Isolation and Identification of Polioviruses from Stool Specimens of Suspected Cases

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Abstract

Background: To study the isolation and identification of polioviruses from the stool specimen of suspected cases of poliomyelitis (polio).

Methods: In this descriptive study two poliovirus-sensitive cell lines of human origin HEP-2, and RD were used for poliovirus isolation. Two hundred and two samples of stool specimen were processed according to the WHO recommendations. Supernatant of stool specimen were inoculated on HEP-2, and RD for poliovirus culture, and incubated at 36°C. Cultures were initially set up in growth medium supplemented with serum. After the formation of a confluent monolayer from the cells, cultures were changed to maintenance medium, designed to maintain cultures in healthy state without stimulating growth. The tubes were incubated, and put in racks at inclined angle of 5°, with line (blue for HEP-2, and black for RD cell line) of tube in upper position. Cultures were daily examined for color change from pinkish to yellow, and for appearance of cytopathic effects (CPEs) under the inverted microscope for 10-14 days before discarding as negative. The yellowish coloration indicated the absence of poliovirus while no color change was indicative of presence of poliovirus, and presence of characteristic CPEs production of poliovirus i.e. rounding of cells, and detachment of cells from culture tubes. Tubes showing CPEs were stored at -20°C. Identification of poliovirus isolates was done by mixing the diluted isolates samples, with equal volumes of a set of antisera of known poliovirus serotypes. The antiserum/poliovirus mixture was incubated for 2 hr to allow binding of antibodies to poliovirus. Afterwards the mixtures were inoculated into cell culture tubes, and examined daily for CPEs. Prevention of the development of CPEs by the specific antiserum, indicated the identity of the poliovirus serotype. The identification of both isolates from RD as well as Hep-2 cell cultures, was carried on as poliovirus susceptibility of these two lines varies.

Results: Majority of cases (38.1%) were of 3-12 months, followed by 13-24 months (34.6%). Male to female ratio was 1.4:1.0. Among the suspected cases, 94% were vaccinated, while about 2% were partially vaccinated. Poliovirus was isolated from 28.2% stool specimens of suspected cases of polio. The poliovirus type 1 was found 75.4%, one of the most common among positive cases. 35% cases were found from Punjab and 28% from KPK.

Conclusion: Most prevalent type of poliovirus is type-1, and the maximum number of polio is found in the province Punjab. The most vulnerable age group for polio was 3-12 months, and the target age less than 5 years, that covered the percentage of total number of cases upto 99%. Moreover, 94.06% cases of polio were unvaccinated.

Key Words: Polio, RD, HEP-2, GPEI.

Introduction

Polio is the most serious viral disease. It presents as an acute febrile illness with sudden onset of weakness of extremities. It is caused by poliovirus type 1, 2, and 3, and spreads most commonly by faecal-oral route, particularly in area of poor sanitation. The disease is preventable through high immunization coverage with polio vaccines. Best source of poliovirus is stool, in which excretion may prolong for period of a fortnight or more. Isolation and identification of poliovirus from the stool is the best to confirm the diagnosis of polio. In May 1988, the World Health Assembly (WHHA) declared that the WHO was committed to the Global Eradication of Poliomyelitis by the year 2000. In the current global effort to eradicate polio, the laboratory has leading role in identifying poliovirus isolates. The initiative for the global eradication of Polio was being coordinated by the EPI, established by WHA in 1974. The initial goal of EPI was to provide immunization services to all the children of the world by 1990, during the first year of life against diphtheria,
tetanus, pertussis, measles, tuberculosis and polio. Polio was the first disease whose eradication was the goal of EPI.\textsuperscript{10} The eradication of Smallpox provided the EPI a model of success.\textsuperscript{9-12} Pakistan was the co-sponsor of the resolution of the subject, in the Eastern Mediterranean Region (EMR).\textsuperscript{13} Culture of polioviruses from clinical samples was the gold-standard method for virological surveillance in the world-wide initiative to eradicate wild polioviruses (WPV), and two poliovirus-sensitive cell lines of human origin HEp-2, and RD were used originally by the laboratories of the WHO global poliovirus network.\textsuperscript{14} The number of reported polio cases has fallen worldwide from 350,000 to less than 300 in 2012 since the GPEI in 1988. Pakistan had reduced, from 20,000 polio cases in 1994, to just 58 in 2012.\textsuperscript{15} Pakistan seemed to be very near to success, because polio cases remarkably decreased from 198 in 2011 to only 58 in 2012.\textsuperscript{16} Polio is close to being completely eradicated, with less than 300 new cases being reported globally in 2012.\textsuperscript{17} Now Pakistan is one of the three polio endemic countries, and is posing a real threat to the success of GPEI.\textsuperscript{15} Fortunately Afghanistan, Nigeria, and Pakistan (three polio endemic countries), those have yet to end polio transmission have restricted the virus to lesser areas than ever before.\textsuperscript{18} In 2012, the WHA declared ending polio a “programmatic emergency for global public health”, and called on the WHO Director-General to develop and finalize a comprehensive polio endgame strategy, considering India’s success, the threat to the global community of ongoing poliovirus transmission in three polio endemic countries, and the growing knowledge about circulating vaccine-derived polioviruses (cVDPVs), which can cause outbreaks of paralytic disease.\textsuperscript{19} Recently, the EPI has given the target to win over the race of eradication of poliovirus in Pakistan.\textsuperscript{16} In 2013, the GPEI launched the Eradication and Endgame Strategic Plan, to end polio by 2018. More than 450 scientists voiced their support for the GPEI’s plan, and more than US$4 billion was pledged for its implementation from the donors.\textsuperscript{19}

Polio today is under control due to GPEI worldwide, but the situation will be changed very rapidly if eradication is not completed, because polio has an inbuilt epidemic tendency, which warrants its entire eradication to save polio-free areas, from the threat of polio transmission through three endemic countries.\textsuperscript{19} The Polio Eradication and Endgame Strategic Plan 2013-2018 addresses the eradication of all polio disease, whether caused by WPV or cVDPV. Pakistan is the only endemic country where cases rose last year, with 93 cases in 2013 compared to 58 in 2012.\textsuperscript{18} The WHO’s Executive Board met in Geneva in April 2014 and discussed progress against the Strategic Plan to end polio by 2018, priorities for 2014, and growing concerns over the risk of major outbreaks in polio-free countries. The Member States requested the WHO Director-General to convene experts to advise on the public health benefits of global vaccination requirements for travellers from polio-infected areas. Three new wild poliovirus type 1 (WPV1) cases were reported in May 2014 from North Waziristan, Federally Administered Tribal Areas(FATA), Pakistan, making the total number of cases for 2014 to 39. Despite all these challenges, still it is expected that Pakistan is very much capable of addressing the issues related to reaching children and administering vaccine. The need is to ensure access to the affected population in security compromised areas, improving, and enhancing campaign quality and routine immunization coverage, plus addressing social and cultural constraints, and to develop pinpoint strategies against poliovirus, to fulfil global commitment of implementing WHO’s global Polio emergency Action Plan 2013-18, a long period since May 1988, when WHA passed the resolution of GPEI in the hope of best by the year 2000.

**Patients and Methods**

Two poliovirus-sensitive cell lines of human origin HEp-2, and RD were used at Virology department, NIH Islamabad, from January 1994 to December 1995, for poliovirus isolation. Two hundred and two samples of stool specimen were processed according to the WHO recommendations. The supernatant after centrifugation of each stool sample, was equally divided into two parts in appropriately labeled screw capped vial, one for poliovirus isolation, and one as back up at -20°C for future possible transfer to reference laboratory. The supernatant of stool specimens were inoculated on HEp-2, and RD for poliovirus culture, and incubated at 36°C, according to the WHO guidelines for cell culture technique for poliovirus isolation. Cultures were initially set up in growth medium supplemented with serum. After the formation of a confluent monolayer from the cells, cultures were changed to maintenance medium, designed to maintain cultures in healthy state without stimulating growth. The tubes were incubated , and put in racks in inclined angle of 5°, with line (blue for HEp-2, and black for RD cell line) of tube in upper position. One uninoculated tube of each cell type was also included a control. Cultures were daily examined
for color change from pinkish to yellow, and for appearance of cytopathic effects (CPEs) under the inverted microscope for 10-14 days before discarding as negative. The yellowish coloration indicated the absence of poliovirus, while no color change was indicative of presence of poliovirus, and presence of characteristic CPEs production of poliovirus i.e. rounding of cells, and detachment of cells from culture tubes. Tubes showing CPEs were stored at -20°C. Identification of poliovirus isolates was done by mixing the diluted isolates samples, with equal volumes of a set of antisera of known poliovirus serotypes. The antiserum/poliovirus mixture was incubated for 2 hr to allow binding of antibodies to poliovirus. Afterwards the mixtures were inoculated into cell culture tubes, and the examined daily for CPEs. Prevention of the development of CPEs by the specific antiserum, indicated the identity of the poliovirus serotype. The identification of both isolates from RD as well as Hep-2 cell cultures, was carried on because poliovirus susceptibility of these two lines varies.

Results

Majority were in the age group 3-12 months (Table 1). Among 202 subjects, 118 (58.42%) were males, while 84 (41.48%) were females. Male to female ratio was 1.4:1. Among 202 subjects, 77 (38.12%) belonged to the province Punjab, and 56 (27.7%) belonged to Islamabad. From Khyber Pakhtunkhwa (KPK) 37 (18.3%) specimens were collected while 24 (11.9%) cases from Sindh. Six (3%) cases were collected from Baluchistan while only 2 (1%) stool specimen came from Azad Jammu and Kashmir (AJK). Out of 202 subjects, 4 (1.98%) children were partially vaccinated and 8 cases (3.96%) were regularly vaccinated while the remaining 190 (94.06%) children were unvaccinated. Among the 57 culture positive cases 3 (5.26%) were vaccinated, 1 (1.75%) partially vaccinated whereas 53 (92.98%) cases were unvaccinated.

Out of 202 specimens, poliovirus could be isolated from 57 (28.2%) cases. No virus was isolated in the remaining 145 (71.8%) cases. Out of 77 children upto 12 months of age 25 (32.47%), and among 70 between 13-24 months 22 (31.43%) were polioviruses positive. Between the age 25-36 months in 7 (25%), and in age group 37-48 months, no poliovirus was isolated. In age group 49-60 months poliovirus was isolated in 3 (27.27%). Above the age of 60 months, in all of the 3, poliovirus negative culture obtained. Among 57 positive cases, 43 (75.4%) were poliovirus type 1 (P1) and 4 (7%) were poliovirus type 2 (P2), while 10 (17.6%) cases were of poliovirus type 3 (P3)(Table 2). Out of total 57 positive cases, 20 (35.08%) were from Punjab, 8 (14.04%) from Islamabad, 16 (28.08%) from KPK, 9 (15.79%) from Sindh and 4 (7.02%) from Baluchistan. Out of 2 stool specimens collected from AJKashmir, none was positive. Calculating the frequency of positive cases in different regions, it turned out to be 25.97% for province of Punjab, 14.28% for Islamabad, 43.24% for KPK, 37.50% for Sindh and 66.66% for Baluchistan. No positive case was recorded from AJK. From 20 cases of province of Punjab, in 18 (90%) the poliovirus isolated was P1, and in 2 (10%) cases it was P3 while no isolate belonged to P2. Among 16 positive cases from KPK 13 (81.25%) cases turned out to be of P1 while 1 (6.25%) of P2 and 2 (12.5%) P3. Among 14 positive cases from Baluchistan all (100%) belonged to P1 and no case of P2 or P3 was detected. No positive case was recorded out of 2 cases from AJK. Out of 9 positive cases from the province of Sindh, 4 (44.44%) were of P1 while 2 (22.22%) were P2 and 3 (33.33%) of P3.

Discussion

Collectively 72.7% belonged to 3-24 months of age group. The rest (less than 1/3rd of the total) were belonging to 25-96 months age group (Table 1). These results are in accordance with findings of Hull et al.11 They reported polio, predominantly a disease of very young (upto 90% cases occurring in less than 3 years). Isomura et al reported that majority of children were between 6 months to 2 years of age.20 These results are also in agreement with our study. Label reported male to female ratio of 1.4:1 exactly in agreement with our

<table>
<thead>
<tr>
<th>Age group (months)</th>
<th>Number of subject</th>
<th>Percentage of subject</th>
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<tbody>
<tr>
<td>3-12</td>
<td>77</td>
<td>38.1</td>
</tr>
<tr>
<td>13-24</td>
<td>70</td>
<td>34.6</td>
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<td>5.4</td>
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<td>&gt;60</td>
<td>3</td>
<td>1.4</td>
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<table>
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<tr>
<th>Poliovirus type</th>
<th>Number of subject</th>
<th>Percentage of the subject</th>
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<tbody>
<tr>
<td>P1</td>
<td>43</td>
<td>75.4</td>
</tr>
<tr>
<td>P2</td>
<td>4</td>
<td>7.0</td>
</tr>
<tr>
<td>P3</td>
<td>10</td>
<td>17.6</td>
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Majority of children (94.06%) were unvaccinated. Failure of vaccination is an issue when, whole of the target age community is addressed, and even then polio is occurring, but here the situation is other way round. It is impossible that polio is not eradicated, if serious effort is made for mass immunization during NIDs throughout Pakistan, being a preventable disease. As regards the failure of vaccination, it can be overcome with strict administrative controls, by adopting pin point strategies to make the GPEI a success. It can only be achieved, by reaching every unvaccinated child of Pakistan for polio vaccination.

In this study poliovirus was isolated from 28.2% cases, which is in agreement with the findings of Sokhey et al.22 Among positive cases 75.4% were of P1, 7% of P2, and 17.6% of P3 (Table-2), these results are in line with Sokhey et al, and Ahuja et al.22,23 A local study found, one of the most prevalent poliovirus type with 44.45% in 90% different geographical areas of Pakistan. This finding is consistent with Isomura et al.20

Conclusion
1. Most prevalent poliovirus type is type-1, and the maximum number of polio cases were found in the province of Punjab.
2. The most vulnerable age group for polio was 3-12 months, and the target age (less than 5 years) covered the percentage of cases upto 99%.
3. Majority (94.06%) cases of polio were unvaccinated.
4. There is a dire need for collective efforts of health professionals, political representatives, and media persons, to create an conducive environment to motivate people and convince those who have reservations about the vaccination campaign.

References
15. Ather F. Polio eradication: The biggest public health intervention. KMJ. 2012;