Detection of MEF-1 laboratory reference strain of poliovirus type 2 in children with poliomyelitis in India in 2002 & 2003

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Background & objectives: Significant progress has been made towards eradication of poliomyelitis in India. Surveillance for acute flaccid paralysis (AFP) has reached high standards. Among the 3 types of polioviruses, type 2 had been eliminated in India and eradicated globally as of October 1999. However, we isolated wild poliovirus type 2 from a small number of polio cases in northern India in 2000 and again during December 2002 to February 2003. Using molecular tools the origin, of the wild type 2 poliovirus was investigated.

Methods: Polioviruses isolated from stool samples collected from patients with AFP were differentiated as wild virus or Sabin vaccine-like by ELISA and probe hybridization assays. Complete VP1 gene nucleotide sequences of the wild type 2 poliovirus isolates were determined by reverse transcriptase polymerase chain reaction (RT-PCR), followed by cycle sequencing. VP1 nucleotide sequences were compared with those of wild type 2 polioviruses that were indigenous in India in the past as well as prototype/laboratory strains and the GenBank database.

Results: Wild poliovirus type 2 was detected in stool samples from 6 patients with AFP in western Uttar Pradesh and 1 in Gujarat. In addition, the virus was isolated from one healthy contact child and from environmental sewage sample in Moradabad where three of these patients were reported. These isolates were identified as genetically closely related to laboratory reference strain MEF-1. Molecular characterization of the isolates confirmed that there was no evidence of extensive person-to-person transmission of the virus in the community.

Interpretation & conclusion: Laboratory reference strain (MEF-1) of poliovirus type 2 caused paralytic poliomyelitis in 10 patients in September 2000 and November 2002 to February 2003. The origin of the virus was some laboratory as yet not identified. This episode highlights the urgent need for stringent containment of wild poliovirus containing materials in the laboratories across the country in order to prevent recurrence of such incidents.

Key words Acute flaccid paralysis (AFP) surveillance - nucleotide sequencing - poliovirus - polio eradication
circulating for three years and re-emerged subsequently? Or, was this strain reintroduced from an extraneous source? If the former explanation was true, then type 2 virus had not been eradicated and also that three years of absence of detection of virus by the current methods of surveillance were not long enough for certifying eradication. This would have meant a more serious setback to the global polio eradication than the one described above. On the other hand, if the source of the virus was not the community, then this finding has quite different implications for the eradication programme. If it was from a laboratory source, then stringent laboratory containment has to be introduced in the country without delay in order to prevent such incidents happening again\textsuperscript{7,8}.

In the present study, this question was investigated by genetic characterization of the type 2 polio virus strains. Since the nucleotide sequence information on the local wild type 2 strains of the past and laboratory reference strains used widely in laboratories was already available, it was compared with that of the current virus isolates to arrive at a conclusion regarding the source of these strains.

\section*{Material & Methods}

\textit{Clinical specimens:} Surveillance for acute flaccid paralysis (AFP) is carried out by the National Polio Surveillance Project, a joint operation by the Government of India and the World Health Organization (WHO)\textsuperscript{9}. In India, more than 7000 AFP cases are reported each year of which 85 to 90 per cent cases are investigated for poliovirus isolation by the polio laboratories. Stool samples are collected from AFP cases by the surveillance staff, or under their supervision, and sent using ‘reverse cold chain’ to the laboratory designated to serve the place of residence of the case. In the present study, children in western Uttar Pradesh (UP) and Gujarat were involved. The stool samples from UP were analysed in the global specialized polio laboratory, Enterovirus Research Centre, Mumbai and those from Gujarat in the Behramji Jeejibhoy (BJ) Medical College, Ahmedabad.

\textit{Supplementary surveillance samples:} Stool samples collected from 375 healthy children (145, 93 and 137 for the Aligarh, Mathura and Moradabad district cases, respectively) living in the same localities as the first 3 children (AFP cases) were received for virus isolation. One environmental sample was collected from local sewage drains from each of the three places for virus isolation studies. Supplementary surveillance samples were collected during December 29, 2002 to January 9, 2003 for Mathura and Aligarh cases and January 12-28, 2003 for Moradabad case.

\textit{Virus isolation and identification:} Standard laboratory techniques were used for virus isolation\textsuperscript{10}. Briefly, stool suspension (10\% w/v) was treated with chloroform (Sigma, USA) and inoculated into RD and L20B cell lines\textsuperscript{11}. The viral isolates were identified as poliovirus types by virus neutralization tests using type-specific equine antisera (RIVM, The Netherlands). The sewage samples were processed as described earlier\textsuperscript{12}.

\textit{Intratypic differentiation between wild and vaccine strains:} Every poliovirus isolate was subjected to enzyme linked immunosorbent assay (ELISA) using strain-specific antisera for wild and vaccine virus types 1, 2 and 3\textsuperscript{13}. The necessary reagents were supplied by the National Institute of Public Health and Environmental Protection (known as RIVM), Bilthoven, The Netherlands. Each isolate was classified as wild-like or vaccine-like. A second test, namely, nucleic acid probe dot-blot hybridization, was also applied to each isolate for confirming intratypic differentiation (ITD)\textsuperscript{14}. Both the tests were performed as previously described\textsuperscript{10}. The probes were specific for wild virus (Indian type 2 wild poliovirus genotype) and for vaccine virus. The reagents for the dot-blot hybridization test were supplied by the Centers for Disease Control and Prevention (CDC), Atlanta, USA.

\textit{Amplification of VP1 region of poliovirus genome:} All type 2 poliovirus isolates that were identified as wild-like in either or both ITD tests, were subjected to further genomic characterization. The poliovirus infected RD cell cultures were frozen-thawed and RNA was extracted using TriPure reagent (GIBCO-BRL). Using the RNA as template and specific primers, cDNA was synthesized and amplified in reverse transcriptase polymerase chain reaction (RT-PCR) in a single tube protocol\textsuperscript{*} as described earlier\textsuperscript{15}. Amplification was applied
to the VP1 region with sense primer Y7 (nucleotides 2399 to 2421) and antisense primer S1 (nucleotides 2987 to 3006) as well as sense primer S2 or S2E (nucleotides 2852 to 2871) and antisense primer Q8 (nucleotides 3485 to 3504). All primers were obtained from the CDC, Atlanta. The primer S2E was a modified version of S2, designed in our laboratory. The PCR mixtures (50µl) contained 2µl RNA, 10 pmole of sense and antisense primers, 15mM Tris-HCl (pH 9.0), 50 mM KC1, 2.5 mM MgCl2, 2.5mM dithiothreitol (Sigma, USA), 0.1 percent triton X100, 200µM (each) dNTPs (Roche Diagnostics, Germany), 10U RNase inhibitor (Roche Diagnostics, Germany), 6U AMV reverse transcriptase (Roche Diagnostics, Germany) and 2.5U of Taq DNA polymerase (Roche Diagnostics, Germany). Reverse transcription at 42ºC for 60 min was followed by 30 cycles of programmed PCR amplification with denaturation at 95ºC for 1 min, annealing at 55ºC for 1 min and extension at 72ºC for 2 min, in a thermal cycler.

Nucleotide sequencing: The nucleotide sequence of VP1 region was determined in two parts, in cycle-sequencing reactions containing fluorescent-dye-labelled dideoxynucleotides as chain terminators, using protocols supplied by the manufacturer (ABI Prism Big-Dye Terminator Cycle Sequencing Ready reaction kit version 2, part No. 4303237E, Applied Biosystems, USA). Thirty cycles of denaturation: 95ºC for 10 sec, annealing: 50ºC for 5 sec and extension: 60ºC for 4 min were carried out in a thermal cycler.

Phylogenetic analysis: The nucleotide sequences of nine isolates of type 2 poliovirus obtained in 2002 and 2003 and laboratory reference strain MEF-1 obtained from the CDC and RIVM were included in genetic comparison. Three isolates of type 2 virus obtained in 2000 identified as MEF-1 laboratory strain (unpublished) were also included in the genetic comparison. Phylogenetic tree of evolutionary relationship of VP1 region (903 nucleotides) was constructed under a maximum likelihood algorithm and 1000 steps of quartet puzzling, using the computer programme Tree Puzzle 4.0 and presented as a dendrogram. This programme estimates evolutionary distances using HYK model of nucleotide substitutions. Neighbour-joining method was used for constructing the phylogenetic tree.

Results

Since October 1999 most isolates of type 2 polioviruses from children with AFP were found to be of vaccine origin. However, three strains in 2000 and 7 strains isolated during December 2002 to February 2003, from children with AFP turned out to be wild and not vaccine-like. One virus isolate each from among healthy contacts and sewage samples collected from Sambhal block, Moradabad district, UP was also identified as wild type 2 poliovirus. All of these were wild-like in ELISA for ITD. However, none of these reacted with either the Indian wild type 2-specific nucleic acid probe or the vaccine-virus-specific probe. Due to this discordance of ITD test results, the VP1 gene nucleotide sequences of our isolates were compared with those of other known type 2 virus strains and thus we identified these as very closely related to the laboratory reference strain known as MEF-1.

The clinical and demographic features of the children with polio due to MEF-1 virus were noted (Table I). Of the 7 cases, 6 were from three districts of western UP and the last one from Gujarat state.

Nucleotide substitutions within the VP1 gene sequences of the 12 virus isolates from the published sequences of two MEF-1 reference strains (CDC and RIVM) were studied (Table II). The CDC and RIVM strains differed by two 3rd position synonymous nucleotide substitutions (nt 99 and 300). All sequences were very closely related (with only 3 to 4 substitutions, hence with 99.5-100% sequence similarity). Nucleotide substitutions were observed at 13 sites that included eight 3rd codon position transitions and one transversion. Three isolates from cases and one from the environmental sewage (Sarai Tareen, Sambhal block, Moradabad district) had sequences identical to an isolate detected previously in 2000 in Banda district. VP1 sequence of one isolate (UPMTR02038) was identical to the RIVM strain.

Two clusters of MEF-1 were observed in the dendrogram showing the genetic relationship of the 12
Discussion

It has been shown clearly in the present study that the isolates of type 2 wild poliovirus in September 2000 and November to February 2002-2003 from 10 children with AFP, one healthy contact and one environmental sample, were quite unrelated to all previous type 2 wild viruses found in India that were genetically characterized in our laboratory. These isolates were identified as laboratory reference type 2 poliovirus strain MEF-1. In the second episode, cases had onset in November to February, but virus isolations and identification were made in the laboratory during December to March. Since it was clearly not a community derived wild strain, but a laboratory reference strain, we concluded that it was introduced from a laboratory source.

Table I. Epidemiological details of MEF-1 wild poliovirus infected children with acute flaccid paralysis

<table>
<thead>
<tr>
<th>Case ID No.</th>
<th>Block/District/State</th>
<th>Age/sex</th>
<th>Date onset of OPV paralysis</th>
<th>Date of stool samples</th>
<th>60 days follow up examination</th>
<th>Virus isolation laboratory</th>
</tr>
</thead>
<tbody>
<tr>
<td>UPMTR02038</td>
<td>Mathura/UP</td>
<td>7/F</td>
<td>10-11-02</td>
<td>25-11-02</td>
<td>RW present</td>
<td>ERC</td>
</tr>
<tr>
<td>UPMRD02144</td>
<td>Sambhal/UP</td>
<td>15/M</td>
<td>12-11-02</td>
<td>30-11-02</td>
<td>RW present</td>
<td>ERC</td>
</tr>
<tr>
<td>UPGALG02056</td>
<td>Moradabad/UP</td>
<td>8/M</td>
<td>16-11-02</td>
<td>21-11-02</td>
<td>RW present</td>
<td>ERC</td>
</tr>
<tr>
<td>UPMRD02150</td>
<td>Moradabad/UP</td>
<td>8/M</td>
<td>06-12-02</td>
<td>21-12-02</td>
<td>RW present</td>
<td>ERC</td>
</tr>
<tr>
<td>UPMRD02157</td>
<td>Moradabad/UP</td>
<td>6/M</td>
<td>18-12-02</td>
<td>21-01-02</td>
<td>RW present</td>
<td>ERC</td>
</tr>
<tr>
<td>UPMG03003</td>
<td>Jawan/</td>
<td>2/F</td>
<td>01-01-03</td>
<td>10-01-03</td>
<td>RW present</td>
<td>ERC</td>
</tr>
<tr>
<td>GUJMC03003</td>
<td>Kalyanpur/Gujarat</td>
<td>25/M</td>
<td>04-02-03</td>
<td>12-02-03</td>
<td>RW present</td>
<td>BJMC</td>
</tr>
<tr>
<td>UPSTP00013</td>
<td>Sitapur/UP</td>
<td>54/F</td>
<td>05-09-00</td>
<td>17-09-00</td>
<td>RW present</td>
<td>SGPGI</td>
</tr>
<tr>
<td>UPBNA00027</td>
<td>Chitrakoot/UP</td>
<td>42/M</td>
<td>21-09-00</td>
<td>28-09-00</td>
<td>RW absent</td>
<td>SGPGI</td>
</tr>
<tr>
<td>BIBXR00017</td>
<td>Buxar/Bihar</td>
<td>84/M</td>
<td>19-07-00</td>
<td>28-09-00</td>
<td>RW present</td>
<td>SGPGI</td>
</tr>
</tbody>
</table>

*Age in months; RW, residual weakness; ERC, Enterovirus Research Centre, Mumbai; BJMC, B. J. Medical College, Ahmedabad; SGPGI, Sanjay Gandhi Post Graduate Institute of Medical Sciences, Lucknow.

AFP cases are given unique identification numbers indicating state (two letter code: UP, BI, GU), district (three letter code: MRD for Moradabad, MTR for Mathura, ALG for Aligarh, BAN for Banda, SIT for Sitapur, BXR for Buxar, JMC for Jamnagar Corporation), year (two digits; 02 for 2002) and case number (three digits; 001).
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Second, there was a discrepancy between the results of the two ITD tests, ELISA showing it as wild, but dot-blot hybridization failing to identify it as wild (or vaccine-derived). Since our probe was based on the gene sequence of indigenous Indian wild type 2 virus, this discrepancy was clearly due to the fact that its parental lineage was not of Indian origin. Therefore a search was done for its ancestral relatives and it was identified as MEF-1.

Possibility of contamination of samples occurring between the points of collection in the field and the isolation and identification of the virus in the laboratory was excluded by the fact that the specimens were collected at different times and places by different personnel. The specimens were transported through different routes. Contamination could occur in the laboratory where the stool was processed and inoculated in cell cultures. In this case, the initial virus isolation and identification had been done in three different laboratories, namely the Sanjay Gandhi Post Graduate Institute of Medical Sciences (SGPGI), Lucknow (in 2000), polio laboratory at the ERC, Mumbai (in 2002-2003) and the BJ Medical College, Ahmedabad (2003). As soon as the detection of laboratory reference strain from two stool samples was informed to us, our laboratory practices

Table II. Nucleotide substitutions in VP1 (903 nt) region of MEF-1 isolates

<table>
<thead>
<tr>
<th>Strain</th>
<th>VP1 nucleotide number</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEF-1 CDC, Reference</td>
<td>66 95 99 122 174 223 300 384 387 555 669 711 827</td>
</tr>
<tr>
<td>UPALG02056</td>
<td>A C C C C A T T A G C A C C</td>
</tr>
<tr>
<td>UPMRD02157</td>
<td>A C C C C A G T A G C A C C</td>
</tr>
<tr>
<td>UPALG03003</td>
<td>A C C C C A G T A G C A C C</td>
</tr>
<tr>
<td>UPMRD02144-ENVIRON</td>
<td>A C C C C A G T A G C A C C</td>
</tr>
<tr>
<td>UPBNA00027</td>
<td>A C C C C A G T A G C A C C</td>
</tr>
<tr>
<td>BIBXR00017</td>
<td>A C C C C G G T A G C A C C</td>
</tr>
<tr>
<td>MEF-1 RIVM, Reference</td>
<td>A C T C C A T C A G C A C C</td>
</tr>
<tr>
<td>UPMTR02038</td>
<td>A C T C C A T C A G C A C C</td>
</tr>
<tr>
<td>UPMRD02144</td>
<td>A C T T T A T C A G C A C C</td>
</tr>
<tr>
<td>UPMRD02150</td>
<td>R T T T T A T C A G C A C C</td>
</tr>
<tr>
<td>UPMRD02144-C104*</td>
<td>A C T T C A T C A G C A C C</td>
</tr>
<tr>
<td>GJUAC03003</td>
<td>A C T T T A T C A G C A C C</td>
</tr>
<tr>
<td>UPSTP00013</td>
<td>A C T C C A T C A G C A T C</td>
</tr>
</tbody>
</table>

R, Adenine/Guanine (Purine); Y, Cytosine/Thymidine (Pyrimidine); *Healthy child contact of case UPMRD02144
were reviewed and scrutinized in great detail and no breach of discipline to account for any possible laboratory contamination of specimens or cell cultures was found. Similar conclusions were drawn by a WHO review team. The subsequent events like, isolation of MEF-1 in the laboratory in Ahmedabad and re-confirmation of virus isolation from original specimens in a third laboratory (data not presented), showed clearly that laboratory contamination at any stage could not explain the finding of this strain of virus in several specimens.

The first time (in 2000) it caused very restricted circulation in time and space (resulting in 3 cases) and died out quickly, but the second time it caused larger but still limited circulation during the three months. Six cases were reported in three districts of western UP, one in Gujarat that was clearly the result of importation, but without any detectable circulation locally. As the source of this strain was determined as introduction from some laboratory, though not yet identified, there is no evidence that indigenous wild type 2 poliovirus had remained silently in human communities and later re-emerged. Since the introduced virus caused very limited circulation, these episodes should not affect the elimination status of wild type 2 poliovirus in India and its eradication status globally.

The virus strain designated as MEF-1 was isolated from polio cases occurring in 1941-42 in the Middle East Forces of the British Army17. Apparently post mortem specimens of two or more cases were pooled for virus isolation, first in monkeys and later passed in cell cultures. It had become a laboratory standard or reference strain for raising antibodies and for challenge in virus neutralization test to detect and assay antibody in serum. It is also used as the seed stock virus for preparing inactivated poliovirus vaccine (IPV)18. Thus, it had been widely distributed throughout the world and many laboratories still maintain it in the freezers. However, to the best of our knowledge it had not caused laboratory-associated infection or nosocomial polio paralysis, at least in recent years. It must also be pointed out that it could be identified only using specific molecular investigations, which had become available only in recent years. Therefore, any episode of breach of safety in a laboratory in the past would not have been recognized.

The question that whether the MEF-1 strain that infected children in 2000 remained in silent circulation and resurfaced in 2002 could be answered in the negative by the gene sequencing data. Mutation rates in the range of \(10^{-5}\) to \(10^{-4}\) per base pair per replication have been reported for different regions of poliovirus genome19,20. Poliovirus genome would incorporate 1 to 2 per cent nucleotide substitutions within VP1 (~900 nt) during 1 yr of person-to-person transmission21. There was no evidence of evolutionary progression due to and during community circulation by the presence of cumulative mutations amongst the two clusters of viruses. Therefore, more than one introduction would have occurred in one part of UP in August and/or September 2000, with very limited spread, with one importation into Bihar. Multiple introductions happened in another part of UP in October and/or November 2002, and again in December 2002 and/or January 2003. In the light of these findings one could be reasonably certain that each of the introductions resulted only in extremely limited transmission. It is known that any given laboratory stock of MEF-1 is made up of a quasispecies22. The CDC and RIVM strains are such variants. Of the two clusters in the Fig., one was closer to the CDC strain and the other closer to the RIVM strain.

One of the main reasons for the lack of wide circulation of the introduced viruses is likely to be the level of herd immunity defined as the proportion of children with immunity to polioviruses23. Moreover, four doses of OPV were administered to children in the annual pulse polio immunization campaigns (29 September and 17 November, 2002 and 5 January and 9th February 2003). Among the 3 types of viruses in the oral polio vaccine (OPV), type 2 is the most immunogenic, both in terms of the frequency of antibody induction in vaccinated children (seroconversion frequency) and the height of antibody response (antibody titre). Therefore, poliovirus type 2 was the first to be eradicated globally due to the efforts for the eradication of wild polioviruses types 1, 2 and 3.

The laboratory or institutional source of this virus introduction into the community remains to be determined. Obviously, stringent measures for establishing laboratory containment of all polioviruses in stock, either as reference strains, or as old isolates or even virus containing stool specimens need to be undertaken immediately in order to prevent any recurrence of a similar introduction of any type of poliovirus. This experience highlights the critical need of
molecular virology investigations and expertise for the complete success of polio eradication efforts, as long as live virus vaccine is in use and any laboratory keeps neurovirulent polioviruses in stock.

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References


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