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

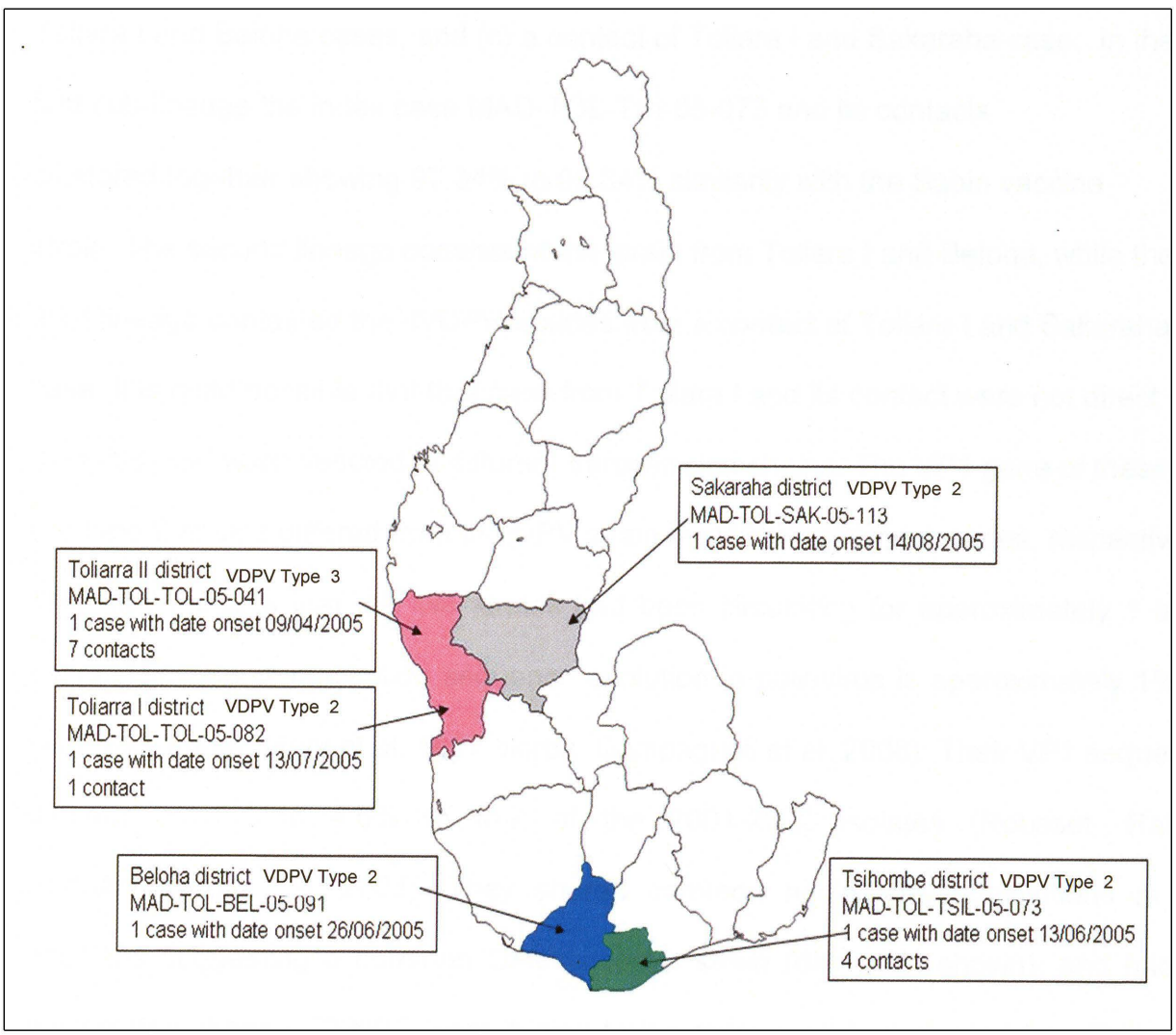

Figure 4.1 A Madagascar map showing geographical distribution of the cases.

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.

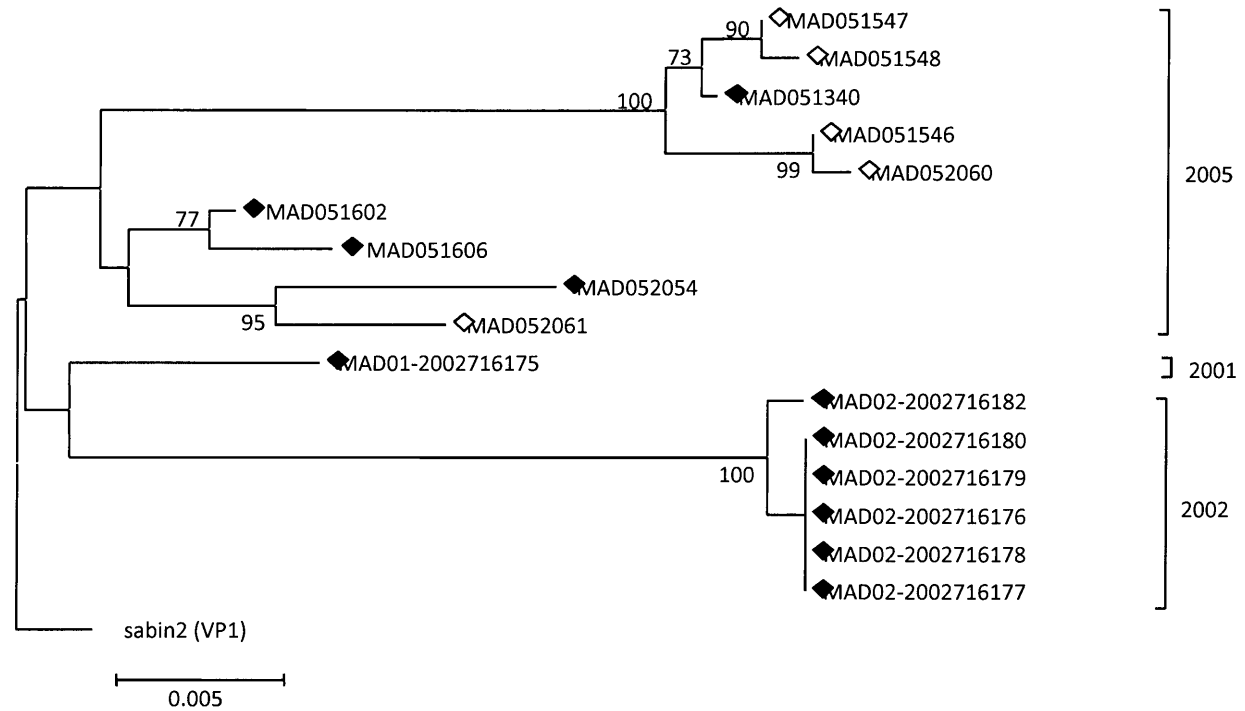

Figure 4.2. Neighbour-joining phylogenetic tree of circulating vaccine-derived polioviruses VP1 gene nucleotide sequences (903 bp). Numbers at branches nodes refer to the number of bootstrap repetitions (of 1000) at which the distal sequences grouped together. Closed diamonds ◆ represent AFP cases, open 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%.

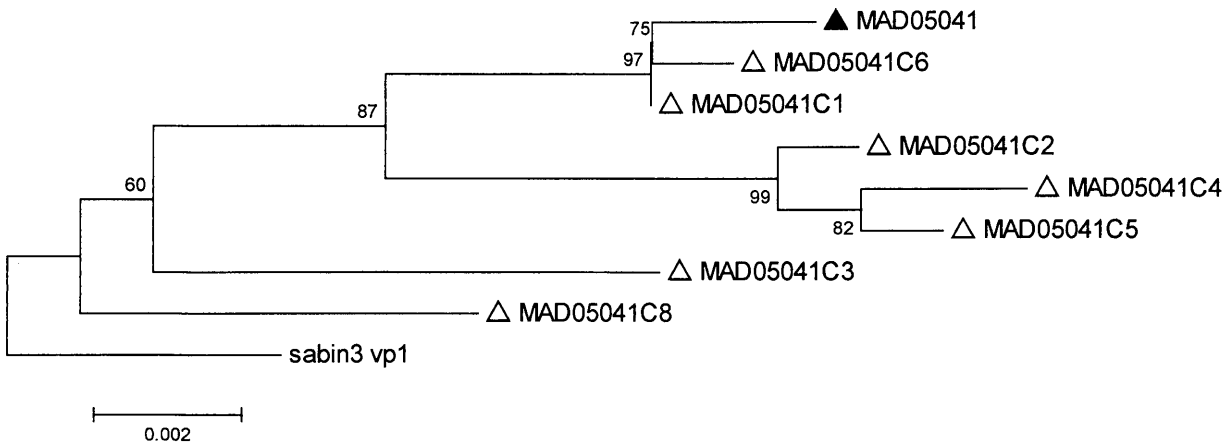

Figure 4.3. Neighbour-joining phylogenetic tree of circulating vaccine-derived polioviruses VP1 gene nucleotide sequences (900 bp). Numbers at branches nodes refer to the number of bootstrap repetitions (of 1000) at which the distal sequences grouped together. Closed diamonds ▲ represent AFP cases, open 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

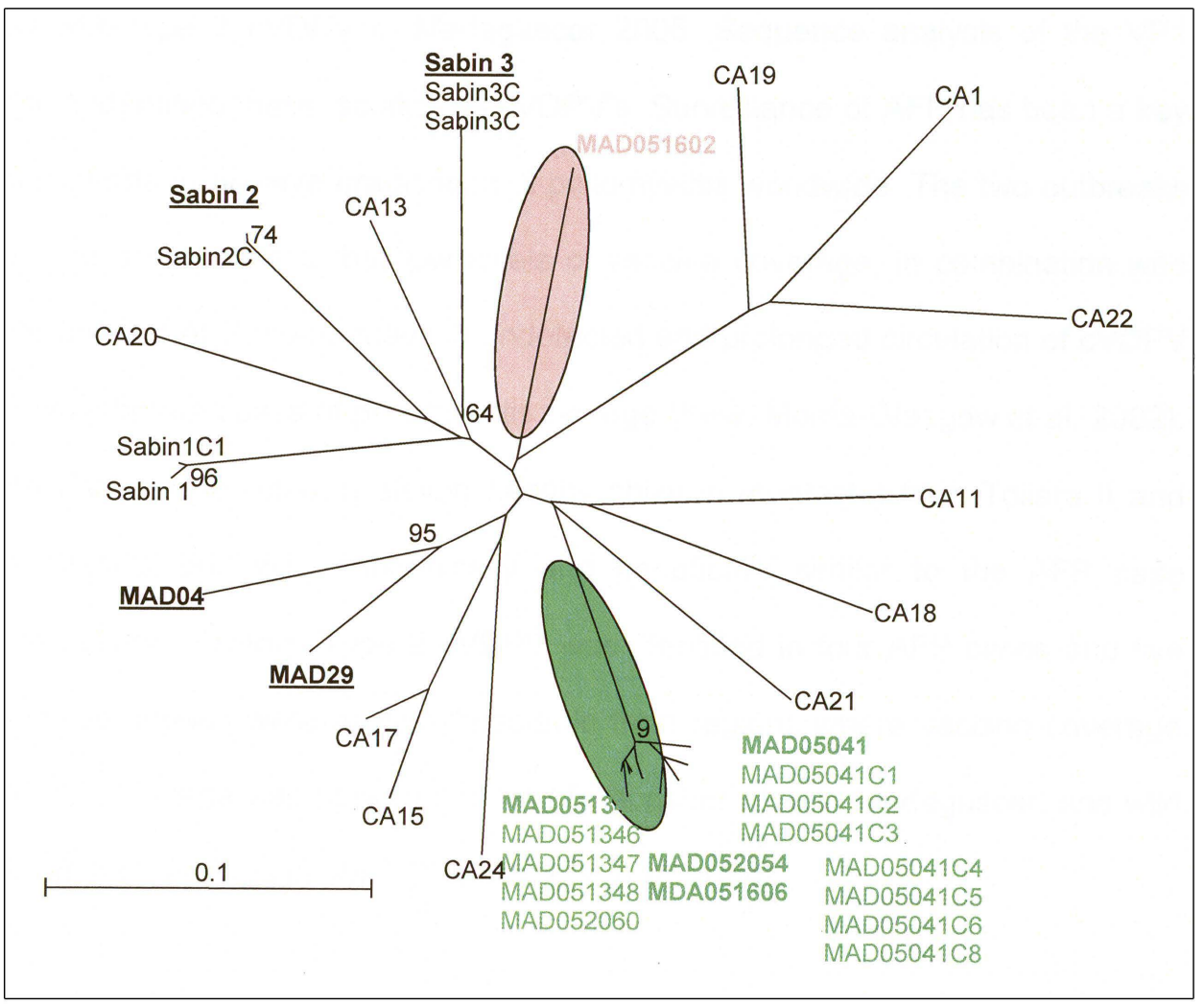

Figure 4.5. The unrooted neighbour-joining tree of the 3D region (nt 6145 – 6507) demonstrating relationship with unknown human enterovirus C species (HEV-C).GenBank accession numbers HEV-C used are: AF499635=CA1; AF499636=CA11; AF499637=CA13; AF499638=15; AF499639=CA17; AF499640=CA18; AF499641=CA19;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