a cysteine for phenylalanine substitution at this position. A unique proline for alanine substitution at residue 8 of protease 2A was evident for the most recent strains from Ghana (001GHA97 and 002GHA97) within the West African genotype, and for isolate 24CAR93 within the Middle Eastern genotype.

Several strains had other substitutions scattered within the 50 amino acid region under investigation. Isolates 9475ZAI, 20CAR93 and 24CAR93 differed from Sabin 1 by as many as 4 (8%) and 5 (10%) residues respectively.

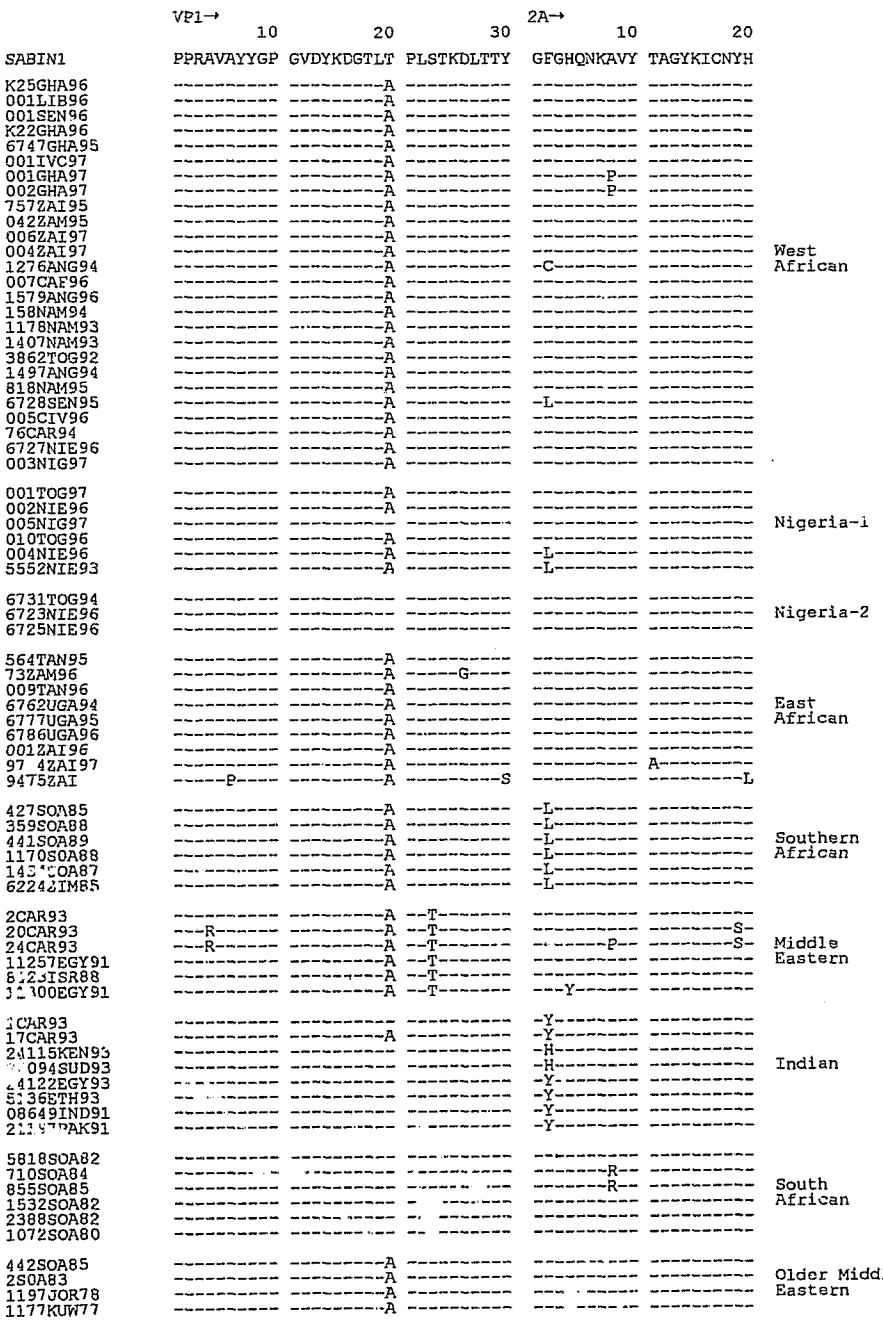

Figure 5. 5 Predicted amino acid sequences of the 150 bp VP1/2A interval for representative wild-type 1 polioviruses belonging to the West African, Nigeria-1, Nigeria-2, East African, Southern African, Middle Eastern, Indian, South African (1980-1985) and older Middle Eastern (1977-1985) genotypes. Dashes represent amino acids which are identical to those of Sabin 1.

5.4 DISCUSSION

Partial genomic sequence analysis was employed to study the molecular epidemiology of wild-type 1 polioviruses circulating throughout sub-Saharan Africa in recent years. The 150 bp region spanning the VP1/2A junction was chosen for sequence analysis, as it has been shown that although this region represents only 2% of the poliovirus genome, this is sufficient to obtain a reasonable picture of the natural distribution and transmission of wild-type polioviruses (Rico-Hesse *et al.*, 1987). This region codes for part of the viral capsid protein VP1 and for part of 2A, which has known protease function, so that differences in mutation rates for structural and non-structural proteins, if any, can be determined.

The majority of wild-type polioviruses circulating in Africa during the past 20 years were found to be type 1. Wild-type 3 has been isolated recently in CAR (Gouandjika *et al.*, 1995; also isolated in 1996 and 1997), Cameroun (1993), Togo (1996) and Madagascar (1995, 1997). Wild-type 2 was isolated in Madagascar during 1980. The wild-type 2 and 3 strains were sent to the NIV late in 1997, towards the completion of this study, and although sequence analysis of these isolates was performed, it was not possible to accurately determine regions of endemicity and routes of transmission, and thus the molecular epidemiology of these serotypes will not be discussed. No evidence of recombinant genomes (wild-type/Sabin or inter-serotype recombinants), characterised by VP1 and 2A sequences derived from 2 different genotypes/serotypes, was found. The rates of mutation of the sequence intervals coding for VP1 and 2A were found to be uniform.

To date, 9 major polio type 1 genotypes have been found in sub-Saharan Africa (Figures 4.2, 5.1 and 5.2). The geographic distribution of these genotypes in Africa is presented in Figure 5.6. The West African genotype, which has been in circulation since at least 1980, has the widest distribution, covering western, south western, central and southern Africa. This genotype may well have been circulating still earlier in Africa, since older isolates (1970-77) from Senegal and Cameroun also just fall within this genotype (Rico-Hesse *et al.*, 1987). Within the West African genotype are several smaller clusters which show a high degree of sequence diversity from each other and may represent newly emerging genotypes. They appear to be segregated geographically, indicating independent sustained circulation of these lineages in different geographical regions. Cluster A appears to be restricted to the west African countries of Senegal, Liberia, Cote D'Ivoire, Burkina Faso, Ghana and Togo. The relatively low degree of sequence diversity between strains within this cluster (with the exception of strains 001GHA97 and 002GHA97), suggests frequent cross-border transmission between countries. The most recent isolates from Ghana, strains

001GHA97 and 002GHA97, isolated after the NID's held in 1996, diverge by approximately 11% from strains isolated in the region in 1995-96; the absence of genetic similarity to any other strains rules out importation into the area, but rather is suggestive of the presence within the country of a previously undetected reservoir sustaining independent circulation of these strains. The NID's thus appear to have halted the circulation of the major lineage circulating within the country, but were not fully successful in reaching all of the reservoirs sustaining independent poliovirus circulation.

Cluster B includes isolates from central and south-western Africa. The high degree of sequence similarity (≥96%) between isolates from D.R.Congo, CAR, and Angola suggests possible cross-border transmission between D.R.Congo and Angola, and D.R.Congo and CAR between 1992 and 1997, although the direction of transmission cannot be established. The separate branching pattern displayed by isolates from D.R.Congo, Angola and CAR, however, suggests that sustained independent circulation subsequently occurred within the individual countries. The 99% sequence similarity between strains from the D.R.Congo epidemic and strains isolated from Zambia and Tanzania several months later indicates a definitive epidemiological link between cases and evidence for direct transmission of this genotype into Zambia and Tanzania. The absence of any further isolates belonging to this genotype from Zambia and Tanzania may indicate that further transmission of this strain in both countries was successfully interrupted by effective control measures.

A common link between cases from central Angola in 1994 and isolates from the 1993 epidemic in Namibia is also evident, possibly indicative of importation of the epidemic strain from Angola into previously polio-free Namibia, although the 6% divergence between strains isolated only months apart points to the introduction and silent transmission of this strain into Namibia at least 3 - 4 years earlier. The earlier presence of the West African genotype within Namibia is evidenced by the isolation of 4926NAM82 (Figure 4.2), which diverged from the later epidemic isolates by 14%. Since the availability of sequence data for strains isolated in Namibia prior to 1993 is limited to that for 4926NAM82, it is not possible to determine whether this strain represents a progenitor of the later 1993 epidemic isolates, or a strain imported into Namibia from western Africa.

In northern Namibia, the isolation in early 1995 of strains exhibiting ≥98% sequence similarity with those isolated several months earlier from southern Angola is evidence of a definitive epidemiological link between the cases, and of the direct introduction of the viruses from southern Angola into northern Namibia. Theses strains diverge by 12% from strains isolated in the interior of either country, indicative of the presence of a second reservoir of susceptibles, capable of prolonged sustained transmission of a separate

lineage of the West African genotype (cluster C) along the Angola-Namibia border.

Displacement of endemically circulating genotypes by an imported genotype, favoured by conditions of low vaccine coverage allowing the transmission of the imported strain amongst pockets of susceptible individuals (Rico-Hesse *et al.*, 1987), occurred both in the CAR and in South Africa. This phenomenon has also been reported for developing countries in South America (Rico-Hesse *et al.*, 1987). In the CAR, the West African genotype displaced the imported Indian and Middle Eastern genotypes in 1993. Strains belonging to this genotype fall within cluster F. Although strains from Cameroun, Nigeria and Niger are also included within this cluster, except for a direct link between strains in the CAR and Cameroun, there appears to be no evidence for direct transmission between these countries. In South Africa, the southern African genotype appears to have been introduced in 1985, most probably from countries north of the border (most likely Zimbabwe). This genotype went on to displace the endemically circulating genotypes, as evidenced by the subsequent isolation of strains belonging to the Southern African genotype only.

The presence, within a single country, of several reservoirs capable of independent transmission of polioviruses for prolonged periods of time, as evidenced by the concurrent circulation of more than one poliovirus genotype, was seen in Nigeria, Togo, CAR and South Africa. In Nigeria and Togo, 3 genotypes, the West African, Nigeria-1 and Nigeria-2 genotypes were co-circulating between 1993 and 1997. The unavailability of demographic information relating to strains within these genotypes makes it impossible to determine whether the genotypes circulated in different geographical niches or whether all three circulated within the same communities. In the CAR, 2 poliovirus genotypes, the Indian and Middle Eastern, were co-circulating during 1992-93. Viruses belonging to the Indian genotype represent strains most likely imported into the CAR from Sudan. Importation of strains from Sudan to the north of Kenya and to the CAR is probably the result of movements of large populations due to political unrest (Morvan *et al.*, 1997). The origins of this genotype, which is equivalent to genotype 4 described by Mulders *et al.* (1995), are, as the name suggests, in the Indian subcontinent. Strains belonging to this genotype have been associated with several outbreaks and have circulated for over 10 years in Europe, the Middle East and the Indian subcontinent (Mulders *et al.*, 1995; Huovilainen *et al.*, 1995). The Middle Eastern genotype represents strains originally circulating in the Middle East and probably imported into the CAR via Egypt. This genotype has also been previously described, associated with cases of poliomyelitis in Middle Eastern countries (Mulders *et al.*, 1995). The high degree of diversity (9%) between isolates from the CAR and Egypt, separated by a time interval of only 2 years, suggests continued silent transmission of this genotype over a period of several years.

In South Africa, 3 independent poliovirus genotypes, the South African, West African and older Middle Eastern genotypes were in circulation between 1982 and 1985 (see Chapter 4 for more detailed discussion). Five strains belonging to the West African genotype and displaying 87% sequence similarity to strains isolated in west Africa, were isolated from a wide geographical area in the country, suggesting that these strains were not originally endemic to the country, but represent imported strains, most probably from countries in western Africa. Similarly, strains belonging to the Middle Eastern genotype represent importation from the Middle East. What cannot be ascertained is whether theses genotypes were introduced directly from west Africa or the Middle East, or whether their geographical range also included other African countries neighbouring South Africa, from which the introduction may have taken place. The sequence heterogeneity between the South African strains belonging to these genotypes is suggestive of progenitor infections in the late 1970's. The absence of any epidemiological relationships between strains from the older Middle Eastern genotype, circulating in the Middle East in the 1970's, and the more recent Middle Eastern genotype circulating in the late 1980's, suggests that the more recent Middle Eastern genotype was not derived simply by evolution of the older genotype, but represents an independent genotype. The South African genotype was endemic in South Africa since at least 1980. The high degree of sequence divergence between South African strains within each genotype suggests widespread circulation and sustained endemic transmission of individual strains along separate pathways for extended periods.

Simultaneous co-circulation of more than 1 genotype in regions with sub-optimal health services has also been reported from China (Zheng *et al.*, 1993), the former Soviet Union (Lipskaya *et al.*, 1995) the Middle East and Pakistan (Mulders *et al.*, 1995).

The identification of a definitive epidemiological link between cases from Tanzania and Zambia illustrates the resolving power of molecular epidemiology in establishing otherwise unrecognised links. The high degree of sequence similarity between isolates from these countries is indicative of direct importation of the Tanzanian strain (East African genotype) into Lusaka. The isolation in Lusaka of strains displaying highly similar nucleotide sequences (99 - 100%) suggests rapid epidemic transmission amongst inadequately immunised individuals. The 4% divergence between a strain isolated in Uganda in 1994 and the 1995-6 Tanzania/Zambia isolates suggests that the East African genotype was originally introduced into Tanzania from Uganda. The presence of a second East African cluster in Uganda, differing from the 1994 Uganda and 1995-6 Tanzania/Zambia strains by approximately 7%, suggests that at least 2 reservoirs sustaining independent wild-type poliovirus circulation existed in Uganda. Included in the East African genotype are strains from D.R.Congo. They branch separately within the genotype, indicating independent

evolution of the genotype within the country. The presence of strain 9475ZAI, isolated in D.R.Congo some time prior to 1987, suggests that the East African genotype has been in circulation in central Africa for several years and that the route of transmission may have been from D.R.Congo into neighbouring Uganda.

Strains from the East and Southern African genotypes exhibit an average of 84% sequence similarity (sequence divergence of 14%, however, is observed between 50ZAM95 and 6226ZIM85, and between 50ZAM95 and 1170SOA88). Although this exceeds the 15 % divergence limit set for identification of relationships based on epidemiological grounds, it is possible that the two genotypes, which are separated temporally by about 10 years and which cover neighbouring geographical areas may represent separate evolution of the same progenitor genotype along two different transmission pathways. Results of bootstrap analysis of the sequence data in the construction of the dendrogram indicate that the branching pattern connecting the two genotypes is significant, occurring in >76% of trees sampled.

Accurate determination of relationships between type 1 polioviruses based on their amino acid sequences was not possible, as most of the base differences between strains were silent and produced synonymous codons. Consistent with published data (Rico-Hesse *et al.*,1987), the most frequent substitution was an alanine for threonine at position 292 of VP1 (residue 20, Figure 5.5); this mutation appears to be common for all strains except those originating from the Indian subcontinent, and those belonging to the Nigeria-1, Nigeria-2 and South African genotypes. Close analysis of the amino acid sequences revealed that there appears to be a correlation between the amino acid substitutions and specific genotypes. The double substitution of alanine for threonine and threonine for serine at residues 20 and 23 of VP1 respectively was found only in strains that fell into the Middle Eastern genotype. Analysis of the published amino acid sequences of wild-type 1 strains from a wider geographical region (Rico-Hesse *et al.*, 1987) revealed that strains obtained between 1980 and 1984 from Venezuela, introduced into the country from the Middle East, also contained the same 2 substitutions. Isolates obtained 10 years apart from the CAR and from Venezuela exhibit 86-88% sequence similarity (results not shown), indicative of their relatedness to the same progenitor strain from the Middle East. Consistent with published data for type 1 strains from Pakistan (Huovilainen *et al.*, 1995) and from Tajikistan, north of the Pakistan border (Lipskaya *et al.*, 1995), substitution of a tyrosine or histidine for phenylalanine at position 2 of 2A was seen for strains belonging to the Indian genotype. A unique leucine for phenylalanine substitution at the same position was evident for strains belonging to the Southern African genotype.

The limited number of amino acid substitutions in the VP1/2A region may indicate a requirement for polypeptide structure conservation in both the carboxyl terminus of VP1 and the amino terminus of protease 2A. There does, however, appear to be a tendency for the conservation of unique substitutions associated with certain genotypes, although the significance of this observation is unclear. None of the mutations observed were found to occur at sites shown by mutational analysis of the substrate recognition determinants of protease 2A to be important for autocatalytic cleavage at the VP1/2A junction (Hellen *et al.*,1992); variation at residue 2 of 2A, exhibited by isolates in the Southern African and Indian genotypes, has been found to be tolerated, and the critical threonine and glycine residues at positions 29 of VP1 and 1 of 2A were conserved for all strains examined.

In summary, the distribution and molecular epidemiology of wild-type 1 poliovirus genotypes in sub-Saharan Africa has been described, based on the poliovirus isolates /sequence data available for analysis. Although by no means complete, due to the unavailability of isolates from many countries and of demographic data for many of the available strains, the results provide an overview of the past and present wild-type 1 poliovirus circulation across most of the African continent. It must be noted, however, that the results presented here are based on the analysis of strains / sequence data which were available at the NIV, and may thus not accurately represent both the number and geographic distribution of all genotypes in circulation. Of the 9 genotypes described here, 3 , previously circulating in southern Africa (the South African, older Middle Eastern and the Southern African) appear to have been eliminated, and 2 imported genotypes (more recent Middle Eastern and Indian) appear to have been displaced, at least from the Central African Republic. The distribution of the East African genotype appears to be limited to D.R.Congo, Uganda, Tanzania and Zambia, although the absence of recent strains from the latter 3 countries may indicate that the circulation of this genotype within these countries has been effectively controlled. The West African genotype continues to circulate endemically throughout most of west and central Africa.

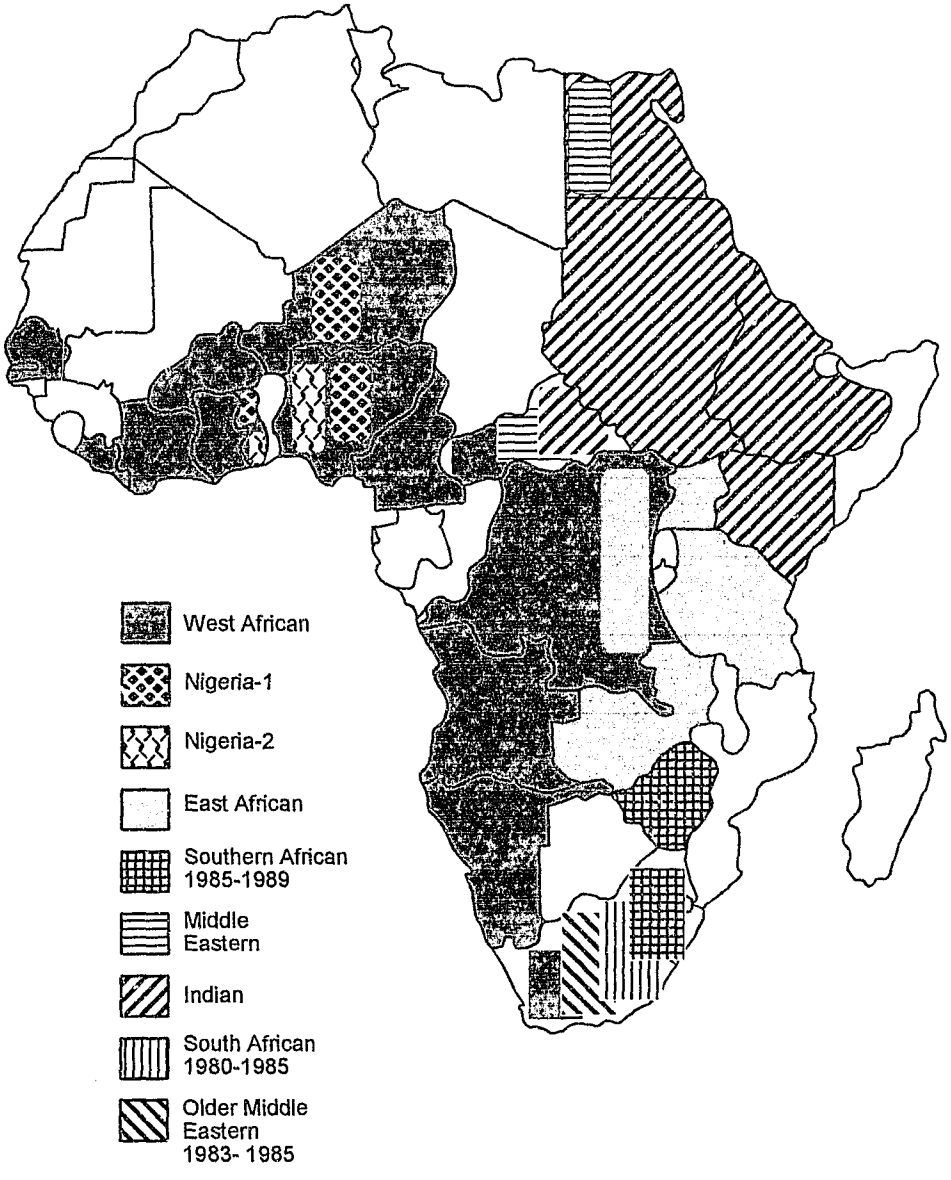

Figure 5.6 Geographic distribution of poliovirus type 1 genotypes in Africa, 1980-1997, based on specimens / sequence data available for analysis at the NIV. Unshaded areas represent countries for which no data is available.

6. GENOTYPE-SPECIFIC AMPLIFICATION OF WILD-TYPE 1 POLIOVIRUSES FROM SUB-SAHARAN AFRICA

6.1 INTRODUCTION

The identification and characterisation of the major wild poliovirus genotypes has paved the way for the development of molecular reagents for the rapid and sensitive direct detection and identification of specific wild-type virus strains. Since laboratory confirmation of wild-type poliovirus infection is a critical aspect of the PEI, direct identification of wild-type poliovirus infection offers enhanced diagnostic reliability. Genotype-specific molecular reagents are also especially useful for monitoring silent infections and transmission, and for monitoring environmental contamination by wild-type viruses. Genotype-specific probes and PCR primer sets have been used extensively for monitoring and surveillance of wild-type poliovirus circulation in high risk communities, either through surveillance of healthy individuals, community wastewater, or drinking and recreational water samples (Da Silva *et al.*, 1991; Yang *et al.*, 1992; Tambini *et al.*, 1993; De *et al.*, 1997; Mulders *et al.*, 1995c).

PCR analysis in particular offers the advantages of speed and exceptional sensitivity and selectivity. PCR primer sets have been developed that can detect sequences of specific wild poliovirus genotypes in samples containing large stoichiometric excesses (up to 10^6-fold) of vaccine-related RNA's (Yang *et al.*, 1992). Poliovirus group- and serotype-specific PCR primer sets have also been developed (Kilpatrick *et al.*, 1996, 1998).

The design of genotype-specific reagents is only feasible when all genotypes circulating in a particular area have been identified, and when effective control strategies have reduced both the number of circulating genotypes and the extent of sequence heterogeneity between strains within individual genotypes. In the previous chapters, the poliovirus type 1 genotypes circulating in sub-Saharan Africa have been described. The majority of wild-type polio 1 strains presently circulating in sub-Saharan Africa have been found to belong to the indigenous West and East African genotypes. In this chapter, the development of PCR reagents for the efficient and sensitive detection of wild-type 1 polioviruses belonging to the genotypes indigenous to Africa will be described.

6.2 MATERIALS AND METHODS

6.2.1 Viruses

Twenty eight wild type 1 poliovirus strains (Table 6.1), representative of the West and East African genotypes, as well as the Southern African, South African and older Middle Eastern genotypes, were selected for sequence analysis of the amino- terminus of VP1 for the subsequent design of genotype-specific primers. All strains had been previously characterised by Sabin-specific PCR and partial genomic sequencing across the VP1/2A region.

6.2.2 Sequence analysis of the amino- terminus of VP1

Viral RNA was extracted as described in Section 3.2.2a. RNA concentrations were determined spectrophotometrically using the following formula:

$OD_{260} \approx 1$ corresponds to approximately 40 μg RNA per millilitre (Sambrook *et al.*, 1989)

DNA sequencing templates were generated by reverse transcription and amplification of the viral RNA with primers VP3-1 and VP1-1 (Table 3.1), as described in Section 3.2.7. Dideoxy sequencing was performed using the Sequenase PCR-product sequencing kit (United States Biochemicals) with ^{35}S-dATP. Prior to sequencing, amplified templates (5 μl per sequencing reaction) were purified by digestion at 37°C for 15 minutes with 10 units Exonuclease 1, and 2 units Shrimp Alkaline Phosphatase (provided in the sequencing kit). Following digestion, the enzymes were denatured by incubating the reactions at 80°C for 15 minutes. Purified templates were sequenced in both directions using primers VP3-1 and VP1-1 as sequencing primers. Sequencing products were resolved on 8% acrylamide gels containing 7M urea, and visualized by autoradiography. Sequence data was analysed using DNASIS 2.5 software (Hitachi).

A dendrogram representing sequence divergence between strains (amino- terminus of VP1, nt 2479-2858) was generated as described in Section 3.2.6.

Table 6.1 Poliovirus type 1 strains selected for sequence analysis of the amino- terminus of VP1

Strain	Country in which isolated	Year of isolation	Genotype
1531SOA82	South Africa	1982	South African
1325SOA82	South Africa	1982	South African
1677SOA82	South Africa	1982	South African
3043SOA84	South Africa	1984	South African
5918SOA82	South Africa	1982	South African
442SOA85	South Africa	1985	older Middle Eastern
72NAM95	Namibia	1995	West African
289NAM95	Namibia	1995	West African
284NAM95	Namibia	1995	West African
266NAM95	Namibia	1995	West African
1587ANG96	Angola	1996	West African
1579ANG96	Angola	1996	West African
1277ANG94	Angola	1994	West African
1281NAM93	Namibia	1993	West African
1266NAM93	Namibia	1993	West African
1227NAM93	Namibia	1993	West African
1177NAM93	Namibia	1993	West African
042ZAM95 [a]	**Zambia**	**1995**	**West African**
65ZAM96	Zambia	1996	East African
564TAN95 [b]	**Tanzania**	**1995**	**East African**
50ZAM95	Zambia	1995	East African
MpoTAN95	Tanzania	1995	East African
63ZAM95	Zambia	1995	East African
73ZAM96	Zambia	1996	East African
009TAN96	Tanzania	1996	East African
359SOA88	South Africa	1988	Southern African
181SOA88	South Africa	1988	Southern African
364SOA88	South Africa	1988	Southern African

[a] Reference strain for design of the West African genotype-specific primers R-WA2/F-WA5.

[b] Reference strain for design of the East African genotype-specific primers R-EA1/F-EA1.

6.2.3 Genotype-specific primers

Primer pairs for the specific amplification of poliovirus type 1 strains belonging to the West and East African genotypes, and for the amplification of both genotypes (pan-African) are listed in the following table (Table 6.2)

Table 6.2 Primer pairs for the specific amplification of poliovirus type 1 strains belonging to the West and East African genotypes, and for amplification of both genotypes (pan-African)

Primer	Position in genome*	Sequence (5' 3')	Amplicon size (bp)	Specificity
R-EA1 (reverse)	2712-2736	GGA TTC CAC ACT AGA CTC CGA CCT C	248 bp	East African genotype
F-EA1 (forward)	2489-2505	CAC TAA CAC AGG GAC TG		
R-WA2 (reverse)	2784-2782	TTT GGA (TC)GT GGT GGA TGC C	254 bp	West African genotype
F-WA5 (forward)	2550-2568	CAT AGG (CT)GC TTC (AG)AC CTC C		
R-WA1 (reverse)	2769-2787	TGC CGA GTT GTC CAC GGT C	239 bp	East and West African genotypes
F-AF1 (forward)	2550-2571	CAT AGG (TCG)GC (TG)TC (AG)AC (AC)TC (AC)AG G		(also South and Southern African genotypes)

* Position in the genome of Sabin 1. Numbering according to Toyoda *et al.*, 1984.

6.2.4 Genotype-specific amplification

The specificity of each set of genotype-specific primer pairs was evaluated using the poliovirus strains listed in Table 6.3. RNA was extracted from the poliovirus isolates as described in section 3.2.2a.

Genotype-specific amplification was performed in two steps (reverse transcription followed by PCR amplification) as follows:
Reverse transcription was carried out for 45 minutes at 42 °C in a 20μl reaction containing 50 mM Tris-HCl, pH 8.5, 30 mM KCl, 8 mM $MgCl_2$, 1 mM DTT, 0.5 mM of each deoxynucleotide triphosphate, 20 units RNase inhibitor (Boehringer Mannheim), 12.25 units AMV reverse transcriptase (Boehringer Mannheim), 10 pmol of the reverse downstream primer, and 5μl RNA. Upon completion of cDNA synthesis, reactions were heated to 95 °C for 3 minutes to denature the reverse transcriptase, and cooled on ice. PCR was performed using 5 μl cDNA mixture as template, which was added to 95 μl of PCR master mix containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each deoxynucleotide triphosphate, 20 pmol each of the forward and reverse primers, and 2.5 units of *Taq* polymerase (Boehringer Mannheim). Amplification was performed using the following programme on a Stratagene Robocycler Gradient 40 automatic cycler: 1 cycle of 95 °C for 2 minutes; 30 cycles of 95 °C for 1 minute, optimal annealing temperature (see below) for 1 minute, and 72 °C for 90 seconds; 1 cycle of 72 °C for 8 minutes. Amplified products were detected by electrophoresis through 2% agarose gels containing ethidium bromide (0.1μg /ml).

Optimal annealing temperatures for the genotype-specific primer pairs were as follows:

R-EA1 + F-EA1	54 °C
R-WA2 + F-WA5	54 C
R-WA1 + F-AF1	48 °C

Table 6.3 Poliovirus strains used to evaluate the specificity of the genotype-specific primer pairs

Strain	Genotype	Amplification		
		R-EA1/F-EA1	R-WA2/F-WA5	R-WA1/F-AF1
Sabin 1		-	-	-
Sabin 2		-	-	-
Sabin 3		-	-	-
001SEN96	West African - A	-	+	+
1227NAM93	West African - B	-	+	+
1277ANG94	West African - B	-	+	+
765ZAI95	West African - B	-	+	+
042ZAM95	West African - B	-	+	+
005CAF97	West African - B	-	+	+
72NAM95	West African - C	-	+	+
1587ANG96	West African - C	-	+	+
1CAR94	West African - F	-	+	+
564TAN95	East African	+	-	+
1199TAN95	East African	+	-	+
MpoTAN95	East African	+	-	+
50ZAM95	East African	+	-	+
ChansaZAM95	East African	+	-	+
HanyZAM95	East African	+	-	+
73ZAM96	East African	+	-	+
009TAN96	East African	+	-	+
012TAN96	East African	+	-	+
13CAR93	Middle Eastern	-	-	-
442SOA85	older Middle Eastern	-	-	-
17CAR93	Indian	-	-	-
364SOA88	Southern African	-	-	+
1325SOA82	South African	-	-	+

6.3 RESULTS

6.3.1 Design of genotype-specific primer pairs

Primer pairs were designed for the specific amplification of the 2 major poliovirus type 1 genotypes circulating in Africa, namely the East and West African genotypes. In addition, a separate primer pair was designed for the amplification of all indigenous African poliovirus type 1 genotypes (West, East, Southern and South African). Table 6.2 lists the primer sequences, their positions in the poliovirus genome, and the expected size of amplicon products. The specificity of each set of genotype-specific primer pairs was evaluated using the poliovirus strains listed in Table 6.3.

In order to select optimal primer binding sites, the amino- terminus of VP1 of representative strains from each of the poliovirus type 1 genotypes circulating in Africa at present or in the past was sequenced. Strains belonging to the Nigeria-1 and Nigeria-2 genotypes were not included, however, since they had not yet been identified when this section of the study was initiated, and strains belonging to the Nigeria-2 genotype were not available for sequence analysis (VP1/2A sequence data only was kindly provided for these strains; see Chapter 5 for details). Strains for sequence analysis were selected to represent individual lineages within the individual genotypes (particularly in the case of the West African genotype), such that the selected primers sets would efficiently amplify related strains displaying a high degree of sequence heterogeneity.

A 380 bp interval coding for the amino- terminus of VP1 was compared for the selected representative strains. Nucleotide substitutions appeared to be evenly distributed throughout the 380 bp interval. To ensure that comparison of the nucleotide sequences within this interval accurately reflected comparison of the genotypes defined by sequence analysis of the VP1/2A region, a dendrogram of sequence divergence between the representative strains was generated (Figure 6.1). The branching pattern between and within genotypes was found to be directly comparable to that generated by comparing VP1/2A sequences. Strains 564TAN95 (East African genctype) and 042ZAM95 (West African genotype) were chosen as reference strain for the selection of optimal primer binding sites. These strains differed from Sabin 1 at 84 (22%) and 76 (20%) of the 380 positions respectively, and from each other at 87 of the 380 positions (23%).

Because of the extent of nucleotide sequence heterogeneity between all strains compared, it was not possible, by direct comparison of the nucleotide sequence data, to accurately identify sequence intervals that were specific to individual genotypes.

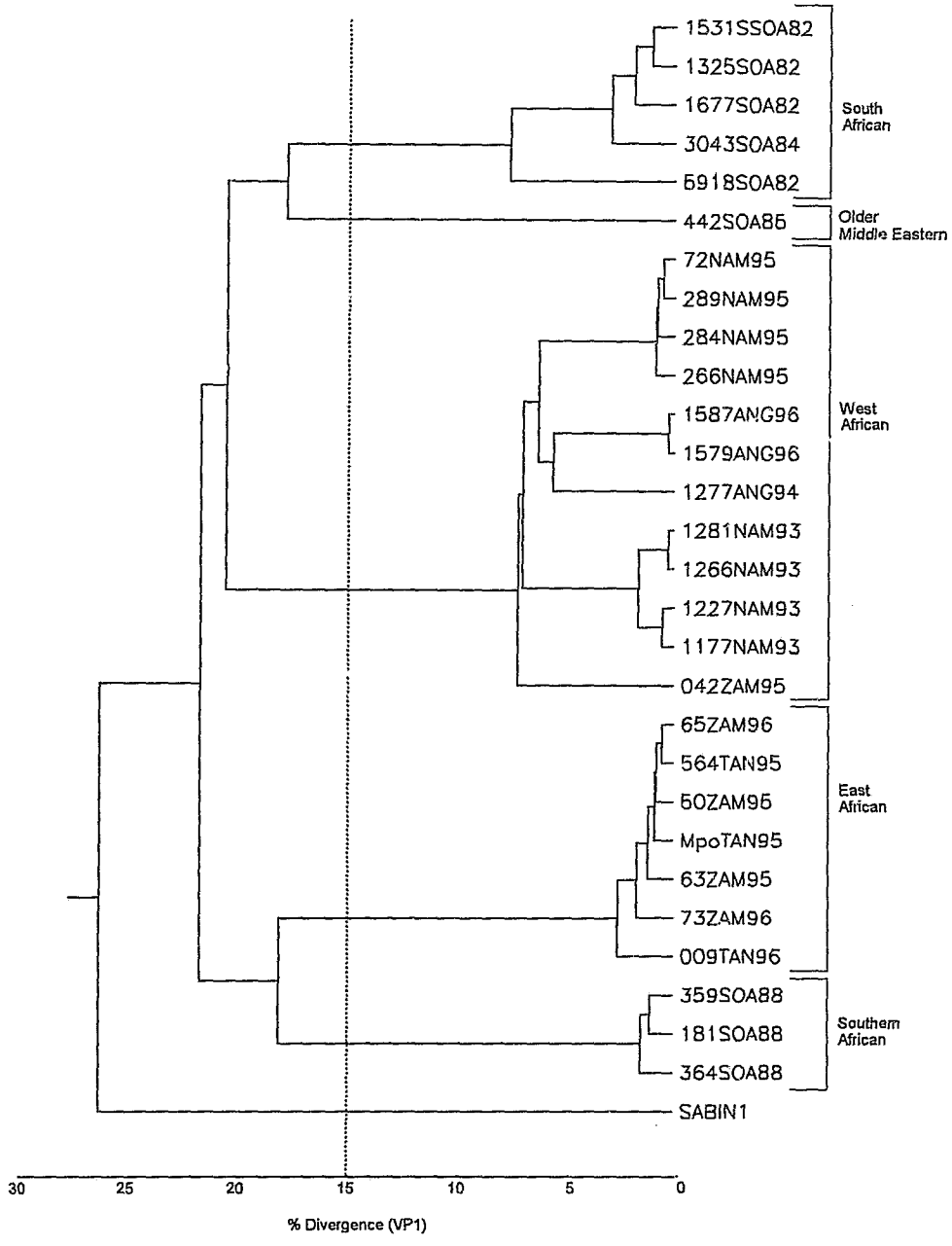

Figure 6.1 Dendrogram of sequence divergence (amino- terminus of VP1, nt 2479-2858) between representative type 1 polioviruses from Africa. Country abbreviations are listed in the Preface.

The amino acids encoded by the 380 bp region were thus predicted. Although the majority of the nucleotide mutations were silent, generating synonymous codons, as many as 14 (11%) amino acid differences from those of Sabin 1 were found within the 127 amino acid interval (data not shown). Strains 564TAN95 and 042ZAM95 differed from Sabin 1 by 12 and 10 amino acids respectively (data not shown). Intervals encoding amino acids unique for the individual genotypes were selected for primer binding sites, which were targeted to intervals displaying multiple nucleotide mismatches with both the Sabin 1 template and other heterologous wild-type templates (Figure 6.2). The East African genotype-specific primers F-EA1 and R-EA1 mismatch the Sabin 1 template by 5 of 17 and 7 of 25 nucleotide positions respectively, and mismatch the West African genotype reference strain 042ZAM95 by 7 of 17 and 6 of 25 nucleotide positions respectively. Similarly, the West African genotype-specific primers F-WA5 and R-WA2 mismatch the Sabin 1 template by 8 of 16 and 6 of 19 nucleotide positions respectively, and mismatch the East African genotype reference strain 564TAN95 by 4 of 17 and 3 of 19 nucleotide positions respectively. Because of the extent of sequence heterogeneity between strains within the West African genotype, mixed (degenerate) bases were included at positions of highest degeneracy within the West African-specific primers R-WA2 and F-WA5.

The pan-African primers F-AF1 and R-WA1 were targeted to regions of multiple mismatches with the Sabin 1 template (10 of 22 and 5 of 19 positions respectively), but which exhibited minimal mismatches with strains belonging to East, West, Southern and South African genotypes. Mixed bases were also included in the pan-African forward primer F-AF1 in order to increase the binding efficiency of the primer to highly heterologous targets.

All the primers were designed with the 3' terminal base matched to a third position wobble base on the homologous reference template, and all 3' terminal bases comprise G 's or C's. Mismatches at the 3' terminal base, especially those involving A:G, G:A and C:C have the greatest potential to reduce amplification efficiency, whereas mismatches of T with either G, C or T have a minimal effect on PCR product yield (Kwok *et al.*, 1990).

Primer binding sites were spaced along the target genomes so that the amplification products (246 bp for the East African-specific, 254 bp for the West African-specific, and 239 bp for the pan-African primers) could be easily differentiated from the amplicons generated with the Sabin-specific primer pairs (Sabin 1, 97 bp; Sabin 2, 71 bp; Sabin 3, 53 bp; see Chapter 3).

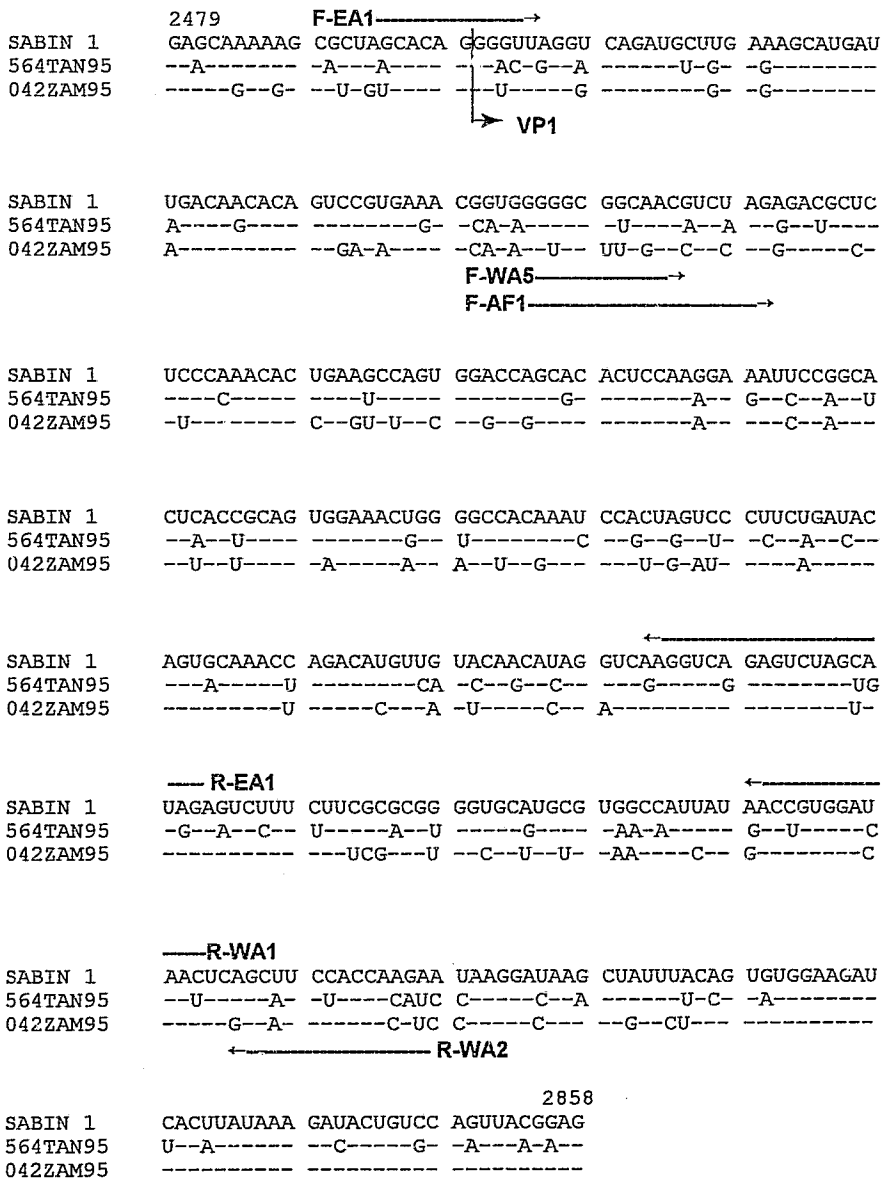

Figure 6.2 Comparison of VP1 sequences (nt 2479-2858) of Sabin 1, 564TAN95 (reference strain for the East African genotype-specific primers) and 042ZAM95 (reference strain for the West African genotype-specific primers). Dashes represent nucleotides that are identical to those of Sabin 1. The positions of the primers are indicated above and below the nucleotide sequences. The Sabin 1 nucleotide positions are numbered according to Toyoda *et al.* (1984).

6.3.2 Specificity of the East African genotype-specific primers

The East African-specific primer pair R-EA1/F-EA1 efficiently amplified all tested strains belonging to the East African genotype (Table 6.3; Figure 6.3). All amplification products were of the expected size (248 bp). The Sabin 1, 2 and 3 reference strains, and strains belonging to the West, Southern and South African genotypes, as well as those belonging to the Indian, Middle Eastern and older Middle Eastern genotypes, failed to amplify with the East African genotype-specific primer pair.

6.3.3 Specificity of the West African genotype-specific primers

The West African-specific primer pair R-WA2/F-WA5 efficiently amplified all tested strains belonging to the West African genotype (Table 6.3; Figure 6.4). All amplification products were of the expected size (254 bp). The Sabin 1, 2 and 3 reference strains, and strains belonging to the East, Southern and South African genotypes, as well as those belonging to the Indian, Middle Eastern and older Middle Eastern genotypes, failed to amplify with the West African genotype-specific primer pair.

6.3.4 Specificity of the pan-African primers

The results of amplification using the pan-African primer pair R-WA1/F-AF1 are presented in Table 6.3 and Figure 6.5. All strains representing genotypes indigenous to Africa (West, East, Southern and South African) were successfully amplified using the pan-African primers. No amplification of strains representing genotypes introduced from other regions (Indian, Middle Eastern and older Middle Eastern genotypes) occurred.

6.3.5 Selective amplification of East and West African genotype strains in samples containing mixtures of wild-type and vaccine-like strains

To evaluate the ability to detect wild-type templates in samples containing mixtures of wild-type and vaccine-like strains, a constant quantity (50 pg) of RNA from strain 564TAN95 (East African genotype) or 042ZAM95 (West African genotype) was mixed with different concentrations of Sabin 1 RNA (50 pg to 5 μg in 10-fold increments), such that mixtures containing wild-type:vaccine-like template ratios of 1:1 to 1:10^5 were obtained. The mixtures were then amplified using the East African-specific primer pair R-EA1/F-EA1 or the West African-specific primer pair R-WA2/F-WA5. The results of the amplification reactions are presented in Figure 6.6. The presence of wild-type RNA templates was detectable, using the genotype-specific primers, in mixtures containing a 10^5-fold excess of Sabin 1 RNA.

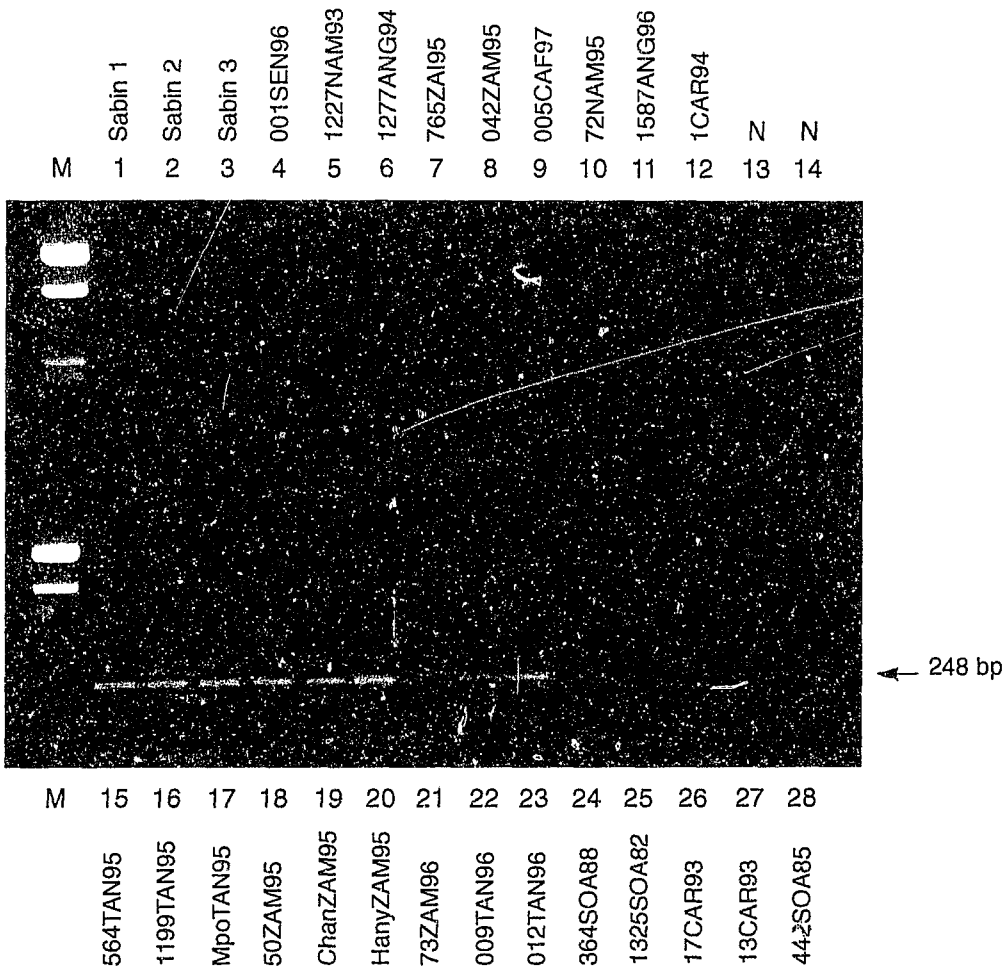

Figure 6.3 Specific amplification, using the R-EA1/F-EA1 primer pair, of poliovirus type 1 strains belonging to the East African genotype. The Sabin poliovirus strains and wild-type strains belonging to other genotypes fail to amplify with the East African-specific primers. M = *TaqI* digest of ΦX174. N = control reactions without RNA.

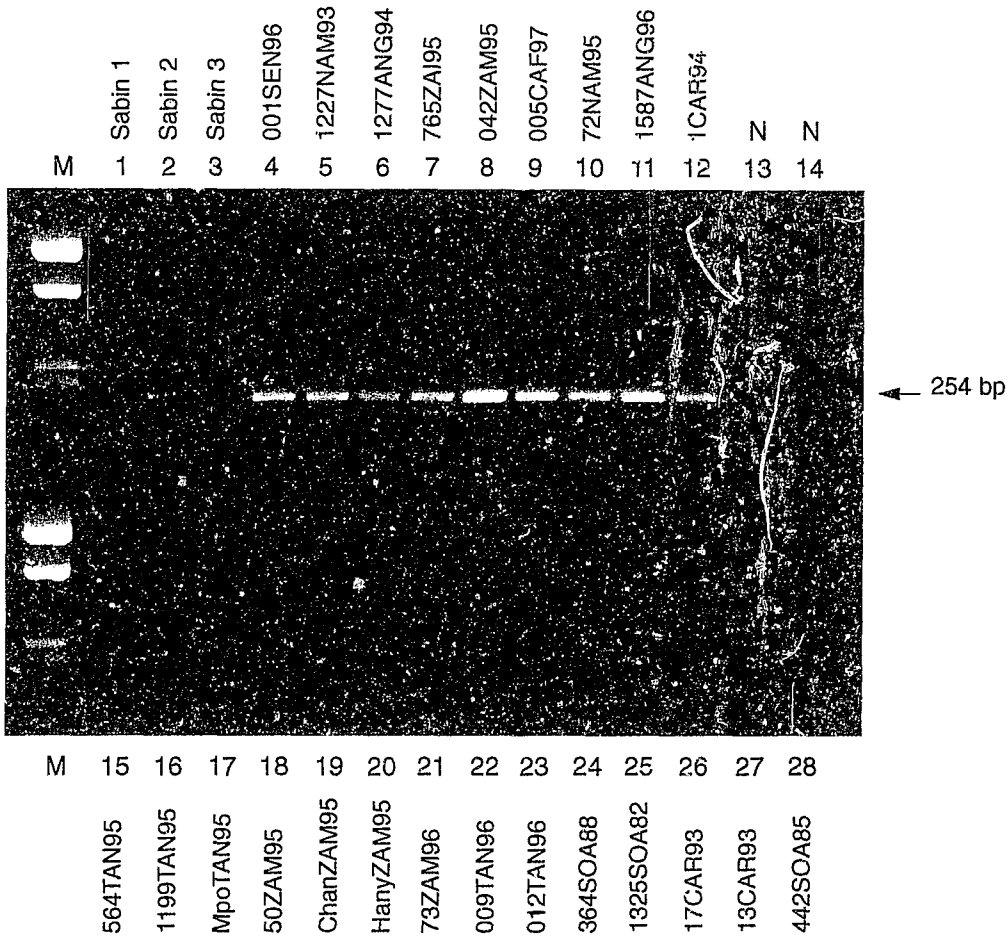

Figure 6.4 Specific amplification, using the R-WA2/F-WA5 primer pair, of poliovirus type 1 strains belonging to the West African genotype. The Sabin poliovirus strains and wild-type strains belonging to other genotypes fail to amplify with the West African-specific primers. M = *TaqI* digest of ΦX174. N = control reactions without RNA.

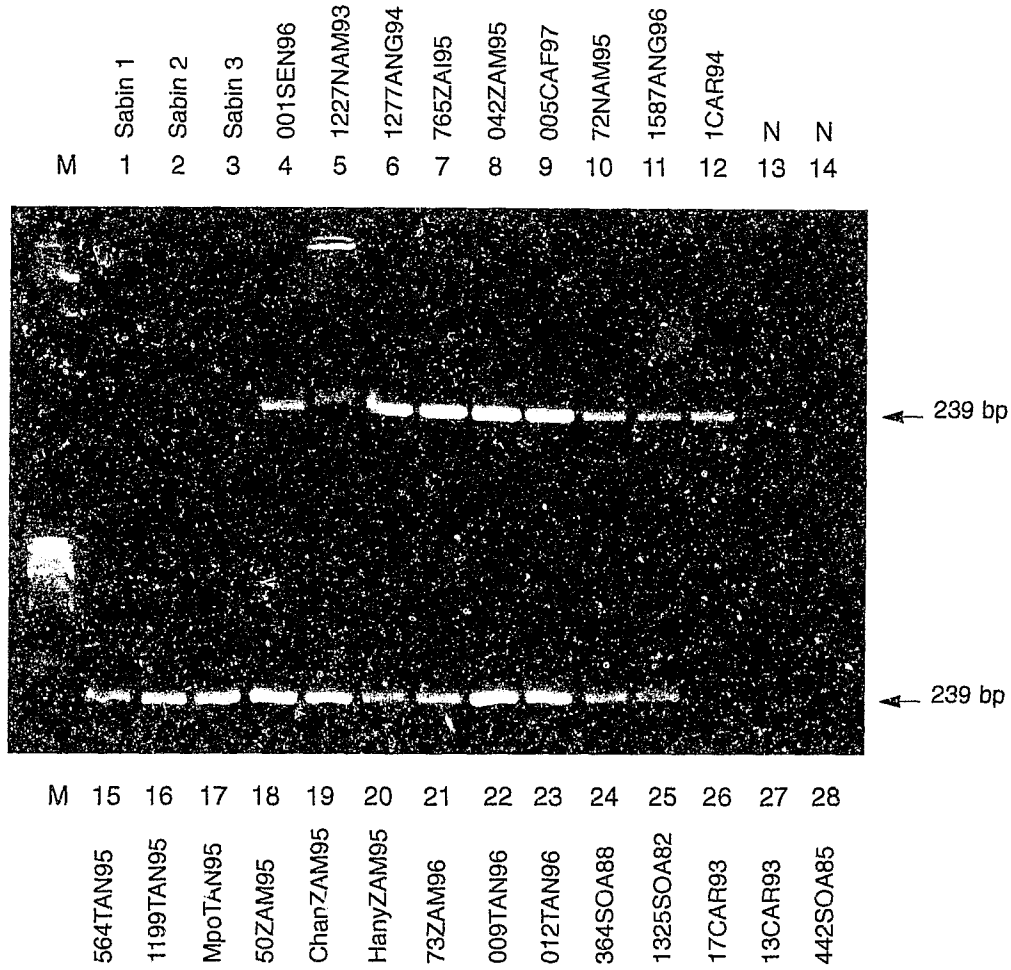

Figure 6.5 Amplification, using the pan-African R-WA1/F-AF1 primer pair, of poliovirus type 1 strains belonging to the East, West, Southern and South African genotypes. Poliovirus strains belonging to the Indian, Middle Eastern and older Middle Eastern genotypes fail to amplify with the pan-African primers. M = *TaqI* digest of ΦX174. N = control reactions without RNA.

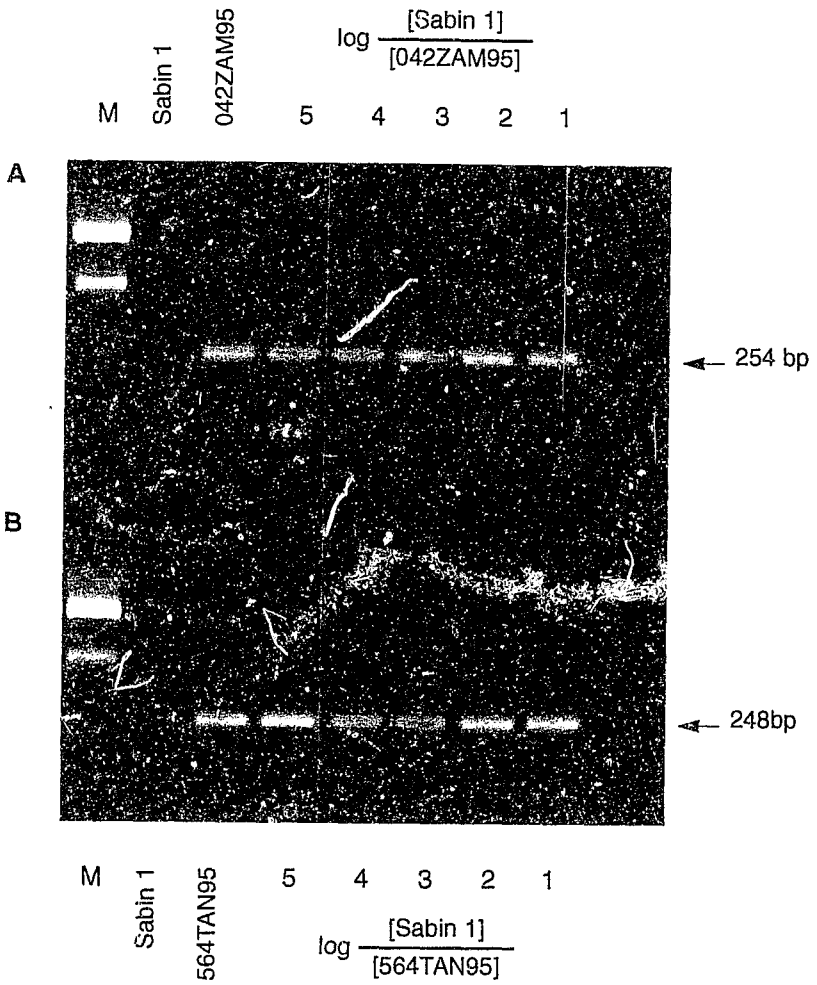

Figure 6.6 Selective detection of wild-type polio 1 strains in the presence of excess Sabin 1 template. **Panel A**: amplification of 042ZAM95 (West African genotype) with the primer pair R-WA2/F-WA5. **Panel B**: amplification of 564TAN95 (East African genotype) with primer pair R-EA1/F-EA1. The logarithm of the molar ratio of Sabin 1 template to that of each of the wild-types is indicated above (panel A) or below (panel B) each lane. M = *TaqI* digest of ΦX174. N = control reactions without RNA.

6.4 DISCUSSION

PCR reagents have been developed for the rapid and sensitive detection of the two major wild-type 1 poliovirus genotypes presently circulating in Africa, namely the West and East African genotypes. In addition, a primer pair for the amplification of all poliovirus type 1 genotypes indigenous to Africa has also been designed.

The majority of wild-type polio 1 strains circulating in sub-Saharan Africa have been found to belong to the West and East African genotypes; although strains belonging to the Middle Eastern and Indian genotypes also co-circulated in central and north-east Africa for several years, no strains belonging to these genotypes have been obtained in recent years. Thus the described primer sets should allow for surveillance and screening for wild-type 1 polioviruses of the presently known genotypes, except perhaps for those belonging to the Nigeria-1 and Nigeria-2 genotypes. These genotypes, to date known only to be circulating in Nigeria, Niger and Togo, were identified only after this study was already in progress. In addition, strains belonging to the Nigeria-2 genotype were not available for sequence analysis. For these reasons, reagents specific to these 2 genotypes could not be designed. If the distribution of these genotypes is contained, the unavailability of reagents specific for the Nigeria-1 and Nigeria-2 genotypes will not pose too big a problem, as alternative techniques can be employed for rapid molecular characterisation. Should these genotypes predominate in Africa, then specific reagents may be required for effective screening and surveillance.

The majority of wild-type 1 strains presently circulating in west and central Africa belong to the West African genotype. A strain from central Africa, 042ZAM95, was chosen as the reference strain for the design of West African-specific primers, since central Africa remains one of the major reservoirs of wild-type viruses in Africa. The extent of sequence heterogeneity between strains within the West African genotype is very high (approaching 15%), thus complicating the design of primers for the sensitive and efficient amplification of widely divergent, although related, strains. In order to compensate for the high degeneracy of the targeted codons between strains within this genotype, primers were designed to contain mixed-base residues. In this way it was possible to amplify all heterologous strains within the West African genotype. However, if independent evolution of lineages within this genotype continues, the usefulness of the West-African-specific primers may be limited to the next few years only.

Strains within the East African genotype exhibit far less genomic variation between them, such that the use of degenerate bases in the design of the East African-specific primers was not required.

The amino- terminus of VP1 was selected as the region of choice for genotype-specific primer design for the following reasons: (1) nucleotide and amino acid sequences within this region vary widely among different poliovirus genotypes, and both PCR primers (Yang *et al.*, 1991; Yang *et al.*, 1992), synthetic oligonucleotide probes (Da Silva *et al.*, 1991; De *et al.*, 1995) and RNA probes (De *et al.*, 1997) complementary to this region have been found to be highly specific for genotype; (2) the amino- terminal VP1 domains are folded within the interiors of native virions (Hogle *et al.*, 1985), and thus their evolution is probably not driven by strong immune selective pressures. None of the genotype-specific primers described here is located within the known type 1 antigenic sites (Minor *et al.*, 1986a).

The sensitive detection, using the genotype-specific primers, of wild-type templates in virus mixtures containing vast excesses of vaccine-like templates makes the primers particularly useful for monitoring of silent infections, and for screening for wild-type viruses in regions where the use of OPV has been recently intensified and during extensive NID campaigns, when co-infection with both wild-type and vaccine strains is likely to occur, and when the wild-type strains may be missed using conventional intratypic differentiation techniques.

Although the sensitivity of the primers was tested only in mixtures containing wild-type 1 and Sabin 1 templates, similar sensitive detection of wild-type templates in mixtures containing wild-type 1 and Sabin 2 or 3 templates, or mixtures of wild-type 1 and multiple Sabin serotypes, are expected. Since the sensitivity of the wild-type 1 specific primers was not compromised by the presence of vast stoichiometric excesses of Sabin 1, with which the primers have limited sequence homology, it is not expected that Sabin 2 or 3 templates, with which the primers have highly reduced homology, would interfere with the sensitive amplification of wild-type 1 templates.

The extreme selectivity and sensitivity for wild-type polioviruses in mixtures containing vast excesses of vaccine-like strains renders genotype-specific amplification particularly useful for environmental monitoring for wild-type virus circulation, especially in communities immunised with OPV. It has been suggested that because the generation of amplification products of specific chain lengths may be the only evidence for the presence of wild polioviruses in environmental samples, 2 independent primer pairs should be used, one set for the initial screening and the second set for confirmation (Yang *et al.*, 1992). To this end, initial screening could be performed with the pan-African primers described in this study,

followed by specific confirmatory amplification with the East or West African genotype-specific primers.

In conclusion, the development of PCR reagents for the sensitive and specific amplification of wild-type 1 polioviruses indigenous to Africa has been described. These reagents may prove to be useful additions to the library of molecular reagents already available for the detection of wild-type viruses and monitoring of silent infections and transmission.

7. GENETIC STABILITY OF ORAL POLIO VACCINE PREPARED ON PRIMARY MONKEY KIDNEY CELLS OR VERO CELLS - EFFECTS OF PASSAGE IN THE HUMAN GASTROINTESTINAL TRACT AND IN CELL CULTURE

7.1 INTRODUCTION

The Sabin live attenuated oral polio vaccines have been used widely and successfully to control paralytic poliomyelitis, and mass vaccination with OPV is a major component in the WHO's campaign for the global eradication of this disease. OPV, traditionally prepared on primary monkey kidney cells, reproduces the mechanism of natural infection, replicating in the gastrointestinal tract of vaccinees, and inducing local intestinal as well as long-lived systemic immunity. The greatest concern with the use of live attenuated vaccines is their potential for reversion to neurovirulence. Despite the excellent safety record of OPV, vaccine-associated paralytic poliomyelitis (VAPP) may occur in OPV recipients at a frequency of 1 to 2 cases per 2 500 000 doses (Joce *et al.*, 1992; CDC, 1997a).

The genetic basis for the attenuation of the 3 Sabin OPV strains has been studied extensively, and although the mechanisms for attenuation have not yet been fully elucidated, single-base mutations that are strongly associated with attenuation and reversion to neurovirulence have been identified. The genomes of the Sabin 1, 2 and 3 attenuated poliovirus strains differ from their neurovirulent progenitors at 57 (Nomoto *et al.*, 1982), 23 (Moss *et al.*, 1989; Pollard *et al.*, 1989) and 10 (Stanway *et al.*, 1984) positions respectively. Major attenuating mutations for all 3 serotypes have been identified in the 5' non-coding region (NCR), at position 480 for type 1(Kawamura *et al.*, 1989), 481 for type 2 (Equestre *et al.*, 1991; Macadam *et al.*, 1991b; Ren *et al.*, 1991), and 472 for type 3 (Cann *et al.*, 1984; Evans *et al.*, 1985; Westrop *et al.*, 1989). These mutations have been found to be unstable and rapidly lost during replication in the human gastrointestinal tract (Evans *et al.*, 1985; Minor and Dunn, 1988; Macadam *et al.*, 1989; Dunn *et al.*, 1990; Tatem *et al.*, 1991). For type 3, an additional point mutation associated with both attenuation and temperature sensitivity has been identified at position 2034 in the VP3 capsid gene (Westrop *et al.*, 1989; Minor *et al.*, 1989).

The availability of primary monkey kidney cells for OPV production is strictly dependent on a regular supply of healthy monkeys, and in 1989, a major vaccine supplier, Pasteur

Merieux Serums et Vaccins, France, switched to preparing OPV on a validated working bank of Vero cells to overcome monkey supply problems (Montagnon, 1989; HeleneTixier, Pasteur Merieux Connaught, personal communication). At the National Institute for Virology (NIV), OPV has been produced on primary vervet monkey kidney cells (VK) since the early 1960's. However, in 1989 it was decided to produce the vaccine using a continuous Vero cell line because of the prohibitive level of contamination of VK vaccine bulks with adventitious monkey viruses. Because of the change in cell substrate, additional safety tests were conducted, as advised by the WHO Expert Committee on Biological Standardisation (WHO, 1990c). The phenotypic characteristics (monkey neurovirulence and replicative capacity at supraoptimal temperature) of OPV produced and passaged on the VK or Vero substrates were compared, and it was found that all 3 types were further attenuated by passage in Vero cells, and that type 3 poliovirus became more heat stable after passage in Vero cells (Dommann, 1994).

What was not known was whether the genetic stability of OPV produced on the Vero cell substrates was in any way altered during passage in cell culture and in the intestinal tract of vaccinees. Differences in the stability of the vaccines might have direct bearing on the safety of the vaccine and on the number of cases of VAPP cases occurring both in South Africa as well as in other African countries where Vero cell-produced OPV is used for routine and mass immunisations. This study was thus undertaken to compare the genetic stability of OPV produced on VK or Vero cell substrates, when passaged in cell culture and the gastrointestinal tract of vaccinees, with respect to mutations at the sites considered most important for attenuation.

7.2 MATERIALS AND METHODS

7.2.1 Study design

The study was conducted in Edenvale, Johannesburg, South Africa. Twenty one healthy infants aged 3 months were selected from those attending the Edenvale Child Health Centre. Inclusion criteria included infants who had not yet received their first dose of trivalent OPV, did not have gastroenteritis at the time of selection and immunisation, and who were well at birth with a normal birth weight. Written informed consent was obtained from the parents of infants participating in the project. The infants were randomly selected to receive 3 drops of OPV prepared either on VK or Vero cells. Vaccine bottles were opened at the time of enrolment and any vaccine remaining after immunisation was discarded.

7.2.2 Oral polio vaccines

Infants received 1 of 2 licensed OPV's (prepared either on VK or Vero cells), produced by the Vaccine Unit of the NIV and used in routine immunisation of infants in South Africa. The formulation ratio of both vaccines was 10:1:3 (10^6 $TCID_{50}$ Sabin 1, 10^5 $TCID_{50}$ Sabin 2, 3×10^5 $TCID_{50}$ Sabin 3).

For passage in VK or Vero cells, the following Sabin attenuated poliovaccine strains were used:
type 1 LS-c, 2ab/KP_2 (P1/Sabin); type 2 P712, Ch, 2ab/KP_2 (P2/Sabin); and type 3 Leon 12 a_1b/KP3 (P3/Sabin). The viruses used for passage were 3 passages from the Sabin original (SO +3) for types 1 and 2, and 2 passages from the original (SO + 2) for type 3, which are the passage levels of the seed virus used to inoculate cell cultures for the production of OPV.

7.2.3 Stool collection

Stool specimens were collected from each vaccinee on days 2, 7, 14 and 21 post vaccination, and transported to the NIV laboratories within 24 hours.

7.2.4 Cell cultures

VK cells were prepared by Dr. C. Dommann, Vaccine Unit, NIV. The kidneys from an anaesthetized vervet monkey were removed aseptically, cells were dispersed as described in Kuchler (1977), and planted using Hanks' lactalbumin containing 3% sterile filtered foetal calf serum (FCS). When the monolayers were confluent the cells were trypsinised, resuspended in FCS with 10% dimethylsulphoxide and stored in liquid nitrogen until required for use. Cells were subsequently planted in RPMI 1640 plus 10% FCS. The VK cells had therefore been passaged once before use; this ensured a consistent supply of cells for experimental work.

Vero cells were obtained frozen from the Centre for Applied Microbiology and Research, Porton Down, UK, at the 134th passage level. The cells were further passaged in RPMI 1640 containing 10% FCS to the 141st passage level, then frozen down and stored in liquid nitrogen until required.

7.2.5 Passage of poliovaccine strains in cell culture

Passaging of the poliovaccine strains was performed by Dr. C. Dommann, Vaccine Unit, NIV. VK or Vero cells were seeded in 150 cm^2 plastic flasks using 100 ml RPMI 1640 with 10% FCS. When cells were 90% confluent, the spent medium was removed and the cell monolayers washed once with RPMI 1640. Seed virus was diluted 1:1000 in RPMI 1640 and 1 ml inoculated onto the cell monolayer and allowed to absorb at room temperature for 10 minutes. The flasks were refed with serum-free RPMI 1640 and incubated at 33°C for 3 to 4 days before harvesting the virus-containing medium. If the harvested material was not used immediately to inoculate cells for a further passage it was stored at -70 °C until used. For each successive passage, harvest samples were diluted 1:1000 with RPMI 1640 before inoculation onto cells. All 3 virus serotypes were identified at passage level 4 and again at passage level 10 by the microneutralization test (Lenette, 1992) to ensure that no cross-contamination had occurred.

7.2.6 Isolation of poliovaccine strains from stool specimens

For each stool specimen, a 20% (weight/volume) suspension was prepared in gelatin-buffered saline. Two hundred microlitres of suspension were used to inoculate VK cell

monolayers for isolation of polioviruses. For each positive isolate, individual poliovirus serotypes were recovered by a further passage in the presence of 32 units of polyclonal antisera against 2 of the 3 types.

7.2.7 Isolation of individual poliovaccine viruses from the OPV preparations used for immunisation.

The individual components of the trivalent OPV were isolated by passaging a 200 μl aliquot of OPV twice in VK cells, with polyclonal antisera against 2 of the 3 types included during the second passage.

7.2.8 Intertypic differentiation of excreted polioviruses

The presence and identity of each poliovirus serotype isolated from each stool sample was confirmed by PCR using Sabin-specific primers (Yang *et al.* 1991), as described in section 3.2.3.

Isolates that were found to contain more than 1 Sabin poliovirus serotype by PCR analysis were subjected to an additional passage in the presence of antisera to suppress 1 or more type.

7.2.9 Sequence analysis of poliovirus isolates

The nucleotides at position 480 (Sabin 1), 481 (Sabin 2), 472 (Sabin 3) and 2034 (Sabin 3) were analysed by partial sequence analysis of the 5' non-coding and VP3 regions. Sequencing templates were generated by RT-PCR.

Primers: For amplification of the 5' NCR, the general enterovirus primers Ent1 and Ent3 described by Rotbart (1990) were used (Table 7.1). These primers amplify a 154 bp region which includes the nucleotides at position 472, 480 and 481 in the 5' NCR. The forward primer Ent3 was modified slightly by shortening to facilitate analysis of the nucleotide at position 472 of Sabin 3. For analysis of position 2034 in the VP3 region of Sabin 3, primers S3VP3-1 and S3VP3-2 (Table 7.1) were designed to amplify a 170 bp region spanning the nucleotide of interest.

RNA extraction: RNA was extracted from 160 μl of virus-containing cell culture supernatants. Forty microlitres of 5X lysis buffer (250 mM Tris-HCl pH 8.3, 350 mM KCl, 25 mM $MgCl_2$, 2.5% NP-40) were added to each tube, and the tubes incubated on ice for 15 minutes. Nucleic acids were extracted once with phenol, once with phenol/chloroform (1:1), and once with chloroform/isoamyl alcohol (24:1). The aqueous supernatants were used as templates for PCR reactions and subsequently stored at -70 °C.

Reverse transcription and PCR. RT-PCR was performed in a single step using the following conditions: 100 μl reaction volume; 5 μl RNA; 20 mM Tris-HCl pH 8.75; 10 mM KCl; 10 mM $(NH_4)_2SO_4$; 2 mM $MgCl_2$; 0.1% Triton X-100; 0.1 mg/ml BSA; 200 μM of each dNTP; 10 units RNase inhibitor (Boehringer Mannheim); 5 units AMV-reverse transcriptase (Boehringer Mannheim); 2.5 units *Pfu* DNA polymerase (Stratagene) and 20 pmol of each primer. Thermal cycling for RT-PCR was performed on a Biometra Trioblock using the following programme: 1 cycle of reverse transcription (42 °C, 45 minutes); 1 cycle of denaturation (95 °C, 3 minutes); 30 cycles of denaturation (95 °C, 30 seconds), annealing (Ent1/Ent3 50 °C, 45 seconds; S3VP3-1/S3VP3-2 56 °C 45 seconds), and elongation (72 °C, 1 minute); and 1 final cycle of elongation (72 °C, 7 minutes). Amplified products were analysed by electrophoresis through 2.5% Nusieve agarose gels (FMC Bioproducts) containing 0.4 μg/ml ethidium bromide.

Table 7.1 Primers for amplification of the poliovirus 5' NCR and VP3

Primer	Position in genome*	Sequence (5'→ 3')	Amplicon size (bp)
Ent1	5' NCR 584-603	ATT GTC ACC ATA AGC AGC CA	154
Ent3	5' NCR 449-467	CCT CCG GCC CCT GAA TGC	
S3VP3-2	VP3 2139-2160	CAT CAT TGA ACC ACA GAA CAG G	170
S3VP3-1	VP3 1991-2012	GAG TTA CTC TGA GCG ACA GTG C	

* Position in the genome of Sabin 1. Numbering according to Toyoda *et al.* (1984).

7.3 RESULTS

7.3.1 Poliovirus excretion by vaccinees

A total of 252 stool specimens were analysed for the presence of polioviruses. Specimens were available from all children on days 2, 7, 14 and 21 after vaccination with trivalent OPV manufactured on VK (n = 10) or Vero (n = 11) cells. The poliovirus excretion patterns of vaccinees are presented in Table 7.2. Excretion of type 1 was evident for only 3 infants in the VK group and 2 infants in the Vero group. All infants had been previously vaccinated with type 1 monovalent OPV at birth, and the absence of excreted type 1 from the majority of infants in both groups suggests that efficient immunisation with monovalent type 1 had occurred. Except for 2 infants in the Vero group (11 and 18), all vaccinees showed evidence of infection by type 2. For type 3, 1 infant in each group (child 10 VK and child 15 Vero) did not excrete virus, but all other infants displayed evidence that type 3 had taken.

The duration and frequency of excretion of each poliovirus serotype were compared for each vaccine group. Type 1 was excreted only for a very short period of time (2 days post vaccination) by 1 infant in each vaccine group (child 4 VK and child 11 Vero). Prolonged excretion for 21 days after vaccination was seen only for 1 infant (child 15) in the Vero group, and for child 2 in the VK group, who only began shedding type 1 virus 21 days after vaccination. For type 2, all recipients of the VK vaccine excreted virus within one day of vaccination. Eight of 10 VK vaccine recipients excreted type 2 virus 7 days after vaccination, and by day 21 virus was being excreted by only 4 of 10 recipients. In the Vero group, 7 of 11 recipients excreted type 2 virus 2 days after vaccination; by day 21, only 2 of these were still excreting virus. Type 3 virus was shed consistently by the majority of infants in both vaccine groups throughout the 21-day post-vaccination study period. Seven of 10 infants in the VK group and 10 of 11 in the Vero group were still excreting type 3 virus 21 days after vaccination. Statistical analysis of the data (Fisher's exact test) revealed that there were no significant differences between the two vaccine groups with respect to the number of isolates obtained at each time point after vaccination. When the total number of viruses of each serotype excreted by the VK or Vero recipients over the 21-day post-vaccination period were compared, the VK vaccine recipients were found to excrete type 2 more frequently than the Vero vaccine recipients ($p = 0.035$, Fisher's exact test).

Table 7.2 Excretion of Sabin-like and revertant polioviruses by primary vaccinees

	Days post vaccination															
Infant	Type 1(n480)				Type 2 (n481)				Type 3 (n472)				Type 3 (n2034)			
	2	7	14	21	2	7	14	21	2	7	14	21	2	7	14	21
VK-cell vaccine																
1	S	-	S	-	S	R	R	R	-	-	R	R	-	-	S	S
2	-	-	-	R	R	-	R	-	S	-	R	R	S	-	S	R
3	-	-	-	-	S	-	-	-	R	-	R	-	S	-	S	-
4	S	-	-	-	S	R	-	-	R	R	R	R	S	S	S	R
5	-	-	-	-	S	R	-	-	R	R	R	R	S	S	S	S
6	-	-	-	-	S	R	-	-	S	R	R	R	S	S	R	R
7	-	-	-	-	S	R	R	R	R	R	R	R	S	S	R	R
8	-	-	-	-	S	S	-	-	-	-	S	R	-	-	S	S
9	-	-	-	-	S	R	R	R	R	-	-	-	S	-	-	-
10	-	-	-	-	S	R	R	R	-	-	-	-	-	-	-	-
Vero-cell vaccine																
11	S	-	-	-	-	-	-	-	-	-	R	R	-	-	S	S
12	-	-	-	-	S	S	-	-	R	R	R	R	S	S	S	S
13	-	-	-	-	-	R	-	-	R	R	R	R	S	S	S	S
14	-	-	-	-	S	R	R	R	R	R	R	R	S	S	S	S
15	S	R	R	R	R	R	R	-	-	-	-	-	-	-	-	-
16	-	-	-	-	S	R	-	-	S	-	R	R	S	-	S	R
17	-	-	-	-	-	R	-	-	-	R	R	R	-	S	S	S
18	-	-	-	-	-	-	-	-	S	R	R	R	S	S	S	R
19	-	-	-	-	S	S	-	R	S	-	S	R	S	-	S	S
20	-	-	-	-	S	R	-	-	R	R	R	R	S	S	R	R
21	-	-	-	-	S	R	-	-	R	R	R	R	S	R	R	R

- = No virus isolated

S = Sabin-like (S1 - G_{480}; S2 - A_{481}; S3 - U_{472}, U_{2034})

R= Revertant (S1 - A_{480}; S2 - G_{481}; S3 - C_{472}, C_{2034})

7.3.2 Reversion of Sabin poliovirus strains during passage in the gastrointestinal tract of vaccinees

Attenuation of the Sabin poliovaccine strains has been associated with base changes at positions 480 (Sabin 1), 481 (Sabin 2), 472 (Sabin 3) and 2034 (Sabin 3). These bases have been found to revert rapidly to those found in neurovirulent wild-type viruses upon replication both in the human gut and in cell culture. In order to compare the rates of reversion of the Sabin vaccines produced on VK versus Vero cells during replication in the human gastrointestinal tract, the viruses excreted by vaccinated infants were examined for the presence of Sabin-like or revertant bases at the positions mentioned above. To control for reversions that might occur during the cell culture passages required for isolation of viruses from stool specimens, the individual type 1, type 2 and type 3 components of the VK or Vero cell OPV administered to the infants in this study were isolated under the same cell culture conditions employed for isolation of viruses from stool specimens. The results of sequence analysis indicated that all 3 poliovaccine strains isolated from the VK or Vero OPV used for immunisation contained exclusively Sabin-like bases at the positions examined (Sabin 1, G_{480}; Sabin 2, A_{481}; Sabin 3, U_{472}; Sabin 3, U_{2034}). Thus the two passages in cell culture required for isolation of viruses from stool specimens did not appear to influence reversion rates at these positions in any significant manner.

Sequence analysis results of the viruses excreted by the infants receiving VK or Vero cell vaccine are presented in Table 7.2. For type 1, the rates and frequency of reversion of viruses excreted from infants in the 2 vaccine groups could not accurately be compared as only 3 infants in the VK group and 2 infants in the Vero group excreted type 1. In the VK group, 1 infant (infant 2) only began shedding type 1, which was found to be revertant, 21 days after vaccination. In the Vero group, infant 15 shed type 1 throughout the 21-day investigation period, and all strains isolated after day 7 were found to be revertants.

For type 2, only 1 of 10 and 1 of 7 viruses excreted on day 2 by infants in the VK and Vero groups respectively were found to have reverted. By day 7 after vaccination, 7 of 8 viruses isolated from the VK group and 7 of 9 isolated from the Vero group had reverted. All viruses excreted after day 7 were revertants.

The majority of type 3 viruses excreted between 7 and 21 days after vaccination with either VK or Vero vaccine had reverted at position 472. In the VK group, 5 of 7 (71%) of viruses excreted just 2 days after vaccination had already reverted. Similarly in the Vero group, 5 of 8 (62%) of viruses excreted on day 2 had reverted. Only 1 infant in each vaccine group excreted Sabin-like type 3 14 days after vaccination; in the VK group, infant 8 only began

shedding Sabin-like type 3 on day 14, and in the Vero group, infant 19 excreted Sabin-like virus on days 2 and 14 post vaccination. The type 3 viruses excreted by both these infants 21 days after vaccination were revertants.

Reversion of type 3 at position 2034 occurred later than that at position 472 in both vaccine groups (Table 7.2). Fourteen days after vaccination with VK or Vero vaccine, only 2 of 8 and 2 of 10 viruses excreted by infants in each respective group were revertants. By day 21, 4 of 7 infants in the VK group and 4 of 10 in the Vero group shed revertant viruses.

The rate (time of reversion after vaccination) and frequency (total number of revertants) of reversion for type 2 (position 481) and type 3 (positions 472 and 2034) viruses excreted by infants in the VK or Vero group were not found to be significantly different (Fisher's exact test). In both groups, there was no evidence of the re-emergence of Sabin-like viruses after initial detection of revertant viruses.

7.3.3 Reversion of Sabin poliovirus strains during passage in cell culture

The rates of reversion of the 3 poliovaccine strains during passage in VK or Vero cells are presented in Table 7.3. Ten consecutive passages of viruses were performed in VK or Vero cells, and an aliquot of virus from each passage level was sequenced to determine the presence of Sabin-like or revertant bases at positions 480 (Sabin 1), 481 (Sabin 2), 472 (Sabin 3) and 2094 (Sabin 3). Type 1 and type 3 viruses were found to contain mixtures of Sabin-like and revertant strains in the same sample. In order to determine the approximate proportions of the 2 strains, mixtures containing 0%, 10%, 25%, 50%, 75% and 100% revertants were prepared for types 1 and 3, and the mixtures sequenced. The relative proportions of Sabin-like and revertant viruses in the vaccine passages were then determined by visual comparison of the Sabin-like and revertant nucleotide band intensities with those of mixtures containing known ratios of Sabin-like to revertant viruses.

For Sabin 1, revertants began accumulating after 4 passages in both VK and Vero cells. At this passage level, both VK and Vero preparations contained approximately equal proportions of revertant viruses (10%). Mixtures of Sabin-like and revertant viruses were present at each passage after level 4 in both cell types. By the tenth passage on VK cells, approximately 30% of the viruses in the mixtures were revertants. In comparison, the proportion of revertants in the Vero cell preparations remained constant at 10% between passages 4 and 10.

Sabin 2 was found to be stable with respect to reversion at position 481 during passage on both VK or Vero cells. Viruses present at each passage level in both cell types were found to be exclusively Sabin-like.

For Sabin 3 passaged in VK cells, approximately 20% reverted from U to C at position 472 by passage 3. The proportion of revertant viruses increased with each passage, and by passage 10, approximately 50% of the viruses had reverted. On passage in Vero cells, approximately 10% of type 3 viruses had reverted after 5 passages. The proportion of revertants increased with each passage, reaching a maximum of approximately 30% by the tenth passage. Position 2034 appeared to be stable in type 3 viruses passaged on either VK or Vero cells, as no revertants were detected in virus preparations after 10 passages on either cell line.

Table 7.3 Reversion of poliovirus vaccine strains passaged in cell culture

Strain		Passage number									
		1	2	3	4	5	6	7	8	9	10
Sabin 1 n480	VK	S	S	S	S+R(10%)	S+R	S+R	S+R	S+R	S+R	S+R(30%)
	Vero	S	S	S	S+R(10%)	S+R	S+R	S+R	S+R	S+R	S+R(10%)
Sabin 2 n481	VK	S	S	S	S	S	S	S	S	S	S
	Vero	S	S	S	S	S	S	S	S	S	S
Sabin 3 n472	VK	S	S	S+R(20%)	S+R	S+R	S+R	S+R	S+R	S+R	S+R(50%)
	Vero	S	S	S	S	S+R(10%)	S+R	S+R(20%)	S+R	S+R	S+R(30%)
Sabin 3 n2034	VK	S	S	S	S	S	S	S	S	S	S
	Vero	S	S	S	S	S	S	S	S	S	S

S = Sabin-like (S1 - G_{480}; S2 - A_{481}; S3 - U_{472}; S3 - U_{2034})

R = Revertant (S1 - A_{480}; S2 - G_{481}; S3 - C_{472}; S3 - C_{2034})

S+R = Mixed Sabin-like / Revertant (figures in parentheses refer to the % revertants in the mixture)

7.4 DISCUSSION

The bases at position 480 (Sabin 1), 481 (Sabin 2) and 472 (Sabin 3) in the poliovirus 5' NCR have been found to be of major importance in the attenuated phenotype of the Sabin polio vaccine strains. The poliovirus 5' NCR has been found to act as an internal ribosome entry site for the initiation of translation (Pelletier and Sonnenberg, 1988). Attenuation appears to result from the destabilisation of the secondary hairpin loop structure in this region (Skinner *et al.*, 1989; Macadam *et al.*, 1992), resulting in altered interactions with cellular factors mediating ribosome binding (Pelletier and Sonnenberg, 1989; Pestova *et al.*, 1991; Gebhard and Ehrenfeld, 1992) and a subsequent reduction in translation efficiency (Svitkin *et al.*, 1985; Svitkin *et al.*, 1988; Svitkin *et al.*, 1990). Reversion at the abovementioned positions has been documented both during passage in the human gastrointestinal tract (Evans *et al.*, 1985; Minor and Dunn, 1988; Macadam *et al.*, 1989; Dunn *et al.*, 1990; Tatem *et al.*, 1991; Mallet *et al.*, 1997) and in cell culture, where conditions such as cell substrate, passage number, and incubation temperature have been found to influence the genetic stability of all 3 serotypes of Sabin OPV (Chumakov *et al.*, 1992; Chumakov *et al.*, 1994; Rezapkin *et al.*, 1994; Rezapkin *et al.*, 1995; Taffs *et al.*, 1995). The recent use of Vero cells as an alternative to primary monkey kidney cells for the manufacture of OPV prompted this investigation to compare the genetic stability of the 3 Sabin OPV strains produced on VK or Vero cell substrates, by examining the kinetics of reversion of the bases associated with attenuation during passage *in vivo* in the gut of vaccinees and *in vitro* in VK or Vero cells. However, because all infants had received monovalent type 1 vaccine at birth, shedding of type 1 was limited to very few infants, and thus the frequency of excretion and rate of reversion could not accurately be compared for this serotype.

PCR and sequencing were employed to analyse the presence of Sabin-like or revertant bases at positions 480, 481 and 472 in the 5' NCR of Sabin 1, Sabin 2 and Sabin 3 respectively, and at position 2034 in VP3 of S3. The thermostable *Pfu* polymerase was used in preference to *Taq* polymerase in the amplification reactions, as this enzyme has been shown to exhibit a 12-fold increase in fidelity of DNA synthesis over *Taq* DNA polymerase, thus reducing the possibility that mutations at the positions under investigation were the result of enzyme-induced errors. The finding that all 3 serotypes of OPV reverted rapidly with respect to the mutations in the 5' NCR during passage in the gut of vaccinees is consistent with reports from the UK (Minor and Dunn, 1988; Dunn *et al.*, 1990), USA (Tatem *et al.*,1991) and France (Mallet *et al.*,1997). Results of this study confirm that there is strong selective pressure against these attenuating mutations in the gastrointestinal tract, and that

revertants are rapidly generated. No significant differences were observed in either the frequency of excretion or the rate of reversion between Sabin vaccine viruses of all 3 serotypes produced on either VK or Vero cell substrates. The finding that the substrate on which the OPV is produced does not significantly affect the frequency of excretion or rate of reversion in vaccinees is in agreement with that of Mallet *et al.* (1997) who also compared OPV produced on VK and Vero substrates, and Dunn *et al.* (1990), who compared OPV produced on VK and human diploid cells.

Results of this study show that the majority of isolates of all 3 serotypes obtained 7 days after immunisation were revertants. This finding is consistent with the suggestion that for all 3 serotypes, longer periods of virus excretion may be associated with higher frequencies of reversion (Dunn *et al.*, 1990). However, it was found that the duration of excretion of type 2 and 3, whether Sabin-like or revertant, differed from those of other studies, in that less than half the infants in both the VK and Vero vaccine group were shedding type 2 14 days after vaccination, in contrast to the results of Dunn *et al.* (1990), who found that virtually all infants in their study shed type 2 14 days after vaccination, and about half continued to shed type 2 28 days after vaccination.

Dunn *et al.* (1990) and Tatem *et al.* (1991) found that the majority of vaccinees stopped excreting type 3 virus, whether reverted or not, by day 5 after vaccination. Results of this study indicate more rapid reversion, where all isolates obtained on day 2 and thereafter were revertants, and prolonged excretion (up to 21 days) of reverted type 3 by the majority of infants in both the VK and Vero vaccine groups. These findings are similar to those of an early study by Minor and Dunn (1988), where it was found that all excreted type 3 viruses had reverted by day 4, and that excretion continued for at least 25 days.

The rate and frequency of reversion at position 2034 of type 3 were also found to be the same in both vaccine groups. In comparison to the rapid selection of revertants at position 472, mutants at position 2034 appeared less rapidly, with only half the viruses excreted on day 21 being revertants. These results suggest that in the gastrointestinal tract mutation at position 2034 is better tolerated than at position 472, although, as for position 472, prolonged excretion appears to be associated with reversion at this position too.

Results of this study show, consistent with other published reports, that the 3 serotypes of OPV are relatively unstable during passage in cell culture, and that the cell substrate plays a role in the rate of accumulation of revertants. The reversion rate was found to be higher in viruses passaged in VK cells than in Vero cells, suggesting that the OPV produced in Vero cells is more stable than that produced in VK cells. The results of monkey

neurovirulence tests with seed virus after 10 passages in VK and Vero cell cultures (Dommann, 1994) support this suggestion, as all 3 serotypes passaged in Vero cells consistently produced lower mean lesion scores and were less neurovirulent than those passaged in VK cells. These findings are in contrast to those of Chumakov *et al.* (1992; 1994), Rezapkin *et al.* (1994; 1995), and Taffs *et al.* (1995), who observed that mutation rates were higher in all 3 serotypes passaged in Vero cells, and appear to contradict the suggestion (Rezapkin *et al.*, 1995) that since the 3 Sabin OPV strains were derived from primary monkey kidney cell cultures, the strains are better adapted to the VK substrate, and that switching to a new substrate may require the virus to change. The finding by Dommann (1994) that the tenth Vero passage type 1 and type 3 passed the neurovirulence test was, however, surprising, since results of the present study demonstrate that the levels of revertant viruses present after 10 passages are far higher than the estimated threshold level for monkey neurovirulence (approximately 4.1% for type 1, Rezapkin *et al.*, 1994; and 1% for type 3, Chumakov *et al.*, 1992). These results re-enforce the notion that attenuation is a complex phenomenon which appears not to be solely mediated by mutations in the 5' NCR.

It has previously been shown that the rate of mutant accumulation is dependent not only on the type of cell substrate, but also on the condition of the substrate and the incubation temperature, with mutants being generated more rapidly in overconfluent cultures, and in cells incubated at 37°C rather than at 34°C (Chumakov *et al.*, 1992; Chumakov *et al.*, 1994; Rezapkin *et al.*, 1994; Rezapkin *et al.*, 1995; Taffs *et al.*, 1995). In the present study, all passages were performed on subconfluent cells at 33°C. The results for all 3 Sabin serotypes passaged in Vero cells, although not directly comparable because the sequencing technique that was employed for the detection of revertants is less sensitive than mutant analysis by PCR and restriction enzyme cleavage (Chumakov *et al.*, 1991), are similar to those obtained by others under the same culture conditions. For type 1, Chumakov *et al.* (1994) and Rezapkin *et al.* (1994) observed that the proportion of mutants remained steady at about 10% between passages 2 and 10. Similarly, in this study a constant level of approximately 10% revertants was detected between passages 4 and 10. For type 2 passaged in Vero cells, Chumakov *et al.* (1994) and Taffs *et al.* (1995) detected a maximum level of only 1.4% G481-mutant accumulation. No type 2 revertants were detected under the same passage conditions in this study, but this might be explained by the inability to detect revertants which constituted less than 10% of the virus population by using sequence analysis as the detection method. For type 3, approximately 30% reversion was detected at position 481 after 10 passages in Vero cells, comparable to the 20% reversion observed by Chumakov *et al.* (1994).

In agreement with the findings of Chumakov *et al.* (1992) and Lu *et al.* (1996), no reversion was detected at position 2034 of Sabin 3 after 10 consecutive passages in both VK and Vero cells. Westrop *et al.* (1989) found that substitution at position 2034 of Sabin 3 resulted in increased neurovirulence. The results of this study indicate that this position appears to be stable during passage in cell culture, but that revertants are selected for during prolonged replication in the gut, suggesting that selective forces present in cell cultures differ from those in the gastrointestinal tract.

In summary, findings presented in this chapter confirm previous reports that all 3 serotypes of OPV are unstable, to a greater or lesser extent, both *in vitro* and *in vivo* with respect to the attenuating mutations in the 5' NCR. The use of Vero cells as opposed to VK cells as substrate for OPV manufacture, however, does not appear to influence the genetic stability of the vaccine *in vivo*, and appears to result in increased stability *in vitro*.

8. CONCLUDING REMARKS

Paralytic poliomyelitis continues to remain a serious public health problem in many African countries, and its elimination from Africa remains one of the major challenges to achieving the goal of global eradication by the year 2000. Despite intensive efforts by the WHO to control poliomyelitis through sustained high vaccination coverages and good AFP surveillance, in 1996 reported OPV3 coverage reached only 54% for the African region overall, and 15 countries, including 4 of the largest and epidemiologically most important countries - Angola, Ethiopia, D.R.Congo and Nigeria - reported that less than 15% of children were routinely immunised with 3 doses of OPV (WHO, 1998c). The AFP surveillance throughout Africa is also suboptimal, with a rate of only 0.2 AFP cases reported per 100 000 children under the age of 15 (WHO, 1998c).

Of the 4074 cases of poliomyelitis reported to WHO in 1996, 1949 (approximately 48%) were reported from Africa, the majority from countries in central and western Africa (WHO, 1998c). Figures for 1997 are not yet complete, but stood at 219 confirmed cases as of May 1998, again reported mostly from western and central Africa (WHO, 1998a). Countries in the southern African epidemiological block have reported good vaccine coverages and very few or no confirmed cases of poliomyelitis over the last few years, and WHO indications suggest that a polio-free zone may exist in southern Africa (WHO, 1994). Countries that are polio-free are, however, at risk of re-introduction of poliomyelitis from polio-endemic areas, as a result of the influx of immigrants and refugees from poverty-stricken or war-torn countries.

In addition to the sporadic cases which occurred in Africa in recent years, outbreaks of poliomyelitis have also recently occurred in several African countries: in Namibia and in the Central African Republic during 1993-94, and in D.R.Congo and Zambia in 1995. Two major outbreaks also occurred in South Africa during 1982 and 1988.

If the goal of elimination of poliomyelitis from Africa is to be achieved, the regions where polioviruses continue to circulate endemically need to be identified and the patterns of transmission of wild-type viruses determined, so that improved strategies for the interruption of transmission may be designed and implemented. In an attempt to answer these questions, the molecular epidemiological characteristics of poliovirus strains associated with cases and outbreaks of poliomyelitis in sub-Saharan Africa were investigated.

The laboratory diagnosis of poliomyelitis is a critical component of the WHO 's initiative for

poliomyelitis eradication, as it ultimately determines the specificity of AFP surveillance. The need for rapid and accurate identification of polioviruses in clinical or environmental specimens will become increasingly important as the goal of poliomyelitis eradication is approached and circulation of wild-type polioviruses decreases. To this end, the poliovirus-specific PCR was developed for the rapid and sensitive identification of polioviruses in tissue culture harvests and clinical specimens (Chapter 3). The primers described are specific for both vaccine-like and wild-type polioviruses of all 3 serotypes, but do not react with non-polio enteroviruses. In addition, the primers span the 150 bp VP1/2A region commonly sequenced for molecular epidemiological studies, so that the primers are useful not only for poliovirus identification, but also for preparation of large quantities of DNA templates which can be readily sequenced.

The determination of the vaccine or non-vaccine origins of poliovirus strains associated with poliomyelitis cases is an important aspect of laboratory diagnosis. The available WHO-approved techniques for intratypic differentiation between poliovirus strains are based on either antigenic or genomic differences between poliovirus strains (van der Avoort *et al.*, 1995). However, disadvantages exist for all of the currently available methods. Differentiation based on antigenic characteristics is not always definitive, because the antigenic properties of the viruses are not always stable and type-specific epitopes may be lost. Differentiation based on genomic differences between vaccine-like and wild-type templates may also prove equivocal: in the RFLP, mutations may lead to the gain or loss of a restriction enzyme site, leading to incorrect results; probe hybridisation and PCR with Sabin-specific reagents, while highly sensitive for detecting vaccine-related strains, may miss the wild-type component in isolates containing mixtures of vaccine-like and wild-type strains. A novel technique, the HMA, was thus developed in order to accurately differentiate between vaccine-like and wild-type strains, both in isolates containing homotypic strains or mixtures of vaccine-like and wild-type strains (Chapter 3). The HMA is based on the detection of mismatches between double stranded DNA containing one strand of known sequence composition and a complementary strand with an altered nucleotide sequence. The mismatched bases between the two strands cause structural distortions (bulges) in the resulting double-stranded molecule (heteroduplex). These structural distortions result in a decrease in the electrophoretic mobility of the heteroduplex compared to that of ds DNA made up of perfectly complementary strands (homoduplexes). Thus sequence differences between strains can be detected simply by noting a shift in the electrophoretic mobility of heteroduplexes formed following the denaturation and reannealing of PCR-amplified fragments from a reference strain of known sequence and that of an unknown type. The 3 Sabin poliovaccine strains were used as reference strains in the HMA. Using this technique, all specimens which had previously been typed as vaccine-like or wild-type using Sabin-

specific PCR (and confirmed by partial sequence analysis) also typed as vaccine-like or wild-type by HMA. In addition, the presence of both vaccine-like and wild-type strains in the same specimen could also be detected. The HMA technique could thus prove to be a useful addition to the presently available techniques for intratypic differentiation.

For detailed characterisation of strains identified as wild-type, the existence of a molecular clock for poliovirus evolution during passage in humans has meant that the potential resolving power of molecular epidemiological studies based on sequence comparisons, rather than comparisons of antigenic properties, is very high. By far the most powerful approach to molecular epidemiological investigations has been found to be comparative genomic sequencing (Rico-Hesse *et al.*, 1987). Sequence comparisons of poliovirus strains has revealed the existence, for each poliovirus serotype, of numerous genotypes, defined as groups of polioviruses sharing ≥85% nucleotide similarity within a defined genomic interval (Rico-Hesse *et al.*, 1987; Kew *et al.*, 1990). Genotypes have been found to be distributed geographically and to be endemic to different regions of the world (Rico-Hesse *et al.*, 1987; Kew *et al.*, 1990, 1995). Sequence diversity within a genotype has been found to be reduced by epidemics (as 1 lineage predominates), as well as by intensive immunisation (as lineages are eliminated) (Kew *et al.*, 1995).

In South Africa (Chapter 4) , analysis of all poliovirus strains obtained between 1980 and the present day indicates that the last confirmed case of poliomyelitis due to wild-type infection took place in 1989, and that all strains isolated in the country since then have been vaccine-like. Analysis of the poliovirus strains associated with the 1982 outbreak in Gazankulu and the 1988 outbreak in Kwazulu-Natal suggests that the molecular epidemiological characteristics of each outbreak were different: although both outbreaks originated from a single source, and both were caused by viruses already present in the outbreak areas prior to the outbreaks, the Gazankulu outbreak was caused by a genotype unique to South Africa (termed South African genotype), which had been circulating in the country since at last 1980, whereas the Kwazulu-Natal outbreak was caused by a different genotype, termed Southern African, which was not indigenous to the country and was probably introduced into South Africa from Zimbabwe in 1985. Three genotypes were found to have co-circulated concurrently between 1980 and 1985: the South African genotype, plus 2 additional genotypes probably originally introduced from the Middle East and west Africa. These 3 genotypes were displaced in 1985 by the Southern African genotype - the displacement of the locally circulating genotypes by the imported genotype reflects the transition from endemic to epidemic poliovirus circulation as the vaccine coverage in the country increased, but a sufficiently large pool of susceptibles, which permitted the local establishment of the imported genotype, remained.

Analysis of poliovirus strains obtained from countries in sub-Saharan Africa (Chapter 5) revealed the presence of a total of 9 genotypes circulating in sub-Saharan Africa during the past 2 decades (included in these 9 are the South African, Southern African, older Middle Eastern and West African genotypes circulating in South Africa between 1980 and 1989). The South African, Southern African and older Middle Eastern genotypes appear to have been effectively eliminated, as they have not been detected since 1985 (South African, older Middle Eastern) and 1989 (Southern African).

The West African genotype, which has been in circulation since at least 1980, but which may well have been circulating still earlier in Africa, since older isolates (1970-77) from Senegal and Cameroun also just fall within this genotype (Rico-Hesse *et al.*, 1987), appears to be the most widespread geographically, covering western, south western, central and southern Africa. Within the West African genotype are several distinct lineages, which display a high degree of sequence diversity from each other and may represent newly emerging genotypes. These lineages appear to be segregated geographically, indicating independent sustained endemic circulation of these lineages in west, central and southern Africa.

The Nigeria-1 and Nigeria-2 genotypes have to date been found only in Nigeria, Niger and Togo. Their co-circulation concurrently with the West African genotype in these countries is evidence of at least 3 pockets, within the individual countries, of reservoirs sustaining independent circulation of all 3 genotypes.

Evidence for the endemic establishment of genotypes originally introduced from the Middle East (Middle Eastern genotype) and the Indian subcontinent (Indian genotype) can be found in Egypt, Ethiopia, Sudan, Kenya, and as far into central Africa as the Central African Republic, where they were associated with an outbreak in 1993. In the Central African Republic, these genotypes were displaced by the West African genotype in 1994 - effective vaccination controlled the outbreak and eliminated the genotypes associated with it, but a sufficiently large reservoir of susceptibles remained for the continued circulation of the West African genotype in the country. The unavailability of specimens from countries in north-eastern and east Africa makes it impossible to determine whether the Middle Eastern and Indian genotypes are still in circulation or have been effectively eliminated.

The East African genotype appears to be geographically restricted to central (D.R.Congo) and eastern Africa (Uganda, Tanzania, Zambia). This genotype has not been isolated recently (during the second half of 1997, and during 1998), prompting speculation that effective control measures in east African countries may have successfully eliminated this

genotype.

The potential resolving power of molecular epidemiological investigations in establishing otherwise unrecognised epidemiological links is illustrated in the identification of links between the 1993 outbreak in Namibia and earlier cases in Angola, and subsequent cases in Angola and the 1995 outbreak in D.R.Congo, and similarly, by the demonstration of the direct introduction of the strain associated with D.R.Congo outbreak into the Central African Republic, Tanzania and Zambia in 1995, the direct introduction of strains from southern Angola into susceptible populations in northen Namibia in 1994-95, and the introduction of strains from Uganda into Tanzania and subsequently into Zambia in 1995.

The outbreaks in Namibia in 1993 and Zambia in 1995 both occurred in countries which were previously polio-free, and both were the result of the introduction of strains from polio-endemic areas. This highlights the very real risk of re-introduction of wild-type viruses in countries which are free of polio, and underscores the need for maintaining high immunisation coverages, with vaccines of good quality, to ensure that populations of susceptibles do not build up.

The intensive efforts to control poliomyelitis in Africa have led to a dramatic reduction in the number of cases reported from most countries . The two most important reservoirs of wild poliovirus are Nigeria and D.R.Congo (WHO, 1998c). As the eradication programme progresses, sequence heterogeneity within the West African genotype is expected to be reduced, with most lineages disappearing except perhaps for those associated with cases in central Africa and Nigeria. While vaccination coverage in Nigeria continues to be sub-optimal, the circulation of both the Nigeria-1 and Nigeria-2 genotypes will probably continue, and it is possible that one of these genotypes may be introduced and become endemic in other west or central African countries (other than those directly neighbouring Nigeria) if optimal immunisation coverages are not maintained.

The identification and characterisation of the major wild poliovirus genotypes has paved the way for the development of molecular reagents for the rapid and sensitive direct detection and identification of specific wild-type virus strains. Since laboratory confirmation of wild-type poliovirus infection is a critical aspect of the eradication initiative, direct identification of wild-type poliovirus infection can offer enhanced diagnostic reliability. Genotype-specific molecular reagents are also especially useful for monitoring silent infections and transmission, and for monitoring environmental contamination by wild-type viruses. PCR analysis in particular offers the advantages of speed and exceptional sensitivity and selectivity, and PCR primer sets have been developed that can detect sequences of

specific wild poliovirus genotypes in samples containing large stoichiometric excesses (up to 10^6-fold) of vaccine-related RNA's (Yang *et al.*, 1992).

Since the majority of wild-type polio 1 strains presently circulating in sub-Saharan Africa have been found to belong to the indigenous West and East African genotypes, PCR primers for the efficient and sensitive detection of these genotypes were thus designed (Chapter 6). The specific detection, using the genotype-specific primers, of wild-type templates in virus mixtures containing 10^5-fold excesses of vaccine-like templates renders these primers particularly useful for monitoring of silent infections, and for screening for wild-type viruses in regions where the use of OPV has been recently intensified and during extensive NID campaigns, when co-infection with both wild-type and vaccine strains is likely to occur and when identification of the wild-type strains may be missed using conventional intratypic differentiation techniques.

In addition to laboratory-based surveillance, vaccination with OPV is one of the major strategies employed by the PEI for the eventual eradication of poliomyelitis. The substrate for OPV production has recently changed, from primary monkey kidney cells to continuous cell lines, due to the limited availability of monkeys and the presence of adventitious monkey viruses in monkey tissue. Whether the genetic stability of OPV produced on Vero cells was in any way altered during passage in cell culture and in the intestinal tract of vaccinees was not known. Since the stability of the vaccines might have direct bearing on the safety of the vaccines and on the number of cases of VAPP occurring in African countries where Vero cell-produced OPV is used for routine and mass immunisation, the genetic stability of Vero or primary monkey kidney cell- produced OPV was thus compared, with respect to reversion at the nucleotide positions considered most important for attenuation (Chapter 7). Results of this study confirm previous reports that all 3 serotypes of OPV are unstable, with respect to the attenuating mutations located within the 5'NCR, when passaged in the gastrointestinal tract or in cell culture, but that the use of Vero cells, as opposed to primary monkey kidney cells as substrates for OPV manufacture, does not appear to influence the genetic stability of OPV *in vivo*, and appears to result in increased stability *in vitro*.

What of the future? Despite the many problems in controlling poliomyelitis in Africa, the progress achieved to date suggests that the target of global eradication by the year 2000 remains achievable (WHO, 1998c). Certification of polio eradication will require critical evidence that all endemic wild poliovirus circulation has ceased. At present, requirements for certification include the achievement of target AFP surveillance rates and the maintenance of a polio-free status for at least 3 years (Henderson, 1989). However,

computer simulations which relate case-free periods to the presence or absence of silent infections suggest that even after five years without cases, the probability of silent poliovirus transmission can still be in the range of 0.1-1.0% (Eicher and Dietz, 1996). Molecular epidemiological investigations will be central to the certification process: they will provide evidence of a reduction in wild-type sequence heterogeneity and the eventual disappearance of all circulating genotypes; in addition, if evidence of absence of wild poliovirus circulation in the environment is required, then molecular methods for wild-type poliovirus detection are likely to be the only techniques with the sensitivity required to permit the detection of minute amounts of wild-type virus in a sea of vaccine-like strains.

As global eradication approaches, the circulation of wild-type polioviruses will steadily decrease and will eventually cease, so that the only strains that will continue to circulate will be Sabin vaccine-like. Research now needs to be focussed on investigating whether the Sabin strains can persist for extended periods, particularly in immunocompromised individuals, and whether they can evolve to the extent that the vaccine strains themselves can pose a risk to the eradication programme.

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