

Intratypic Differentiation of Polioviruses Isolated from Suspected Cases of Poliomyelitis in Brazil during the Period of 1990 to 1993

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This study analyzed 3129 fecal samples derived from 1626 patients with sudden onset acute flaccid paralysis clinically compatible with poliomyelitis. The samples were collected in the period ranging from January 1990 to September 1993 in all regions of Brazil. Among the 1626 cases studied, 196 had isolation of poliovirus. Nevertheless, it was observed that some factors influenced the isolation rate and the intratypic characterization of these polioviruses. No cases of acute flaccid paralysis has been found to be etiologically related with wild polioviruses.

Key words: poliovirus - intratypic characterization - molecular hybridization - polymerase chain reaction

In Brazil, poliomyelitis started being observed more often in the beginning of this century, even though there are references of sporadic cases in the final decades of last century (Scorzelli 1966).

The upcoming of antipoliomyelitic vaccines, in the 50's and 60's, has changed the scenario of poliomyelitis in the world, so that today approximately 30% of the world population lives in regions which are considered to be free of native transmission of the wild poliovirus (Lemon & Robertson 1991).

Brazil introduced systematic activities of vaccination against poliomyelitis in 1961, with the inclusion of the oral vaccine in public health services, which was not very successful, for the disease was hyperendemic until 1980, when 21203 cases had been recorded (Technical Advisory Group-TAG/1992). In the same year, the routine vaccination strategy suffered an important change, with the introduction of mass vaccination on the "National Day of Vaccination". With this change, Brazil saw a dramatic reduction in the number of cases of poliomyelitis, which in practice meant going from 2564 confirmed cases in 1979 to 45 cases in 1983 (Risi 1984).

In spite of the health officials' willingness to eradicate the native transmission of the wild poliovirus, the number of cases of poliomyelitis rose to 130 in 1984, 329 in 1985 and 612 in 1986 (Poliomielite 1988) because of a drop down in the

percentage of vaccinal coverage. Since the problematic areas concentrated in the northeast of Brazil, in 1986 the Northeastern Day of Poliomyelitis Vaccination was created.

As the percentage of vaccinal coverage increased in the following years, the number of cases decreased, reaching 35 confirmed cases in 1989 in the whole country.

From 1986 to 1989 all cases of sudden onset acute flaccid paralysis (AFP) in all ages were investigated in Brazil, as well as cases of peripheral facial paralysis.

In 1990, with the reduction of the wild poliovirus circulation in the Americas, TAG of the expanded program of immunization, from Panamerican Health Organization (PAHO), proposed a new final classification for the investigated cases of AFP. This classification was accepted by all countries of the Americas, including Brazil, which then began to investigate only the cases of AFP in under 15-years-old, as it was observed that of the 72 wild poliovirus isolations obtained between 1987 and 1989, 76.4% had struck children under 5-years-old (Poliomielite 1991).

According to the new classification, only the cases in which there is isolation of wild polioviruses in feces are confirmed as poliomyelitis. This classification aims at reducing the number of "false positive" cases. Cases compatible with poliomyelitis are those in which feces samples were not properly collected, as the correct collecting takes two feces samples within the first 14 days after the beginning of motor deficit, with an interval of at least 24 hr between collecting. Furthermore, the

samples amount must be 8-10g and must be sent to analysis in ideal conditions of temperature (2-8°C). Cases compatible with poliomyelitis are those in which there was not proper feces collecting and which present compatible sequel after 60 days of the beginning of motor deficit. Non-compatible cases are the AFP cases without sequel or with non-compatible sequel after 60 days of the beginning of motor deficit, or else those in which, in spite of compatible sequel, a new analysis of the feces in another laboratory using a special treatment in the feces confirms the absence of wild polioviruses.

In 1990 a new criterion was included, the vaccine associated cases criterion, which includes all AFP cases in which there is isolation of vaccinal polioviruses in the feces sample(s) and the presence of a sequel compatible with poliomyelitis 60 days after the beginning of motor deficit, besides proper feces collecting. It was also established that in all cases with improper feces samples or non-collecting, a coprologic research will be carried out immediately after the noticing of the fact, only in the cases of higher risk (children under six who had fever in the beginning of the motor deficit).

Considering the above mentioned criteria and Brazil's willingness to eradicate wild polioviruses, laboratorial diagnosis has played a fundamental role in this context. Therefore, the main purpose of our study has been to intratypically characterize polioviruses which were isolated from AFP suspected cases, in order to identify whether the etiological agent responsible for these paralysis is a wild poliovirus. The methods used were molecular hybridization and Polymerase Chain Reaction (PCR).

MATERIALS AND METHODS

Feces - Three thousands one hundred twenty nine samples of 1626 cases of AFP suspected of poliomyelitis was received from all Brazilian regions. The samples were processed according to PAHO/WHO laboratorial procedure handbook (WHO/EPI/CDS/Polio/90.1).

Cells - Hep-2C (made available by the National Institute for Quality Control of Health at the Fundação Oswaldo Cruz, Rio de Janeiro, Brazil); RD (made available by Centers for Disease Control, Atlanta, Georgia, USA). Both human origin and continuous lineage cells were cultivated in glass tubes and maintained in Eagle Essential Medium (MEM) supplemented with 5% bovine fetal serum.

Isolation, titration and identification of the virus - The methods used in this stage were those recommended by PAHO/WHO laboratorial procedure handbook.

Intratypic characterization - Molecular hybridization and PCR using synthetic oligonucleotides probes and primers specifically designed to recognize vaccine-related polioviruses sequences (Sabin 1, Sabin 2, Sabin 3), Brazilian wild polioviruses types 1 and 3 genotypes which were usually endemic in the Northeastern Region of Brazil until recent years and a group probes for enterovirus detection, has been described (da Silva et al. 1991, Yang et al. 1991).

Molecular hybridization - The procedure is the same already described by da Silva et al. (1991).

PCR procedures to in vitro amplification - Freeze-thaw lysates of RD or Hep-2C polio positive cell cultures were clarified at 12000 x g/15 min, and 4 µl of each supernatant were denatured by heating (95°C/5 min) with a mixture containing 50 mM Tris-HCL/pH 8.3, 70 mM KCl, 5mM MgCl₂, 10 mM (dithiothreitol), PCR-1 and PCR-2 primers (20 pmoles each), 200 µl each of dATP, dCTP, dGTP, dTTP (Pharmacia) and two drops of mineral oil.

The cDNA transcription was carried out by incubation (42°C, 30 min) of denatured RNA templates in a mixture containing to a final volume of 100 µl, 10 U placental ribonuclease inhibitor (Boehringer Mannheim Biochemicals, Indianapolis, IN), 2.5U of reverse transcriptase from avian myeloblastosis virus (AMV) and 2.5U of the thermostable DNA polymerase from *Thermus aquaticus* (Taq DNA polymerase; Perkin Elmer-Cetus, Norwalk, CT). Amplification cycles were performed for 30 cycles in a programmable DNA thermal cycler (Perkin Elmer-Cetus) (denaturation: 94°C, 45 sec; annealing: 60°C, 45 sec; extension: 72°C, 60 sec). The PCR products were visualized in ethidium bromide stained 12% acrylamide gels.

RESULTS

Virus isolation - From 3129 fecal suspensions inoculated in Hep-2C and RD cells, the presence of the virus was observed due to the cytopathic effect (CPE) in 693 (22%) samples. In most of the positive cultures CPE occurred between the 1st and 5th day after inoculation. The culture which proved to be most sensitive to the virus isolation was RD; from 196 polioviruses isolated, 106 (54%), were isolated in both cells, 71 (36%) only in RD and 19 (10%) only in Hep-2C. The same happened with the non-polio (NP) from 266 isolated, 39 (15%) were isolated in both cells, 154 (58%) only in RD and 73 (27%) in Hep-2C. That sensitivity was also proven by way of the number of passages necessary for the isolation of the virus.

Even though the feces sample recommendation is two per patient, from 1626 cases, 1503 (92%) had two feces samples collected, while in

123 cases (8%) just one sample was collected. Considering that the interval between the beginning of the motor deficit and the collection of the samples varied between 0 and 65 days, we selected all fecal samples which were collected during the period of 0 to 41 days after the beginning of the motor deficit in order to observe whether the virus excretion rate varied with the beginning of the motor deficit and the date of the sample collection, this period was chosen to have a great number of samples collected. Our results showed a high virus excretion during the 1st and the 14th day after the beginning of motor deficit. The percentage of virus excretion correlated to this two factors is on Table I and Fig. 1.

Virus identification - From 693 positive samples, 200 (29%) were selected to be titrated; only the samples in which the CPE occurred between the first and sixth day after inoculation of the second passage in the used cultures were selected. The samples title varied from 10^1 to 10^8 TCID₅₀/0.05 ml. However, we observed that, of the 200 samples, 167 (84%) presented an endpoint varying from 10^4 to 10^6 . These results were important to carry out the test for the identification of the isolated virus, since in such test three dilutions of the isolated viruses were chosen (10^{-4} ,

10^{-5} , 10^{-6}). By the results obtained from the previously titrated samples, it was estimated that in one of the three dilutions we would have approximately 100 TCID₅₀, which means enough particles to be neutralized by 40U of poliovirus-specific antiserum.

The results obtained in the identification tests demonstrate that the clinic samples inoculated in cell cultures make possible not only the isolation of poliovirus, but also of other viruses infecting the gastrointestinal tract as well. However, as the main purpose of this study was to investigate the etiological role played by the three serotypes of poliovirus in the flaccid paralysis occurring in Brazil in the last four years, the polioviruses isolated were selected to be characterized genetically. In spite of the relevant number of samples with isolation of other viruses, they were classified as being just NP.

Of the 1503 with two fecal samples collected, 452 (30%) were virus positive; from these, 193 (43%) had isolation of poliovirus and 259 (57%) had isolation of NP (Fig. 2). Besides, 61 (32%) had isolation of poliovirus in both samples, 73 (38%) just in the first one and 59 (30%) in the second sample. Those with NP isolation had 165 cases (64%) with isolation of NP in both feces samples, 57 cases (22%) just in the first sample and 37 cases (14%) in the second one (Fig. 3). Of the 123 cases with only one sample collected, 10 (8%) were virus positive, from these 3 (30%) had isolation of poliovirus and 7 (70%) had isolation of NP (Fig. 4).

According to our results, the poliovirus isolated the most in the last four years was that of serotype 3. Of the 196 isolated poliovirus, 60 (30%) were of serotype 1, 47 (24%) of serotype 2 and 89 (45%) of serotype 3. Among the isolated polioviruses, 152 (78%) were found as single isolates, 36 associated among them (18%) and 8 (4%) with other NP viruses.

Intratypic characterization - All the polioviruses identified as being of serotype 1 hybridized positively with the specific Sabin 1 probe and the group probe; those of serotype 2, with the probe

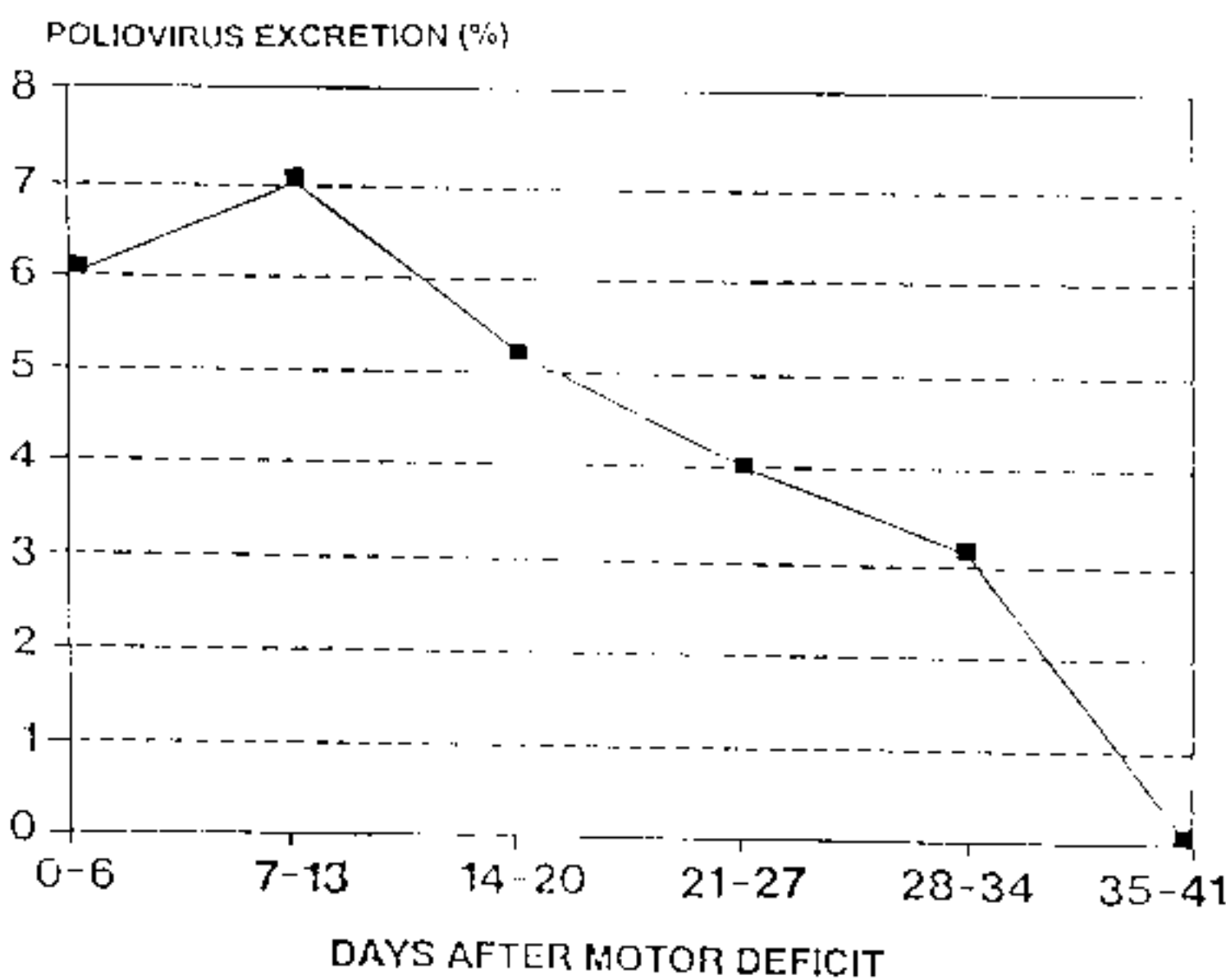


Fig. 1: poliovirus excretion rate related to the specimen collecting date after motor deficit.

TABLE I

Virus excretion rate related to the specimen collecting date and the beginning of motor deficit

Virus isolation	Days after motor deficit					
	0 - 6	7 - 13	14 - 20	21 - 27	28 - 34	35 - 41
Polio	61 (6%)	73 (7%)	23 (5.2%)	10 (4%)	4 (3.1%)	0
Non polio	170 (16%)	152 (14.7%)	56 (12.6%)	24 (9.6%)	14 (10.9%)	2 (4%)
Negatives	817 (78%)	811 (78.3%)	365 (82.2%)	215 (86.4%)	111 (86%)	48 (96%)
Total	1048	1036	444	249	129	50

specific for Sabin 2 and the group probe, and those of serotype 3 hybridized with the probe specific for Sabin 3 and group. None of the polioviruses hybridized with the wild genotype specific probes.

During the hybridization test, some polioviruses which had been isolated as mixtures (Polio 1+2 ,

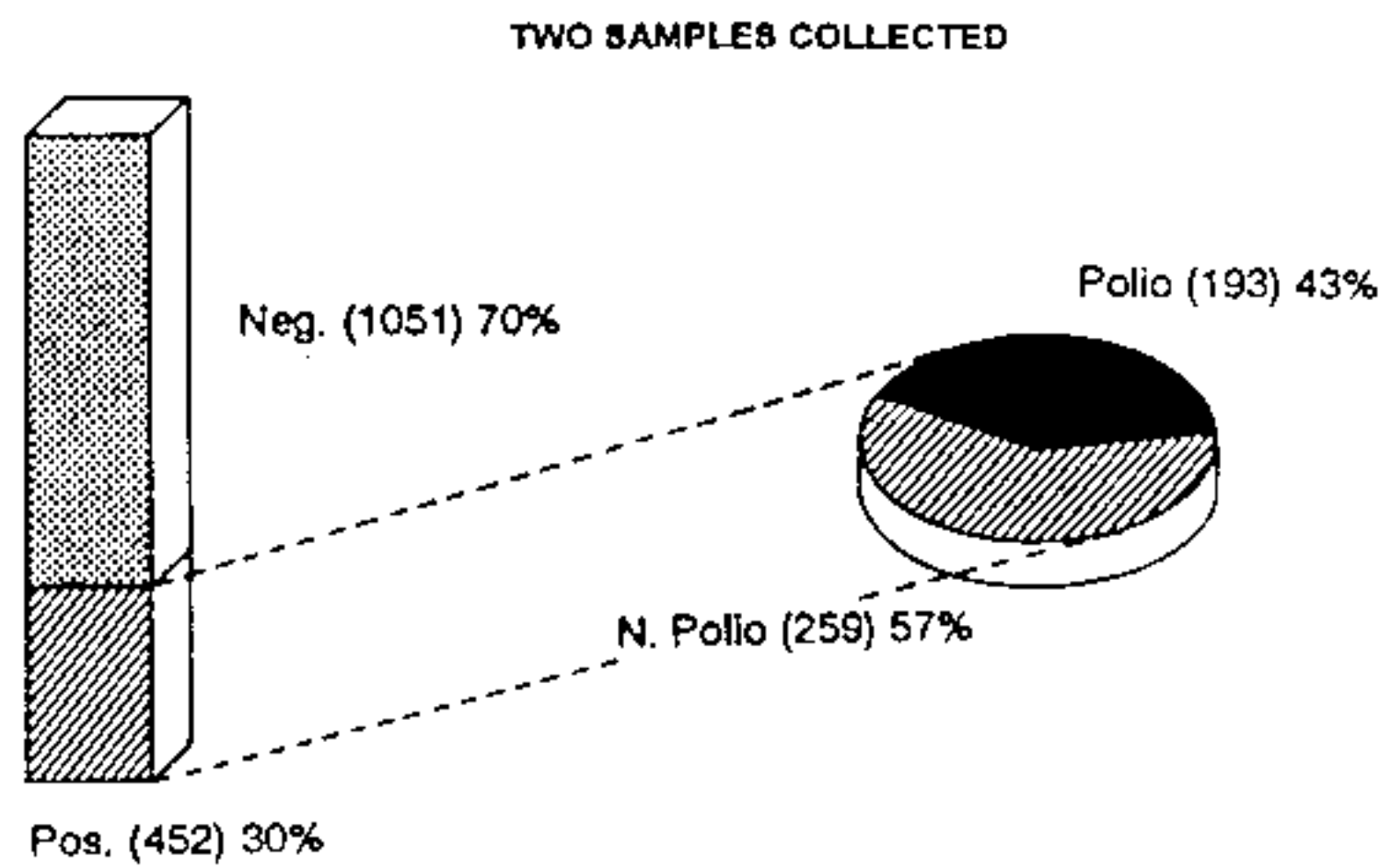


Fig. 2: isolation rate of poliovirus and non polio in cases with two samples collected.

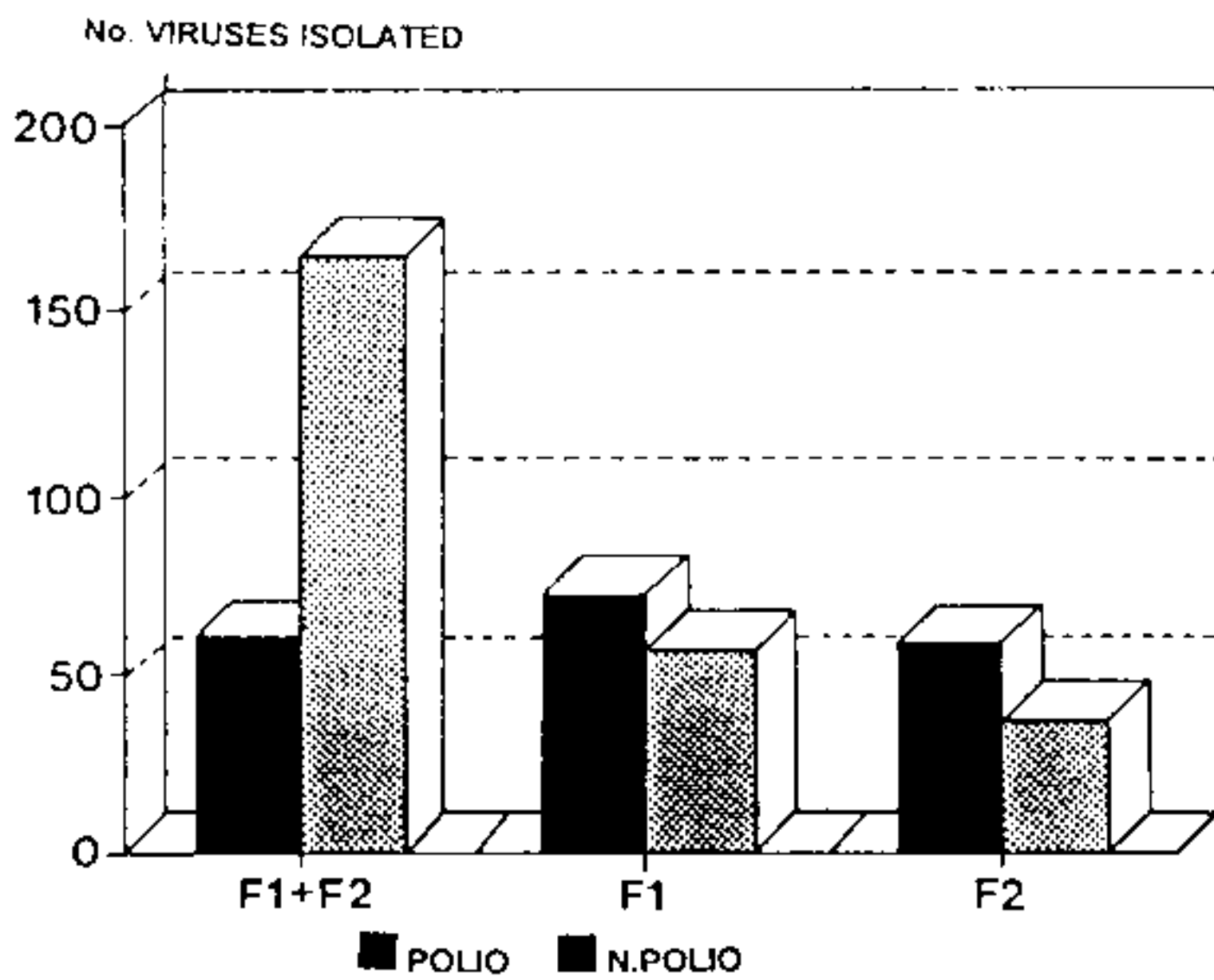


Fig. 3: distribution of virus isolated in first and second feces sample collected.

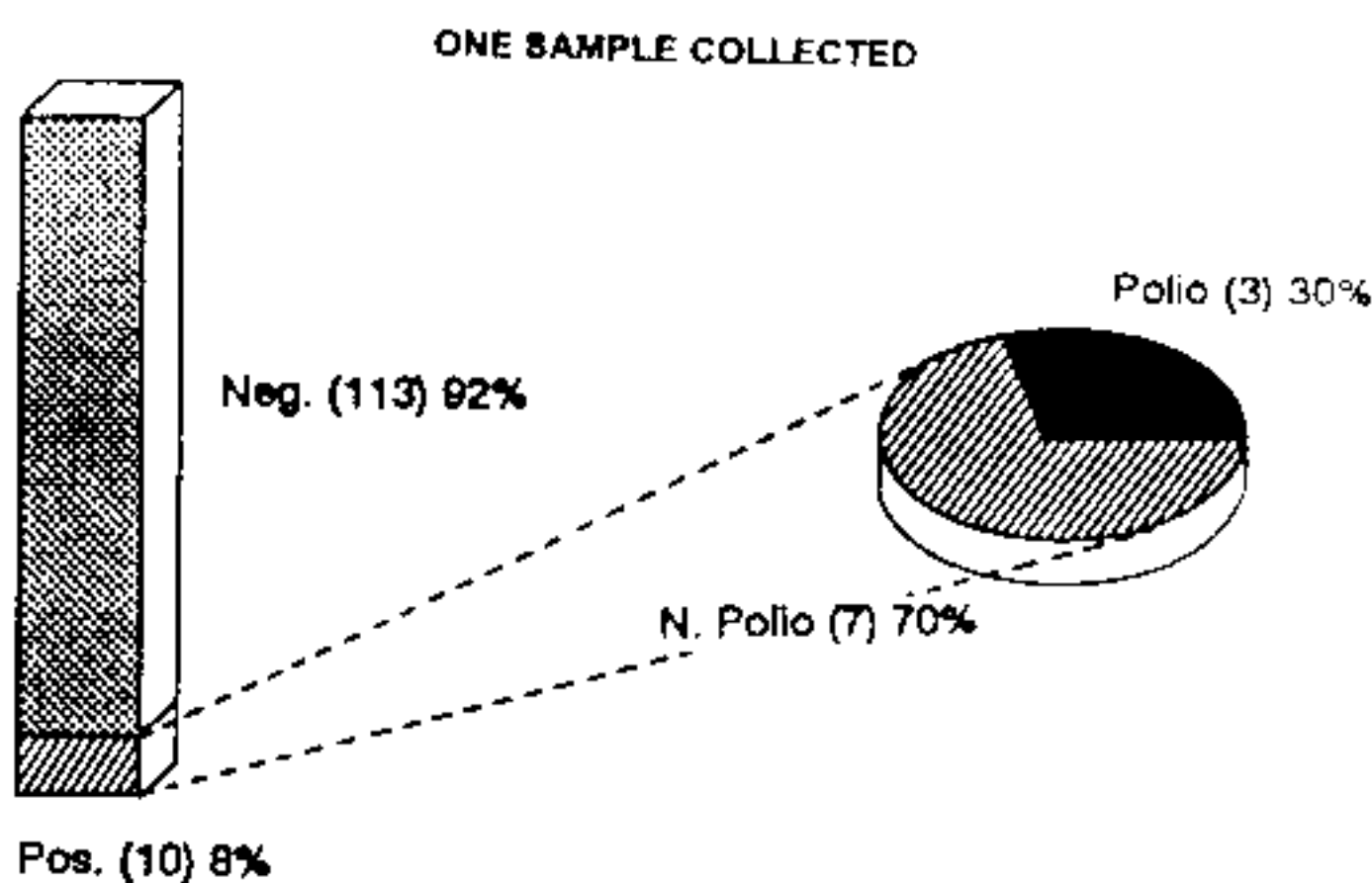


Fig. 4: isolation rate of poliovirus and non polio in cases with one sample collected.

2+3, 1+3, 1+2+3) or even separately did not hybridized with any of the used probes. In some cases of virus mixture, only one virus hybridized with the corresponding probe, while the other did not hybridize with any of the probes. These samples were serotyped once more in order to confirm the previous results, and the results obtained were the same as the previous reaction. We titrated these viruses and observed that their titles did not exceed 10^4 TCID₅₀, thus the polioviruses which did not react with the used probes in hybridization were submitted to the PCR method. The synthetic oligonucleotides used in this method, as well as those used in hybridization, recognize all three poliovirus serotypes and differentiate them between wild and vaccine ones. The results obtained through PCR show that all the polioviruses which had not reacted in hybridization were of vaccinal origin, since they were amplified using vaccine poliovirus specific primers.

DISCUSSION

Due to the methodology employed in this study and the results obtained, it was possible to clarify some virological aspects about the poliomyelitis suspected AFP cases in Brazil and to use more safely the criteria established by the case classification system. Besides, it was also possible to find out the types of poliovirus circulating in the country.

We observed that some, such as the type of tissue cultures used, factors the onset of the paralysis related to the date of the collection of the feces and the number of samples per patient, influenced the polioviruses isolation rate. When we compare the polioviruses isolated in both cells, we can see that the isolation rate in RD cells was higher than in Hep-2C independent of the number of passages, the same happened with the NP enteroviruses.

In spite of the low virus isolation rate in Hep-2C cell cultures, use of both cultures is important because 10% of the polioviruses that were isolated only in Hep-2C could have been lost if we had used just RD cells.

When we correlate the beginning of the paralysis to the date of the feces collection, we find out that the highest virus excretion rate occurred during the first fourteen days after the onset of the motor deficit.

In spite of the recommendation to collect two feces samples for each patient, 8% of the analyzed cases had only one sample collected. Knowing that the poliovirus elimination in feces is intermittent, we could observe that the virus isolation rate was quite reduced in the cases with just one sample and, as it has been shown, 59 (30.6%) polio-positive cases could have been lost if the second feces sample had not been collected.

In order to have a good poliovirus isolation rate, you should collect two feces samples of poliomyelitis suspect cases on no later than the 14th day after the beginning of the motor deficit.

Due to the methods used to differentiate among vaccine related and wild poliovirus samples (Yang et al. 1991, da Silva et al. 1991), the Dot blot hybridization method presented lower sensitivity (Rotbart et al. 1984, 1985, Preston et al. 1990) for the the poliovirus detection when the viruses had a title lower than 10^4 . That happened mostly in the samples which contained a heterologous mixture of polioviruses, because in such condition the two viruses in the sample are hardly ever in the same concentration. That has been shown, when samples containing two or three polioviruses were submitted to the hybridization test and just one or two, depending on the mixture, were detected by the probes. This low sensitivity is probably due to the fact that the probes are terminally labeled using $Y\text{-}^{32}\text{P}\text{-ATP}$ and also due to their length (≈ 23 bp oligos). Thus in order to obtain a strong signal in hybridization, several viral RNA molecules must be present in the sample to hybridize with the labelled probes.

The PCR method confirmed the above mentioned, when the samples that did not hybridize with the $Y\text{-}^{32}\text{P}\text{-ATP}$ labelled probes were submitted to amplification, reacted to the primers specific to all enteroviruses and to those specific to vaccine related polioviruses.

The probes and primers used in the hybridization reactions and PCR which identify wild polioviruses reacted only to the wild controls used in the tests, thus proving their specificity and confirming the absence of wild polioviruses in the analyzed samples.

This study did not use neither probe nor primer specific for wild poliovirus serotype 2 because this virus has not been detected in Brazil for more than eight years. Up to the present, all polioviruses 2 isolated from clinic samples have reacted with vaccine probes or vaccine primers.

Even though vaccine related polioviruses have been isolated from 196 suspected cases of poliomyelitis, we cannot classify them all as vaccine associated without some previous considerations, such as: was there exposition to vaccine? Is it a case of temporary paralysis or does the paralysis persist for more than 60 days? Does the affected child present any kind of immunodeficiency? And did the vaccine isolated virus keep its initial phenotypic characteristics? (Kew & Nottay 1984a). The last factor is not taken into account by the technicians assessing such cases. One of the reasons for such is probably because the pathogenesis of the poliomyelitis cases associated with the vac-

cine has not been clarified up to the present moment (Lipskaya et al. 1991). However, we know that two factor groups are important: (1) defects in the host's defense mechanism (such as immunodeficiencies) and (2) the appearance of variants with increased neurovirulence during replication of viruses in the human intestines (Crainic et al. 1984, Kew & Nottay 1984a,b, Almond 1987, Racaniello 1988). Studies have identified several punctual mutations that could significantly increase the virus neurovirulence (Almond 1987). Nevertheless, it is very difficult to assess the real contribution of these two factors, namely, host and infectious agent, because the same mutations were observed in polioviruses strains excreted by vaccinated healthy children and in cases classified as vaccine associated paralytic poliomyelitis (Kew et al. 1981, Macadam et al. 1989, Tatem et al. 1991, Friedrich 1993). Taking into consideration just the isolation of vaccine related poliovirus, the presence of poliomyelitis-compatible sequel 60 days after the beginning of the motor deficit and the vaccine history, of the 196 cases only 12 were classified as vaccine associated poliomyelitis. In spite of the vaccinal poliovirus isolation and permanent sequel, only one case was classified as poliomyelitis-compatible because the child had been vaccinated five days after the beginning of the paralysis and the feces had been collected after vaccination. The other 183 cases with vaccinal poliovirus isolation were discarded or received other diagnosis such as Guillan-Barré syndrome, transverse myelitis, trauma and others, since they did not present poliomyelitis-compatible sequel 60 days after the beginning of the motor deficit.

Of the 1164 negative cases, 1025 (88%) were discarded because they did not present compatible sequel, 23 (2%) were considered poliomyelitis-compatible and 116 (10%), which presented poliomyelitis-compatible sequel, had their feces samples reexamined using ultracentrifugation in order to concentrate the material; this method is only used for the reexamination of these materials, due to its complexity. Of the above mentioned 116 cases, 110 (95%) were discarded, because of NP isolation or negative result confirmed, and 3 (3%) were classified as vaccine associated, since they had vaccinal poliovirus isolation. The 3 (3%) remaining ones were classified as poliomyelitis-compatible because they did not have virus isolation and proper feces collecting.

Of the 266 cases with NP isolation, 2 (0.8%) were classified as poliomyelitis-compatible and the remaining 264 (99.2%) were discarded, or received other diagnosis for not present poliomyelitis-compatible sequel. According to the results obtained in this study, we can say that the acute flaccid

TABLE II

Final classification of poliomyelitis correlating isolation results

	Cases	Virus isolation	
		+	-
Polio confirmed	0	0	0
Polio associated	15	15	0
Polio compatible	29	3	26
Non polio (others) ^a	1582	447	1135
Total	1626	465	1161

^a: Guillan-Barre' Syndrome, trauma, etc.

paralysis cases which occurred from January 1990 to September 1993 did not have Brazilian wild poliovirus as etiological agent. The final classification of studied cases is presented in Table II.

The high excretion rate of vaccinal polioviruses due to high vaccinal coverages and NP enteroviruses found in poliomyelitis suspect patients is a strong evidence that the circulation of wild polioviruses in Brazil is coming to a halt.

The poliovirus which was isolated most in the last four years (from 1990 until 1993), was that of vaccinal serotype 3.

The last wild poliovirus isolated in clinic samples from two paralytic poliomyelitis suspect cases was of serotype 1, in march 1989, in the Northeast Region of Brazil. With these preliminary results (till September 1993), we can say that Brazil will complete four years without indigenous wild poliovirus isolation.

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