

Molecular epidemiology of silent introduction and sustained transmission of wild poliovirus type 1, Israel, 2013

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Poliovirus vaccine coverage in Israel is over 90%. The last nine birth cohorts have been vaccinated exclusively with inactivated polio vaccine (IPV). However, between February and July 2013 type 1 wild poliovirus (WPV1) was detected persistently in 10 and intermittently in 8 of 47 environmental surveillance sites in southern and central Israel and in 30 stool samples collected during July from healthy individuals in southern Israel. We report results of sequence and phylogenetic analyses of genes encoding capsid proteins to determine the source and transmission mode of the virus. WPV1 capsid protein 1 nucleotide sequences were most closely related to South Asia (SOAS) cluster R3A polioviruses circulating in Pakistan in 2012 and isolated from Egyptian sewage in December 2012. There was no noticeable geographical clustering within WPV1-positive sites. Uniform codon usage among isolates from Pakistan, Egypt and Israel showed no signs of optimisation or deoptimisation. Bayesian phylogenetic time clock analysis of the entire capsid coding region (2,643 nt) with a 1.1% evolutionary rate indicated that Israeli and Egyptian WPV1-SOAS lineages diverged in September 2012, while Israeli isolates split into two sub-branches after January 2013. This suggests one or more introduction events into Israel with subsequent silent circulation despite high population immunity.

Introduction

Two major lineages of type 1 wild poliovirus (WPV1) are currently circulating in endemic countries: the South Asia (SOAS) lineage in Pakistan and Afghanistan, and a West African lineage (WEAFB1 and WEAFFB2) in Nigeria [1]. Both of these lineages occasionally spread to other

countries with a high percentage of non-immunised children and have caused cases of acute flaccid paralysis (AFP) [2]. Individuals in populations with high vaccination coverage are generally protected from disease [3,4]. In such countries, detection of poliovirus importation, subsequent transmission and interruption of chains of transmission requires a high level of integration of multiple surveillance strategies performed in parallel over extended periods of time to increase reliability and strengthen interpretation of data [5].

The World Health Organization (WHO) European Region, which includes Israel, was declared poliovirus-free on 21 June 2002 [6]. Afterwards, there was only a single importation of WPV1 to the region – into Tajikistan in April 2010 from a reservoir in India. This introduction resulted in a local outbreak of poliomyelitis that subsequently spread to central Asia and Russia [7].

The last cases of poliomyelitis in Israel occurred during an outbreak in 1987–88 [8,9]. Israel has been free of poliomyelitis since 1989, as a consequence of high immunisation coverage [9,10]. Between 1990 and 2005, infants in Israel received three doses of enhanced inactivated polio vaccine (eIPV) and three doses of live-trivalent attenuated oral polio vaccine (OPV) by the age of 15 months [11], followed by an OPV booster at age five to six years (first grade of school). After 2005, the routine immunisation schedule was changed, consisting of four eIPV doses by 15 months and an eIPV booster at age five to six years. Vaccination coverage since 1990 has ranged between 92% and 95% and within individual health districts from 81% to 100% (Emelia Anis,

Epidemiology Division, Israel Ministry of Health, personal communication, 31 December 2013).

Classical AFP surveillance [12] and routine monthly environmental (sewage) surveillance in catchment areas covering 30–40% of the entire population [13,14] performed routinely since 1989 revealed silent importation of WPV1 into Ashdod, Israel, in 1996 and routine surveillance samples supplied by the Palestinian Ministry of Health, in 1994–96, 1999 and 2002, revealed repeated introductions of WPV1 into nearby Gaza [5,10].

In May 2013, a virus isolated from a sewage sample collected on 19 April from Rahat, Israel, was identified as WPV1 [15,16]. A sensitive quantitative reverse transcription polymerase chain reaction (qRT-PCR) assay [17] was used for retrospective and prospective screening of sewage. A sample collected from Beer Sheva in February 2013 was the earliest to contain WPV1. In June 2013, the frequency of sampling and the number of environmental surveillance sites was expanded to 80 (from fewer than 15), especially in catchment areas in southern Israel near sites that were found to be positive for WPV1 [15,16]. Between February and July 2013, a total of 10 sites in south and central Israel were persistently positive for WPV1. In addition, WPV1 was found intermittently in an additional eight sites out of 47 environmental surveillance sites in southern and central Israel and in some stool suspensions from stool samples collected in July 2013 in southern Israel from healthy individuals (data not shown).

The first response to these findings targeted completing immunisation with IPV of all children in the southern districts, raising full coverage from 90% to above 99%. This was followed, from 8 August 2013 onwards, by one round of supplementary immunisation with bivalent OPV (types 1 and 3) targeting all children up to the age of nine years, i.e. those previously immunised exclusively with IPV. As of 15 September 2013, approximately 750,000 of about 1,200,000 children eligible for bivalent OPV (63%, interhealth district range: 45–83%) had been vaccinated nationwide [12].

Here we report on the genotype of WPV1 isolated between February and July of 2013 and its ability to circulate in the highly immunised population of Israel, and assess the possible impact of such isolates on polio eradication in Israel and other countries using an IPV-only vaccination programme. To the best of our knowledge, this is the first in-depth molecular analysis of a wild poliovirus responsible for introduction and sustained silent circulation in a highly vaccinated population.

Methods

Ethics statement

The Ethical Review Board of the Sheba Medical Center, Tel Hashomer, approved this study (SMC-0774-13) and

exempted it from a requirement to obtain informed consent. All links to personal details pertaining to, or which could be used to identify individual patients, were removed and data was analysed anonymously.

Isolation of poliovirus from sewage samples

In-line automatic samplers at the point of entry into sewage treatment plants in Israel [5,18] were supplemented with portable automatic composite samplers for collecting sewage at upstream sites. Poliovirus in the sewage was concentrated according to the Israel Sewage Surveillance Protocol as described elsewhere [13]. Monolayers of L20B cells in 10 cm Petri dishes were challenged with aliquots of processed sewage (2 mL from 1 L of sewage concentrated to 30 mL) under plaque-forming conditions (1 hour incubation at room temperature followed by incubation at 37 °C for ≥2 days until staining after addition of 12.5 mL of culture medium containing 0.9% agar, staining with neutral red to visualise plaques.

Viral loads are the number of plaque-forming units per ml of processed sewage. Plaque isolates were re-grown in HEp2C tube cultures incubated at 37 °C. In parallel, four replicate L20B tube cultures were challenged with aliquots (0.3 mL) of processed sewage and grown for five days or until full cytopathic effect (CPE) developed. To enable higher throughput and reduce workload, replicate supernatants from CPE-positive L20B tube cultures challenged with aliquots of processed sewage were pooled before RNA extraction.

Isolation of poliovirus from stool suspensions

Stool samples that had been collected from healthy children and adults in catchment areas with low and high viral loads in July 2013 were tested for the presence of WPV1.

Virus isolation: two strategies were used to isolate WPV1 from stool suspensions. The United States Centers for Disease Control and Prevention (CDC) isolated poliovirus from (10%) stool suspensions using standard WHO protocols involving two passages in tissue culture of L20B (a mouse cell line expressing the human cellular receptor for poliovirus) and RD (a cell line derived from human rhabdomyosarcoma) [19]. In parallel, the Central Virology Laboratory (CVL), Tel Hashomer, Israel, screened RNA extracted from replicate stool suspensions from these samples using quantitative real-time RT-PCR (qRT-PCR) with primers specific for WPV1 [17] to identify WPV1-positive suspensions. WPV1 was isolated by challenging L20B tube cultures with 0.3 mL aliquots of the WPV1-positive stool suspensions. As OPV does not grow well at elevated temperatures [20], we used this feature to select for non-vaccine-derived polioviruses (14) until the first discovery of WPV1 in May 2013 since none of these plaques grew at 40 °C. VP1 capsid genes were sequenced from WPV1 isolated from 30 SOAS-positive stool suspensions from asymptomatic individuals.

Sequencing

Forward primers Y7 or Y7R and reverse primer Q8 were used to amplify and sequence RNA from WPV1 VP1 templates by RT-PCR [21]. At CDC, both strands of amplicons were sequenced on an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, United States) using an ABI PRISM BigDye Terminator Cycle Sequencing kit (Applied Biosystems) and appropriate primers [21]. At the CVL, both strands of the amplicons were sequenced on an ABI 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, United States) using an ABI PRISM BigDye Terminator Cycle Sequencing kit (Applied Biosystems) and Y7 and Q8 primers. Complete P1 and 3D polymerase sequences were determined for some isolates using additional primers as described in Yang et al. [22].

Phylogenetic analysis

Poliovirus isolates with the closest matches to the initial partial VP1 sequence from the plaque from the original Israeli environmental sample from which WPV1 was first isolated (from sewage collected from Rahat on 9 April 2013) were identified by Basic Local Alignment Search Tool (BLAST) [23] analysis of the DDBJ/EMBL/GenBank (DNA Data Bank of Japan/EMBL-Bank, European Nucleotide Archive, United Kingdom/GenBank, National Center for Biotechnology Information (NCBI) United States) and databases at the National Institute of Health-Pakistan and CDC. Representative related sequences were included in the phylogenetic and time clock analyses.

Neighbor-joining [24] phylogenetic analysis was performed with MEGA5.22 [25] using the Tamura-Nei substitution model [26].

A Bayesian Markov chain Monte Carlo (MCMC) tree was inferred from complete P1 capsid sequences (2,634 nt) using BEAST v1.7.5 [27]. The TN93 + Γ was the best-fitting model of evolution as estimated by MODELTEST [28]. Two independent chains of 40 million steps each were run under the strict clock model, assuming a constant substitution rate of 0.011 substitutions per site per year [29] and including the sample's collection date as temporal data. Effective sample size values were monitored for consistency using Tracer v1.5 [30]. A maximum credibility tree was obtained using TreeAnnotator (included in BEAST) and visualised in FigTree v1.4 [31].

The sequences of Israeli WPV1-SOAS isolates have been deposited in the DDBJ/EMBL/GenBank and have been assigned accession numbers KJ019831 to KJ019833, KJ013419 to KJ013424, KJ013425 to KJ013498, and KJ155485 to KJ155493. Sequences from Pakistan had DDBJ/EMBL/GenBank accession numbers JQ906456, and KF990615 to KF990620, and those from Egypt had accession numbers KJ155495 to KJ155500. Israeli isolate names (e.g. PV1_8062-PL1_ISR13) indicate the serotype (PV1), the ID of the sewage sample or stool suspension (8062); the source of the isolate (-PLn for

plaques where 'n' indicates the number of the plaque, -tt for pool of mass tube cultures, or -s for stool suspension) and the country from which the sample was collected (ISR for Israel), with the year in which it was collected (13, short for 2013). For brevity, within the manuscript, this name has been shortened to the ID of the sample and the source (e.g. 8062-PL1).

Results

Molecular characterisation of environmental and stool survey isolates

Concentrated environmental surveillance samples from 11 March 2013, from routine monthly sampling of sewage from Rahat (8082) and Beer Sheva (8083), yielded respectively 25.4 and 3.7 plaques per mL of sewage on L20B cells. By 9 April, this had increased to 2,600 and 6.2 per mL, respectively (samples 8099 and 8098).

Partial VP1 sequences of five plaques chosen for enterovirus genotype identification were compared with sequences in the DDBJ/EMBL/GenBank by BLAST. The closest match (95.3%) was a WPV1 belonging to the SOAS lineage isolated in Pakistan in 2006. We notified the WHO Regional office for Europe that WPV1-SOAS had been isolated from sewage. The complete VP1 sequence of isolate 8099-PL9, sent to WHO and CDC for comparison with more contemporary wild poliovirus isolates, was most closely related to WPV1-SOAS cluster R3A isolated from environmental samples collected in Pakistan in 2012 and from sewage in Egypt in December 2012. Retrospective analysis using WPV1-SOAS-specific primers and probes revealed that sample 8062 from Beer Sheva collected on 6 February 2013 was the earliest to contain WPV1-SOAS.

The complete VP1 sequence (906 nt) was obtained for viruses from WPV1-SOAS qRT-PCR-positive cultures from sewage and stool suspensions collected between February and July 2013. The sequences confirmed them as WPV1-SOAS. The maximum pairwise sequence divergence was 2.1%. This rate was twice as high as the expected 1.1% per year [29] and suggested possible multiple introductions. However a Bayesian time clock analytical comparison with a substitution rate of 1.1% per year was consistent with the dates of isolation when nucleotide sequences of the complete P1 capsid region (2,643 nt) for isolate 8062-PL1 (Beer Sheva; 6 February), 8099-PL9 (Rahat; 9 April), three Israeli sewage isolates with VP1 sequences that were the most divergent from that of 8099-PL9, stool isolates from three healthy Israeli children collected in July and environmental isolates from Pakistan and Egypt were analysed (Figure 1). Branch support for the tree was robust; the major branches linking the Pakistani, Egyptian and Israeli isolates had very high posteriors (>0.99). The estimated mean value for the total length of the tree was about 1.16 years (95% confidence intervals: 1.09–1.23), placing the date of the root around 10 May 2012. The estimated dates for the nodes, and bars

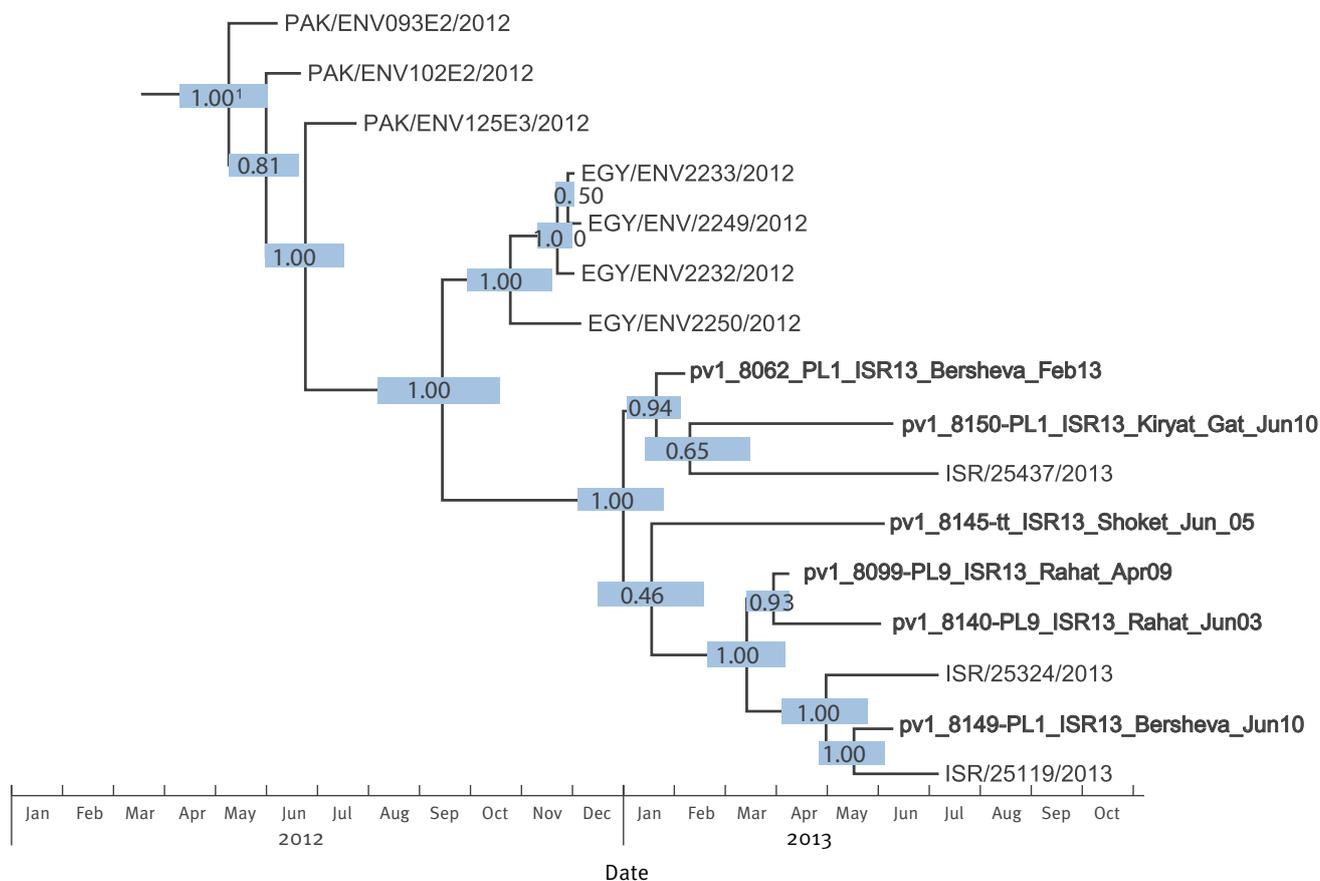
representing 95% highest posterior density intervals for the estimated dates, are shown in Figure 1.

A neighbor-joining, Tamura-Nei model tree for VP1 sequences (906 nt) from 31 stool isolates, 21 tube cultures and 28 plaques is shown in Figure 2. Some non-plaque purified isolates contained mixtures of WPV1-SOAS as indicated by more than one nucleotide for a given nucleotide position. A unique, majority call identity was assigned for these positions when the same base predominated on both strands. It was still not possible to assign a majority call for one or more positions in 34 sequences from isolates from environmental samples collected between February and July 2014: these sequences were not included in the phylogenetic analysis. With the inclusion of the genetically related samples from Pakistan and Egypt, both a model- and topology-dependent (maximum likelihood, Tamura-Nei model) and a model- and topology-independent (Tjima's test) analyses indicated that the accumulation and diversity of nucleotide substitutions among Israeli isolates did not rule out evolution according to the expected evolutionary clock for

poliovirus. In the former analysis, the shape parameter for the discrete gamma distribution was 0.3239 and p value for rejection of the molecular clock was 0.07. For the latter analysis, the equality of evolutionary rate between sequences 8191-tt (Kiryat Gat Jul03) and EGY12-2013712250, with PAK12-ENV093E2 was used as an outgroup in Tajima's relative rate test [32]. The chi-squared test statistic was 3.00 ($p=0.08326$ with one degree of freedom). P values less than 0.05 were used to reject the null hypothesis of equal rates between lineages. Codon positions included were first + second + third + noncoding. None of the positions contained gaps or missing data. No signs of codon usage optimisation or de-optimisation were detected among the Pakistani, Egyptian and Israeli datasets. Tree branch topology indicated co-circulation of a number of different sublineages. Members of sublineages were dispersed throughout the catchment areas in central and southern Israel that were positive for WPV1-SOAS, e.g. there was no noticeable geographical clustering of related isolates within the catchment areas. There was also no evidence of recombination with other polio or non-polio enterovirus serotypes during the period

FIGURE 1

Bayesian phylogenetic tree of P1 capsid sequences (2,634 nt) from WPV1-SOAS environmental samples from Pakistan (2012), Egypt (December 2013) and Israel (February to July, 2013)

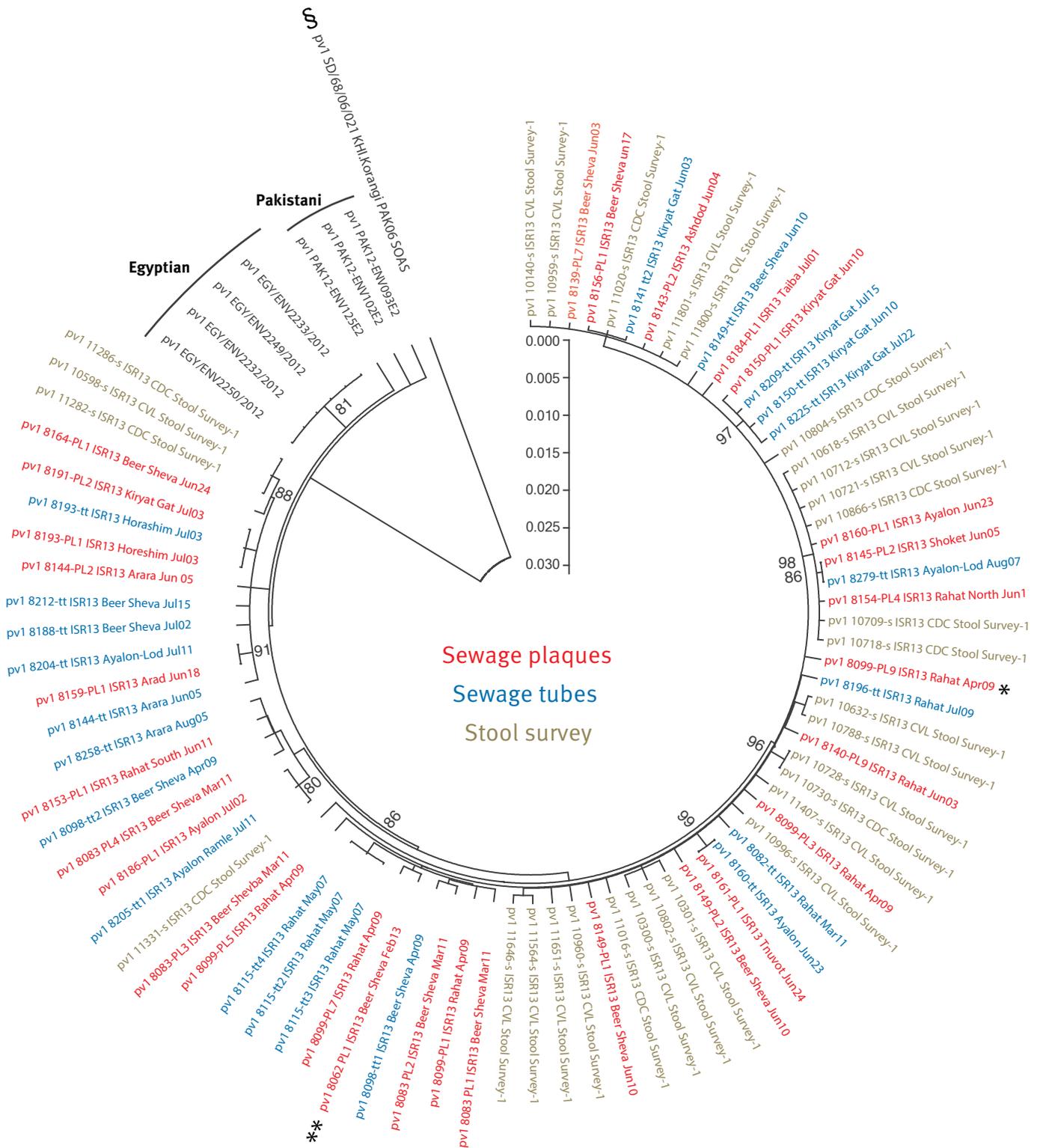


SOAS: South Asia; WPV1: type 1 wild poliovirus.

The tree includes sequences from stool cultures from three healthy Israeli individuals (ISR/25437/2013, ISR/25324/2013, AND ISR/25119/2013) in addition to environmental sequences. Branch lengths are proportional to time (years) assuming a constant substitution rate of 0.011 substitutions per site per year. Sample collection dates have been appended to the name of each isolate. The 95% confidence intervals for each estimated node age are shown in light blue bars. Branch support at each node is shown as posterior value within the bars.

FIGURE 2

Neighbor-joining phylogenetic tree of VP1 sequences from WPV1-SOAS recovered from environmental samples from Pakistan (2012), Egypt (December 2012) and Israel (between February and July 2013) and from stools from healthy individuals in Israel (July 2013)



SOAS: South Asia; WPV1: type 1 wild poliovirus.

§ indicates the sequence used as an outgroup, i.e. the sequence isolated from Pakistan in 2006.

* indicates the sequence from the original Israeli environmental sample from which WPV1-SOAS was first isolated.

** indicates the earliest Israeli environmental sample found to contain WPV1-SOAS by retrospective screening.

The evolutionary history was inferred with MEGA5.22 using the neighbor-joining method. The figure represents the bootstrap consensus tree inferred for complete VP1 sequences (906 nt) from 1,000 pseudo-replicates. Branches corresponding to partitions reproduced in fewer than 75% bootstrap replicates were collapsed. The evolutionary distances were computed using the Tamura-Nei method and are in the units of the number of base substitutions per site. The rate variation among sites was modelled with a gamma distribution (shape parameter = 0.3). None of the sequences contained gaps, degenerate bases (from mixture of closely related isolates in tissue culture) or missing data. The names of the isolates have been colour-coded to indicate their origin (red for plaques isolated from environmental samples, blue for WPV1 from tube cultures challenged with processed sewage and brown for WPV1 isolated from stool suspensions from healthy individuals).

of study based on 3D polymerase sequence analysis of two Pakistani, four Egyptian and eight Israeli WPV1 SOAS sequences, although a stool survey indicated a five-fold higher point prevalence of enteroviruses among children in southern Israel as compared with that of WPV1 (data not shown).

Discussion

Sequence and phylogenetic analyses are essential epidemiological tools that have been used to trace the origin and routes of transmission of polioviruses from endemic regions to poliovirus-free regions and to characterise the evolution of poliovirus during person-to-person transmission during outbreaks [6,24,27]. This report describes the sequence analysis of progeny of a WPV1 that was introduced into Israel and then established a sustained silent spread within a population that started with an average vaccine coverage of more than 95% and where the last nine birth cohorts were vaccinated exclusively with IPV.

Sequence comparisons established that the introduced WPV1 strain was related to WPV1-SOAS cluster R3A polioviruses endemic in Pakistan in 2012 and polioviruses isolated from sewage in Egypt in December 2012. The epicentre of the virus circulation was in Bedouin communities in southern Israel (16) and the main reservoir of infected individuals in this community was the cohort of IPV-vaccinated children under the age of nine years (data not shown). By including sequences from WPV1-SOAS cluster R3A isolated from Pakistan in 2012 and from Egypt in December 2012, the dates of isolation of the Israeli isolates were consistent with the 1.1% evolutionary rate observed for other wild polioviruses [24]. Indigenous WPV1-SOAS cluster R3A from Pakistan in 2012 was most likely transmitted to Egypt and Israel through a common reservoir (the location of which is unknown). Egyptian and Israeli lineages separated around mid-September 2012 and a subsequent major divergence within the Israel lineage occurred before January 2013. This divergence may have occurred shortly after the virus was introduced into Israel when it was at low abundance (single introduction) or while it still circulated in the external reservoir (multiple introductions of closely related viruses from the same reservoir). The low viral load of WPV1 in sewage sample 8o62 (Beer Sheva, 6 February 2013), a single WPV1 plaque, lends support to this timeline. A 1.1% substitution rate during transmission from Pakistan to the Middle East, with no evidence for optimisation or de-optimisation of codon usage in Pakistani, Egyptian and Israeli isolates, is consistent with a natural rate of accumulation of the observed single nucleotide substitutions during subsequent person-to-person transmission and makes deliberate modification and release unlikely [5].

The distribution of isolates with different sequences within individual catchment areas was similar to that for the entire geographical and temporal distribution of the virus in Israel. Diversity, together with the

widespread and sustained chains of transmission implicit in the observed high and sustained viral loads recovered from environmental samples [16], is consistent with co-circulation of different isolates within the same community and probably reflects the frequent movement of many infected individuals between communities. As expected, sequence variation in RNA from virus isolated from sewage and stools from the same catchment had a similar distribution pattern. This strengthens the argument for use of environmental surveillance as a surrogate for stool surveillance during outbreaks. However, two confounding factors must be kept in mind when comparing transmission patterns: (i) the frequency between sampling and the different dates for initiating sampling at different surveillance sites; and (ii) differences arising from the types of samples sequenced in relation to the quasi-species nature of poliovirus infections [33]. Sequences from plaque isolates are unambiguous at all nucleotide positions but the individual plaque isolate may not represent the most common variant in the quasi-species. In contrast, sequences of RNA from mass tube cultures from environmental samples and stool suspensions represent the consensus sequence of the quasi-species, but may contain non-unique bases at certain positions when minor species variants are present in the quasi-species at a frequency of greater than 20%. The phylogenetic analysis shown in Figure 2 was performed using sequences in which all nucleotides were unique.

The Israeli experience suggests that the risk for an outbreak of poliomyelitis in high-vaccination coverage countries is low. However, closely related WPV1-SOAS strains have caused AFP cases in Syria in 2013) and Pakistan (2012) [34,35]. These AFP cases and neurovirulence of WPV1-SOAS isolates from Israel in transgenic mice (data not shown) clearly indicate a risk of disease if WPV1-SOAS R3A viruses, such as those circulating in Israel, are transmitted to unprotected or under-protected individuals. The last nine birth cohorts in Israel were vaccinated exclusively with eIPV and coverage was greater than 90%. Persistent WPV1 viral loads in the environment of a highly immune population indicate the potential for sustained transmission in countries exclusively using IPV and for large outbreaks in countries where OPV and/or IPV vaccine coverage is low. They also reinforce arguments for terminating the chain of transmission as rapidly as possible, even in highly immune populations, since protection against the disease is never 100%. Furthermore, the sustained transmission in Israel in the absence of cases points to the risk of undocumented extensive silent circulation in other countries with highly vaccinated populations using IPV exclusively and relying only on AFP surveillance for detecting circulation of non-vaccine-derived polioviruses. Finally, undocumented circulation creates potential problems for laboratory containment of wild polioviruses [36] under the Global Poliovirus Eradication Initiative [37] since clinical samples collected during undocumented, silent poliovirus circulation may

unknowingly contain wild poliovirus and be potential sources for post-eradication re-emergence.

In conclusion, sequence analysis of the evolution of VP1 sequences of the WPV1-SOAS cluster R3A circulating in Israel in 2013 indicated that the pattern and rates of nucleotide substitutions were consistent with natural introduction and circulation of WPV1. Our findings illustrate the importance of intensive environmental surveillance to rapidly detect and follow this event and for detecting similar events that may occur elsewhere. As long as there are countries where endemic transmission of wild poliovirus continues uninterrupted, there remains the potential for wild poliovirus to spread to poliovirus-free regions, including those with very high vaccine coverage. Phenotypic characterisation of WPV1-SOAS in relation to individual and population immunity, examination of vaccination histories of identified healthy excretors and analysis of socio-economic factors are needed to better understand the current silent transmission of WPV1 in Israel and to evaluate the risk of such events occurring in other countries.

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Conflict of interest

None declared.

Authors' contributions

Lester M Shulman: planned the laboratory algorithm for environmental surveillance and molecular characterisation of WPV1 isolates, analysed results of the sewage surveillance, supervised and conducted typing, molecular, and phylogenetic analyses, coordinated the collaborative study and wrote the manuscript. Eugene Gavrilin: analysed the phylogenetic relationships between WPV1 SOAS isolates from Israel, Pakistan and Egypt; participated in the analysis of the data and in writing of the manuscript. Jaume Jorba: led the group

preparing the Bayesian clock tree analytical comparison of sequences of WPV1 isolates from Israel, Pakistan and Egypt; participated in the writing of the manuscript. Javier Martin: critically reviewed the molecular analysis, directed the genotypic and phenotypic characterization of some of the WPV1 isolates, and participated in the writing of the manuscript. Cara C Burns: supervised the molecular characterisation of WPV1 isolates from WPV1 isolated from stools of healthy individuals from Israel, supervised and conducted typing and molecular analyses of WPV1 from Israel, Egypt and Pakistan at the CDC. Yossi Manor: planned conducted and analysed results of the sewage surveillance; participated in writing of the manuscript. Jacob Moran-Gilad: led and guided the validation of the real-time RT-PCR specific assays for SOAS that were used to identify and provide preliminary characterisation of the environmental WPV1 isolates described in this report and coordinated the collection of stool samples from healthy individuals from which WPV1 were isolated; participated in drafting of the manuscript. Danit Sofer: conducted virus isolations in tube cultures and participated in the validation of the sensitivity and specificity of the real-time RT-PCR specific assays and in data analysis; participated in writing of the manuscript. Musa Y. Hindiyeh: developed and validated the real-time RT-PCR assays for the WPV1 SOAS, conducted all the real-time RT-PCR testing and analysed the results; participated in writing of the manuscript. Ronni Gamzo: was involved in evaluation and in routine consultations regarding the environmental surveillance sampling programme and stool collection and results. Ella Mendelson: coordinated and supervised the laboratory groups at the Central Virology Laboratory, participated in planning of the environmental surveillance, development and validation of the real-time RT-PCR assay, and data analysis; participated in writing the manuscript. Itamar Grotto: participated and supervised the environmental and stool surveillance activity in all districts, and in data analysis and manuscript preparation. The Genotype - Phenotype Identification Group (GPI) group: the additional members of the GPI group consulted, participated in the planning of the experiments, the analysis of the data and in formulating the reports at the various collaborating institutions that constitute the basis of the information presented in this manuscript; they also participated in review of the drafts of the manuscript.

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