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.

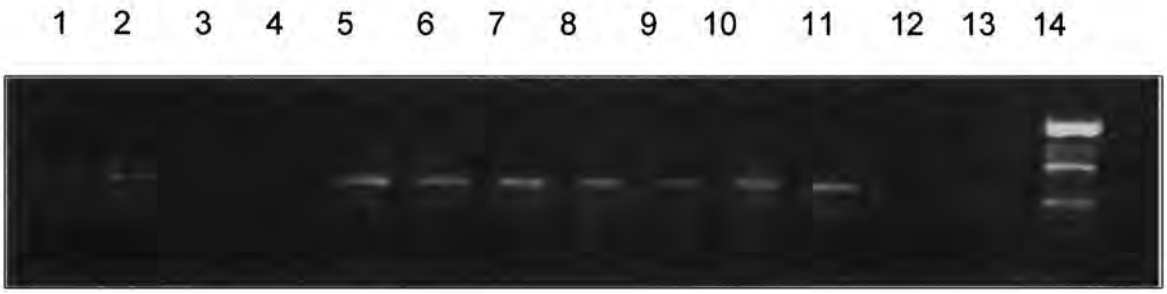
Year	LabID	ITD_Results
1995	D/95/1380	Sabin1
1996	D/96/0803	Sabin1
1996	D/96/0382	Sabin3
1996	PMOL961374	Sabin3
1996	D/96/1379	Sabin3
1996	D/96/1417	Sabin3
1996	D/96/1419	Sabin3
1996	PMOL961431	Sabin3
1996	D/96/1432	Sabin3
1996	D/96/1288	Sabin2
1997	D/97/2303	Sabin2
1997	D/97/2306	Sabin2
1998	D/98/0005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

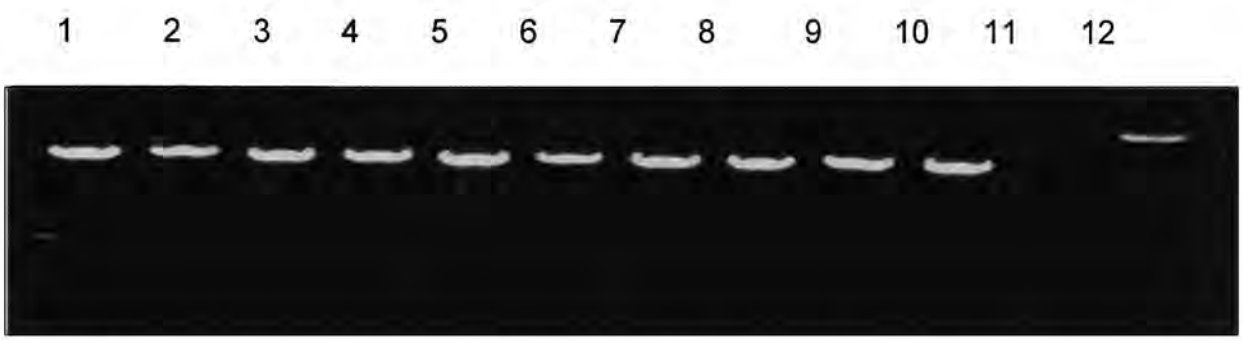


Figure 7.1. Ethidium bromide-stained 1.5% agarose gel showing amplification of the (A) 5' NTR and (B) VP1 regions of poliovirus type 3 strains. Sabin 3 used as a positive controls (+ve) and negative controls (-ve) are reactions without template. Lanes: 1. PMOL070911, 2. PMOL100360, 3. PMOL080728, 4. 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	

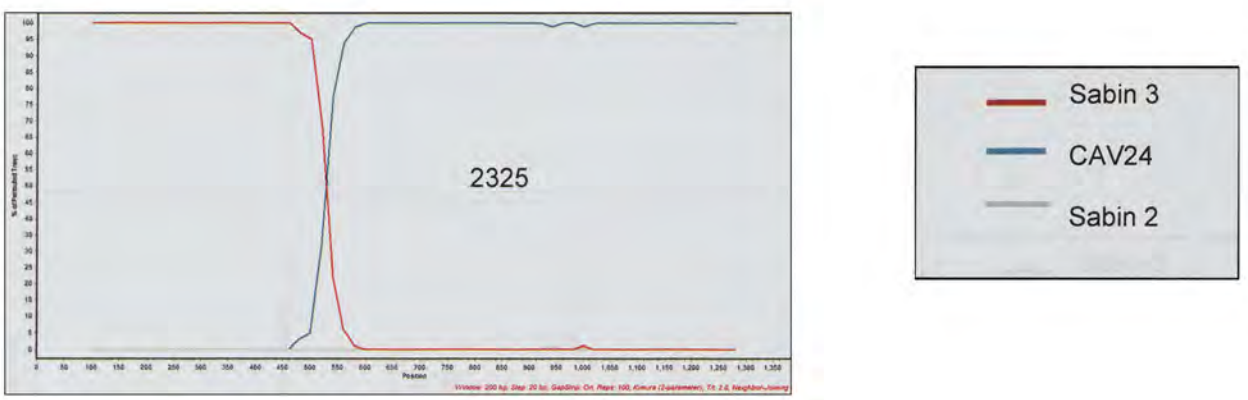
The Sabin reference strains for the 3D region were obtained from GenBank. Both RDP3 and SimPlot identified putative positions of recombination by plotting either percentage bootstrap support (RDP3, not shown) or percentage of permutated trees (SimPlot). Sequences were considered to have strong evidence 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.

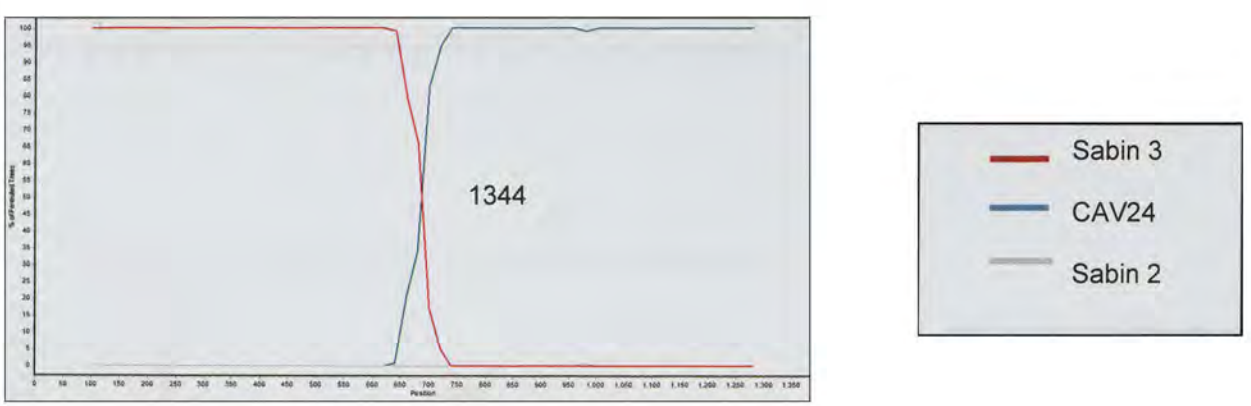
7.3.4 Pattern of recombination

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.

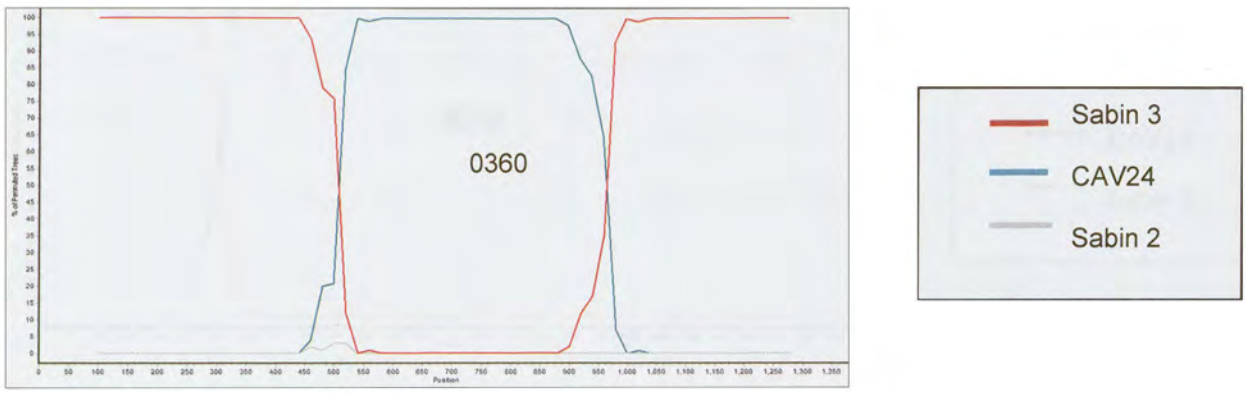
A



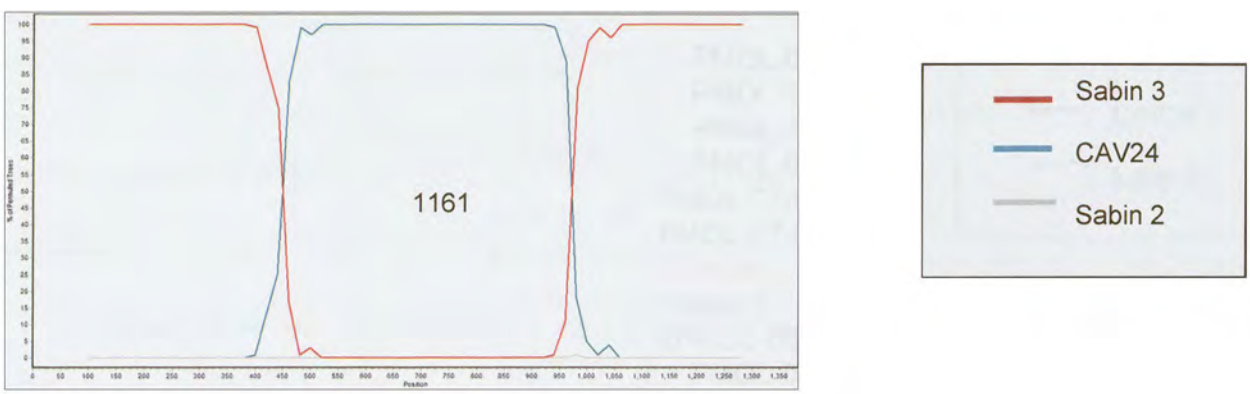
B



C


0360
Sabin 3
CAV24
Sabin 2

D


1161
Sabin 3
CAV24
Sabin 2

E


0269
Sabin 3
CAV24
Sabin 2

F

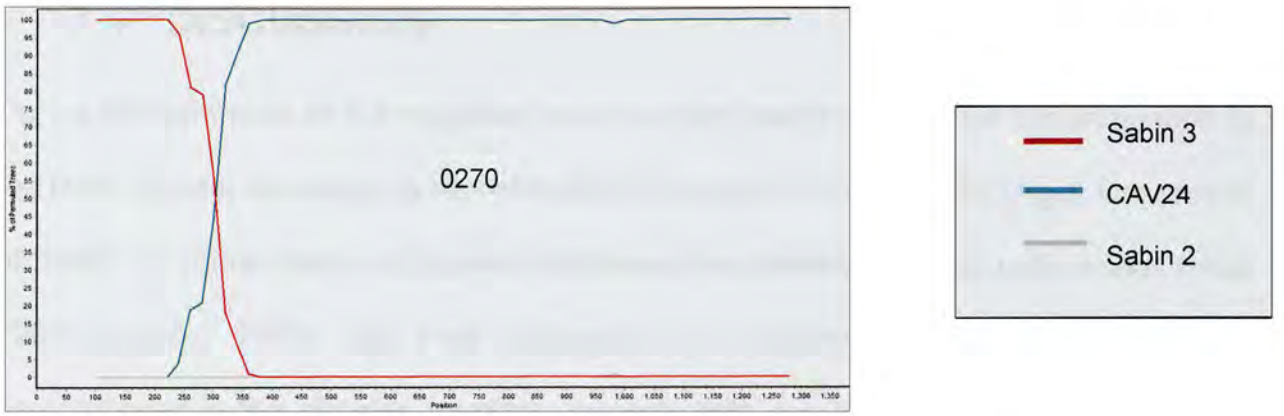


Figure 7.2 (A,B,C,D,E and F): Bootscanning results for South Africa viruses with strong evidence of recombination. The percentage of permuted trees is shown along y axis and the position is shown along the length of the sequence (x axis). The initial bootscan results were obtained using Sabin 3 reference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

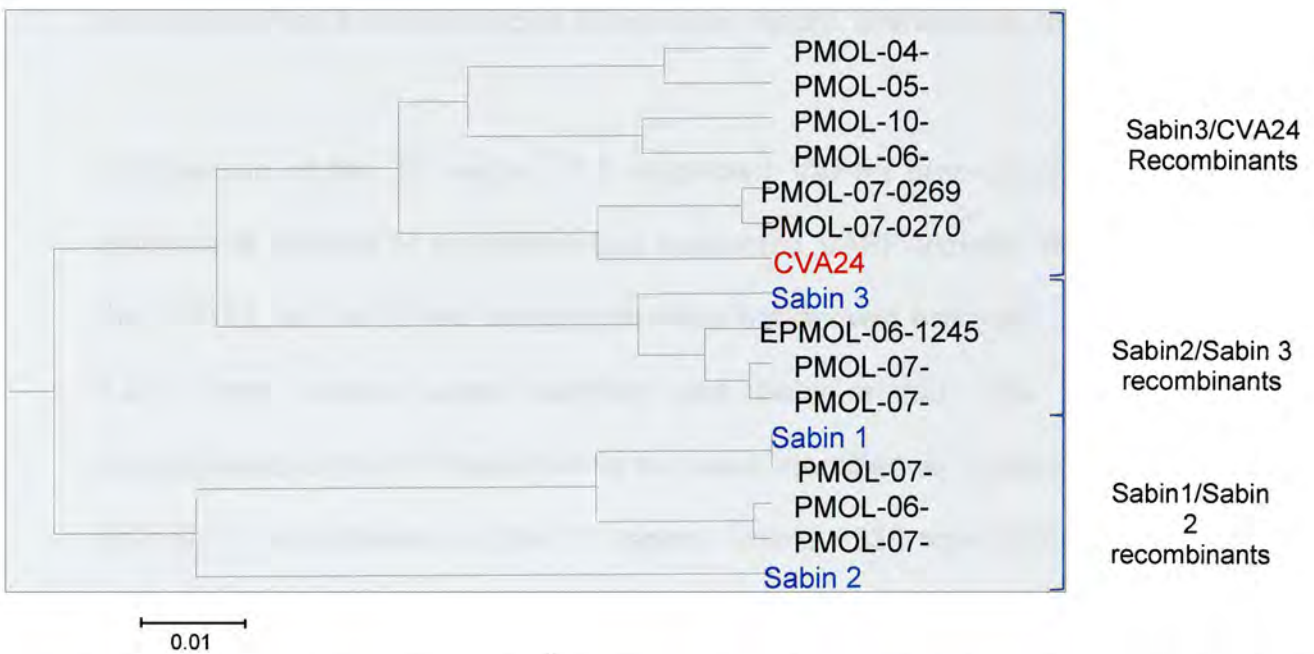


Figure 7. 3. Maximum-likelihood (PHYML) phylogenetic tree based on the 3D gene sequence of South African Sabins with recombination. Type of recombination is indicated on the right hand side. Coxsackie A 24 (CVA24) strain is highlighted in red and the Sabin reference strains are highlighted in blue.

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).