Advanced environmental surveillance and molecular analyses indicate separate importations rather than endemic circulation of wild type 1 poliovirus in Gaza district in 2002.

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Running title: Advanced Poliovirus Environmental Surveillance
Abstract

An improved sewage surveillance algorithm (sample acquisition - processing – molecular analysis) for wild and vaccine-derived polioviruses was developed and validated. It was based on plaque isolation with sensitive and high throughput methods. The molecular analysis included sequencing, a comparison of type, rate, and distribution of nucleotide substitutions with a Profile for Outbreaks Evolving from a Single Progenitor (POESP), and phylogenetic analysis for Relative Similarity (RS). The analyses revealed that two environmental wild type 1 isolates from Gaza district in 2002 were imported separately, most likely from Egyptian southern governorates, and were not linked by endemic circulation. These findings illustrate the continuous spreading potential of wild type poliovirus and underscore the value of extensive environmental surveillance employing advanced molecular analysis to monitor wild poliovirus in poliomyelitis free regions.

Keywords: Poliovirus, environmental surveillance, molecular intratypic differentiation [ITD], molecular epidemiology.
Most wild poliovirus infections are inapparent (7). Sewage surveillance employing manual or automatic sampling coupled with molecular analysis carried out in well-equipped central laboratories is the method of choice for monitoring silent poliovirus circulation within large populations before acute flaccid paralysis appears (10, 24). Models such as that of Ranta et al, (16) describe the influence of various factors on detection probability. The combined surveillance process (sample acquisition – processing - analysis) must be economical and allow high throughput without compromising sensitivity and specificity. It should be capable of identifying vaccine-derived (VDPV) and wild polioviruses in the absence or presence of high vaccine virus background in sewage from populations vaccinated with live attenuated oral poliovaccine (OPV). An isolate is defined as vaccine, VDPV or wild if its capsid protein VP1 has nucleotide homology with the corresponding Sabin vaccine serotype, of 99-100%, 85-99%, or < 85%, respectively (3). Vaccine and VDPV may cause vaccine-associated paralytic poliomyelitis. However unlike vaccine, VDPVs may accumulate sufficient amino acid diversity in neutralizing antigenic sites to render them less well recognized even by large segments of highly immunized populations (19), and may cause poliomyelitis outbreaks in communities with low immunization coverage (11). Furthermore, documented continued isolations of VDPVs from the environment may indicate silent circulation, chronically infected individuals, or re-importations. Each of these possibilities has important implications for poliovirus containment and vaccination policies after global poliomyelitis eradication will be achieved (11, 19).
Environmental Sampling and Processing. Israel initiated environmental surveillance to provide early warning of non-vaccine circulation before appearance of poliomyelitis (12). From 1989-99, sewage was analyzed monthly from 20-36 sentinel communities in Israel and the Palestinian Authority (the West Bank, and Gaza district). After 1999, the number of sampling sites was reduced to 15 covering mainly high-risk sites. As expected, most (>98%) polioviruses isolated from sewage samples between 1989 and 2005 were identified as vaccine strains. Exceptions included 17 related, highly diverged VDPVs isolated between 1998 and 2006 in Israel (18-20) and wild polioviruses isolated in 1990, 1994, 1995-6, 1999, and 2002 almost exclusively from the Gaza district (13).

Wherever possible, untreated sewage aliquots were collected every 30 to 60 minutes over 24 hours by in-line or external automatic composite samplers. Elsewhere, including the Gaza district, aliquots were collected manually every half hour during 3 hours of peak usage in the morning. A cold chain (approximately 4°C) was maintained during sample transport to the lab and storage (<1 week) and processing. The pH of a 1L aliquot from the approximately 1.5 L pooled total volume was adjusted to 7.2. Stirring continued for 30 to 60 minutes after addition of 80 grams of polyethylene glycol 6000 (PEG 6000, Sigma or Merck) and 17.5 grams of NaCl. Samples were centrifuged (10,000xg, 1 hour, 4°C) after an overnight incubation (4°C). The pellet was vigorously agitated in 15 ml of Dulbecco’s calcium and magnesium free phosphate buffered saline containing 0.1% Tween 80 and 15 ml of chloroform. The virus suspension was clarified by centrifugation (1400xg, 15 minutes, 4°C). Virus trapped in the pellet was recovered by centrifugation (1400xg, 15 minutes, 4°C) after vigorously shaking it in 5 ml of 3%
beef extract (pH=7.2). Both supernatants were pooled, antibiotics added (for each 50 to
60 ml of supernatant, 0.5 ml of penicillin stock (50 mg/ml), 0.5 ml of mycostatin stock
(6250 U/ml) and 0.8 ml of PSMy stock (penicillin 50 mg/ml, streptomycin (50000
U/ml) and mycostatin 6,50 U/ml) were added) and samples frozen at -20°C until assay.

There were 2.7±1.5 fold fewer plaques/liter recovered with this PEG method than with
the previously used Freon-based method (12) in 7 samples where poliovirus was
recovered by both methods and no plaques in 4 samples where there were ≤2 plaques
recovered with the Freon method. A mean of twelve percent (15%, or 12% and 8.8%,
respectively) was recovered from three samples spiked with Sabin 3 (100 TCID50) or
Sabin 1 (2x10^4 TCID50).

**Virus isolation:** Viruses in processed sewage were isolated under plaquing
conditions (1 hr challenge, followed by a 2% agarose overlay, 48 hr incubation at 37°C.
Plaque purification of environmental isolates allowed analysis of all recovered
polioviruses. This contrasts with the variable results obtained by methodologies using
mass culture (6, 9) for replicate aliquots from individual sewage concentrates within and
between laboratories (9) which can occur when different isolates dominate and out
replicate the remaining isolates.

We have evaluated different cell lines and growth temperatures for the lowest
input/output ratio as follows: Polioviruses were isolated from processed sewage on
monolayers of murine L20B cells expressing the human poliovirus receptor (15, 25) and
on BGM cells (4). The total number of plaques that required analytic processing was 4.2
fold lower with L20B selection then with growth on BGM cells (12) followed by polio
identification (Table 1). OPV does not grow well at elevated temperatures (14) and we
have used this to select for non-vaccine polioviruses as previously reported (12).

Temperature selection, i.e., restricting analysis to plaques that produced \( \geq 50\% \) CPE within 5 days at \( 40^\circ C \) in 2 ml tube cultures of HEp2C cells, was systematically re-
validated in 2002. Aliquots of poliovirus from each plaque were grown in 2 ml HEp2C
(ATTCCCL23) tube cultures at \( 40^\circ C \) for 5 days and at \( 37^\circ C \) until complete CPE (\( \leq 3 \) days). Uniformly high titers of isolates that grew at \( 40^\circ C \) were prepared for further
characterization by passage on 2 ml HEp2C tube cultures (\( 37^\circ C \); 24 hours).

Poliovirus plaques isolated from 51 of 103 (49.5\%) consecutive sewage samples
were characterized (Table 2). Two were wild type. All the remaining isolates were
vaccine and 27.5\% of them grew at \( 40^\circ C \). Most (92\%) vaccine isolates that grew at \( 40^\circ C \)
were of the same serotype as virus from the same plaque cultured at \( 37^\circ C \).

Occasionally two or more virus particles remained physically associated during passage
through the sewer system, subsequent treatment, and challenge. Mixed serotype
microclumping was confirmed by selective outgrowth of each serotype after
neutralization of the other type(s). Microclumping frequency was underestimated since
only micro-clumps containing roughly equal amounts of different serotypes would have
been detected. Temperature selection reduced the number of mixed-serotype plaques
because of the low probability that >1 isolate was capable of growth at \( 40^\circ C \).

Microclumping was further reduced by vigorous agitation during processing, however
this increased the number of isolates with identical or near identical substitutions.

At \( 37^\circ C \), all 3 poliovirus serotypes were recovered in near equal numbers (38\% type 1, 30\% type 2 and 31\% type 3). In contrast type 2 isolates predominated (62\%)
among isolates recovered after growth at \( 40^\circ C \), as found previously (18).
Quality for sewage collection and processing was assured using quantitative,
plaque isolation of enterovirus from processed sewage on either BGM or HEp2C
monolayers.

**Typic (TD) and intratypic (ITD) differentiation of poliovirus isolates.**

Poliovirus serotypes (TD) were determined by micro-neutralization (2) or immuno-
fluorescence using type-specific monoclonal antibodies (Chemicon International, Inc.
Temecula, CA., cat. nos. 3331, 3332, and 3335). Isolates were characterized as Sabin-like
or non-Sabin-like (ITD) by micro-neutralization using monoclonal and or cross-adsorbed
polyclonal anti-Sabin vaccine antisera (12).

TD and ITD were also inferred by dot blot hybridization to Sabin vaccine-specific
RNA probes (5) and/or by sequence analysis(17). For sequence analysis, viral RNA was
amplified (Ready-to-Go RT-PCR beads; Amersham-Pharmacia Biotech LTD,
Buckinghamshire, England), purified (QIAquick Gel Extraction Kits; QIAGEN GmbH,
Hilden, Germany or HighPure PCR Product Purification Kits; Roche Diagnostics,
Indianapolis, IN, USA) with Y7 sense and Q8b anti-sense primers and sequenced
automatically using the Q8b anti-sense primer (17). Molecular TD was provided with the
Sequencher sequence analysis program (Gencodes Corporation, Ann Arbor, MI, USA).

When parameters were set for ≥85 % homology, the anti-sense strand VP1/2A isolate
sequence (≥500 nt) only formed a contig with the actual sequences (both strands) of its
corresponding Sabin serotype. A contig is a continuous sequence of bases derived from a
set of aligned sequence fragments. Furthermore, the % nucleotide difference between
query and Sabin reference within this contig provided ITD (e.g., ≤1% for vaccine, >1%
for VDPVs). Both strands were sequenced whenever the difference exceeded 0.5%.
Isolate sequences failed to form contigs when they were of poor quality, represented poliovirus mixtures, were from non-polioviruses, or were from wild poliovirus (divergence >15%). Inspection of the graphic output distinguished among the possibilities. TD of wild viruses was obtained by reiteratively decreasing the % homology until a contig was formed.

Wild polioviruses, PV1/5763-1T/PAL02 and PV1/5816-2T/PAL02 (access numbers AM056055 and AM056056, respectively), were isolated from sewage samples no. 5763 and 5816 collected from Gaza district on Feb 16th and Aug 4th, 2002 (Table 2). They were revealed to be type 1 by iteratively increasing the allowed maximum divergence in Sequencher by 2% steps until a contig formed with Sabin 1. Both strands were sequenced. These isolates grew at 40°C, were non-OPV by dot blot hybridization, and were not neutralized by anti-Sabin monoclonal or polyclonal anti-polio vaccine antisera.

**Epidemiological characterization of the 2 wild type viruses.** Empirical observations (Table 3, central column) of three parameters: the maximum rate of substitutions, the distribution of substitutions along the poliovirus genome and within codons, and the transitions:transversion ratio for isolates from the one-year 1987-88 poliovirus outbreak in Israel (17) were used to develop a Profile for Outbreaks Evolving from a Single Polioviral-progenitor (POESP). The POESP (Table 3) was formulated from an outbreak of short duration so that it would most closely represent evolutionary changes expected to occur during a short interval between ≥2 environmental and/or clinical isolations. Similar values (not shown) were extracted from an outbreak of longer duration (9). Of the three parameters, the rate of substitution carries the most weight for
determining whether isolates were directly related through silent endemic circulation or introduced separately from ≥1 external reservoir. The confidence for ruling out a direct chain of transmission increases the higher the substitution rates is above POESP expectations especially when the interval between isolations is short. Thus it is very unlikely that PV1/5763-1T/PAL02 and PV1/5816-2T/PAL02 collected 6 months apart in Gaza district were directly related by endemic circulation since the substitution rate was 17-fold higher than expected (Table 3). Poliovirus genomic RNA frequently recombines with RNA from other polioviruses or non-polio enteroviruses (1, 8). Identical recombination patterns, like those described for Israeli VDPV’s (19), are strong indications of a direct relationship to a common ancestor (Section 2, Table 3).

Relative similarity (RS), the % nucleotide similarity between local isolates divided by the % similarity between either and its most similar non-local isolate, provides another guideline for distinguishing separate introductions from endemic circulation. Sequences from databases of the Global Specialized Poliovirus Reference Laboratories at the CDC and Pasteur Institute, and from the WHO supported (TSA/18/181/526) project on environmental surveillance in Egypt at the KTL Laboratory were screened for similarity to the Gaza district sequences. Egyptian environmental isolates from Minya (AF531118 and AY923837) shared highest homology with PV1/5763-1T/PAL02, while isolates from Asyut (AF545125-6) and Abu Qurqas (AF545121) shared highest homology with PV1/5816-2T/PAL02. An RS <1 for both 2002 Gaza district isolates compared with these Egyptian isolates (Table 3) indicated that both local isolates were more closely related to isolates from external reservoirs than they were related to each other. This strengthened confidence for separate introductions as indicated by POESP as
did a consensus neighbor-joining phylogenetic tree constructed using the ClustalX (22) program with sequence data bootstrapped 1000 times (Fig. 1.).

In conclusion, we have documented the effectiveness of our improved sewage surveillance algorithm (isolation - treatment - analysis) for identifying viral circulation before appearance of clinical cases, and have provided a paradigm for identifying the location of remote reservoirs from which the local isolates were most likely imported. Sewage treatment and viral isolation modifications described here significantly reduced laboratory costs and hands-on time. The surveillance algorithm worked well for sewage with high OPV background in the environment when the Israel and Palestinian immunization programs included OPV (21, 23) and in the absence of OPV when Israel switched to exclusive use of inactivated poliovaccine, IPV (19). Finally our results illustrate the significant international contribution this algorithmic approach can provide for the Global Poliomyelitis Eradication Initiative. Specifically, a poor fit with the POESP and a strong RS linkage between local isolates and recent external isolates should trigger surveillance and possibly active intervention at the remote reservoir where the virus lineage may also still circulate silently.
Acknowledgements

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Accession numbers for wild type 1 environmental isolate sequences PV1/5763-1T/PAL02 and PV1/5816-2T/PAL02 are AM056055 and AM056056, respectively. The Egyptian environmental isolates (KTL database) are derived from a WHO supported (TSA/18/181/526) project on environment surveillance in Egypt and have accession numbers: AF545121, AF545125-26, AY531188, and AY923837.

Conflict of Interest

None declared.


Figure Legends

**Fig. 1** Phylogenetic relationship between wild poliovirus environmental isolates from Gaza and Southern Governates in Egypt.

Consensus neighbor-joining phylogenetic trees were constructed using the ClustalX (22) program with sequence data bootstrapped 1000 times. Trees were visualized using njplot (M. Gouy, Laboratoire de Biometrie et Biologie Evolutive, U. Lyon, CNRS, France, URL [http://pbiol.univlyon1.fr/software/njplot.html](http://pbiol.univlyon1.fr/software/njplot.html)).

Table Legends

**Table 1.** Comparison of poliovirus identification from processed sewage after plaque isolation on L20B or BGM cell monolayers.

| * | Remaining plaques could not be analyzed due to their overlap with ≥1 other plaque. |
| ** | Poliovirus isolates recovered at 37°C and/or 40°C. |
| *** | Potential isolates of VDPV or wild poliovirus. |

Cell medium for all lines was DMEM, 10% fetal bovine serum, penicillin (160 U/ml), streptomycin (0.32 gm/ml) and mycostatin 20 U/ml.

**Table 2.** Comparison of characterized polioviruses from viral stocks prepared from L20B plaques amplified at 37°C or after selection at 40°C for 5 days and re-amplification at 37°C.
• Includes 3 plaques with >1 type at one temperature, 1 of which matched the type at the other temperature.
** Includes the additional serotyped isolates from plaques containing more than one serotype.
*** Total number of isolates from both plaque categories characterized by ITD as either Sabin 1 (S1), Sabin 2 (S2), Sabin 3 (S3), or non-Sabin like (NS).
**** Both characterized as wild type-1 polioviruses by sequencing, dot blot hybridization, and micro-neutralization with monoclonal and polyclonal anti-Sabin antibodies.

Cell medium for all lines was DMEM, 10% fetal bovine serum, penicillin (160 U/ml), streptomycin (0.32 gm/ml) and mycostatin 20 U/ml).

Table 3. POESP and RS analyses of wild type 1 poliovirus isolates from 2 sewage samples from the Gaza district collected 4 months apart.

* Multiple substitutions at any nucleotide position can only be identified if the sequence of a progenitor is available.
** Absent or Present, but if present, then present in all progeny unless replaced by a subsequent recombination further towards the 5’ end of the genome.
*** Ratio of the % similarity between local isolates divided by the % similarity between at least one local isolate and the isolate from an external reservoir with the highest % similarity.
<table>
<thead>
<tr>
<th>Sewage Samples Analyzed</th>
<th>Primary Isolation</th>
<th>Samples Containing Enteroviruses (%)</th>
<th>Samples Containing Polioviruses (%)</th>
<th>Total Number of Plaques</th>
<th>Total Number of Plaques Analyzed</th>
<th>Poliovirus Isolates Growing at 40°C ***</th>
</tr>
</thead>
<tbody>
<tr>
<td>68</td>
<td>BGM</td>
<td>48 (71%)</td>
<td>29 (43%)</td>
<td>860</td>
<td>464 (54%)**</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>L20B</td>
<td>28 (41%)</td>
<td>28 (41%)</td>
<td>110</td>
<td>9 (9%)</td>
<td>110 (100%)</td>
<td>**</td>
</tr>
</tbody>
</table>
### A. Typic Differentiation of Poliovirus Isolates

<table>
<thead>
<tr>
<th></th>
<th>Challenge at 37°C</th>
<th>Challenge at 40°C</th>
<th>Identical Serotype at 37°C &amp; 40°C</th>
<th>Non-identical Serotypes at 37°C &amp; 40°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poliovirus Positive Sewage Samples per Total Analyzed</td>
<td>51 of 103</td>
<td>20 of 103</td>
<td>n.a.</td>
<td>n.a</td>
</tr>
<tr>
<td>Poliovirus Plaques Typed</td>
<td>273</td>
<td>75</td>
<td>69*</td>
<td>6</td>
</tr>
<tr>
<td>Total Number of Poliovirus Isolates Typed**</td>
<td>306</td>
<td>78</td>
<td>n.a.</td>
<td>n.a</td>
</tr>
</tbody>
</table>

### B. Intratypic Differentiation of Typed Poliovirus Isolates**

<table>
<thead>
<tr>
<th>ITD of All Isolates in Each Plaque</th>
<th>Number of Plaques</th>
<th>Total Number of Isolates***</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>37°C</td>
<td>40°C</td>
</tr>
<tr>
<td>Plaques with 1 Serotype</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S1</td>
<td>98</td>
<td>3</td>
</tr>
<tr>
<td>S2</td>
<td>77</td>
<td>45</td>
</tr>
<tr>
<td>S3</td>
<td>72</td>
<td>22</td>
</tr>
<tr>
<td>NS</td>
<td>2***</td>
<td>2****</td>
</tr>
<tr>
<td>Plaques with &gt; 1 Serotype</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S1 S2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>S1 S3</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>S2 S3</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>S1 S2 S3</td>
<td>9</td>
<td>0</td>
</tr>
</tbody>
</table>

Total Number of Non-vaccine Isolates per Total Analyzed | 2 per 306 | 2 per 78
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Expected Values for Local Transmission</th>
<th>Actual Values for PV1A compared to PV1B</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. POESP</td>
<td>random, uniform &gt; 80% 3rd position</td>
<td>random, uniform 91.5% 3rd position</td>
</tr>
<tr>
<td>Nucleotide Substitutions</td>
<td>1.2 x 10^{-2}</td>
<td>2.0 x 10^{-2}</td>
</tr>
<tr>
<td>Distribution by position</td>
<td>3.5 - 3.8 x 10^{-2}</td>
<td>1.9 x 10^{-2}</td>
</tr>
<tr>
<td>by nucleotide position</td>
<td>&lt; 1.5 x 10^{-4}</td>
<td>progenitor unavailable</td>
</tr>
<tr>
<td>by codon position</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rate: substitutions/yr/site</td>
<td></td>
<td></td>
</tr>
<tr>
<td>any position</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3rd codon positions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>multiple at same site *</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type of substitution</td>
<td>&gt; 90%</td>
<td>91.5%</td>
</tr>
<tr>
<td>synonymous</td>
<td>5-15%</td>
<td>11.3%</td>
</tr>
<tr>
<td>% transversions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Genomic Recombination**</td>
<td>Absent or Present</td>
<td>not determined</td>
</tr>
<tr>
<td>3. RS Relative Similarity***</td>
<td>&gt;1.0</td>
<td>0.94 - 0.95</td>
</tr>
</tbody>
</table>