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 circulating WPV1-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 2×10^8 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 (Waltham, 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'-TACGTGATTTCCACACTGA-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 Dreier 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 (2×10^8 pfu/mL) of extracted WPV1-SOAS RNA in H₂O 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 (2×10^8 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 (2×10^8 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 WPV1-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 2×10^8 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 2×10^8 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 r^2 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 circulating strain 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. Danit Sofer 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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