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Distribution of wild poliovirus genotypes in India

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In order to study the genetic relationships and epidemiological links between strains circulating in the Vellore region of India, 70 wild type poliovirus strains isolated from paralytic poliomyelitis cases were sequenced at the VP1-2A junction region of the viral genome. This showed that three genotypes of types 1 and 2 and four genotypes of type 3 poliovirus were circulating in India at least since 1985. Different clusters were identified within wild genotypes. This study demonstrates the endemic circulation and wide genetic variation of all three serotypes in Southern India. Knowledge of wild poliovirus distribution before NIDs beginning could be helpful for a critical monitoring of poliomyelitis control and further evaluation of the NIDs impact on wild poliovirus transmission in India.

Key words: Poliovirus, poliomyelitis, genotype, molecular epidemiology, eradication, surveillance.

INTRODUCTION

In 1988, the World Health Assembly established the goal of global poliomyelitis eradication by the year 2000. Since then, substantial progress has been reported from all WHO regions as a result of the implementation of WHO recommended strategy. In the South-east Asia region, the number of reported cases decreased by 96% from 25711 cases in 1988 to 1116 in 1996. This sharp decrease in reported cases primarily reflects improved control of poliomyelitis in India, 1996 population, 952 969 000, that is, 76% of the region's population (Anonymous, 1997a). During the implementation of India's first NIDs, an astronomical 87.8 million and 93.6 million children received one dose of OPV on December 9th, 1995 and chain reaction; RT, reverse transcription; PV, polio virus; January 20th, 1996, respectively (Misra and Banerjee, 1996). Following this, reported cases decreased by 69% from 3263 cases in 1995 to 1005 cases in 1996 (Anonymous, 1997b).

While the trend of increasing immunization coverage of OPV or IPV in most countries will continue and lead to improved control of poliomyelitis, the goal of eradication might be delayed due to one of the following problems : i) Outbreaks of poliomyelitis due to importation of alien strains or emergence of new variants among native strains (Hull et al., 1994), ii) vaccine failures in immunized children due to variant wild type strains which are significantly antigenically distinct from vaccine strains, iii) Vaccine associated paralysis in a small proportion of OPV vaccines (Almond et al., 1987; Racaniello, 1988).

Conventional laboratory based techniques are limited in addressing the problems mentioned previously. Detailed genetic characterization of poliovirus isolates is important to define the predominant circulating strains, natural genetic variation in and between geographical areas and the circulation of poliovirus strains in human populations. Documentation of these factors in every geographical area will aid in investigations of the origin and nature of outbreaks of poliomyelitis. The epidemiological significance

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Abbreviations: IPV, Inactivated polio vaccine; **OPV,** oral polio vaccine; **WHO,** World Health Organization; **RFLP,** restriction fragment length polymorphism; **PCR,** polymerase chain reaction; **NID,** national immunization day.

Table 1. Detail of Pasteur Institute monoclonal antibodies for polioviruses

Mab to poliovirus	Mab specific to Serotype (K)	Strain (V)	
		Sabin (VS)	Wild (VW)
Type 1	1c	1o	Io
	2b		Ic
	C3		Id
			Ia
Type 2	IIb	2d	IIo
	2b	2c	IIa
	2o		
	2a		
Type 3	3a	3o	IIIo
	3b		
	IIIa		

MAbs Ic, Ib and C3 were serotype specific and raised against the antigenic site 2c, 2a and 1a, respectively. MAbs IIb and 2b were serotype specific and raised against the antigenic site 1a. Mabs 3a and IIIa were specific for type 3 Sabin vaccine strain and prototype wild strain saukett respectively. The Sabin 3 specific MAbMab 3o was raised against the antigenic site 3b. For the MAbMab IIIo antigenic site specification was not known.

of antigenic variation in poliovirus strains was documented during the type 3 poliovirus outbreak in Finland in 1984 (Hovi, 1993). It is also important for differentiating the wild or vaccine origin of a particular isolate from a polio affected child.

Since 1988, important progress has been made in polio eradication programs in India. If the goal of elimination of poliomyelitis from India is to be achieved, states where polioviruses continue to circulate endemically need to be identified and their patterns of spread of wild type viruses determined. Antigenic and genomic characterization of wild type virus before beginning of NIDs would be helpful for both monitoring of poliomyelitis control and valuation of the NIDs impact on wild poliovirus transmission in India for determining if strains have emerged from a local reservoir or have been imported.

In this study, we report the antigenic and genetic characterization of polioviruses isolates from 1985 to 1993 in various part of South India using neutralization with serotype specific monoclonal antibodies. We examine circulation of wild type polioviruses strains through PCR-RFLP screening addressing poliovirus genome segments in the capsid protein VP1 (RFLP1, 480 nt fragment). This was followed by encoding region and nucleotide sequencing to determine relatedness between RFLP patterns and genomic sequences of the 1987 epidemic isolates and non epidemic strains isolated from 1985 to 1993. Relatedness to other wild type polioviruses isolated in this region and from elsewhere is subsequently analyzed.

MATERIALS AND METHODS

From January 1985 to December 1993, a total of 540 specimens (stool, throat or rectal swab) from children with acute flaccid paralysis were submitted for virologic investigations to the Department of Virology, Christian Medical College and Hospital, Vellore, Tamilnadu.

Virus isolation and characterisation

Primary isolation of the virus was done in primary bonnet monkey (*Macaca radiata*) kidney cells and primary identification and serotyping of PV were done by neutralization with type specific hyperimmune sera as previously described (Melnick, 1990). The neutralizing MAbs panels for all three types used in this study were obtained from Pasteur Institute, Paris, and from the National Institute for Biological Standard and Control, London (NIBSC). A detailed description of the MAbs and their specific reaction patterns has been reported elsewhere (Crainic et al., 1981; Fergusson et al., 1982) and are summarized in Table 1. Two other MAbs provided by the NIBSC were used for the antigenic typing. The MAb 11 is Sabin strain - specific while the MAb 16 is serotype specific. A panel of 5 Indian MAbs namely In3-1, In3-6, In3-7, In35-1 and In35-4 were raised against a dominant circulating poliovirus type 1 wild strain isolated in the study area. For genomic analysis, the original isolates were passed once or twice in HEp2c cells.

Table 2. Age distribution of children with poliomyelitis.

Age (months)	Reported cases in					
	1985-1989			1990-1993		
	N°	(%)	Cum. (%)	N°	(%)	Cum. (%)
0 - 11	96	35.2	35.2	21	22.6	22.6
12 - 23	74	27.1	62.3	24	25.8	48.4
24 - 35	46	16.8	79.1	16	17.2	65.6
36 - 47	28	10.3	89.4	9	9.7	75.3
48 - 56	8	2,9	92.3	10	10.8	86.1
>60	21	7.7	100.0	13	14.0	100.0

Table 3. Immunization status of children with poliomyelitis (1985 - 1993).

OPV doses	Reported cases with paralytic poliomyelitis in			
	1985-1989		1990-1993	
	N°	(%)	N°	(%)
0	116	42.5	20	21.5
1	41	15.0	13	14.0
2	29	10.6	9	9.7
3	87	31.9	51	54.8
Total	273	100.0	93	100.0

Genomic analysis

RFLP assays were carried out as described by Balanant et al. (1991). Sequence alignment was done using the Clustal W program (Gibson et al., 1994). Different dendrograms of sequence relatedness were constructed using distance matrix, maximum parsimony and maximum likelihood methods. Distance matrix was calculated using the Kimura 2 and Kimura 10 parameters method as implemented in the program DNADIST. The rate of transition/tranversion was set as 2.0. Different tree reconstruction procedures were done using UPGMA, FITCH least-squares method with evolutionary clock and Neighbor Joining methods. The statistical significance of the branching order was estimated by performing 100 replications of bootstrap resampling of the original alignment using SEQBOOT and synthesizing the resulting set of trees using CONSENSE. Phylogenic tree was drawn using TreeView (Page, 1996). All phylogenic analysis programs are included in the PHYLIP 3.5 package (Felsenstein, 1993).

RESULTS

The majority of cases were from Tamil Nadu (80%), Andhrapradesh and neighboring states. The age distribution of 366 cases is given in Table 2 and immunization status of the children with poliomyelitis is shown in Table 3.

From 540 specimens collected from children with acute flaccid paralysis in Southern India from 1985 to 1993, poliovirus was isolated in 286 cases. PV type 1, type 2 and type 3 were isolated in 218, 23 and 45 cases, respectively.

One hundred and twenty poliovirus type 1, 19 type 2 and 31 type 3 isolates were obtained from all sources and were characterized either as wild-type or vaccine-derived by MAb neutralizing test and RFLP. All type 1 strains isolated from paralytic cases were classified as wild type strains, based on their antigenic properties, except for four strains isolated in 1992. Non concordant neutralization index was observed for thirteen strains, while using MAbs raised again an Indian wild strain; these strains were not neutralized by these MAbs (Table 4). Two Sabin 2 like strains were isolated in 1989 and the remaining 17 were classified as wild type. Two Sabin 3 like strains were isolated within the type 3 strains in 1991. Except these two strains, all the others tested strains were classified as wild strains. These results were confirmed when the genomes of the strains were characterized by three different RFLP tests which analyze genomic segments corresponding to the capsid protein VP1 (RFLP-1) and VP1-2A junction (RFLP1-2A).

For determining PV relationship and genealogy, comparative genomic sequencing of a 150 nucleotide segment in the VP1-2A coding region has been considered to be a powerful approach. Strains showing a sequence identity of more than 85% are considered to belong to the same genotype whereas a sequence identity of at least 98% characterize strains with direct epidemiological link (Rico-Hesse et al., 1987).

A graphical representation of the sequence relationships between wild polioviruses determined by the genetic distance between strains is shown in Figures 1, 2 and 3 for type 1, 2 and 3, respectively. Bootstrap values greater than 70% were considered significant and are mentioned in Figure 1, 2 and 3. All the genotypes in the VP1-2A tree were stable with bootstrapping value at greater than 70%. In a second step of our analysis, we applied different tree reconstruction procedure using UPGMA and Neighbor Joining methods, synthesizing the resulting set of trees using CONSENSE and drawing phylogenic trees with TREEVIEW program. However tree reconstruction method chosen, the general topology of different trees is quiet identical. High bootstrap values have been obtained with type 2 tree.

Three major genotypes (1 - 3) are found among wild type1 strains. Genotype 1 with an average of 99% homology clusters strains are very closed to the prototype Sabin 1 and Mahoney strains. These strains had restriction patterns similar to those of Sabin 1. Partial genomic sequencing was performed to analyze further these 13 strains (Dahourou, 2002). Wild strains are clustered into two genotypes. Strains grouped in the first genotype were isolated in various years from 1986 to

Table 4. Neutralization epitope pattern of Sabin-like strains isolated in Southern India (1985 to 1992).

	NIBSC			PASTEUR					VELLORE				
	S	B	K	V									
				S	W								
Strains	11	16	1c	1o	Io	Ic	Id	Ia	3-1	3-5	3-7	35-1	35-4
Sabin 1	6.0	7.0	6.0	4.0	0.0	0.0	0.0	0.0	0.0	0.5	1.0	0.5	1.5
Mahoney	0.0	5.0	3.5	1.5	4.0	3.5	4.0	2.5	0.0	0.5	0.5	0.0	0.5
89/1492	1.0	5.5	4.5	0.0	1.5	0.5	1.0	1.0	4.0	4.0	4.0	4.0	3.0
87/0827	0.0	3.5	4.5	1.5	3.5	4..5	4.5	4.5	0.25	1.0	0.5	0.25	0.0
87/0831	1.5	5.0	5.0	3.0	2.5	5.0	5.0	5.0	1.25	1.25	1.5	1.25	1.0
87/0874	0.0	5.5	5.0	2.5	5.0	4.0	4.0	5.0	0.75	0.5	0.25	0.25	0.75
87/0880	0.0	5.5	4.0	1.0	3.5	3.5	4.0	5.5	1.25	1.0	0.75	1.0	1.0
87/0884	0.0	5.5	4.0	2.0	3.5	4.0	5.5	5.5	1.5	0.75	0.75	1.0	0.75
87/0896	0.0	4.5	3.5	1.0	3.5	4.5	4.5	4.5	0.5	0.5	1.0	0.5	0.5
87/0988	0.5	4.5	3.5	1.0	4.0	3.5	4.0	3.5	1.0	0.5	0.0	0.5	1.0

1993. Within this genotype, isolates were classified into 2 clusters differing from each other by between 2 and 10%.

Cluster 1 group strains isolated in 1991 and 1992. Some of them had more than 98% homology in the VP-1-2A genomic sequence, demonstrating an epidemiological link. Strains grouped in cluster 2 are closely related to other wild strains isolated elsewhere in India (08645ind92 and 16006ind82) and in neighboring countries such as in Pakistan (21198pak95) and Nepal (6117nep92) (Figure 1).

All type 2 isolates except strain 285/92 displayed more than 85% sequence identity in the VP1- 2A region (Figure 2) and thus belong to the same genotype. This indicated the presence of a dominant genotype throughout the years studied. Within this unique genotype, the isolates could be classified into 3 clusters differing from each other by 8 to 10%.

Sequencing grouped type 3 strains into 4 major genotypes (1 to 4). Genotype 1 with an average of 98% similarity clustered very close to the prototype Sabin 3 strain. Wild strains were clustered into 3 genotypes. Genotype 1 clustered strains isolated from 1988 to 1992 and is divided into three subclusters (Figure 3). These strains were closely related to other epidemic strains isolated in India in 1984 (0005ind84) and in 1991 (8670ind91) and to imported strains from India to the Netherlands in 1986 (16873net86) and 1988 (14815net88). Strains belonging to genotype 2 were isolated in 3 consecutive years (1986-87-88). They are closed to strains isolated in 1980 (39-OJind80), 1982 (15250ind82) and 1986 (7095ind86) elsewhere in India, and also to imported wild poliovirus isolated in the Netherlands in 1979 (22290net79), 1983 (05598net83) and 1984 (09134net84). Three strains isolated in 1987 and 1989 have been clustered in genotype 3. Sequencing results confirm RFLP patterns, as same clustering is observed for both assays.

The deduced amino acid sequences encoded by the 150 bp VP1/2A sequences are shown in Figure 4 for type 1 and type 2 PV. All of the nucleotides substitutions were silent, producing synonymous codons. There were no more than three substitutions in fifty amino acid sequence in each group. At antigenic site 3A, all type 3 wild strains differed totally from the Sabin 3 vaccine strain. They have Lys-Asp-Gly-Leu-Ala (KDGLA) at position 280 instead of Arg-Asp-Asp-Leu-Asp (RNNLD). Most of them presented at position 2 of the viral protease a leu for phe (L > F) substitution.

DISCUSSION

Clinical, epidemiological and virological features of children with paralytic poliomyelitis seen in Vellore and in other districts in Tamil Nadu and other South Indian states between 1967 and 1979 have been described (Ratnaswani et al., 1973; Maiya et al., 1981). In this study, we have documented the general epidemiological features of paralytic poliomyelitis cases reported to our laboratory during the 9 years from 1985 to 1993.

The age distribution of cases (Table 2) shows that paralytic poliomyelitis remains an illness of infants and young children in India. From 1967 to 1985, more than 87% of the cases were reported in children below 4 years, whereas only 75% of cases were reported in the same age group during the recent years (1990 - 1993). These patterns have been observed in industrialized countries, where most cases occurred among persons aged more than four years old, many of them adults, while in developing countries, cases occurred predominately among infants below 2 years of age (Patriarca et al., 1991). A striking feature of the past

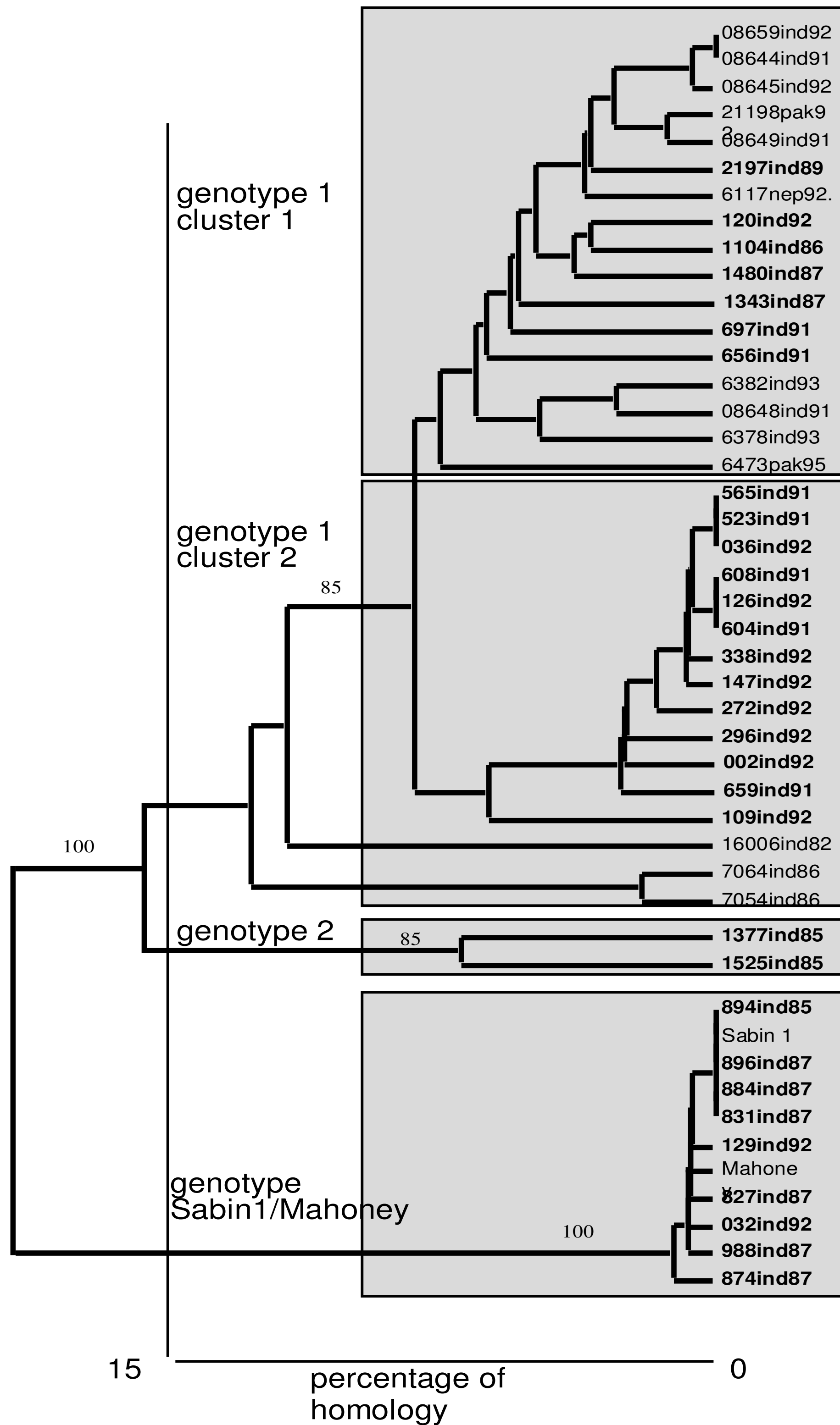

Figure 1. Phylogenetic analysis of PV type 1 strains from India and from different part of Asi. Dendogram is based on relatedness between nucleotide sequences in the VP1/2A encoding region. Studied Indian strains are written in bold characters. Country abbreviations are: ind = India; pak = Pakistan; nep = Nepal. EMBL nucleotide sequence bank accession numbers are AJ248493 to AJ248523.

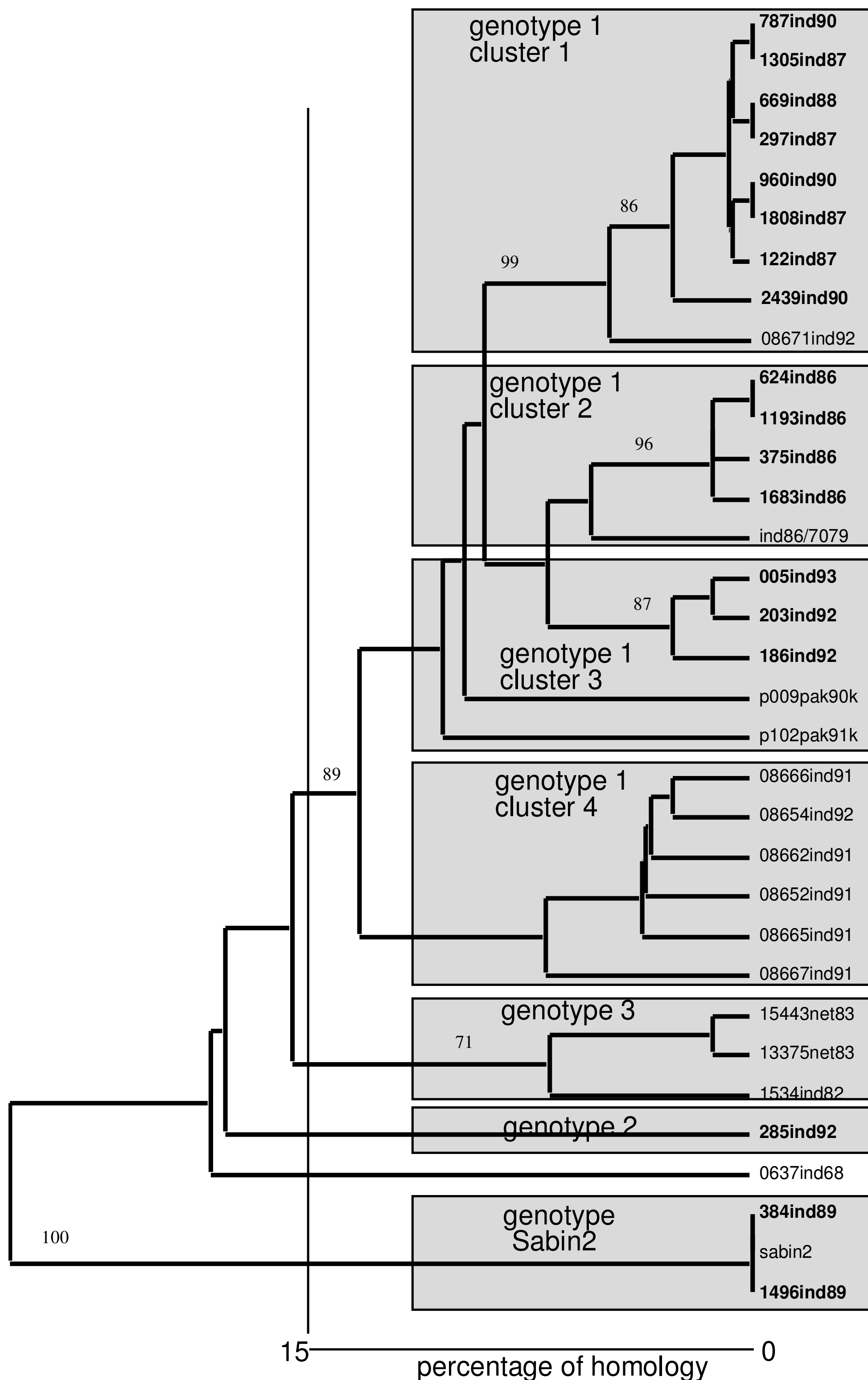

Figure 2. Phylogenetic analysis of PV type 2 strains from India and from different part of the world. Dendogram is based on relatedness between nucleotide sequences in the VP1/2A encoding region. Studied Indian strains are written in bold characters. Country abbreviations are: ind = India; pak = Pakistan; net = the Netherlands. EMBL nucleotide sequence bank accession numbers are AJ248475 to AJ248492.

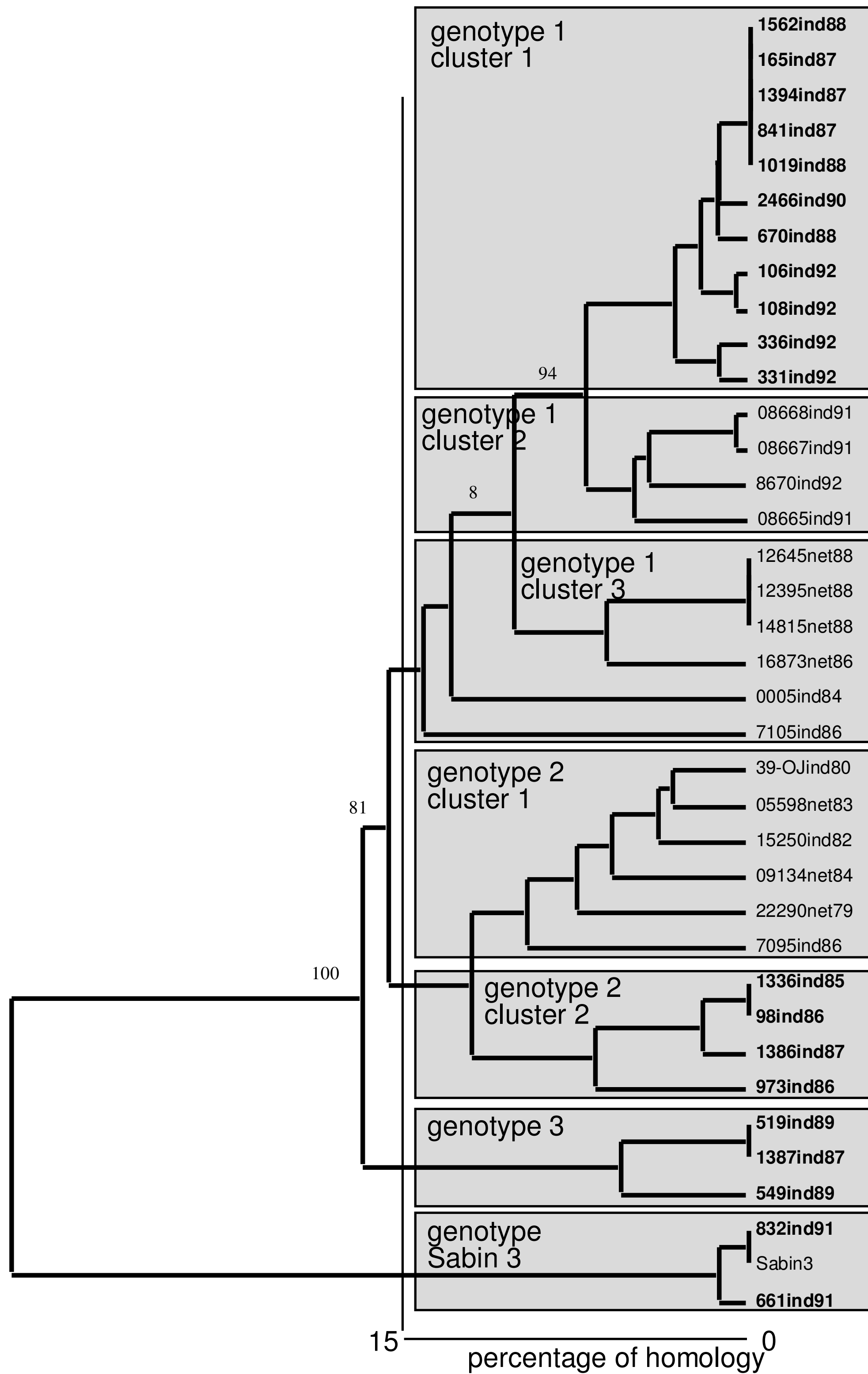

Figure 3. Phylogenetic analysis of PV type 3 strains from India and from other part of the world. Dendogram is based on relatedness between nucleotide sequences in the VP1/2A encoding region. Studied Indian strains are written in bold characters. Country abbreviations are: ind = India; net = the Netherlands. EMBL nucleotide sequence bank accession numbers are AJ248524 to AJ248545.

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                VP1                                      2A
Sabin_1         PPRAVAYYGP GVDYKDGTLT PLSTKDLTTY         GFGHQNKAVY TAGYKICNYH
Mahoney         ---------- ---------- ----------         ---------- ----------
87/827          ---------- ---------- ----------         ---------- ----------
92/032          ---------- ---------- -F--------         ---------- ----------
85/894          ---------- ---------- ----------         ---------- ----------
87/831          ---------- ---------- ----------         ---------- ----------
87/874          ---------- ---------- ----------         ---------- ----------
87/884          ---------- ---------- ----------         ---------- ----------
87/896          ---------- ---------- ----------         ---------- ----------
87/988          ---------- ---------- ----------         ---------- -------K--
92/129          ---------- ---------- ----------         ---------- ----------
87/1343         ---------- ---------- ----------         -Y-------- --------F-
87/1480         ---------- ---------- -F--------         -H-------- ----------
91/659          ---------- ---------- ----------         -Z-------- ------S---
91/656          ---------- ---------- ----------         -Y-------- ------S-C-
92/272          ---------- ---------- -F--------         -Y-------- ----------
92/109          ---------- ---------- -F--------         -Y-------- ----------
92/147          ---------- ---------- -F--------         -Y-------- ----------
92/296          ---------- ---------- -F--------         -Y-------- ----------
89/2197         ---------- ---------- -F--------         -Y-------- ----------
91/697          ---------- ---------- -F--------         -Y-------- ----------
92/126          ---------- ---------- -F--------         -Y-------- ----------
86/1104         ---------- -----E---- ---I------         -Y-------- ----------
91/523          ---------- ---------- ----------         -Y-------- ----------
91/565          ---------- ---------- ----------         -Y-------- ----------
91/608          ---------- ---------- -F--------         -Y-------- ----------
92/002          ---------- ---------- ----------         -Y-------- ----------
92/036          ---------- ---------- ----------         -Y-------- ----------
85/1525         ---------- ---------- ---I------         -L-------- ----------
91/604          ---------- ---------- -F--------         -Y-------- ----------
92/120          ---------- ---------- ---I------         -Y-------- ----------
85/1377         ---------- ---------- -F-I------         -L-------- ----------
92/338          ---------- ---------- ----------         -Y-------- ----------

Sabin2          RPPRAVPYFG PGVDYKDGLT PLPEKGLTTY         GFGHQNKAVY TAGYKICNYH
89/384          ---------- ---------- ----------         ---------- ----------
89/1496         ---------- ---------- ----------         ---------- ----------
Indian wild     --------Y- ---------- ----------         ---------- ----------
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Figure 4. Alignment of deduced amino acid sequenced of the VP1/2A interval. Dashes indicate aa identity with Sabins 1 and 2.

outbreak of poliomyelitis in Albania (1996) is that children aged 1 - 10 years were largely unaffected (Prevots et al., 1997), in contrast to other outbreaks in Europe which priorily, affected children, albeit primarily those unvaccinated or inadequately vaccinated (Anonymous, 1992; Oblapenko and Sutter, 1997; Sutter et al., 1997).

There is thus a shift in occurrence of polio towards the older children above 4 years. This shift in age may be due to the high immunization coverage among the younger age groups in recent years. These results provide evidence of effectiveness of the North Arcot District Polio Control Program targeted to children born after the beginning of the program. Another possible reason for this shift could be the fact that, after years of routine immunization wild poliovirus circulation is often reduced with concomitant dispersion of inadequate vaccinated children leading to outbreaks in older children (Patriarca et al., 1997).

The immunization status of the cases is shown in Table 3. Whereas only 22% of the cases from 1990 to 1993 were unimmunized, as many as 43% were unimmunized during 1985 to 1989 period. In earlier studies, it has beenshown that 93% of the cases were unimmunized during the period 1967 to 1972 (Ratnaswani et al., 1973) and 85% during the period of 1973 to 1979 (Crainic et al., 1981). It is of importance to note that from 1990 to 1993, 55% of cases were children immunized with 3 or more doses of OPV or IPV.

In 1993 alone, 63% of children with paralytic poliomyelitis had received 3 or more doses of OPV/IPV.

One reason for the high rate among immunized children could be because of vaccine failure in the setting of increased immunization coverage rate. Poor seroconversion rates have been established especially for type 1 poliovirus (John and Jayabal, 1972) but also in type 3 (Anonymous, 1990; Triki et al., 1997). Two major factors have been reported for poor seroconversion in the developing countries. First, geographical variations in the response to and efficacy of oral poliovirus vaccine occur in developing countries. Second, the power of poliovirus transmission is stronger in many developing countries than in developed countries (John, 1993).

The presence of Sabin 1 specific epitopes and Mahoney strain specific epitopes in 13 strains suggest that Sabin type 1 like strains could also be in circulation. These strains may have been derived from type 1 Sabin strain and may have regained their wild parent (Mahoney strain) specific epitope during *in vivo* passage in humans. We found that heterologous epitopes (Sabin-type and wild-type) could co-exist in the same strain and that transition from one to another was possible (Dahourou, 2002).

Circulation of wild type 2 poliovirus in vaccinated populations is considered as a sign of vaccination failure, since circulation of type 2 poliovirus is supposed to be best inhibited by oral polio vaccine. Even until 1998, circulation of wild type PV2 continued to occur only in Africa (Benin) and in South East Asia (India, Pakistan and Afghanistan) (Anonymous, 1997). Different genotypes were described by several authors within all three serotype worldwide (Rico-Hesse et al., 1987; Kew et al., 1990, 1995; Mulders et al., 1995a, b; Huovilainen et al., 1995) and two (or more) of them in each serotype were endemic in the Indian subcontinent. All studied strains isolated from this region belonged to these genotypes. We have described in this study two different wild genotype for type 1 and type 2 and three genotypes in type 3 in this region with separate clusters, possibly representing strains in the process of evolving into new genotypes during the monitored period. This is not surprising because people in India, especially in the state of Tamil Nadu constantly move from one area to another within that region or all the country, to visit family, go to temples or look for work. Christian Medical Center of Vellore is a tertiary care center were people from all over India come for medical care. Simultaneous co-circulation of more than one genotype in region with sub-optimal health service and environmental hygiene has been reported in China (Zheng et al., 1993), the former Soviet Union (Lipskaya et al., 1995), in Pakistan (Huovilainen, 1995) and in South Africa (Chezzi et al., 1997).

We compared the genomic sequences of these Indian wild isolates with previously published poliovirus genomic sequences from different parts of the world including an isolate obtained from India three decades ago. This comparison shows that current wild type strains have been in circulation since at least 1985, when the testing began. Consistent to the published data, there are many wild genotypes all over the world and two of each serotype 1 and 2 and three of serotype 3 are endemic in the Indian subcontinent. For all the present strains, these main genotypes were observed.

The antigenic site 3A is considered to be important only for type 3 poliovirus. The amino acid compound of protease 2A and especially that of its N terminal part is highly conserved among known poliovirus (Minor et al., 1986). Consistent with the previous published data (Rico-Hesse et al., 1987; Huovilainen et al., 1995; Chezzi et al., 1997) we found that amino acid at position 2 is especially variable in type 1 and 3 polioviruses.

Conclusion

We have analyzed the genetic relationships of all three poliovirus types in South India. Our result show that wild poliovirus remain endemic in the Indian subcontinent and indicate simultaneous co-circulation of more than one genotype in this region. Strains belonging to all genotypes have been previously described elsewhere. Genetic analysis of these strains show that wild type 2 strains was in prolonged circulation in this region despite increasing vaccine coverage and establish epidemiologic links with strains studied by other investigators (Rico-Hesse et al., 1987; Kew et al., 1990; Mulders et al., 1995b; Huovilainen et al., 1995). We observed a shift in the occurrence of polio paralysis towards older children, a high rate of the disease among infants in the recent past years and that Sabin/Mahoney-derived strains was circulating in this region during the study period.

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