MOLECULAR CHARACTERISATION OF WILD AND SABIN-LIKE POLIOVIRUSES CIRCULATING IN AFRICA AFTER 2000

By

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DECLARATION

I declare that this thesis is my own, unaided work. It is submitted for the degree of Doctor of Philosophy at the University of Pretoria, Pretoria. It has not been submitted before for any degree or examination at this or any other university.

.....

Heronyma N Gumede-Moeletsi

On this..... . day of..... 2012

To my family

My husband Joe and my sons Lesego and Thato with sincere thanks

for their love, encouragement and understanding

during the writing of this thesis

"Nothing is too high for a man to reach, but he must climb with care and confidence".

Hans Christian Andersen

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MOLECULAR CHARACTERISATION OF WILD AND SABIN-LIKE POLIOVIRUSES CIRCULATING IN AFRICA AFTER 2000

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SUMMARY

Polioviruses have been around for a long time in man's history. Before the development of killed and live virus vaccines in the 1960's, poliomyelitis was a serious problem in public health. Since then paralytic poliomyelitis remains a threat in certain underdeveloped countries but has been considered a conquered disease in the developed world.

The molecular epidemiology of wild-type 1 polioviruses (WPV1) isolated in Angola in 2005, the Democratic Republic of the Congo (DRC) in 2006-2008 and Namibia in 2006 were investigated by sequence analysis of the complete VP1 gene of all isolates. All outbreak viruses clustered with the Indian type 1 genotype (SOAS) which was unique to India circulating endemically in the Uttar Pradesh (UP) and Bihar provinces in Northern

India. Epidemiological and virological analyses suggested that the Namibia outbreak virus had been circulating without detection for at least one year in Angola.

Four cases of acute flaccid paralysis (AFP) occurred in children in Madagascar in 2005. Molecular analysis confirmed cVDPV type 2 and 3 in affected patients. The first case, occurred in Toliara II district, on 09 April 2005. The last two cases were in the Toliara I and Beloha districts and paralysis onset on 26 June and 13 July 2005 respectively. Partial genomic sequencing of the poliovirus isolates revealed considerable divergence from the prototype Sabin strain in all cases. This is the second time that type 2 cVDPV is associated with an outbreak of AFP in Madagascar, and to our knowledge the first time that a type 3 cVDPV is identified in Madagascar.

A total of fifty-six children with AFP were found to excrete VDPVs of serotype 2 in the DRC between 2005 and 2010. These viruses represent at least three emergences and at least two outbreaks. Partial genomic sequencing of the poliovirus isolates revealed considerable nucleotide sequence divergence of between 1.1% to 2% from the prototype Sabin strain in the VP1 region of the viral genome. This was the first time that a type 2 cVDPV outbreak was detected in the DRC.

In total, 89 viral isolates obtained from Ethiopia during 2007 to 2010 and partial sequencing analysis confirmed that 13 isolates were VDPV's. Seven AFP cases were type 3, 4 AFP cases were type 2 and 2 contacts for type 3. Partial genomic sequencing of the poliovirus isolates revealed considerable divergence from the prototype Sabin strain in all cases.

Finally, cases of AFP where only Sabin-like viruses were identified were investigated in South Africa with 11 possible VAPP cases identified with recombinant events in the 3D region and also revealing a mutation that restore the original stem-loop structure in the internal ribosomal entry site (IRES) in the 5' Non-Translated Region (NTR).

In this study, the molecular epidemiology of poliovirus outbreaks that occurred in Angola, Namibia, and the DRC is described that were associated with wild polio 1 and 3. Investigation of Sabin-like vaccine strains in the DRC, Madagascar and Ethiopia identified vaccine-derived polioviruses in AFP cases as well as possible vaccine-associated paralytic poliovirus in South Africa.

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SELECTED ABBREVIATIONS USED IN THE TEXT

AFP	Acute flaccid paralysis
BDD	Bandundu
CDC	Centers for Disease Control and Prevention
cVDPV	Circulating vaccine-derived polioviruses
CVID	Common variable immunodeficiency disorder
CPE	Cytopathic effect
DNA	Deoxyribose nucleic acid
EQT	Equateur
GPLN	Global Polio Laboratory Network
RD	Human Rhabdomyosarcoma
iVDPV	Immunodeficiency vaccine-derived polioviruses
IPV	Inactivated poliovirus vaccine
IRES	Internal Ribosomal Entry Site
SOAS	South Asia genotype (Indian genotype)
ITD	Intratypic Differentiation
KOC	Kasai Occidental
KAT	Katanga
mOPV	Monovalent Oral Poliovirus Vaccine
NIDs	National Immunization Days
NICD	National Institute for Communicable Diseases
NTR	Non-Translated Region
OPV	Oral Poliovirus Vaccine strains
ORT	Orientale

PCR	Polymerase chain reaction
PEI	Polio Eradication Initiative
PV	Polio Virus
PVR	Polio Virus Receptor
RT-PCR	Reverse transcription PCR
RNA	Ribose nucleic acid
SNNPR	Southern Nations Nationalities people Region
SNIDs	Sub-National Immunization Days
SKV	Sud- Kivu
DRC	the Democratic Republic of the Congo
P-distance	The nucleotide pairwise distance
tOPV	Trivalent Oral Poliovirus Vaccine
UP	Uttar Pradesh
VAPP	Vaccine-associated paralytic poliomyelitis
VDPV	Vaccine-derived Polioviruses
WEAF-A	Western African A or African genotype
WEAF-B	Western African B or African genotype
WPV	Wild poliovirus
WPV1	Wild poliovirus 1

CHAPTER 1

Literature Review

1.1. INTRODUCTION

Poliovirus, a member of the Enterovirus genus of the family *Picornaviridae*, is the cause of paralytic poliomyelitis which occurs only in a small proportion (<1%) of poliovirus infections of susceptible individuals (Kew, Sutter et al. 2005). Poliovirus has a predilection for the motor nerve cells of the brain and spinal cord and infection results in their destruction and paralysis of the muscles supplied by the affected cells (Gear 1993). Some infected people experience minor illness of several days and a symptom-free interval of 1 to 3 days, followed by acute onset of flaccid paralysis with fever. Within few days, paralysis which is usually asymmetric, progresses depending upon the sites of virus replication in the central nervous system and may affect skeletal muscles (spinal poliomyelitis), respiratory muscles (bulbar poliomyelitis), or both (bulbo-spinal poliomyelitis) (Racaniello 1996). Polioviruses have been around for a long time in man's history with >99% reduction of cases since 1988 (WHO 2004). Before the development of killed in the 1950s and live virus vaccines in the 1960's, poliomyelitis was a serious problem in public health. Since then paralytic poliomyelitis remains a threat in certain underdeveloped countries but has been considered a conquered disease in the developed world (Melnick 1983). Two different poliovaccines have been developed, a), the inactivated poliovirus vaccine (IPV) of Salk and Youngner (Salk 1954; Plotkin 2004) and b) the live, attenuated oral poliovirus vaccine (OPV) of Sabin (Sutter, Caceres et al.

2004). Both IPV and OPV; have their particular advantages and disadvantages, OPV needs 4 or more doses, while IPV only need 2 or 3 doses. OPV has high intestinal immunity while IPV low intestinal immunity. OPV is cheap compared to IPV (Melnick 1978).

Active vaccination campaigns have been implemented since 1980 with the original aim of eradicating polio by the year 2000. This has led to a reduction in poliovirus cases worldwide with many developed and developing countries being declared polio-free. In parts of the developing world however, certain regions have remained endemic. Major setbacks have been experienced since 2002 with importations and outbreaks especially in Africa.

1.2. POLIOVIRUSES

1.2.1. *Classification*

Polioviruses are members of the Picornaviridae family, a group of non-enveloped positive-strand RNA viruses (Martin, Dunn et al. 2000). They group into three distinct serotypes, type 1, type 2 and type 3, and replicate mainly in the gut. Viruses within a serotype exhibit greater than 70% homology at the nucleotide level and 88% at the amino acid level. Poliovirus has an icosahedral structure, which consists of 60 identical asymmetrical protomers. These protomers are arranged along fivefold, threefold and two fold axes (Rueckert, Dunker et al. 1969). Each protomer is composed of virion proteins: VP1, VP2, VP3 and VP4. VP1 exhibits the greatest sequence variability, and plays a key role in receptor attachment (Rueckert, Dunker et al. 1969). VP1, VP2 and VP3 are very similar in tertiary structure but differ in size and in their amino acid level (Hogle, Chow et al. 1985). VP4 is the smallest structural protein and is located on the

VP3 are very similar in tertiary structure but differ in size and in their amino acid level (Hogle, Chow et al. 1985). VP4 is the smallest structural protein and is located on the inner surface of the capsid (Minor 1992). Each capsid protein present a common structure of an 8-stranded antiparallel β-barrel core but differ both in N- and C- terminals and in their size and loop structure that connect the outer strands of the β-barrels (Figure 1.1). The loops represent the major antigenic sites of the virus. The N- terminal are intertwined internally forming a connected network that contributes to its stable structure (Hogle, Chow et al. 1985).

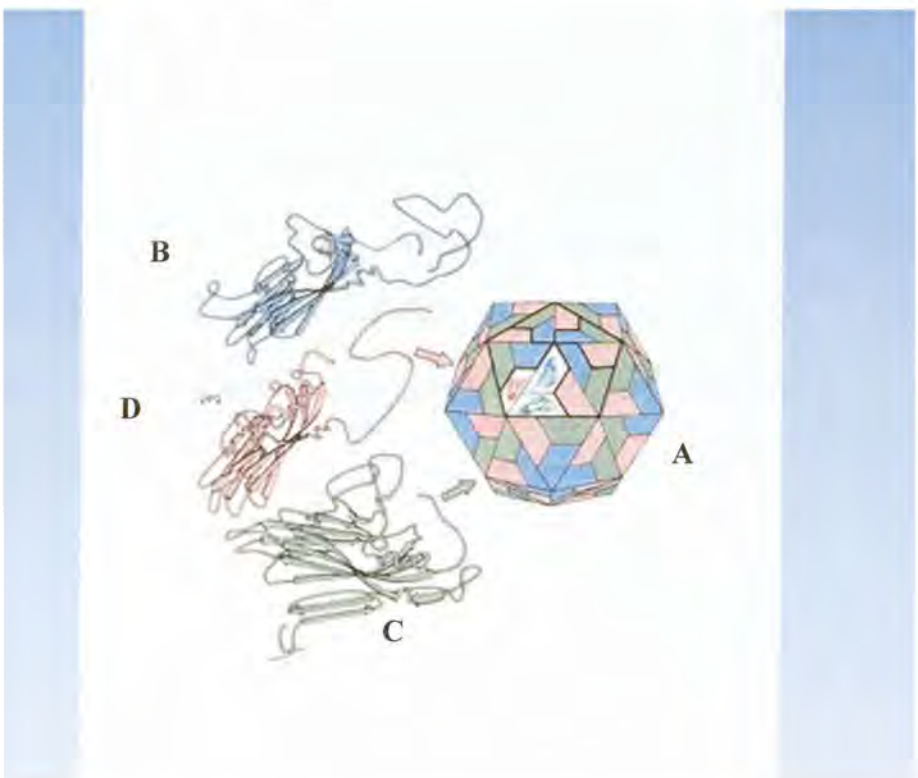


Figure 1.1: (A) Schematic representative of the icosahedral capsid structure of poliovirus, (B), (C) and (D) represent VP1, VP2 and VP3 proteins respectively in their tertiary configuration. (reproduced from http://www.biol.vt.edu/faculty/lederman/biol4664/text/images/).

1.2.2. *Genome organization and Replication*

The genomic RNA is approximately 7500bp long and terminates at the 3'-end in a polyadenylated tail of 70-100 nucleotides, and is covalently bound at the 5'-end to a small protein known as VPg (Figure 1.2). A 5' non-translated region (NTR) of about 740 nucleotides precedes a single large open reading frame. The coding region is translated as a single polyprotein that is post-translationally processed to generate the viral capsid that consist of VP4, VP2, VP3 which are products of proteolytic processing of the precursor, P1. The N- termini of VP0, VP4 and P1 are myristylated. Proteolytic processing of P2 and P3 non-structural proteins give rise to polypeptides $2A^{pro}$, 2B, 2C, 3A, 3B, $3C^{pro}$ and $3D^{pol}$ (Wimmer and Nomoto 1993)). Non-structural proteins have two or more distinct functions. For example, the 3C region code for a protease ($3C^{pro}$) which is responsible for the maturation cleavages in the precursor polyproteins. The 3D regions code for an RNA-dependent RNA polymerase ($3D^{pol}$) which has regions of homology with many DNA and RNA polymerases (Porter, Ansardi et al. 1993) (Figure 1. 2).

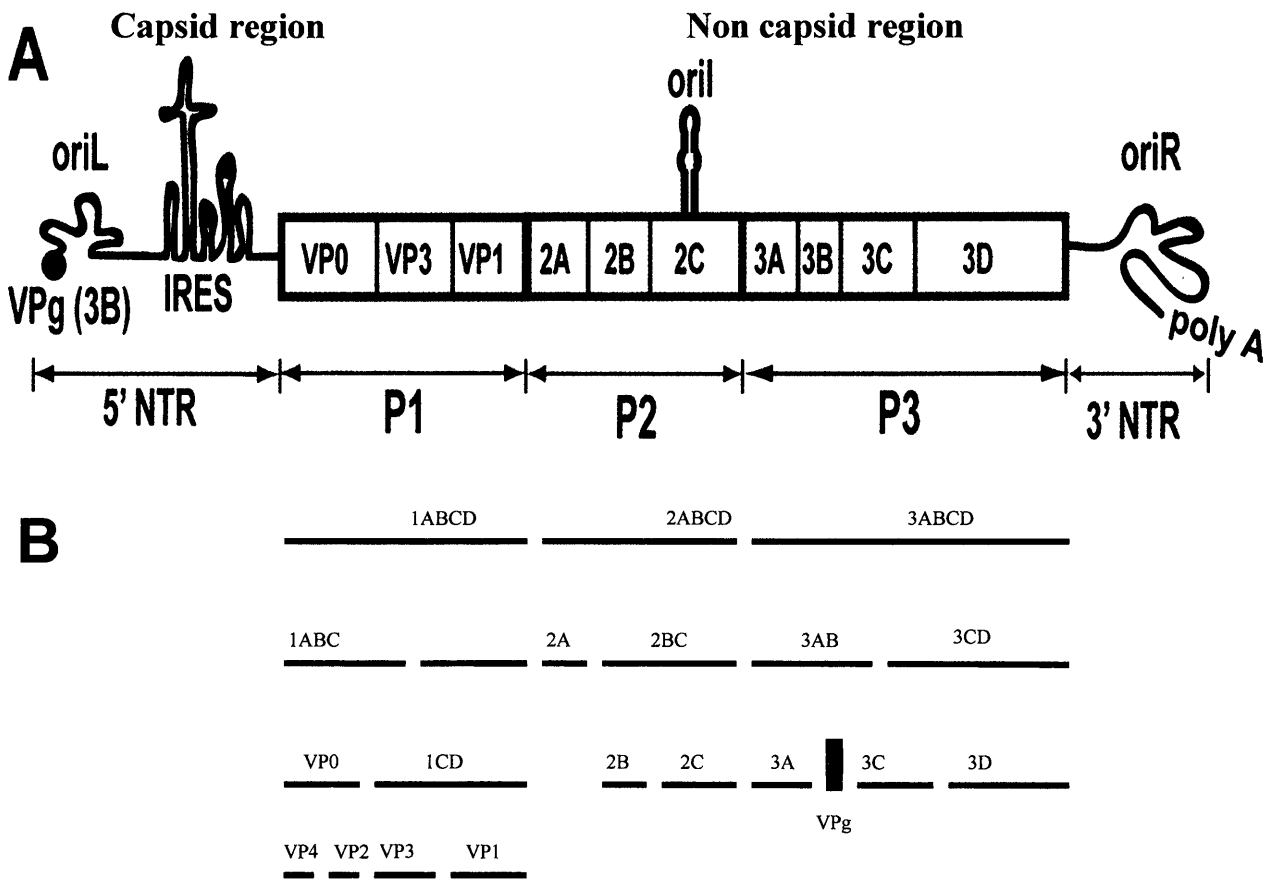


Figure 1.2. (A) Poliovirus genome organization and (B) processing pathway (reproduced from (Oh, Pathak et al. 2009).

The proteolytic processing can be divided into three stages, (i) the cleavage of P1 capsid protein precursor from the nascent polypeptide, at a junction of VP1 and 2A which is catalysed by the viral protease $2A^{pro}$(Toyoda, Nicklin et al. 1986), (ii) $3C^{pro}$ and $3CD^{pro}$ catalyse the processing of the capsid and non-capsid precursors. 3AB gives rise to 3B which represents VPg and thought to be involved in initiation of RNA synthesis (Pallansch, Kew et al. 1980). 3D is responsible for elongating nascent RNA chain from the RNA template (Flanegan, Petterson et al. 1977). 2B and 2C have key support functions in RNA replication (Bernstein, Sarnow et al. 1986);(Li and Baltimore 1988). 2A

is involved with abolishing of cap-dependent translation and also involved with cap-independent translation (Bernstein, Sonenberg et al. 1985);(Macadam, Stone et al. 1994), and (iii) during encapsidation of the viral RNA, VP0 is cleaved into VP4 and VP2 (Rueckert, Dunker et al. 1969).

The non-translated regions on each side of the protein coding region consist of highly conserved sequences. The initiation of translation takes place at the 5' NTR while initiation of RNA synthesis is at the 3' end of the positive and negative sense strand respectively (Rueckert, Dunker et al. 1969). Most eukaryotic mRNAs have a m^7GpppNp cap structure at the 5' nontranslated end, a characteristic that is missing in the poliovirus secondary structure. The 5' NTR is about 740 nucleotides in length (Kitamura, Semler et al. 1981);(Racaniello and Baltimore 1981). The 5' NTR contains a stem loop structure and has an unusually large number (8) of AUGs prior to the authentic start at nucleotide 743 (Figure 1.3) (Rivera, Welsh et al. 1988); (Pilipenko, Blinov et al. 1989); (Skinner, Racaniello et al. 1989). The 5'-end of the genome forms a cloverleaf structure with 90 nucleotides that interacts with VPg protein and viral protein 3CD to form a complex for RNA synthesis (Skinner, Racaniello et al. 1989); (Andino, Rieckhof et al. 1990); (Harris, Xiang et al. 1994); (Xiang, Harris et al. 1995).

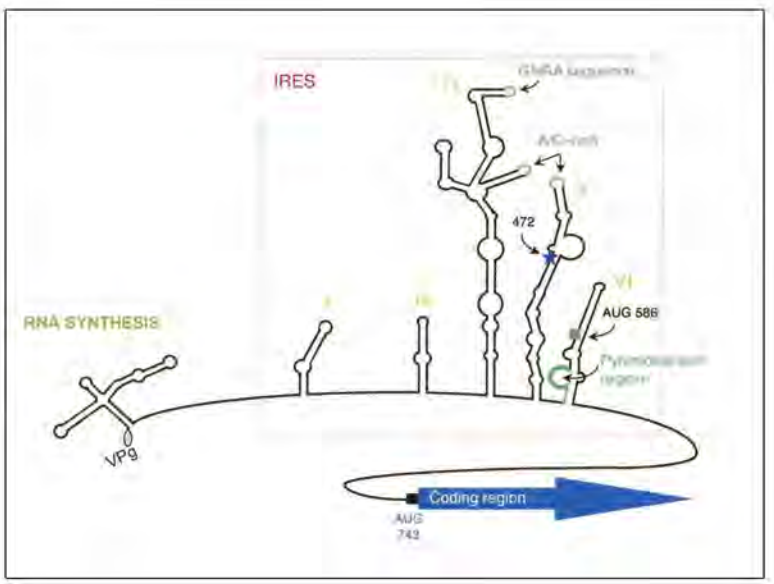


Figure 1.3: Predicted RNA secondary structure of the poliovirus 5′ NTR (Semler 2004). Computer prediction and chemical and enzymatic RNA-structure probing were used to deduce a consensus RNA conformation. Conserved sequences among picornaviruses include a NRA tetraloop (thought to function in tertiary interactions of RNAs and in protein binding), A/C_rich loops, and a pyrimidine-rich region just upstream of the conserved AUG codon. The IRES domain is boxed by red lines. (reproduced from (Semler 2004)).

For cap-independent initiation of translation, it has been found that 5' NTR acts as a internal ribosomal (IRES) (Pelletier and Sonenberg 1988). Domains III, VI, and V are the main important cis-acting elements in the 5' NTR (Nicholson, Pelletier et al. 1991);(Percy, Belsham et al. 1992) and the AUG triplet which has shown to be complementary to conserved sequences in the 18S ribosomal RNA (Nicholson, Pelletier et al. 1991).

A common feature of all 3 Sabin strains is the specific mutation at the 5' NTR which in Sabin 1 is located at position 480 (Kawamura, Kohara et al. 1989)), 481 for Sabin 2 (Equestre, Genovese et al. 1991); (Macadam, Ferguson et al. 1991); (Ren, Moss et al. 1991) and 472 for Sabin 3 (Cann, Stanway et al. 1984); (Evans, Dunn et al. 1985);(Westrop, Wareham et al. 1989). The attenuation mutations in the IRES of the 5' NTR alter the stem loop structure and reduce the initiation of translation of mutant poliovirus RNA (Svitkin, Pestova et al. 1988); (Ehrenfeld and Gebhard 1994); (Gutierrez, Denova-Ocampo et al. 1997).

1.2.3. ***Poliovirus infection cycle***

The poliovirus replication starts with the interaction of the poliovirus with host cell's surface protein and the poliovirus receptor (PVR) called: CD-155. The PVR has been identified as a member of a family of proteins called the immunoglobulin (Ig) superfamily. The PVR has three loops expressed outside the cell arranged as follows: V-C2-C2, where V is variable and C is constant (Mendelsohn, Wimmer et al. 1989). The protein extends through the bilayer cell membrane as represented in figure 1.4. The initial event involved the attachment of the virion to the PVR (step 1), this triggers the conformational change in the virus capsid that involves the loss of the internally located VP4 protein (De Sena and Mandel 1977), extrusion of the VP1 protein (Fricks and Hogle 1990) and to the delivery of viral RNA across the membrane by the process of endocytosis (step 2) leading to the process of translation (step 3). The viral RNA strand is copied to make the negative strand genomic RNA in order to form the complementary new plus strands (step 4), which takes place in the endoplasmic reticulum (Caliguiri and Tamm 1970). The newly synthesized plus strand recycled over and over in additional

replication centres leading to a higher levels of viral protein production (steps 5, 6, and 7). The newly synthesized RNA molecules are packaged into virions (Baltimore, Jacobson et al. 1969). For virions assembly to take place, coat precursor protein P1 must be cleaved to form immature protomers composed of VP0, VP3 and VP1. With an increasing of proteinase activity, the protomers are assembled to form provirions which are packaged to uninfectious provirions (step 8).

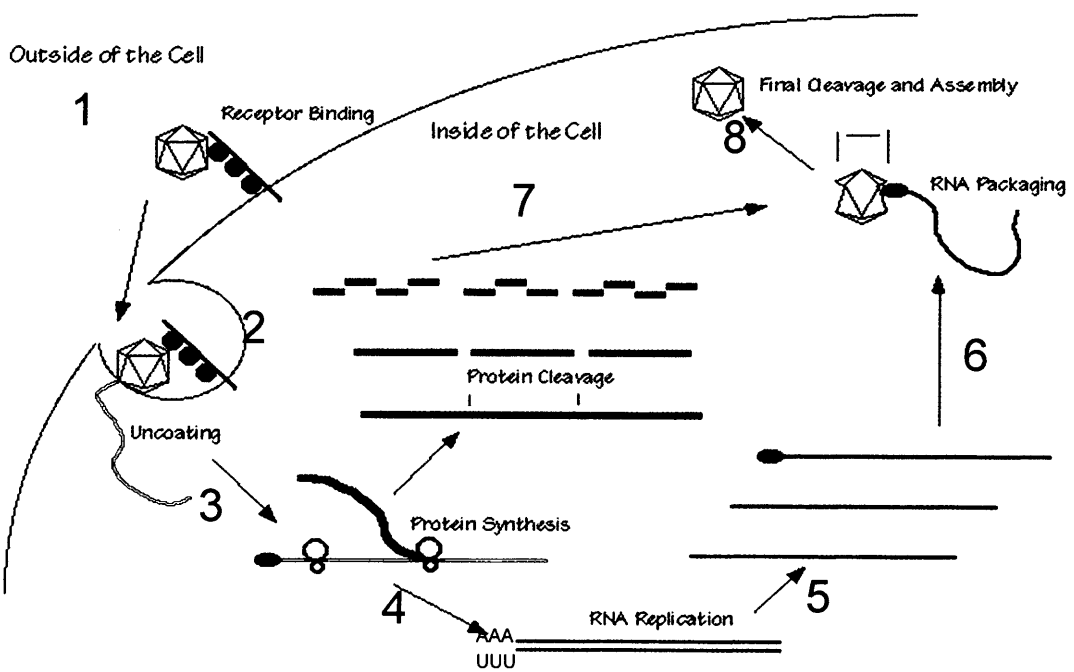


Figure 1.4. Poliovirus infection cycle (reproduced from the Polio Information Center Online, http://microbiology.columbia.edu/pico/Chapters/cellular.html

1.2.4. *Poliovirus vaccine*

Poliovirus vaccination with oral poliovirus vaccine (OPV), consisting of live attenuated strains of each of the three poliovirus serotypes (Nathanson and Fine 2002), has resulted in a worldwide decrease in the circulation of the wild polioviruses (WPV) (Wood, Sutter et al. 2000). The oral vaccine originally developed by Sabin in the 1960's and the inactivated poliovirus vaccine (IPV) developed by Salk in the 1950s (Salk, Krech et al. 1954) have made poliomyelitis a preventable and eradicable disease. Despite the success in the developed world, over 4000 cases were reported in Africa from January 2000 until now. The only remaining endemic country in the region is Nigeria, but additional outbreaks in several countries have resulted from importations from this reservoir and from outside the region (Taren, Nesheim et al. 1987). The advantages of OPV over IPV are that it can be easily administered by mouth, thereby, facilitating its widespread use; it induces intestinal immunity, making recent OPV recipients resistant to infection by polioviruses and effectively blocking wild poliovirus transmission when used in mass campaigns; and it provides long–term protection against polio through durable humoral immunity (Nathanson and Fine 2002; Dowdle, De Gourville et al. 2003). Despite its many advantages, OPV use carries certain risks (Fine and Carneiro 1999). OPV virus can spread from OPV recipients to unvaccinated contacts (Heymann 2004). The appearance of cases of vaccine-associated paralytic poliomyelitis (VAPP) was the evidence of clinical disease following genetic changes of OPV (Kim, Kim et al. 2007).

One of the adverse events associated with OPV use is vaccine-associated paralytic poliomyelitis (VAPP), which is characterized by clinical signs typical of paralytic poliomyelitis and also isolates shed from VAPP cases that typically contain neurovirulent

vaccine-related virus (Sutter, Caceres et al. 2004). Despite the extraordinary safety record of OPV, cases of VAPP demonstrate the clinical consequence of the genetic instability of OPV strains. There are ~250-500 annual VAPP cases worldwide and most of them occur in countries already free of polio (Sutter, Caceres et al. 2004). The incidence of VAPP is highest in immunodeficient people (iVAPP), particularly among B-cell immunodeficiencies. Laboratory findings suggest that Sabin 1 vaccine is rarely associated with VAPP cases among immunocompetent individuals, whereas Sabin 3 and Sabin 2 are more frequently associated to iVAPP cases. Isolates from VAPP cases share the genetic reversion to the neurovirulent phenotype and many of them are vaccine/vaccine recombinants. Regardless of the extent of divergence between VAPP isolates and their parental Sabin vaccine, VAPP isolates are attenuation revertants and VAPP cases are clinically indistinguishable from cases associated with WPV. The minimum difference separating wild polioviruses from Sabin strains is >15% divergence at the VP1 region. Closely related (<1% VP1 divergence) Sabin-like strains are classified as OPV-like strains.

Although cases of VAPP have long been recognized, more recently two additional OPV-related problems have been identified that have a significant impact on polio eradication. These are the long-term excretion of vaccine-derived polioviruses (VDPVs) in persons with primary humoral immunodeficiencies, so-called immunodeficiency vaccine-derived poliovirus (iVDPV) and polio outbreaks associated with circulating vaccine-derived poliovirus (cVDPV) in areas with low rates of OPV coverage(Kew, Sutter et al. 2005). Poliovirus isolates sharing between 1% and 15 % of VP1 divergence from Sabin reference strains are VDPVs.

VDPVs are classified into three categories, namely: 1) the immunodeficiency vaccine-derived polioviruses (iVDPV) obtained from person with defects in antibody production (Bellmunt, May et al. 1999); (Kew, Sutter et al. 1998); (Yoneyama, Hagiwara et al. 1982), 2) circulating vaccine-derived polioviruses (cVDPV) associated with person-to-person transmission (Yang, Naguib et al. 2003) and 3) ambiguous vaccine-derived polioviruses (aVDPVs) isolated from environmental isolates whose ultimate source has not been identified (Kew, Sutter et al. 2005).

The immediate public health importance of cVDPV was underscored by episodes of cVDPV in Egypt (1988-93) (Yang, Naguib et al. 2003), Hispaniola (2000-01) (Kew, Morris-Glasgow et al. 2002) (CDC. 2001d) (CDC. 2001e), Philippines (Kew, Morris-Glasgow et al. 2002), Madagascar (2001-02, 2005) (Rakoto-Andrianarivelo, Gumede et al. 2008) (CDC. 2002b and Nigeria (2005-2011) (CDC.2009b). Recent molecular studies have also detected the likely localized spread of OPV-derived virus in previous decades in Belarus (1965-66) (Korotkova, Park et al. 2003). cVDPVs show a significant sequence drift which indicates a prolonged replication of the vaccine strain and thereby acquiring the phenotypic property of transmissibility and circulation in the community. A better understanding of VDPV persistence and circulation is very important for decision making, about when and how to stop immunization with oral poliovirus vaccine (OPV) after the global eradication of poliomyelitis (Fine and Carneiro 1999; Wood, Sutter et al. 2000; CID.2002). In addition persons with primarily immunodeficiency are at risk to become chronic excretors of polio vaccine viruses and may represent a potential reservoir for poliovirus reintroduction after polio eradication. Once exposed to OPV, immunocompetent persons excrete polio vaccine viruses for up to 2 – 3 months (Alexander, Gary et al. 1997; Jorba, Campagnoli et al. 2008) although cases of the

prolonged excretion of VDPV by immunodeficient persons have been reported (MacCallum 1971);(Lopez, Biggar et al. 1974);(Hara, Saito et al. 1981);(Misbah, Lawrence et al. 1991);(Kew, Sutter et al. 1998);(Bellmunt, May et al. 1999);(Martin, Dunn et al. 2000). This could also influence the decision on polio vaccination cessation if poliovirus eradication is achieved.

To increase the sensitivity of detecting VDPV's, the Global Polio Laboratory Network (GPLN) implemented additional testing requirements in 2001 for all isolates under investigation (World Health Organization recommendations to Polio Reference Centers, 2002): This includes an ELISA test to identify non-Sabin-like strains and a PCR test to identify Sabin-related strains. Isolates that showed a discrepancy between the two tests are considered to be potential VDPV's. It is known that ELISA does not detect all VDPVs, therefore a new VDPV screening method known as real-time assay has been introduced into the GPLN. All potential VDPV's are therefore also confirmed by sequencing. The potential risk of cVDPV emergence has increased dramatically in recent years as wild poliovirus circulation ceased in most of the world and vaccine coverage dropped in some countries. The risk appears highest for the type 2 OPV strains because of its greater tendency to spread to contacts.

Although the mutations associated with reversion of Sabin to virulent wild virus are known, a predictive scoring system that will allow the identification of high risk Sabin drift towards VDPVs will be invaluable for the early detection and control of VDPV outbreaks. It has been shown that recombination events between circulating enteroviruses and polio vaccine were associated with some of the current VDPVs.

The control of poliovirus in the post-eradication era remains a big challenge. One of the suggested options for the complete eradication of live poliovirus from the world is to stop the use of OPV worldwide at some point after the world has been certified free from circulating wild polioviruses (Cherkasova, Korotkova et al. 2002). This may limit the circulation of derivatives of the Sabin strains, and it is assured that the derivatives will be unable to survive in nature long enough to evolve into highly transmissible neurovirulent strains (Fine and Carneiro 1999); (Wood, Sutter et al. 2000). The long-term persistence of vaccine derivatives in immunocompromised persons and the ability of the evolved variants to cause paralytic disease are well-established phenomena (Bellmunt, May et al. 1999);(Yoneyama, Hagiwara et al. 1982); (Kew, Sutter et al. 1998). This and low vaccine coverage in a population my result in the emergence of a new setback to polio eradication namely, virulent vaccine-derived polioviruses.

1.3. **EPIDEMIOLOGY**

The most neurovirulent of the three serotypes is poliovirus type1 (Salk 1956) which causes most epidemics followed by type 3 and type 2 (Salk 1956). Poliovirus is transmitted through fecal-oral route and the infection remains 7 to 14 days after the onset of paralysis. Poliovirus is excreted in the feces for approximately 3 to 6 weeks (Gelfand, Fox et al. 1957). Susceptible households have high secondary infection rate (Gelfand, Fox et al. 1957). The period of 3 to 6 days is enough for incubation between infection and first symptoms of minor illnesses, and from infection to the date of onset of paralysis usually 7 to 21 days (Horstmann and Paul 1947). Previously, an immunocompetent person was thought to excrete poliovirus for a period of 4 to 6 weeks

and an immunocompromised person can excrete from 3 years to 7 years as reported for a patient with common variable immunodeficiency disorder (CVID) who acquired vaccine-associated paralytic poliomyelitis (CDC. 1997a); (Kew, Sutter et al. 1998).

Forty-eight previously reported outbreaks occurred between 1976 and 1995 involving approximately 17000 unvaccinated and inadequately vaccinated cases, caused by poliovirus type 1 (Patriarca, Sutter et al. 1997). In the developing countries these cases were amongst children of 0 - 2 years and in industrialised countries occurred in older people (Sutter, Jafari et al. 2008). In developing countries, lower socioeconomic conditions have been shown to be one of the risk factors for paralytic poliomyelitis due to vaccine failure after OPV administration because of concurrent enterovirus infections (Bernkopf, Medalie et al. 1957).

Wild animals such as great apes, chimpanzees, gorillas and orangutans can experience paralytic disease after poliovirus infection, and such outbreaks had been reported in literatures by Allmond et al. and Dowdle et al.(Allmond, Froeschle et al. 1967; Dowdle and Birmingham 1997). In contrast, monkeys cannot be infected by OPV and cannot participate in the chain of transmission; therefore animals are not thought to be reservoir for poliovirus (Dowdle and Birmingham 1997).

The epidemiology of poliomyelitis has been observed in three patterns; 1) endemic, 2) epidemic and 3) vaccine era (Kew, Ryves et al. 1997; Sutter, Jafari et al. 2008). In the 19th century and the beginning of the 20th century, a change from endemic to epidemic was observed in the countries such as United States, Norway and Sweden. In developing countries such as Nigeria, Pakistan, Afghanistan and India an endemic

pattern is dominating while transmission of indigenous wild poliovirus was interrupted in other countries by year 2006 (CDC. 2010a) .

In 2011, reported polio cases amounted to ~618 (only from serotypes 1 and 3), a >99% decline since 1988 in which the number of polio cases reached an estimated number of ~350,000 (from all three serotypes) while the number of endemic countries declined from 125 to 4 during the same period of time (Figures 1.5 and 1.6). Three WHO Regions have already been declared polio free: 1) the Americas Region in 1994, 2) the Western Pacific Region in 2000, and 3) the European Region in 2002. However, there are several immediate challenges facing the GPEI, among them: (a) the re-introduction of wild polioviruses in polio free countries like Angola, Chad, Democratic Republic of the Congo and Sudan (CDC. 2009b) and (b) appearance of polio outbreaks due to the transmission of VDPVs (Kew, Sutter et al. 2005).

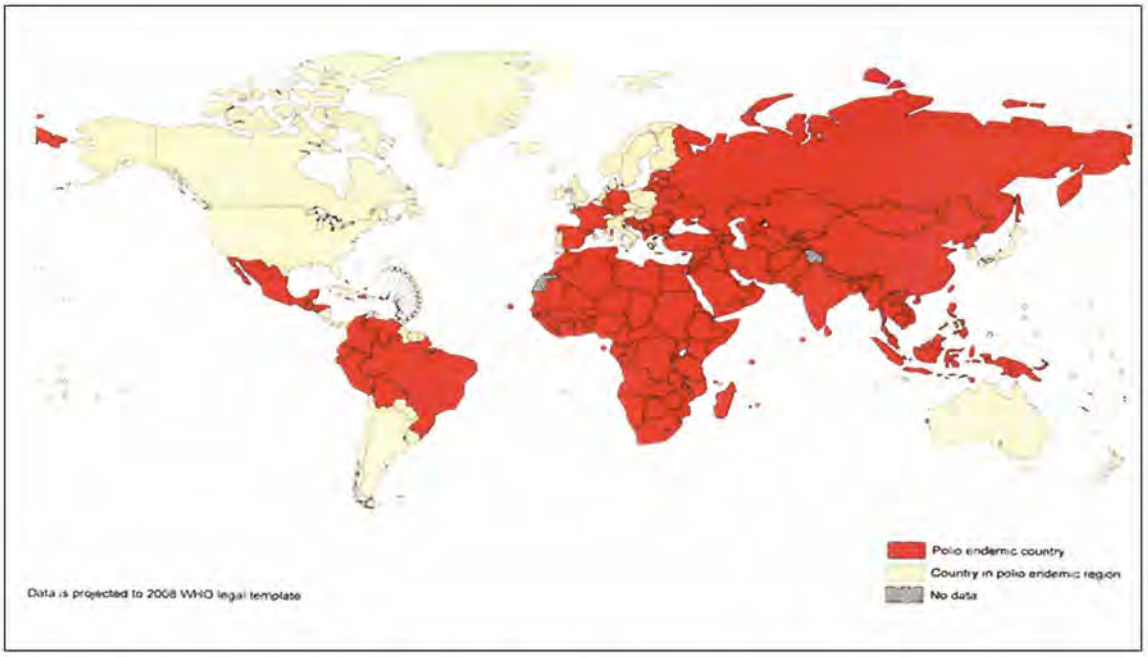


Figure 1.5: Countries in red with reported cases of poliomyelitis in 1988. Source: WHO

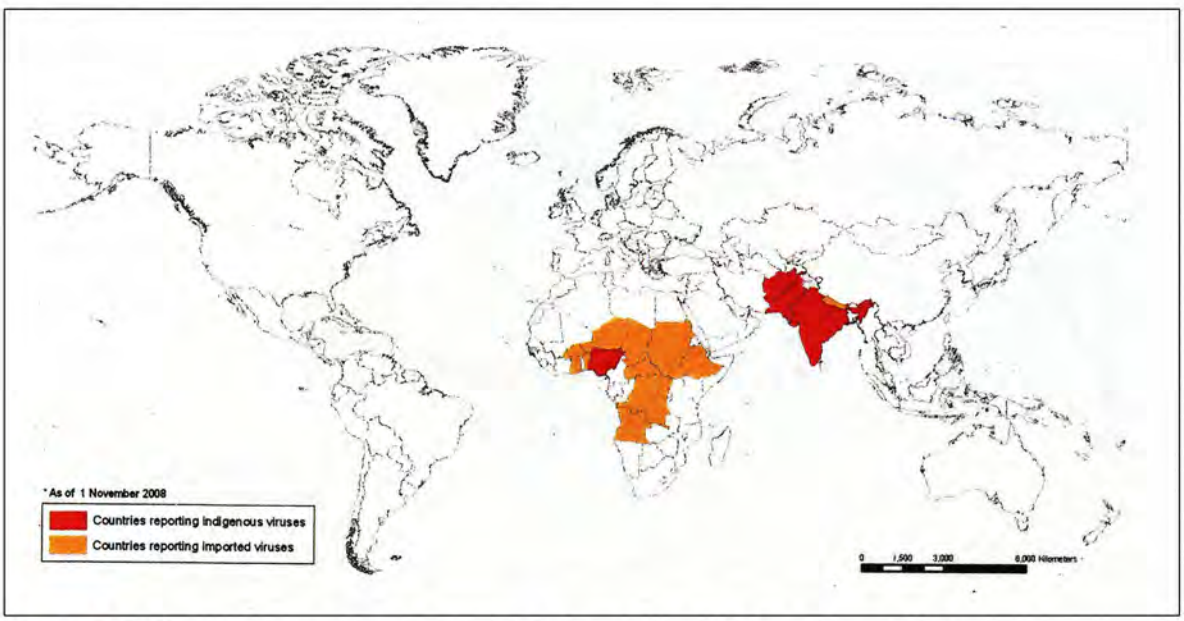


Figure 1.6: Countries with reported cases of poliomyelitis in 2010. Source: WHO

The recurring polio outbreaks especially in sub-Saharan Africa have been a setback in the eradication campaign. Many of these are re-infected countries, particularly in sub-Saharan Africa, at risk due to low routine immunization coverage (<80%), suboptimal outbreak response, weak health systems, and importations originating from the West Africa, into central Africa and to the Horn of Africa. By mid-2009, Angola, Chad, the DRC and southern Sudan had persistent for >12 months leading to their designation as having re-established transmission while other countries suffered new importations (Figure 1.7) (Jenkins, Aylward et al.).

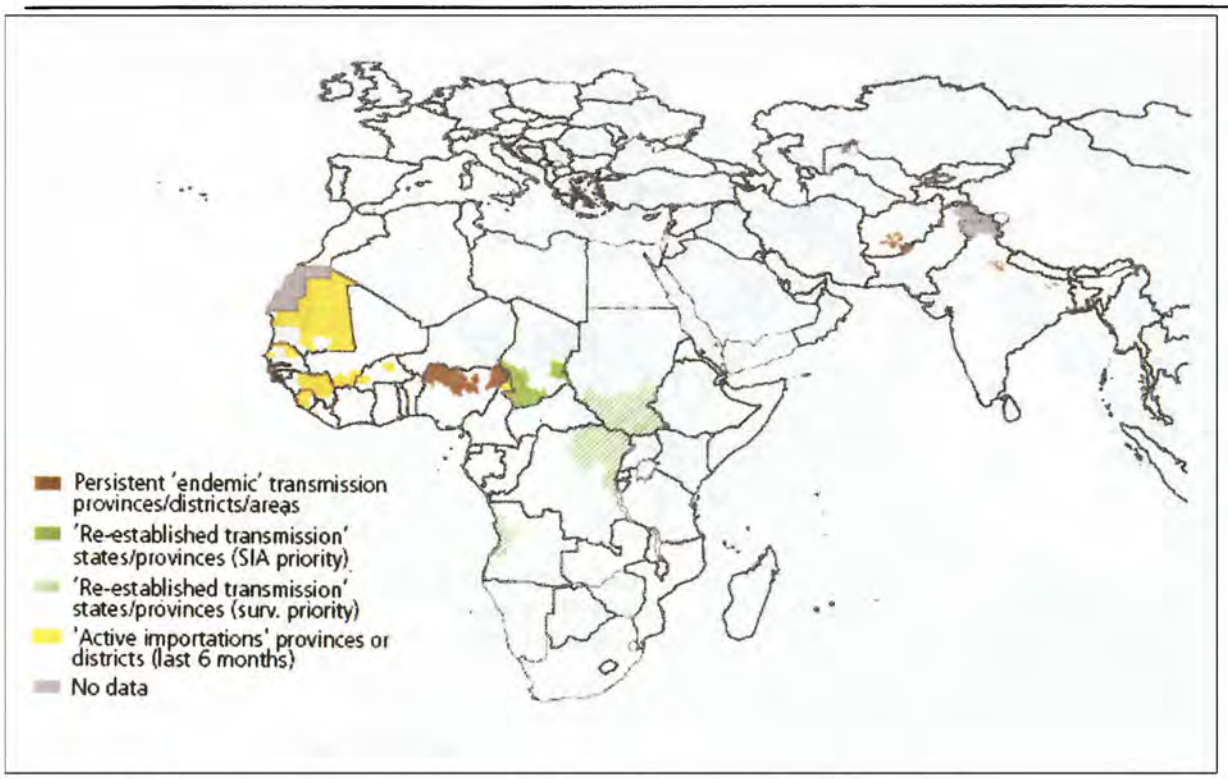


Figure 1.7: Countries with reported cases of poliomyelitis in 2010. Source: WHO

Over 500 AFP cases associated with VDPVs have been reported globally (Figure 1.8). Nigeria had the largest outbreak affecting neighbouring countries like Niger and Chad. The first VDPV case was identified from a child in 2002 (Adu, Iber et al. 2007). In Nigeria, the outbreak continued into 2011 and a total of 355 cases were reported (CDC. 2011).

As of July 2011, new outbreaks of VDPVs were identified in Afghanistan and India ((CDC. 2011). Ethiopia and the DRC also experienced emerging of VDPVs as described in Chapter 5 and Chapter 6.

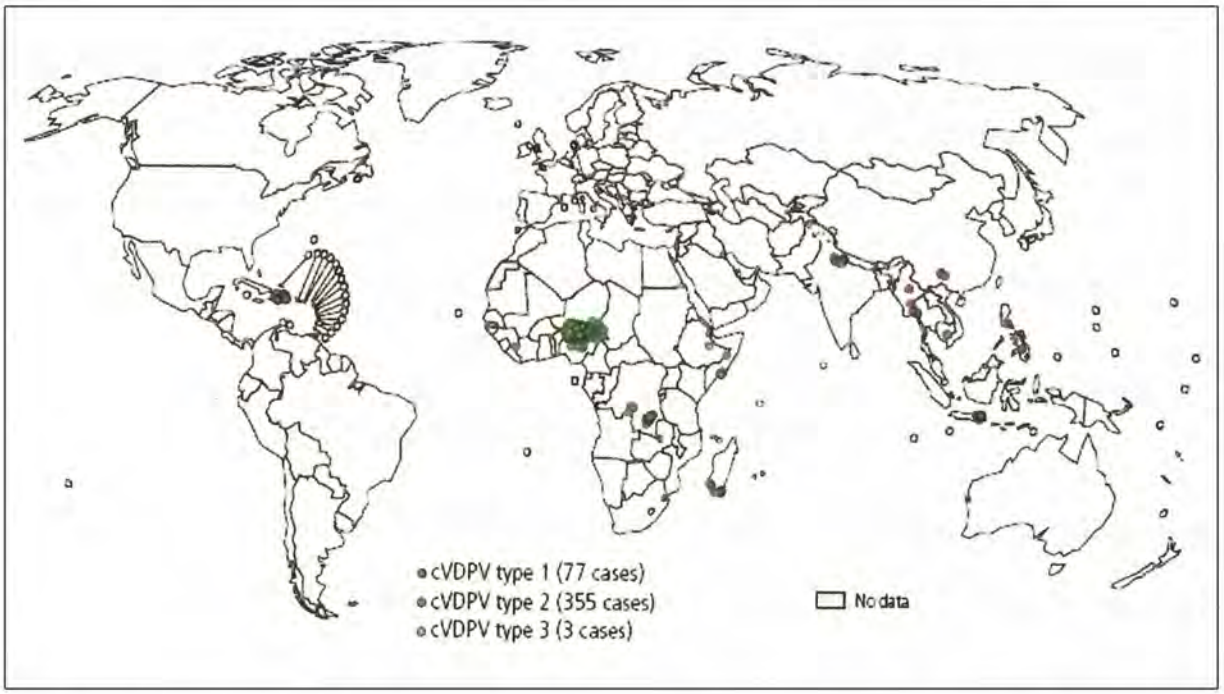


Figure 1.8: Countries with reported cases of vaccine derived polioviruses as of 2010. Source: WHO

Exposure to poliovirus occurred early in life but earlier theories suggested that the poliomyelitis was not a health burden as infants are protected from paralysis by maternal antibodies but this has been recently reviewed (Sutter, Jafari et al. 2008).

In the absence of vaccination, there would be more cases of paralysis per year according to WHO estimation and the vaccine coverage has resulted in some countries from the developing world to shift from endemic to epidemic pattern (Patriarca, Sutter et al. 1997), (Otten, Deming et al. 1992),(Afif, Sutter et al. 1997) (Reichler, Abbas et al. 1997).

National immunization campaigns were not developed in many developing countries until 1970s and 1980s and in 1990s the global OPV coverage with three doses reached 80% in children of 1 year old

(www.who.int/vaccines.../GlobalSummary/GlobalSummary.pdf.WHO/IVB/2006).

In order to achieve high vaccine coverage and control of poliomyelitis, supplemental doses of OPV in addition to routine coverage are needed (www.who.int/vaccines.../GlobalSummary/GlobalSummary.pdf.WHO/IVB/2006).

1.4. MOLECULAR EPIDEMIOLOGY

The Global Poliomyelitis Eradication Initiative (GPEI) was initiated in 1988, after the declaration of the eradication of smallpox in 1980 by the World Health Assembly, to eradicate poliomyelitis globally by 2000 (CDC. 2009a). The GPEI has been successful in reducing cases and by the end of 2010, cases had been reduced from 350,000 cases in 1988 to fewer than 1000 cases by the end of 2011, and Pakistan, Afghanistan and Nigeria the only remain endemic countries (CDC.2006c) .

Any case of new onset of hypotonic weakness in a child aged less than 15 years of age is defined as acute flaccid or floppy paralysis (AFP) which include Guillian-Barre' syndrome, transverse myelitis, enterovirus infections and traumatic neuritis. To make sure not to miss any polio case, AFP surveillance is targeting an AFP symptom rather that a specific disease (CDC. 2000a). AFP surveillance is a GPEI strategy to detect poliovirus circulation, re-importation of wild poliovirus into polio free-areas or regions and emerging VDPVs. As polio eradication is approaching, it is crucial to maintain high quality AFP surveillance

(www.hpsc.ie/hpsc/A-Z/VaccinePreventable/Polio/.../File,2461,en.pdf).

In 1996, National Immunization Days (NIDs) and AFP surveillance was implemented by the African Region (AFR) (CDC.2000c)(. WHO indicator of AFP surveillance, which is a measure of surveillance system sensitivity, is targeted at >/= 2 nonpolio AFP case per 100,000 population aged <15 years (CDC. 2000c). By 1999, AFP surveillance had improved and as a result of adequate surveillance, AFP cases were 4999 compared to 1754 reported in 1998 (CDC. 2000c). Wild poliovirus surveillance is based on AFP case investigation and virological studies (Kew, Sutter et al. 2005), as was the case of this study. There are different etiologies associated with AFP, hence a need of combining AFP surveillance with virological studies to investigate the role of wild polioviruses (Kew, Sutter et al. 2005), VDPVs and VAPP. In support of GPEI, a Global Polio Laboratory Network has been established by WHO to apply virological tools of poliovirus surveillance (Kew, Sutter et al. 2005). Methods of isolating virus from stool specimen (www.who.int/vaccines/en/poliolab/WHO-Polio-Manual-9.pdf.WHO/IVB/04.10); intratypic differentiation of vaccine from a wild poliovirus by the use of PCR (Kilpatrick, Nottay et al. 1996; Kilpatrick, Nottay et al. 1998) and ELISA (van der Avoort, Hull et al. 1995), and by real-time (Kilpatrick, Yang et al. 2009) and the molecular techniques were developed and implemented in the GPLN.

Polioviruses recombine with each other (Cooper 1968); (Cammack, Phillips et al. 1988); (Kilpatrick, Ching et al. 2004) and with human enteroviruses of species C (Guillot, Caro et al. 2000); (Liu, Zheng et al. 2003); (Shimizu, Thorley et al. 2004). For closely related isolates the nature and recombination junction provide markers for the resolution of poliovirus lineages (Liu, Zheng et al. 2003);(Cherkasova, Yakovenko et al. 2005).

The finding that polioviruses evolve at a rate of approximately 1% per year(Jorba, Campagnoli et al. 2008), has meant that the sequence analysis provides the resolving power for molecular epidemiological studies that has confirmed the existence of different genotypes or lineages within each outbreak (Liu, Zheng et al. 2000). Genotypes are found in different regions world-wide and are distributed geographically (Rico-Hesse, Pallansch et al. 1987) (Figure 1.9 and Figure 1.10). For WPV1, 10 genotypes were circulating worldwide between 1999 and 2004 with 8 out of 10 affecting the East, North and West African region. The remaining two affecting India, Pakistan and Afghanistan which are still endemic countries. For the same period, WPV3 had 5 genotypes that affected Africa and Asia, with 4 out of these circulating in the African region.

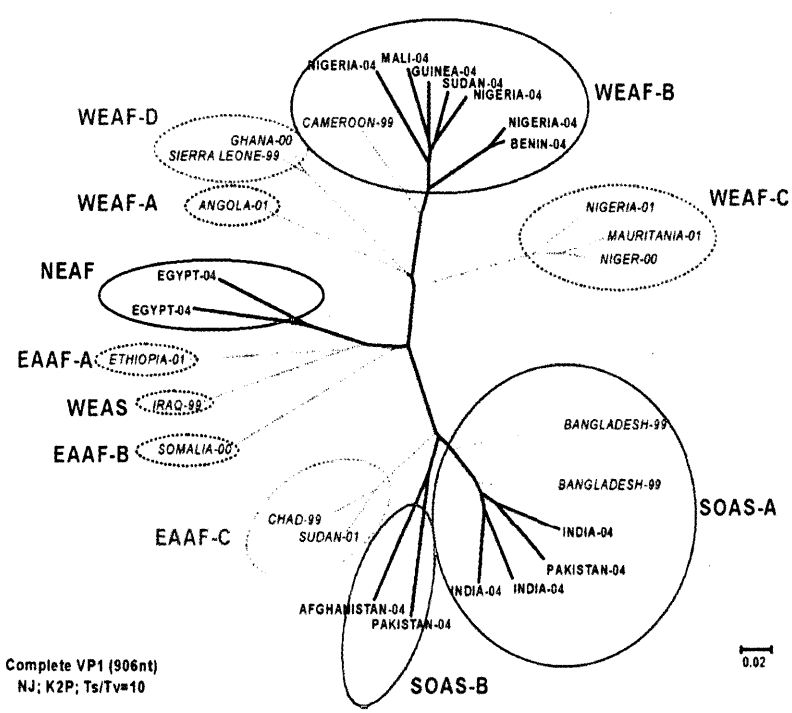


Figure 1.9: Neighbour-joining tree of WPV1 showing different genotypes from different regions between 1999 – 2004. Source: CDC.

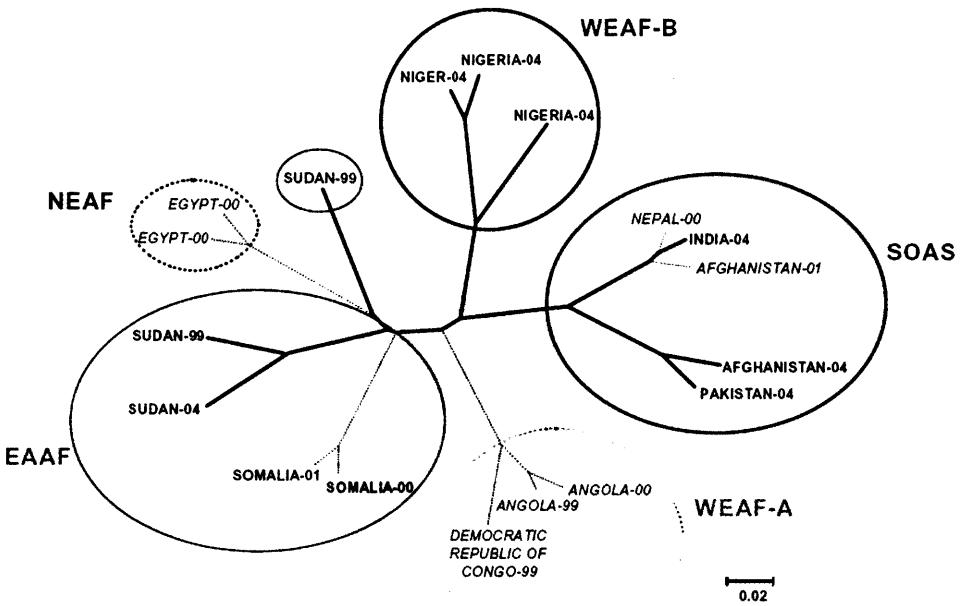


Figure 1.10: Neighbour-joining tree of WPV3 showing different genotypes from different regions between. 1999 – 2004. Source: CDC

Molecular epidemiology has been used to identify the poliovirus reservoirs, the pathway of poliovirus transmission, the tracing the sourcing of importation, the development of new molecular techniques and finally the progress towards the eradication of polioviruses (Kew and Nottay 1984).

Genomic sequencing and phylogenetic analysis of polioviruses are powerful molecular tools that help to understand the epidemiology of poliomyelitis disease, therefore poliovirus transmission and the characterisation of importation from endemic countries can be identified. These molecular methods have managed to identify and distinguish between different poliovirus endemic genotypes in outbreaks and locate them to their indigenous location (Shulman, Handsher et al. 2000; Kew, Morris-Glasgow et al. 2002).

They also help to identify different outbreaks of VDPVs where recombination with other enteroviruses have been identified.

Different genotypes of poliovirus can co circulate and cause poliomyelitis in the same region (Afif, Sutter et al. 1997). There are only two remaining genotypes circulating worldwide and have been localized in areas with low OPV coverage. These genotypes are SOAS and WEAF-B and both for serotype 1 and serotype 3 respectively.

Although the initial target date for polio eradication in the world was the year 2000, several factors and polio vaccine coverage has been a challenge globally over the past years and wild polioviruses have reemerged and VDPV has emerged in several countries. Therefore, evaluation of this situation using molecular and bioinformatics techniques remains a very important goal.

1.5. AIM AND STUDY OBJECTIVES

Aim:

Identification and molecular characterisation of wild-type, Sabin-like and vaccine derived polioviruses from the African Region.

To achieve this aim the study was divided into different sections and the following objectives set:

1) To investigate wild polio outbreaks in the African region that occurred after 2000 with specific reference to:

- Molecular epidemiological investigation of poliovirus outbreaks in Namibia and Angola in 2005-2008 through phylogenetic analysis of isolates. (Chapter 2)
- To identify the poliovirus genotype in Angola and Namibia. (Chapter 2)
- To determine the epidemiological pattern of poliovirus strain the Democratic Republic of Congo (DRC). (Chapter 3).
- To analyse the distribution of strains in different countries. (Chapter 2 and 3)

2) To investigate Vaccine-derived poliovirus (VDPV) outbreaks in the African region that occurred after 2000 with specific reference to:

- Identification and molecular epidemiological investigation of VDPV outbreaks in Africa:
    - Phylogenetic analysis of VDPVs outbreaks in Madagascar. (Chapter 4)
    - Phylogenetic analysis of VDPVs outbreaks in the DRC. (Chapter 5)
    - Phylogenetic analysis of VDPVs outbreaks in Ethiopia. (Chapter 6)

3) To investigate Vaccine-associated paralytic poliomyelitis (VAPP) outbreaks in South Africa.

- Identification of cases that fit the clinical criteria of VAPP from Southern Africa since 2000 and PCR amplification of the 5'-NTR region of Sabin-like strains that fit the VAPP criteria to identify loss of attenuation mutations by sequencing. (Chapter 7)
- Identification of recombination events across the genome of Sabin-like strains that fit VAPP criteria by both real-time PCR and sequencing. (Chapter 7).

CHAPTER 2

Identification and molecular characterisation of an Indian genotype of poliovirus type 1 isolated during consecutive outbreaks of poliomyelitis in Angola and Namibia from 2005- 2009

2.1 INTRODUCTION

Great progress has been made in the World Health Organization's (WHO's) program for the eradication of poliomyelitis with only four countries still remaining endemic for wild-type poliovirus namely; Nigeria, India, Afghanistan and Pakistan (CDC. 2006b). Most countries in the WHO southern Africa region have been reporting a high vaccine coverage with three doses of oral poliovirus vaccine (OPV) in children under one year and were expected to be certified as a polio-free region (CDC. 1994). Unfortunately, outbreaks of poliomyelitis continue to be reported from hitherto polio-free regions as a result of importation from endemic countries (van Niekerk, Vries et al. 1994). If the elimination of poliomyelitis is to be achieved in Africa, there is a need for characterizing the patterns of spread of wild-type viruses to improve strategies of interrupting virus transmission (Chezzi, Blackburn et al. 1997; Chezzi, Blackburn et al. 1997). A major component of the WHO strategy of eradicating poliomyelitis is laboratory surveillance of Acute Flaccid Paralysis (AFP) cases in order to identify the presence of wild-type virus infections (Chezzi, Blackburn et al. 1997; Chezzi, Blackburn et al. 1997).

The rate of nucleotide sequence evolution in poliovirus is approximately 1% per year (Alexander, Gary et al. 1997); (Jorba, Campagnoli et al. 2008) and this has given rise to different genetic strains of wild-type polioviruses, termed genotypes which can be linked to specific geographic regions (Chezzi, Blackburn et al. 1997; Chezzi, Blackburn et al. 1997). Sequence analysis of the full VP1 900 nucleotides (nt) gene and molecular epidemiological comparisons of isolates in outbreaks can be used to answer epidemiological questions regarding the likely location of endemic virus reservoirs, patterns of virus transmission or source of imported strains.

Over 10000 AFP cases of both poliovirus type 1 and 3 were reported in Africa from January 2000 until 2010. Although most cases were concentrated in Nigeria, importation has resulted in outbreaks in a number of neighbouring and distant countries in West, East and Central Africa (CDC. 2002d). In 2005, ten cases of AFP associated with type 1 poliovirus and one contact were reported from various districts of Angola. The last wild-type poliovirus 1 (PV1) case in Angola was reported in 2001 in the Province of Lunda Sul. Following the outbreak in Angola in 2005, Namibia also experienced outbreaks by the same strain. The last cases of wild-type polio occurred in Namibia in 1993 following a wild-type PV1 outbreak with 53 cases (van Niekerk, Vries et al. 1994). Vaccine coverage in both countries has been inadequate with routine coverage of only approximately 50% (CDC. 2000a), especially in the regions affected by the outbreak.

Here the genetic characterization of the wild-type PV1 isolated during these outbreaks of poliomyelitis in Angola and Namibia is reported.

2.2 MATERIAL AND METHODS

2.2.1 Viruses

Virus Isolates from original stool specimen of suspected poliovirus AFP cases were submitted to the National Institute for Communicable Diseases (NICD) in South Africa for diagnostic reverse transcription polymerase chain reaction (RT-PCR), ELISA and partial genomic sequencing from Angola and Namibia as part of the poliovirus WHO Network (Table 2.1). The original stool specimen of suspected poliovirus were also sent to the NICD for confirmation by virus culture and micro-neutralization with antiserum pools from the National Institute for Public Health and Environment Protection (RIVM), Bilthoven, The Netherlands (van der Avoort, Hull et al. 1995). Virological investigation identified polioviruses of serotype 1.

2.2.2 Laboratory Diagnosis

Intratypic differentiation of vaccine and wild-type strains was carried out by RT-PCR as described previously (Yang, De et al. 1991); (Chezzi and Schoub 1996); (Kilpatrick, Nottay et al. 1996);(Kilpatrick, Nottay et al. 1998), and ELISA (van der Avoort, Hull et al. 1995)as recommended by the World Health Organization (WHO) .

2.2.2.1 Diagnostic RT-PCR

For the intratypic differentiation, all viral cultures that showed cytopathic effect (CPE) were tested for poliovirus using an RT-PCR kit supplied by the Centres for Disease Control and Prevention, Atlanta, USA (CDC, Atlanta) that included separate reactions with primers for pan-enterovirus, pan-poliovirus, serotype-specific (Kilpatrick, Nottay et

al. 1996); (Kilpatrick, Nottay et al. 1998), and specific for the Sabin type 1, 2 and 3 viruses (Yang, De et al. 1991). In brief, 1ul of the 1:4 diluted virus isolates was added to the master mix containing 19µl of primer and 5 µl of buffer B (containing 2.8ul of 1M Dithiothreitol (Roche Diagnostic GmbH, Mannheim, Germany), 14.4ul of 5U/ul myeloblastosis virus Reverse Transcriptase (Roche Diagnostic GmbH, Mannheim, Germany), 27.6ul of 40U/ul Rnase Inhibitor (Roche Diagnostic GmbH, Mannheim, Germany) and 54.8ul of 5U/ul Taq Polymerase (Roche Diagnostic GmbH, Mannheim, Germany)). Reverse transcription was carried out at 42°C for 20 minutes in a GeneAmp 9700 thermocycler (Applied Biosystem, Foster City, CA), followed by 95°C for 3 minutes (95°C for 45 seconds, 42°C for 45 seconds and 60°C 45 seconds) x30 cycle. Amplicons were separated on a 10% polyacrylamide gel and visualized by ethidium bromide staining.

2.2.2.2 ELISA

An ELISA developed by National Institute of Public Health and the Environment, Bilthoven, Netherlands, was used for identification of Sabin-like and wildtype strains using highly specific cross-adsorbed antisera (van der Avoort, Hull et al. 1995). In brief, wells of microtitre plates are coated with bovine IgG antibodies to poiliovirus type 1, type 2 and type 3 and incubated with the identified and typed poliovirus strain to be tested. Incubation is then carried out with the type specific, cross-adsorbed rabbit antisera. After washing off any unbound rabbit sera, a peroxidase-labelled anti-rabbit IgG antibody is added to detect bound rabbit sera. The OD readings which are measured at 450nm by

loading the plate on a spectrophotometer are used to calculate a ratio which is in turn used to determine whether the virus is either Sabin-like strain or Non-Sabin–like strain.

2.2.2.3 RNA Extraction

RNA was extracted from 140µl of clarified stool or cell culture supernatant using QIAamp Viral RNA extraction kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's recommendations. In brief, 140µl of tissue culture supernatant was added to 560µl of prepared Buffer AVL and incubated at room temperature for 10 minutes. At the end of incubation 560µl of ethanol (96 – 100%) was added to the sample mix. The mixture was transferred to the QIAamp spin column and centrifuged at 8000rpm for 1 minute. The spin column was washed twice by adding 500µl of Buffer AW1 followed by adding 500µl of Buffer AW2. RNA was eluted by adding 60µl of Buffer AVE.

2.2.2.4 RT-PCR for sequence analysis

RT-PCR was performed in a single step as described before (Yang, De et al. 1991); (Chezzi and Schoub 1996; Kilpatrick, Nottay et al. 1996; Kilpatrick, Nottay et al. 1998). Briefly, the extracted RNA (10 µl) was added to 90 µl of the amplification mixture containing 10 µl standard 10 × reaction buffer, 100 µM of each dNTP (Roche Diagnostics GmbH, Mannheim, Germany), 10 mM dithiothreitol, 80 pmol of each primer sets ((Y7R: (5'GGTTTTGTGTCAGCITGYAAYGA3') and Q8: (5'AAGAGGTCTCTRTTCCACAT3')) (Manor, Handsher et al. 1999), 20 U of placental RNase inhibitor (Roche Diagnostics GmbH, Mannheim, Germany), 12. 5 U of myeloblastosis

virus reverse transcriptase (Roche Diagnostics GmbH, Mannheim, Germany), 5U of Taq DNA polymerase (Roche Diagnostics GmbH, Mannheim, Germany). Reverse transcription was carried out at 42°C for 60 min in a GeneAmp 9700 thermocycler (Applied Biosystems, Foster City, CA) followed by 95°C for 3 min (95°C for 30 sec, 42°C for 30 sec, and 60°C for 2 min) x30 cycles

2.2.2.5 Cycle sequencing

The complete VP1 gene (nucleotides 2480 to 3385) was sequenced. The primers used for sequencing are specific to the 5' ends of the VP1 region and were as follows:

Y7R (5'GGTTTTGTGTCAGCITGYAAYGA3'); PV1A TTIAIIGCRTGICCRTTRTT3');

PV2S (CITAITCIMGITTYGAYATG 3') and Q8 (5'AAGAGGTCTCTRTTCCACAT3').

Nucleotide sequencing was carried out on the sense and anti-sense strands using the ABI Prism^{R}BigDye^{TM} Terminator Cycle Ready Reaction kit v3. 1 (PE Biosystems, Foster City, USÁ) according to the manufacturer's recommendations. The reaction was carried out in a 10 µl reaction: In brief, 4 µl of double distilled water was mixed with approximately 1 µl of 5–20 ng of PCR product; 2.0 µl Terminator Ready Reaction Mix (A, C, G and T-big dye terminators; (PE Biosystems, Foster City, USA), 2 µl of 5X Sequencing Buffer and 1 µl of 3.2 pmol primer. Cycle sequencing was performed according to the protocol specified for the GeneAmp 2400 thermocycler: 94°C for 1 minute; (94°C for 10 seconds, 50°C for 5 seconds, 60°C for 4 minutes) for 25 cycles; and finally 4°C infinity.

2.2.2.6 *Sequence Analysis*

The complete VP1 gene (nucleotides 2480 to 3385) was sequenced as described (Liu, Zheng et al. 2000) using cycle sequencing with the Big Dye Terminator Cycle sequencing kit version 3. 1 (Applied Biosystems, Foster City, USA) on the Gene Amp 9700 (Applied Biosystems). The DNA sequence was determined using the ABI 3100 GeneticAnalyzer, version 3. 1 (Perkin Elmer Applied Biosystems, Foster City, USA). Raw data was edited using the Sequencher™ software package version 4. 1. 4.

2.2.2.7 *Phylogenetic Analysis*

Phylogenetic analysis was carried out on the complete VP1 gene, which corresponds to 906 nt for all isolates from patients with AFP. Sequences were aligned with Clustal X (Thompson, Gibson et al. 1997). Neighbour-joining tree was constructed with MEGA version 4.0 (Kumar, Tamura et al. 2004; Tamura, Dudley et al. 2007; Kumar, Nei et al. 2008). Bayesian analysis was carried out with the programme BEAST version 1.4 using the actual sampling date of each specimen to estimate the rates of evolution (Drummond and Rambaut 2007). The GTR + G substitution model was used to allow different sites in the alignment to evolve at different rates and the substitution rate calculated from the data assuming a molecular clock. The Markov chain Monte Carlo search was set at a chain length of 10 000 000. The log and tree files were analysed with Logcombiner and the Treeannotator programmes and visualised with Figtree version 1. 1. 2 (http://tree. bio. ed. ac. uk/software/figtree))(Drummond and Rambaut 2007).

The alignments (nucleotide and translated amino acid sequences) were analysed with Genedoc version 2. 6001 (www. psc. edu/biomed/genedoc) to identify specific mutations and positive selection.

Table 2.1: Selected wild poliovirus type 1 isolated in Angola and Namibia between 2005 and 2009.

Epidemiological Number (EPID)	Country	Year of onset of paralysis
ANG-BEN-LOB-05-003	ANGOLA	2005
ANG-BEN-LOB-05-008	ANGOLA	2005
ANG-LSL-SAU-05-001	ANGOLA	2005
ANG-LSL-SAU-05-002	ANGOLA	2005
ANG-LUA-CAC-05-003	ANGOLA	2005
ANG-MOX-MOX-05-001	ANGOLA	2005
NAM-HAR-ARA-06-015	NAMIBIA	2006
NAM-KHO-WHK-06-018	NAMIBIA	2006
NAM-KHO-WHK-06-020	NAMIBIA	2006
NAM-KHO-WHK-06-022	NAMIBIA	2006
NAM-KHO-WHK-06-024	NAMIBIA	2006
NAM-KHO-WHK-06-027	NAMIBIA	2006
NAM-KHO-WHK-06-029	NAMIBIA	2006
NAM-KHO-WHK-06-031	NAMIBIA	2006
NAM-KHO-WHK-06-036	NAMIBIA	2006
NAM-KHO-WHK-06-039	NAMIBIA	2006
NAM-KHO-WHK-06-062	NAMIBIA	2006
NAM-KHO-WHK-06-067	NAMIBIA	2006
NAM-KHO-WHK-06-123	NAMIBIA	2006
NAM-KHO-WHK-06-171	NAMIBIA	2006
NAM-KHO-WHK-06-194	NAMIBIA	2006
NAM-OHA-ENG-06-054	NAMIBIA	2006
NAM-OHN-OSH-06-102	NAMIBIA	2006
NAM-OHN-OSH-06-137	NAMIBIA	2006
NAM-OMU-OKA-06-059	NAMIBIA	2006
ANG-LUA-CAZ-07-004	ANGOLA	2007
ANG-LUA-CAZ-07-005	ANGOLA	2007
ANG-LUA-KIL-07-003	ANGOLA	2007

2.3 RESULTS

2.3.1 Identification of wild poliovirus 1:

Viral isolates obtained from Angola, and Namibia in 2005, 2006 and 2007 were tested by RT-PCR using pan-enterovirus, pan-poliovirus, serotype-specific, and Sabin types 1, 2 and 3 virus specific primers. All isolates were positive for poliovirus type 1. The RT-PCR test was confirmed by ELISA and both techniques identified the isolates as wild-type poliovirus. These isolates were characterised by sequencing of 906 nt representative of the complete VP1 gene and all strains were found to be wild-type 1.

2.3.2 Outbreak description:

From 2005-2007 of AFP cases associated with poliovirus 1 have been reported from 2 countries in southern Africa.

2.3.2.1 Angola 2005-2007

In 2005, ten cases of AFP associated with type 1 poliovirus and one contact were reported from a number of districts of Angola. The first case (onset in April 2005) was a 17-month old girl who had a previous history of oral polio vaccine from the urban district of Cacuaco. She developed fever and paralysis on the 25 April 2005, in Luanda province. The patient and her family had no travel history. The Cacuaco district reports routine polio vaccine coverage of 50%. Two further cases were reported in Angola in 2006 with paralysis setting on the 14 November 2006 in the final case. In 2007, 8 cases

had been reported with the first onset in May while the last case developed paralysis on the 8th of July 2007.

2.3.2.2 Namibia 2006

In 2006 the first polio outbreak in more than 10 years was reported in Namibia affecting mainly adults. The index case was a 39 year old man from the Hardap region, south-east of the capital Windhoek, who had an onset of AFP on the 6th of May 2006 (CDC. 2006a). As of October 2, 2006, 19 AFP cases were reported, and confirmed as wild-type poliovirus type 1 and six of the confirmed cases died. The last case had an onset in June 2006. Wild-type polio virus was also identified in AFP cases in Windhoek in the Khomas region and in a northern area bordering Angola as well as with three regions adjacent to the border of Angola, Omusati, Oshana and Ohangwena. These are the most populated areas in the country.

2.3.3 Phylogenetic and P-distance analysis:

Blast search analysis of all polioviruses isolated in the current investigation identified the Indian genotype (SOAS) as being the most closely related genotype to strains identified in Angola and Namibia. Initial analysis of all wild-type PV1 genotypes facilitated the classification of the Angola and Namibia strains to the SAOS genotype. Figure 2.1 illustrates the relationship between selected representative wild-type PV1 isolates from southern Africa and India. In order to investigate this relationship and evolution over time unrooted tree was constructed using Bayesian analysis on all Angola and Namibian

isolates from the current outbreak as well as the African strains belonging to the African genotype (WEAF-B) isolated in the last prior outbreak, and Indian strains isolated in 2004 belonging to the SOAS genotype. Strains from India that were identified by BLAST seem to be the closest to the described outbreaks were included in the analysis. All polioviruses investigated fell within the same genotypes, i.e. SOAS (India genotype). Figure 2.2 shows the Bayesian tree rooted at the closest related Indian strain demonstrating the evolution that has occurred since the introduction of this genotype into Africa using Bayesian analysis with the assumption of a molecular clock at a set rate for poliovirus of 1 substitution per year.

Since all previous poliomyelitis cases in both countries were associated with the African genotype (WEAF-B), the homology of all isolates identified in the outbreaks in Angola and Namibia was investigated by full VP1 gene sequence analysis and included in these phylogenetic trees.

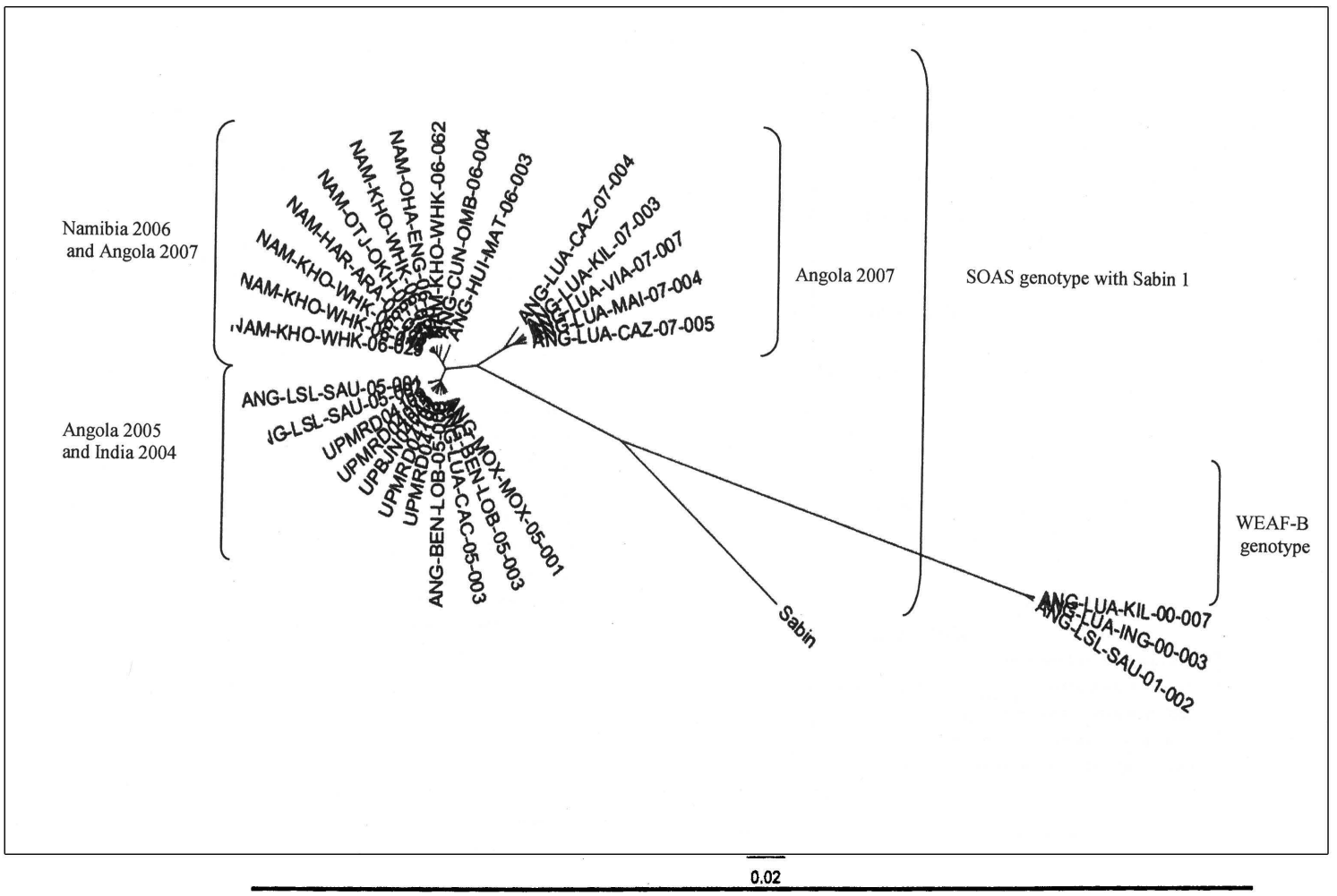


Figure 2.1. An unrooted tree showing the close relationship of the Angola and Namibia isolates to Indian strains. Sabin 1 reference strain also added

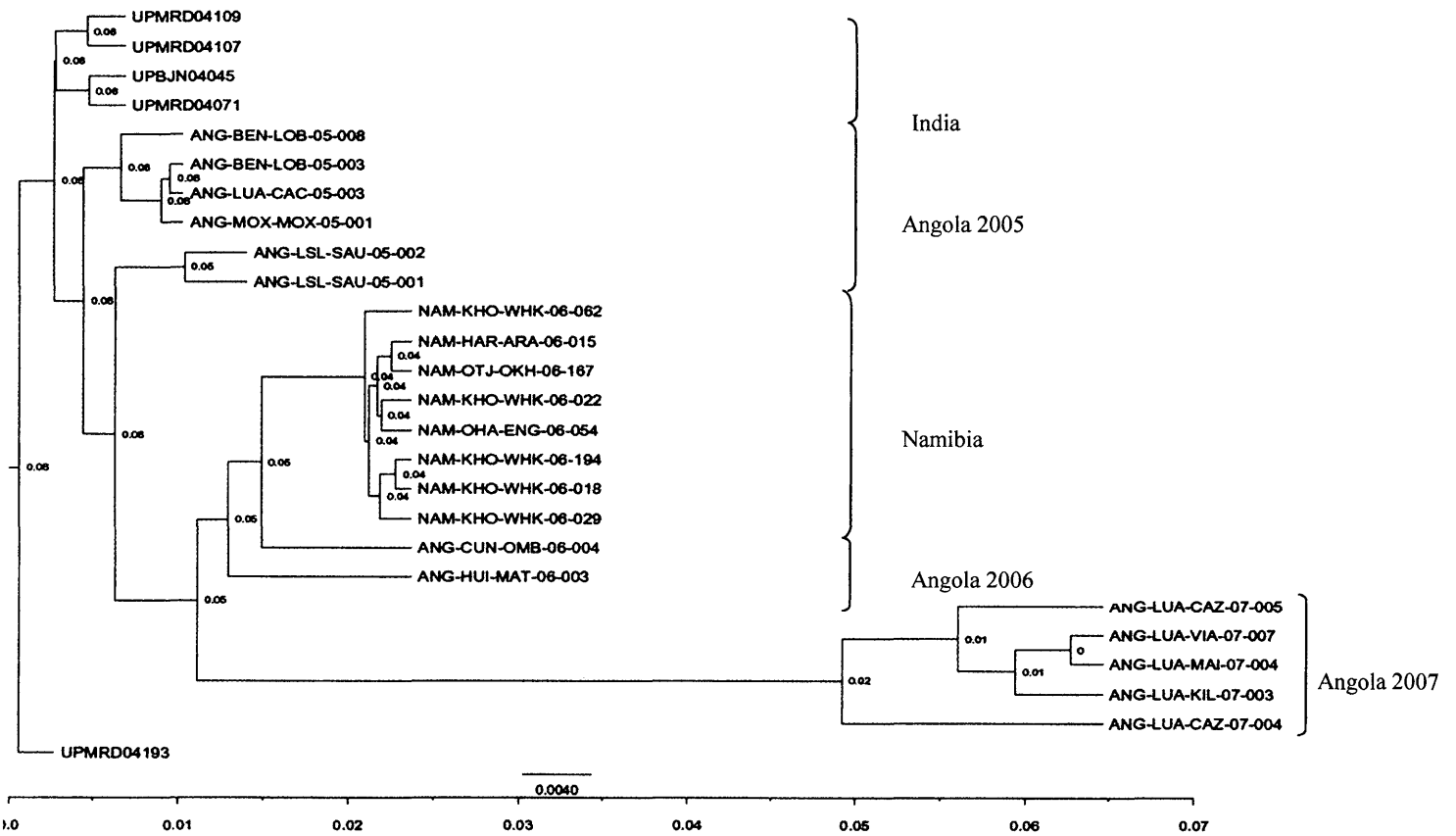


Figure 2.2: A Bayesian tree of wild poliovirus VP1 gene rooted at India strain demonstrating the evolution that has occurred since introduction of India genotype into Africa at a set rate for poliovirus of 1% per year.

2.3.4 Calculation of circulation time of poliovirus strains in Africa

The nucleotide pairwise distance (P-distances) of the sequenced VP1 region within India strain ranged from 0% to 1%, Angola strains from 0% to 2% and within the Namibia strains ranged from 0% to 1%. The calculated P-distance between the closest branched

strain isolated in Angola in 2005 and the Namibian strains was 2%. The rate of nucleotide sequence evolution in poliovirus is approximately 1% per year (Alexander, Gary et al. 1997; Jorba, Campagnoli et al. 2008). This suggests that the virus that caused the outbreak in Namibia had been circulating in Angola for at least a year undetected. The calculated P-distance between the Namibian, Angola and Indian strains are listed in Table 2.2.

Table 2. 2: The nucleotide pairwise distance of the SOAS (Indian) genotype in Africa. The P-distance range within each country is bold and the differences between each country are below.

COUNTRY	INDIA	NAMIBIA	ANGOLA
INDIA	**(0-1%)**		
NAMIBIA	(2-3%)	**(0-1%)**	
ANGOLA	(0-6%)	(2-7%)	**(0-7%)**

2.3.5 Amino acid substitutions in the VP1 region

Comparisons of the VP1 amino acids sequences relative to Sabin 1 are shown in the alignment in Figure 2.3. Most substitutions resulted in silent mutations. Both genotypes (WEAF-B and SOAS) have 10 identical substitutions relative to Sabin 1: An alanine at

position 21 of Sabin is replaced by a serine, indicated as A21S; as well as a A32S; V67I; A88T; I90M; K99T; N100S; T106S; V138I and T292A.

The first outbreak in Angola in 2005 was caused by a strain that had an identical amino acid sequence to a strain from India, suggesting the origin to be by importation from India. Angola 2006 strains maintained the same identity with the exception of a change at position 168 (E168G). The Angola strains for 2007 have a unique substitution at position 77 relative to Sabin 1 (I77V) which was not present in the other groups.

Namibia strains of 2006 had additional changes which were not observed in previous SOAS strains. Changes in positions 17 and 215 (T17A and V215I) indicates further genetic drift from the original virus from India which were not observed in the Angola strains. One strain from Namibia contained an additional substitution of A222V.

Genotype WEAF-B displayed its own distinctive changes relative to the Namibia isolates at position 215 (V215I). All SOAS strains share the same six substitutions relative to Sabin 1 that is not present in some of WEAF-B strains.

STRAINS		VARIABLE POSITIONS																	
		17		21	32	67	77	88	90	99	100	106	110	138	168	169	215	222	223	292
	Sabin1(VP1)	T	G	A	A	V	I	A	I	K	N	T	I		E	K	V	A		T
India	UPBJN04045																			
	UPMRD04071			S	S	I		T	M	T	S	S		I					E	A
	UPMRD04107			S	S	I		T	M	T	S	S		I					E	A
	UPMRD04109			S	S	I		T	M	T	S	S		I					E	A
	UPMRD04193			S	S	I		T	M	M	S	S		I					E	A
Angola	ANG-LSL-SAU-05-001			S	S	I		T	M	T	S	S		I					E	A
	ANG-BEN-LOB-05-008			S	S	I		T	M	T	S	S		I					E	A
	ANG-LUA-CAC-05-003			S	S	I		T	M	T	S	S		I	G				E	A
	ANG-LSL-SAU-05-002			S	S	I		T	M	T	S	S		I					E	A
	ANG-MOX-MOX-05-001			S	S	I		T	M	T	S	S		I					E	A
	ANG-BEN-LOB-05-003			S	S	I		T	M	T	S	S		I					E	A
	ANG-CUN-OMB-06-004			S	P	I		T	M	T	S	S		I	G				E	A
	ANG-HUI-MAT-06-003			S	S	I		T	M	T	S	S		I	G				E	A
	ANG-LUA-CAZ-07-005			S	S	I	V	T	M	T	S	S		I					E	A
	ANG-LUA-VIA-07-007			S	S	I	V	T	M	T	S	S		I					E	A
	ANG-LUA-CAZ-07-004			S	S	I	V	T	M	T	S	S		I					E	A
	ANG-LUA-KIL-07-003			S	S	I	V	T	M	T	S	S		I					E	A
	ANG-LUA-MAI-07-004			S	S	I	V	T	M	T	S	S		I					E	A
Namibia	NAM-HAR-ARA-06-015	A		S	S	I			M	T	S	S		I	G		I		E	A
	NAM-KHO-WHK-06-018	A		S	S	I		T	M	T	S	S		I	G		I		E	A
	NAM-KHO-WHK-06-022	A		S	S	I		T	M	T	S	S		I	G		I		E	A
	NAM-KHO-WHK-06-029	A		S	S	I		T	M	T	S	S		I	G		I		E	A
	NAM-KHO-WHK-06-062	A		S	S	I		T	M	T	S	S		I	G		I		E	A
	NAM-KHO-WHK-06-194	A		S	S	I		T	M	T	S	S		I	G		I	V	E	A
	NAM-OHA-ENG-06-054	A		S	S	I		T	M	T	S	S		I	G		I		E	A
	NAM-OTJ-OKH-06-167	A		S	S	I		T	M	T	S	S		I	G		I		E	A
WEAF-B	ANG-LUA-ING-00-003			S	S	I		T	M	T	S	S		I			I			A
	ANG-LUA-KIL-00-007			S	S	I		T	M	T	S	S		I			I			A
	ANG-LSL-SAU-01-002			S	S	I		T	M	T	S	S		I	G	E	I			A

Figure 2. 3: Amino acids comparison of VP1 region of the genome for wild-type polioviruses from Africa. Only variable positions are shown. The countries are abbreviated as follows: ANG, Angola; UP, Uttar Pradesh, province of India and NAM, Namibia. The previous African genotype WEAF-B is shown at the bottom for Angola.

2.4 DISCUSSION

In this study the complete VP1 gene of wild-type polio strains causing AFP during outbreaks in Angola and Namibia was sequenced to investigate the molecular epidemiology of these strains in Africa. In the past, the VP1/2A junction of 150 nt was used to study the epidemiology and circulation of wild-type polioviruses worldwide (Zheng, Zhang et al. 1993); (Huovilainen, Mulders et al. 1995);(Lipskaya, Chervonskaya et al. 1995);(Mulders, Lipskaya et al. 1995). The complete VP1 region has since been chosen for molecular epidemiological investigations as it exhibits the greatest sequence variability, and plays a key role in receptor attachment (Wimmer, Hellen et al. 1993).

The first AFP cases associated with wild-type polio virus were identified in April 2005 in Angola along the coast of the Luanda province and had a 99.34% identify to isolates from Uttar Pradesh, in northern India. Isolates from Angola and Namibia grouped with isolates from India, (Figure 2.1). The 2005 index strain (ANG-LUA-CAC-05-003) from Angola was most closely related to the Indian genotype with a P-distance (P) of 0.01. The Namibia strains were closest to India strain by 0.02 – 0.03 P distance range. The Bayesian tree (Figure 2.2) which assumes a molecular clock demonstrates the drift that had occurred from the introduction of the Indian strains in Angola in 2005 to 2007 suggests that the Angola 2006 strain was most probable the source for the 2006 outbreak in Namibia. All 2006 strains from Angola grouped with isolates from the border of Namibia that separate the two countries suggesting the possible route of introduction

into Namibia. The similarity in nucleotide sequences of the Namibia strains suggests rapid epidemic transmission amongst inadequately immunized individuals.

P-distances and substitutions over time of the Angola strains relative to those of India suggest a time of circulation of 2 to 3 years. This is consistent with the recent undetected circulation and surveillance gaps in Angola in 2006 and 2007. Surveillance of AFP has been a key factor in efforts to achieve eradication of poliomyelitis worldwide. The outbreaks in Angola and Namibia demonstrated that low levels of vaccines coverage, in combination with poor surveillance of AFP, could result in undetected and prolonged circulation of poliovirus in a community before cases of poliomyelitis are evident (Kew, Morris-Glasgow et al. 2002). Low vaccine coverage may have allowed the transmission of the imported strain amongst pockets of unvaccinated individuals.

Displacement of an endemic circulating genotype by an imported genotype has also been reported in South America (Rico-Hesse, Pallansch et al. 1987). In Africa, the WEAF-B genotype which was last detected in September 2001 in Angola was replaced by the imported SOAS genotype in 2005. This is the first time that AFP cases associated with the Indian genotype were identified in Africa. The 2005 importation in Angola lasted until 2007 and led to outbreaks in Namibia, the DRC, Central African Republic and Burundi (CDC. 2006e). Re-established transmission in Angola has continued into 2011. This emphasises the vulnerability of regions with suboptimal vaccination coverage for importation and reintroduction of wild-type polio virus from the remaining endemic countries. Namibia has been free of polio more than 11 years before

the 2006 Indian genotype outbreak. Until the establishment of the Namibian Expanded Program on Immunization in 1990, Namibia public health services had been disrupted by conflict during 1966 to 1989 (CDC. 2006d) . Since 1990, vaccine increased from 37% in 1989 to 76% in 2000. During 2006 outbreak, all confirmed WPV1 cases occurred in persons aged >14 who either had not been vaccinated for polio or had incomplete polio vaccine schedule (CDC. 2006d) . In response to the outbreak, the Namibia Ministry of Health and Social Services (MoHSS) activated the National Health Emergency Committee to coordinate activities and several SIAs were conducted (CDC. 2006d) .

The Indian genotype has been associated with several outbreaks and has circulated for years in India. This genotype has also been associated with cases in Bangladesh (CDC. 2002c). The urgency of reaching the goal of global polio eradication had been underscored by the importation of wild polioviruses into these countries (Angola and Namibia). This can only be achieved by the elimination of remaining reservoirs of wild polioviruses endemicity in South Asia and Sub-Saharan Africa (CDC. 2002c; CDC. 2002d; CID. 2002).

CHAPTER 3

Identification and molecular characterisation of an Indian genotype of poliovirus type 1 isolated during outbreaks of poliomyelitis in the Democratic Republic of Congo (DRC) from 2006-2008.

3.1 INTRODUCTION

Poliomyelitis is caused by one of the wild type poliovirus serotypes 1, 2 and 3 and the great progress has been made by the Global Polio Eradication Initiative (GPEI) in interrupting these strains worldwide (CDC. 2006b by using three doses of live oral polio vaccine (OPV) in children under one year. This approach has stopped transmission of indigenous wild poliovirus (WPV) in all but 4 countries, Nigeria, India, Pakistan and Afghanistan (CDC. 2009a). The last case of WPV type 2 was detected in India in 1999 (CDC. 2001b). Unfortunately due to low vaccination coverage, cases of poliomyelitis continue to be reported in other regions as results of importations from endemic countries (van Niekerk, Vries et al. 1994) or emerging of vaccine derived polioviruses (VDPVs). OPV has been associated with adverse events as a results it causes vaccine associated paralytic polio (VAPP).

Laboratory surveillance has played an important role to provide to provide rapid information about the circulating WPV. Molecular tools have been developed and

implemented to help identify the serotype of the poliovirus and further perform molecular characterisation of wild strains. These tools have help to identify the presence of wild poliovirus infections and characteristic patterns of their distribution and spread (Chezzi, Blackburn et al. 1997). As described by Jorba et al., that the rate of poliovirus substitution is estimated at approximately 1% per year (Jorba, Campagnoli et al. 2008), this has given rise to genotype associated with specific regions(Chezzi, Blackburn et al. 1997). Molecular analysis has help to answer questions associated with location of endemic virus reservoirs, patterns of virus transmission or source of imported poliovirus.

Over 8500 AFP cases of both poliovirus type 1 and 3 were reported in Africa from January 2000 until 2008. Although most cases were concentrated in Nigeria, importation has resulted in outbreaks in a number of neighbouring and distant countries in West, East and Central Africa (CDC. 2002d).

In 2006, thirteen wild poliovirus1 cases and 3 contacts were reported in DRC. In 2007 this number increased to 41 before dropping back to 5 cases in 2008. The routine vaccine coverage in the DRC was estimated at 50% in 2008 (CDC. 2000a). This was even lower in regions that had reported wild poliovirus outbreaks.

We report the genetic characterization of the wild poliovirus 1 cases isolated during the period 2006-2008 in the DRC. The outbreak in the DRC originated from Angola, as suggested by genetic relatedness to wild poliovirus that circulated in Angola beginning in 2005. The Angola outbreak was due to imported virus of Indian genotype (SOAS)

(CDC.2006a; CDC. 2006d). This is the first time that AFP cases associated with the Indian genotype were identified in the DRC. This finding emphasises the vulnerability of regions with suboptimal vaccination coverage to the reintroduction of wild poliovirus from the remaining endemic countries.

3.2 MATERIAL AND METHODS

3.2.1 Viruses

Isolates from original stool specimens of suspected poliovirus AFP cases were submitted to the National Institute for Communicable Diseases (NICD) in South Africa for diagnostic reverse transcription polymerase chain reaction (RT-PCR), ELISA and partial genomic sequencing as described in Chapter 2. Virological investigation identified polioviruses of type 1.

3.2.2 Laboratory Diagnosis

Refer to Chapter 2

3.2.2.1 *Diagnostic RT-PCR*

Refer to Chapter 2

3.2.2.2 *ELISA*

Refer to Chapter 2

3.2.2.3 *RNA Extraction*

Refer to Chapter 2

3.2.2.4 *RT-PCR for sequence analysis*

Refer to Chapter 2

3.2.2.5 *Sequence Analysis*

Refer to Chapter 2

3.2.2.6 Phylogenetic Analysis

Phylogenetic analysis was carried out on the complete VP1 gene, which corresponds to 906 nt for all isolates from patients with AFP. Sequences were aligned with Clustal X (Thompson, Gibson et al. 1997) and analyzed using the software package Geneious [http://www.geneious.com]. Phylogenetic trees were inferred using Neighbour-Joining (Tamura, Dudley et al. 2007) , Maximum likelihood (Guindon, Lethiec et al. 2005) and Bayesian (Drummond and Rambaut 2007) methods as implemented in Geneious. Genetic distances were estimated under models of evolution correcting for multiple substitutions at a site and for unequal transition and transversion rates. Statistical support at each node of the inferred trees was assessed using common procedures for each method. Estimation of the evolutionary rate of VP1 sequences was calculated using Bayesian Markov chain Monte Carlo (MCMC) as implemented in BEAST v1.6 (Drummond and Rambaut 2007). Onset dates were used for estimating the evolutionary rate under the strict clock model (Drummond and Rambaut 2007). Briefly, two independent chains consisting of 40,000,000 steps each were run under the GTR+gamma model of evolution and the Coalescent Bayesian Skyline model as a tree prior. The GTR + G substitution model was used to allow different sites in the alignment to evolve at different rates and the substitution rate calculated from the data assuming a molecular clock. The Coalescent Bayesian Skyline model was used to estimate the posterior distribution of the population size through time. Sample correlation was assessed checking the Effective Size Samples (ESS) statistic. A maximum credibility tree was inferred by BEAST in which branch lengths were scaled to time according to

the estimated mean substitution rate. The annotated tree file was visualised with Figtree version 1.3.1 (http://tree.bio.ed.ac. uk/software/figtree).(Drummond and Rambaut 2007).

3.3 RESULTS

3.3.1 Outbreak description:

In the Democratic Republic of the Congo the first case of wild poliovirus was confirmed in Boma district in Bas Congo (BCG) province, at the border with Angola in February 2006. This case had a date of onset of paralysis on the 27th of February 2006 and was followed by another with an onset on the 24th of April 2006, from Sekebanza district in the same province of DRC. By the end of 2006, a total of 13 cases and 3 contacts had been reported in DRC from nine districts spread in four provinces. Most of these cases were from Kasai Occidental (KOC) province. Following the index case in the Boma district, no further circulation was reported from this district.

In 2007 additional 41 wild poliovirus cases plus 5 contacts were reported. Équateur (EQT) province reported more than 50% of these cases, whereas Orientale (ORT) province had 13 wild poliovirus cases, followed by Bandundu (BDD) province with 5 cases. Orientale province was not affected in 2006, but Bandundu province's outbreak continued until March 2007. In total, 17 districts were affected. The first wild poliovirus case in 2007 had an onset of paralysis on the 1st of January 2007 whereas the last case confirmed the paralysis on the 20th of November 2007. The spread of the wild poliovirus in DRC followed the traditional transport route of the river Congo. All poliovirus cases reported in 2006 and 2007 were confirmed as wild poliovirus 1.

In 2008, only 5 wild poliovirus PV1 cases were reported, affecting 4 provinces and 5 districts. In total, 58 AFP cases of wild poliovirus 1 were reported in the DRC during period of 2006 to 2008.

3.3.2 Prevalence and properties of the India strain in Africa:

Viral isolates obtained from DRC cases from 2006, 2007 and 2008 were tested by RT-PCR using pan-enterovirus, pan poliovirus, serotype specific, and Sabin type 1, 2 and 3 virus specific primers. All isolates were identified as wild poliovirus1. The RT-PCR results were also confirmed by ELISA. These isolates were characterised by sequencing of 906 bases comprising the complete VP1 gene.

3.3.3 Phylogenetic analysis:

Since all previous poliomyelitis cases in the DRC were associated with the African genotype WEAF-A, the genetic variability of all isolates identified in the outbreaks in the DRC was investigated by complete VP1 gene sequence analysis and included in the phylogenetic trees.

Blast search analysis of all polioviruses isolated in the current investigation identified them as SOAS (Indian) genotype. Comparison to reference strains of all wild poliovirus 1 genotypes confirmed the classification of the DRC strains to the SOAS-A genotype. Indian strains that were demonstrated to be the closest to the described outbreaks were included in the analysis. All polioviruses investigated fell within the SOAS-A genotype within the B2D1B genetic cluster (Table 3.1).

Table 3.1: Laboratory data of wild poliovirus strains detected in acute flaccid paralysis (AFP) from the DRC. Epid Number indicates Epidemiological number (EPID Number).

EPID Number	Onset date	Percentage	Best Match	Accession Numbers
RDC-SKV-MTM-08-001	2008.217	98.68	RDC-ORT-KIS-07-100	GU951803
RDC-SKV-KLH-08-003	2008.161	99.45	RDC-EQT-BOE-07-006	GU951802
RDC-NKV-RUT-08-002	2008.282	99.12	RDC-EQT-BOE-07-006	-
RDC-ORT-LUB-08-004	2008.45	99.45	RDC-EQT-BOE-07-006	GU951800
RDC-ORT-BAN-07-019	2007.324	99.23	RDC-ORT-KIS-07-094	GU951799
RDC-ORT-BAS-07-027	2007.286	99.23	RDC-EQT-BOE-07-006	GU951798
RDC-ORT-YAK-07-008	2007.287	99.34	RDC-ORT-BAS-07-017	-
RDC-ORT-KIS-07-100	2007.278	99.89	RDC-ORT-KIS-07-094	-
RDC-ORT-KIS-07-098	2007.273	99.56	RDC-ORT-KIS-07-094	-
RDC-ORT-BAS-07-023	2007.269	99.34	RDC-ORT-YAH-07-003	-
RDC-ORT-BAS-07-020	2007.242	99.45	RDC-EQT-BOE-07-001	-
RDC-ORT-BAS-07-019	2007.261	99.45	RDC-EQT-BOE-07-001	-
RDC-ORT-KIS-07-094	2007.267	99.12	RDC-EQT-BOE-07-001	-
RDC-ORT-BAS-07-017	2007.252	99.78	RDC-ORT-YAH-07-003	GU951797
RDC-ORT-BAS-07-016	2007.237	99.45	RDC-ORT-BAS-07-016	GU951796
RDC-ORT-YAH-07-003	2007.219	99.22	RDC-ORT-YAH-07-003	GU951795
RDC-EQT-BIK-07-013	2007.200	98.90	RDC-EQT-MBD-06-013	GU951794
RDC-EQT-BIN-07-009	2007.233	99.44	RDC-EQT-BOM-07-004	GU951793
RDC-BDD-BOL-07-006	2007.173	99.22	RDC-EQT-BOE-07-001	-
RDC-EQT-PIM-07-003	2007.143	99.67	RDC-EQT-BOE-07-001	-
RDC-EQT-PIM-07-002	2007.178	99.12	RDC-EQT-MBD-07-001	-
RDC-EQT-LIS-07-029	2007.157	98.90	RDC-EQT-BOE-07-001	-
RDC-EQT-LIS-07-030	2007.157	99.23	RDC-EQT-BOE-07-001	-
RDC-EQT-BOE-07-006	2007.138	99.78	RDC-EQT-BOE-07-001	-
RDC-EQT-BIK-07-009	2007.144	99.23	RDC-EQT-BOE-07-001	GU951792
RDC-EQT-ING-07-007	2007.102	98.90	RDC-BDD-INO-06-013	GU951791
RDC-EQT-MBD-07-018	2007.126	99.45	RDC-EQT-MBD-07-012	-
RDC-BDD-KIR-07-004	2007.67	99.12	RDC-BDD-INO-06-013	GU951790
RDC-BDD-KIR-07-003	2007.64	99.12	RDC-BDD-INO-07-002	GU951789
RDC-EQT-MBD-07-012	2007.53	99.45	RDC-EQT-BOE-07-001	-
RDC-EQT-BLB-07-002	2007.62	99.34	RDC-EQT-BOE-07-001	-
RDC-EQT-BLB-07-001	2007.44	99.45	RDC-EQT-MBD-07-001	-
RDC-BDD-KIR-07-001	2007.44	99.00	RDC-BDD-INO-06-013	-
RDC-BDD-INO-07-002	2007.10	99.89	RDC-BDD-INO-06-013	GU951788
RDC-KOC-LUE-06-004	2006.225	99.23	RDC-KOC-TKP-06-004	GU951785
RDC-BCG-MAT-06-001	2006.170	99.01	RDC-BCG-SEK-06-004	GU951784
RDC-KOC-TKP-06-006	2006.187	99.01	ANG-LSL-SAU-05-002	GU951783
RDC-KOC-KAO-06-005	2006.161	98.45	ANG-BEN-LOB-05-003	GU951782
RDC-EQT-LUK-07-005	2007.173	99.22	RDC-EQT-BOE-07-001	-
RDC-KOC-KAM-06-005	2006.140	98.34	ANG-LSL-SAU-05-002	GU951781
RDC-KOC-TKP-06-004	2006.125	98.34	ANG05-2006706767	GU951780
RDC-BCG-SEK-06-004	2006.114	99.56	RDC-BCG-BOM-06-001	GU951779
RDC-BCG-BOM-06-001	2006.58	98.68	ANG-BEN-LOB-05-003	GU951778
ANG-BEN-LOB-05-003	2005.139	99.45	ANG-LUA-CAC-05-003	EU046204

Phylogenetic relationships among 58 DRC sequences and 3 ANG related sequences are summarized in a Bayesian MCMC tree using complete VP1 (906 nt) sequences (Figure 3.1). The geographical and temporal clustering of the sequences in the tree suggests local circulation of multiple DRC lineages after importation of ANG strains. The topology of the tree is consistent with at least three distinct geographic lineages (A, B, and C). Lineage A groups sequences mainly from BCG and BDD provinces and it contains the first case dated in early 2006. The majority of cases belonged to lineage B comprising viruses found in EQT and ORT provinces. Two clearly defined groups defined lineage B; one group with sequences from EQT and the other group with cases mainly from EQT and ORT provinces. Viruses circulating exclusively in the KOC province during 2006 grouped in lineage C.

Figure 3.1 shows the Bayesian tree with an estimated root splitting the three lineages, including the related Angola strains, demonstrating the evolution that has occurred since the introduction of this genotype into Africa. Branch lengths from the tree were estimated under the strict clock model and displayed in time units (Figure 3.1). The inferred evolutionary rate was 1.11×10^{-2} substitutions per site per year (s/s/y) with estimated 95% highest posterior densities (95% HPD) ranging from 0.89×10^{-2} to 1.37×10^{-2} s/s/y. The estimated rate for the DRC lineages is consistent with rates estimated for other wild poliovirus genotypes and vaccine-derived poliovirus outbreaks (Jorba, Campagnoli et al. 2008). The estimated age of the most common recent ancestor to DRC and related ANG sequences was 3.54 years (95% HPD ranging from 3.32 to 3.70 years), approximately one year before the first DRC case and approximately 3 months before the first related ANG sequence. The topology of the tree is consistent with three main

lineages that spread widely over DRC. However, local multiple chains of transmission are inferred from each lineage. Long branches and/or low branch support (posterior values) characterize the three lineages. Date estimates inferred in internal nodes showed wide confidence intervals (not shown) because of gaps in phylogenetic signal indicative of gaps in surveillance.

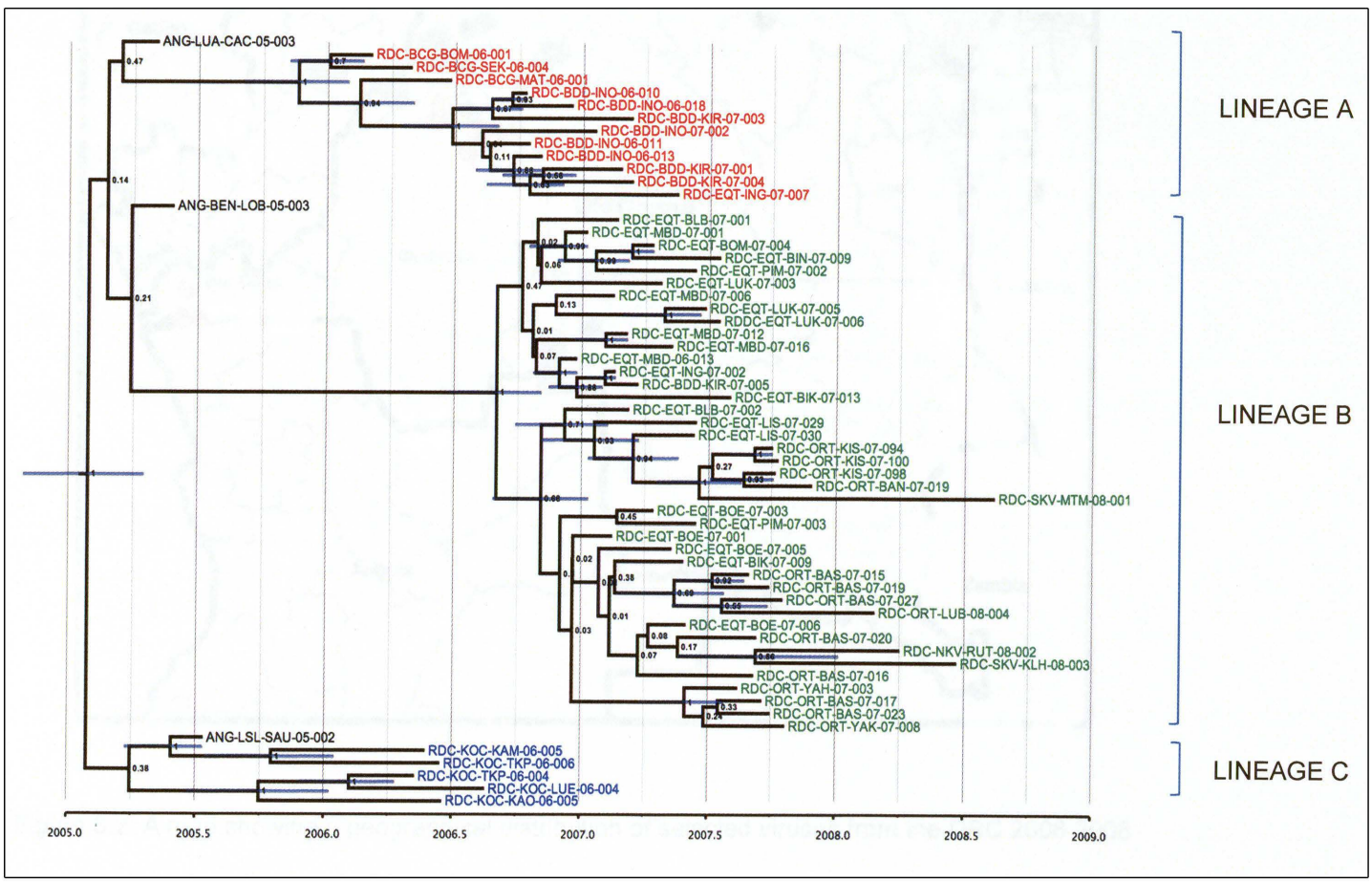


Figure 3.1: A Bayesian tree with an estimated root demonstrating the evolution over time. The scale indicate the time of first isolation (2005) to the last (2008) at a rate of 1% per year as demonstrated for poliovirus.

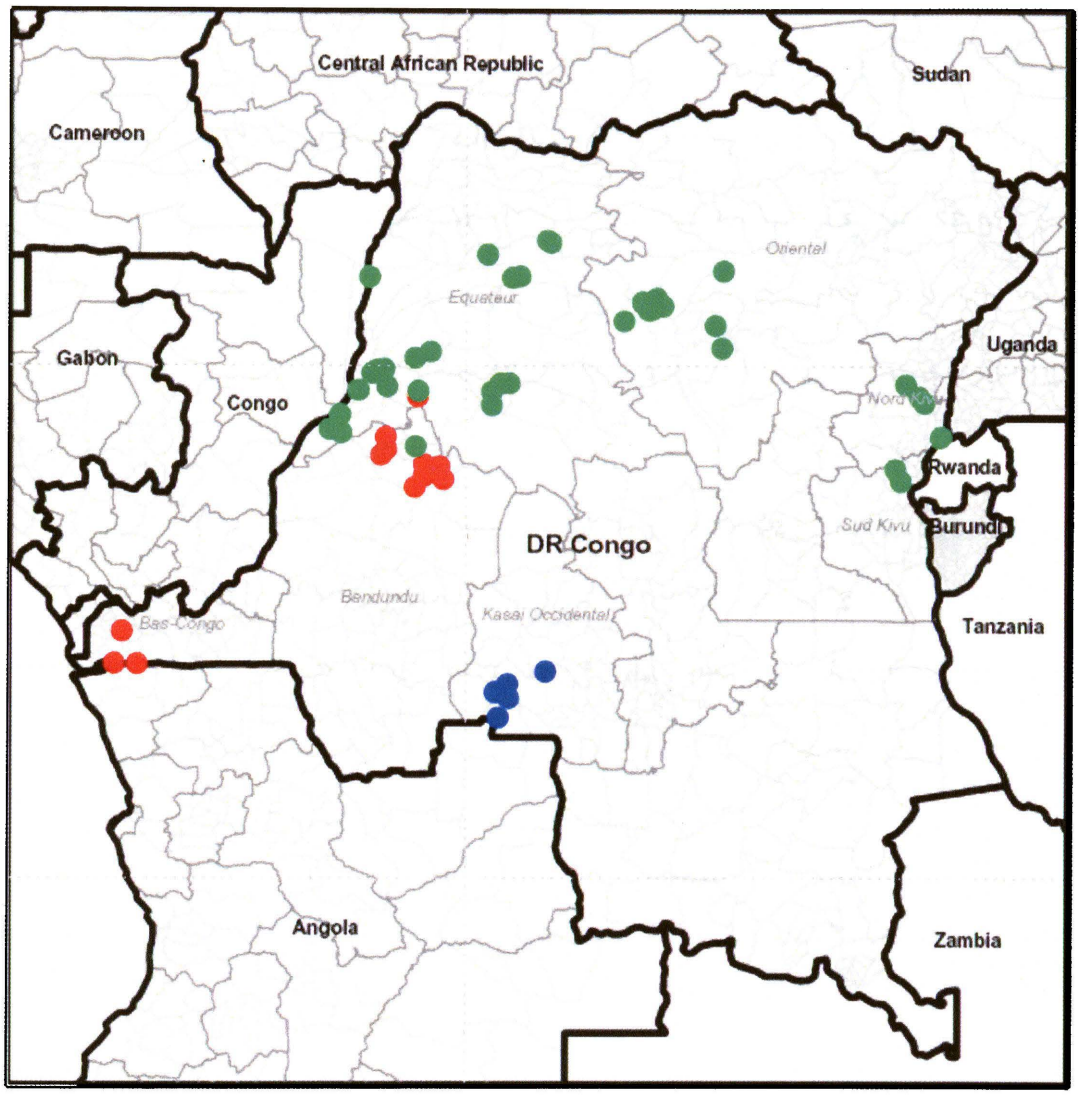


Figure 3.2: A map showing a geographical distribution of selected viruses from the DRC 2006-2008 outbreak. Red dots, lineage A, Green dots, lineage B and Blue dots lineage C.

Mapping and visualization of genetic lineages (Figure 3.2) was consistent with phylogenetic results. Separate importations from ANG into southern DRC (KOC and

BCG provinces) were detected during 2006, at least half a year after their closest ANG sequence. While cases in the KOC province circulated within the province (lineage C), lineage A spread north and east into the BDD and EQT provinces during more than 6 months. Interestingly, lineage C had higher nucleotide diversity (0.023±0.004 substitutions per site, n=5) than lineage A (0.011±0.002 substitutions per site, n=12) and lineage B (0.013±0.001 substitutions per site, n=41). The earliest isolates in lineage B clustered in the EQT province. Further inference about timing and source of importation events in lineage B were undetermined due to lack of sequence record during at least one year. Lineage B spread north and east covering an extended geographical range reaching in 2008 communities bordering with Uganda, Rwanda, and Burundi. In addition, local circulation across the interprovincial borders of EQT and BDD were inferred and visualized in both the tree and map.

3.4 DISCUSSION

In this study the complete VP1 gene of wild PV1 polio strains causing poliomyelitis during outbreaks in the DRC was sequenced to investigate the molecular epidemiology. The complete VP1 region has since been chosen for molecular epidemiological investigations as it exhibits the greatest sequence variability, and plays a key role in receptor attachment. (Wimmer, Hellen et al. 1993).

The first case associated with wild poliovirus was identified in February 2006 in the Boma district of the DRC. The Bayesian tree (Figure 3.1) which assumes a molecular clock demonstrates the drift that had occurred from the introduction of the Angola strains in the DRC in 2006 and strongly suggests that Angolan strains were the source for the DRC outbreaks. Most isolates from DRC were from the border provinces between the DRC and Angola, namely Equateur, Bandundu and Kasai Occidental. The DRC viruses from these border regions form separate virus lineages to those viruses circulating in Angola.

Wild poliovirus type 1 of SOAS genotype was first identified in the DRC in 2006. Three to four possible independent poliovirus lineages were identified, and they could be grouped according to their geographical location in the country. Viruses from Bas Congo and Bandundu formed lineage A, which were detected from 2006 to 2007. Kasai Occidental (KOC) viruses formed lineage B, which circulated in 2006 and was not detected thereafter. The viruses from the Equateur (EQT) province and viruses

from ORT formed lineage C. These results demonstrate widespread circulation and sustained transmission of individual strains along separate pathways.

Surveillance of AFP has been a key factor in efforts to achieve global eradication of poliomyelitis worldwide. The outbreaks in the DRC demonstrated that low levels of vaccine coverage, in combination with poor surveillance of AFP, could result in undetected and prolonged circulation of poliovirus in a community before cases of poliomyelitis are evident (Kew, Morris-Glasgow et al. 2002).

In South America an endemic circulating genotype was displaced by an important genotype due to low vaccine coverage (Rico-Hesse, Pallansch et al. 1987). In Africa, the WEAF-A genotype which was last detected in September 2000 in DRC was followed by the imported SOAS genotype in 2006. The DRC had been free of polio for the 6 years prior to the 2006 outbreak. The Indian genotype has been circulating for years and same strain has been reported in Bangladesh, (CDC. 2002c) and additional importations were reported in Nepal and Myanmar (CDC.1999).

As mentioned previously in Chapter 2, importations had underscored the global polio eradication goal and this can only be achieved by the elimination of remaining reservoirs of wild poliovirus endemicity in South Asia and Sub-Saharan Africa (CID. 2002).

In conclusion, this study describes the distribution and molecular epidemiology of wild PV1 SOAS genotype in southern and central Africa. The SOAS strains identified in the DRC were unique to Africa and are estimated to have circulated about 1 year in the

DRC or Angola before being detected. The DRC outbreak was caused by the SOAS genotype, but was introduced from neighbouring Angola, most likely through frequent cross-border movement among population groups living on both sides of the border. On the African continent, so far the SOAS genotype appears to be limited to certain countries in Southern and Central regions although virus circulation is still ongoing.

CHAPTER 4

Characterisation of type 2 and type 3 circulating vaccine derived polioviruses isolated during an outbreak of Poliomyelitis in Madagascar in 2005

4.1 INTRODUCTION

Great progress has been made in the World Health Organization's (WHO's) program for the eradication of poliomyelitis with extensive use of the trivalent oral polio vaccine (OPV), only four countries still remaining endemic for wild poliovirus namely, Nigeria, India, Afghanistan and Pakistan (CDC. 2006b). However, during replication in humans, OPV strains frequently undergo mutations which may cause vaccine-associated paralytic poliomyelitis (VAPP) (Zimmerman and Spann 1999) and recombination which contributes to the variability of polioviruses (Cuervo, Guillot et al. 2001; Dahourou, Guillot et al. 2002; Liu, Zheng et al. 2003). These two genetic characteristics seem the underlying mechanism for the occurrence of poliomyelitis outbreaks associated with circulating vaccine-derived poliovirus (cVDPV) (Kew, Wright et al. 2004; Kew, Sutter et al. 2005).

Between 2000 and 2005, six cVDPV outbreaks have been reported in Haiti/Dominican republics, the Philippines, Madagascar, China, Cambodia and Indonesia (Kew, Morris-Glasgow et al. 2002; Rousset, Rakoto-Andrianarivelo et al. 2003; Shimizu, Thorley et al.

2004). From 1988 to 1993, evidence of VDPV circulation was retrospectively identified in Egypt (Yang, Naguib et al. 2003). In all these outbreaks, the affected patients were partially or not vaccinated against polioviruses, and except for Cambodia, the implicated viruses have been recombinants with *Human Enterovirus C* species (HEV-C). In addition, indigenous wild polioviruses have been eliminated in countries or regions where those outbreaks occurred. These factors – low population immunity and HEV-C circulation - appear thus to be associated in most cases with the emergence of vaccine-derived strains (Kew, Wright et al. 2004; Kew, Sutter et al. 2005).

In Madagascar, where the last wild type poliovirus reported in October, 1997 (World, Health et al.) a type 2 cVDPV outbreak occurred in the southern province of Toliara in December, 2001 and March-April, 2002 (Rousset, Rakoto-Andrianarivelo et al. 2003). To interrupt VDPV circulation, the Ministry of Health of Madagascar conducted nationwide mass vaccination campaign in fall 2002. However, three years later, a second outbreak re-emerged in the same province from April to August, 2005.

Here, we report the genetic characterization of these type 2 and type 3 vaccine-derived polioviruses isolated during the outbreak of poliomyelitis in Madagascar in 2005. This was the first time that an AFP case associated with cVDPV type 3 was identified in Madagascar. In addition four cases of type 2 cVDPV associated AFP were reported that were significantly different from the strains identified in 2001.

4.2 MATERIALS AND METHODS

4.2.1 Viruses

Virus isolates from original stool specimen of suspected poliovirus AFP cases were submitted to the National Institute for Communicable Diseases (NICD) in South Africa as explained in Chapter 2.

4.2.2 Epidemiological investigation

To identify the causative agent, stool specimens were collected from five patients who developed Acute Flaccid Paralysis (AFP) in 2005. Clinical signs were paralysis of one or two lower limbs associated with fever. Figure 4.1 shows the geographic distribution of the cases. Between April 21 and September 9, 2005, two stool specimens were collected at 24-48 hours interval for each case (n = 10), and eight additional specimens were obtained at approximately one month interval (total, n = 18).

Two hundred and eighteen stool specimens were collected among children living in the district of Toliara II on May 20, and Jul 28, in the district of Toliara I on May 24, and Aug 28, and in the district of Tsihombe on Jul 17, and 27. This investigation was conducted as part of the national AFP surveillance. The last data analysis on routine immunization and AFP surveillance from Toliara province was also reported (data not shown).

4.2.3 Virus isolation and intratypic differentiation

Extracts of stool specimens were treated with chloroform and cultured on RD and HEp-2 cell lines used for enterovirus isolation, and L20B mouse L-cells expressing the human poliovirus receptor used specifically for poliovirus isolation. Any specimen inducing cytopathic effect on RD or/and HEp-2 but not on L20B cell line was re-inoculated on L20B cells. All L20B poliovirus isolates were identified using microneutralization serotyping test. Isolates showing cytopathic effect only on HEp-2 or RD cell lines were considered to be non-polio enteroviruses (NPEV) and further analyzed only by molecular methods.

To distinguish if the poliovirus isolates were of vaccine or wild origin, ELISA and PCR (Yang, De et al. 1991) tests were performed targeting the VP1 coding region (van der Avoort, Hull et al. 1995). In addition, to detect mutant and recombinant poliovirus vaccine strains, we performed real-time assay(Kilpatrick, Ching et al. 2004; Kilpatrick, Yang et al. 2009) .

4.2.4 Reverse Transcription PCR for sequence analysis.

Reverse transcription PCR (RT-PCR) was performed as detailed in Chapter 2.

4.2.5 Sequence Analysis.

Prior to sequencing, the RT-PCR products were purified as detailed in Chapter 3.

To analyze and compare the genetic structures and the genetic relationships among isolates, we sequenced different regions of the viral genomes. We extracted viral RNA

from the supernatant of virus-infected cells with the QIAamp Viral Mini Kit (Qiagen, Chatsworth, CA, USA).

The Primers: Y7R (5'GGTTTTGTGTCAGCITGYAAYGA3');PV1A (TTIAIIGCRTGICCRTTRTT3'); PV2S (CITAITCIMGITTYGAYATG 3') and Q8 (5'AAGAGGTCTCTRTTCCACAT3') were used to generate a complete VP1-coding region region. Subsequently, poliovirus subgenomic regions were amplified by RT-PCR: the 5' nontranslated region (5' NTR), and the 3D-3'NC (Guillot, Caro et al. 2000). The sequences generated by our study were deposited in GenBank (accession numbers EF420856 – EF420871). The following primer sets were used to analyse 5'NTR and 3D regions.

Primer	Position*	Gene	Sequence (5' – 3')
EV2S	446	5' NTR	TCCGGCCCCTDAATGCGGCTAATCC
EV1A	559	5' NTR	ACACGGACACCCAAAGTAGTCGGTTCC
DK234S	5915	3D	ATGCAYGTIGGIGGIAAYGG
DK235	6570	3D	TAIAGRTTICCRAAIGCCAT

* Position in the genome of Sabin 1. Numbering according to (Kilpatrick, Nottay et al. 1996; Kilpatrick, Nottay et al. 1998)

4.2.6 Phylogenetic Analysis.

Phylogenetic analysis was carried out on the complete VP1 gene, which corresponds to 903bp for poliovirus type 2 and 900bp for poliovirus type 3. To determine VP1 nucleotide diversity we compared all complete VP1 sequences of isolates from patients with AFP, their contacts and the Sabin type 2 and type 3 OPV reference strains. Nucleotides

sequences were aligned with Clustal X (Thompson, Gibson et al. 1997). Neighbour-joining trees were constructed with MEGA version 4.0 (Tamura, Dudley et al. 2007). The alignments (nucleotide and translated amino acid sequences) were analysed with Genedoc version 2. 6001

(www. psc. edu/biomed/genedoc).

4.3 RESULTS

4.3.1 Prevalence and properties of circulating vaccine-derived polioviruses from AFP cases in Madagascar.

In total, 49 viral isolates obtained from Madagascar during May to September 2005 were tested by RT-PCR using pan-enterovirus, pan-poliovirus, serotype-specific, and Sabin type 1, 2 and 3 virus specific primers. Five isolates were positive for poliovirus type 1, 22 for poliovirus type 2 and 22 for poliovirus type 3. The RT-PCR test was confirmed by ELISA and both techniques identified the isolates as Sabin-like poliovirus. Because the last poliomyelitis cases in Madagascar were associated with a derivative of the type 2 Sabin strain (Rousset, Rakoto-Andrianarivelo et al. 2003) the genetic variability of all isolates was further investigated by nucleotide sequence analysis of the VP1 region. Partial sequencing and P-distance analysis confirmed that 17 isolates were VDPV's with > 1% divergence from the parental Sabin strain (Table 4.1) i.e., cVDPV and 9 isolates were Sabin–like with <1% divergence (data not shown). In total, 9 VDPV type 2 were identified consisting of four AFP cases and 5 contacts. Figure 4.1 is showing geographical distribution of the cases. The four AFP cases had 98.9% - 97.3% sequence identity in the VP1 gene to the Sabin reference strain and 99.9 – 96.6 sequence identity to each other. The VP1 sequences of the cVDPV type 3 consisting of one AFP case and 7 contacts had 99.0% - 98.2% sequence identity to Sabin reference strain and 99.7% – 98.1% to each other.

Table 4. 1. Laboratory data of selected circulating vaccine-derived polioviruses and Sabin-like strains detected in acute flaccid paralysis (AFP) and contacts samples from Madagascar.

Laboratory ID	EPID Number	Age (yrs)	Nucleotide difference relative to Vaccine Reference strain	Accession Number	Serotype
MAD050781	MAD-TOL-TOL-05-041	2	1. 44	EF420856	3
MAD050994	Contact 1	2	1. 22	EF420857	3
MAD050995	Contact 2	4	1. 56	EF420858	3
MAD050997	Contact 3	1. 8	1. 22	EF420859	3
MAD051342	Contact 4	4	1. 78	EF420860	3
MAD051344	Contact 5	3	1. 67	EF420861	3
MAD051345	Contact 6	0. 7	1. 33	EF420862	3
MAD051340	MAD-TOL-TSI-05-073	2	2. 33	EF420864	2
MAD051346	Contact 1	1. 5	2. 44	EF420865	2
MAD051347	Contact 2	3	2. 55	EF420866	2
MAD051348	Contact 3	2	2. 66	EF420867	2
MAD052060	Contact 4	2	0. 66	EF420868	2
MAD051602	MAD-TOL-TOL-05-082	2	1. 11	EF420869	2
MAD051606	MAD-TOL-BEL-05-091	3	1. 11	EF420870	2
MAD052054	MAD-TOL-SAK-05-113	2	1. 33	EF420871	2

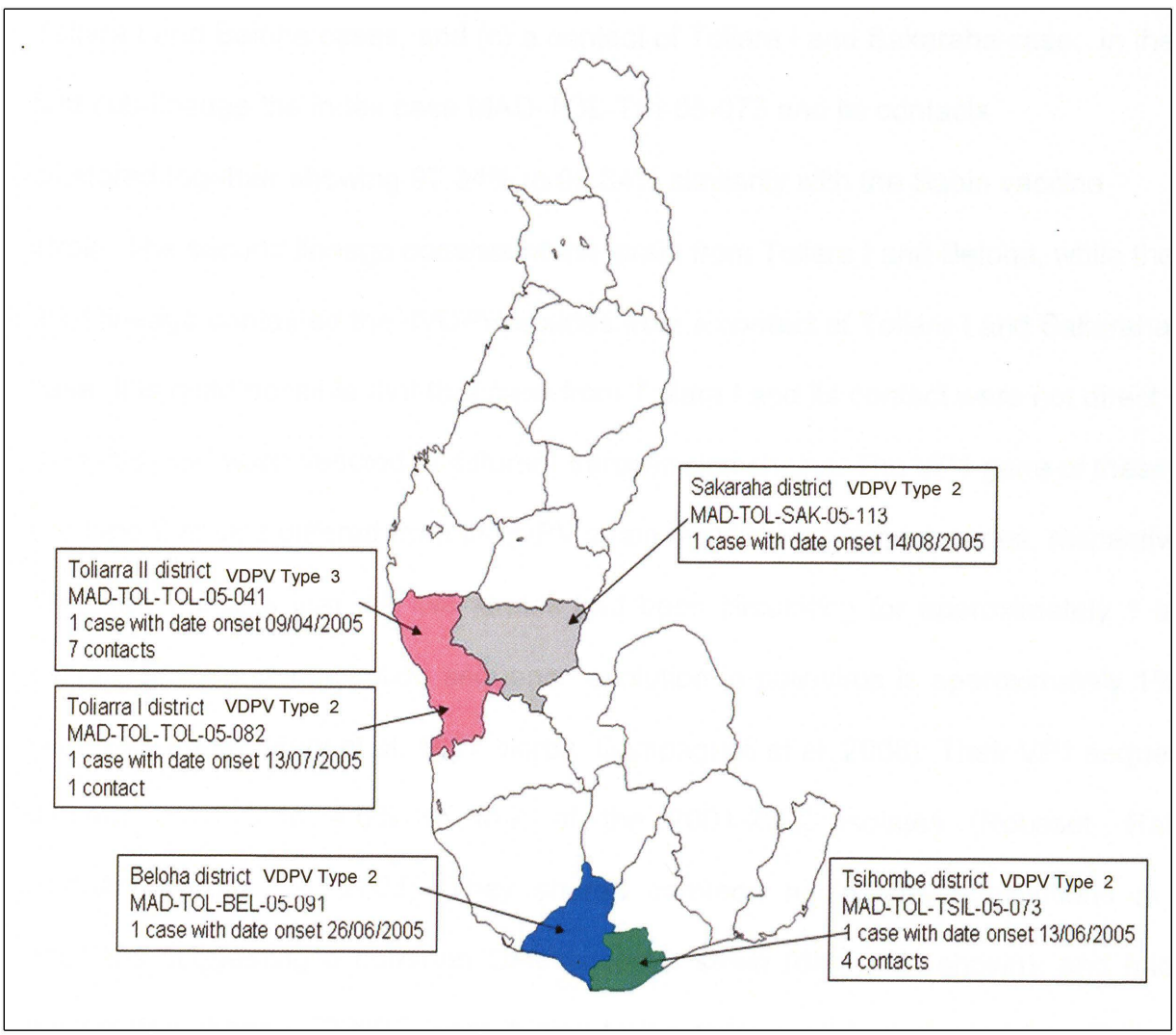


Figure 4.1 A Madagascar map showing geographical distribution of the cases.

4.3.2 Phylogenetic analysis of the different regions of the cVDPV

The poliovirus type 2 cVDPVs characterized in this study clustered into 3 distinct groups (Figure 4.2) and were compared to those isolated in 2001 in Toliara I and 2002 in Tolagnaro. The 2001 and 2002 isolates formed 2 independent lineages which are genetically distinct from the 2005 isolates. The 2005 type 2 cVDPV isolates formed three separated sub-lineages: (a) Tsihombe case and contacts; (b)

Toliara I and Beloha cases; and (c) a contact of Toliara I and Sakaraha case. In the first sub-lineage the index case MAD-TOL-TSI-05-073 and its contacts clustered together showing 97.34% to 99.34% similarity with the Sabin vaccine strain. The second lineage consists of the strain from Toliara I and Beloha, while the third lineage contained the cVDPV isolates from a contact of Toliara I and Sakaraha case. It is quite possible that that case from Toliara I and its contact were not direct contacts, and were infected in different transmission chains. The VP1 gene of these two type 2 strains differed from the OPV strain by 1% to 2.6% nucleotides, respectively. This may indicate that the two strains had been circulating for approximately 1 and 2 years. The rate of nucleotide sequence evolution in poliovirus is approximately 1% per year (Alexander, Gary et al. 1997; Jorba, Campagnoli et al. 2008). Their VP1 sequences differed by 1.7 to 4.6% to that of the 2001-2002 isolates (Rousset, Rakoto-Andrianarivelo et al. 2003). They shared common nucleotide substitutions at four positions suggesting a common OPV ancestor strain (data not shown), and had the nucleotides change T2909C, contributing to the reversion of attenuated type 2 strain to neurovirulence.

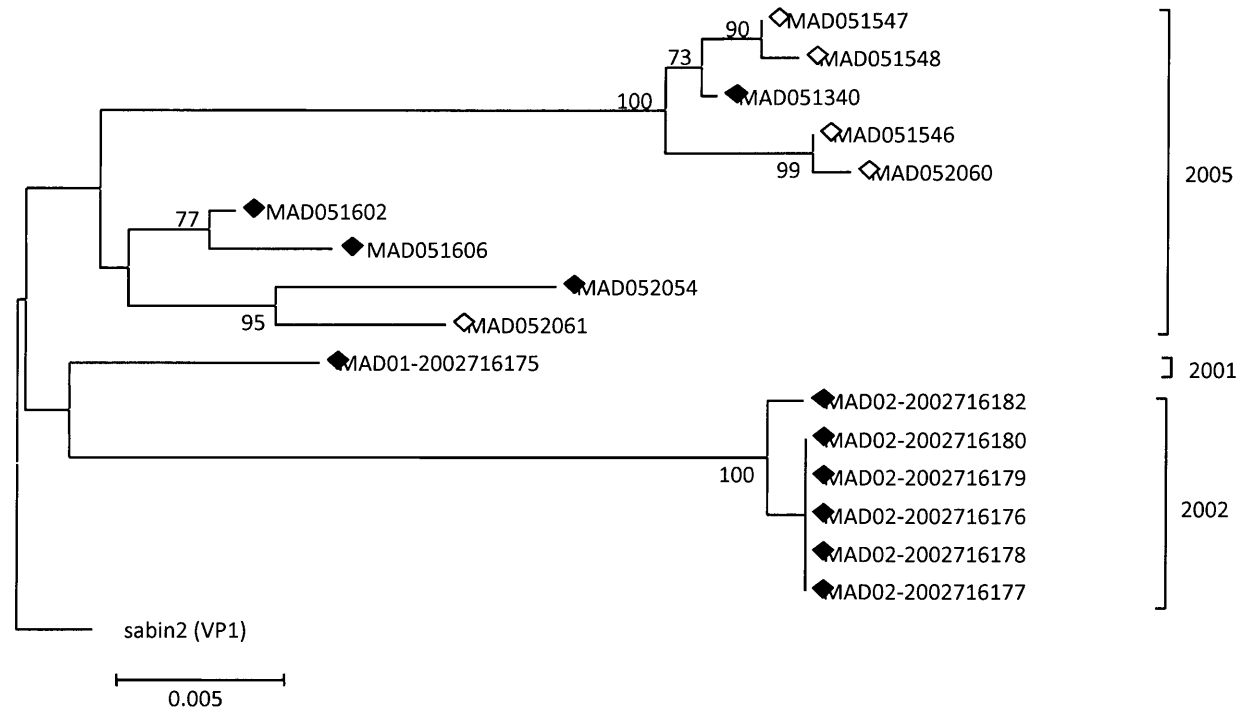


Figure 4.2. Neighbour-joining phylogenetic tree of circulating vaccine-derived polioviruses VP1 gene nucleotide sequences (903 bp). Numbers at branches nodes refer to the number of bootstrap repetitions (of 1000) at which the distal sequences grouped together. Closed diamonds ◆ represent AFP cases, open diamonds ◇ represent contacts. Sabin type 2 virus was used as an out-group. Genetic clusters of strains that were isolated from 3 years are indicated.

Only one AFP case of cVDPV type 3 was identified (Figure 4.3). This case had eleven contacts of which three were 100% identical to the OPV reference strain. The VDPV type 3 case was 1.4% different from the OPV strain which indicate that the strain had been multiplying for the past 1.3 years according to the evolution rate of 1.1% per site per year (Jorba, Campagnoli et al. 2008). No wild PV strains have been isolated in Madagascar since 1997 despite AFP surveillance performance of 91%.

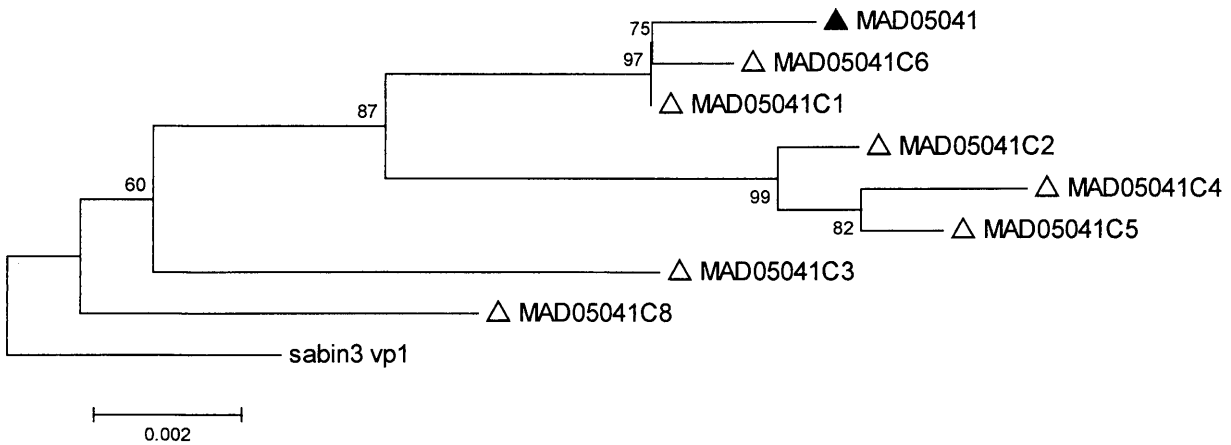


Figure 4.3. Neighbour-joining phylogenetic tree of circulating vaccine-derived polioviruses VP1 gene nucleotide sequences (900 bp). Numbers at branches nodes refer to the number of bootstrap repetitions (of 1000) at which the distal sequences grouped together. Closed diamonds ▲ represent AFP cases, open diamonds △ represent contacts. Sabin type 3 virus was used as an out-group.

4.3.3 Recombinant features of VDPVs

The 5' NTR sequences of the 11 type 3 VDPVs were closely related to those of Sabin 3 (Figure 4.4), but exhibited the T472C nucleotide substitution associated with the reversion to neurovirulence. In contrast, the 5' NTR sequences of the type 2 VDPVs were apparently derived from non-OPV viruses. The sequences of the 5' part of the 3D-coding region (Figure 4.5) of the type 2 and 3 VDPVs isolates gather in 2 distinct genetic clusters related to HEV-C but different from OPV sequences. In contrast, the sequences of the 3' part of the 3D-coding region and the 3'NTR region of most VDPVs were clearly derived from Sabin 3 (97.2 to 99.1% nucleotide identities) data not shown. Non-OPV sequences present in these VDPVs presented 81% to 87% nucleotide identities with HEV-C sequences available in GenBank and were probably derived from co-circulating

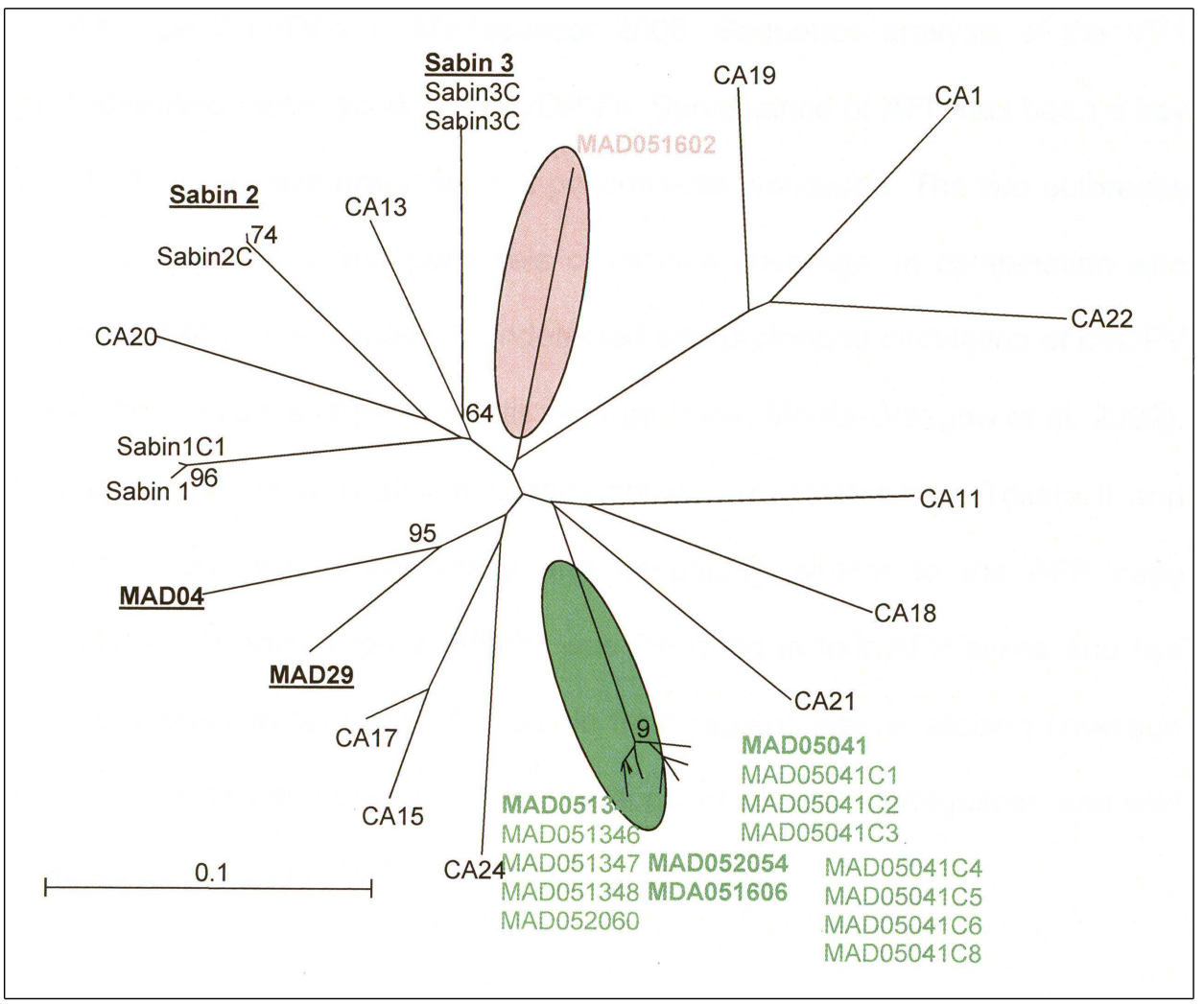


Figure 4.5. The unrooted neighbour-joining tree of the 3D region (nt 6145 – 6507) demonstrating relationship with unknown human enterovirus C species (HEV-C).GenBank accession numbers HEV-C used are: AF499635=CA1; AF499636=CA11; AF499637=CA13; AF499638=15; AF499639=CA17; AF499640=CA18; AF499641=CA19; AF499642=CA20; AF546702=CA21; AF499643=CA22 and D90457=CA24. All cases are bolded.

4.4 DISCUSSION

In this study we identified 4 AFP cases associated with type 2 cVDPV and 1 AFP case associated with type 3 cVDPV in Madagascar 2005. Sequence analysis of the VP1 coding region identified these isolates as cVDPV's. Surveillance of AFP has been a key factor in the efforts to achieve eradication of poliomyelitis worldwide. The two outbreaks in Madagascar demonstrated that low levels of vaccine coverage, in combination with poor surveillance of AFP, could result in undetected and prolonged circulation of cVDPV in a community before cases of poliomyelitis emerge (Kew, Morris-Glasgow et al. 2002). Type 3 cVDPV was detected in eleven healthy children (contacts) from Toliara II and Tsihombe districts and was antigenically and genetically similar to the AFP case identified in Toliary 1 district. Type 2 cVDPV was identified in four AFP cases and five contacts. These viruses were readily detectable from regions where vaccine coverage was low. OPV coverage was between 40 and 68% nationwide in Madagascar, and wild poliovirus was last reported in 1997 (Kew, Wright et al. 2004).

All outbreaks identified in this study for two serotypes have been recombinants with HEV-C confirming the circulating VDPVs are associated with co-circulation of HEV-C and genetic exchange between this species and OPV viruses. The impact of genetic exchange between poliovirus and HEV-C in evolution and pathogenicity remained to be further investigated.

Considering VP1 region, the type 2 viruses had diverged from the common OPV ancestor and multiply and circulate along multiple chains of transmission, whereas the

type 3 spread along an independent chain. On the 5' NTR region, the type 2 and HEV-C similarities suggest that this part for both strains derived from the common ancestor. The lack of genetic relationship between 2001-2002 and 2005 outbreaks suggests that the 2005 outbreak originated independently from an OPV strain. Relationship between all cases indicates an intense co-circulation and a rapid co-evolution between OPV strain and indigenous HEV-C. This report emphasise the rapid evolution between poliovirus and HEV-C by multi recombination events in a short period of time and restricted geographical area.

The detection of several distinct cVDPV lineages in Madagascar suggest that cVDPV's can emerge independently in locations where there is inadequate immunity to poliovirus (Kew, Wright et al. 2004). The rapid spread and evolution of OPV to VDPVs can be explained by the existence of population immunity gaps and this confirms that the risk for VDPV outbreaks in low population immunity (Dowdle, De Gourville et al. 2003). The cVDPV retained the two most important biological properties of wild polioviruses, namely, (i) the capacity to cause paralytic disease in humans and (ii) the capacity for continuous person-to-person transmission.

The origin of cVDPV in Madagascar was the result of low population immunity to poliovirus, due to a combination of low OPV coverage in many communities and the prior elimination of the indigenous wild poliovirus. The risk factors appear to be the same as for wild poliovirus circulation and include crowding, high birth rates, poor hygiene and sanitation, and a tropical climate (Nathanson and Martin 1979);(Fine and Carneiro

1999). Under such conditions, the cVDPV established several independent foci of endemicity in separate communities.

In conclusion, two outbreaks occurred successively in 2001-2002 and in 2005 in the Toliara province. Our findings have important implications for the future of the polio eradication program and the associated viral surveillance. In both cases these outbreaks emerged after efficient OPV mass campaigns and subsequent three years low vaccine coverage periods. Therefore, the priorities should be placed on (i) achieving and maintaining high population immunity to ensure definitely VDPV transmission, (ii) reconsidering the strategy and use of poliovaccines to get rid of the last wild and VDPV associated poliomyelitis cases, (iii) increasing the performance of surveillance, and (iv) conducting detailed epidemiologic, clinical and laboratory study to better understand the risk factors for VDPV and to prevent its re-emergence.

CHAPTER 5

Vaccine-derived Polioviruses (VDPV) threatening eradication of polio in the Democratic Republic of Congo (DRC)

5.1 INTRODUCTION

Four countries remain endemic for indigenous wild type poliovirus namely, Nigeria, India, Afghanistan and Pakistan (CDC. 2006b). Several African countries have experienced transmission in 2010 following an importation of poliovirus. Worldwide, a 19% decrease of cases was reported in 2010 compared to 2009 (Zheng, Zhang et al. 1993). Live, attenuated oral poliovirus vaccine (OPV) is still the vaccine of choice for developing countries. However, reversion to virulence may occur during OPV replication in humans and may result in the rare cases of vaccine-associated paralytic poliomyelitis (VAPP) in OPV recipients and their close contacts. Although cases of VAPP have long been recognized, two additional OPV-related problems that may have a significant impact on polio eradication have been identified. These are long-term persistent infection with OPV-derived viruses in persons with primary humoral immunodeficiencies (so-called immunodeficiency-associated vaccine-derived polioviruses, iVDPVs) and polio outbreaks caused by person-to-person spread and circulation of vaccine-derived polioviruses (cVDPV) in areas with low rates of vaccine coverage (Kew, Sutter et al.

2005). VDPV strains are defined as follows: a) VDPV strains of types 1 and 3 have less than 99% nucleotide sequence identity to the capsid VP1 coding region to Sabin reference strain, and b) VDPV strains of type 2 have < 99.4% sequence identity to the Sabin reference VP1 (Kew, Sutter et al. 2005) and http://www.polioeradication.org/GPLN_meeting_recommendation_2010.pdf).

cVDPVs show significant sequence drift, indicating prolonged replication of the vaccine strain in human hosts and consequent changes in phenotypic properties of neurovirulence and transmissibility. Poliomyelitis outbreaks associated with cVDPVs have been reported in several countries, such as Egypt (retrospectively detected for the period 1982-1993); Haiti (2000-2001); Dominican Republic (2000-2001) (Kew, Morris-Glasgow et al. 2002); Philippines (2001) (Kew, Morris-Glasgow et al. 2002); China (2004) (Tong, Zhang et al. 2005; Liang, Zhang et al. 2006); Cambodia (2005-2006); Indonesia (2005) (Estivariz, Watkins et al. 2008); Madagascar (2002 and 2005) (Rakoto-Andrianarivelo, Gumede et al. 2008); and Nigeria 2005-2010 (Jenkins, Aylward et al.; Wassilak, Pate et al.). As a result of the accumulating evidence about the emergence and spread of cVDPV, there are plans for synchronized cessation of the use of OPV and implementation of more widespread use of IPV (Kew, Morris-Glasgow et al. 2002; Rousset D 2003; Yang, Naguib et al. 2003; Jarzabek 2005).

In addition, some persons with primary immunodeficiency have been found to be persistently infected with VDPV. Once exposed to OPV, immunocompetent persons usually excrete polio vaccine viruses for up to 2 – 3 months (Alexander, Gary et al. 1997). In contrast, a few cases of prolonged excretion of VDPV for 6 months to more

than 10 years have been found in some immunodeficient persons (MacCallum 1971; Lopez, Biggar et al. 1974; Davis, Bodian et al. 1977; Hara, Saito et al. 1981; Misbah, Lawrence et al. 1991; Kew, Sutter et al. 1998; Bellmunt, May et al. 1999; Martin, Dunn et al. 2000). A better understanding of VDPV persistence and circulation is very important for decision making about when and how to stop immunization with oral poliovirus vaccine (OPV) after the global eradication of wild polioviruses (Fine and Carneiro 1999; Wood, Sutter et al. 2000).

This chapter presents the genetic characterization of the first cVDPV outbreaks detected in DRC. A total of 58 children with AFP were found to excrete VDPVs of serotype 2 in the DRC between 2004 and 2010. This is the first description of AFP cases associated with VDPVs of serotype 2 identified in the DRC.

5.2 MATERIALS AND METHODS

5.2.1 Viruses.

Virus isolates from faecal specimens of AFP cases were referred as described in Chapter 2 (van der Avoort, Hull et al. 1995). The original stool specimens from which these isolates were obtained were also referred to NICD for confirmation of virus isolation results using methods recommended by the World Health Organization (http://www.who.int/immunization_monitoring/Supplement_polio_lab_manual.pdf).

5.2.2 Diagnosis

Polio Virus (PVs) isolates were determined to be Sabin-like or wild-type strains by Intratypic differentiation (ITD) using a PCR-based technique (Yang, De et al. 1991; Kilpatrick, Nottay et al. 1996; Kilpatrick, Nottay et al. 1998), and ELISA as recommended by the World Health Organization (WHO) (Yang, De et al. 1991; Chezzi 1996; Kilpatrick, Nottay et al. 1996; Kilpatrick, Nottay et al. 1998).

5.2.2.1 Diagnostic reverse-transcription polymerase chain reaction (RT-PCR) for poliovirus

Refer to Chapter 2

5.2.2.2 ELISA for poliovirus

Refer to Chapter 2

5.2.2.3 Real-Time PCR for Sabin-like strains

A real-time assay developed by CDC, Atlanta was used for screening Sabin and wild-type strains, (Kilpatrick, Yang et al. 2009) and for screening VDPV strains (David Kilpatrick, personal communication).

5.2.2.4 RNA Extraction

Refer to Chapter 2

5.2.2.5 Reverse Transcription PCR for sequence analysis

Refer to Chapter 2

5.2.2.6 Sequence Analysis

Before sequencing, the RT-PCR products were purified with the QIAquick PCR purification kit (Qiagen). The complete VP1 gene (nucleotides 2480 to 3385) was sequenced as described in Chapter 2. In 2010, the Global Polio Laboratory Network (GPLN) established a new definition for VDPVs of type 2, based on accumulating data that showed the inadequacy of the current definition. The current definition is based on the detection of more than 1 AFP case with genetically related viruses showing > 10 nucleotide (nt) changes from VP1 nucleotide sequence of the Sabin reference strain of the same serotype. However the accumulated data from several countries point to situations that have programmatic implications, yet do not meet the current definition for circulation, including amongst others: Sabin-like viruses from different individuals that cluster by serotype, time or place and have VP1 sequences suggestive of a common evolutionary pathway (lineage) based on accumulation of mutations (some of which are

shared) yet sequence divergence from Sabin does not exceed 1%. This situation has been seen most frequently with serotype 2 viruses. Therefore, inadequacies of the current cVDPV definition used in the Polio Eradication Initiative programme (PEI) may delay the characterization or underestimate the scope of some outbreaks. According to the new definition, VDPVs of type 2 are those that have more than 5 nucleotide differences from Sabin 2 in the VP1 coding region.

(http://www.polioeradication.org/GPLN_meeting_recommendation_2010.pdf).

5.2.2.7 Phylogenetic Analysis

Phylogenetic analysis was carried out on the complete VP1 gene,(903bp). To determine VP1 nucleotide diversity we compared all complete VP1 sequences of isolates from AFP cases and their contacts with that of the Sabin type 2 OPV reference strain. Nucleotide sequences were aligned with Clustal X (Thompson, Gibson et al. 1997). A Neighbour-joining was constructed with MEGA version 4.0 (Kumar, Tamura et al. 2004; Tamura, Dudley et al. 2007; Kumar, Nei et al. 2008) rooted at the Sabin reference strain to demonstrate the evolution since the introduction of the VDPV into Africa. The alignments (nucleotide and translated amino acid sequences) were analyzed with Genedoc version 2.6001 (www.psc.edu/biomed/genedoc) and MEGA version 4 to identify specific mutations and positive selection (Tamura, Dudley et al. 2007).

5.3 RESULTS

5.3.1 Identification of circulating vaccine-derived polioviruses from AFP cases in the DRC

In total, over 600 viral isolates obtained from the DRC from 2003 to 2010 were tested by RT-PCR using pan-enterovirus, pan-poliovirus, serotype-specific, and Sabin type 1, 2 and 3 virus specific primers. Some isolates were further tested by ELISA, and both techniques identified the isolates as Sabin-like poliovirus. All isolates were further screened by Real-Time RT-PCR (rRT-PCR), which included a screen for VDPV as has been implemented in the GPLN (Taren, Nesheim et al. 1987; CDC. 2009a) . The genetic variability of virus isolates from 58 AFP cases was further investigated by performing nucleotide sequence analysis of the VP1 coding region. Partial genomic sequencing confirmed that 58 cases had $\leq$ 99.4% VP1 sequence identity to the parental type 2 Sabin strain, and the isolates were classified as VDPVs.

The polio cases were found in several regions of DRC during this time period (Figure 5.1). Altogether 27 cases occurred in Katanga (KAT) province, 5 cases in Kasai Occidental (KOC), 2 cases in Bandundu (BDD), 1 case in Sud Kivu (SKV), 6 case in Orientale (ORT), 11 cases in Maniema (MAN) and 4 cases in Équateur (EQT). The first case occurred in Inongo district of Bandundu province in September of 2005 and the last case was in the Tshikapa (TKP) district of KOC province and paralysis set in on 24th of September 2010.

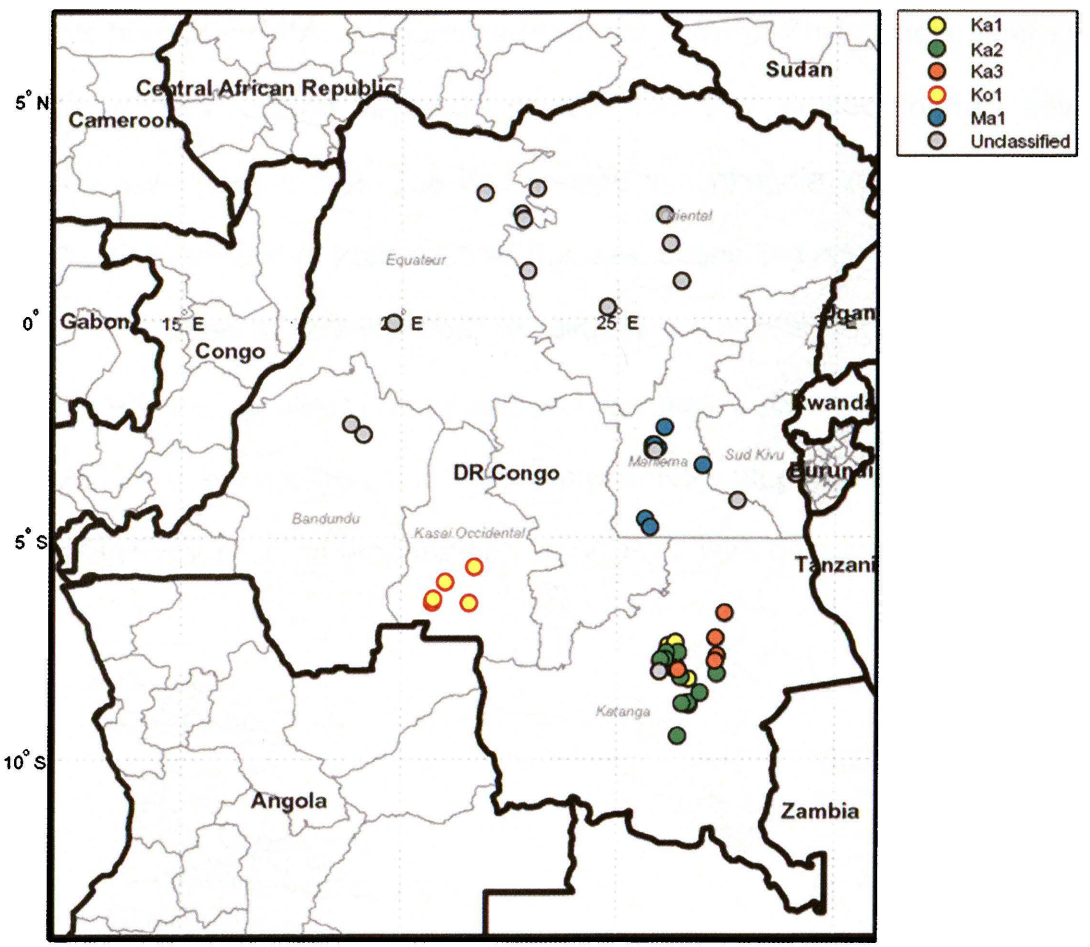
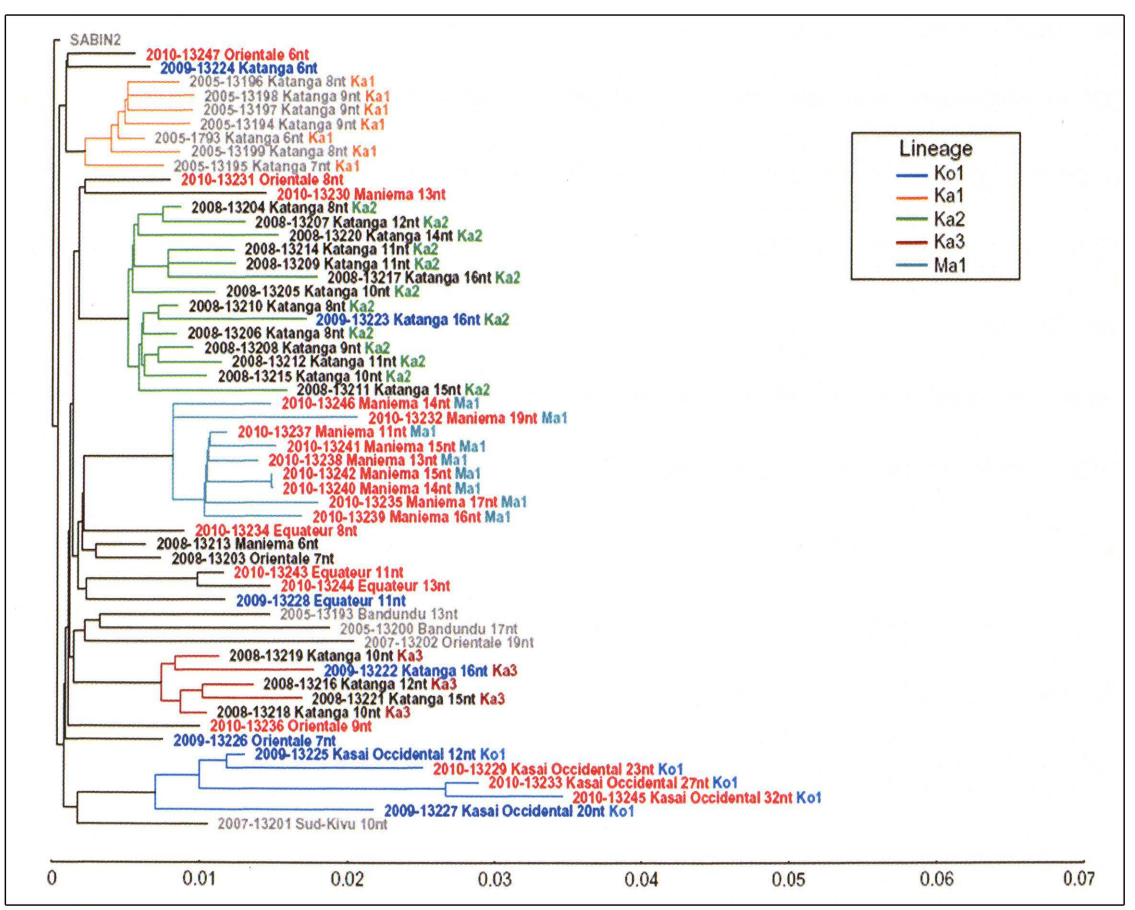


Figure 5.1: A map showing a geographical distribution of VDPV from the DRC

5.3.2 Phylogenetic analysis of the VP1 region of the VDPVs

The DRC type 2 cVDPVs characterized in this study were found in the southern part of the DRC and classified into 5 lineages (Ka1, Ka2, Ka3, Ko1 and Ma1) and several were referred as unclassified group identified in Bandundu, Equateur and Oriental provinces (Figure 5.1). We found that the DRC VDPVs were not closely related to previously isolated VDPVs from other African countries (data not shown). Phylogenetic analysis of the DRC capsid VP1 coding regions showed that the viruses formed several

(Figure 5.1). We found that the DRC VDPVs were not closely related to previously isolated VDPVs from other African countries (data not shown). Phylogenetic analysis of the DRC capsid VP1 coding regions showed that the viruses formed several independent lineages (Figure 5.2). The Ka1 lineage in Kinkondja and Malemba-Nkulu consisted of 7 cVDPVs isolated from 2005 AFP cases, circulating primarily in Kinkondja. Circulation of this lineage apparently stopped abruptly in 2005, as no additional related viruses were detected. The other four viruses isolated from 2005 and 2007 cases were not closely related to each other, and they represented independent emergences of VDPV from OPV (Shaw et al., in preparation). No VDPVs were detected in 2006.



In 2008, two concurrent cVDPV2 outbreaks occurred in Katanga. Lineages Ka2 and Ka3 were represented by 15 and 5 viruses, respectively. Both Ka2 and Ka3 outbreaks continued into 2009. Ka3 circulated primarily in Mulongo and Manono districts, whereas the Ka2 lineage circulated primarily in Kinkondja, Mukanga and Butumba. Viruses from these lineages were not detected after 2009. These two outbreaks accounted for 20 cVDPVs, almost one third of the total number of VDPVs detected. Those in the KAT province showed 99.20% to 98.2% similarity to Sabin type 2.

In 2008 and 2009, several independent emergences of VDPVs occurred in the provinces of MAN, ORT, and EQT. These sporadic detections were scattered in districts such as Kabondon-Dianda, Yahuma, Yambuku, Bumba, Monieka, Aketi, Salamabila, Banalia throughout the 3 provinces. In 2009, an outbreak was detected in KOC, and it continued into 2010 (Ko1 lineage). In 2010, an outbreak (Ma1 lineage) occurred in the MAN province in several districts (last case = RDC-MAN-KAM-10-008 onset 26 October 2010). The virus from this case had accumulated up to 14 nucleotide differences from Sabin type 2 in VP1 region. The viruse from MAN province showed 99.34% to 97.9% similarity to Sabin type 2. The 0.66-3.5% VP1 sequence divergence of the DRC lineages indicates circulation of approximately 6 months to 3.5 years, based on the previous published estimates of a rate of 1% per year for PV nucleotide sequence evolution (Alexander, Gary et al. 1997; Jorba, Campagnoli et al. 2008). The Ko1 lineage exhibited the highest sequence divergence, with the VP1 sequences showing 98.67% to 96.5% similarity to Sabin type 2. The sequence with the highest divergence from Sabin type 2 was RDC-KOC-TKP-10-008, with 32 nucleotide differences. Based on the rate of nucleotide evolution, it is likely that this lineage emerged and started to circulate in 2008.

5.4 DISCUSSION

In this study, we identified 58 AFP cases associated with type 2 VDPVs in the DRC in the period 2004 to 2010. Sequence analysis of the VP1 coding region showed that 41 of these isolates represented 5 outbreaks of cVDPV's, and 17 others represented several separate independent VDPV emergences. Although one third of the VDPVs were isolated from cases in a single province in DRC, favorable conditions for VDPV emergence existed throughout much of DRC.

The VDPV2 definition was changed recently in response to mounting evidence that viruses with less than 10 nucleotide differences were circulating and causing paralysis in human populations. In order to detect cVDPV and respond rapidly by immunization, it was necessary to develop a new definition for type 2 VDPV. For example, viruses in the Ka1 lineage had 6 – 9 nt. differences in VP1 and would not have been considered to be VDPV according to the old VDPV2 definition. The need to classify these programmatically relevant polioviruses as VDPV was shown by the genetic evidence of circulation, their ability to cause paralysis, and loss of the genetic markers of attenuation. Likewise, four of the viruses in the Ka2 lineage have 7 - 8 nt. differences from Sabin and are closely related to viruses that have more than 9 nt. differences from Sabin 2.

AFP surveillance has been a key strategy used to achieve eradication of poliomyelitis world-wide. The poliomyelitis outbreaks in the DRC demonstrated sub-optimal vaccine coverage, however, which led to outbreaks caused by both imported wild-type poliovirus and emergence and circulation of VDPVs during the period discussed. The Katanga province had more VDPV cases than the other provinces; this could be due to sub-

optimal vaccine coverage or better AFP surveillance than elsewhere in the country. Follow up investigations revealed that immunization coverage had been low (43%) at the beginning of the study conducted by Odusanya et al in 2006 (Grais, X et al. 2006). Vaccine coverage was low nationwide in the DRC, and wild type poliovirus is still circulating in the DRC (CDC. 2009a). The cVDPV2 outbreaks in MAN and KOC might still be ongoing.

A high level of geographic clustering was observed for viruses within a lineage. For example, the 9 AFP cases in the Ma1 lineage were found in one province, in 7 districts. Likewise, six of the seven viruses in the Ka1 lineage were from the Kinkondja district; the remaining virus was from nearby district Malemba-Nkulu, all in Katanga province. The extent of geographic clustering varies by serotype for poliovirus, although this has been studied primarily for types 1 and 3 poliovirus.

The detection of several distinct cVDPV lineages in the DRC suggest that cVDPVs can emerge independently in locations where there is inadequate immunity to poliovirus (Kew, Wright et al. 2004); (Wassilak, Pate et al.). The cVDPVs recovered the two most important biological properties of wild polioviruses, namely, (i) the capacity to cause paralytic disease in humans and (ii) the capacity for continuous person-to-person transmission. VP1 amino acid changes in position 143 associated with the reversion to virulence were identified. The origin of cVDPV2 in the DRC was probably the result of low population immunity to poliovirus, due to a combination of low vaccine coverage in some communities and the prior elimination of the indigenous wild poliovirus of the same serotype. Such risk factors are the same as for wild-type poliovirus (WPV) circulation, (Nathanson and Martin 1979; Fine and Carneiro 1999) and imported wild

polioviruses circulated in RDC during this time period. Poor hygiene and sanitation and tropical climate were probably additional factors that facilitated circulation of both WPV and VDPVs in some communities. The occurrence of WPV outbreaks during the same period emphasizes the need to maintain high vaccine coverage and AFP surveillance to minimize the risk of emergence of VDPVs or circulation of imported WPVs. Because similar conditions may exist elsewhere in Africa, ongoing high-quality surveillance will be essential for the achievement of polio eradication in Africa.

CHAPTER 6

Identification of type 2 and type 3 circulating vaccine-derived polioviruses isolated during an outbreak of poliomyelitis in Ethiopia in 2008-2010

6.1 INTRODUCTION

Poliomyelitis is highly contagious, and wild polioviruses in endemic areas infect virtually the entire population (Gear 1993). Polio is a seasonal disease, with peak transmission in the summer and autumn and reduced transmission during winter. Seasonal differences in transmission are far less pronounced in tropical areas (Nathanson and Martin 1979; Sabin 1985). Paralytic attack rates vary by serotype, with the highest rates (~0.5%) associated with poliovirus type 1 and the lowest rates (<0.05%) associated with poliovirus type 2 (Nathanson and Martin 1979). Poliovirus is transmitted person to person by the fecal-oral route and also by the oral-oral route, especially in areas with good sanitation and hygiene. Major risk factors for poliovirus transmission include poor sanitation and hygiene, tropical and subtropical conditions, arge birth cohorts, and high population densities (Nathanson and Martin 1979); (Sabin 1985). In Ethiopia, as in other developing countries, poliomyelitis was a major public health problem affecting mainly preschool children (Cockburn and Drozdov 1970). A study conducted at a major pediatric hospital in Addis Ababa demonstrated an increase in new cases between 1971 and 1981. Eighty-nine per cent of the cases with residual paralysis were 2-year-old

and 1981. Eighty-nine per cent of the cases with residual paralysis were 2-year-old children. There was no seasonal variation. A study in 1979 involving over a quarter of a million school age children in Addis Ababa indicating a prevalence of 2.7 per 1,000, with an estimated annual incidence of 7.8 per 100,000 (Maru, Getahun et al. 1988). Another study, conducted from February to July in 1993, indicated that the prevalence among children aged 5-9 years was 7.3 per 1,000 (Maru, Getahun et al. 1988). Other school and institutional based studies revealed a higher prevalence (Hull, Ward et al. 1994)

In 1988, the World Health Assembly resolved to eradicate poliomyelitis worldwide. Subsequently, the Global Polio Eradication Initiative (GPEI) of the World Health Organization (WHO) reduced the global incidence of polio associated with wild polioviruses (WPVs) from an estimated 350,000 cases in 125 countries in 1988 to 1,651 reported cases in 2008 and reduced the number of countries that have never interrupted WPV transmission to four endemic countries (Afghanistan, India, Nigeria, and Pakistan) (Maru, Getahun et al. 1988). However, because vaccine-derived polioviruses (VDPVs) can produce polio outbreaks in areas with low rates of oral poliovirus vaccine (OPV) coverage and can replicate for years in immunodeficient persons, enhanced strategies are needed to limit emergence of VDPVs and stop all use of OPV once WPV transmission is eliminated.

The polio eradication initiative in Ethiopia started in 1996 following the signing of the Yaoundé Declaration on Polio Eradication in Africa in 1996. Since then, Ethiopia has accelerated implementation of polio eradication strategies. Acute Flaccid Paralysis (AFP) surveillance, the detection and reporting cases of AFP in children less than 15

means for detecting circulating wild poliovirus in a population and therefore is crucial to the global polio eradication effect.

Ethiopia conducted the first sub-National Immunization Days (sNIDs) in 1996 and then two rounds of National Immunization Days (NIDs) every year thereafter. House-to-house immunization started in 1999 and contributed to the increased immunization coverage and AFP surveillance rates in parallel.

The surveillance quality at National level continues to show progressive improvement from year to year. Achieving uniform quality of surveillance at the lower level of the administration system in the country remains a great challenge. Uniform nationwide surveillance is crucial for ruling out undetected transmission of poliovirus. The key to evaluating the status of polio eradication is combining epidemiological and laboratory investigation of AFP among infants and children (Hull, Ward et al. 1994). An AFP surveillance indicator is detection of two AFP case per 100,000 children under 15 years of age. From 1997 to 2001, Ethiopia made remarkable progression on the rate of collection of two stool specimen within 14 days after onset of paralysis cases and met the surveillance indicator of WHO (Appendix A).

During this period five indigenous cases of WPV1 were isolated. The first isolate was in 1999 from Oromia Region East Shewa zone. In the year 2000 two cases from Southern Nations Nationalities people region (SNNPR) and one case from North Gonder zone in Amhara region were isolated. Three of these patients had not received OPV. Residual paralysis was observed in four of the five patients. Genetic sequencing revealed that the wild isolates identified in 1999-2001 were not related to other viruses isolated in neighboring countries or elsewhere. It is unlikely that the virus was imported from

outside. The last indigenous of WPV1 case in 2001 was in SNNPR which is southern part of Ethiopia (Mentaye, Tomori et al. 2002).

Ethiopia has achieved tremendous progress in its Polio Eradication Initiative activities since it commenced in 1996. The AFP surveillance system proved to be sensitive enough to detect imported WPV outbreaks and OPV coverage rates have increased from less than 400,000 children in 1996 to more than 14 million in 2001 leading to reduced transmission of the virus. From January 2002 to 2003 no WPV was detected in Ethiopia and the country was categorized as an area with low transmission. However, following a massive polio outbreak in Sudan that paralyzed 132 Sudanese children in May 2004, the virus spread to Ethiopia. The first two confirmed cases of WPV1 were isolated in the Tigray region and genetic sequencing data confirmed that the two cases are linked to polio circulating in Sudan.

In this report, we describe the detection of type 2 and type 3 circulating VDPVs from 2007 to 2010. Like other outbreaks in Hispaniola (Kew, Morris-Glasgow et al. 2002), the Philippines and Madagascar (Rakoto-Andrianarivelo, Gumede et al. 2008) the circulation of VDPVs occurred after the elimination of the corresponding serotype of wild poliovirus at a time when the rate of vaccine coverage was insufficient.

6.2 MATERIALS AND METHODS

6.2.1 Diagnostic reverse-transcription polymerase chain reaction (RT-PCR) for poliovirus

Refer to 2.2.2.1

6.2.2 ELISA for poliovirus

Refer to 2.2.2.2

6.2.3 RNA Extraction

Refer to 2.2.2.3

6.2.4 Reverse Transcription PCR for sequence analysis.

Reverse transcription PCR (RT-PCR) was performed in a single step as described previously (Yang, De et al. 1991; Chezzi and Schoub 1996; Kilpatrick, Nottay et al. 1996; Kilpatrick, Nottay et al. 1998) and as explained in chapter 2.

6.2.5 RT-PCR product analysis

PCR products were analysed by agarose gel electrophoresis. The DNA was visualised by addition of 6 µl of 10 mg/ml ethidium Bromide. A 100 bp DNA ladder was used as the molecular weight marker (DNA molecular marker XIV, Roche diagnostics, Mannheim Germany). The gels were visualized and photographed on UVP trans-illuminator using Grab IT annotating Grabber 2. 51 software (UVP (Inc), USA).

6.2.6 PCR product purification

PCR products were purified with the Qiaquick PCR purification kit (QIAGEN, Hilden, Germany) according to the manufacturer's recommendations. The Qiaquick columns have a silica-gel membrane with selective binding properties. In short, five volumes of PB buffer (guanidine hydrochloride, isopropanol) were added to 1 volume of PCR reaction. Primers and impurities are removed by washing with 750 µl PE buffer containing 88% ethanol. PCR product was eluted with distilled water.

6.2.7 Cycle sequencing

As described in 2.2.2.5

6.2.8 Sequence Analysis

Refer to Chapter 2.

6.2.9 Phylogenetic Analysis

The alignments (nucleotide and translated amino acid sequences) were analyzed with Genedoc version 2. 6001 (www. psc. edu/biomed/genedoc) and MEGA version 4 to identify specific mutations and positive selection (Kumar, Nei et al. 2008).

6.3 RESULTS

6.3.1 Prevalence and properties of circulating vaccine-derived polioviruses from AFP cases in Ethiopia

In total, 89 viral isolates obtained from Ethiopia during 2007 to 2010 were tested by intratypic differentiation tests to screen out Sabin-like strains from wild type strains using both RT-PCR and ELISA. All confirmed Sabin-like strains were further analysed by real-time assay. The genetic variability of all isolates was further investigated by nucleotide sequence analysis of the VP1 region. Partial sequencing and P-distance analysis confirmed that 13 isolates were VDPV's with > 1% divergence from the parental Sabin strain (Table 6.1).

Table .6.1: List of selected VDPV cases detected in Ethiopia since 2008. Epid Number is an epidemiology number given to a case or contact.

Epid Number	OPV doses given	Date Of Onset of paralysis	Serotype	Nucleotide difference to Sabin reference strain
ETH-ORO-BAL-10-034	1	06/01/2010	3	2.33%
ETH-ORO-BAL-10-136	1	17/02/2010	3	2.57%
ETH-ORO-BAL-10-330	3	08/04/2010	3	2.56%
ETH-ORO-BAL-10-430	3	17/05/2010	3	2.78%
ETH-ORO-BAL-10-C30	3	Not available	3	2.78
ETH-ORO-EHA-09-258	3	27/04/2009	3	1.33%
ETH-ORO-EHA-09-108	2	06/02/2009	2	1.11%
ETH-ORO-EHA-08-1033	1	14/12/2008	2	1.11%
ETH-ORO-EHA-08-727	0	04/10/2008	2	1.22%
ETH-ORO-EHA-08-925	3	17/11/2008	2	1.22%
ETH-SOM-DEG-10-251	2	05/02/2010	3	2.56%
ETH-SOU-BEN-10-937	1	11/04/2010	3	2.12%

In total, seven AFP cases for type 3, 4 AFP cases for type 2 and 2 contacts for type 3 were detected. The four AFP cases of type 2 had 98.9% - 98.78% sequence identity in the VP1 gene to the Sabin reference strain and 99.78% - 99.10% sequence identity to each other. The VP1 sequence of the cVDPV type 3 had 98.70% – 96.90% sequence identity to the Sabin reference strain and 99.00% to 95.60% to each other.

6.3.2 Amino acid substitutions in the VP1 region

Comparisons of the VP1 amino acids sequences of the VDPV type 2 from Ethiopia relative to Sabin 2 are shown in the alignment in Figure 6.1. Most substitutions resulted in silent mutations and occurred on the 5' end of the VP1 region. A proline at position 21 of Sabin 3 is replaced by a leucine, indicated as P21L; as well as I143T. T143I encoded by C2909U nucleotide appear to be responsible for the attenuated phenotype of Sabin 2 (Ren, Moss et al. 1991; Macadam, Pollard et al. 1993). Any back mutation may cause neurovirulence (Sabin 1985) as in the case of Ethiopia strains which demonstrated the same base as it is with wild type 2 strains.

The VDPV type 3 had three substitutions that were observed at positions, 12, 53 and 106, i.e., G12S, A53V and M106T (Figure 6.2). ETH-10-10-251 had an A to S substitution at position 144 (A144S) and D24M which was not observed in other Ethiopia strains which indicates further genetic drift from the original virus from Sabin reference strain. One strain from Ethiopia (ETH-10-10-258) contained an additional substitution of E8D. Most of Ethiopia strains share the same substitutions relative to Sabin 2 and 3.

STRAINS **VARIABLE POSITIONS**

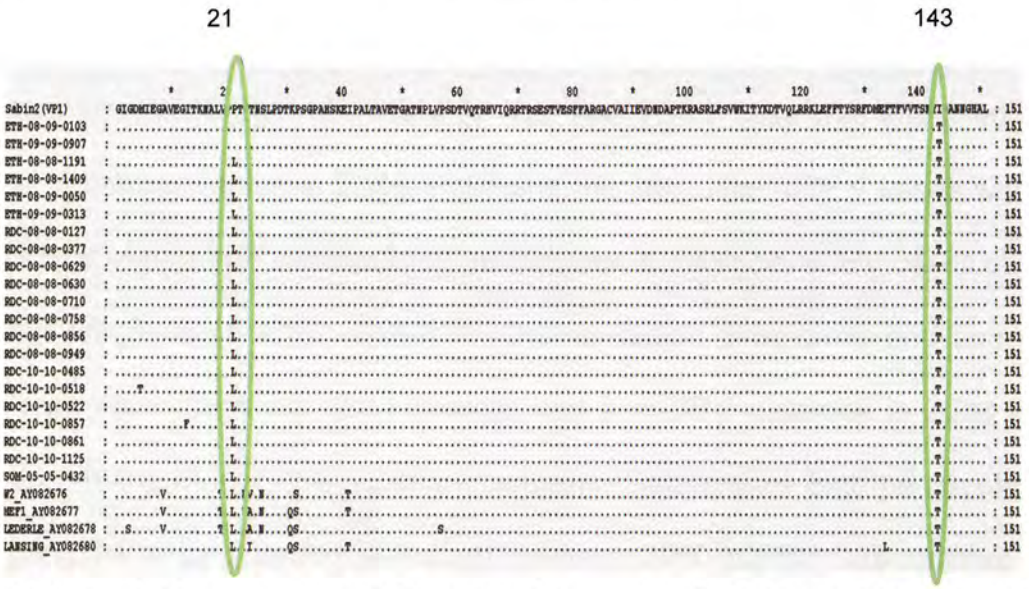


Figure 6.1 Amino Acids comparison of VP1 region of the genome for Sabin 2, wild type 2 and VDPVs type 2 from Africa. W2_AY082676, MEF_AY082677, Laderle_AY082678 and Lansing_AY082680 are from the genebank. RDC strains are from the DRC (detailed in Chapter 4) and ETH strains are from Ethiopia.

STRAINS **VARIABLE POSITIONS**

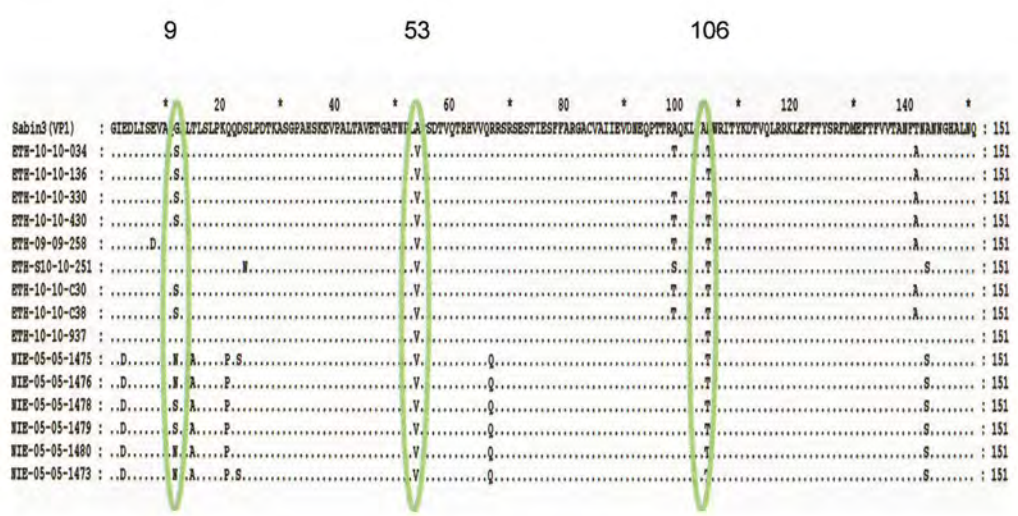


Figure 6.2. Amino Acids comparison of VP1 region of the genome for Sabin 3, wild type 3 and VDPVs type 3 from Africa. NIE strains are from the Nigeria and ETH strains are from Ethiopia.

6.3.3 VDPV Distribution in Ethiopia, 2008 – 2010

A total of nine VDPV cases were identified from two regions Oromia and one from Somali from 2008-2010 (Figure 6.3). The first VDPV case was identified in East Harge zone in Oromia region in 2008. Following this two other VDPV cases were identified in the same region and zone in 2009. In the year 2010 three VDPV cases one from Somali region Degehabour zone, one from Oromia region Bale zone and another from East Harerge were identified. East Hararge had a cVDPV outbreak in 2008 and 2009 with similar finding of low immunization coverage. Data shows that the circulation has been ongoing for a long period. Although trivalent OPV (tOPV) Sub-national Immunization Activities (SIAs) were conducted right around the identification of the first case in E. Hararge, the virus may have already entered Bale. The case in Somali Region is right at the border with Somalia.

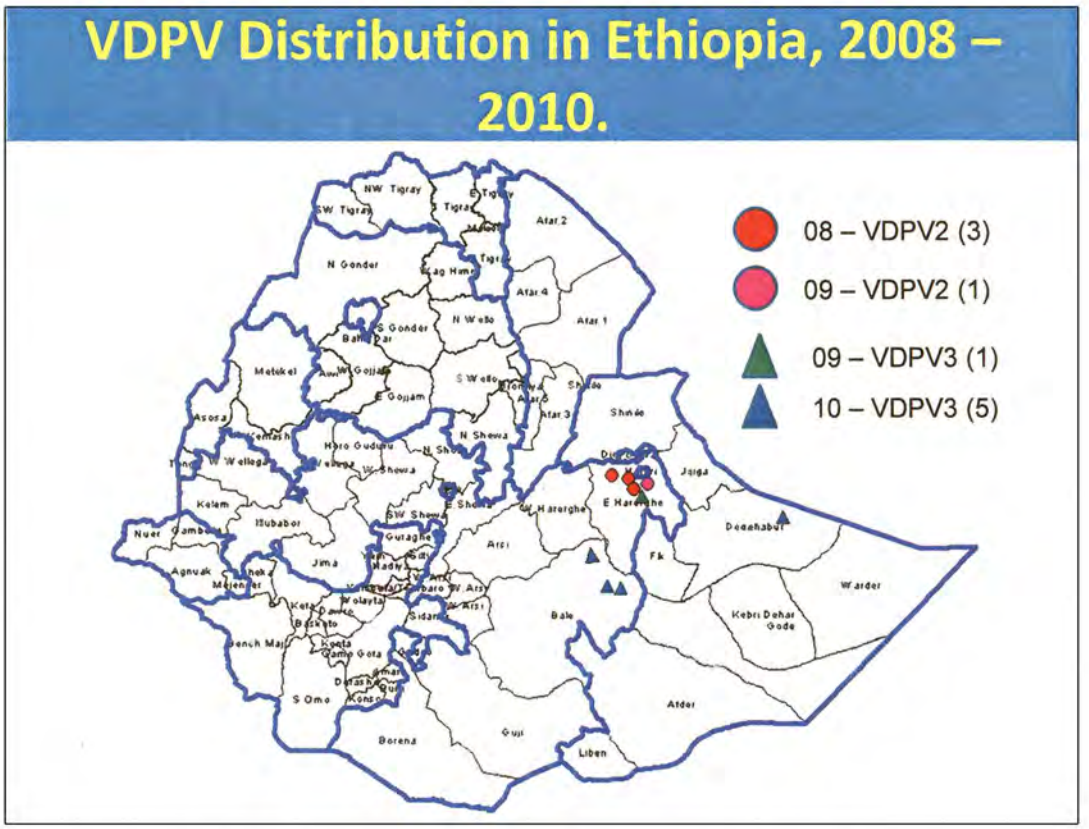


Figure 6. 3.: Geographical distribution of VDVP cases in Ethiopia in 2008 to 2010

6.4 DISCUSSION

VDPVs can cause paralytic polio in humans and has the potential for sustained circulation. VDPVs resemble WPVs biologically and differ from the majority of Sabin vaccine-related poliovirus isolates by having genetic properties consistent with prolonged replication or transmission. Because poliovirus genomes evolve at a rate of approximately 1% per year (Jorba, Campagnoli et al. 2008), Sabin vaccine-related isolates that differ from the corresponding OPV strain by more than 1% of nucleotide positions (usually determined by sequencing the genomic region encoding the major viral surface protein, VP1) are estimated to have replicated for at least 1 year after administration of an OPV dose. This is substantially longer than the normal period of vaccine virus excretion of 4-6 weeks.

The transmission of vaccine-related virus from vaccinees to susceptible close contacts was recognized prior to the introduction of oral polio vaccine. Vaccine viruses were transmitted from person to person in families, but less readily than wild poliovirus strains (Hull, Ward et al. 1994). The overriding factor for the emergence of all VDPVs is the same as for WPV circulation, low immunity levels in a population. Where routine coverage with OPV is poor or where there is an absence of high quality supplementary immunization activities, a population can become susceptible to the emergence of VDPV. This is because the virus has time to change, replicate and exchange genetic material with other enteroviruses, while spreading through a population (Rakoto-Andrianarivelo, Gumede et al. 2008). Importantly, if a population is fully immunized

against polio, it will be protected against the spread of both wild and vaccine strains of poliovirus (Rakoto-Andrianarivelo, Gumede et al. 2008).

Studying each outbreak has shown that most were either self-limiting or rapidly stopped with OPV campaigns. As long as wild poliovirus circulates anywhere in the world, it is vital to maintain high population immunity levels. Maintaining high population immunity will also minimize the risk of cVDPVs occurring.

A vaccine-derived poliovirus is a rare strain of poliovirus, genetically mutated from the reference Sabin strain. More cases have been reported since the start of the VDPV2 outbreak in Madagascar in 2002 and 2005 (Rakoto-Andrianarivelo, Gumede et al. 2008), 58 detected in the DRC (chapter 5), and 11 from Ethiopia reported in here (Gumede et al, in preparation).

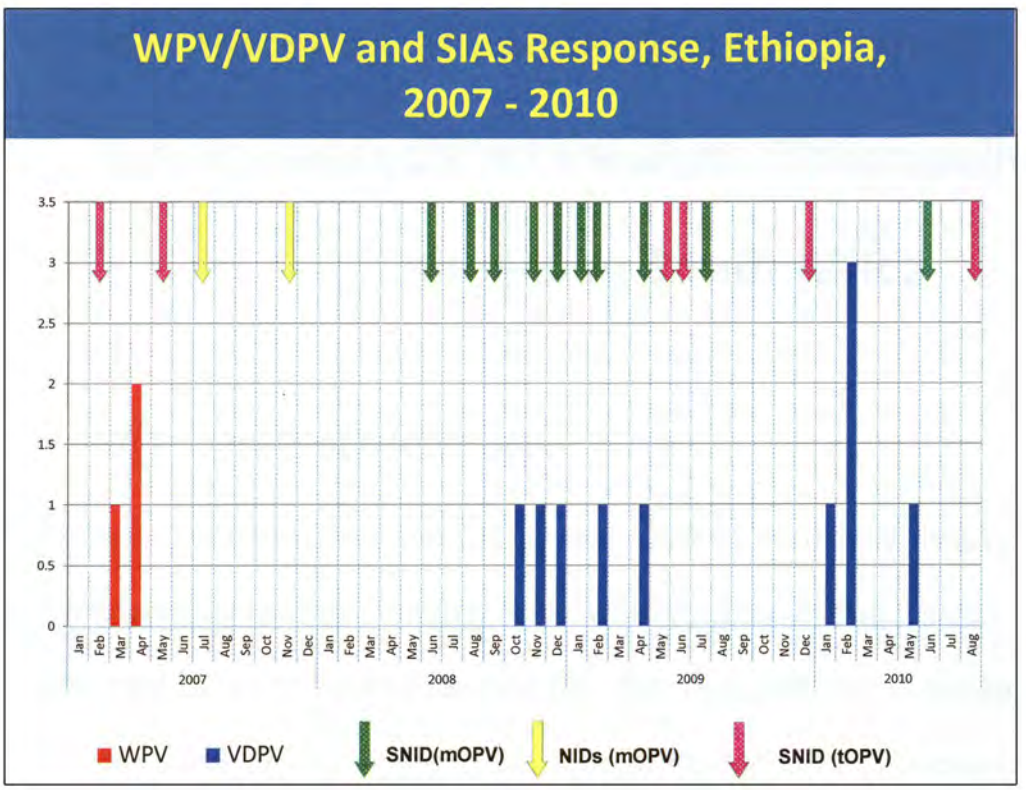
Amino acid changes in the VP1 gene that were similar to wild-type strains but different to Sabin could be identified which may be associated with the reversion to virulence, as demonstrated in Figure 6.1 and Figure 6.2. The origin of cVDPV2 in Ethiopia was probably the result of low population immunity to poliovirus, due to a combination of low OPV coverage in some communities and the prior elimination of the indigenous wild poliovirus of the same serotype. Such risk factors are same as for wild poliovirus (WPV) circulation, and imported wild polioviruses of circulated in the same country during the period 2005 to 2008 (Nathanson and Martin 1979; Fine and Carneiro 1999). Poor hygiene and sanitation and tropical climate were probably additional factors that facilitated circulation of both WPV and VDPVs in some communities.

The management of cVDPVs is a necessary part of the global polio eradication effort, and is similar to management of wild poliovirus outbreaks; i.e., by rapid implementation of large-scale, high-quality SIAs. OPV is the polio vaccine by which to achieve the eradication of wild polioviruses worldwide. Once wild poliovirus transmission has been interrupted globally, however, OPV use in routine immunization programmes will eventually be stopped, in order to eliminate also the rare risks posed by VDPVs and as recommended by the Advisory Committee on Polio Eradication.

Studies have also been proposed to measure the prevalence and duration of poliovirus excretion among human immunodeficiency virus-infected children in developing countries (Ochoa and Lago 1987).

The emergence of a cVDPVs in Ethiopia reaffirms that not enough children are protected from poliovirus (wild or vaccine-derived) and that much more must be done to reach all children with vaccine.

In response to recent outbreak in Bale Zone of Oromia and Degahabour Zone of Somali two rounds of campaign using mOPV3 and tOPV was conducted in June and August 2010 during SNIDs (Figure 6.4) (personal communication with Epidemiology group from Ethiopia).



Key

WPV:	Wild poliovirus
VDPV:	Vaccine-derived poliovirus
SNID:	Sub-National Immunization Day
mOPV:	Monovalent Oral Poliovirus Vaccine
NIDs:	National Immunization Days
tOPV:	Trivalent Oral Poliovirus Vaccine

Figure 6. 4: The graph showing outbreak response in Ethiopia.

CHAPTER 7

Identification of and molecular characterisation of Vaccine-associated Paralytic Poliomyelitis (VAPP) viruses in South Africa

7.1 INTRODUCTION

Paralytic poliomyelitis is caused by poliovirus of the family *Picornaviridae*, a group of nonenveloped positive strand RNA viruses. The coding region of the genome is preceded by a 749 nucleotide long non coding region (NCR) containing a determinants of virulence while the 3' end of the genome contain a 70 nucleotide also playing part in RNA replication (Minor 1997). The coding region is translated as a single polyprotein which is further processed to viral capsid (VP1 to VP4) and the nonstructural proteins (Minor 1990). During the poliovirus evolution, genomic RNA recombination is a common event. The recombination of polio vaccine and wild poliovirus and/or recombination with the other enteroviruses have been described (Friedrich, Da-Silva et al. 1996). Of great concern are the outbreaks associated with vaccine-derived polioviruses as described in chapter 4, 5 and 6. The Madagascar episode as described by Rakoto et al., (Rakoto-Andrianarivelo, Gumede et al. 2008) state that the epidemic strains have been identified as recombinant polioviruses containing sequences of human enterovirus species C. These recombination junctions are found in genomic coding region of the nonstructural protein.

The polio eradication programme has dramatically reduced the number of cases since 1988 however the risk of paralytic poliomyelitis will still exist even after eradication of wild polioviruses. The normal period of OPV excretion is between 4 to 8 weeks (Alexander, Gary et al. 1997). OPV has been associated with few adverse events and the most common is recognized as VAPP, different from polio caused by wild poliovirus (Kew, Sutter et al. 2005). Within the year of OPV licensure, the first cases of VAPP were identified and all associated with the Sabin 3 strains. The rate of VAPP is very rare and is similar in most countries (Esteves 1988; Kohler, Banerjee et al. 2002). The first-dose OPV is at a higher risk than a subsequent doses and the VAPP cases occur both in the OPV recipient and unimmunised contacts (Kew, Sutter et al. 2005). VAPP are most frequently associated with Sabin 3 followed by Sabin 2 where Sabin 1 is rarely associated with VAPP (Strebel, Sutter et al. 1992).

A collaborative study to obtain the information related to risk associated with OPV was coordinated by WHO in 1969 in 13 countries (CDC. 1997b). The data revealed that the risk of VAPP was 1 case per 3.3 million doses of OPV administered and following supported that the vaccine as causative: poliomyelitis clinical syndromes; identification of vaccine strains from all cases; vaccine exposure being evident; evidence of clustering of cases and their contacts; mutation of shed viruses towards neurovirulence and immunodeficient patient with B-cell deficiencies having a higher incidence of VAPP which is also higher for risk of poliomyelitis caused by wild poliovirus. In patients with VAPP, back-mutations that restore original stem loop structure leading to neurovirulence are frequently observed, Sabin 1: G480A; Sabin 2: A481G and Sabin 3:T472C (Minor and Dunn 1988) (Muzychenko, Lipskaya et al. 1991).

In South Africa, the vaccine coverage in South Africa was reported to be >80%. The last indigenous wild poliovirus was isolated in 1989 (Chezzi, Blackburn et al. 1997; Chezzi, Blackburn et al. 1997).

To determine if cases of VAPP have been missed in South Africa, a molecular investigation of all Sabin-like strains was conducted. The results emphasize the risk posed by vaccine-associated paralytic polioviruses and the implication of designing efficient vaccination and surveillance strategies to succumb the emerging of VAPP.. In addition, continued vaccination with OPV will increase the risk of and occurrence of VAPP.

7.2 MATERIALS AND METHODS

7.2.1 Case history

A suspected case of a possible VAPP was identified in a 2 year old female child born on 12 June 2008 in KwaZulu Natal province in South Africa. The child was brought to Mbonwa clinic in Harding with difficulties in walking on 15 April 2010 after being immunised on the 13 of April 2010. On examination, weakness of both lower limbs was observed and the child was transferred to St Andrews Hospital for further examination. On the 16 April 2010 was transferred to Port Shepstone Regional Hospital for paralysis on both limbs as admission diagnosis. Neurological examination confirmed a weakness in the lower limbs. The rest of the cases were in a ratio of 2:1 (girls: boys) under the age of 5 years from different provinces in South Africa. Faecal specimens for virological examination were obtained within 14 days after the onset of paralysis and 48 hours apart as per World Health

Organization (WHO) recommendation.

7.2.2 Virus Isolation

The fecal specimens of South African Sabin-like strains were processed at the National Polio Laboratory at the National Institute for Communicable Diseases (NICD) in South Africa according to the WHO protocol for virus isolation as per WHO laboratory manual version 4 (145). Briefly, tube cultures of L20B and RD cell lines were inoculated with 0.2ml of specimen extract and incubated in the stationery sloped (5^0) position at 36^0C. Cultures were monitored daily using standard or inverted microscope for the appearance of cytopathic effect (CPE). All cell lines with characteristics of enterovirus CPE were stored at 20^oC for a second passage in a tube containing 2ml of medium. Second

passage material was pooled for typing. Virological investigation identified polioviruses of serotype 1, serotype 2 and serotype 3.

7.2.3 Real time reverse-transcription polymerase chain reaction for intratypic differentiation (rRT-PCR ITD) and for vaccine-derived polioviruses (rRT-PCRVDPV) for poliovirus

All serotyped polioviruses (PVs) or viral isolates that had shown cytopathic effect (CPE) in L20B or Human Rhabdomyosarcoma (RD) cell line were tested using an rRT-PCR kit supplied by the Centers for Disease Control and Prevention, Atlanta, USA (CDC, Atlanta) that included separate reactions with primers for pan-enterovirus, pan-poliovirus, PV serotypes 1,2 and 3 (Kilpatrick, Nottay et al. 1996; Kilpatrick, Nottay et al. 1998), and multiplexed primers for Sabin type 1, 2 and 3 PVs (Kilpatrick, Yang et al. 2009) and further screening for VDPVs strains (Kilpatrick, personnel communication). In brief, 1ul of the 1:4 diluted virus isolates was added to the master mix containing 19µl of primer and 5 µl of buffer B (containing 2.8ul of 1M Dithiothreitol (Roche Diagnostic GmbH, Mannheim, Germany), 14.4ul of 5U/ul myeloblastosis virus Reverse Transcriptase (Roche Diagnostic GmbH, Mannheim, Germany), 27.6ul of 40U/ul Rnase Inhibitor (Roche Diagnostic GmbH, Mannheim, Germany) and 54.8ul of 5U/ul Taq Polymerase (Roche Diagnostic GmbH, Mannheim, Germany)). Reverse transcription was carried out at 42°C for 20 minutes in a GeneAmp 9700 thermocycler (Applied Biosystem, Foster City, CA), followed by 95°C for 3 minutes (95°C for 45 seconds, 42°C for 45 seconds and 60°C 45 seconds) x30 cycle.

7.2.4 RNA Extraction

Refer to 2.2.2.3.

7.2.5 Reverse Transcription PCR for sequence analysis

Reverse transcription PCR (RT-PCR) was performed in a single step as described previously in chapter 2. The primers used are described in Table 7.1.

Table 7.1 Prime pair used for amplification of and sequencing of the different regions of the poliovirus genome.

Primer	Position*	Gene	Sequence (5' – 3')
EV2S	446	5' NTR	TCCGGCCCCTDAATGCGGCTAATCC
EV1A	559	5' NTR	ACACGGACACCCAAAGTAGTCGGTTCC
Y7R	2421	VP1	GGTTTTGTGTCAGCITGYAAYGA
PV1A	2954	VP1	TTIAIIGCRTGICCRTTRTT
PV2S	2875	VP1	CITAITCIMGITTYGAYATG
PV8A	3460	VP1	AAGAGGTCTCTRTTCCACAT
DK234S	5915	3D	ATGCAYGTIGGIGGIAAYGG
DK235	6570	3D	TAIAGRTTICCRAAIGCCAT

* Position in the genome of Sabin 1. Numbering according to (Kilpatrick, Nottay et al. 1996; Kilpatrick, Nottay et al. 1998).

Reverse transcription PCR (RT-PCR) was performed in a single step as described previously in chapter 2.

7.2.6 RT-PCR product analysis

Refer to 5.2.5

7.2.7 PCR product purification

Refer to 5.2.6.

7.2.8 Cycle sequencing

Nucleotide sequencing was carried out on the sense and anti-sense strands using the ABI Prism^{R}BigDye^{TM} Terminator Cycle Ready Reaction kit v3. 1 as detailed in Chapter 5.

7.2.9 Sequence Analysis

Two programs, Recombination Detection Program 3 (RDP3) (http://Darwin.uvigo.es/rdp/rdp.html) (Martin and Rybicki 2000) and SimPlot (http://sray.med.som.jhml.edu/SCRoftware) were used to identify sites of recombination. RDP3 statistically identifies and characterises historical recombination events and rapidly analyse these with a range of powerful non-parametric recombination detection methods such as Bootscan (Martin, Posada et al. 2005). This program identifies possible recombination breaks points, and further identifies parental and recombinant sequences. Simplot calculate and plots the identity of the query sequence against the panel of reference sequences using a sliding window size across the alignment in steps (Salminen, Carr et al. 1995). In this study two different versions of SimPlot were used to determine the relationship of the query sequence and the reference sequences using the bootstrap resampling (bootscanning). One hundred bootstrap replicates generated

by neighbor–joining method were obtained using sliding window of 200bp overlapping by a 20bp step.

7.2.10 Phylogenetic Analysis

Phylogenetic analysis was carried out on different genes, NTR, VP1 and 3D for poliovirus type 1, 2 and 3. To determine nucleotide diversity we compared all complete sequences of isolates from AFP cases and their contacts with that of the Sabin OPV reference strains. Nucleotides sequences were aligned with Clustal X (Thompson, Gibson et al. 1997). Maximum-likelihood (ML) phylogenetic tree was constructed using PHYML (Guindon, Lethiec et al. 2005) with GTR+I+G nucleotide substitution model selected as most appropriate by RDP3 (Martin, Lemey et al.).

7.3 RESULTS

7.3.1 Prevalence and properties of vaccine-associated paralytic polioviruses from AFP cases in South Africa

In total, 71 viral isolates that had a case history that could fit VAPP were obtained from South Africa from 1995 until 2010 were tested by rRT-PCR using pan-enterovirus, pan-poliovirus, serotype specific, and Sabin type 1, 2 and 3 virus specific primers. Twenty-three isolates were positive for poliovirus type 1, 23 for poliovirus type 2 and 25 for poliovirus type 3 (Table 7.2).

Table 7.2 AFP Poliovirus Sabin strains selected for screening of VAPP's in South Africa.

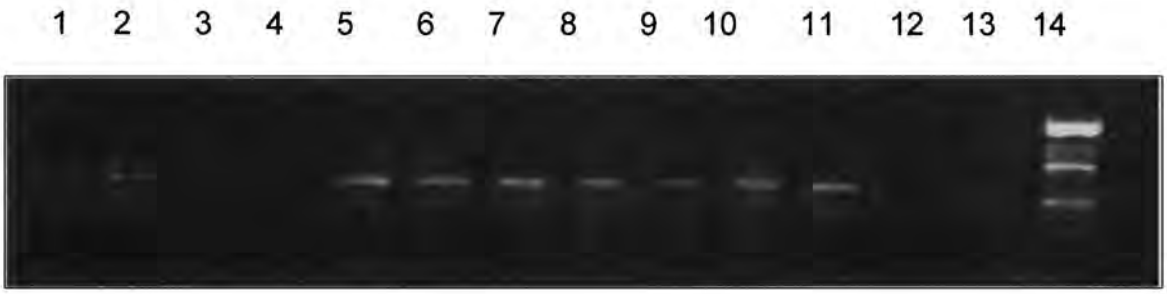
Year	LabID	ITD_Results
1995	D/95/1380	Sabin1
1996	D/96/0803	Sabin1
1996	D/96/0382	Sabin3
1996	PMOL961374	Sabin3
1996	D/96/1379	Sabin3
1996	D/96/1417	Sabin3
1996	D/96/1419	Sabin3
1996	PMOL961431	Sabin3
1996	D/96/1432	Sabin3
1996	D/96/1288	Sabin2
1997	D/97/2303	Sabin2
1997	D/97/2306	Sabin2
1998	D/98/0005	Sabin1
1998	D/98/0155	Sabin1
1998	VE/98/0173	Sabin1

1998	VE/98/0512	Sabin1
1998	VE/98/0513	Sabin1
1998	VE/98/0526	Sabin1
1998	D/98/0801	Sabin2
1999	VE/99/0822	Sabin1
1999	VE/99/0823	Sabin1
1999	VE/99/0824	Sabin1
1999	VE/99/0825	Sabin1
1999	VE/99/0085	Sabin2
2000	VE/00/0839	Sabin1
2000	VE/00/0971	Sabin3
2000	VE/00/0020	Sabin2
2000	VE/00/0058	Sabin2
2000	VE/00/0113	Sabin2
2000	VE/00/0113	Sabin2
2003	VE/03/1269	Sabin1
2003	VE/03/0216	Sabin3
2003	VE/03/0459	Sabin3
2003	VE/03/1183	Sabin2
2004	VE/04/0952	Sabin1
2004	VE/04/1022	Sabin1
2004	VE/04/1050	Sabin1
2004	VE/04/1052	Sabin1
2004	PMOL040953	Sabin3
2004	VE/04/1021	Sabin3
2004	VE/04/1071	Sabin1
2004	VE/04/0292	Sabin2
2004	VE/04/0790	Sabin2
2004	VE/04/0791	Sabin2
2004	VE/04/0870	Sabin2
2004	VE/04/0874	Sabin2
2004	VE/04/1231	Sabin2

2005	VE/05/0545	Sabin3
2006	VE/06/0602	Sabin2
2006	VE/06/2239	Sabin2
2006	VE/06/2049	Sabin3
2006	VE/06/1416	Sabin1
2006	VE/06/2115	Sabin1
2007	VE/07/0797	Sabin2
2007	VE/07/0886	Sabin2
2007	VE/07/1818	Sabin2
2007	VE/07/0125	Sabin2
2007	PMOL070521	Sabin3
2007	PMOL070678	Sabin3
2007	PMOL070795	Sabin3
2007	PMOL070798	Sabin3
2007	PMOL070805	Sabin3
2007	PMOL070899	Sabin3
2007	PMOL071294	Sabin3
2007	PMOL071500	Sabin3
2007	VE/07/1210	Sabin1
2007	VE/07/0637	Sabin1
2008	PMOL080724	Sabin3
2008	PMOL080391	Sabin3
2009	PM1336/2009	Sabin2
2010	PMOL100360	Sabin3

7.3.2 RT-PCR

Isolates were selected from all Sabin-like strains identified in South Africa. For the VP1 gene all positive isolates were identified by a specific1500bp band for VP1, a 400bp band for 5' NTR region and a 1383bp for the 3D region confirming the specificity and sensitivity of the selected primers (Table 7.1) as described by Kilpatrick et al., (Kilpatrick, Nottay et al. 1996; Kilpatrick, Nottay et al. 1998). A.



B.

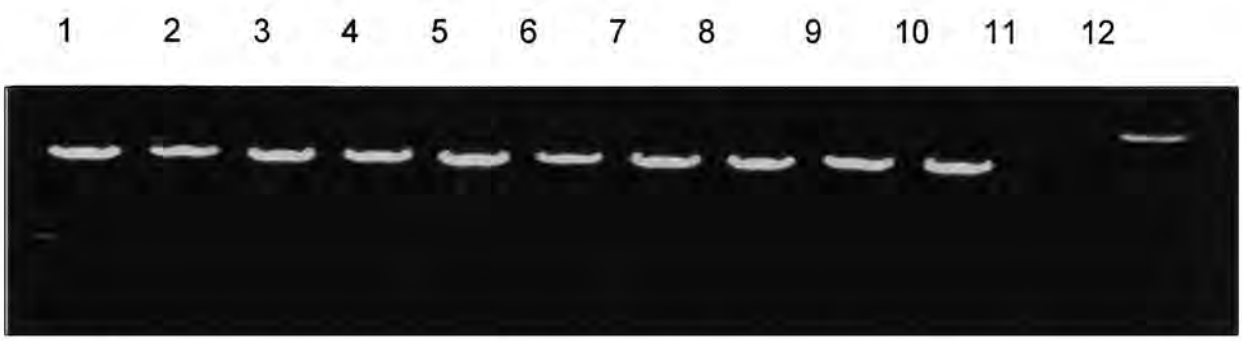


Figure 7.1. Ethidium bromide-stained 1.5% agarose gel showing amplification of the (A) 5' NTR and (B) VP1 regions of poliovirus type 3 strains. Sabin 3 used as a positive controls (+ve) and negative controls (-ve) are reactions without template. Lanes: 1. PMOL070911, 2. PMOL100360, 3. PMOL080728, 4. PMOL070899, 5. PMOL070805, 6. PMOL070678, 7. PMOL070769, 8. PMOL070798, 9. PMOL070521, 10. PMOL040953, 11. PMOL080391 in panel A and negative control in panel B. Lane 12 and 13, negative control in panel A and Molecular weight marker in panel B. Last lane is a molecular weight marker for panel A.

7.3.3 Nucleotide sequence analysis

All Sabin-like strains were sequenced at 3 different regions of the genome, 5' NTR, VP1 and 3D. For 7 viruses the sequence analysis revealed a mutation at nucleotide 472 of the 5' NTR, a critical attenuating mutation feature for Sabin 3 (Table 7.3). This substitution in the internal ribosomal site (IRES) restores the original structure of the stem loop and permitting the initiation of translation of the poliovirus RNA template (Muzychenko, Lipskaya et al. 1991; Haller, Stewart et al. 1996; Gromeier, Bossert et al. 1999). One virus demonstrated a nucleotide change at position 480, an important determinant of the attenuated phenotype of Sabin 1 (Muzychenko, Lipskaya et al. 1991; Haller, Stewart et al. 1996; Gromeier, Bossert et al. 1999) and only two viruses demonstrate a change at position 481 of Sabin 2. Its contribution to the attenuated phenotype of Sabin 2 remain unclear since there is no evidence in the IRES of the wild poliovirus type 2 (Rezapkin, Fan et al. 1999).

At a 3D region, the sequence analysis revealed a recombinant event resulted in the insertion of nucleotide sequence from the human enterovirus species C (HEVC) as shown in figures 7.2 and also on neighbour-joining tree figure 7.3.

Table 7.3: Nucleotide changes between South African Sabin viruses and Sabin reference strains.

Origin	Region	Position	Nucleotide in Sabin	Nucleotide mutations in viruses identified in suspected VAPP cases in each year								
				1996	2000	2003	2004	2006	2007	2008	2009	2010
	5' NTR	472	U	C	C	C	C		C	C		C
	5' NTR	481	A					G			G	
	5' NTR	480	G								A	

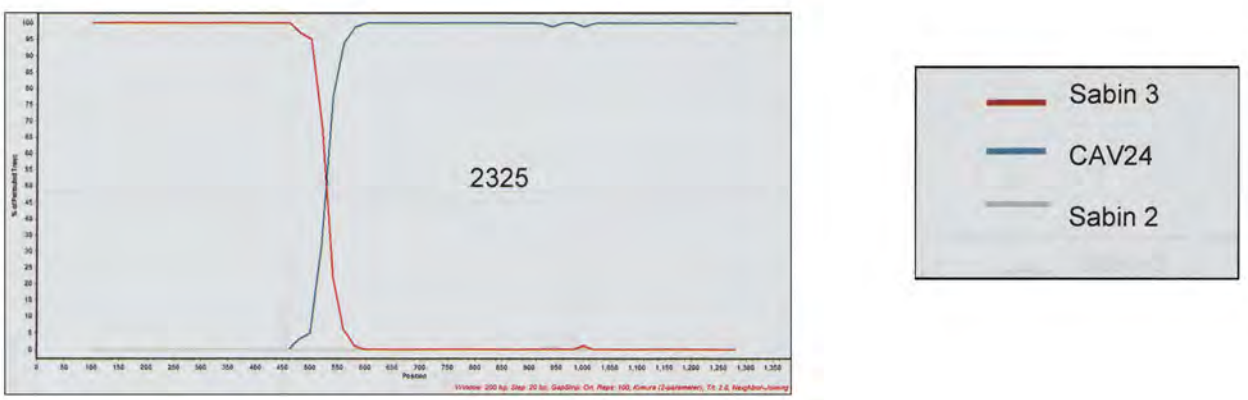
The Sabin reference strains for the 3D region were obtained from GenBank. Both RDP3 and SimPlot identified putative positions of recombination by plotting either percentage bootstrap support (RDP3, not shown) or percentage of permutated trees (SimPlot). Sequences were considered to have strong evidence of recombination if recombination were identified using a window size of 200 with a maximum bootstrap of at least 90%. Sequences identified using a window size of 100 with bootstrap percentage of <70% were considered having weak evidence of recombination.

Maximum Likelihood (PhyML) tree using Kimura two-parameter nucleotide substitution model was created. To determine the reliability of the tree topology, bootstrap resampling was performed 1000 times for each segment.

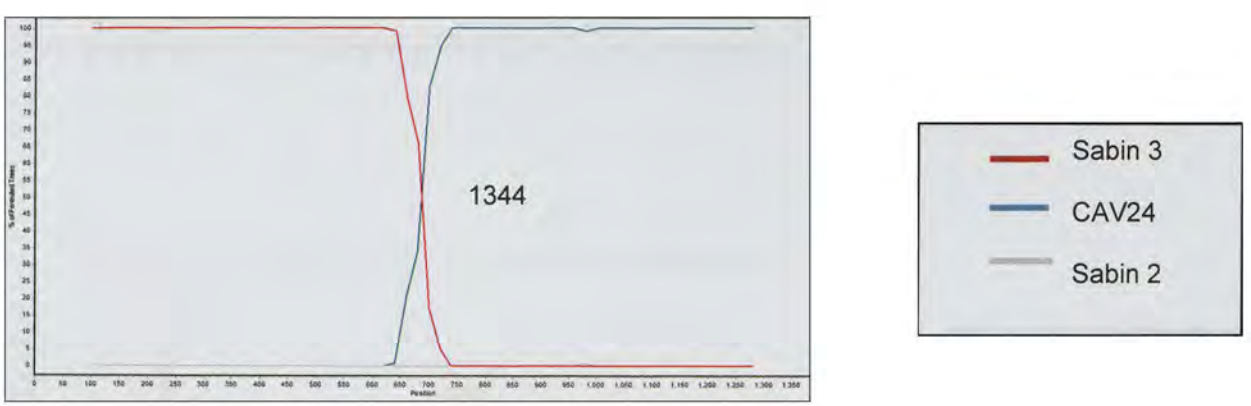
7.3.4 Pattern of recombination

Among the 6 isolates that have strong evidence of recombination, 3 patterns were observed (Figure 7.2). Isolates 2325 and 1344 shared evidence at 450 base pair (bp) and 650bp. Two other isolates (0360 and 1161) shared evidence at two sites, 400bp – 550bp and 900bp – 1030bp. Isolates 0269 and 0270 share evidence at position 200bp. Base pair position and distance options are shown along the x axis.

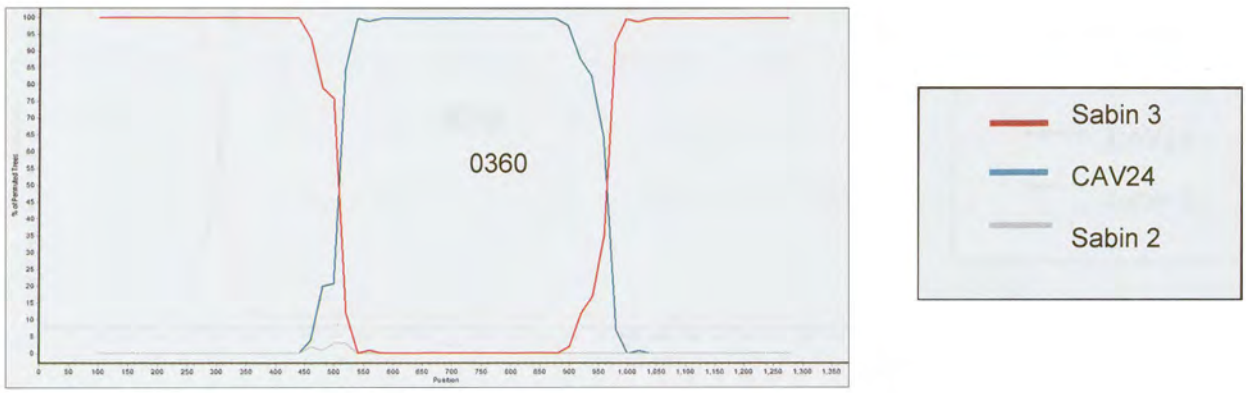
A



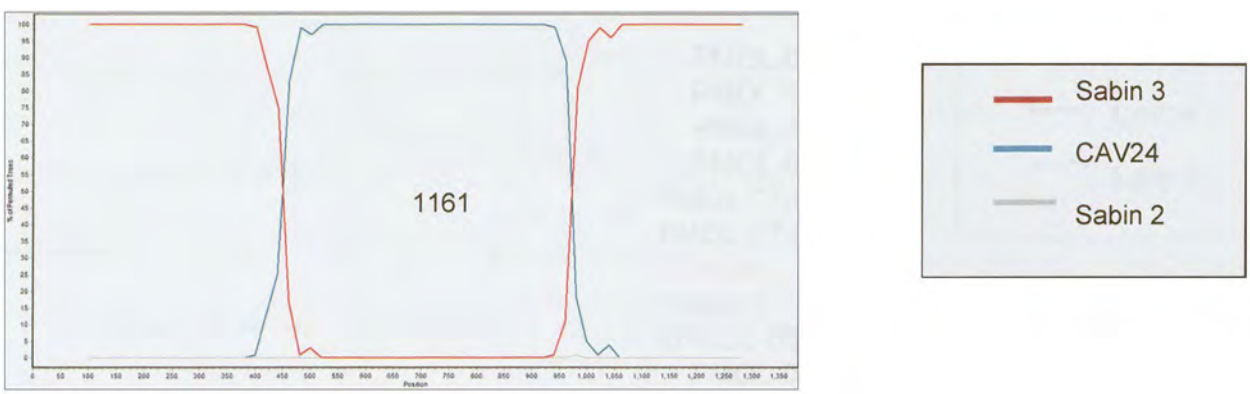
B



C


0360
Sabin 3
CAV24
Sabin 2

D


1161
Sabin 3
CAV24
Sabin 2

E


0269
Sabin 3
CAV24
Sabin 2

F

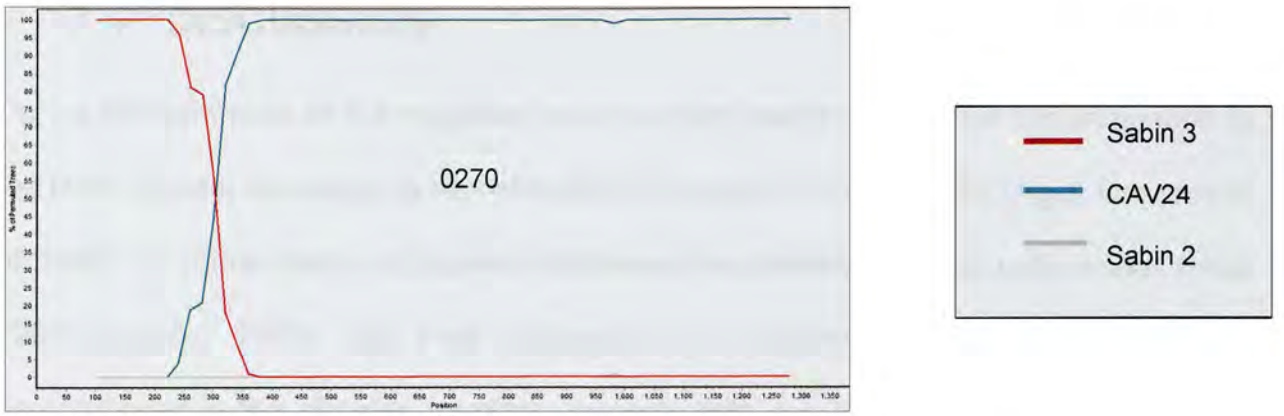


Figure 7.2 (A,B,C,D,E and F): Bootscanning results for South Africa viruses with strong evidence of recombination. The percentage of permuted trees is shown along y axis and the position is shown along the length of the sequence (x axis). The initial bootscan results were obtained using Sabin 3 reference strain. The bootscan was redone using the Coxsackie A24 (CAV24) and Sabin 2 (used as a control).

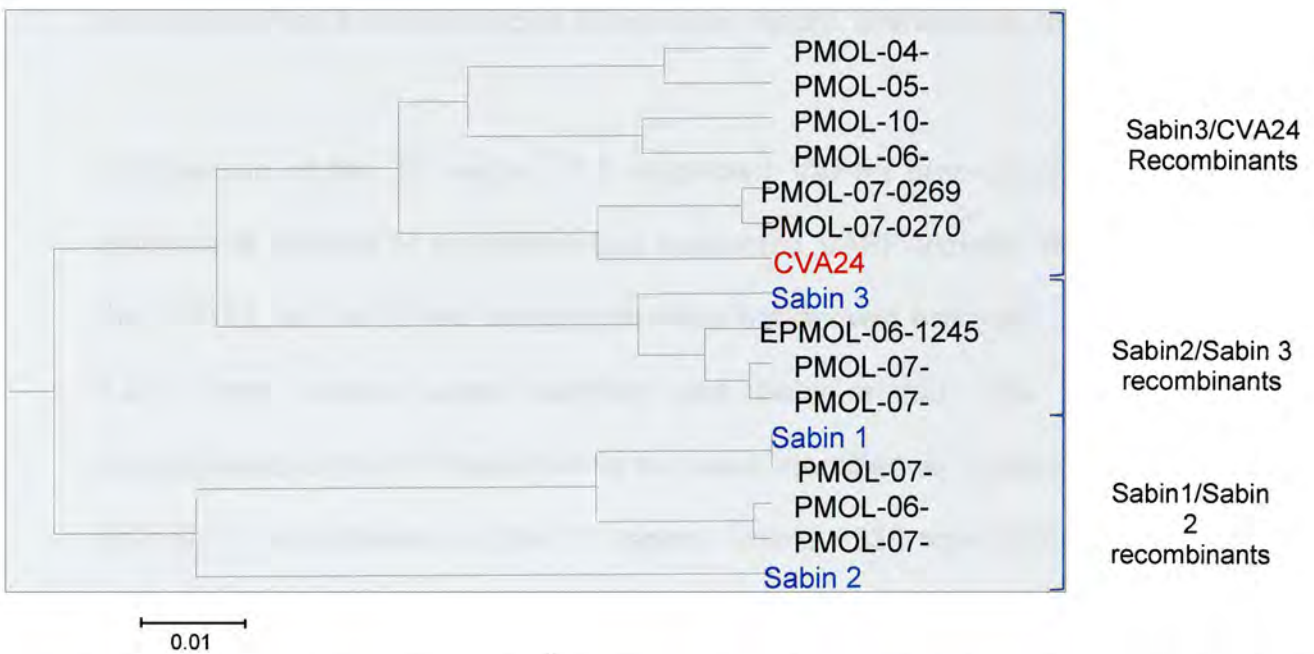


Figure 7. 3. Maximum-likelihood (PHYML) phylogenetic tree based on the 3D gene sequence of South African Sabins with recombination. Type of recombination is indicated on the right hand side. Coxsackie A 24 (CVA24) strain is highlighted in red and the Sabin reference strains are highlighted in blue.

7.4 DISCUSSION

During the synthesis of the negative strands which lead to replicative recombination in the RNA viruses, homologous recombination do occur, as reviewed by (Agol, Drozdov et al. 1985). In 1960s Hirst and Ledinko discovered the recombination of polioviruses (Hirst 1962);(Ledinko 1963) and their participation in recombination events have been demonstrated in this chapter. Intertypic recombinants can be reproducibly isolated from vaccines immunised with OPV as observed in this chapter and also explained by Minor and Cammack (Minor, John et al. 1986) (Cammack, Phillips et al. 1988). Polioviruses were not classified with HEV-C previously, and due to their genetically close relation are now reclassified into this species (Savolainen-Kopra, Samoilovich et al. 2009).

Comparison of the 3D region of 6 suspected VAPPs from South Africa with human enterovirus species C suggested that suspected VAPP viruses have recombined with the CVA24, as the 3D pol sequences were not derived from any Sabin strains (Figure 7.2). Three viruses were identified asa Sabin-related type 2/type 3 poliovirus recombinants at the 3D region while two were identified as Sabin-related type 1/type 2 poliovirus recombinants at the 3D region. Isolate 0335 was 100% similar to Sabin 1 (Figure 7.3).

The attenuated phenotype in all three Sabin strains is determined by multiple substitutions and Sabin 1 and Sabin 3 are mostly affected by the substitution in the IRES. These mutations are frequently found in vaccine–related isolates from healthy

patients (Minor and Dunn 1988; Yoshida, Horie et al. 2002) and also from patients with VAPP(Yoshida, Horie et al. 2002).

In some of the isolates describe in this chapter, a mutation at position 480 of Sabin 1, position 481 of Sabin 2 and position of 472 of Sabin 3 was observed. The recombinant virus lost the attenuated phenotype characteristics of Sabin most likely due to direct reversion of the change at these positions (Kew, Sutter et al. 2005).

As in this chapter, Sabin 3 has been associated with highest rate of suspected VAPP, probably as results of low genetic stability of the attenuating substitution, low immunogenicity (Nathanson and Martin 1979) and an intermediate paralytic attack (Strebel, Sutter et al. 1992).

Each VAPP case is not associated with the other, all cases are independent. VAPP cases occur in countries free of circulating wild poliovirus, as is estimated to 250 – 500 cases worldwide (Kew, Sutter et al. 2005). VAPP are caused by instability of the Sabin OPV strains and can be prevented by stopping of OPV usage (Alexander, Seward et al. 2004).

CHAPTER 8

CONCLUDING REMARKS

The genetic pattern of the poliovirus transmission is permitted by the rapid rate of mutation (Liu, Zheng et al. 2000; Shulman, Handsher et al. 2000), which has caused poliovirus to be one of the most rapidly evolving viruses known (Nottay, Kew et al. 1981; Gavrilin, Cherkasova et al. 2000; Liu, Zheng et al. 2000). The overall rate of virus evolution is determined by several factors, including the replication error rates, the virus population size and growth rate, the frequency of genetic bottlenecks and the mechanism of genetic exchange (Domingo and Holland 1997). Wimmer et al. has estimated error rates to be 10^4 to 10^5 per site per replication (Drake 1993; Wimmer, Hellen et al. 1993). Estimates of the rates of the total nucleotide substitution of which most are synonymous in the coding region accumulate at overall rate of approximately 10^{-2} substitution per site per year (Georgescu, Delpeyroux et al. 1995; Bellmunt, May et al. 1999; Gavrilin, Cherkasova et al. 2000; Kew, Morris-Glasgow et al. 2002; Jorba, Campagnoli et al. 2008). Evolution rates appear to be similar across all three poliovirus serotypes and between wild and vaccine-derived polioviruses. Many poliovirus clinical samples are recombinants (Cammack, Phillips et al. 1988; Kinnunen, Huovilainen et al. 1990; Liu, Zheng et al. 2000), and frequently heterotrophic recombinants are from trivalent OPV recipients (Georgescu, Delpeyroux et al. 1994). The recombination is most common in the 3D gene of the non-capsid region and less common in other regions (Georgescu, Delpeyroux et al. 1995). Molecular clock can be used to estimate the dates of the common ancestors to WPVs as in the case of Chapter 3 and VDPVs (Jorba, Campagnoli et al. 2008).

Rapid evolution appear to occur during replication in the human intestine and genomic evolution has help to facilitate molecular epidemiologic studies, permitting the identification of imported cases (Chiba, Murakami et al. 2000) and VDVPs (Gavrilin, Cherkasova et al. 2000; Cherkasova, Yakovenko et al. 2005) and to resolve different lineages during outbreaks (Shulman, Handsher et al. 2000; Shimizu, Thorley et al. 2004) as in the case of this study.

In an attempt to answer these questions, the molecular epidemiological characteristics of polioviruses associated with outbreaks of VDPVs, wild polioviruses and identification of suspected VAPPs in Africa after 2000 were investigated. This involved reporting of 1091 cases of wild polioviruses in 2005 in Africa, including 10 imported cases of India genotype to Angola, 13 cases identified in the DRC and 19 cases reported in Namibia (Chapter 2 and Chapter 3). In this study we have described the distribution and molecular epidemiology of wild-type 1 poliovirus SOAS genotype in southern Africa. The SOAS strains identified in Angola, the DRC and Namibia were unique to Africa and is estimated to have circulated at least since 2005 in Angola. Both the 2006 Namibia outbreak and the DRC outbreaks were caused by the SOAS genotype, but were introduced from neighbouring Angola most likely through frequent cross-border movement among population groups living on both sides of the border. So far the SOAS genotype appears to be limited in Africa to these three countries. Since 2006 no wild-type viruses have been reported in Namibia and it thus appears that the transmission of wild-type polioviruses had been effectively controlled by the subsequent mass vaccination campaigns. New polio cases have, however, been recently reported in both Angola and the DRC suggesting that poliovirus circulation in the other 2 countries may still be occurring.

The Angola outbreak was caused by a genotype previously identified in India, while the outbreaks from the Namibia most likely originating from Angola. This is the first time that AFP cases associated with the Indian genotype were identified in Africa. This emphasises the vulnerability of regions with suboptimal vaccination coverage for importation and reintroduction of wild-type polio virus from the remaining endemic countries.

In Madagascar (Chapter 4), analysis of all poliovirus strains confirmed cases of cVDPVs in 2001/2002 and 2005 and suggest that the molecular epidemiological characteristics of each outbreak were different. The circulation of cVDPV in Madagascar differs from previous cVDPV outbreaks in that it was caused by both type 2 and type 3. This is the second time that type 2 cVDPV had caused an outbreak in Madagascar, and to our knowledge the first time that a type 3 cVDPV has been identified through AFP in Madagascar. The additional finding of type 3 cVDPV further emphasizes the serious implications for the Global Polio Eradication Initiative for stopping immunization once eradication has been achieved. In Madagascar and other countries affected by cVDPV outbreaks, OPV coverage rates were particularly low and nearly all of the case patients and contacts were unimmunized or incompletely immunized children (Kew, Morris-Glasgow et al. 2002) (Rousset, Rakoto-Andrianarivelo et al. 2003).

Following the Madagascar cVDPV, the DRC reported 32 confirmed cVDPV cases (Chapter 5). This is the first time that type 2 cVDPVs were detected in the DRC. The occurrence of VDPV outbreaks during the same period with WPV emphasize the need to maintain high OPV coverage and AFP surveillance in order to minimize the risk of emergence of VDPVs or of circulation of imported WPVs.

To summarise, Chapter 4, 5 and 6 reported vaccine-derived polioviruses (having > 1% VP1 nucleotide sequence divergence from the Sabin parental strain) from Madagascar, the DRC and Ethiopia since 2005 until 2011. Failure to interrupt these outbreaks jeopardizes the most significant achievement of the Polio Eradication Initiative to date, the global interruption of transmission of wild serotype 2 viruses since 1999. In terms of virology, the VDPVs show reversion of mutations associated with attenuation of the Sabin virus, and transmission now represents uninterrupted circulation and evolution of the wild serotype 2 parental strain.

In Ethiopia two outbreaks of both cVDPVs type 2 and cVDPVs type 3 were in this study. The era of wild polioviruses is rapidly drawing to a close. In a short time it appears likely that the only source of poliovirus infection worldwide will be from OPV. Successful navigation from the current pre-eradication era to the imminent post-OPV era and beyond requires surmounting an unprecedented series of public health challenges. The first step is the elimination of the last reservoirs of wild poliovirus circulation. Soon thereafter, implementation of the post eradication endgame strategy can begin. Implementation of this crucial phase of polio eradication requires a more detailed assessment of the risks of VDPV emergence in various settings (especially those at highest risk); a clearer understanding of the biological properties of VDPVs; reinforcement of global poliovirus surveillance; development of effective means to clear long-term iVDPV infections; establishment of appropriately formulated, sized, and positioned OPV stockpiles; and completion of poliovirus containment worldwide. Moving forward will continue to require the best efforts of the global public health and scientific communities.

The cVDPV findings have important implications for the Global Polio Eradication Initiative and for future policies about OPV immunization (Kew, Morris-Glasgow et al. 2002; Rousset, Rakoto-Andrianarivelo et al. 2003) (Yang, Naguib et al. 2003). OPV has been very effective in decreasing poliomyelitis cases, however its concerns regarding collateral effects has been increasing in recent years due to identification of cVDPV.

In Chapter 7, 10 cases of possible VAPP and one suspicious case of possible VAPP were identified. Sabin-like cases described in this chapter had mutation at the 5' NTR at position 480, 481 and 472 for Sabin 1, Sabin 2 and Sabin 3 respectively. The VP1 gene revealed similar sequences to that of their respective Sabin reference strain (data not shown). Recombination with either CAV24 and other Sabin-like strains have been confirmed in some of the cases. Relationship between all cases indicates an intense co-circulation and a rapid co-evolution between OPV strain and indigenous CAV24. As in Chapter 4, this report emphasise the rapid evolution between polioviruses and CAV24 by multi recombination events.

What need to be done:

The molecular data collected in this study has helped in dealing with the outbreak identified during this study, but there is still a need of studies to be conducted to estimate the potential for VDPV to persist among immunodeficient persons in the developing world. Such studies should measure the prevalence and duration of chronic poliovirus excretion in children with recurrent infections.

It has not yet been determined how often recombination occurs in the Sabin-like viruses identified in areas with low vaccine coverage in countries where VDPVs have not yet been identified.

Knowledge of the frequency of these events may help to further plan strategies to prevent emerging VDPVs from causing renewed outbreaks after termination of the oral poliovirus vaccination program.

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APPENDIX A

Summary of AFP surveillance performance indicators, Ethiopia, 2006 -2010

Indicators	Target	2006	2007	2008	2009	2010
NP-AFP rate per 100,000 < 15 Yrs	2	2. 2	2. 4	2. 9	2. 6	2. 4
Stool adequacy	80%	88%	87%	86%	86%	78%
Investigated < 2 days of notification	80%	97%	96%	95%	98%	99%
Specimen arriving at lab < 3 days	80%	98%	98%	99%	99%	99%
Specimen arriving in good condition	90%	99%	100%	99%	100%	85%
Non-polio enterovirus isolation rate	10%	15%	20%	10%	10%	5%
Timely Lab result within 14 days of receipt	80%	99%	97%	88%	90%	99%

APPENDIX B

Poliovirus sequences submitted to the GenBank

(Chapter 2)

ISOLATE	ACCESSION NO.
ANG-LUA-KIL-07-003	EU046199
ANG-LUA-MAI-07-004	EU046200
ANG-HUI-MAT-06-003	EU046201
ANG-CUN-OMB-06-004	EU046202
ANG-LUA-CAC-05-003	EU046203
ANG-BEN-LOB-05-003	EU046204
ANG-BEN-LOB-05-008	EU046206
NAM-KHO-WHK-06-062	EU046207
NAM-KHO-WHK-06-029	EU046208
NAM-KHO-WHK-06-194	EU046209
NAM-KHO-WHK-06-018	EU046210
NAM-OTJ-OKH-06-167	EU046211
NAM-HAR-ARA-06-015	EU046212
NAM-OHA-ENG-06-054	EU046213
NAM-KHO-WHK-06-022	EU046214
RDC-KOC-TKP-06-004	EU046215
RDC-BDD-KIR-07-004	EU046217

RDC-EQT-MBD-07-001	EU046218
RDC-EQT-ING-07-002	EU046219
RDC-EQT-BOE-07-001	EU046220
RDC-EQT-BLB-07-001	EU046221
RDC-BDD-KIR-07-001	EU046222
RDC-BDD-INO-07-002	EU046223
RDC-BDD-INO-06-013	EU046224
RDC-BCG-BOM-06-001	EU046225
RDC-BCG-SEK-06-004	EU046226
RDC-BCG-MAT-06-001	EU046227

(Chapter 2)

ISOLATE	**ACCESSION NO.**
PMOL060399	GU951778
PMOL060531	GU951779
PMOL060545	GU951780
PMOL060625	GU951781
PMOL060694	GU951782
PMOL060698	GU951783
PMOL060701	GU951784
PMOL061008	GU951785
MOL061189	GU951786

PMOL070092	GU951787
PMOL070125	GU951788
PMOL070248	GU951789
PMOL070250	GU951790
PMOL070507	GU951791
PMOL070538	GU951792
PMOL070791	GU951793
PMOL070798	GU951794
PMOL070850	GU951795
PMOL070917	GU951796
PMOL070936	GU951797
PMOL071053	GU951798
PMOL071247	GU951799
PMOL080167	GU951800
PMOL080333	GU951801
PMOL080766	GU951802
PMOL080975	GU951803

Chapter 3)

ISOLATE	ACCESSION NO.
PMOL/05/0781	EF420856
PMOL/05/0994	EF420857
PMOL/05/0995	EF420858
PMOL/05/0997	EF420859
PMOL/05/1342	EF420860
PMOL/05/1344	EF420861
PMOL/05/1345	EF420862
PMOL/05/1340	EF420864
PMOL/05/1346	EF420865
PMOL/05/1347	EF420866
PMOL/05/1348	EF420867
PMOL/05/2060	EF420868
PMOL/05/1602	EF420869
PMOL/05/1606	EF420870
PMOL/05/2054	EF420871

ISOLATE	ACCESSION NO.
RDCKA80948	HM134095
RDCKA80758	HM134096
RDCKA80629	HM134097
RDCKA80760	HM134098
RDCKA80921	HM134099
RDCKA81093	HM134100
RDCKA90587	HM134101
RDCKO80379	HM134102
RDCKA80374	HM134103
RDCKA81301	HM134104
RDCKA80526	HM134105
RDCSK70269	HM134106
RDCEQ90167	HM134107
RDCBD52225	HM134108
RDCBD52491	HM134109
RDCOR70043	HM134110
RDCKA81088	HM134111
RDCKA80134	HM134112
RDCKA81208	HM134113
RDCKA81304	HM134114
RDCKA90585	HM134115

APPENDIX C

ETHICS APPROVAL

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

* **FWA** 00002567, Approved dd 22 May 2002 and Expires 13 Jan 2012.
* **IRB** 0000 2235 IORG0001762 Approved dd 13/04/2011 and Expires 13/04/2014.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee
Fakulteit Gesondheidswetenskappe Navorsingsetiekkomitee

DATE: 29/07/2011

PROTOCOL NO.	**109/2011**
PROTOCOL TITLE	Molecular characterisation of wild-type and Sabin -like polioviruses circulating in Africa after the year 2000
INVESTIGATOR	**Principal Investigator:** Heronyma Nelisiwe "Nicksy" Gumede-Moeletsi
SUBINVESTIGATOR	Not Applicable
SUPERVISOR	Prof M Venter **E-Mail:** marietjiev@nicd.ac.za
DEPARTMENT	**Dept: Molecular Virology** **Phone:** 0113866390 **Fax:** 0115550531 **E-Mail:** nicksyg@nicd.ac.za **Cell:** 0836622708
STUDY DEGREE	PhD (Medical Virology)
SPONSOR	None - UP postgraduate Scholarship
MEETING DATE	**27/07/2011**

The Protocol was approved on 27/07/2011 by a properly constituted meeting of the Ethics Committee subject to the following conditions:

1. The approval is valid for 1 year period [till the end of December 2012] . and
2. The approval is conditional on the receipt of 6 monthly written Progress Reports, and
3. The approval is conditional on the research being conducted as stipulated by the details of the documents submitted to and approved by the Committee. In the event that a need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

Members of the Research Ethics Committee:

Prof M J Bester	(female)BSc (Chemistry and Biochemistry); BSc (Hons)(Biochemistry); MSc(Biochemistry); PhD (Medical Biochemistry)
Prof R Delport	(female)BA et Scien. B Curationis (Hons) (Intensive care Nursing). M Sc (Physiology). PhD (Medicine), M Ed Computer Assisted Education
Prof JA Ker	MBChB; MMed(Int); MD – Vice-Dean (ex officio)
Dr NK Likibi	MBB HM – Representing Gauteng Department of Health) MPH
Prof TS Marcus	(female) BSc(LSE), PhD (University of Lodz, Poland) – Social scientist
Dr MP Mathebula	(female)Deputy CEO: Steve Biko Academic Hospital; MBCHB, PDM, HM
Prof A Nienaber	(female) BA(Hons)(Wits); LLB; LLM; LLD(UP); PhD; Dipl.Datametrics(UNISA) – Legal advisor
Mrs MC Nzeku	(female) BSc(NUL); MSc(Biochem)(UCL, UK) – Community representative
Prof L M Ntlhe	MbChB (Natal) FCS (SA)
Snr Sr J Phatoli	(female) BCur(Eet.A), BTec(Oncology Nursing Science) – Nursing representative
Dr R Reynders	MBChB (Pret). FCPaed (CMSA) MRCPCH (Lon) Cert Med. Onc (CMSA)
Dr T Rossouw	(female) MBChB (cum laude); M.Phil (Applied Ethics) (cum laude), MPH (Biostatistics and Epidemiology (cum laude), D.Phil

Dr L Schoeman	(female) B.Pharm, BA(Hons)(Psych), PhD – Chairperson: Subcommittee for students' research
Mr Y Sikweyiya	MPH; SARETI Fellowship in Research Ethics; SARETI ERCTP; BSc(Health Promotion)Postgraduate Dip (Health Promotion) – Community representative
Dr R Sommers	(female) MBChB; MMed(Int); MPharmMed – **Deputy Chairperson**
Prof TJP Swart	BChD, MSc (Odont), MChD (Oral Path), PGCHE – School of Dentistry representative
Prof C W van Staden	MBChB; MMed (Psych); MD; FCPsych; FTCL; UPLM - **Chairperson**

DR R SOMMERS; MBChB; MMed(Int); MPharmMed
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria