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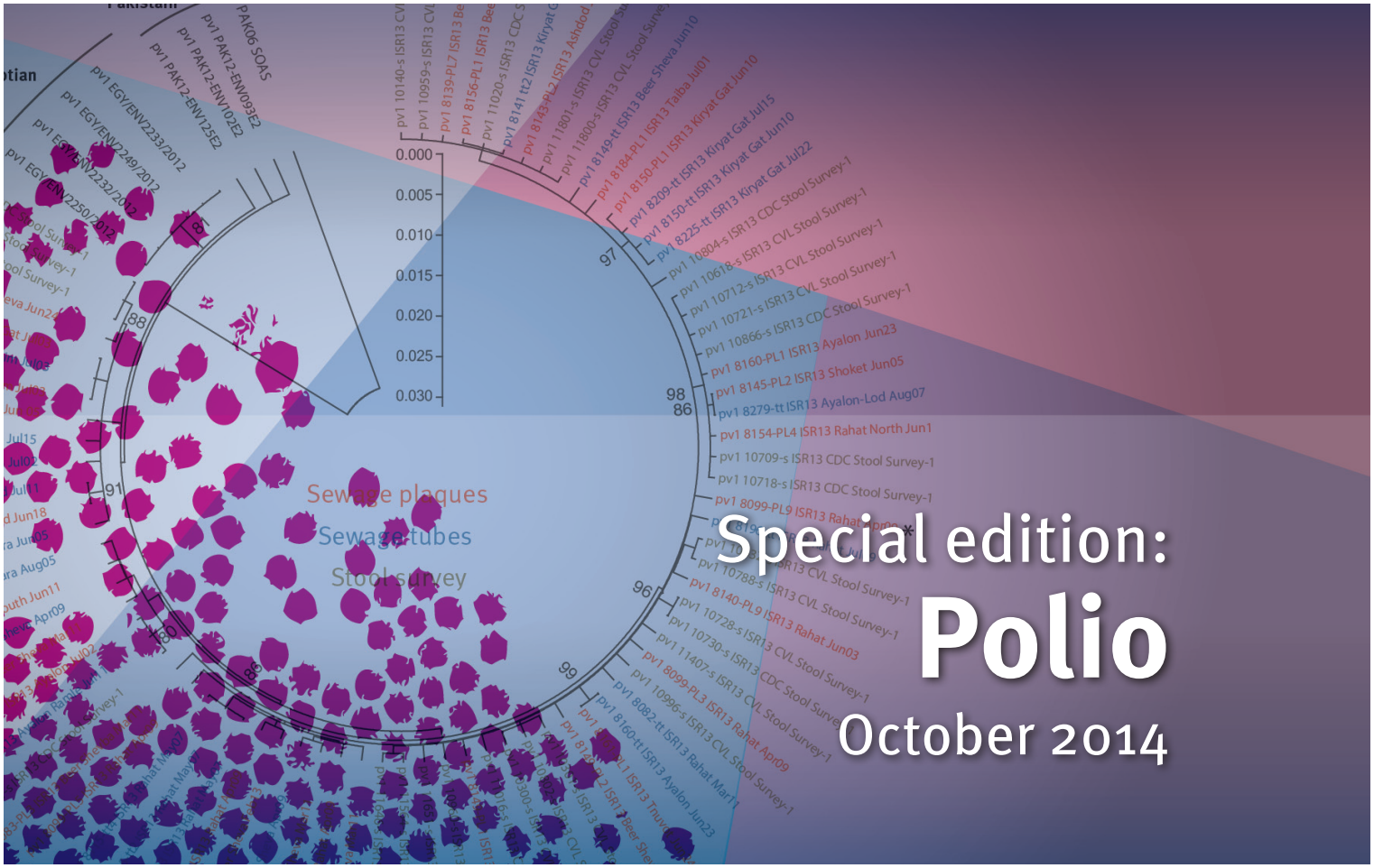

Special edition:

Polio

October 2014

Featuring

- **The polio eradication end game: what it means for Europe**
- **Molecular epidemiology of silent introduction and sustained transmission of wild poliovirus type 1, Israel, 2013**
- **The 2010 outbreak of poliomyelitis in Tajikistan: epidemiology and lessons learnt**

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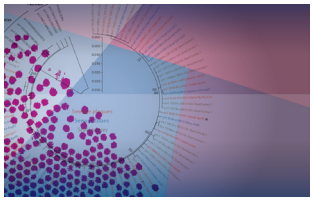

Illustration of polio viruses, phylogenic tree

The polio eradication end game: what it means for Europe

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This edition of *Eurosurveillance* provides a series of articles related to polio that present a microcosm of some of the issues that have plagued polio eradication since the programme first began, and it also provides many of the solutions.

Had these and other issues been clearly understood in 1988 when the World Health Assembly passed the resolution that committed all countries to polio eradication by the year 2000, the decision to eradicate would have been more difficult. But fortunately, buoyed by the then recent success in the eradication of smallpox, active debate on whether to use inactivated or live polio vaccines, awareness that many countries had already interrupted polio transmission, and unawareness of the difficult eradication end game, the resolution was passed by consensus of the World Health Organization (WHO)'s member states [1]. Although progress towards eradication has been slower than anticipated in 1988, paralytic polio has decreased from an estimated 1,000 children per day during 1988, to approximately 400 during 2013. Today there remain only three countries with endemic polio, Afghanistan, Nigeria and Pakistan, and the recent risk assessment from the European Centre for Disease Prevention and Control (ECDC) reminds us that Europe must remain vigilant with strong surveillance and sustained laboratory capacity [2].

The series of polio articles in this edition begins with the article by Hindiyeh et al. [3] describing direct sewage testing for wild poliovirus antigen, using a multiplex quantitative reverse-transcription PCR (qRT-PCR) for rapid detection of the virus, directly on concentrated sewage samples. When compared to cell culture of the same sewage specimens, which is the initial process in the gold standard testing protocol for confirmation of polio, sensitivity and specificity of the multiplex system were shown to be high. Results were obtained in 24 to 48 hours, rather than the usual five to seven days required for the culture-based protocol.

The time from collection of a stool sample to analysis for polio virus in polio eradication programmes has often been weeks, not days, causing delays in response, more widespread transmission, and greater and more costly containment efforts. Recently though, times from specimen collection to outbreak control have decreased considerably by strengthening transport systems from the field to the laboratory, and modifying the testing protocol [4]. At the same time, there is active research and development of new testing algorithms that can provide more rapid results [5]. Hindiyeh et al. have concluded that their qRT-PCR system could be a promising application for testing of RNA extracted directly from processed stool samples from children with acute flaccid paralysis (AFP), and it remains to be seen whether further study will be conducted along these lines [3].

The article by Manor et al. [6] describes the discovery of a silent introduction of wild poliovirus, in the absence of detection of AFP in children (the standard surveillance methodology), by what they describe as an early warning system of sewage monitoring for poliovirus. They point out that this silent introduction occurred in a highly immune population in which inactivated polio vaccine (IPV) has been used exclusively since 2005, and that AFP surveillance alone had not detected this introduction and circulation. The authors suggest that there is a fundamental role for environmental surveillance in routine monitoring as an early warning system in polio-free countries, possibly more sensitive than surveillance for AFP. Shulman et al. [7] add greater perspective in their report on genetic sequencing of these wild polioviruses. It suggests that they were linked to strains that were circulating in South Asia and Egypt in 2012 and concludes that there had been one, or perhaps more than one, importation event.

Indeed, environmental monitoring in sewage has been used by many countries during the past decade, and it has identified wild poliovirus imported in 2007 to Switzerland from Chad, and in 2013 to Egypt from Pakistan [8,9]. Environmental surveillance has been a mainstay of polio eradication in several developing countries as well, for example in Egypt and India, and

its wider role in the polio eradication end game continues to be assessed [10].

Van der Maas et al. [11] and Yakovenko et al. [12] discuss the importance of maintaining high polio immunity levels in order to prevent re-establishment of circulation of wild poliovirus, and the vulnerability of countries with lower than optimal population immunity in the general population or with pockets of low coverage such as those in certain religious and other groups. They clearly call attention to the fact that the circulation of wild poliovirus in one country is a threat to all others, and that vaccination coverage, using either IVP or oral polio vaccine (OPV), must be maintained until the circulation of all wild poliovirus has been interrupted. Yakovenko et al. also underscore the fact that adults are at risk from imported polio virus, having isolated wild poliovirus from adults with AFP in the Tajikistan outbreak [12].

A recent polio outbreak also provided a clear demonstration that adults are at risk of paralytic polio during outbreaks. Because adult populations in Namibia had not been vaccinated against polio in the period before independence, and had not developed immunity by exposure to poliovirus because of high quality water and sanitation systems, an importation of wild poliovirus led to an outbreak of paralytic polio in adults in 2006 [13].

Other issues that have been important to polio eradication such as circulating vaccine derived poliovirus (cVDPD) are not discussed in this series, although the ease with which the poliovirus recombines in nature was demonstrated by studies of viral sequences in the Tajikistan outbreak [12]. However, this omission from the series does not minimise the importance of cVDPV as a challenge to polio eradication that the end game will take into account [14]. The decision in Israel to reintroduce OPV after failure to interrupt transmission with IPV, foretells the complexity the end game will face in the event of a reintroduction after eradication has occurred. Outbreak containment strategies for all countries are actively being assessed for application during the post-eradication period [15].

Although solutions to most of the technical problems in polio eradication are either available or under development, risk communication and gaining trust in polio vaccination in the absence of paralytic disease remain a major challenge. This is clearly demonstrated by the experience described by Kaliner et al. [16] in developing trust and paving the way for the supplementary immunisation activities that reintroduce OPV several years after having switched to IPV in routine vaccination programmes.

The importance of trust and risk communication has likewise been clearly demonstrated in the past, when in 2003 polio vaccination was stopped in northern Nigeria because of false rumours, many of which were circulating on the world wide web, that the vaccine was being used in a plot by some Western nations to permanently sterilise young Muslim girls, and in some instances that it was associated with the introduction of AIDS [17]. Although there may have been other reasons than concern over vaccine safety, the governor of one northern state in Nigeria interrupted polio eradication activities, and other northern Nigerian states followed. Within months, polio had spread from Nigeria to neighbouring countries, to Saudi Arabia and Yemen, and from there on to Indonesia [18].

All of the countries affected were members of the Organization of the Islamic Conference who, at their October 2003 summit in Malaysia, adopted a resolution that endorsed and promoted stronger polio eradication activities [19]. Religious leaders became involved as well, and promoted polio vaccination through a series of fatwas and other declarations. Countries that had been free of polio, again had children paralysed by poliovirus, and increased their surveillance and conducted supplementary immunisation activities to prevent the virus from becoming endemic again. They were successful in containing wild poliovirus and interrupting its transmission, but at great financial cost to the countries and the polio partnership.

This series of articles on polio is a timely reminder that polio eradication has not yet been completed, and they confirm that eradication is technically feasible. But obstacles to polio eradication remain. Killings of polio workers in Pakistan and northern Nigeria have caused fear among polio workers, and pose a risk to the life of those who vaccinate door to door. These incidents have prompted a call for action from the Muslim world to counter opposition to the polio eradication programme recently published in *The Lancet* [20].

Civil unrest, such as that caused by the killing of polio workers, has occurred in the past, but was never targeted specifically at polio eradication. In Sudan in 2005, for example, the United Nations called for days of tranquillity so that polio and other vaccinations could continue [21]. But the solution to violence targeted at the global eradication of polio and at vaccination programmes in general, requires more than vaccine supplies, door-to-door vaccination, and meticulous surveillance. It requires collective ownership and solidarity by all countries, and it may need a prominent and accepted figure in all countries where opposition has been observed, to step forward as a leader and bring polio eradication to completion.

In the meantime, European countries must continue to maintain high levels of polio vaccination coverage, and sustained surveillance of AFP with laboratory support, in order to ensure that wild poliovirus, if imported, is rapidly detected and completely contained.

Conflict of interest

None declared

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Intensified environmental surveillance supporting the response to wild poliovirus type 1 silent circulation in Israel, 2013

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An emergency response was triggered by recovery of wild poliovirus type 1 (WPV1) of the South Asia (SOAS) lineage from sewage in southern Israel in April 2013 during routine environmental surveillance. Public health risk assessment necessitated intensification of environmental surveillance in order to facilitate countrywide monitoring of WPV1-SOAS circulation. This involved increasing sampling frequency and broadening the geographical area, for better coverage of the population at risk, as well as modifying sewage testing algorithms to accommodate a newly developed WPV1-SOAS-specific quantitative real-time RT-PCR assay for screening of RNA extracted directly from sewage concentrates, in addition to standard virus isolation. Intensified surveillance in 74 sites across Israel between 1 February and 31 August 2013 documented a sustained high viral load of WPV1-SOAS in sewage samples from six Bedouin settlements and two cities with Jewish and Arab populations in the South district. Lower viral loads and intermittent detection were documented in sampling sites representing 14 mixed communities in three of the five health districts in central and northern Israel. Environmental surveillance plays a fundamental role in routine monitoring of WPV circulation in polio-free countries. The rapid assay specific for the circulating strain facilitated implementation of intensified surveillance and informed the public health response and decision-making.

Introduction

In the drive towards global eradication of poliomyelitis, as of 2013, only three countries remained endemic for wild-type poliovirus (WPV) infection: Afghanistan, Pakistan and Nigeria, with recent introduction of the virus resulting in paralytic cases to countries with suboptimal immunisation coverage in Africa (Somalia, Kenya, Ethiopia and Cameroon) and in Asia (Syria) [1]. Only WPV type 1 (WPV1) is currently circulating, consisting of two major lineages: the South Asia (SOAS) lineage, which is indigenous to Pakistan and Afghanistan, and the West Africa (WEAF) B lineage, which is indigenous to Nigeria [2].

Poliovirus circulation in highly immune populations is far less likely to be detected via identification of clinical paralytic poliomyelitis cases, which are expected to be very rare or absent (silent circulation), and thus environmental surveillance for poliovirus has become a very useful tool for population-based alert and monitoring of WPV activity. This approach has been implemented by several countries, both for early detection of WPV introduction and transmission as well as for detection of vaccine-derived neurovirulent polioviruses (VDPVs) that emerge following the use of oral poliovirus vaccines (OPVs) [3,4].

Israel has been free of poliomyelitis since the last outbreak caused by WPV1 in 1988, which resulted in 15 paralytic cases [5,6]. The outbreak strain originated in Egypt and arrived in 1987 from Gaza to Rahat and spread in 1988 from Rahat to central Israel. A routine environmental surveillance for poliovirus programme has been implemented since then in Israel, Gaza and the West Bank, which monitors sentinel sites that represent large populations (such as the Shaf-Dan, a sewage treatment facility in the metropolitan Tel Aviv area) and populations (such as Rahat) considered at high risk for introduction of WPV from other countries. A combined inactivated poliovirus vaccine (IPV)/trivalent oral poliovirus vaccine (tOPV) routine vaccination schedule was in place until 2005, when it was replaced by an IPV-only schedule [13].

Over the years, the environmental surveillance programme has detected several introductions of WPV into Gaza and Israel, but subsequent circulation in the

local community occurred only once in Gaza in 1994–45 [7-9]. In addition, it has detected two lineages of highly diverged type 2 VDPV in the Tel Aviv sewage system, excreted by single individuals [9-11], demonstrating the high sensitivity of environmental surveillance for monitoring large populations. Laboratory methods for sample treatment and poliovirus isolation, including the plaque formation approach, are also a major factor in the sensitivity of the environmental surveillance, as reviewed by Hovi et al. [3]. Plaque formation allows a rough estimation of the virus circulation intensity since the number of viral plaque-forming units (PFU) present in the original sewage sample can be deduced based on spiking experiments [8].

In December 2012, WPV1-SOAS was detected in sewage collected from Cairo, Egypt [11], where systematic environmental surveillance for poliovirus has been in place since 2000. A large immunisation campaign initiated as a response led to the disappearance of the virus from the sewage and, by implication, from the population at large [12].

In April 2013, a surge in the number of plaques recovered on L20B cells from a sewage sample collected in Rahat and Beer Sheva, two major cities in southern Israel, occurred. Identification of the plaques as WPV1 suggested an importation and possible circulation of WPV1 in the region [13]. These alarming findings prompted an urgent assessment and response by the Public Health Services of the Ministry of Health. Notably, intensification of environmental surveillance for poliovirus played a key role in monitoring the spread of the virus. Here we describe the modification and enhancement of the environmental sampling and laboratory methods used in order to meet the increased demand for processing of sewage samples and generation of surveillance data that will inform public health response and incident management.

Methods

Sampling sites and sample collection

Since 1989, composite sewage samples have been collected monthly from sentinel sites covering 30–40% of the Israeli population using computerised automatic inline samplers located at the mouth of sewage treatment facilities [8]. These samplers collect and pool aliquots hourly over a 24-hour period to a final volume of 1 L. Sample collection in sites without automatic inline samplers and at upstream lines (carrying sewage from around the city or from different communities into the main sewage line that enters the sewage treatment facility) was carried out using computerised automatic portable samplers (Sigma SD900 portable samplers, HACH, Loveland, CO, United States). Samples were transported and analysed at the Ministry of Health Central Virology Laboratory.

Poliovirus purification and isolation from sewage samples

The methods used for sample concentration and virus purification from sewage samples during the routine surveillance period have been previously described in detail [11]. Poliovirus was plaque isolated in L20B cells (2 mL of sample inoculated into four 10 cm tissue culture plates) and the number of plaques isolated on each plate was recorded. Each plaque was further propagated in tube cultures of Hep2C cells to obtain high amount of virus for identification and characterisation. Plaques from Gaza, the West Bank and Rahat were propagated at 40 °C to select against Sabin strains originating from OPV, which is included in the Palestinian routine immunisation schedule. These strains were common in Rahat due to family relationships and close contact of Israeli Bedouins with their relatives in Gaza. Plaques from all other sites of Israel were propagated at 37 °C as OPV strains are very rarely found there because of the exclusive use of IPV in Israel. Supernatants from cultures showing cytopathic effects (CPEs) were then subjected to molecular analysis for virus identification and sequencing [11].

Following the detection of WPV1 in Rahat in April 2013 that failed to grow at 40 °C and the subsequent implementation of highly intensified countrywide environmental surveillance for poliovirus, laboratory protocols were amended as following: for poliovirus isolation in tissue culture, the 40 °C selective growth of plaque-purified viruses on Hep2C tube cultures was replaced with propagation on L20B tube cultures at 37 °C. In addition, four L20B tube cultures were each inoculated with 0.3 mL aliquots of processed sewage and incubated at 37 °C for 5 days or until full CPEs developed. This step yielded mass cultures and was performed in parallel to the inoculation of tissue culture plates for plaque formation. The number of CPE-positive tubes was recorded, RNA was extracted from a pool of the CPE-positive culture tubes and was subjected to molecular analysis according to standard protocols of the Central Virology Laboratory [14].

qRT-PCR assay specific for WPV1-SOAS circulating in Israel

The development and validation of a real-time RT-PCR (qRT-PCR) assay specific for the circulating virus for rapid detection of the virus directly in concentrated sewage samples is described elsewhere [15]. Briefly, two sets of primers and probes were designed based on the viral protein 1 (VP1) sequences of five plaque-purified isolates from sample number 8099 collected on 9 April 2013 in Rahat. The analytical sensitivity and specificity of the assay, as well as its positive predictive value and negative predictive value, were found to be 100% compared with virus isolation in 50 sewage samples tested, of which 40% were positive and 60% were negative, with high correlation of the cycle threshold (Ct) values with the number of plaques obtained for each sample.

qRT-PCR assays for intratypic differentiation of poliovirus isolates and for non-poliovirus enteroviruses

The Sabin 1, 2 and 3 multiplex and pan poliovirus types 1, 2 and 3 components of the intratypic differentiation assay developed and provided to the Global Polio Laboratory Network (GPLN) by the United States Centers for Disease Control and Prevention (CDC) was used [16]. The assay identified the presence of WPV1 and indicated whether vaccine strains of the same serotype or other serotypes were present. In addition, an in-house pan-enterovirus qRT-PCR assay [14,17] was used when polio type-specific primers were not available, for example, for identification of wild poliovirus of unknown type.

Sequence analysis of virus isolates

Molecular epidemiological and phylogenetic characterisation based on complete VP1 capsid protein sequence for all virus isolates were performed as previously reported [12].

Results

Detection of SOAS-WPV1 importation to southern Israel and intensified environmental surveillance

Sewage samples collected in Rahat as part of the routine environmental surveillance in April 2013 yielded 20,800 plaques on L20B cells. Sequence analysis identified the virus isolates as WPV1 belonging to the SOAS lineage, with closest match to the virus isolated from sewage in Egypt in December 2012 [18]. The viral load in the sewage sample was calculated from the number of plaques isolated: by taking into consideration of the size of the sampled population, the number of faecal excretors of the virus could be roughly estimated to be tens or even hundreds of excretors, indicating ongoing indigenous circulation. Although the sample collected in April turned out to be a grab sample (taken at one time point) that yielded an 'off-scale' number of plaques, this finding was still considered alarming. Retrospective examination of all sewage samples from January 2013 revealed 203 plaques of the WPV1-SOAS in a sample collected in Rahat in March, and 3 and 29 WPV1-SOAS plaques in samples collected in Beer Sheva in February and March, respectively.

The investigation and response [13] included highly intensified environmental surveillance, first in other communities in southern Israel and subsequently nationwide. The sampling strategy included mostly sample collection at STFs but on several occasions, particularly when STFs represented separate communities, additional samples were collected from upstream sewage lines by a mobile laboratory operated by the Central Virology Laboratory using portable samplers. The number of samples obtained monthly rose from May 2013 (12 samples), with 47, 57, 145 and 53 samples in June, July, August and September, respectively, and with average weekly numbers of 12–36 samples. This increase in demand for sample analysis necessitated the development of new algorithms for sample processing and testing in order to meet the challenge.

Incorporation of a novel sewage-testing algorithm

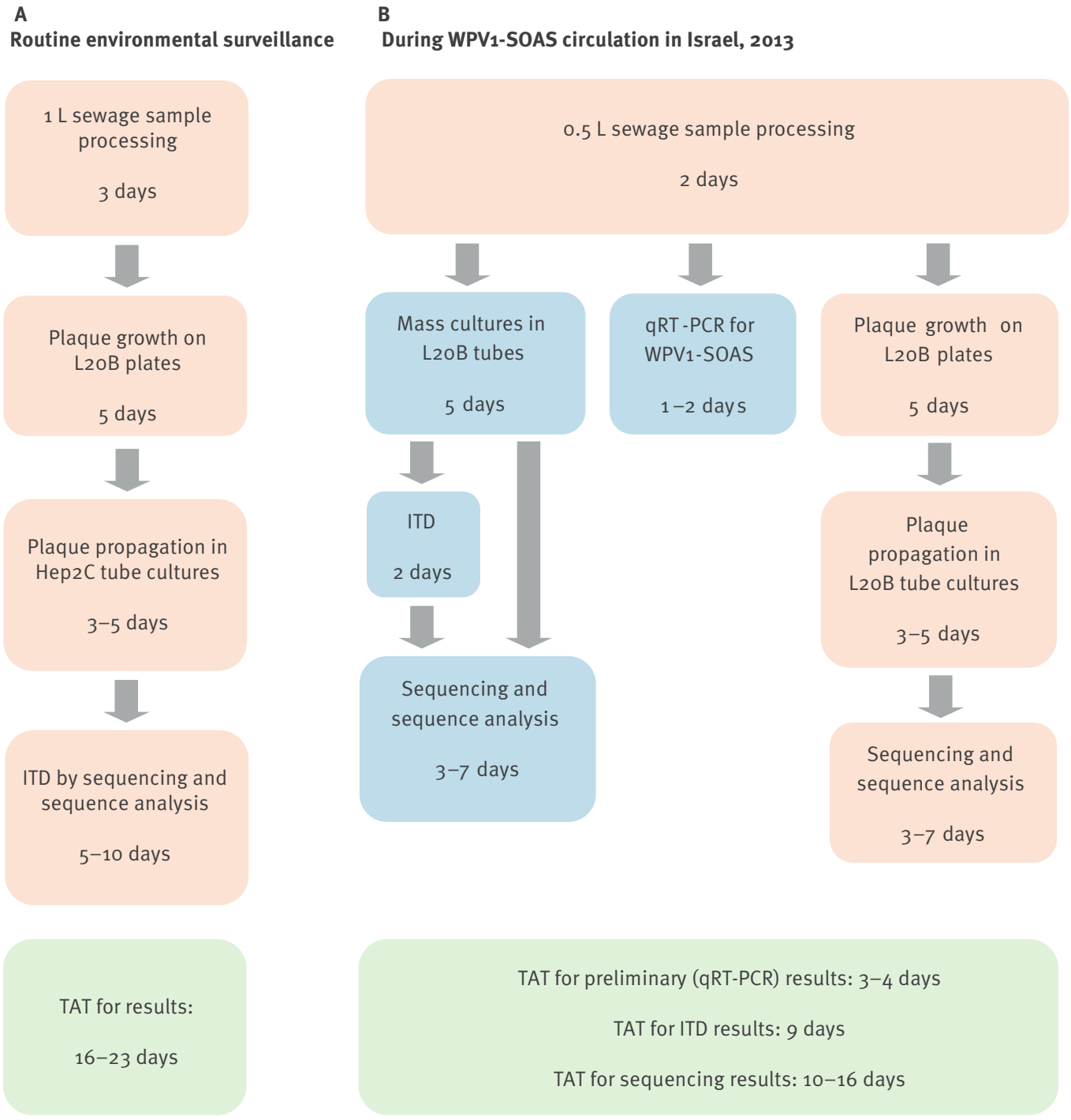
A new testing algorithm was implemented in order to cope with the large number of samples and the need for rapid results. It provided the following advantages: firstly, a fourfold increase in the number of sewage samples processed daily, by concentrating 500 mL of raw sewage instead of 1 L, as done in the routine ESP, and recovering the virus in 15 mL instead of 30 mL final volume to preserve the concentration factor. This allowed us to overcome the initial bottleneck of large-volume centrifugation and to assign for the process two workers with staggered starting times. Secondly, rapid identification of WPV1-positive samples was possible using a novel approach that included testing of RNA extracted directly from concentrated sewage by a qRT-PCR screening assay specific for the circulating virus [15]. Thirdly, inoculation of four L20B tube cultures to produce a mass culture of all viruses in the sample, in parallel with the plaque-formation assay, thereby shortened the time for virus molecular analysis if and when indicated. A comparison of the two algorithms is shown in Figure 1. The original algorithm produced results only after 16–23 days by sequence analysis of isolates whereas the modified algorithm produced preliminary WPV1-SOAS positive/negative and qRT-PCR results within 3–4 days, confirmation by intratypic differentiation within 9 days and full sequencing within 10–16 days. This approach allowed us to immediately identify communities at risk and to prioritise samples for further analysis and plan site re-sampling. Thus, all three arms of the new testing algorithm supported each other and allowed a higher throughput (up to 50 sewage samples per week) and shorter turnaround time for results. Notably, the quantification of viral load by the qRT-PCR assay correlated well with the plaque number obtained for each sample, as was found during the validation process of the assay [15].

Poliovirus isolation and identification algorithms employed during routine environmental surveillance (A) and after discovery of WPV1-SOAS (B). The algorithm shown in panel A confirms isolation of any poliovirus type by sequence analysis within 16 to 23 days and allows processing of 6 to 12 samples per week. Quantitative evaluation of plaque number is done after the final identification. The algorithm shown in panel B, incorporating additional steps (shown in blue), allows primary detection and quantification of WPV1-SOAS by qRT-PCR within 3 to 4 days, confirmation by intratypic differentiation and quantitative evaluation of plaque number within 9 days and full sequence results within 10 to 16 days. It also allows processing of up to 50 samples per week.

Altogether, between early January and the end of August 2013, the Central Virology Laboratory received

Figure 1

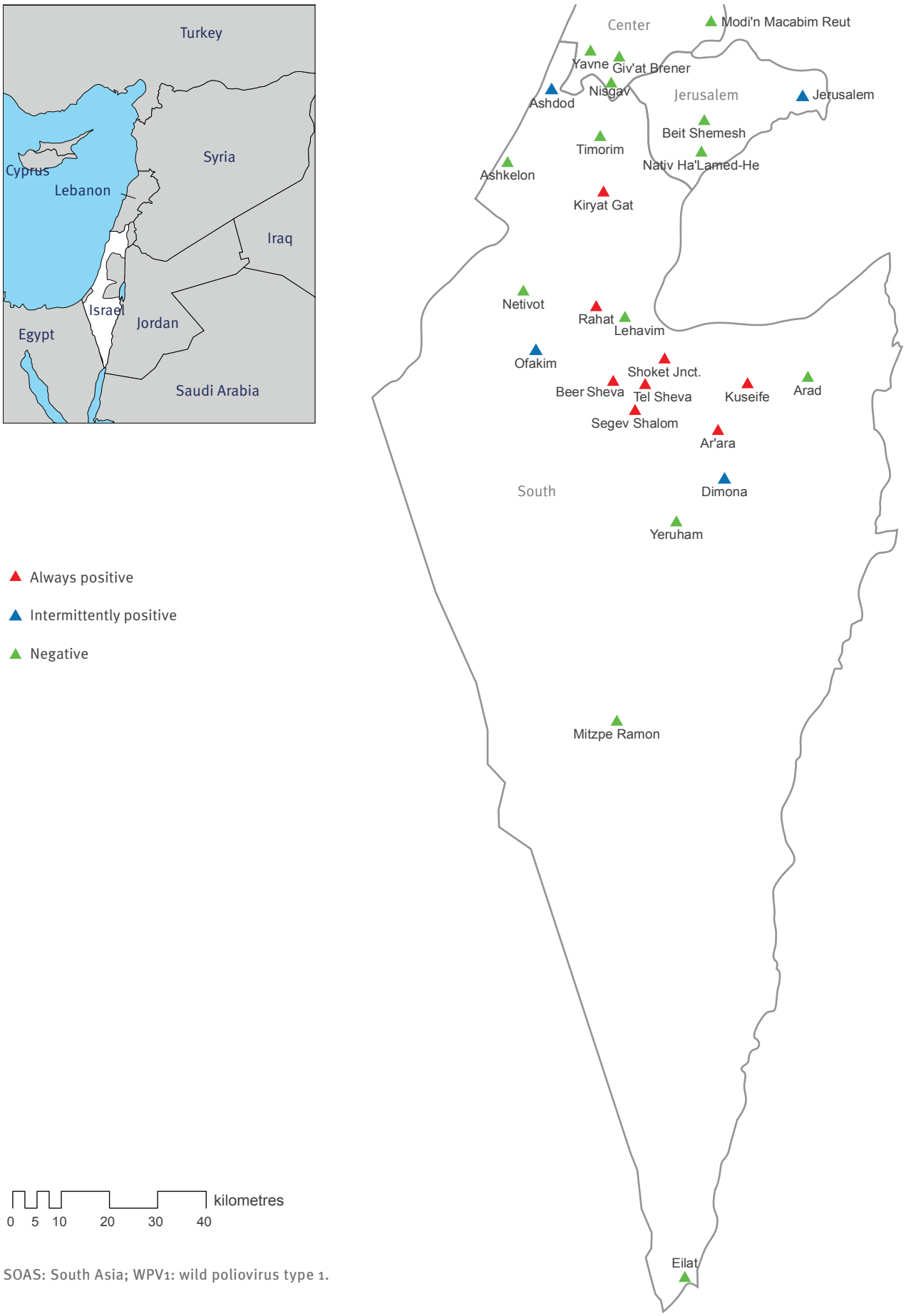
Sewage processing and testing algorithm in routine environmental surveillance for poliovirus (A) and during WPV1-SOAS circulation (B), Israel, 2013



ITD: intratypic differentiation; q: quantative; SOAS: South Asia; TAT: turnaround time; WPV1: wild poliovirus type 1.

Figure 2

Location of sewage sampling sites with WPV1-SOAS testing results in the South, Jerusalem and Centre (part) health districts, Israel, February–August 2013



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

and tested 262 sewage samples, most of them between mid-July and mid-August. These comprised 192 samples obtained from 75 sites across Israel, as well as 70 samples obtained from the Palestinian Ministry of Health.

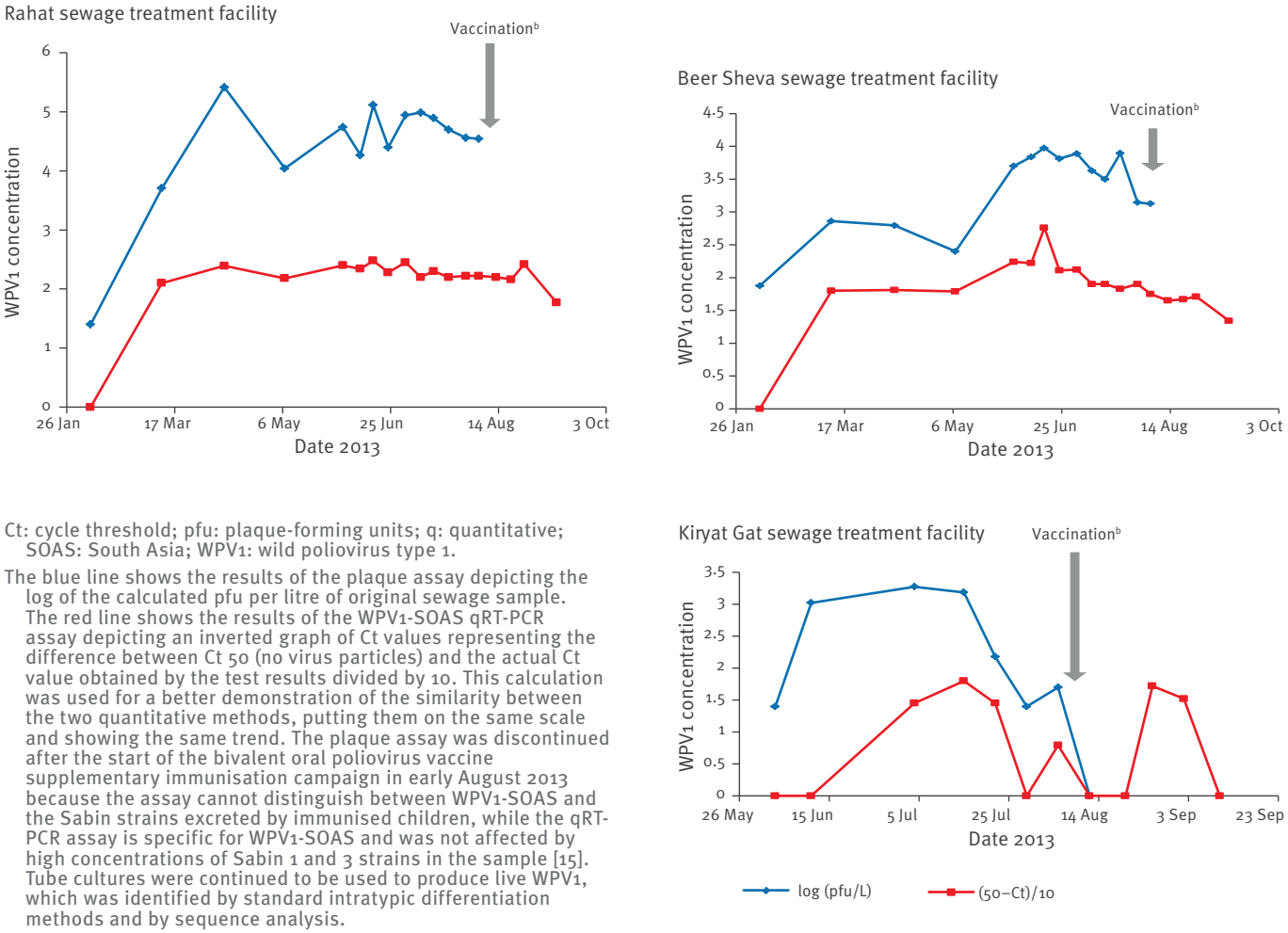
Circulation of WPV1-SOAS in the South health district

Of the 69 sites sampled, 23 were in the South health district, with catchment areas covering Bedouin cities and villages, semi-nomadic Bedouin communities, Jewish cities and combinations of Jewish and Bedouin cities or villages. Sampling intervals were one or two weeks for sites belonging to one or more of the following categories: (i) WPV1-SOAS-positive sites; (ii) positive or negative sites from major cities; or (iii) high-risk sites representing populations having close contacts (close family relationships as well as intensive work and social contacts) with the southern WPV1-SOAS-positive communities. Sites not belonging to any of these categories were sampled once every few weeks or only once. Results were categorised by the relative number of plaques obtained during the study period, as shown in Figure 2, which illustrates the geographical location of 19 of 23 sampling sites in the South health district (excluding two upstream lines of Beer Sheba sewage treatment facility and the Arad sewage treatment facility) and the respective findings. Eight of these sites were persistently positive over the study period according to most samples collected, six covering Bedouin communities, one covering Jewish community (Kiriat Gat) and one covering Jewish and Bedouin communities (Beer Sheva). Three sites covering Jewish communities were intermittently positive (steady or occasional).

A total of 10 sites, eight covering Jewish cities (all shown in Figure 2) and two covering neighbourhoods

Figure 3

Dynamics over time of sewage WPV1-SOAS testing results in three sampling sites[a] in southern Israel, February–August 2013



Ct: cycle threshold; pfu: plaque-forming units; q: quantitative; SOAS: South Asia; WPV1: wild poliovirus type 1.

The blue line shows the results of the plaque assay depicting the log of the calculated pfu per litre of original sewage sample. The red line shows the results of the WPV1-SOAS qRT-PCR assay depicting an inverted graph of Ct values representing the difference between Ct 50 (no virus particles) and the actual Ct value obtained by the test results divided by 10. This calculation was used for a better demonstration of the similarity between the two quantitative methods, putting them on the same scale and showing the same trend. The plaque assay was discontinued after the start of the bivalent oral poliovirus vaccine supplementary immunisation campaign in early August 2013 because the assay cannot distinguish between WPV1-SOAS and the Sabin strains excreted by immunised children, while the qRT-PCR assay is specific for WPV1-SOAS and was not affected by high concentrations of Sabin 1 and 3 strains in the sample [15]. Tube cultures were continued to be used to produce live WPV1, which was identified by standard intratypic differentiation methods and by sequence analysis.

[a] Three sampling sites are shown: the sewage treatment facilities of Rahat (a Bedouin city), Beer Sheva (Jewish and Bedouin communities) and Kiryat Gat (Jewish city).

[b] Bivalent oral polio vaccine was used in a supplementary immunisation campaign starting in early August 2013 and lasting till the end of October.

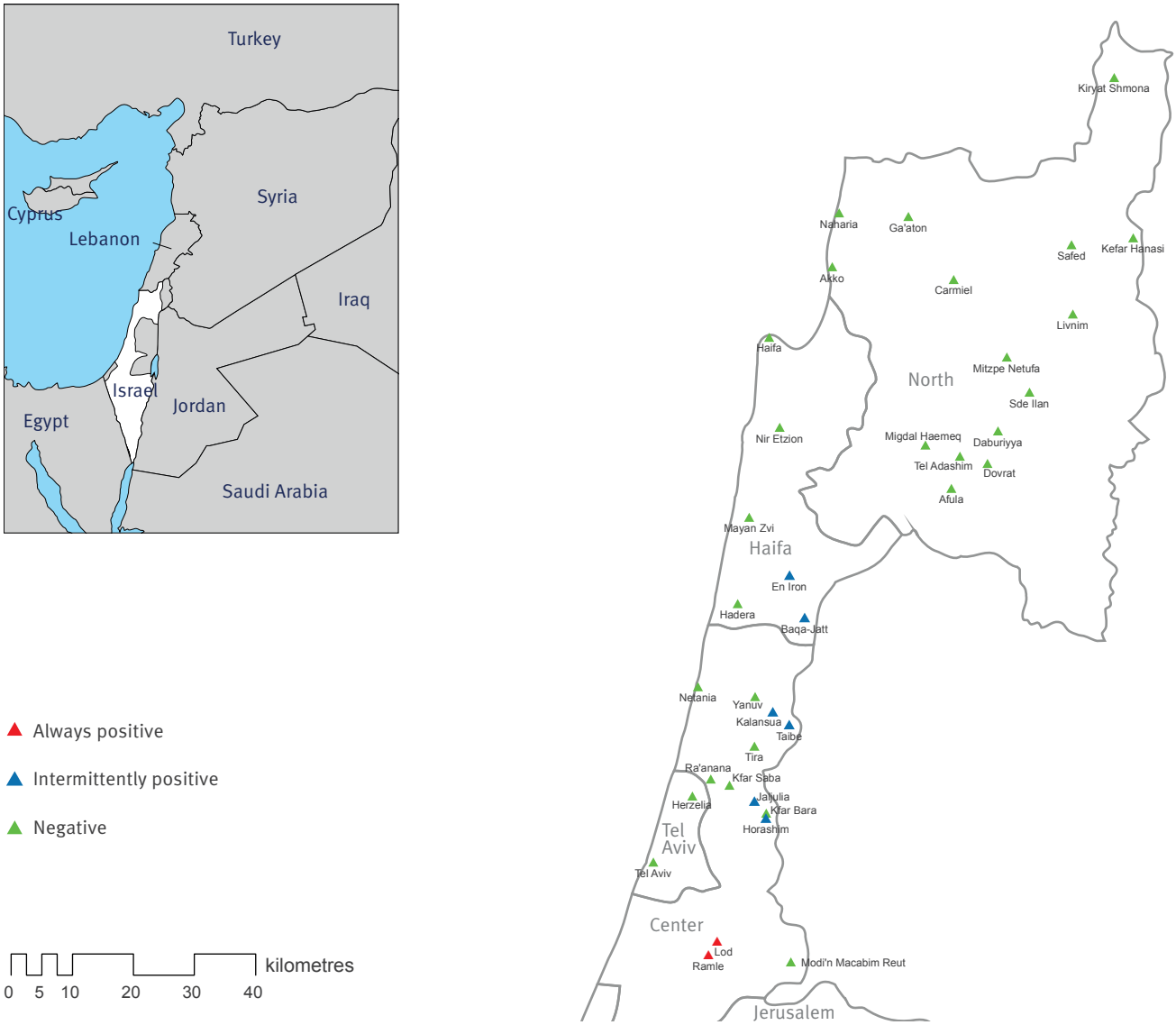
inside Beer Sheva (not shown in Figure 2), which were sampled through sewage lines upstream of Beer Sheva sewage treatment facility were negative once and were not sampled again.

The dynamics over time of the plaque assays and the WPV1-SOAS qRT-PCR Ct values in three sampling sites with distinctive characteristics are shown (Figure 3): Rahat sewage treatment facility, which collects sewage only from the Bedouin city of Rahat; the Beer Sheva sewage treatment facility, which collects sewage from Jewish cities (Beer Sheva and Ofakim) and from Bedouin communities (Tel-Sheva and Segev Shalom); and the Kiryat Gat sewage treatment facility, which collects sewage only from the Jewish city of Kiryat Gat. The plaque assay was discontinued after the beginning of the bivalent OPV (bOPV) supplementary immunisation campaign in early August 2013 [13] because of interference from the high amounts of Sabin strains excreted by immunised children, while the qRT-PCR assay was not affected by high concentrations of Sabin 1 and 3 strains [15]. Live poliovirus continued to be isolated by mass culture in L20B cells, as outlined in Figure 1.

Testing of the sites (Figure 3) reflected the epidemiological situation in southern Israel: rapid increase and persistence of high WPV1-SOAS viral load in the sewage of Beer Sheva and Rahat, indicating sustained transmission, and gradual decrease, which began in Rahat only after the beginning of the immunisation with bOPV in August, and in Beer Sheva since June. In contrast, in Kiriat Gat, WPV1-SOAS was detected intermittently and this site became negative even before bOPV vaccination began but repeatedly tested positive again afterwards. This may suggest non-persistent transmission in that area. Some of the other sites were found positive once or twice: for example, the port city

Figure 4

Location of sewage sampling sites in Centre (part), Tel Aviv, Haifa and North health districts in Israel with WPV1-SOAS test results, February–August 2013



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

of Ashdod was found positive on 4 and 17 June and was negative in subsequent sampling in July and August.

These results indicate a high and persistent circulation of WPV1-SOAS in the Bedouin communities and low or no circulation in the Jewish communities, according to city of residence. Our results also demonstrate an excellent correlation between the Ct values of the direct qRT-PCR test and the plaque assay (Figure 3), suggesting that direct testing of processed sewage samples by qRT-PCR is a reliable approach that can be used for rapid response under emergency situations associated with WPV circulation.

Spread of WPV1-SOAS to central Israel

Towards the end of June 2013, routine surveillance in central and northern Israel was enhanced from 6 to to 46 sampling sites including sewage treatment facilities and upstream sewage lines representing major cities and populations of Israel in all five health districts: Jerusalem, Centre, Tel Aviv, Haifa and North. The sampling intervals for each site were according to the results obtained for these sites and the type of the populations. Small communities negative for WPV1-SOAS or low-risk communities without frequent contacts with populations from WPV1-SOAS-positive communities were sampled once during August. In contrast, major cities, cities positive for WPV1-SOAS or communities at high-risk due to frequent contacts with populations from WPV1-SOAS -positive communities or due to geographical proximity to other positive sites in densely populated areas were sampled several times. A total of 14 positive samples (two always positive and 12 intermittently positive) were found in three health districts: Center, Jerusalem and Haifa, while Tel Aviv and the North districts remained negative for WPV1-SOAS. Much lower numbers of plaque-forming polioviruses were recovered from these sites compared with the sites covering the Bedouin cities in the South district. The geographical location and poliovirus circulation status for most of these sites is shown in Figures 2 and 4. Nine positive sites were found in the Centre district (six are shown in Figure 4), of which only two cities, with mixed Jewish and Arab populations, were always positive (Lod and Ramle). Three intermittently positive sites were found in Jerusalem district (shown in Figure 2 as one triangle) representing Jewish and Arab mixed populations, and two intermittently positive sites were found in Haifa district (shown in Figure 4), one representing an Arab population and one representing a mixed Jewish and Arab population.

The finding of positive sewage samples outside of the Southern District provided evidence for continuous spread of the WPV1-SOAS and supported the decision to expand the supplementary immunisation activity using bOPV which began on 5 August in children in the South to children in the rest of the country (commencing 18 August).

Discussion

Our study highlights the critical role of environmental surveillance for monitoring global WPV circulation. In countries with high vaccination coverage (about 90–95%), acute flaccid paralysis (AFP) surveillance might not detect virus introduction and circulation as occurred in Israel: enhanced AFP surveillance and aseptic meningitis surveillance implemented in response to the WPV1-SOAS detection in sewage did not identify any polio-associated illness from early June to late August [13] and later (data not shown). In contrast to previous introductions of WPV1 to Israel and Gaza detected by the environmental surveillance [11], after which the virus disappeared without the need for supplemental immunisation (in Israel) or following national immunisation days (in Gaza) [7], in 2013, virus importation resulted in sustained circulation [13], which eventually necessitated supplemental immunisation activity with bOPV.

The intensified environmental surveillance for polio, which was essential for management of the event, led to the development of a novel approach, implementation of a qRT-PCR assay specific to the outbreak virus. Together with modifying the testing algorithm, this allowed testing a large number of samples to be tested and result produced within a few days. The plaque and qRT-PCR assays are very precise and quantitative when used on pure viral stocks or on spiked negative sewage samples [15]. Although they are less precise when used on positive sewage samples, because of their variable contents [3], they were still very useful in assessing the intensity of virus circulation in different communities. This approach had not been used in previous outbreak investigations and allowed identification of the most affected communities and the epicentre of the silent outbreak. For example, in Rahat we continuously obtained a few hundred plaques per mL of concentrated sewage (after correcting for the dilution factor) while in Kiryat Gat the numbers ranged between less than 10 and up to 80. Thus, we estimated that the number of WPV1 excretors in Rahat may reach hundreds and may be much higher than in Kiryat Gat. These estimates were later confirmed by a stool survey that assessed the prevalence of WPV1 excretion among subpopulations (data not shown). WPV1 circulation was probably propagated by the accumulation of a large cohort of children who had been immunised only with IPV, and not with a combination of IPV and OPV, since 2005 [13]. The qRT-PCR assay replaced the plaque assay for quantification of WPV1-SOAS excretion after the beginning of the immunisation campaign since the plaque assay was unable to distinguish between WPV1-SOAS and the Sabin 1 and 3 vaccine strains found at high concentration in the sewage.

While no paralytic poliomyelitis cases were identified, the epidemiological picture that unfolded by the intensified environmental surveillance, including sample collection from sewage upstream lines, was very detailed with regard to virus circulation in different

communities. This included persistently positive sites (mostly with a high number of plaques and corresponding low Ct values in the qRT-PCR), with sustained virus transmission rates and intermittently positive sites (lower number of plaques and higher Ct values), which indicated either a lower rate of virus transmission or occasional importations by visitors or dayworkers from Rahat (involved in various types of work), who we assume were a possible source of the virus found in the sewage. On the basis of intensive sewage surveillance using semi-quantitative methods, we speculated that the epicentre of the outbreak was in the Bedouin communities in southern Israel, in which sustained transmission has occurred. These findings have prompted an IPV catch-up campaign among Bedouin communities to minimise the already low risk for clinical poliomyelitis and to initiate bOPV supplemental immunisation to communities in southern Israel before introducing the campaign to the rest of the country.

Our report highlights the importance of environmental surveillance, which is the most sensitive and efficient approach for detection of WPV introduction and circulation in highly immunised populations. It requires systematic composite sample collection and experienced laboratories, but under these conditions, it has the highest 'population sensitivity', compared with other methods [3]. The routine environmental surveillance programme implemented in Israel since 1989 included monthly sampling of large cities and populations at high risk of virus penetration, covering around 40–50% of the Israeli population, which now counts around 8 million people [19]. During the silent WPV1 outbreak, coverage of the programme was increased, to around 70% of the population, focusing on communities with circulating virus. Other supplementary surveillance approaches addressing the general population rather than only AFP cases, which are currently in use in countries in Europe and elsewhere and which can detect subclinical circulation, are stool surveys or general testing of enterovirus PCR-positive stools from patients without poliomyelitis symptoms for the presence of poliovirus by culture on L20B cells. France, the Netherlands and Australia, for example, implement enterovirus surveillance that includes testing for poliovirus [20-22]. However, none of the surveillance methods used is comparable to environmental surveillance in efficiently covering large populations. For example, in France 192,598 samples were tested over five years, between 2000 and 2004 [20], which on an average yearly basis represent roughly less than 0.3% of the French population [23], while in Israel, routine surveillance before 2013 covered 30–40% of the population. Environmental surveillance could be very useful for monitoring and detecting WPV introduction and silent circulation in countries that use only IPV.

The WPV1-SOAS specific qRT-PCR assay is limited to the detection of this particular virus lineage and may lose its sensitivity even if there are minor mutations, which may occur naturally during WPV evolution. It cannot detect other poliovirus types or strains such as the WEAF lineage or type 2 vaccine-derived polioviruses (VDPV2), which circulate in Africa. Therefore, adoption of this method should take these limitations into consideration.

In conclusion, our study provides a proof of concept for the rapid implementation of qRT-PCR in the framework of an outbreak in which short turnaround times and high-throughput testing are essential for incident management, while maintaining confirmatory culture-based methods. In addition, molecular assays capable of directly detecting a wider range of wild polioviruses and VDPVs currently in global circulation should be developed for routine surveillance and emergency response.

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Conflict of interest

None declared.

Authors' contributions

Yosef Manor: responsible for conducting the polio sewage surveillance, virus isolation and data analysis. Planned conducted and analysed results of the sewage surveillance; participated in writing of the manuscript.

Lester M Shulman: .planned the laboratory algorithm, analysed results of the sewage surveillance, supervised and conducted typing and molecular analyses, participated in writing of the manuscript.

Ehud Kaliner: was in charge of supervising the environmental surveillance activities, sampling programme and participated in data analysis.

Musa Hindiyeh: in charge of assay development and performance of clinical testing. Developed and validated the real-time RT-PCR assays for the wild poliovirus type 1 (SOAS) and for Sabin 1 and Sabin 3 strains. Conducted all the real-time RT-PCR testing and analysed the results.

Daniela Ram: participated in development and validation of the real-time RT-PCR specific assay for SOAS, Sabin 1 and Sabin 3 and, participated in testing and data analysis.

Jacob Moran Gilad: led and guided the validation of the real-time RT-PCR specific assays for SOAS, Sabin 1 and Sabin 3. Participated in data analysis and 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.

Boaz Lev: participated in supervision and evaluation of the surveillance activities and results on a national level.
Itamar Grotto: participated and supervised the environmental surveillance activity in all districts, and in data analysis and manuscript preparation.
Roni Gamzu: was involved in evaluation and in routine consultations regarding the environmental surveillance sampling programme and results.
Ella Mendelson: coordinated and supervised the laboratory groups' work, participated in planning of the environmental surveillance, development and validation of the real-time RT-PCR assay, and data analysis, wrote the manuscript.

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The 2010 outbreak of poliomyelitis in Tajikistan: epidemiology and lessons learnt

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A large outbreak of poliomyelitis, with 463 laboratory-confirmed and 47 polio-compatible cases, took place in 2010 in Tajikistan. Phylogenetic analysis of the viral VP1 gene suggested a single importation of wild poliovirus type 1 from India in late 2009, its further circulation in Tajikistan and expansion into neighbouring countries, namely Kazakhstan, Russia, Turkmenistan and Uzbekistan. Whole-genome sequencing of 14 isolates revealed recombination events with enterovirus C with cross-overs within the P2 region. Viruses with one class of recombinant genomes co-circulated with the parental virus, and representatives of both caused paralytic poliomyelitis. Serological analysis of 327 sera from acute flaccid paralysis cases as well as from patients with other diagnoses and from healthy people demonstrated inadequate immunity against polio in the years preceding the outbreak. Evidence was obtained suggesting that vaccination against poliomyelitis, in rare cases, may not prevent the disease. Factors contributing to the peculiarities of this outbreak are discussed. The outbreak emphasises the necessity of continued vaccination against polio and the need, at least in risk areas, of quality control of this vaccination through well planned serological surveillance.

Introduction

A tremendous decrease in the incidence of paralytic poliomyelitis has occurred since 1988, when the World Health Organization (WHO) launched the Global Polio Eradication Initiative [1]. Nevertheless, the originally set goal to eradicate polio worldwide by the year 2000 was not achieved. Two major problems impede the accomplishment of this task, (i) continuous circulation of wild polioviruses (WPV) in a few countries and importation of these viruses to other countries [2], and (ii) the ability of Sabin vaccine viruses to circulate for a long time, with the propensity to acquire phenotypical properties, including neurovirulence and transmissibility, similar to those of their wild-type counterparts [3].

An outbreak of poliomyelitis occurred in Tajikistan in 2010 [4]. An increase in the incidence of acute flaccid paralysis (AFP) was registered, starting early in that year. The number of AFP cases increased modestly in February and March, and peaked between mid-April and mid-May (Figure 1A). Twenty-nine fatal cases were registered. The outbreak was caused by WPV1 originating from India. To curb the outbreak, four rounds of supplementary immunisation activities with monovalent type 1 oral poliovirus vaccine (mOPV) were carried out in May (4–8 and 18–22 May) and June (1–5 and 15–19 June), followed by two rounds of immunisation with trivalent OPV (tOPV) on 4–8 October and 8–12 November 2010. In addition, mop-up vaccination with mOPV was carried out in 34 districts on 13–17 September 2010 [5]. As a result, the AFP incidence declined, and the last WPV1 was isolated from an AFP patient on 4 July 2010. The total number of reported AFP cases in 2010 was 715. The most affected cohort (43.8%) were one to five year-old children. The majority of the cases were registered in the most populated south-western districts of Tajikistan (Figure 2A). From Tajikistan, the outbreak spread to neighbouring countries in the same year (Kazkhstan, Russia, Turkmenistan and Uzbekistan). This polio epidemic was one of the largest among those recently caused by importation of WPV.

Here we present a molecular-epidemiological analysis of this outbreak. The nature and the origin of the virus were ascertained and its evolution was characterised. We have established the conditions favouring the outbreak and discuss implications for the worldwide efforts to combat poliomyelitis.

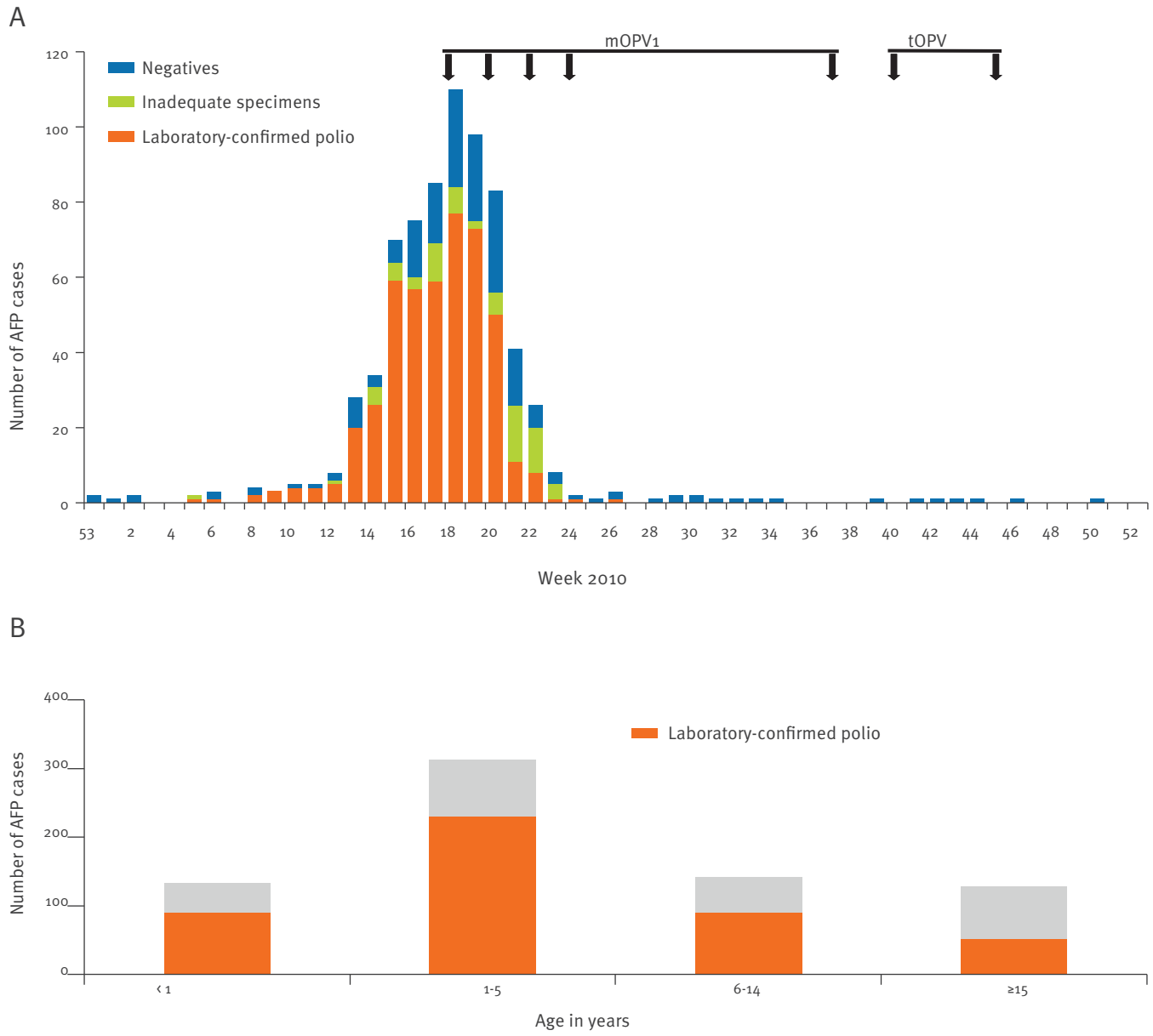
Methods

Virological investigations

Virus isolation from stools and typing were performed by standard methods [6]. Intratypic differentiation (ITD) was carried out by ELISA with cross-absorbed

Figure 1

Cases of acute flaccid paralysis and laboratory-confirmed poliomyelitis, as deduced from our virological analyses and epidemiological information accompanying faecal samples, Tajikistan, 2010 (n=715)



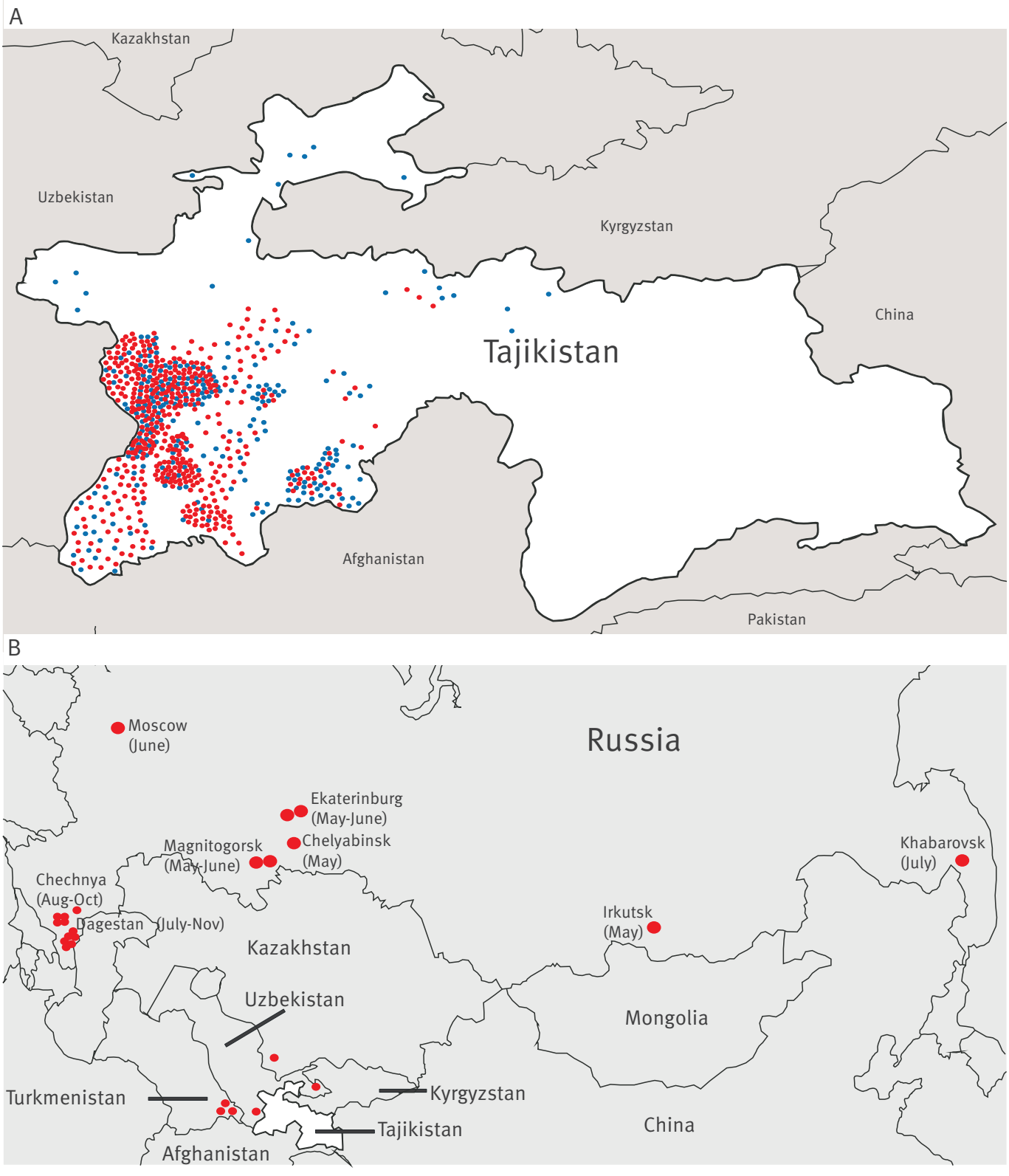
AFP: acute flaccid paralysis; mOPV: monovalent type 1 Sabin vaccine; tOPV: trivalent Sabin vaccine.

(A) The time course of acute flaccid paralysis incidence and laboratory-confirmed poliomyelitis. Arrows indicate timing of mass immunisation with monovalent type 1 and trivalent Sabin vaccines [5]. The target groups during the first two rounds were children up to the age of six years, and in the subsequent rounds children under the age of 15 years.

(B) Age distribution of acute flaccid paralysis and confirmed poliomyelitis cases.

Figure 2

Geographical distribution of acute flaccid paralysis and poliomyelitis cases in Tajikistan (n=715) and neighbouring countries (n=25) in 2010



(A) Laboratory-confirmed cases due to wild poliovirus type 1 and polio-compatible (red dots) and other acute flaccid paralysis (blue dots) cases in Tajikistan. The dots are placed randomly within each district (borders not shown).

(B) Polio cases in Russia and other neighbouring countries due to wild poliovirus type 1 importation (red dots, with unspecific location within Kazakhstan, Turkmenistan and Uzbekistan or within each federal subject in the case of Russia).

The territory of Tajikistan is marked in white and represents the epicentre of the outbreak.

polyclonal antisera (kindly provided by Dr HGAM van der Avoort, National Institute of Public Health and Environmental Protection (RIVM), the Netherlands) [6,7] and real-time RT-PCR [8]. For sequencing, poliovirus RNA was extracted by phenol-chloroform from infected cells and was reverse-transcribed using random hexamer primers (Syntol) and SuperScriptII reverse transcriptase (Invitrogen). The cDNA of the region encoding the capsid protein VP1 was amplified by PCR using primers Y7R and Q8 [9]. For full-genome sequencing, cDNA of eight overlapping genomic fragments were amplified by PCR (the primer sequences are available upon request). The products were purified with QiaQuick DNA Purification System (Qiagen). Sequencing was performed by using a Beckman Coulter Seq 8000 or ABI 3130 Genetic Analyzer.

Serological assays

Poliovirus-neutralising antibodies in human sera were determined by microneutralisation [10]. Sera with neutralising antibody titres <1:8 were considered negative. Sabin 1-specific IgM and IgG were detected by ELISA [11].

Origin of samples

Stool samples from AFP cases were collected in Kazakhstan, Russia, Tajikistan, Turkmenistan, and Uzbekistan in accordance with the WHO Guidelines [10]. Stool samples from healthy close contacts of cases in Russia were collected following the national recommendations for investigation of suspected polio cases. The stool and sera specimens were collected [10] by local public health workers. Personal information (clinical data, vaccination status, age, location, etc) were retrieved from the documents accompanying the samples.

Bioinformatics methods

Multiple alignments, the estimation of the degree of synonymous nucleotide divergence and of the similarity of deduced amino acid sequences with the WPV sequences available in the GenBank were performed as described [12]. Bayesian phylogenetic analysis was conducted using the software BEAST 1.7.4 [13]. The general time-reversible substitution model with four gamma categories and invariant sites was used for calculations. Codons were grouped into three partitions and the substitution model was unlinked across codon positions. Markov chains were run for $2x10^8$ generations, sampled every 5,000 generations, and executed three times to ensure adequate mixing and stationarity. The first 4,000 samples were discarded. The effective sample size, inspected with Tracer v1.5 (http://tree.bio.ed.ac.uk/software/tracer/), of all parameters was larger than 200, indicating that stationarity was achieved. An analysis of the marginal likelihoods indicated that the constant molecular clock and expansion population growth model ($log^{10}BF>2$) was the best for our data set. For the SimPlot analysis, default settings were used [14].

Nucleotide sequence accession numbers

The nucleotide sequences determined in this study are available from GenBank, accession numbers KC800662–KC800683, KC812248–KC812257 and KC880365–KC880521.

Results

The causative agent of the outbreak, its origin and evolution

Faecal samples (n=1,003) from 644 AFP cases were investigated, and WPV1 was isolated from 463 patients (Figure 1A), who were therefore diagnosed as poliomyelitis cases. Of those 463, 29 cases were fatal. The age distribution of poliomyelitis cases paralleled that of AFP cases (Figure 1B). It is noteworthy that a significant proportion of poliomyelitis victims (including three fatal cases) were 15 years-old or older.

Specimens (two faecal samples each) from 181 AFP cases were poliovirus-negative. Specimens from an additional 69 AFP cases were unavailable and two specimens were delivered in inadequate condition. Forty-seven of these cases were eventually classified as 'polio-compatible' by the National Expert Committee in Tajikistan.

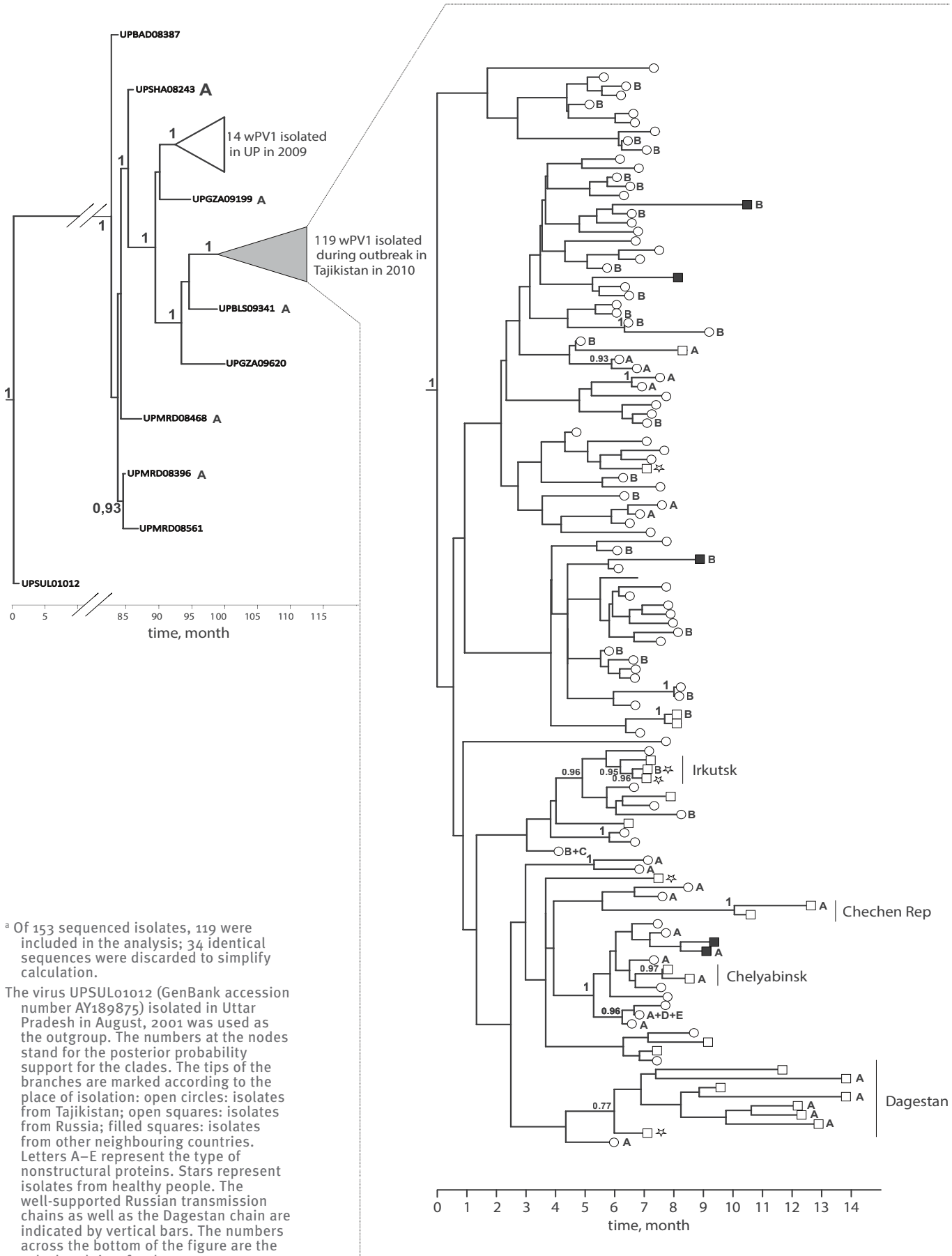
Non-polio enteroviruses were isolated from seven AFP cases, including five polio-negative cases (Echovirus 7, Coxsackievirus B1 and three non-typable human enteroviruses) and two victims of poliomyelitis (Echovirus 13 and one non-typable, both in mixtures with WPV1).

We sequenced the capsid protein VP1 coding regions of 116 WPV1 isolated from poliomyelitis cases from February to July 2010 in Tajikistan as well as the 37 isolates obtained from 25 poliomyelitis cases and eight healthy persons in April to November 2010 in neighbouring countries (see below). They were found to represent a monophyletic group most closely related to the viruses isolated in Uttar Pradesh (India) from two polio cases with disease onset on 1 October and 6 November 2009 (Figure 3). Phylogenetic analysis suggested that the outbreak in Tajikistan resulted from a single importation event. Consistent with a rapid development of the outbreak, the majority of the isolates exhibited a low level of genetic divergence and did not cluster into distinct, statistically supported lineages, although the maximum VP1 nucleotide divergence between the WPV1 isolated in Tajikistan was 1.7%.

The VP1 diversity between the most diverged members of this monophyletic population, RUS38492 (Dagestan, 18 September 2010) and RUS38655 (Chechnya, 6 October 2010) was 2.3%. On the basis of the phylogenetic reconstruction, it may be estimated that these viruses had independently evolved from a common ancestor ca 14 months earlier (with 95% highest probability density confidence interval (HPD) of 12.3–15.8 months) (Figure 3). The mean rate of nucleotide substitutions comprised ca 1.28% (95% HPD: 1.05–1.51%) per

Figure 3

Maximum clade credibility tree for the VP1 coding region of wild polioviruses isolated during the outbreak in Tajikistan and neighbouring countries, 2010 (n=119[a]) and of isolates circulating in Uttar Pradesh in 2008 (n=5) and 2009 (n=17)

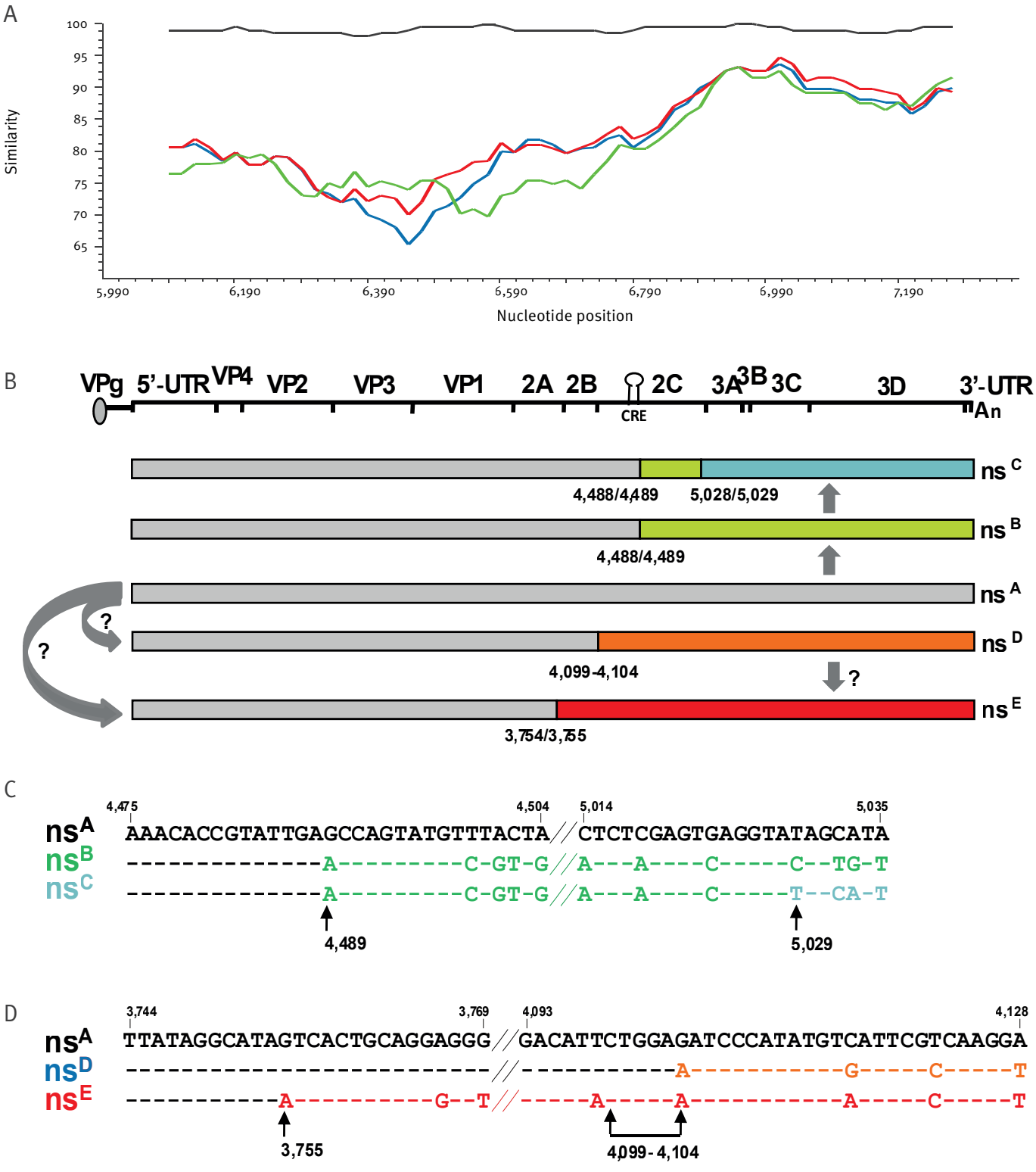


[a] Of 153 sequenced isolates, 119 were included in the analysis; 34 identical sequences were discarded to simplify calculation.

The virus UPSUL01012 (GenBank accession number AY189875) isolated in Uttar Pradesh in August, 2001 was used as the outgroup. The numbers at the nodes stand for the posterior probability support for the clades. The tips of the branches are marked according to the place of isolation: open circles: isolates from Tajikistan; open squares: isolates from Russia; filled squares: isolates from other neighbouring countries. Letters A–E represent the type of nonstructural proteins. Stars represent isolates from healthy people. The well-supported Russian transmission chains as well as the Dagestan chain are indicated by vertical bars. The numbers across the bottom of the figure are the calculated time for the most common recent ancestor.

Figure 4

Recombinant genomes of polioviruses isolated during the outbreak in Tajikistan and neighbouring countries, 2010



ns: non-structural; UTR: untranslated region.

(A) Similarity plots of a1,383 nt long genome segment (position 5,990–7,372) of ns^A (gray), ns^B (green), ns^C (blue), ns^D and ns^E (red) isolates compared with the sequence of wild poliovirus type 1 UPBSL09341 isolated in Uttar Pradesh in 2009. The nsA variant was inherited from an Indian predecessor, while other variants appeared to originate in Tajikistan.

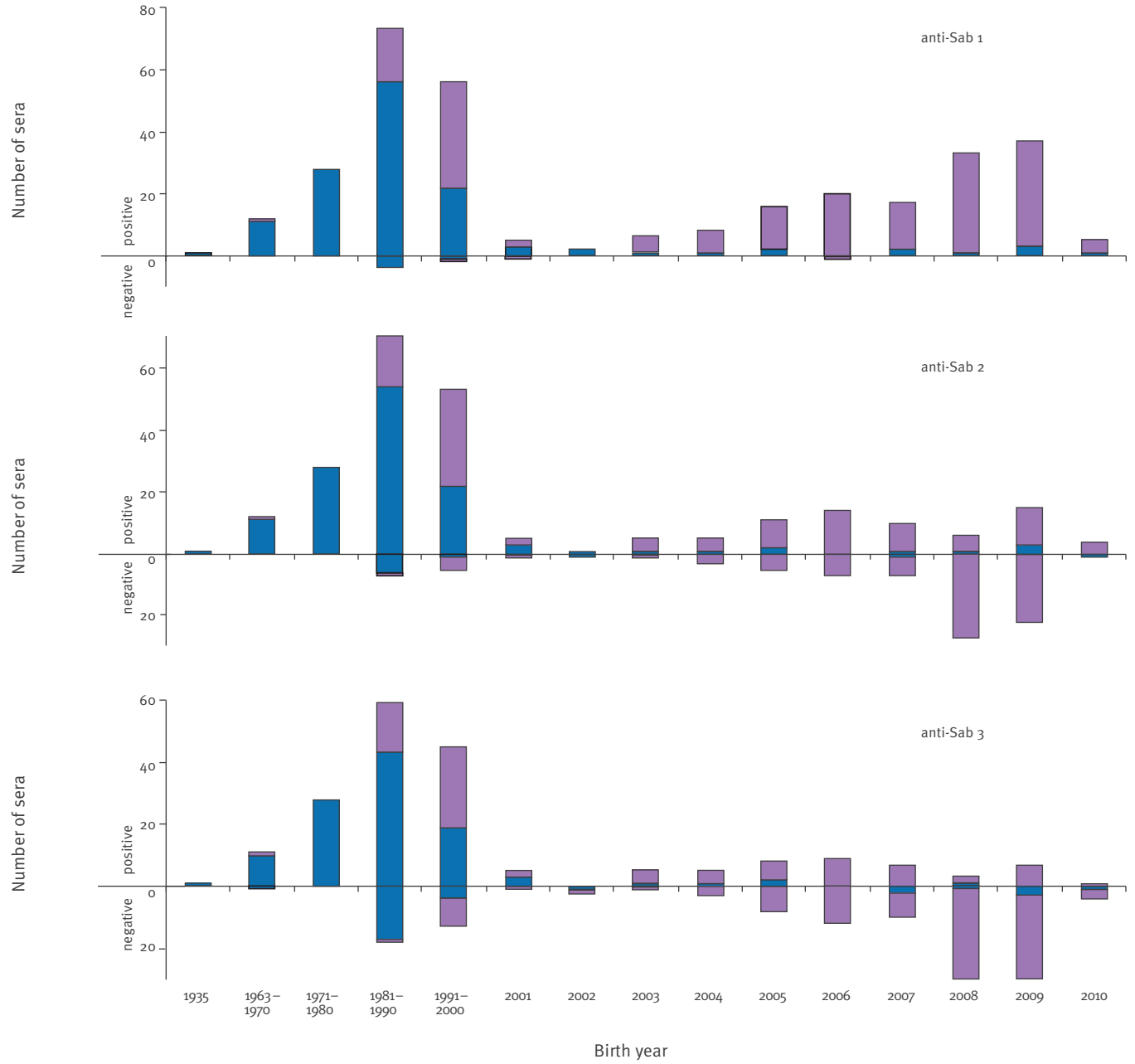
(B) Schematic representation of five types of the non-structural genomic region. Possible relations between different variants are indicated by arrows.

(C) Nucleotide sequences in the proximity of the crossover points of ns^B and ns^C recombinants compared with the parental ns^A sequence.

(D) Nucleotide sequences in the proximity of crossover points of ns^D and ns^E recombinants compared with the parental ns^A sequence. Identical nucleotides are marked by dashes.

Figure 5

Population immunity to polioviruses in Tajikistan, 2010 (n=327)



Sera collected from patients with AFP (blue bars) as well as from healthy people or patients with other diagnoses (purple bars) were tested in microneutralisation assay with type 1, type 2, and type 3 Sabin strains (panels presented as anti-Sab1, anti-Sab2, anti-Sab3). Year of birth of sera donors is indicated below the corresponding bars. Positive and negative sera correspond to samples containing ≥1:8 and <1:8 titres of neutralising antibodies respectively.

year, i.e. close to the earlier estimates by Gavrilin et al [15] and Jorba et al [16].

Nucleotide variability in the VP1 coding region mostly affected synonymous sites (147 synonymous substitutions per 182 changed positions). The majority of non-synonymous mutations were sporadic and only one, Glutamine to Arginine at position VP1-004 (the amino acid residues are numbered starting at VP1-001) was observed in 12 of 153 analysed WPV1 sequences. This and several other mutations, which mapped to, or close to, antigenic sites and/or sites involved in interaction of poliovirus with its receptor, did not deviate from the VP1 consensus for WPV deposited in the GenBank and appeared to reflect 'permitted' variability with little or no adaptive significance.

Whole genome sequencing

Full genome sequences of 14 WPV1 isolates from Tajikistan (n=6), Russia (n=6), Kazakhstan (n=1) and Turkmenistan (n=1), were determined. Several amino acid residues of the capsid proteins other than VP1 were found to be unique (not present in the GenBank) and two of them appeared to map to the antigenic/receptor-interacting regions. However, neither of them was consistently fixed in a significant number of isolates or reflected a substantial change in the physical-chemical properties of the residues, again suggesting that this variability was of a non-adaptive nature.

These sequences as well as partial sequences of the non-structural region of 37 additional isolates (32 from Tajikistan, four from Russia and one from Turkmenistan) revealed, in addition to mutations, several recombination events. Comparative analysis indicated that one set of nonstructural proteins (designated ns^A) was inherited from the parental Indian strain (Figures 3 and 4), whereas another (ns^B) appeared to be acquired by recombination with an unknown enterovirus C (Figure 4) before February 2010, because the very first WPV1 isolate in Tajikistan (the case dated 1 February 2010) already was of ns^B type. Since that isolate exhibited sequence heterogeneity, plaque cloning was performed. As a result, an additional related recombinant was identified. The crossover region in the ns^B-harbouring isolate mapped to around nt position 4,488 in the 2C region, whereas the other isolate (ns^C), while sharing ca 540 nt with that of ns^B, had acquired the sequence downstream of position 5,028 from another enterovirus C (Figure 4). Detection of the ns^B set of proteins in numerous WPV1 isolates (Figure 3) suggested widespread co-circulation of these viruses together with ns^A-harbouring viruses. On the other hand, ns^C proteins were detected only in the isolate from a single patient and thus appeared to represent a dead-end population (although it should be admitted that only a limited proportion of isolates (n=51) were sequenced in the non-structural region and all isolates were from cases of poliomyelitis).

Sequencing of a WPV1 isolate from another patient also revealed heterogeneity. Cloned populations of this isolate demonstrated the presence, along with the ns^A-containing virus, of two recombinants possessing distinct, though closely related, sets of non-structural proteins, ns^D and ns^E. The crossing partner(s) of the ns^A-containing virus appeared to be another human enterovirus C (or two related viruses), which donated their sequences downstream of nt positions 3,755 and 4,104, respectively (Figure 4). It is unclear whether there were in this case two independent recombination events, or one recombinant was the predecessor of the other (Figure 4B). Neither ns^D nor ns^E proteins were detected in any other sequenced isolate. Although numerous mutations in the non-structural proteins were detected, we were unable to ascribe to any of them an obvious biological significance.

Table 1

Antibodies in sera of poliovirus-negative cases of acute flaccid paralysis registered before the start of the supplementary immunisation activities, Tajikistan, 2010 (n=8)

Year of birth	Microneutralisation			ELISA (Sab1)	
	anti-Sab1	anti-Sab2	anti-Sab3	IgM	IgG
1986[a]	841	1,024	>1,024	1,600	25,600
1990[a]	299	251	178	100	6,400
1994[a]	1,024	1,024	11	100	3,200
1996[b]	>1,024	>1,024	421	100	25,600
2006[b]	<8	<8	<8	100	100
2008[a]	299	<8	<8	12,800	3,200
2008[b]	299	<8	<8	400	3,200
2010[c]	708	708	<8	1,600	6,400

ELISA: enzyme-linked immunosorbent assay; Sab: Sabin vaccine strain.

[a] Data on the vaccination not available.

[b] Patients reported to be vaccinated before 2010.

[c] Patient reported to be vaccinated late in March, 2010 before the SIAs were started.

The observed variability in the 5'- and 3'-untranslated regions did not appear to affect the stability of their secondary structures (not shown).

Conditions favouring the outbreak

To provide insight into conditions favouring the outbreak, 327 sera obtained before and during the outbreak from 188 randomly selected patients with AFP (144 laboratory-confirmed, 42 WPV-negative and two not investigated) and 139 healthy persons or patients with other diagnoses were assayed for poliovirus antibodies. A low immunity to poliovirus types 2 and 3 was revealed in 67 of 137 children born in 2004 to 2010 (Figure 5). Neutralising antibodies to these serotypes were not detected (<1:8) in 37% of the sera from all AFP cases and in 64% sera of 81 AFP patients born in 2007 to 2009, testifying to insufficient population immunity, which is very likely to have contributed to the outbreak onset and size.

Additional serological features

Of the eight poliovirus-negative patients who developed AFP before the supplementary immunisation activities and from whom sera were collected, at least three seemed to be unvaccinated as they lacked neutralising antibodies to Sabin strains of serotypes 2 and 3. Two of them exhibited high titres of both neutralising antibodies and IgM to poliovirus type 1 (Table 1), consistent with a recent infection with this virus. The data from four patients raised the possibility that even poliovirus-negative AFP patients who possessed neutralising antibodies to all poliovirus serotypes and thus were seemingly effectively vaccinated, might present paralytic disease. Indeed, the sera of one such case had a very high IgM titre against poliovirus 1, suggestive of acute infection. Although non-polio AFP aetiology in some of these cases cannot be rigorously excluded, their appearance during the poliomyelitis outbreak suggests that they were more likely to be caused by WPV1. Remarkably, 13 of the 144 patients with laboratory-confirmed poliomyelitis were serotype-positive against all the three polio serotypes (Table 2) and thus were also likely to be vaccinated.

Table 2

High levels of neutralising antibodies in the laboratory-confirmed poliomyelitis cases registered before the start of the supplementary immunisation activities[a], Tajikistan, 2010 (n=13)

Year of birth	Microneutralisation		
	anti-Sab1	anti-Sab2	anti-Sab3
1990[b]	1,024	355	53
1995[c]	708	708	32
1996[c]	596	355	299
2003[b]	251	501	211
2003[b]	150	355	1,024
2003[b]	1,024	1,024	251
2005[c]	421	1,024	75
2005[c]	1,024	501	45
2006[b]	75	32	1,024
2006[b]	150	45	422
2006[b]	501	1,024	501
2007[b]	89	502	75
2009[b]	422	1,024	211

Sab: Sabin vaccine strain.

[a] Only data for sera with high titres (≥1:32) of neutralising antibodies to the three poliovirus serotypes are included.

[b] Data on the vaccination not available.

[c] Patients vaccinated before 2010.

Export to neighbouring countries

During the outbreak, WPV1 was exported from Tajikistan to several neighbouring countries, Kazakhstan, Russia, Turkmenistan and Uzbekistan. The largest number of disease cases caused by this virus, 19 cases of paralytic poliomyelitis, was observed in Russia (Figure 2B). Seven of these patients had recently come from Tajikistan or Uzbekistan or had known contacts in these countries. Fifteen cases were registered in Russian citizens, predominantly in the North Caucasus region (seven cases in Dagestan and four in Chechnya). The age of the 19 poliomyelitis patients ranged from six months to 32 years, and eight of them had no antibodies against poliovirus types 2 and 3 and were therefore likely to be unvaccinated. Of note, among patients in Dagestan, three were adults (19, 27 and 32 years-old); two of them may have been vaccinated, judging by the presence of antibodies to polioviruses types 2 and 3. WPV1 was also isolated from 39 of 438 investigated healthy contacts, including four persons between 48 and 80 years-old.

Sequencing data indicate that all the isolates from Russia were closely related to the strains circulating in Tajikistan and that there were at least 10 independent virus importations (Figure 3). In some cases, the transmission route from Tajikistan to Russia could be established with reasonable certainty. Thus, the virus with Arg VP1-004 was isolated in adjacent districts of Tajikistan starting from February 2010, and viruses with this marker were later, from May 2010, isolated in Russia as well as in Uzbekistan. The non-structural genomic regions of three of 12 isolates possessing this marker were sequenced and found to share non-structural proteins of ns^{B} type.

Several chains of WPV1 transmission within Russia could also be identified (Figure 3). One was detected in the Irkutsk Region, with the first WPV1 isolated on 4 May from an AFP-diagnosed patient recently arrived from Tajikistan, the second from a healthy contact on 6 May, and the third from an unrelated healthy person on 9 June. A second transmission chain was due to importation to the Chelyabinsk Region around the end of April. Two cases were registered, on 10 May and 2 June, suggesting circulation of the virus for at least three weeks. The importation resulting in the transmission

chain in Chechnya, with onset of the first two polio cases on 4 August (estimated importation date on around 11 July), appeared to occur after the last case of WPV1-caused poliomyelitis was registered in Tajikistan on 4 July. Two subsequent isolations from poliomyelitis victims were on 10 August and 6 October 2010, suggesting circulation for over two months. One more transmission chain may have occurred in Dagestan, where seven polio cases were registered from 15 July to 22 November 2010. However, due to a relatively weak statistical support (0.77), several independent importations to Dagestan cannot be rigorously excluded.

Several WPV1 were isolated from faecal samples collected in 2010 from AFP cases in other neighbouring countries, two in Uzbekistan (six months- and 11 years-old, both vaccinated; dates of onset in April and May), three in Turkmenistan (two-, 11 and 13 years-old, all vaccinated; dates of onset in June), and one in Kazakhstan (seven years-old, vaccinated; date of onset in August). All of them were closely related to the Tajikistan isolates (Figure 3).

Discussion

The viruses isolated during the Tajikistan poliomyelitis outbreak of 2010 were closely related to the strains that circulated in the Indian state of Uttar Pradesh between 1 October and 6 November 2009. A single importation of WPV1 appears to have occurred in October 2009 (mean date with 95% HPD: end of August–early December) (Figure 3), i.e. well before the first isolation of WPV1 in Tajikistan, and the lag between this importation and first detection of the virus in Tajikistan could be explained by a low case:infection ratio for WPV1 infections and/or inadequate AFP surveillance. It may be noted that movement of people exists between the two countries, and this or a similar route of the cross-border poliovirus transmission was recorded also in 1991, when a strain of WPV1 circulating in Tajikistan (so called T-geotype) was found to be closely related to viruses found during the same period in India and Pakistan [15,17]. It may be noted that the VP1 sequences of the T-geotype and the contemporary viruses isolated in India and Tajikistan exhibited only 13–15% similarity. Moreover, the Tajikistan outbreak is a recent example of a long-standing process of importation of WPV from South Asia to Central and East Asia. Other examples include importation into Xinjiang, China in 2011 [18] and in 1999 into Qinghai, China [19].

The size of the outbreak

The number of AFP cases registered in Tajikistan.during the outbreak was 715, of whom 463 were diagnosed as poliomyelitis cases and 47 as polio-compatible cases; 181 WPV-negative patients and 24 patients without adequate faecal samples were classified as non-polio AFP cases. However, there is reason to believe that in reality the outbreak in Tajikistan in 2010 was larger.

Indeed, the number of AFP cases with unavailable or inadequate faecal specimens (n=71 cases) or WPV1-negative specimens (n=181) was very high. The reported mean yearly AFP incidence among children under the age of 15 years from 2000 to 2009.in Tajikistan was 30±8 [20], which corresponds to a rate of slightly above one case of AFP per 100,000 children under 15 years of age. The sharp, ca six-fold, increase in the number of non-polio AFP cases in 2010 was unusual. It is plausible that a proportion of them were associated with WPV1 infection. It is noteworthy that large areas in Tajikistan in 2010 had a significant number of AFP cases with no confirmed poliomyelitis (Figure 2A).

A significant number of poliovirus-negative AFP cases in the context of the 2010 outbreak were classified by the National Expert Committee as polyneuropathy and Guillain–Barré syndrome. However, in-depth clinical investigations such as the cerebrospinal fluid analysis, electromyography and nerve conductance studies had not been performed. Our own clinical observations (data not shown) suggest that at least two lethal AFP cases with unavailable or inadequate faecal specimens were discarded by the National Expert Committee but could, according to the current criteria, be regarded as polio-compatible. The presence of neutralising antibodies and high-titre IgM to type 1 poliovirus in the absence of neutralising antibodies to other poliovirus serotypes in two of the three apparently poliovirus-negative AFP cases may also be considered as an indication for likely recent infection with WPV1.

It cannot be excluded that the unusually high prevalence of AFP cases in 2010 may be partly explained by overreporting during the outbreak or underreporting in the previous years. Nevertheless, we think the available data are largely representative, even if not sampled systematically. If so, this would testify to limitations of the AFP incidence statistics as a key indicator in the current polio surveillance, at least in certain areas.

It is highly surprising that no polio cases were reported from Uzbekistan, as cases occurred across the border in Tajikistan (Figure 2A) and cross-border travel is frequent. Moreover, one patient developed paralysis (and excreted poliovirus closely related to the Tajikistan WPV1) one day after arrival to Moscow from Uzbekistan in the beginning of June, 2010 [21]. WPV1 were also detected in faecal samples from two AFP cases in Uzbekistan.

It may be added that paralytic diseases are known to be presented only by a minority of the infected persons, e.g. one case per 190 for WPV1 [22], suggesting that there may have been tens of thousands infections in Tajikistan in 2010.

Conditions facilitating the outbreak

A marked proportion of AFP victims of the Tajikistan outbreak who were born between 2004 and 2010, had no detectable antibodies to poliovirus serotypes 2 and 3 and hence were most probably not vaccinated. A serological survey conducted by Khetsuriani et al. after

the start of vaccination with mOPV and before tOPV implementation also showed a low level of less than 90% population immunity to type 3 poliovirus [23].

Although observations made in India demonstrate that vaccination may fail to induce adequate antibody response under certain conditions [24], the situation in Tajikistan appears to be different, judging by the effectiveness of vaccinations conducted to curb the outbreak (Figure 1A). The estimated anti-polio vaccination coverage with three doses of OPV in Tajikistan in 2005 to 2008 varied between 76 and 87% [25]. Thus, the inadequate level of immunity before the outbreak appears to have been a consequence of failure to vaccinate rather than of vaccine failure. It may be noted, that many countries, including several European ones, demonstrate similar low, or even lower, polio vaccination coverage [26], a situation requiring urgent measures to diminish the risk of similar outbreaks.

Special attention deserves the circulation of poliovirus among teenagers older than 15 years and adults. This age cohort is rarely targeted by vaccination campaigns, but can play a significant part in transmission of the virus.

Paralytic disease in previously vaccinated people

Although the major factor favouring the development of this outbreak was inadequate population immunity against poliovirus, there is evidence, although circumstantial, that paralytic poliomyelitis may on extremely rare occasions occur in OPV-vaccinated people who have a sufficient level of appropriate antibodies (Table 2). This observation by no means discredits the efficacy or safety of OPV but should be taken into account during epidemiological analyses. It may be added that the possibility of polio infection (without paralytic manifestations) of vaccinated people has been reported previously, e.g. in people with deficient mucosal immunity [27,28].

Epidemiological relevance of recombinants between wild polioviruses and other enteroviruses

Recombination between WPV or vaccine-derived polioviruses with other enteroviruses C is well known [29-31], but its biological relevance remains unclear. Recombination between WPV1 and several human enteroviruses C took place in Tajikistan in 2010. Interestingly, only one of several detected recombinants with newly acquired nonstructural proteins (ns^B) appeared to co-circulate with the originally imported lineage. It could be speculated that the failure of other recombinants to circulate was due to poor mutual compatibility of their genes. Another possibility is that at least some of these other recombinants did circulate but did not cause neurological disease and therefore could not be detected in samples from AFP cases. It should also be kept in mind that non-structural regions were analysed in only a restricted set of isolates.

Do those recombinants that are sufficiently fit (i.e. able to spread in human populations) have any advantage compared to the non-recombinants? An interesting, if speculative, possibility could be a role for non-structural poliovirus proteins in eliciting an immune response. This postulate is partially supported by the detection of antibodies against poliovirus non-structural proteins in some sera of poliovirus-positive AFP cases (data not shown; see also [32]). It is of note that non-structural proteins of other picornaviruses such as foot-and-mouth disease virus [33,34], hepatitis A virus [35], and Theiler's murine encephalomyelitis virus [36,37] do serve as targets for antiviral immunity. If this reasoning is valid, exchanges of non-structural proteins may facilitate immune evasion. We were, however, not able to demonstrate the ability of sera from poliomyelitis patients to discriminate between viruses with different sets of non-structural proteins (ns^A and ns^B). This hypothesis deserves further testing.

Conclusion

Several important lessons can be learnt from the analysis of the outbreak in Tajikistan. The main circumstance permitting the spread of the imported WPV was low population immunity against poliovirus. The effectiveness of the vaccination in stopping the outbreak indicates that there were no factors strongly interfering with the development of immune response against poliovirus and that people with no protective antibodies were most likely not to be (correctly) vaccinated. Serological surveillance can provide objective measures independent of official estimates of polio vaccination coverage. This surveillance should not neglect the immunological status of adults.

This outbreak, as well as an outbreak in the Republic of Congo in 2010 [38], highlight the need for continuous vaccination against poliomyelitis [39], especially taking into account a low level of population immunity against poliovirus in numerous countries. Moreover, the currently adopted vaccination strategy, i.e. a gradual switch from OPV to IPV [40], may need to be reconsidered, particularly in circumstances such as the recent silent transmission of WPV1 in Israel [41]. The possibility of circulation of WPV should be taken into account by the health authorities of the countries that use an IPV-only schedule. Finding the optimal way for maintaining adequate population immunity to poliovirus is an urgent challenge.

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Conflict of interest

None declared.

Authors' contributions

MLY, APG, OEI, VIA – planned the study, MLY, APG, MAP, OVI, JMD – performed molecular analyses, OEI, TPE, OYB - performed initial laboratory analysis of fecal samples, API – performed analyses of sera samples, AKS –conducted clinical investigation of polio cases in Tajikistan at the beginning of the outbreak as a member of expert group, MLY, APG, OEI, TPE, TPE, API, GYL, AKS, OKM, JMD, VIA – discussed the results, MLY, APG, VIA drafted the manuscript, all co-authors reviewed the final version of the manuscript.

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Development and validation of a real time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) assay for investigation of wild poliovirus type 1-South Asian (SOAS) strain reintroduced into Israel, 2013 to 2014

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In February 2013, wild poliovirus type 1 (WPV1) was reintroduced into southern Israel and resulted in continuous silent circulation in the highly immune population. As a part of the public health emergency response, a novel real time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) assay was developed, to allow for the sensitive and specific detection of the circulatingWPV1-South Asian (SOAS) strain. Specific primers and probes derived from the VP-1 region were designed, based on sequenced sewage isolates, and used to simultaneously amplify this WPV1-SOAS sequence together with bacteriophage MS-2 as internal control. High titre WPV1-SOAS stock virus was used for assay optimisation and 50 processed sewage samples collected from southern Israel and tested by reference culture based methods were used for analytical validation of the assay's performance. The limit of detection of the multiplex qRT-PCR (SOAS/MS-2) assay was 0.1 plaque-forming unit (pfu)/reaction (20 pfu/mL) for WPV1-SOAS RNA with 100% sensitivity, specificity, positive and negative predictive values when compared to the culture based method. The turnaround time was rapid, providing results for environmental samples within 24 to 48 hours from completion of sewage processing, instead of five to seven days by culture-based analysis. Direct sewage testing by qRT-PCR assay proved to be a useful tool for rapid detection and environmental surveillance of WPV1-SOAS circulating strain during emergency response. Application of the approach for detection of WPV1-SOAS in stool samples obtained during acute flaccid paralysis (AFP) surveillance or field surveys should be further evaluated.

Introduction

Israel has been free of circulating wild poliovirus (WPV) since 1988 as is evident from the continuous surveillance of children less than 15 years of age presenting with acute flaccid paralysis (AFP) and from routine environmental surveillance [1]. Notably, the environmental surveillance programme that has been running in Israel since 1988 has been successful in detecting sporadic introduction of WPV from neighbouring countries as well as highly diverged vaccine derived strains (VDPVs) [1-5].

In early 2013, WPV type 1 (WPV1) was introduced into southern Israel [6]. WPV1 introduction was initially discovered in April 2013 in sewage samples obtained from the cities of Rahat and Beersheva. The environmental surveillance was subsequently substantially enhanced and the clinical surveillance was widened to include AFP and aseptic meningitis cases of all age groups (data not shown). As of writing this manuscript, no clinical cases of poliomyelitis have been identified or reported. Repeated detection of increasing numbers of WPV1 in sewage in the absence of clinical poliomyelitis suggested ongoing silent circulation of WPV1 in southern Israel. The VP-1 region of the poliovirus genome was sequenced and determined to be closely related to the type 1 South Asian genotype (WPV1-SOAS) currently endemic in Afghanistan and Pakistan [7].

The current approved World Health Organization (WHO) guidelines for the detection of WPV involve, sample preparation for virus isolation on L20B and/or rhabdomyosarcoma (RD) cell lines. Identification of any cultured virus is performed by the Centers for Disease and Control prevention (CDC) intratypic differentiation (ITD)

molecular diagnostic assay, which should be followed by VP-1 complete gene sequencing of suspected WPV isolates [8-10]. These current protocols which cannot be performed on RNA extracted directly from processed sewage or stool samples, have low analytical sensitivity, are labour intensive, with long turnaround time ranging from five to seven days (for a highly positive (>1,000 plaque forming unit (pfu)/mL) stool sample) to two to three weeks (for negative stool samples and for sewage samples). Taking in consideration the laboratory setting requirements and expertise needed to perform these assays, laboratory capacity for culture-based WPV testing is limited in most countries, rendering WPV outbreak investigations very challenging.

In the framework of Israel's emergency response to this silent circulation, data regarding the magnitude of the circulating WPV1-SOAS and its geographical spread were urgently needed. The Ministry of Health's National Poliovirus Center at the Central Virology Laboratory (CVL) of Israel is the only laboratory in Israel accredited and certified for working with WPV. In order to enhance the capacity of the national laboratory and deliver expected outputs, development of a highly specific and reliable real time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) assay for detection of the circulating WPV1-SOAS strain was sought, using MS-2 bacteriophage as an internal control [11,12]. This paper describes the development, optimisation and analytical and field validation of this qRT-PCR (SOAS/MS-2) assay.

Methods

The assay under development was set up according to guidance on in-house molecular assay development and validation [13]. This included assay design and optimisation, analytical validation and environmental field validation.

Sewage sampling

Composite sewage samples were collected by Sigma SD900 automatic samplers (HACH, CO, USA) that were calibrated to collect either 24 samples (400 mL each) per day or 48 samples (200 mL each) per day for a total volume of about 10 litres. One litre of well mixed sewage was transferred to the Israeli CVL for further analysis. Sewage was treated as previously described [1] and concentrated to 20–30 mL. Aliquots from the processed samples were used for WPV isolation in L20B cells as previously described [1]. Plaque purified isolates were inoculated into HEp2 cells tube cultures. Supernatants from cultures showing cytopathic effect (CPE) were then subjected to molecular analysis for virus identification and sequencing as previously described [9].

Culture and quantitation of a circulating wild poliovirus type 1-South Asian stock virus

Circulating WPV1-SOAS virus stock culture was prepared from plaque purified circulating WPV1-SOAS (Isolate-PV-1-8099-PL9-Isr13) on L20B cells in monolayer in tube culture at a multiplicity of infection (MOI) of one. WPV1-SOAS infected cells were incubated at 35°C for 48 hours. Infected cells and supernatants were then subsequently freeze-thawed once at -70°C. The supernatants were then centrifuged at 3,000xg for 10 minutes. The supernatants were then aliquoted and stored at -70°C, pending analysis. WPV1-SOAS stock pfu concentration was determined on L20B cells and adjusted to $2x10^8$ pfu/mL.

Total nucleic acid extraction

Preparation of extraction lysis buffer spiked with MS-2 bacteriophage

MS-2 bacteriophage (ATCC 15597-B1) was chosen as an internal control for the qRT-PCR (SOAS/MS-2). MS-2 stocks maintained at CVL were cultured and titred as previously described [11]. MS-2 was added to the different lysis buffers at a concentration of 10,000 pfu/mL of lysis buffer. The final concentration of MS-2 was 2,000 pfu/qRT-PCR reaction.

Total nucleic acid extraction from sewage samples

Total nucleic acid (NA) was extracted from thoroughly vortexed processed sewage samples using the NucliSENS easyMAG system (bioMérieux, Marcy l'Etoile, France) according to the manufacturer's instructions. Briefly, external lysis was performed on 1 mL of concentrated sewage to inactivate the virus as recommended by the manufacturer. This was followed by NA extraction using the easyMAG extractor. Extracted NA was eluted in 55 µL elution buffer and stored at -70°C pending analysis.

Nucleic acid extraction from stock poliovirus cultures and spiked stool samples

Total NA was extracted from thoroughly vortexed processed stool samples or stock viral cultures using the Thermo Scientific King Fisher System (Walthman, MA, USA) according to manufacturer's instructions. Briefly, external lysis was performed on the maximum aliquot volume allowed (50 µL) to inactivate the virus as recommended by the manufacturer. This was followed by NA extraction using the King Fisher System extractor (RNA extraction kits). Extracted NA was eluted in 50 µL elution buffer and stored at -70°C pending analysis. When obtained NA extracts from stool samples appeared to inhibit the qRT-PCR (SOAS/MS-2), the NA extraction was redone using the easyMAG system.

Real time quantitative reverse transcription-polymerase chain reaction (SOAS/MS-2) assay design and optimisation

WPV1-SOAS specific primers and probes were designed in house based on VP-1 sequences of four isolates obtained from the initial positive sewage sample and confirmed by analysis of isolates from four different regions in southern Israel. Applied Biosystems 7500 sequence detection systems (Life Technology, NY, USA) were used for the amplification and detection of the amplicon (139 bp)

by TaqMan technology. WPV1-SOAS virus forward primer sequence (5'-TCATCCAGCACAGGTCACGA-3'), reverse sequence (5'-TACGTGATTTTCCACACTGA-3') and the probe – labelled with 6-carboxyfluorescein (FAM) and black hole quencher 2 (BHQ-2) – (FAM-5'-AATGACTGTAGACAATTCCGCCT-'3-BHQ2) were commercially prepared (Metabion, Martinsried, Germany) as were the internal control MS-2 primers and VIC-labelled probe sequences (Life Technologies, NY, USA), previously published by Dreir et al. [14] .

The sensitivity of the TaqMan assay for the detection of WPV1-SOAS was optimised by evaluating different concentrations of VP-1 primers (300, 600, and 900 nM) and probe (200, and 300 nM). The concentration of the primers and probe used in this study which yielded the best WPV1-SOAS detection limits were 300 nM for VP-1 forward and reverse primers, and 200 nM for the VP-1 probe, while the internal control primers concentrations were 150 nM each and 50 nM for the probe. The 25 µL volume qRT-PCR reaction mixture contained AgPath-ID One-Step RT PCR (Life Technology, NY, USA) reagents, VP-1 and MS-2 primers and probes and 5 µL of the samples extracted RNA.

Analytical validation

The analytical sensitivity of the qRT-PCR (SOAS/MS-2) assay was determined after serially diluting known concentrations ($2x10^8$ pfu/mL) of extracted WPV1-SOAS RNA in H_2O and running a total of four parallel qRT-PCR reactions for each dilution. This was used to determine the limit of detection (LOD) and precision.

The analytical specificity of the primers and probe for the detection of WPV1-SOAS were evaluated in silico using Basic Local Alignment Search Tool (BLAST) search (available at: http://www.ncbi.nlm.nih.gov) and in vitro when tested against a wide range of poliovirus, enterovirus and other virus reference strains.

The analytical validation of the qRT-PCR (SOAS/MS-2) assay for sewage samples was performed by serially diluting a known quantity of WPV1-SOAS ($2x10^8$ pfu) in pooled processed sewage samples, collected from southern Israel prior to the WPV1-SOAS circulation and negative for WPV1 by tissue culture. A 200 µL aliquot of the well mixed sewage sample with the different spiked virus dilutions was extracted by the easyMAG extractor and tested by the qRT-PCR (SOAS/MS-2) assay. In parallel, 200 µL aliquot of the diluted WPV1-SOAS in sewage was used to infect L20B cell in 90 mm plates. The number of plaques was determined after 48 hours incubation at 35 °C.

The performance of the qRT-PCR (SOAS/MS-2) assay was further challenged with WPV1-SOAS spiked archived stool samples from Israel. The archived stool samples were negative for WPV by tissue culture and by enterovirus qRT-PCR assay [15,16]. This was done in anticipation of mass screening for WPV1-SOAS in stools samples of individuals and the increased testing demand related to AFP surveillance. The analytical sensitivity of the qRT-PCR (SOAS/MS-2) assay was determined after serially diluting a known quantity of WPV1-SOAS ($2x10^8$ pfu) in a mixture of archived stool samples. A 50 µL aliquot of the well mixed stool sample with the different spiked dilutions was extracted by the Thermo Scientific KingFisher System extractor and tested by the qRT-PCR (SOAS/MS-2) assay.

Table

Interpretation criteria of the qRT-PCR (SOAS/MS-2) assay on nucleic acids extracted from sewage and stool samples, Israel, 2013

qRT-PCR (SOAS/MS-2) test result	WPV1-SOAS Ct value	MS-2 relative to MS-2 NC Ct value	Action	Interpretation of the test results
Test result-1	‹37	±3 Cts of MS-2 NC	Report result	Positive for WPV1-SOAS
Test result-2	‹37	›3 Cts of MS-2 NC	Report result	Positive for WPV1-SOAS
Test result-3	›45	±3 Cts of MS-2 NC	Report result	Negative for WPV1-SOAS
Test result-4	›37–‹45	±3 Cts of MS-2 NC	Repeat qRT-PCR in triplicate	Assay considered positive for WPVI-SOAS if 2 of the 3 reactions are positive Negative if only 1 or none positive
Test result-5	›37	›3 Cts of MS-2 NC	Dilute RNA 1:10 and repeat qRT-PCR in triplicate Or Add 0.5% BSA to the qRT-PCR mix and repeat qRT-PCR in triplicate For stool samples re-extract NA using easyMAG and repeat qRT-PCR in triplicate	Follow interpretations above if inhibition resolved Or Report inconclusive if qRT-PCR inhibition not resolved

BSA: bovine serum albumin; NA: nucleic acid; NC: negative control; qRT-PCR: real time quantitative reverse transcription-polymerase chain reaction; qRT-PCR (SOAS/MS-2): qRT-PCR for wild poliovirus type 1-South Asian genotype with MS-2 bacteriophage as internal control; WPV1-SOAS: wild poliovirus type 1 South Asian genotype.

Field validation of sewage testing

Sewage samples (N=50) collected between 29 January 2013 and 25 June 2013 were included in the field validation of the qRT-PCR (SOAS/MS-2) assay. Sewage samples were collected from the two southern cities of Israel, Rahat and Beer-Sheva, where the WPV1-SOAS virus was initially detected and subsequently circulated with high incidence. All samples were simultaneously tested by qRT-PCR (SOAS/MS-2) and by tissue culture on L20B cells [5,17].

Interpretation of real time quantitative reverse transcription-polymerase chain reaction (SOAS/MS2) assay results

A stool or an environmental sewage sample was considered positive for WPV1-SOAS by qRT-PCR (SOAS/MS-2) if the cycle threshold (Ct) for the VP-1 amplification was less than 37 and the sample was considered negative if the Ct value was greater than 45 (Table). This interpretation was valid only after taking into consideration, that no more than a 3 Ct difference in MS-2 value was noted between the tested sample and the negative extraction control. All samples with a Ct value ›37 and ‹45 were re-tested in triplicate and reported as weak positive if two or more of the triplicate analyses gave positive results and the multi-component curve indicated that the Ct value determined by the Sequence Detection System (SDS) software was the result of a true amplification event.

NA from sewage samples which suggested qRT-PCR inhibition (›3Ct difference of MS-2 result in the extracted sewage sample compared to the H2O MS-2 negative control), were reanalysed in triplicate in the presence of 0.5% bovine serum albumin (BSA) or after diluting the extracted NA 1:10 in molecular grade water. A sewage sample result was considered inconclusive if the qRT-PCR inhibition was not resolved. In addition, NA from stool sample which showed signs of qRT-PCR inhibition (›3Ct difference of MS-2 result in the extracted stool sample compared to the H2O control), were re-extracted by the bioMérieux NucliSENS easyMAG and re-evaluated by the qRT-PCR (SOAS/MS-2) assay. If the qRT-PCR inhibition remained, the sample was reanalysed in triplicate in the presence of 0.5% Bovine serum Albumin (BSA) or after 1:10 extracted NA dilution in molecular grade water. A stool sample result was considered inconclusive if the qRT-PCR inhibition was not resolved.

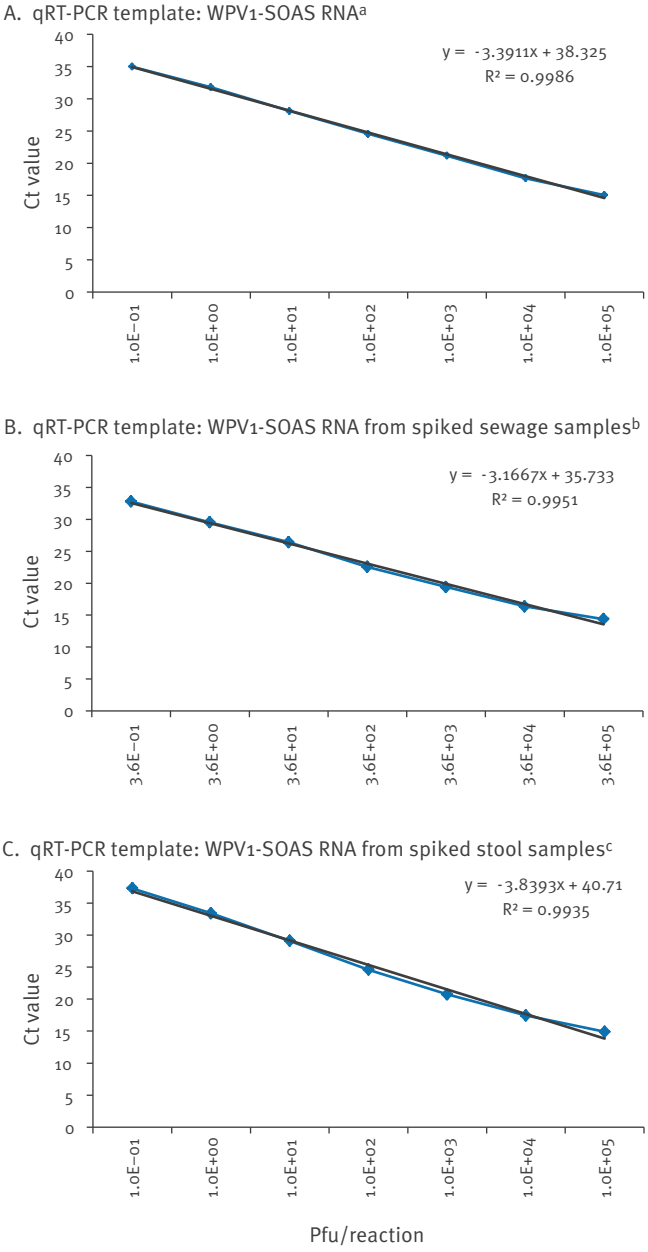
Results

Analytical sensitivity of the real time quantitative reverse transcription-polymerase chain reaction (SOAS/MS-2) assay

The analytical sensitivity of the qRT-PCR (SOAS/MS-2) assay determined after testing serial dilutions of WPV1-SOAS RNA in quadruplet (Figure 1A). The amplification curve was linear over 7 log dilutions (r^2=0.9986; slope: -3.39), and amplification efficiency was 97.3%. The LOD of the qRT-PCR (SOAS/MS-2) assay was 0.1 pfu/

Figure 1

Linear limits of detection of the qRT-PCR (SOAS/MS-2) assay



Ct: cycle threshold value; pfu: plaque forming unit; qRT-PCR: real time quantitative reverse transcription-polymerase chain reaction; qRT-PCR(SOAS/MS-2): qRT-PCR for wild poliovirus type 1-South Asian genotype with MS-2 bacteriophage as internal control; WPV1-SOAS: wild poliovirus type 1 South Asian genotype.

The blue line represents the mean Ct values obtained for each dilution and plotted against the number of pfu per reaction. The black line represents the linear regression. Error bars indicate the Ct standard deviation from the mean for each dilution.

[a] Serial (10-fold) dilutions of WPV1-SOAS RNA were prepared in molecular grade H2O and tested in quadruplet by the qRT-PCR (SOAS/MS-2) assay.

[b] Serial (10-fold) dilutions of WPV1-SOAS at an original concentration of 2x108 pfu/mL were prepared in polio free sewage samples, extracted by the easyMAG extractor and tested in triplicate by the qRT-PCR (SOAS/MS-2) assay.

[c] Serial (10-fold) dilutions of WPV1-SOAS at an original concentration of 2x108 pfu/mL were prepared in polio free stool samples, extracted by the Thermo Scientific KingFisher System, and tested in triplicate by the qRT-PCR (SOAS/MS-2) assay.

reaction (20 pfu/mL). Below 0.1 pfu/reaction, results were not reproducible, most likely due to the well-recognised stochastic properties of q-PCR on highly diluted nucleic acids [18]. The standard deviation for each of the dilutions points as shown by the error bars was less than 0.1 (Figure 1A).

The analytical sensitivity of the assay did not change upon evaluating RNA preparations from three different WPV1-SOAS circulating strains isolated from sewage samples collected from three different Israeli cities. Moreover, the assay's performance did not change upon triplicate runs using three different ABI 7500 instruments.

The performance of the in-house prepared WPV1-SOAS positive RNA control over 30 consecutive runs on different days and by different operators indicated that the qRT-PCR (SOAS/MS-2) assay was highly stable and precise. Overall, the aliquoted WPV1-SOAS RNA control (corresponding to approximately 5–10 pfu/reaction) had a mean Ct value of 33.7, standard deviation (SD) 0.5 and coefficient of variation (CV) 1.5% (data not shown). The precision of the MS-2 internal control assay was also determined on the 30 MS-2 extracted RNA. The mean MS-2 Ct result (corresponding to 2,000 pfu/reaction) was 26.1, while SD and CV were 1.2 and 4.7%, respectively (data not shown).

Analytical specificity of the real time quantitative reverse transcription-polymerase chain reaction (SOAS/MS-2) assay

In silico evaluation of primer and probe specificity did not detect any matching sequences. In addition, the qRT-PCR (SOAS/MS-2) assay was negative with RNA extracted from the following human viral pathogens derived from the national virus repository: Wild poliovirus type 2 and 3, Sabin 1 (4 isolates), Sabin 2 (3 isolates), Sabin 3 (4 isolates), vaccine derived poliovirus1 (aVDPV1) [5], vaccine derived poliovirus2 (aVDPV-2) [19], parechovirus, enterovirus99, echovirus33, echovirus20, echovirus3 coxsackievirus B3, rotavirus, norovirus II, human immunodeficiency virus (HIV), Epstein-Barr virus (EBV), cytomegalovirus (CMV), parvovirus, herpes simplex virus 1 and 2 (HSV), varicella zoster virus (VZV), respiratory syncytial virus A and B (RSV), influenza virus A, influenza virus B, adenovirus, parainfluenza3 and metapneumovirus.

Determination of analytical sensitivity using spiked sewage and stool samples

The LOD of the qRT-PCR (SOAS/MS-2) assay of WPV1 RNA extracted from sewage and ran in triplicate was 0.4 pfu/reaction (20 pfu/mL) and from stool samples 0.1 pfu/reaction (20 pfu/mL). The WPV1 amplification curve of RNA extracted from sewage (r^2=0.9951; slope: –3.1667) and from stool samples (r^2= 0.9935; slope:–3.8393) were linear over 7 logs (Figure 1 B&C). Beyond the detection limits, the results were not reproducible [18]. The standard deviation for each of the dilutions points as shown by the error bars varied between 0.1 and 0.6 (Figure 1 B and &C). The qRT-PCR (SOAS/MS-2) amplification efficiency of RNA extracted from sewage and stool samples were 106.9% and 82% respectively. On the other hand the L20B tissue culture limit of detection of WPV1-SOAS virus in stool spiked samples was 1 pfu/mL.

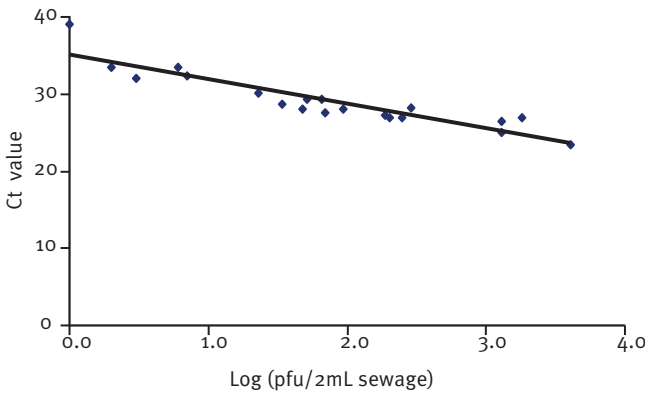
Field validation of environmental sewage samples

Of the 50 sewage samples evaluated for WPV1-SOAS, 20 (40%) were positive by both qRT-PCR (SOAS/MS-2) and culture and 27 (54%) were negative by both qRT-PCR (SOAS/MS-2) and culture. Three samples showed qRT-PCR inhibition as judged by the MS-2 marker results. Upon retesting in the presence of 0.5% BSA, the inhibition was resolved and the samples were reported as negative. Moreover, these three samples were negative by tissue culture. No discrepant results were noted, thus, the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the qRT-PCR (SOAS/MS-2) assay for direct testing of processed sewage samples were all 100%.

Stratifying the qRT-PCR positive results by Ct value showed that 14 (70%) of the positive samples gave Ct results in the range of 25 to 30, while five (25%) gave Ct values in the range of 30 to 35. Only one (5%) of the samples gave a Ct value in the range of 35 to 40. Moreover, the number of plaques obtained from all 20 positive sewage samples correlated well with the Ct values obtained from the qRT-PCR (SOAS/MS-2) assay (Figure 2). Over all, the slope obtained was -3.18; and the r2 value was 0.8445 which indicates a high

Figure 2

Linear limits of detection of the qRT-PCR (SOAS/MS-2) assay



Ct: cycle threshold value; pfu: plaque forming unit; qRT-PCR: real time quantitative reverse transcription-polymerase chain reaction; qRT-PCR (SOAS/MS-2) assay: qRT-PCR for wild poliovirus type 1-South Asian genotype using MS-2 bacteriophage as internal control.

RNA from 20 sewage concentrates were tested by the qRT-PCR (SOAS/MS-2) assay. Ct values were obtained for each sample and plotted against the number of pfu obtained from 2 mL of processed sewage on L20B cells grown in 10 cm tissue culture plates.

correlation between the Ct value and the number of plaques obtained.

Discussion

The emergence and continuous circulation of WPV1-SOAS in Israel has posed a major challenge to the public health services. While traditional methods of poliovirus surveillance still have their place in non-outbreak situations [8,9], in an outbreak setting, a more rapid and sensitive diagnostic modality was clearly needed in order to support epidemiological investigation and incident management.

The qRT-PCR (SOAS/MS-2) multiplex assay was developed after sequencing the VP-1 region of four poliovirus genomes isolated from four different locations in southern Israel. The SOAS/MS-2 assay analytical performance was excellent as the LOD of the assay was 4 pfu/reaction or 80 pfu/mL of extracted sewage, which is superior to that of the currently recommended WHO panPV PCR assay (25–250 pfu) as reported by Kilpatrick et al. [8,9]. The lower LOD of the WPV1-SOAS assay was predictable since non-degenerate primers were used in this assay compared to mixed-base or inosine residues used in the Kilpatrick et al. assay and because the qRT-PCR (SOAS/MS-2) assay was designed for a single sub-lineage branching from the SOAS genotype whereas the assay designed by Kilpatrick et al. needed to recognise a much broader range of targets [8,9]. Because of the degenerate nature of the primers designed by Kilpatrick et al., differences in target concentration, and variability of PCR efficiency due to sequence variations, they recommended that the poliovirus first be amplified to high titre by growth in tissue culture [8,9]. Moreover, complete sequencing of the VP-1 region of 100 WPV1-SOAS viruses isolated from different geographic locations in Israel, 2013, showed minor changes in the qRT-PCR (SOAS/MS-2) VP-1 sequences that did not affect the performance of the assay (data not shown).

The analytical sensitivity was complemented with excellent specificity since none of the viral genomes tested cross-reacted with the qRT-PCR (SOAS/MS-2) assay. Furthermore, several poliovirus vaccine and the Sabin vaccine derived poliovirus strains that were tested did not cross-react in this assay. This was of importance since this assay was anticipated to be used during a subsequent bivalent oral polio vaccine (bOPV) (Sabin-1 and Sabin-3) supplemental immunisation activity in Israel. The qRT-PCR (SOAS/MS-2) assay specificity was further challenged with high concentrations of wild poliovirus type 2 and 3, vaccine derived poliovirus type 1, and other common human pathogens, thus providing further reassurance of assay specificity.

Further to the favourable results obtained with analytical validation using spiked sewage samples, the qRT-PCR (SOAS/MS-2) assay compared well with the WHO Global Polio Laboratory Network tissue culture protocols for detection of poliovirus directly from processed sewage samples [20], exhibiting 100% sensitivity and specificity. Most importantly, the negative and positive predictive values of the assay were 100% compared to the culture based methods, which suggest the newly developed assay may be appropriate as rapid screening and decision-support tool.

Other studies that evaluated the detection of Sabin strains by qRT-PCR in sewage or stool samples did not incorporate any internal controls for the detection of qRT-PCR inhibitors [21]. Setting up the assay with built in internal controls provides further reassurance to the applicability of the assay as both stool and sewage samples may contain PCR inhibitors [11]. MS-2 detected qRT-PCR inhibitors in three (6%) of the sewage samples tested, which were resolved after sample treatment, as previously described [14]. The qRT-PCR ability to minimise false negative results due to inhibition of amplification also support its implementation as a screening tool.

Repeated routine sampling of sewage in the same catchment area increases the confidence in the analytic results and in the comparison of results from different samples collected from the same site. Differences in the physical layout and population in each catchment area are confounding factors when trying to compare results between different sites. Ct values obtained for each positive sewage sample correlated well with the number of plaques counted after culturing the sewage sample on L20B cells. Thus, an additional advantage of the qRT-PCR (SOAS/MS-2) assay was that it also rapidly supplied a semi-quantitative estimation of the virus load in a sewage sample from individual catchment areas. By indicating the relative number of excretors in a given area over time, it provided information on the ongoing transmission of the WPV1-SOAS virus and possibly on the effectiveness of any future intervention.

The analytical performance of the assay using spiked stool samples is very promising with regard to possible applications of the assay for surveillance of AFP via direct testing of clinical faecal samples as well as testing of faecal samples for detection of circulatingstrain excretion in order to delineate the spread of WPV1 in communities and geographical areas and identifying the main reservoir of WPV1 in order to guide vaccination.

In conclusion, the emergence of WPV1-SOAS in a population efficiently protected from paralytic poliomyelitis by high-coverage inactivated polio vaccine (IPV) immunisations mandated a rapid, dynamic and robust virological response in order to meet current and future testing needs in terms of both laboratory capability and capacity. The successful development and validation of a qRT-PCR (SOAS/MS-2) assay specific for the circulating strain has played a vital role as a critical response element that facilitated enhanced testing that informed public health policy and incident management. It also serves as a proof of concept for the need

to develop effective laboratory tools for direct detection of poliovirus as emergence and reintroduction of WPV into polio-free countries is plausible towards the 'end game' of polio eradication. The newly developed assay requires further clinical validation for application in AFP surveillance and faecal excretion surveys.

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Conflict of interest

None declared.

Authors' contributions

Musa Hindiyeh wrote part of the first draft, was part of the study design and conducted part of the work. Jacob Moran-Gilad edited the first draft and was part of the study design group. Yossef Manor edited the first draft, was part of the study design and conducted part of the work. Daniela Ram edited the first draft, was part of the study design group and conducted part of the work. Lester Shulman edited the first draft and was part of the study design group. DanitSofer edited the first draft and was part of the study design group. Ella Mendelson wrote part of the first draft and was part of the study design group.

Erratum

Reference numbers in the manuscript text were corrected on 25 February 2014.

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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 WEAFB2) 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.

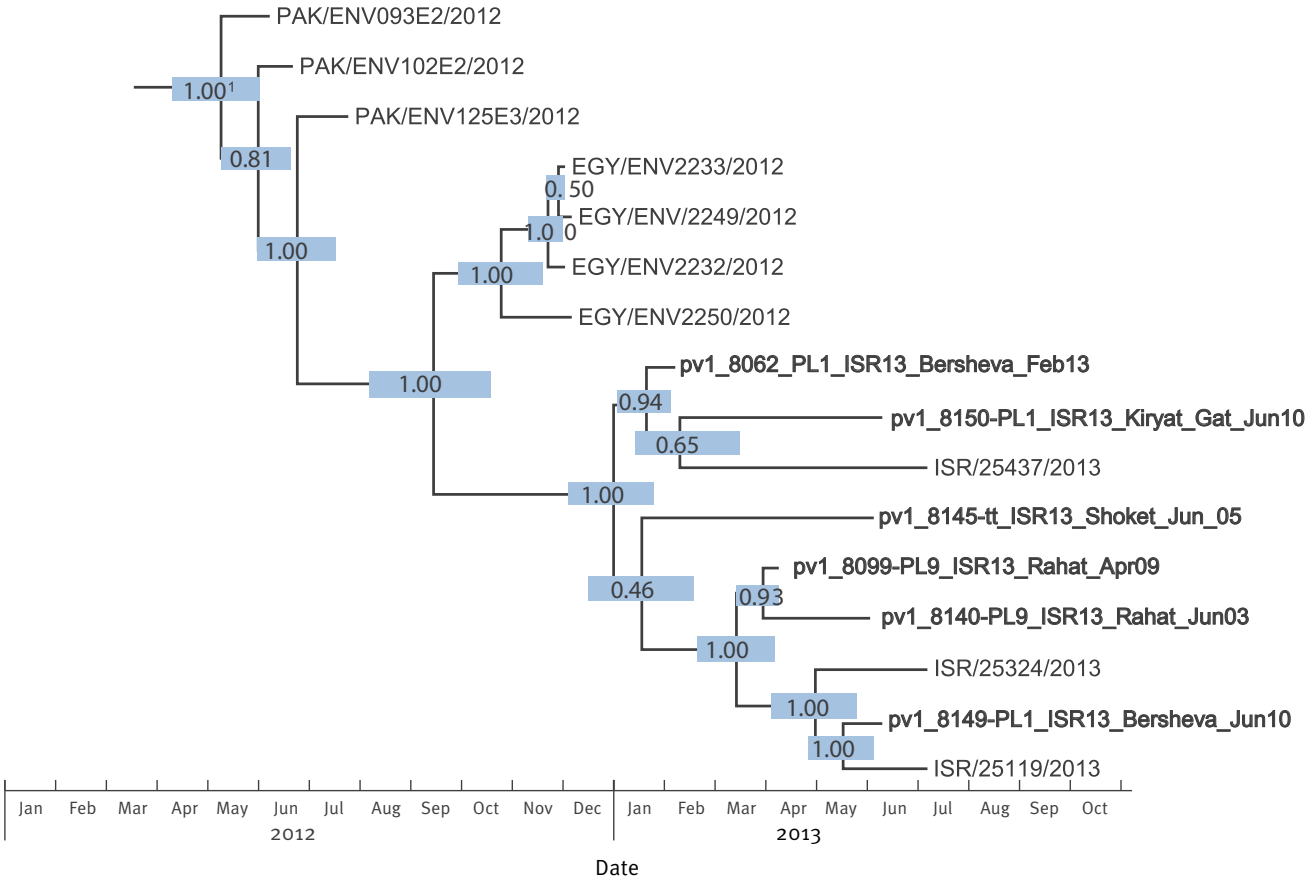
The complete VP1 sequence (906 nt) was obtained for viruses from WPV1-SOAS qRT-PCR-positive cultures from sewage and stool suspensions collected between February and July 2013. The sequences confirmed them as WPV1-SOAS. The maximum pairwise sequence divergence was 2.1%. This rate was twice as high as 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 Julo3) 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)

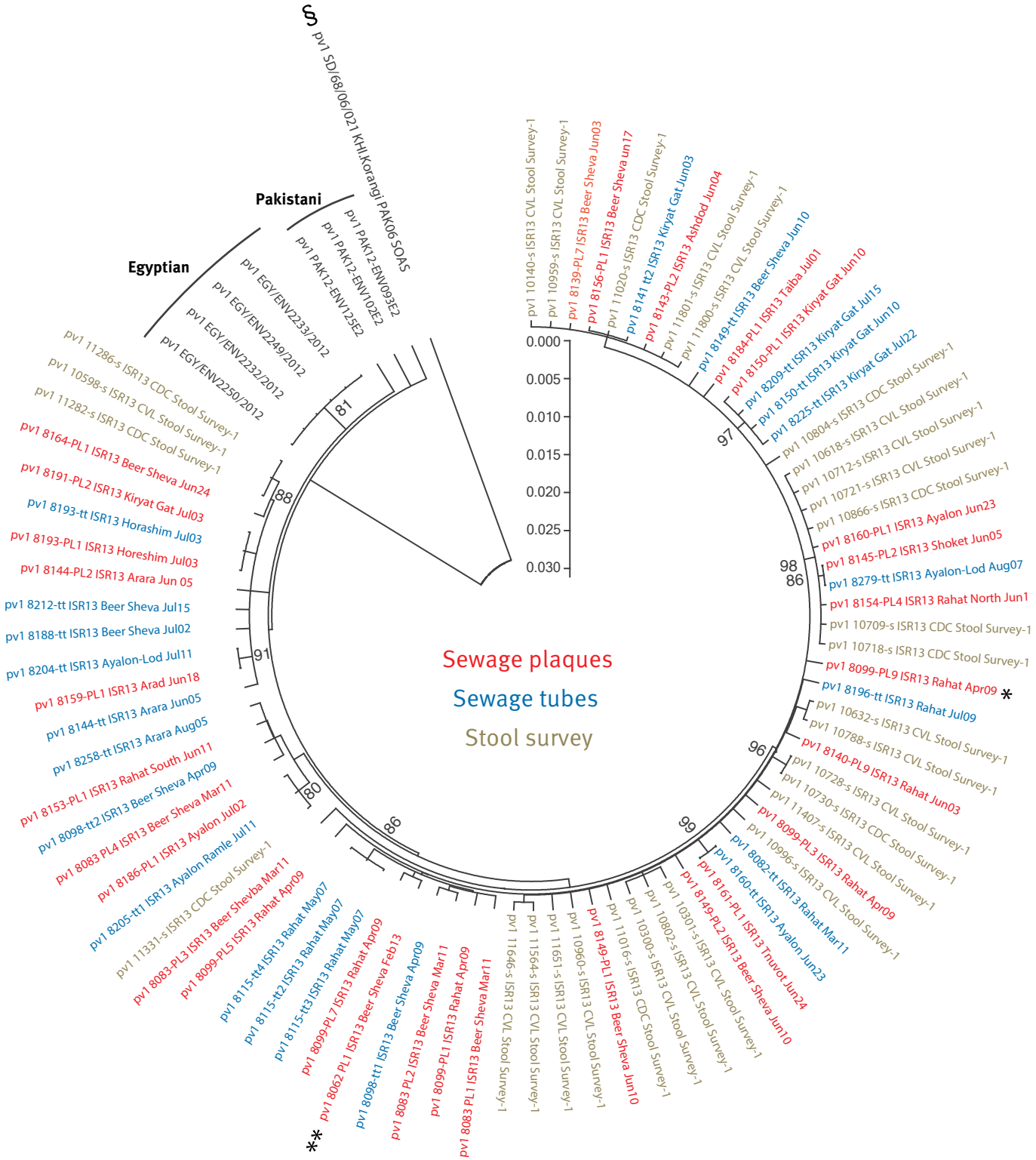


SOAS: South Asia; WPV1: type 1 wild poliovirus.

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 8062 (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 Labortory, 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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Immunity against poliomyelitis in the Netherlands, assessed in 2006 to 2007: the importance of completing a vaccination series

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Europe has been declared polio-free since 2002. Here we describe the seroprotection against poliomyelitis in the Dutch population using banked serum samples. Samples from 1,581 inhabitants of eight municipalities with low vaccination coverage (LVC) and an additional 6,386 samples from a nationwide (NS) group (clinical trial number: ISRCTN20164309; collected in 2006–07) were tested for neutralising antibodies (log² reciprocal titres (GMT); non-protection <3) against all three poliomyelitis serotypes. Demographic and epidemiological data were used for statistical regression analysis. Seroprevalence in the NS was 94.6% (type 1), 91.8% (type 2) and 84.0% (type 3). Infants (0–7 months-old) had ≥80% seroprevalence for all serotypes. The highest seroprevalence was found in children, with type 1 and type 2 in five year-olds and type 3 in nine to 10 year-olds. In the LVC group, orthodox protestants, many of whom refuse vaccination, showed seroprevalence rates of 64.9% (type 1), 61.0% (type 2) and 62.1% (type 3). In the NS group, non-Western immigrants and travellers to non-European continents had higher seroprevalences compared to Western immigrants and travellers within Europe, respectively. The Dutch National Immunisation Programme against poliomyelitis has provided good seroprotection, with high and long-lasting GMTs against all serotypes upon completion. The unvaccinated population remains at risk.

Introduction

Poliomyelitis is a severe infectious disease caused by poliovirus, an enterovirus with three serotypes (types 1, 2 and 3). In 90–95% of cases, the infection remains subclinical. In about 1% of the symptomatic cases, poliovirus invades the central nervous system, leading to muscle weakness and acute flaccid paralysis [1]. In 1988, the World Health Organization (WHO) launched a polio eradication initiative [2]. Since then, large vaccination campaigns have decreased the worldwide number of poliomyelitis cases by more than 99%. However, the WHO's goal of worldwide eradication remains unachieved.

Europe has been classified by the WHO as polio-free since 2002, despite the 2010 epidemic that originated in Tajikistan, because its dissemination was interrupted successfully. The Netherlands, where poliomyelitis has been notifiable since 1924 [3], suffered from a large type1 poliomyelitis epidemic in 1956 and three smaller outbreaks in 1971 (type 1), 1978 (type 1) and 1992–93 (type 3) [4-6]. In all of these smaller outbreaks, export of poliovirus to two polio-free countries, the United States (US) and Canada, occurred, but only during the 1971 and 1978 outbreaks symptomatic cases among unvaccinated individuals in US and Canada were reported. The last two outbreaks were restricted to orthodox protestant individuals, most of whom were refusing vaccination because of religious reasons and living in socio-geographically closely clustered communities. Since 1993, no cases of poliomyelitis have been reported in the Netherlands [8-11]. Currently, the risk of reintroduction of poliovirus to the Netherlands is discussed due to silent circulation of wild poliovirus in Israel and cases of poliomyelitis in Syria [12,13].

Vaccination against poliomyelitis, using trivalent inactivated polio vaccine (IPV), was introduced in the Netherlands in 1957 for all individuals born in 1945 and younger. Vaccination is free of charge for all inhabitants of the Netherlands up to the age of 18 years, and participation in the National Immunisation Programme (NIP) is not mandatory. From 1962 onwards, IPV was

administered in a combination vaccination strategy, along with the diphtheria, tetanus and whole-cell pertussis vaccine (DTwcP-IPV). Booster vaccinations with DT-IPV at four and nine years of age were added to the NIP in 1965. Initially, infant vaccinations were given at three, four and five months of age, followed by a booster at the age of 11 months, but this schedule was changed to two, three and four months of age in 1999, in response to an upsurge in pertussis [14]. This schedule, i.e. six IPV doses, was in use at the time of this survey (2006–07) and still is. In 2005 the infant doses of whole-cell pertussis vaccine were replaced with the safer acellular pertussis vaccine [15]. Vaccination coverage with the infant vaccinations has been continuously high (>95%) since 1957 [16].

A first nationwide seroprevalence study, performed in 1995–96, aimed to monitor the immunity of the Dutch population against diseases included in the NIP [17]. A second study was performed in 2006–07 [18]. Comparison between these studies enables us to assess the impact of changes in the vaccination schedule and (the absence of) circulation of microorganisms targeted by the NIP. The first survey showed good seroprotection rates against poliomyelitis in the general population [19]. Here we describe and discuss the protection against poliomyelitis in a representative Dutch population sample, including individuals from municipalities with low vaccination coverage (LCV), for whom banked serum samples and corresponding demographic and epidemiologic data, retrieved in the second serosurvey in 2006–07, were available.

Methods

Study population and design

A national NIP sera and data bank was established in 2006–07 to estimate age-specific seroprevalence of antibodies against vaccination-targeted diseases, as described previously [18, 20]. The specimen- and database includes male and female Dutch inhabitants, 0–79 years-old, from 40 municipalities nationwide (nationwide sample (NS); n=6,386), with oversampling of the migrant population. In addition, inhabitants from eight LVC municipalities were included (n=1,518). The demographic and epidemiological data included vaccination and travel history, and other known risk factors.

The study protocol was approved by the Medical Ethics Testing Committee of the Foundation of Therapeutic Evaluation of Medicines (METC-STEG) in Almere, the Netherlands (Clinical Trial Number: ISRCTN 20164309). All participants (or the parent/guardian of minors) provided signed informed consent for blood sampling and data gathering via a questionnaire.

Serology

The serum samples, derived from blood by centrifugation (10 min at 1,000 G), were retrieved from –80 °C storage. Poliovirus neutralising antibody titres against serotypes 1, 2, and 3 were determined in a standard neutralisation test (NT) using Sabin vaccine strains as challenge viruses, as recommended by the WHO [21]. Sera were tested in two-fold dilutions series. Quality control samples consisting of virus control and an in-house human serum control were added to each plate. Results were expressed as $\log^2$ reciprocal titres and samples were considered protective if NT titres were ≥8 (i.e. $\log^2$ titre≥3) [22].

Statistical analysis

Seroprevalence and mean $\log^2$ titres, both with 95% confidence intervals (95% CI), were calculated using a sampling weight for each study participant. For the NS group, results were adjusted for age, sex, ethnicity, degree of urbanisation, and a two-stage cluster sampling, taking into account the strata (five regions) and clusters (40 municipalities). For the LVC group, results were adjusted for age and sex; in addition, the LVC population was stratified by vaccination coverage related to religious denomination, as defined by Ruijs et al [23], with orthodox protestants (including Protestant Congregations in the Netherlands, Old Protestant Congregations, Restored Protestant Church and Protestant Congregations) representing low or intermediate vaccination coverage and non-orthodox protestants (including Protestant Bond, Christian Protestant Churches, other Protestant Christians, and other, no or unknown religion) representing moderate to high vaccination coverage.

Linear regression analysis was performed to study the decline of antibodies in individuals who had received the complete NIP vaccination series, consisting of six IPV-containing vaccinations. As such, this analysis was limited to individuals between 10 and 45 years-old, since the sixth IPV-containing vaccination would have been administered at approximately nine years of age and those older than 45 years would have been born before the current NIP strategy.

Logistic regression analysis was performed on the NS group to assess possible risk factors for non-protection (NT<3), using a multivariate model which included all variables with a p-value <0.2 already adjusted for age, sex, degree of urbanisation, and geographic region. To maximise comparability between the three serotypes, the full model was applied without backward selection to each serotype. The results are presented as odds ratios (ORs) with 95% CIs.

All statistical analyses were performed with SAS software (version 9.3) and Microsoft Excel.

Results

Immunity in the nationwide sample

The seroprevalence in the NS sample was 94.6%, 91.8% and 84.0% for poliovirus types 1, 2 and 3, respectively. Mean $\log^2$ titres were 7.39, 6.96 and 6.04 for the three respective types (Table 1).

For one month-old infants, who were too young to be vaccinated, the seroprevalence (reflecting maternal antibodies) was 83.0% (type 1), 79.1% (type 2), and 65.9% (type 3). These percentages were higher for the two to four month-old infants, who were eligible for vaccination (maximum of three IPV doses), with seroprevalences of 89.2%, 83.3% and 85.6% for the three serotypes. Owing to waning of antibodies, the seroprevalence percentages decreased thereafter, with the five to 10 month-olds showing 79.7%, 80.8% and 75.0% for the three serotypes, but increased again in the 11 to 13 month-olds, who were eligible for a booster dose, with seroprevalences of 92.1%, 97.4% and 94.7% for the three serotypes. The highest rates of seroprevalence for type 1 and type 2 (both 100%) were found in five year-old children (after the fifth IPV dose at four years of age), and for type 3 (95.9%) in nine to 10 year-olds (after the sixth IPV dose).

Plotting of the mean $\log^2$ titres of infants and children, stratified by age, indicated the positive effect of booster doses at 11 months and at four and nine years of age (Figure 1). In contrast, plotting of the mean $\log^2$ titres of adults showed a gradually declining trend up to the age of 45–49 years, followed by an increase in mean $\log^2$ titre for people born before 1957, who possibly had been in contact with wild-type poliovirus.

The overall seroprevalence and mean $\log^2$ titres of non-Western immigrants (n=1,069) were higher than those of individuals of Dutch or Western origin (n=5,317). The higher seroprevalence of type 1 and type 2 in the non-Western group was mainly due to higher seroprevalences in children under the age of four years and adults 40 years and older. The increased seroprevalence of type 3 was evenly distributed over the age categories.

Immunity in the low vaccination coverage sample

The seroprevalence in the LVC sample excluding orthodox protestant persons was 92.9%, 90.3% and 86.1% for the three respective serotypes, corresponding to a mean $\log^2$ titre of 7.33 (type 1), 6.66 (type 2) and 6.21 (type 3) (Table 1).

For the orthodox protestant sub-category within the overall LVC sample, both seroprevalence and mean $\log^2$ titres were significantly lower; moreover, analysis of immunity against poliomyelitis in four cohorts in this subcategory, based on date of birth, showed the influence of poliomyelitis outbreaks (Table 2). The results indicated that the youngest cohort, individuals born from 1994 onwards, had not been in contact with wild-type poliovirus, whereas two adult cohorts,

Table 1

Seroprevalence rates and mean $\log^2$ titres for poliovirus types 1, 2 and 3, the Netherlands, 2006–07 (n=7,967)

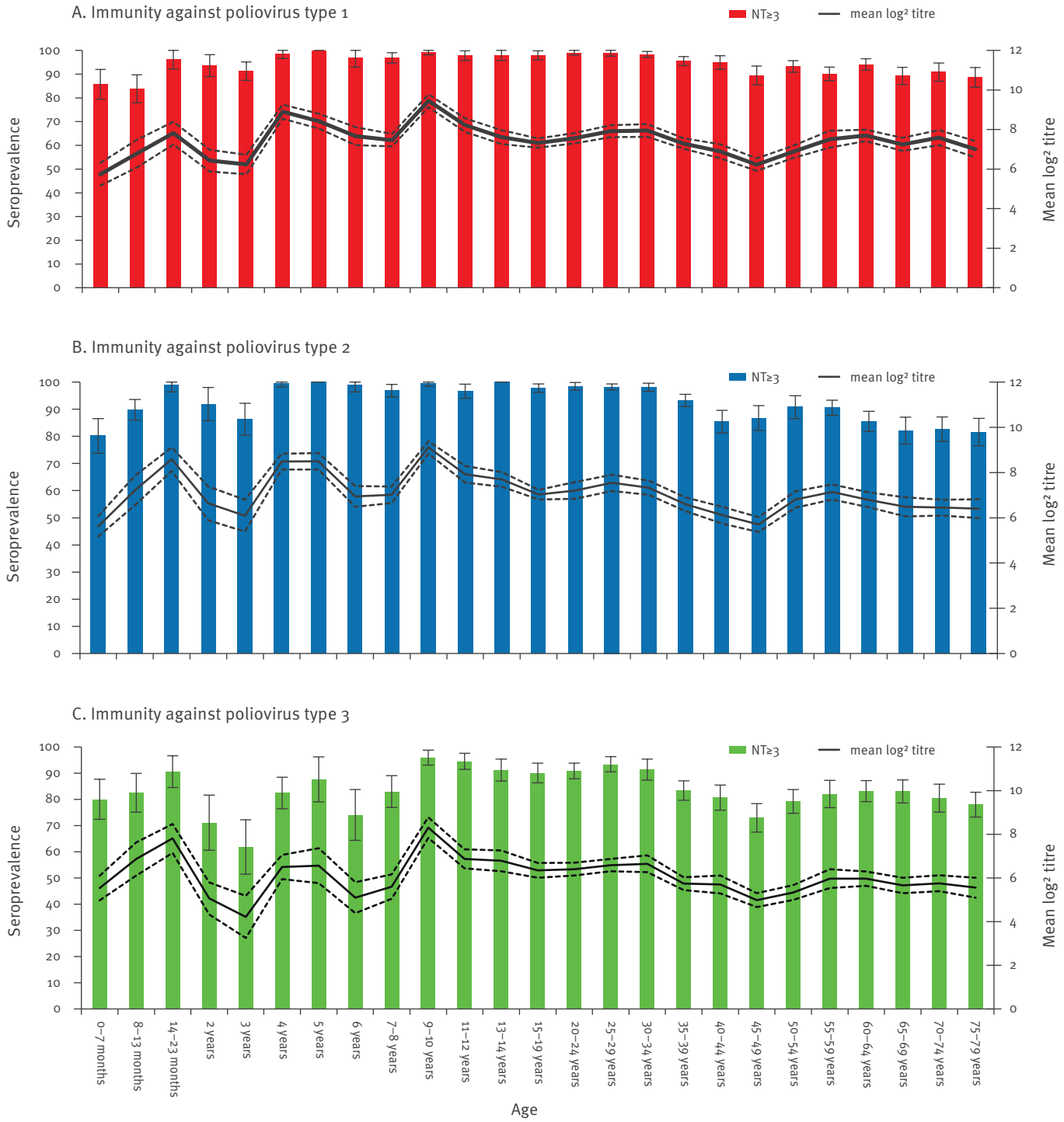
Poliovirus serotype	Sample	NT≥3 % (95% CI)	Mean $\log^2$ titre mean (95% CI)
Type 1	Nationwide sample[a]	94.6 (93.9–95.3)	7.39 (7.32–7.45)
	Dutch citizens and Western immigrants[b]	94.2 (93.4–95.0)	7.30 (7.22–7.37)
	Non-Western immigrants[c]	97.6 (96.7–98.6)	7.83 (7.68–7.98)
	LVC non-orthodox protestants[d]	92.9 (91.7–94.1)	7.33 (7.05–7.60)
	Orthodox protestants[e]	64.9 (57.8–72.1)	5.34 (4.68–6.00)
Type 2	Nationwide sample[a]	91.8 (90.9–92.6)	6.96 (6.89–7.03)
	Dutch citizens and Western immigrants[b]	91.1 (90.3–91.8)	6.84 (6.76–6.92)
	Non-Western immigrants[c]	97.1 (95.8–98.4)	7.57 (7.42–7.72)
	LVC non-orthodox protestants[d]	90.3 (87.8–92.7)	6.66 (6.43–6.88)
	Orthodox protestants[e]	61.0 (50.5–71.4)	4.86 (4.28–5.45)
Type 3	Nationwide sample[a]	84.0 (82.9–85.1)	6.04 (5.96–6.11)
	Dutch citizens and Western immigrants[b]	83.2 (82.2–84.1)	5.91 (5.82–5.99)
	Non-Western immigrants[c]	90.7 (88.9–92.5)	6.68 (6.51–6.86)
	LVC non-orthodox protestants[d]	86.1 (83.6–88.6)	6.21 (5.94–6.48)
	Orthodox protestants[e]	62.1 (54.5–69.7)	4.71 (4.21–5.21)

CI: confidence interval; NT: neutralisation test; LVC: low vaccination coverage.

[a] n=6,386.
[b] n=5,317.
[c] n=1,069.
[d] n=1,038.
[e] n=480.

Figure 1

Seroprevalence and mean log^2 titres for poliovirus types 1, 2 and 3 in the nationwide sample, stratified by age, the Netherlands, 2006–07 (n=6,386)



NT: neutralisation assay.

Table 2

Seroprevalence rates and mean log² titres for poliovirus types 1, 2 and 3 in orthodox protestants, stratified by age, the Netherlands, 2006–07 (n=480)

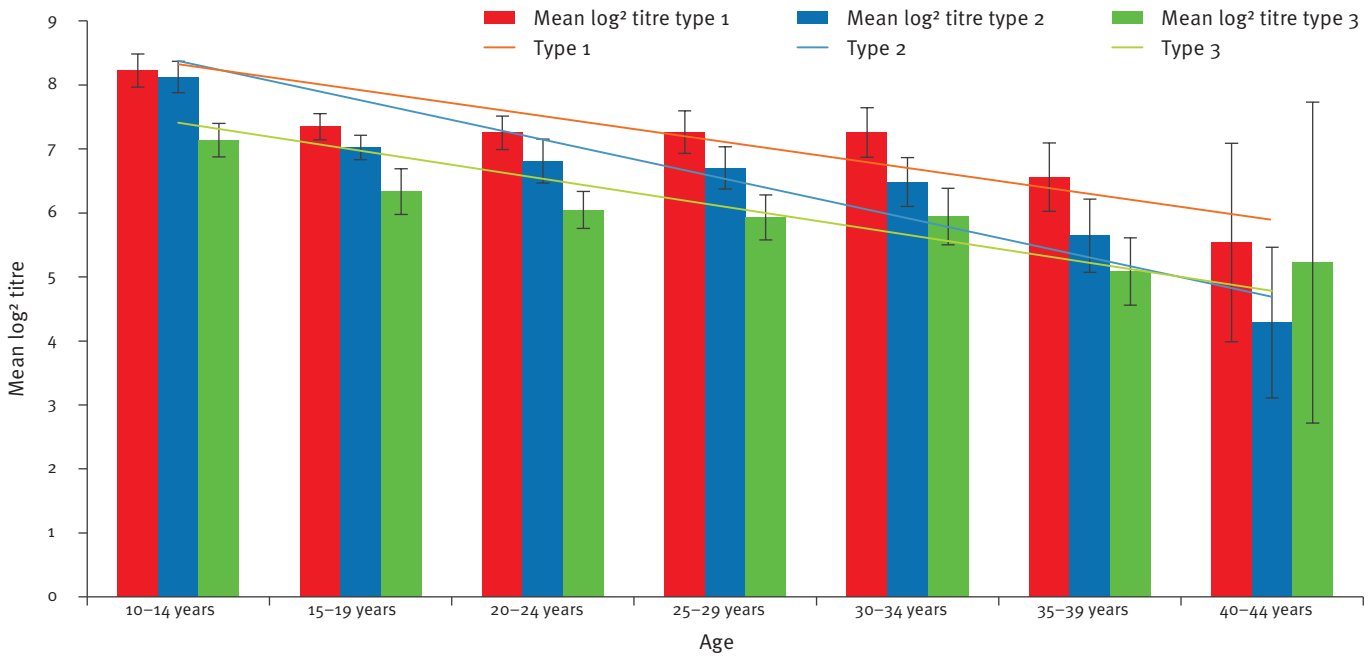
Poliovirus serotype	Cohort, by birth date	NT≥3 % (95% CI)	Log2 titre mean (95% CI)
Type 1	≥1 Jan 1994[a]	49.7 (37.6–61.8)	4.36 (3.59–5.13)
	≥1 Jan1979 and <1 Jan 1994[b]	52.8 (36.9–68.8)	4.59 (3.50–5.68)
	≥1 Jan 1957 and <1 Jan 1979[c]	72.9 (54.6–91.2)	5.77 (4.41–7.14)
	<1 Jan 1957[d]	80.3 (72.2–88.3)	6.40 (5.56–7.24)
Type 2	≥1 Jan 1994[a]	51.0 (39.5–62.5)	4.33 (3.66–5.00)
	≥1 Jan 1979 and <1 Jan 1994[b]	60.8 (45.2–76.4)	4.83 (3.65–6.01)
	≥1 Jan 1957 and <1 Jan 1979[c]	56.2 (41.4–70.9)	4.61 (3.63–5.60)
	<1 Jan 1957[d]	79.0 (63.0–95.0)	5.83 (4.94–6.72)
Type 3	≥1 Jan 1994[a]	48.2 (36.9–59.5)	3.99 (3.14–4.84)
	≥1 Jan 1979 and <1 Jan 1994[b]	68.7 (51.8–85.6)	5.51 (4.40–6.61)
	≥1 Jan 1957 and <1 Jan 1979[c]	61.7 (45.2–78.3)	4.42 (3.57–5.27)
	<1 Jan 1957[d]	71.1 (63.2–79.0)	5.12 (4.47–5.77)

CI: confidence interval; NT: neutralisation test.

[a] n=190, no wild-type virus circulating.
[b] n=87, type 3 outbreak in 1992–93.
[c] n=100, type 1 outbreaks in 1971 and 1978.
[d] n=103, wild-type virus circulating.

Figure 2

Poliovirus mean log² titres in 10 to 44 year-olds in the nationwide sample who completed the NIP vaccination series with no revaccinations, stratified by age, the Netherlands, 2006–07 (n=1,260)



NIP: National Immunisation Programme.

i.e. individuals born 1979–93 and 1957–78, had been in contact with type 3 and type 1. This is in agreement with the recorded serotype-specific outbreaks that occurred in 1992–93 and in 1971 and 1978 [6,9]. These outbreaks were controlled with trivalent (1992–93) and monovalent (1971, 1978) live attenuated oral polio vaccine (OPV), which possibly also contributed to the observed seroprevalence rates. The cohort born before 1957 lived in the era before large-scale immunisation and therefore probably encountered wild-type virus regularly, as reflected in the moderate to high seroprevalence rates.

Persistence of antibodies in relation to completion of the poliovirus vaccination strategy in the Dutch National Immunisation Programme

For the NS cohort of 10 to 44 year-olds who had completed the normal immunisation schedule of six IPV-containing vaccinations by the age of nine years, and had not obtained any extra polio antigen-containing vaccinations (n=1,260), the seroprevalence percentages were 98.8% (type 1), 97.5% (type 2) and 90.9% (type 3). Plotting of the mean $\log^2$ titres showed a decreasing trend with age for all serotypes, but the titres remained well above the protection cut-off of 3, indicating that seroprotection had been achieved (Figure 2). Linear regression analysis to assess the relation between mean $\log^2$ titre and age yielded the following slopes: type 1, −0.06 (95% CI: −0.07 to −0.04), type 2, −0.09 (95% CI: −0.10 to −0.08) and type 3, −0.07 (95% CI: −0.09 to −0.05).

Demographic and vaccination-related risk factors of non-protection

The multivariate logistic regression analysis of risk factors for a non-protective antibody level (NT<3) in the overall NS cohort is presented in Table 3. The lowest seroprevalence for type 1 and type 2 was found in the 0 year-old infants, while the lowest seroprevalence for type 3 was found in the one to four year-olds. Furthermore, non-Western immigrants had higher seroprevalences than Western immigrants or individuals of Dutch origin. Likewise, the cohort who had travelled to Asia, Africa, or South and Central America had higher seroprevalences than the cohort who reported not having travelled to these continents. The cohort that reported practicing a religion that is associated with vaccination refusal had lower seroprevalences than the cohort that did not belong to these religious groups.

The cohort that had received their last polio antigen-containing vaccination more than 10 years (type 3) or more than 20 years (types 1 and 2) before sampling, or who had never received the vaccination, were at increased risk of having a non-protective antibody level compared to the cohort that had received their last vaccination less than one year before sampling. An increased risk for a non-protective antibody level was also found for individuals with medium-level (types 1 and 2) or high-level (types 1 and 3) education, compared with those with low-level education. Finally, individuals who had started but not completed the NIP vaccination series, i.e. those who had received only two to five polio antigen-containing vaccinations, were found to be at higher risk for non-protective antibody levels than the individuals who had completed the NIP vaccination series, although the difference in risk for serotype 2 did not reach statistical significance.

Discussion

The serosurvey described herein, using samples and data obtained in 2006–07 from across the country, showed that the general population of the Netherlands had an overall high poliovirus vaccination coverage, with protective antibody levels (NT≥3) being at >90% for serotypes 1 and 2 and slightly less (84%) for serotype 3. The seroprotection levels in the youngest population showed a trend of good adherence to the recommended initial vaccination schedule in early life; furthermore, analysis of long-term protection against all three serotypes indicated the benefit of completing the recommended vaccination series, starting in early life and receiving boosters in later childhood. Not surprisingly, the demographic feature of belonging to a religious group that is associated with vaccination refusal was identified as a risk factor for a non-protective antibody level.

These collective results are comparable to those of a previous Dutch seroprevalence study done in 1996-97 [19], as well as several seroprevalence studies from other countries [24-28]. In particular, the previous Dutch study showed similar age-related trends in seroprotection that correspond to the vaccination schedule and natural exposure, although the seroprevalences found in the current study were overall slightly lower. When comparing the current results from the Netherlands with those from other nations, no remarkable differences were observed for the age-specific trends in seroprevalence of type 1 or type 2; however, the seroprevalence trends for type 3 did appear to be lower than those reported in the other studies. Unfortunately, substantial differences in study design precluded direct or systematic comparison of the results, as some of the previous studies used for example age-restricted categories and included individuals with unknown vaccination status. One hypothesis is that the lower seroprevalence of type 3 found in the current study may be related to the whole-cell DTP-IPV-Hib vaccine used in the Netherlands until January 2005, which may induce lower antibody titres against serotype 3, but this needs further study. A possible future third serosurvey will provide more insight into this.

The low seroprevalence rates of the orthodox protestants in the Netherlands highlight the continued risk of poliomyelitis in this community. This was also reflected in the composition of affected persons in the last three natural outbreaks (type 1 in 1971 and 1978 and type 3 in 1992–93) that occurred in the Netherlands [11].

Table 3

Multivariate logistic regression analysis of risk factors for non-protection against poliovirus types 1, 2 and 3 in the nationwide sample, the Netherlands, 2006–07 (n=6,386)

Demographic- or vaccination-related factor	Sub-category	Total n	Poliovirus type 1		Poliovirus type 2		Poliovirus type 3	
			NT<3, %	Adjusted OR (95% CI)	NT<3, %	Adjusted OR (95% CI)	NT<3, %	Adjusted OR (95% CI)
Sex	Male	2,912	6.3	Ref	8.8	Ref	16.7	Ref
	Female	3,474	5.6	0.93 (0.75–1.16)	8.2	0.92 (0.76–1.11)	15.2	0.90 (0.78-–1.04)
Age in years	0	348	19.3	Ref	19.3	Ref	21.8	Ref
	1–4	514	5.3	**0.22 (0.12–0.40)**	6.0	**0.35 (0.19–0.64)**	23.5	**0.69 (0.44–1.08)**
	5–9	620	1.8	**0.09 (0.04–0.20)**	1.3	**0.09 (0.04–0.21)**	12.3	**0.33 (0.20–0.55)**
	10–19	730	1.9	**0.14 (0.06–0.37)**	1.9	**0.11 (0.04–0.26)**	7.8	**0.18 (0.10–0.32)**
	20–29	712	1.1	**0.06 (0.03–0.15)**	1.8	**0.06 (0.02–0.13)**	7.9	**0.12 (0.07–0.22)**
	30–39	715	3.4	**0.07 (0.03–0.16)**	4.8	**0.06 (0.03–0.13)**	13.2	**0.16 (0.09–0.28)**
	40–49	641	7.6	**0.08 (0.04–0.17)**	13.1	**0.12 (0.06–0.24)**	23.1	**0.17 (0.10–0.30)**
	50–59	714	7.8	**0.09 (0.04–0.18)**	8.7	**0.08 (0.04–0.16)**	18.6	**0.14 (0.08–0.25)**
	60–69	799	7.5	**0.09 (0.04–0.18)**	15.0	**0.16 (0.08–0.31)**	16.4	**0.13 (0.07–0.22)**
	70–79	593	10.1	**0.11 (0.05–0.23)**	17.9	**0.18 (0.09–0.35)**	20.4	**0.15 (0.09–0.26)**
Geographic region	North-east	1,505	6.6	Ref	9.1	Ref	16.0	Ref
	Central	1,122	7.1	1.06 (0.69–1.63)	9.9	1.06 (0.72–1.55)	16.3	0.97 (0.72–1.31)
	North-west	1,527	5.1	0.93 (0.67–1.30)	7.0	0.91 (0.68–1.21)	14.7	1.03 (0.83–1.27)
	South-west	1,125	5.0	**0.62 (0.42–0.92)**	8.0	0.82 (0.59–1.15)	15.9	0.93 (0.72–1.19)
	South-east	1,107	5.7	0.83-0.58–1.19)	8.5	0.87 (0.64–1.19)	16.8	1.05 (0.83–1.33)
Degree of urbanisation	Very high	1,399	5.2	Ref	6.5	Ref	14.4	Ref
	High	2,848	6.1	0.86 (0.62–1.19)	8.3	1.06 (0.79–1.41)	15.5	0.92 (0.75–1.13)
	Moderately high	804	6.0	0.74 (0.49–1.13)	10.3	1.20 (0.85–1.70)	18.7	1.04 (0.81–1.35)
	Low	589	7.3	0.79 (0.46–1.36)	10.3	1.07 (0.66–1.74)	16.3	0.87 (0.60–1.27)
	Very low	746	5.4	0.63 (0.40–1.00)	9.1	0.99 (0.66–1.48)	16.5	0.89 (0.66–1.19)
Migrant status	Dutch citizens and Western immigrants	5,317	6.6	Ref	9.5	Ref	17.3	Ref
	Non-Western immigrants	1,069	2.4	**0.42 (0.26–0.67)**	3.0	**0.47 (0.31–0.71)**	8.7	**0.47 (0.35–0.62)**
Educational level[a]	Low	730	4.3	Ref	7.5	Ref	12.3	Ref
	Medium	3,138	6.4	**1.57 (1.03–2.40)**	10.0	**1.48 (1.06–2.08)**	16.0	1.21 (0.93–1.57)
	High	2,403	5.9	**1.70 (1.09–2.67)**	6.8	1.26 (0.87–1.82)	16.8	**1.51 (1.14–2.00)**
	Unknown	115	1.7	0.51 (0.12–2.20)	7.0	1.25 (0.55–2.82)	13.9	1.23 (0.68–2.24)
Extent of vaccination refusal according to religious views	None or minor	6,253	5.7	Ref	8.3	Ref	15.6	Ref
	Moderate to strong	133	15.0	**2.86 (1.66–4.91)**	16.5	**1.97 (1.17–3.31)**	27.1	**1.79 (1.18–2.72)**
Duration in years between last polio-containing vaccination and blood sampling	0	503	10.7	Ref	11.1	Ref	12.9	Ref
	1–3	1,201	3.3	1.46 (0.80–2.69)	3.3	1.16 (0.64–2.10)	13.6	**2.86 (1.84–4.44)**
	4–9	946	1.6	1.60 (0.68–3.77)	1.1	0.80 (0.32–1.99)	8.0	**3.69 (2.17–6.26)**
	10–20	735	0.1	0.19 (0.02–1.54)	1.1	0.96 (0.36–2.57)	7.8	**5.25 (2.93–9.39)**
	21–30	407	3.0	**4.47 (1.70–11.78)**	6.1	**5.97 (2.59–26.58)**	12.0	**7.24 (3.87–13.52)**
	>31	264	11.0	**9.31 (4.13–21.00)**	17.1	**12.54 (5.92–26.58)**	31.8	**18.75 (10.42–33.74)**
	Unknown	103	1.0	0.48 (0.06–3.84)	1.9	0.83 (0.19–3.76)	13.6	4.38 (2.11–9.12)
	Not vaccinated	2,227	10.1	**13.58 (5.09–36.24)**	15.9	**5.28 (2.46–11.31)**	22.7	**21.44 (11.18–41.10)**
Number of polio antigen-containing vaccinations	6 (completed NIP)	1,498	1.3	Ref	2.6	Ref	8.6	Ref
	0–1	2,592	9.0	1.29 (0.47–3.55)	14.5	1.95 (0.99–3.85)	20.3	0.85 (0.49–1.47)
	2–5	1,900	6.3	**2.63 (1.31–5.28)**	6.2	1.04 (0.61–1.80)	17.6	**1.61 (1.15–2.26)**
	6, including single IPV or OPV	68	4.4	1.86 (0.47–7.31)	4.4	0.48 (0.13–1.74)	16.2	1.19 (0.56–2.53)
	≥7	328	0.6	0.61 (0.14–2.75)	1.5	0.67 (0.25–1.81)	4.0	0.59 (0.32–1.10)
Travelling to high-risk regions[b]	No	3,956	7.7	Ref	11.5	Ref	20.0	Ref
	Yes	2,430	2.9	**0.61 (0.46–0.82)**	3.5	**0.44 (0.34–0.58)**	9.1	**0.57 (0.47–0.68)**

CI: confidence interval; IPV: inactivated polio vaccine; NIP: National Immunisation Programme; NT: neutralisation test; OPV: oral polio vaccine; OR: odds ratio.

[a] For children younger than 14 years, the mothers' higher educational level was recorded. Low: no education or only primary school; medium: junior technical school, lower general, or intermediate vocational secondary schooling; high: higher vocational, higher general secondary, pre-university, or university schooling.

[b] Asia, Africa, or South and Middle America.

Statistically significant results are indicated by bold font.

Compared with the first survey, seroprevalence rates in orthodox protestant individuals were even lower in this second survey [19]. Since 1993, no notifications of poliomyelitis have been reported in the Netherlands. In addition, nationwide laboratory and environmental surveillance yielded no signal of poliovirus circulation, so it can be assumed that no boosting opportunities with wild-type poliovirus have occurred in the Netherlands since 1993 [9,11]. It is well known that vaccine-derived polioviruses originating from OPV vaccines represent a possible route of disease introduction into communities without established seroprotection. This threat is especially applicable to communities with close socio-geographical clustering, such as the Dutch orthodox protestants.

The current study also found that individuals who had received their last IPV-containing vaccination more than 20 years before sampling, were at increased risk of having antibody levels below the protective threshold. However, Abbink et al. previously demonstrated memory immunity against poliomyelitis in their study of 400 elderly people who were ineligible for vaccination but who were likely to have encountered wild-type infection earlier in life [29]. Assuming that vaccination-induced immunity is as effective as natural immunity suggests that adequate vaccination strategies (including initial and booster doses) will produce sufficient and long-term protection.

Another intriguing finding of the current study is that the non-Western immigrant cohort had higher seroprevalence rates and higher mean $\log^2$ titres than the cohort of Dutch citizens and Western immigrants. In contrast, several other studies found no differences [30,31]. It is possible that the differential results reflect differences in study populations, including variations in age, country of birth, national vaccination strategies (i.e. vaccine type and schedule), and previous exposure.

In the current study, individuals travelling to Asia, Africa, or Central and South America also showed higher seroprevalence rates than those who did not travel to these continents. Indeed, the dT-IPV vaccination is strongly recommended by travel medicine physicians [32]. Assuming that many travellers decide to get this vaccination before travelling, it is possible that the number of polio-antigen containing vaccinations reported in the Dutch NIP registers collected in this study does not accurately reflect the total number of vaccinations an individual has received. In an attempt to address this potential limitation of the current study design, the study participants were requested to provide their personal vaccination booklets that are kept for such travel purposes; however, we cannot exclude that people may have forgotten to bring this certificate.

Finally, the current study found that higher educational level (of the individual or female parent/guardian of a child) was associated with an increased risk for non-protective antibody levels. This finding seems contradictory to the mass of worldwide studies that have shown higher education to be associated with increased healthcare and higher rates of compliance with health-related policies. In particular, a previous analysis of the Dutch NIP questionnaire indicated that lower socio-economic status (which is generally associated with lower education) was associated with lower participation [33]. Two other studies, assessing the uptake of human papilloma virus vaccination and NIP vaccinations in general, reported a similar link between lower socio-economic status and lower vaccination compliance. Thus, the finding from the current study must be investigated in future studies to determine its validity or underlying causes.

The current study design, using data from both a questionnaire and blood sampling of a large random sample of Dutch inhabitants, affords the possibility to extrapolate results to the general population and to assess the potential risk factors of decreased seroprotection. However, any self-reported data (such as vaccination history) carries a risk of being incomplete or incorrect, and may negatively impact study findings [34]. In addition, the current results may have been impacted by assay-related limitations; for example, the neutralisation test used here and in most seroprevalence studies is recommended by the WHO as the gold standard for detecting viral serotypes, but may yield false positives or negatives and preclude direct comparison between laboratories or between studies [35,36]. However, in the current study, the testing of the nearly 8,000 samples was carried out over a period of one year, which may have helped limit the potential influence of day-by-day variability in the testing method and its results. Moreover, an in-house control serum, calibrated to the WHO standard, was included on each test plate. These technical strategies were carried out to help strengthen the results' representation of the current status of immunity against poliomyelitis in the Netherlands.

In conclusion, age-related variations in seroprevalence rates were found in the NS that were related to the Dutch NIP against poliomyelitis. The lowest rates were found in children younger than 14 months, who would be eligible for a maximum of four IPV doses, and the highest rates were found in five year-old children, who would have received five polio-antigen containing vaccinations according to the NIP strategy. Completion of the NIP vaccination series (including all six initial and booster doses) was associated with high and long-lasting seroprotection. In the general population, seroprevalence rates were above the threshold necessary for prevention of poliovirus transmission, estimated at 82–87% given the condition of homogeneous mixing [37], with the exception of one month-old infants and children between five and 10 months of age. Furthermore, the orthodox protestant community remains at high risk due to their refusal of vaccination. Thus, it is important to continue efforts to increase coverage among this population, and

improved approaches should be designed with respect to the particular religious arguments that underlie non-compliance with a vaccination programme [38,39]. Finally, conducting updated nationwide serosurveys is important to monitor the effects of the recent extended period (since 1993) without wild-type virus challenges, and to assess the efficacy of changes in the immunisation schedule.

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Conflict of interest

None declared

Authors' contributions

Nicoline van der Maas, Liesbeth Mollema and Hester de Melker have done the main data analysis. All authors contributed significantly to additional data analyses and to the preparation of the manuscript.

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Silent reintroduction of wild-type poliovirus to Israel, 2013 – risk communication challenges in an argumentative atmosphere

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Israel has been certified as polio-free by the World Health Organization and its routine immunisation schedule consists of inactivated poliovirus vaccine (IPV) only. At the end of May 2013, the Israeli Ministry of Health (MOH) has confirmed the reintroduction of wild-type poliovirus 1 into the country. Documented ongoing human-to-human transmission necessitated a thorough risk assessment followed by a supplemental immunisation campaign using oral polio vaccine (OPV). The unusual situation in which ongoing poliovirus transmission was picked up through an early warning system of sewage monitoring without active polio cases, brought about significant challenges in risk communication. This paper reviews the challenges faced by the MOH and the communication strategy devised, in order to facilitate and optimise the various components of the public health response, particularly vaccination. Lessons learned from our recent experience may inform risk communication approaches in other countries that may face a similar situation as global polio eradication moves towards the 'End game'.

Background

Israel has been certified as free of poliovirus by the World Health Organization (WHO) along with the entire WHO European Region in 2002 and has been using an inactivated polio vaccine (IPV)-only immunisation strategy since 2005, as most European Union (EU) countries do.

At the end of May 2013, the Israeli Ministry of Health (MoH) confirmed the reintroduction of wild-type poliovirus 1 (WPV1) into Israel following its detection in routine environmental surveillance of poliovirus in the sewage system near the town of Rahat, southern Israel. Enhanced surveillance for clinical cases did not detect any cases of paralytic poliomyelitis, most likely due to high vaccine coverage rate of the population, above 95% before WPV1 has first been detected at end of May, and above 98% after the inactivated poliovirus vaccine (IPV) catch-up campaign that was undertaken during June and July, evident from the computerised National Vaccination Registry [1]. On 5 August, following thorough epidemiological and virological investigation and the recommendation of an invited WHO mission, the MoH decided to conduct a supplemental immunisation activity (SIA) to vaccinate all children aged from 0 to 9 years in the southern region of the country (Southern District) who had not received oral polio vaccine (OPV) in the past, with a bivalent oral polio vaccine (bOPV). Two weeks later, following continuous circulation of WPV1, the SIA was extended to cover the entire country [1].

This has been a unique and unprecedented situation and a public health challenge within the global polio eradication efforts. To our knowledge, this has been the first case of detection of WPV by an early warning system of environmental surveillance and not through presentation of polio cases, into a 'polio-free' country that uses 'IPV only' as its routine vaccination regimen [2]. This special situation has made the decision to deploy an SIA with OPV complex in terms of risk communication. This paper reviews the challenges faced by the MoH and the communication strategy formulated in order to facilitate and optimise various components of public health response, particularly vaccination, and lessons learned at the national level.

Risk communication challenges

From the onset, it became clear that risk communication will play a crucial role in building trust among the public and professional stakeholders. Therefore, the Ministry's communication and media experts were full members of the national outbreak control team. The

MoH identified challenges related to risk communication as:

- communicating the risk associated with a silently circulating WPV1 in a highly immunised community;
- communicating the decision and the rationale behind it (need and urgency) for public health action, particularly an SIA, despite the lack of paralytic polio cases;
- communicating the advantage of an SIA using OPV to halt WPV1 circulation, in a community already highly immunised with IPV;
- communicating the benefits of an SIA using OPV taking into consideration individual (prevention of viral shedding), public (eradication of the virus and protection of contacts) and global (polio eradication) health aspects;
- communicating the safety and risks of OPV in general and bOPV in particular;
- communicating the rationale of reintroducing OPV after its use was stopped in 2005.

Communication strategy during early phase of the investigation

In the WHO published guidelines for risk communication in an outbreak situation [3,4], the basic principles are trust, early announcement, transparency, 'the public' and planning. The first three components are difficult to separate. The main goal of communication management is building and maintaining public trust. Routine childhood vaccination programmes in Israel are not obligatory and are generally perceived as having a good reputation, as reflected by the high rate of coverage for all vaccines (over 95%) (unpublished data).

An important tool in maintaining trust is transparency to avoid potential accusations of concealing information by decision makers. This requires that the public receives full information from an official health authority. In accordance with this approach, within one day, after the confirmation of WPV1 circulation in the country, a report using information available to the MoH was made public [5].

The rationale underlying early announcement was to provide information to the media and establish the MoH as the most reliable source while aiming at preventing media reports of rumours and speculations from emerging and spreading, especially via social media [6].

Similarly, the media and the public were informed about any new data generated from environmental surveillance and population surveys and about subsequent public health response, in a timely manner. Except for one instance where preliminary laboratory results were leaked to the media, all information held exclusively by the MoH, was made public via official media briefings. To prevent a situation where the public receives information before healthcare professionals, a major effort was undertaken to ensure that all briefing materials intended for both professionals and lay public were prepared simultaneously and distributed first to healthcare professionals and MoH employees and immediately afterwards to the public.

Communication strategy in preparation for the supplemental immunisation activity

Following consultations with WHO experts during their mission to Israel, preparations for a SIA began as part of a coordinated public health response plan. An integral part of preparedness included a comprehensive communication plan. The Government Advertising Bureau was recruited for this task and a decision was made to design two campaigns – one that would focus on hygiene and be launched before starting the SIA, and another one that would focus on vaccination. As a complementary measure, the MoH sought counselling from a commercial strategic consulting firm to prepare the communication plan and to design key messages for the public. Moreover, the firm was asked to advise on management and monitoring of communication in electronic media, in particular social networks, that were identified during the 2009 influenza A(H1N1) pandemic as having an have an important impact on public opinion [7,8].

In the planning process, we identified a number of potentially critical points. The first and most important was to achieve consensus amongst the medical community regarding the need for a SIA [9]. During the 2009 pandemic in Israel, vaccination campaigns faced difficulties as a sizeable number of physicians did not support or opposed influenza vaccination [10]. In the presented incident, many opinion-leading physicians employed by health organisations outside the MoH, including paediatricians, family physicians, infectious disease and neurology consultants, were fully engaged in the MoH decision regarding vaccination. This was achieved by discussions and briefings of chairs of national medical societies, national medical councils and the Israeli Medical Association and information delivery to the entire medical community. Points of contact at the MoH Public Health Services were established to provide feedback and consultation to frontline physicians [11]. In weeks after the decision to deploy the SIA, dozens of conferences were held by MoH senior representatives in every hospital in the country and in every region. At the political level, the Israeli Government received periodic briefings and the Health Minister was engaged in key decisions. These measures achieved an almost end-to-end consensus amongst the medical community, to the point where local 'anti-vaccine' movements had to seek support from known anti-vaccine doctors in other countries e.g. in the United States and India [12].

A notable example of engagement was the reaction of primary care paediatricians in Israel. Paediatricians' attitudes were monitored by their inputs into the paediatric professional electronic network (IPRONET) that is used by 500 paediatricians across Israel and therefore

served as an indicator to their viewpoints. IPRONET communications as well as small focus groups held prior to the campaign revealed that most paediatricians declared that they were neither going to recommend OPV to their patients, nor vaccinate their own children, due to the perceived low risk-benefit ratio of OPV. This was mitigated by seven informal information papers and daily responses to frequently asked questions (FAQ) by senior paediatricians in their network during the SIA together with direct formal email briefings from the MoH regarding the status of WPV1 circulation and rationale for the SIA, using email contact lists provided by medical organisations and associations. Continuous monitoring of network postings by the site coordinators documented a gradual shift from scepticism to enormous support of the campaign (E Somekh, personal communication, 15 August 2013).

The second critical point was that ideological anti-vaccination groups were expected to take advantage of the situation and attack vaccines in general and the 'new' polio vaccination with bOPV in particular. It was decided not to confront those movements head-on, but to prevent them from negatively influencing the general public as at that point in time, only 55% of parents said they would vaccinate their children against polio although 80% said they do believe an MoH action is needed (Government Advertising Bureau commissioned survey, Y Amikam, personal communication, 10 July 2013). Indeed, even before the campaign was launched, antivaccinists started advocating against it, especially via electronic media.

The third point was the rationale for the SIA using OPV was complicated to deliver and it was even harder to motivate the public to take action and get vaccinated. An SIA had also been carried out in the neighbouring Egypt a few months earlier due to WPV circulation detected through environmental surveillance. However, a profound difference was that Egypt has OPV in routine childhood immunisation programme [13]. The idea of reintroducing a live attenuated vaccine that had been abandoned nearly a decade ago was difficult to understand.

Not only did the fact that there were no paralytic cases make the decision to launch a SIA difficult for policy makers, it certainly also altered the perception of risk among the public. In this respect, the SIA seemed to have become a 'victim' of the success of the environmental surveillance, as much as vaccination has become a victim of its own success [14].

The fact that OPV was to be given to children who were already vaccinated with IPV and hence, protected from paralysis in the event of exposure to WPV1, caused much hesitation amongst parents who subsequently decided not to pursue vaccination with OPV due to the perception of the risk for paralytic polio and risk associated with vaccine administration. Many parents felt that OPV was a 'social' vaccine that builds on herd immunity and compensates for the small percentage of the population that has not received IPV, and felt that administering OPV to their IPV-vaccinated children is merely altruistic. Mindful of that, the message to the public was that the vaccine will protect their family members and close friends and not just the individual or the 'environment' or 'society'. Accordingly, the main slogan of the campaign was 'Just two drops and the family is protected from the risk of polio' (Figure 1).

In the information era, the number of sources of information is immense and as a result, the MoH had to interact or be present in all of them in order to deliver valid information and ensure accessibility of the public to information. The use of a variety of communication measures is an important principle in risk communication plans [8]. Examples of communication channels used by the MoH in the incident under discussion included (i) a new designated official polio MoH website, (ii) an existing official Facebook interface, (iii) an existing national call centre involving over 20 of MoH staff for several weeks reinforcing polio communications (agreed key messages and answering FAQ), (iv) print media (national and sectorial in various languages) and (v) electronic journalism, including, for the first time, various social networks, forums and blogs. Israel has several communities whose first language is not Hebrew but e.g. Arabic, Russian and Tigrigna and information was provided in all common languages spoken in Israel. The relationship and the flow of information between the elements of communication are described in Figure 2.

Figure 1

Campaign slogan for supplemental immunisation activity with bivalent oral polio vaccine during silent reintroduction of wild-type poliovirus 1, Israel, 2013

Communication strategy during the SIA

The SIA was carried out in two phases. First it started in the Southern District which appeared as the epicentre of WPV1 introduction. After two weeks, following ongoing WPV1 activity, the full national phase was launched.

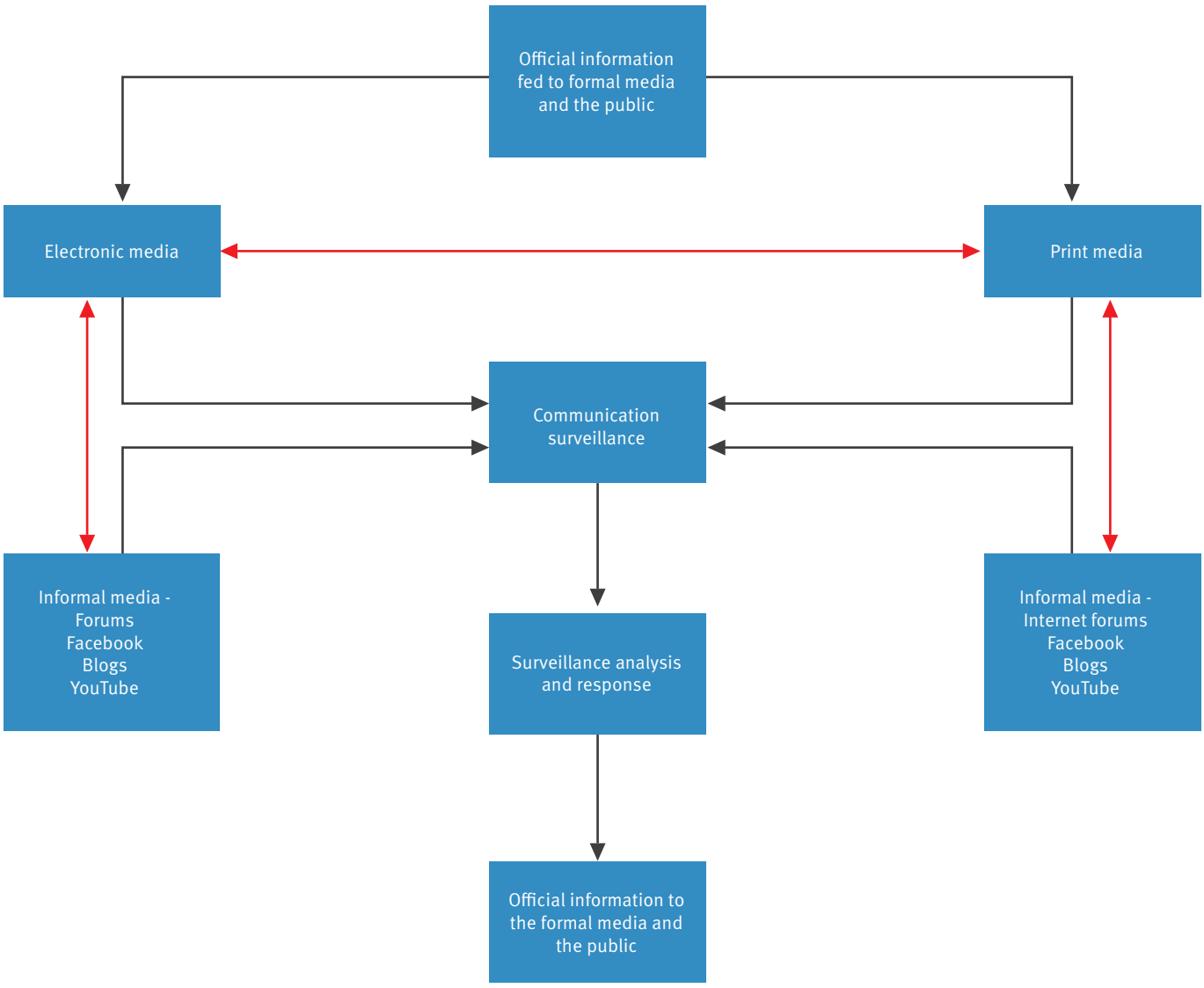
There has been an obvious increase in Internet use for gathering information compared to similar events in the past. Browsing activity monitoring of the official MoH polio campaign website is described in the Table. Notably, most browsing activities were up to three minutes long, and involved single page views ('bounces') in over half of cases.

The MoH decided on a massive and continuous presence of public health professionals who had undergone ad hoc training to answer questions, eliminate ambiguity and rectify disinformation through online and traditional media. This approach proved effective in motivating people to comply with medical recommendations as previously described [15].

One of the problems that arose from deployment of multiple responders, however, was occasional lack of uniformity in messages. Rare cases of contradicting messages (almost always concerning minor issues) were picked up by vaccination opponents and used for attacking the campaign and questioning the MoH credibility. Frequent briefing of professional responders aimed at preventing such situations, but uniform messaging remains a challenge for management of future incidents. By the time the SIA was launched, the continuous sharing of information and transparency had harnessed almost all health journalists to support the SIA and its objectives.

Figure 2

Information flow during silent reintroduction of wild-type poliovirus 1 and supplemental immunisation activity with bivalent oral polio vaccine, Israel, 2013



During the campaign, public figures were recruited to support it, including members of the Knesset, the Israeli parliament, and the president of Israel. MoH physicians were photographed while vaccinating their family members as reassurance to inquiries from members of the public who questioned whether health professionals practise what they preach. At the individual level, phone calls and SMS messages to parents who did not vaccinate their children were used to provide information as needed.

Monitoring of the media was performed throughout the SIA in order to identify the 'hot' issues in the public debates and comment on them e.g. vaccine safety, necessity, side effects, etc. A significant decrease in public interest was observed over time due to 'fatigue' from the 'polio issue', summer leave and Jewish High Holidays in September, as well as regional security concerns. Despite that 'fatigue', fundamental vaccine opponents continued to object to the SIA mostly through certain known anti-vaccine websites and in an active approach MoH responders started conversations over the Internet.

As mentioned above, one of the 'hot' issues was vaccine safety. The public questioned many aspects of OPV such as clinical trial data and evidence base, data from previous OPV use and the risk of vaccine associated paralytic polio (VAPP). One of the arguments of the vaccine opponents was that the MoH yielded to international pressure from WHO and other international health authorities as part of a global campaign and not a local need. The risk perception of the vaccine was enhanced and public anxiety was directed towards VAPP rather than the risk from WPV1 circulation. In order of mitigate those concerns, data from post marketing safety surveillance, the package insert of the vaccine, laboratory testing results of the specific bOPV lots used and clinical trials done with the vaccine were published on the official MoH website and questions in social media answered by a dedicated MoH specialist.

Table

Number of entries to Ministry of Health campaign website 'Just two drops'[a] by topics, Israel, 4 August–16 September 2013

Web page	Visitors (number)
Main homepage	277,290
Poliovirus – general information	94,218
Frequently asked questions (FAQ)	194,897
Vaccination centres (map, addresses, working hours)	170,646
The disease and the vaccine	28,216
Latest updates	19,604
Guidelines for immunocompromised patients[b]	7,873
Hygiene and hand washing	5,799
Vaccination coverage[b]	11,091
Spokesman announcements	14,745
Routine childhood vaccines	9,263
Routine childhood vaccines (in detail)	11,190
Total	**844,832**

[a] URL: http://www.health.gov.il/English/Topics/Vaccination/two_drops/Pages/default.aspx.

[b] Launched on 20 August 2013.

Lessons learned from polio communication strategy

By mid-October, more than 900,000 children of 1.2 million candidates were vaccinated. Seemingly, this coverage rate had a favourable epidemiological effect - as judged by the dramatic reduction in prevalence of WPV1 detected during environmental surveillance and field surveys - but was it successful from a risk communication point of view? How can health authorities improve compliance during SIAs?

First, health authorities need to assess their methods of communication before and during an outbreak of infectious disease [16]. This is certainly true in a major event that lacks concurrent morbidity. Risk communication in relation to vaccine-preventable diseases (VPDs) involves certain unique aspects, especially those related to the safety, efficacy and effectiveness of vaccination, and social factors such as anti-vaccination movements, conspiracy theories and ethical discourse surrounding equality, autonomy and mutual guarantee. The current silent transmission of the poliovirus involved communication challenges related to VPDs in general, but also specific challenges related to public health policy built on early warning signals, perception of disease risk in a vaccinated population and reintroduction of a 'forgotten' pathogen and vaccine.

Second, the importance of the Internet as key media was acknowledged and emphasised in this campaign. However, one cannot abandon traditional media since not everyone has Internet access. Billboards, radio, television and traditional mail messages have proven particularly useful among specific sectors with limited Internet use. This finding is in line with results obtained in a recent EU-wide opinion poll (Eurobarometer), suggesting that television is the main source of information on developments in science and technology, followed by newspapers and websites [17]. Another example is the use of the MoH call centre by the public which reflected public interest and concern over time (Figure 3).

Third, reliable information on vaccines, should preferably be made available routinely and not only when an SIA is contemplated. Such pro-vaccine sites may include narratives and stories of patients or family members thereof who fell ill with VPDs in order to have more impact. It is important to work on the image of the health authority as a credible source of information and judicious decision maker, throughout the year, so that the level of trust in the information provided will be high during public health emergencies.

Health communication interventions can be strengthened through the adoption of a more holistic ecological model of people and their health-related behaviours analysed in the context of larger social, economic, political, and cultural forces [18] .Local stakeholders demand local content for their information feed and messages from a trusted local leader are the most superior forms of communication [19]. Israel is characterised by a heterogeneous population, consisting of social, ethnic or religious communities with distinctive cultural characteristics, occasionally involving sectorial decision making processes. Such communities include the Arabic minority, ultra-orthodox Jews, Ethiopian ethnic group and migrant workers from Africa lacking official status in Israel. For each group, well-phrased messages were delivered taking into account cultural and linguistic barriers as well as direct approach to ensure engagement of community leaders. This attitude proved useful in that vaccine coverage in some of these groups was higher than the coverage in the general population (unpublished data). It is worth mentioning that those communities are far less influenced by the Internet and electronic media.

Many questions arise but still remain unanswered. As public objection to vaccination campaigns as discussed above might increase in the future, there needs to be more engagement of stakeholders in public health decision-making processes. The question is where to draw the line? Is it appropriate to involve non-professionals in a decision making process that is fundamentally professional? Will inclusion of antivaccinist group representatives in the process soften their opposition or just feed information that could be used against vaccination?

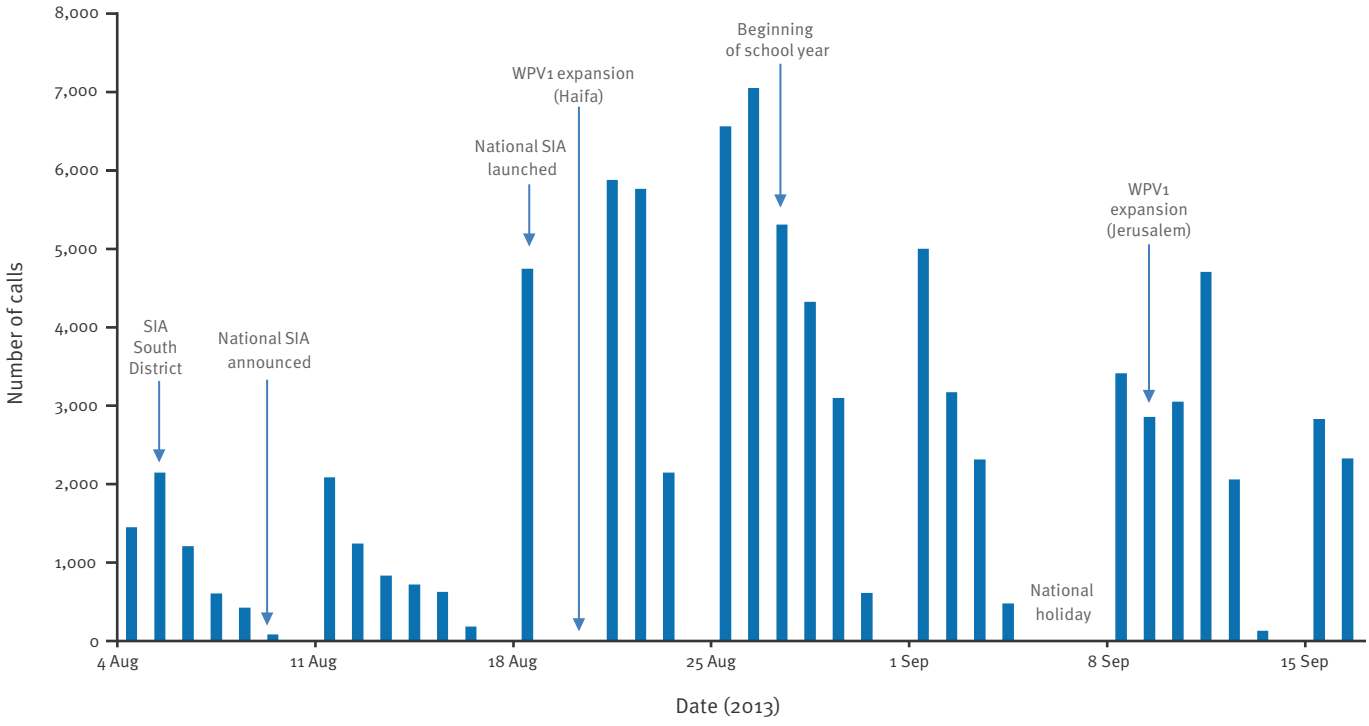
The current campaign, by choice, hardly used negative messages about the risks form the WPV1, although there were voices suggesting it. Is such 'intimidation' a helpful communication technique when risk perception is distorted in parts of the public?

Can there be too much information in public health? Although much information was delivered throughout the event, unprecedented in volume and in number of media channels involved from an Israeli perspective, it did not stop vaccine opponents and other groups to claim that there was not enough information available'. Noteworthy is that this claim was rejected by the Supreme Court of Justice who dealt with a petition filed by antivaccinists against the current polio SIA. Too much information may cause confusion and misunderstanding, especially when messages are complex. The right 'dose' of information is therefore arguable. One example is the paradoxical effect that messages relating to the risk of vaccine safety and efficacy may cause [3].

Routine communication management, health authorities' general image and availability to the public,

Figure 3

Number of calls to call centre and key events during silent reintroduction of wild-type poliovirus 1 and supplemental immunisation activity with bivalent oral polio vaccine, Israel , 4 August–15 September 2013



SIA: supplemental immunisation activity; WPV1: wild-type poliovirus 1.

affects the risk communication in any public health challenge. According to surveys carried out in Israel during the current incident, the level of public trust in the MoH was high and increased over time. A survey conducted by Government Advertising Bureau in early July 2013, found that 75% of respondents thought that the MoH handled the event properly (Y Amikam, personal communication, 10 July 2013). One month later (just before the SIA was launched), this figure rose to 79%.

Interestingly, the links to anti-vaccine sites in the Internet still appear higher up than the MoH official site in Google searches when typing the word 'polio'. This is exemplified by antivaccinist and MoH 'YouTube' movies ranking 1st and 10th in Hebrew Google search of 'polio', respectively, despite the fact that the MoH movie has gained over 200,000 views as compared to only 25,000 for the anti-vaccine movie. Overall, this emphasises the continuous challenge health authorities are facing, even when risk communication is planned thoroughly and performed systematically.

More research that identifies improved methods for communication and sharing of information between public health and healthcare professionals is needed [10]. Communication with professional staff at all levels is critical, and requires planning and sufficient time intervals between transmitting information to professionals and the public, to keep professionals up-to-date in parallel to the process of feeding information to the media. Methods for generating uniform and consistent messages, especially when a large number of responders from various disciplines and organisations are involved, also deserve study.

One limitation of our analysis of risk communication is related to its qualitative nature and the fact that exact quantitative measurements and monitoring in an unexpected and untoward situation may be difficult, especially when most resources are invested in response and monitoring other aspects such as clinical and environmental status.

In conclusion, the Israeli MoH has devised a comprehensive communication strategy in order to facilitate and optimise the various components of the public health response to reintroduction of WPV1 into Israel, particularly the SIA. Lessons learned from our recent experience may inform risk communication approaches in other countries that may face similar situations as global polio eradication moves towards the 'End game'.

Authors' contributions

All authors made substantial contributions to conception, design, acquisition and analysis of data. All authors participated in drafting the article or revising it critically for important intellectual content.

Conflict of interest

None declared.

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