AMERICAN SOCIETY FOR MICROBIOLOGY | Microbiology Spectrum

| Public Health | Research Article

Advancing poliovirus eradication: lessons learned from piloting direct molecular detection of polioviruses in high-risk and priority geographies

Paula L. Marcet,[1] Brandon Short,[1] Ashley Deas,[2] Hong Sun,[1] Chelsea Harrington,[1] Shahzad Shaukat,[3] Muhammad Masroor Alam,[4] Marycelin Baba,[5] Adedayo Faneye,[6] Prossy Namuwulya,[7] Lea Necitas Apostol,[8] Tamer Elshaarawy,[9] John Kofi Odoom,[10] Peter Borus,[11] Shelina Moonsamy,[12] Yogolelo Riziki,[13] Marie Claire Endegue Zanga,[14] Mesfin Tefera,[15] Anfumbom K. W. Kfutwah,[16] Salmaan Sharif,[17] Varja Grabovac,[18] Cara C. Burns,[1] Nancy Gerloff[1]

AUTHOR AFFILIATIONS See affiliation list on p. 11.

ABSTRACT In the Global Polio Laboratory Network (GPLN), poliovirus (PV) screening results from acute flaccid paralysis (AFP) surveillance is based on virus isolation (VI) through cell culture, entailing long turnaround times and the amplification of live poliovirus. An alternative Direct Detection strategy (DD-ITD) for screening viral nucleic acid from stools, bypassing the need for virus culture, has been developed and extensively validated by GPLN partners. A multi-laboratory demonstration project was conceived to field-test the DD-ITD method by GPLN laboratories from the WHO African, Western Pacific, and Eastern Mediterranean regions, where wild serotype 1 or vaccine-derived polioviruses still circulate. Strategically selected laboratories were tasked to simultaneously process stool suspensions with the current gold-standard VI method and the new DD-ITD strategy. Results from 12 laboratories were compiled and analyzed to assess the quality of each RNA extraction and rRT-PCR run. Matched results for both methods of over 10,500 specimens showed an overall method agreement of 91%. All laboratories detected more PV presumptive positive samples with the DD-ITD strategy than with VI, but a large proportion of DD-ITD positive results (72%) were inconclusive or non-typeable, requiring confirmation through sequencing. A total of 298 (2.8%) samples were PV positive using both methods, 828 (7.9%) positive only for DD-ITD, and 62 (0.6%) positive only with VI. The DD-ITD overall performance, quality of results, and agreement between method results varied significantly across participating laboratories. DD-ITD implementation would entail building proficiency in advanced molecular laboratory techniques and data analysis, and increased demand for confirmatory sequencing.

IMPORTANCE Surveillance of acute flaccid paralysis (AFP) and sensitive poliovirus detection are key components of the WHO Global Polio Eradication Strategy. This work summarizes the results of a multi-laboratory evaluation designed to field-test the performance and applicability of a molecular Direct Detection strategy (DD-ITD) that does not require amplification of live poliovirus. AFP samples were processed in parallel with both the DD-ITD and the current gold-standard PV detection methodology, based on virus isolation (VI) through cell culture. All participating laboratories detected more PV presumptive positive samples using the DD-ITD strategy than with virus isolation methodology, although a higher proportion of DD-ITD results required confirmatory sequencing. Significant variability among laboratories was observed in the quality of the results and overall DD-ITD performance. Implementing DD-ITD would entail building proficiency in advanced molecular laboratory techniques and strengthening data analysis skills.

Editor Gabriel I. Parra, U.S. Food and Drug Administration, Silver Spring, Maryland, USA

Address correspondence to Nancy Gerloff, NGerloff@cdc.gov.

The authors declare no conflict of interest.

Received 12 September 2024
Accepted 11 November 2024
Published 12 December 2024

KEYWORDS nucleic acid extraction, poliovirus eradication, acute flaccid paralysis, surveillance, direct detection, real-time RT-PCR, poliovirus

Since the launching of the Global Polio Eradication Initiative (GPEI) in 1988, sustained investment and efforts allowed major achievements in interrupting and reducing poliovirus (PV) transmission, and eradication is closer than ever before (1, 2). Sensitive global surveillance of acute flaccid paralysis (AFP) cases is key to achieve the interruption of PV transmission. AFP refers to weakness or loss of muscle function, typically affecting one or more limbs. AFP can have various causes, including viral infections (such as poliomyelitis caused by PV), nerve damage, and other neurological conditions. The Global Polio Laboratory Network (GPLN), established in 1990, consists of 144 World Health Organization (WHO) accredited polio diagnostic laboratories distributed across 92 countries (2, 3). These specialized polio laboratories screen stools collected from AFP cases following WHO-recommended procedures to detect and characterize polioviruses (4). PV detection from human stools is currently performed by virus isolation (VI) in two different cell lines and is considered the gold standard for GPLN laboratories (5, 6). This method is highly sensitive for detecting both polioviruses and non-polio enteroviruses (NPEVs); however, it is associated with time-sensitive methodological challenges. From the receipt of samples in the laboratory a minimum turnaround time of 6–10 days is required to complete the two consecutive cell passages to confirm a positive result, or 14 days to confirm a negative result. Positive isolates are referred to real-time RT-PCR screening and serotype confirmation which could take 2–3 days. An additional 7 days are required when samples are referred for sequence confirmation (i.e., wild serotype 1 [WPV1], or vaccine-derived polioviruses [VDPV]), which for non-sequencing laboratories could turn into months to obtain a confirmatory result because of delays due to shipments to sequencing laboratories. A decrease in turnaround times could accelerate the release of reportable results that would guide programmatic actions such as the declaration of an outbreak and supplemental immunization campaigns in target communities (7). Additional challenges of the current VI-based methodology, include the amplification of live poliovirus through cell culture, which involves the inherent risk of accidental propagation and/or PV reintroduction, jeopardizing containment and eradication efforts (8).

A long-term objective of the GPEI has been to increase the efficiency of the AFP surveillance testing approach, through direct molecular detection of poliovirus nucleic acid extracts from stools, bypassing the amplification of PV through cell culture without jeopardizing detection sensitivity. The GPLN follows a comprehensive multi-step process to assess any proposed new diagnostic methodology prior to becoming recommended for use in network laboratories. Any new method must prove non-inferior to the current gold standard methodology, be cost-effective, and be easily integrated into the existing testing algorithm. In addition, for a new method to be recommended, it must include a quality assessment and quality assurance (QA/QC) scheme, necessary training must be provided, and global implementation must be feasible in high-risk geographies in low- and middle-income countries (3, 9).

A direct detection method (DD-ITD), extensively validated at CDC in the Polio and Picornavirus Branch, a global specialized poliovirus laboratory, was piloted by several GPLN laboratories (10–12). The method utilizes the Quick-RNA Viral Kit for nucleic acid (NA) extraction (Zymo Research, Irvine, CA), followed by real-time RT-PCR screening to detect and type PV without virus isolation through cell culture (11, 13).

The main objective of this demonstration project was to (i) field-test the direct detection method (DD-ITD) in stools from AFP surveillance, (ii) assess the performance of DD-ITD in parallel with the gold standard VI methodology, and (iii) evaluate the applicability of the DD-ITD methodology for large-scale testing in diverse settings, including laboratories from challenging and high-risk priority countries. The outcomes of this project are discussed, highlighting the challenges and implications for the large-scale implementation of this methodology for poliovirus testing in AFP surveillance.

RESULTS

Training, procurement, and inclusion criteria

GPLN laboratories from three WHO regions (African, Western Pacific, and Eastern Mediterranean) where programmatically relevant polioviruses circulate, and high testing workloads occur, were selected to participate in this demo project (Fig. 1). Participating laboratories were accredited as laboratories and proficient for testing of AFP stool samples through the gold standard virus isolation methodology. Laboratories were also proficient in Intratypic Differentiation (ITD) RT-PCR procedures.

The CDC Polio Molecular Diagnostic Development team, GPLN WHO headquarters (HQ), and the WHO regional polio laboratory coordinators held a series of virtual trainings on DD-ITD methodology for selected laboratories from October 2021 to early months of 2022. Refresher trainings were conducted prior to implementation for some laboratories when required. GPLN WHO-HQ facilitated the procurement and shipment of reagents, equipment, and consumables to participating laboratories. The ITD PCR kits and enzymes used for molecular testing were made available to GPLN-accredited laboratories through the International Reagent Resource (IRR, CDC's Distributor for molecular testing reagents). Supplies and personnel availability in each laboratory determined the actual start date and sample processing capacity.

Data collection, curation, quality control, and validation

Participating laboratories started submitting results in February 2022 and continued until May 2024 (Fig. 1). Twelve laboratories submitted results independently, according to the availability of reagents and labor. The CDC Polio Molecular Diagnostic Development team verified the quality of each result by reviewing the run files for each RT-PCR assay

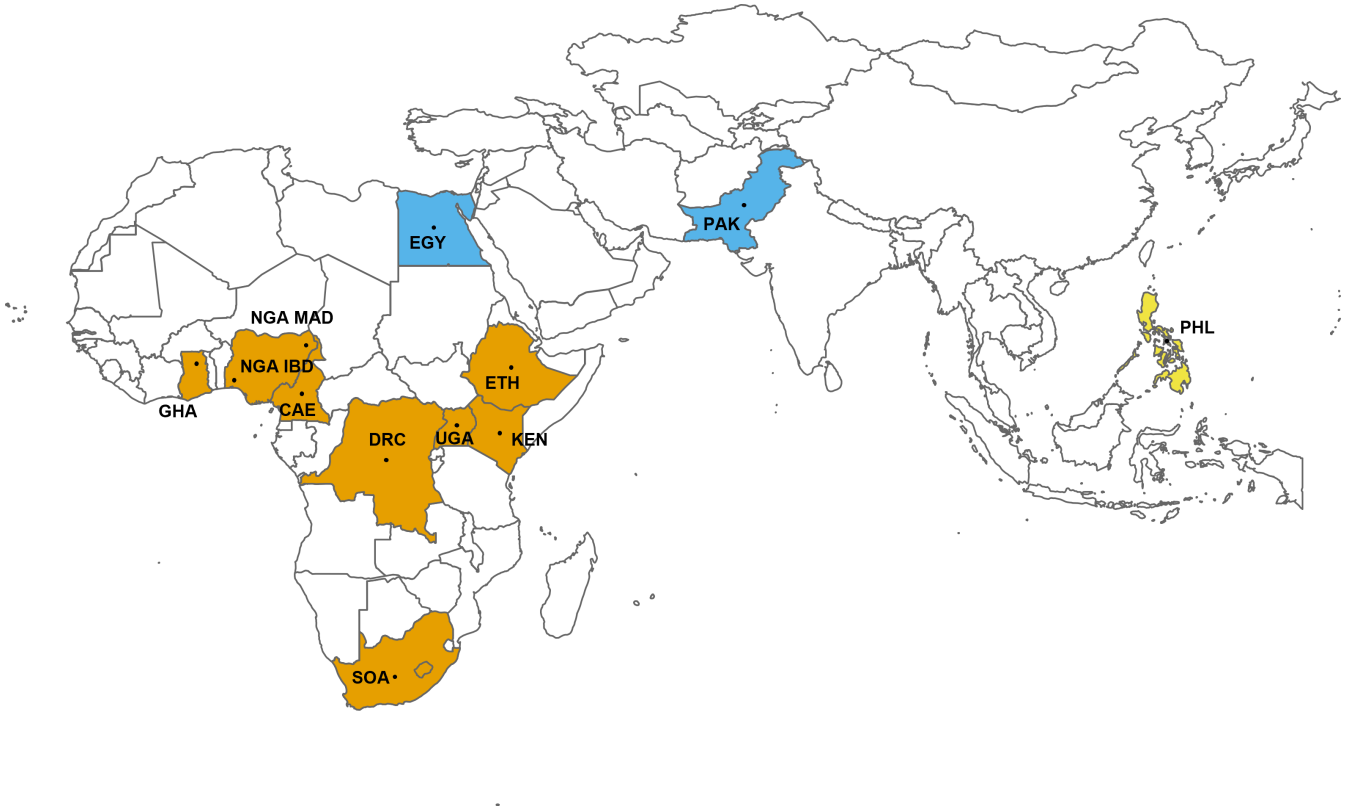

FIG 1 Geographical location of GPLN laboratories participating in the DD-ITD Demo project (February 2022–May 2024). Selected laboratories represent the African (*n* = 9, orange), Eastern Mediterranean (*n* = 2, blue), and Western-Pacific (*n* = 1, yellow) WHO regions. The map was generated in R using the ggmap package (14).

for each sample. The criteria for quality control (QC) for inclusion in subsequent data analysis was determined by Qβ Ct value cut-offs established for each sample and each extraction batch (detailed in methods).

Participating laboratories submitted 13,090 specimen results. However, 1,470 (11.3%) results did not meet the established QC standards and were therefore excluded for analysis (Fig. 2). Results for ITD amplification were matched by sample for both VI and DD-ITD methods, organized according to a standardized nomenclature, and consolidated into a unified database for all laboratories. This database includes RT-PCR results for each of the six ITD assays, comprising specimens, controls, and VI results. After completing the QA screening, the data set for DD-ITD included 11,620 valid specimen results, of which 10,517 had corresponding VI results (matched results, Table 1).

Comparison of VI and the DD-ITD PV screening methods

Results matched for both VI and DD-ITD methods showed an overall method agreement of 91.5%, with a significant difference in method performance (N = 10,517 specimens, McNemar's test, $P < 0.001$) (Table 1). In this data set, a higher proportion of presumptive PV-positive stools was obtained with the DD-ITD method (10.7%) compared to the VI method (3.4%). A total of 298 PV-positive samples were detected by both methods, while 828 (7.9%) were only positive through DD-ITD. The positivity rate of the non-polio-enterovirus (NPEV) obtained with the DD-ITD method in this data set (25.6%, n = 2693) was also higher compared to the VI method (15%, n = 1582).

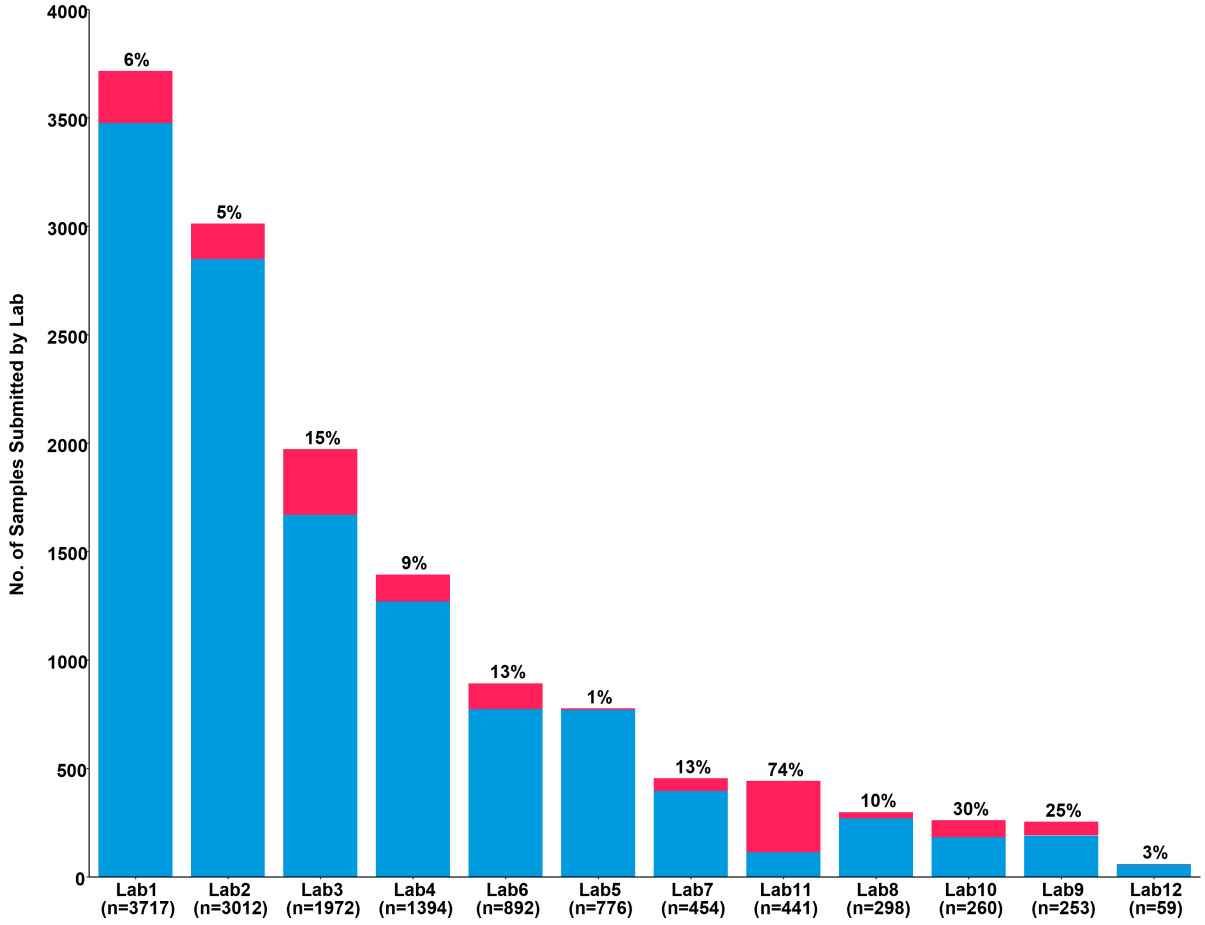

FIG 2 Number of samples submitted by participating laboratories throughout the DD-ITD Demo project. Total number of samples submitted per laboratory (n= # under lab ID), with the number of valid results in blue and rejected in red (results not meeting QA/QC criteria). The percentage of rejected results from the total submitted is shown on top of the bars.

TABLE 1 2 × 2 contingency table for method comparison showing the number of samples with poliovirus (PV) positive or negative results for each method[a]

	DD-ITD PV positive	DD-ITD PV negative	Totals
VI PV positive	298	62	360
VI PV negative	828	9,329	10,157
	1,126	9,391	10,517

[a]VI, Virus isolation; DD-ITD, Direct Direction-Intratypic differentiation.

Despite the higher number of presumptive PV positives obtained with DD-ITD, a total of 62 VI PV-positive samples were not detected by DD-ITD screening in this data set. These VI positive/DD-ITD-negative results represent 0.6% of the total sample analyzed (*n* = 10,517); but were 17.2% of the VI positive samples (*n* = 360). The DD-ITD presumptive poliovirus-positive results (*n* = 1,126) included results with a defined serotype (*n* = 319) but also inconclusive (*n* = 617) and non-typeable (*n* = 190). Among the 807 DD-ITD presumptive positive specimens, 97 were confirmed as PV-positive through VI.

Through the VI and subsequent ITD testing algorithm, 164 PV-positive specimens (1.6% from the total sample *N* = 10,517) would be referred for sequencing confirmation. Based on the DD-ITD testing algorithm, 946 PV-presumptive positive results, representing 9% of the samples tested would be referred for sequencing confirmation.

Variation among laboratories

Detection rates, serotype identification, and outcome in the method performance comparison varied among laboratories. Only one of the 12 participating laboratories met its sample target using the DD-ITD methodology, with less than 1% of data rejected during the submission period. The project's submission timeline was extended to over 2 years, during which only two laboratories achieved 100% of their target number of valid sample results, and a total of five laboratories submitted more than 50% of their target. Additionally, two other laboratories received training for DD-ITD but did not submit results, stating various logistical constraints.

Approximately 12% of the submitted DD-ITD results were rejected due to failure to meet established quality standards. The rejection rate varied widely among laboratories ranging from 1% to 74% (Fig. 2). The proportion of rejected results also varied across different batches submitted by each laboratory; in some instances, entire batches were rejected due to data quality issues. For some laboratories, repeating the ITD assay with RNA extract diluted 10-fold reduced the frequency of background signals and QB amplification anomalies, leading to the confirmation of initial inconclusive or questionable DD-ITD results as negative.

Method agreement was assessed for the six laboratories that submitted more than 600 valid matched results by calculating Cohen's Kappa (k) score for each laboratory (Table 2). The overall DD-ITD positivity rate (poliovirus presumptive positive) reported by each laboratory varied significantly, ranging from 2.3% to 18.6%. VI polio positivity rate

TABLE 2 Summary of valid results submitted by the laboratory, matched for both methods[a]

Submitting lab	valid matched results	Cohen's K	DD-VI concordant		DD-VI discordant		
			Negative	Positive	DD positive VI negative	DD negative VI positive	Total discordant n [%]
Lab1	2,662	0.26	2,142	97	397	26	423 [15.9%]
Lab2	2,476	**0.58**	2,295	77	92	12	104 [4.2%]
Lab3	1,665	0.17	1,521	15	125	4	129 [7.7%]
Lab4	1,248	0.33	1,127	26	92	3	95 [7.6%]
Lab5	743	**0.66**	725	9	8	1	9 [1.2%]
Lab6	693	0.35	648	10	33	2	35 [5.1%]

[a]Only laboratories with more than 600 valid matched results were considered for this comparison. The table shows the number of samples with concordant and discordant results between methods. Method agreement expressed by Cohen's κ score [K ≤ 0 no agreement; <0.20 slight agreement, 0.21–0.40 fair, 0.41–0.60 moderate (bolded), 0.61–0.80 substantial (bolded), and 0.81–1.0 near perfect agreement].

for the same laboratories ranged from 1.1% to 4.6%. The proportion of discordant results between the two methods varied among laboratories from 1.2% to 16%.

DISCUSSION

Effective AFP surveillance is a key component of the success of the GPEI. Assuring high-quality PV testing, maximizing sensitivity, and enhancing timeliness by reducing overall turnaround times (i.e., from sample receipt in the laboratory to obtaining a reportable result) are key priorities for the Global Polio Laboratory Network. A long-standing objective of GPEI has been to replace the traditional virus isolation method, which relies on PV isolation through cell culture, with an alternative detection method with comparable sensitivity but that does not require viral culture. Extensive testing and validation of the DD-ITD method were conducted in controlled conditions in the reference specialized laboratory in the US-CDC and was piloted in other reference laboratories. This demonstration project was conceived to evaluate aspects related to the DD-ITD method implementation in diverse real world, and at times challenging, PV testing scenarios, using real AFP surveillance samples. Throughout the project, various challenges were encountered, and previously unrecognized factors that have an impact on PV detection using DD-ITD testing were identified. These factors must be considered when planning for widespread implementation of molecular-based PV detection methods across GPLN laboratories.

High sensitivity

A higher number of PV-presumptive positive samples were identified using the DD-ITD method by all participating laboratories, compared to VI gold standard methodology, with an average increase of 3.1-fold in the same data set. Moreover, in this data set, the NPEV detection rate was also higher with the DD-ITD method, showing a 1.7-fold increase over the VI method. The virus isolation-based methodology requires an enterovirus to grow in a cell line (e.g., RD or L20B cells), which requires virus viability (i.e., infectivity in cells). By contrast, molecular detection strategies such as RT-PCR specific assays (e.g., enterovirus; PanEV assay) can detect fragmented viral RNA (i.e., non-infectious virions) yielding a positive result; while the non-infectious viral particles from the same specimen would not induce a visible cytopathic effect (i.e., negative VI).

Complex data analysis

Without virus amplification through cell culture, the amount of viral RNA recovered during the RNA extraction is expected to be lower, which limits the amount of template available for RT-PCR amplification. Consequently, amplification results from DD-ITD might show both, lower fluorescence intensity and higher Ct values compared to virus isolates tested in the same real-time PCR assays. Interpretation of DD-ITD PCR results required manual adjustment of analysis parameters (baseline and threshold settings) and additional considerations for the quality assessment of real-time PCR results that were not required for real-time PCR analysis of virus isolates. Low-intensity amplification, commonly observed in DD-ITD assays, can be more challenging to distinguish from possible PCR artifacts (i.e., non-specific amplification, contamination, or background signals). Comprehensive analysis of QB results for each sample including the shape of the curves were critical to validate real-time PCR results.

This demonstration project showed that, despite accreditation of the current gold standard (virus isolation and ITD real-time PCR) methods, some personnel lacked the advanced molecular skillset required for manual data analysis, (i.e., QC assessment by QB analysis results) and algorithm interpretation for DD-ITD reporting. One possible explanation is that in low PV prevalence environments, only a small percentage of virus isolates need PCR testing. Of these, approximately 95% test negative for PV through VI, and when a VI is PV positive, strong rtPCR signals are typically evident. This highlights a key consideration; strengthening data analysis skills among laboratory personnel should be a priority when implementing the DD-ITD method.

Heightened demand for sequence verification

According to the current gold-standard VI and ITD algorithm for detecting and typing PV, any programmatically relevant vaccine-derived PV (VDPV1 or VDPV3), wild poliovirus (WPV1), and poliovirus type 2 (PV2) should be referred for sequencing confirmation (5, 15). The latest global PV surveillance data for 2022–2023 show that 1% of the tested specimens are referred for sequencing confirmation (2). In this data set, 164 poliovirus-positive sample results (1.6% of the total tested, n = 10,517) were referred for sequencing confirmation using the VI testing protocol. Conversely, with the DD-ITD molecular detection algorithm, approximately 9% of the total sample results would be referred for sequencing confirmation, which would result in a significant increase in the number of samples needing sequencing. Among the 807 inconclusive DD-ITD-positive results, 97 (12%) were confirmed as VI positive, highlighting the importance of pursuing confirmation for DD-ITD presumptive PV-positive results.

A limitation of this study was the lack of sequencing confirmation for DD-ITD results, leading to some outcomes being classified as presumptive positives. However, it provides valuable insights for future research, particularly in guiding the incorporation of sequencing protocols into the DD-ITD algorithm to improve its precision. Processing stools with the molecular method (DD-ITD), without virus amplification through culture, might lead to a higher proportion of non-typeable or inconclusive ITD results that would require repetition and/or sequencing confirmation. This can increase both cost and labor efforts. However, in the context of PV eradication, if the probability of PV detection is significantly increased, as demonstrated in this study, the added expenses are strongly justifiable.

Heterogeneity among laboratories: data rejection

The selection of laboratories for this demonstration project aimed to account for regional diversity, recognizing that PV positivity rates and serotypes would vary across laboratories. Each laboratory was assigned a specific target number of samples to process based on the regional PV prevalence and its average sample processing throughput. This demonstration study showed heterogeneity in the quality of performance in the DD-ITD testing arm across the GPLN laboratories.

Throughout the project, laboratory personnel engagement and communication with the CDC-Polio laboratory team varied among laboratories. While some proactively applied the recommendations for improving data quality, others struggled to successfully implement the DD-ITD methodology. Approximately 12% of the DD-ITD PCR results submitted for this project were rejected for not meeting the established QC criteria. Agreement between the method was low (slight) or "fair" (Table 2) for four of the six laboratories that submitted sufficient valid results for both methods (n > 600). The proportion of rejected results varied significantly among participating laboratories and among batches within the same laboratory indicating a need for improved consistency and adherence to protocols.

By contrast, the two laboratories submitting the highest percentage of valid data throughout the project presented substantial and moderate method agreement (Labs 2 and 5, Table 2). These laboratories performed exceptionally well throughout the pilot testing, excelling in data analysis, quality control (QC), and following through with technical feedback for troubleshooting resulting in high data quality and consistency in results submission. Their performance highlights the critical role of rigorous QC practices and effective feedback integration in achieving reliable and consistent results.

Although all laboratories detected more PV-positive specimens with the DD-ITD method, a total of 62 VI-positive samples in this data set were missed by DD-ITD (Table 1). From 12 participating laboratories, eight showed VI positive/DD-ITD negative results, with a proportion ranging from 0.1% to 0.98% of their valid results. The laboratory with the highest number of valid submissions in this project accounted for almost half of these samples (n = 26 of total n = 62), corresponding to less than 1% of the overall results submitted by that laboratory (n = 3,339).

By contrast, one laboratory that submitted less than 200 valid results for this project, had approximately 5% VI positive/DD-ITD-negative results. This laboratory had a high percentage of rejected results (29%) and had not achieved the required quality standards to implement the DD-ITD method, indicating a significant need for in-country training. In addition, a high-performing laboratory that proactively applied feedback provided, performed correct data analysis, and obtained valid DD-ITD results, also reported several VI-positive samples that were negative with DD-ITD (data not shown). The root cause analysis of these observations indicated that the laboratory was processing samples with the DD-ITD method a year or more after processing the same specimens with the VI procedures rather than performing parallel testing. More than 900 results meeting QA standards submitted by this laboratory were removed from the analysis as sample preservation and testing deviated from guidance and sample inclusion criteria. This incident highlights the need for stringent oversight of methodology implementation, ensuring that changes in guidance are promptly reported and addressed.

The manual RNA extraction protocol, using modified Zymo spin columns (11), could have also contributed to the variability in performance among labs and between extraction batches within the same laboratory. Although the method was validated and successfully piloted in reference laboratories, it had yet to be evaluated in laboratories processing a large number of samples. Optimization of automated RNA extraction protocols (9) is a current GPLN priority, with the expectation that the DD-ITD strategy will be improved by reducing operator errors and obtaining higher quality/purified RNA templates, particularly in high-throughput laboratories.

Reagents availability and unforeseen SARS-CoV-2 pandemic-related impact

The global SARS-CoV-2 pandemic (2020–2022) significantly affected international transport, plastics availability, and global commerce, further restricting the availability of reagents for participating laboratories (16). Moreover, shipment delays, particularly due to customs clearance regulations and extensive documentation requirements, were notably experienced in the WHO African region. This factor should be carefully considered when implementing new methodologies within the GPLN to mitigate potential logistical challenges, especially in pandemic settings. In addition, all training for this demonstration project was done remotely due to travel restrictions. This limited the possibility of oversight of DD-ITD initial implementation in each laboratory and to provide in-depth, face-to-face training and troubleshooting. The lack of direct interaction with laboratory personnel may have hindered the understanding of correct data analyses and critical protocol details, affecting the results and method performance.

Conclusions

The piloting of direct detection method for poliovirus in 12 Global Polio Laboratory Network laboratories across various regions of WHO demonstrated promising results. The overall agreement between DD-ITD and the traditional VI gold-standard PV detection methods exceeded 91%, DD-ITD detected threefold more presumptive positive samples than the VI method. However, a larger proportion of DD-ITD presumptive PV-positive samples were inconclusive or non-typeable results, demanding confirmation *via* sequencing.

DD-ITD implementation outcomes varied significantly among the participating laboratories as evidenced by the number of valid results (i.e., those meeting established quality standards) submitted by each laboratory. Factors like fluctuating availability of reagents, the presence of trained personnel for sample processing, proficiency of the PCR data analysis, lack of face-to-face training, and adherence to SOP and guidelines, were factors limiting the number of valid results submitted by laboratories. As PV eradication progresses and the number of PV-positive samples decreases, there will be a need for a reliable sensitive detection workflow to ensure that no positive samples are missed. To effectively adopt molecular-based poliovirus detection methods, ongoing technical oversight, enhanced laboratory training, and improved quality assurance are essential.

Upcoming advancements like regional distribution centers for supplies, adaptation of automated RNA extraction, and integration of refined sequencing protocols will further enhance the DD-ITD method and support global polio eradication goals.

MATERIALS AND METHODS

Parallel processing of stool specimens

AFP surveillance stools were anonymized and assigned an individual processing ID code for this study. Stool suspensions were prepared per GPLN-approved methodology (5, 6). Participating laboratories were tasked to simultaneously process duplicate stool suspensions with both the current gold-standard virus isolation (VI) method and the validated DD-ITD methodology (Fig. 3). Some laboratories stored remaining stool aliquots at −20°C for further testing.

Virus isolation and PCR screening

An aliquot of the 0.5% chloroform (vol/vol) stool suspension underwent virus isolation (VI) following the WHO gold-standard methodology (5, 17): Briefly, 0.2 mL of stool suspensions was inoculated into rhabdomyosarcoma (RD) and L20B recombinant murine cells with poliovirus receptor (PVR). Cells are incubated at 36°C and monitored daily for the appearance of cytopathic effect (CPE) for 7 days. Any isolate showing CPE on L20B cells was considered VI positive and screened for PV with the Intratypic differentiation (ITD) real-time reverse-transcription-PCR suite of assays (explained below). When required per GPLN algorithm, poliovirus-positive samples were referred for sequencing confirmation (18).

DD-ITD testing

On a second stool suspension aliquot, viral RNA was extracted with the modified Zymo Research protocol (2-column parallel extraction) (11). Lyophilized Qβ was provided with

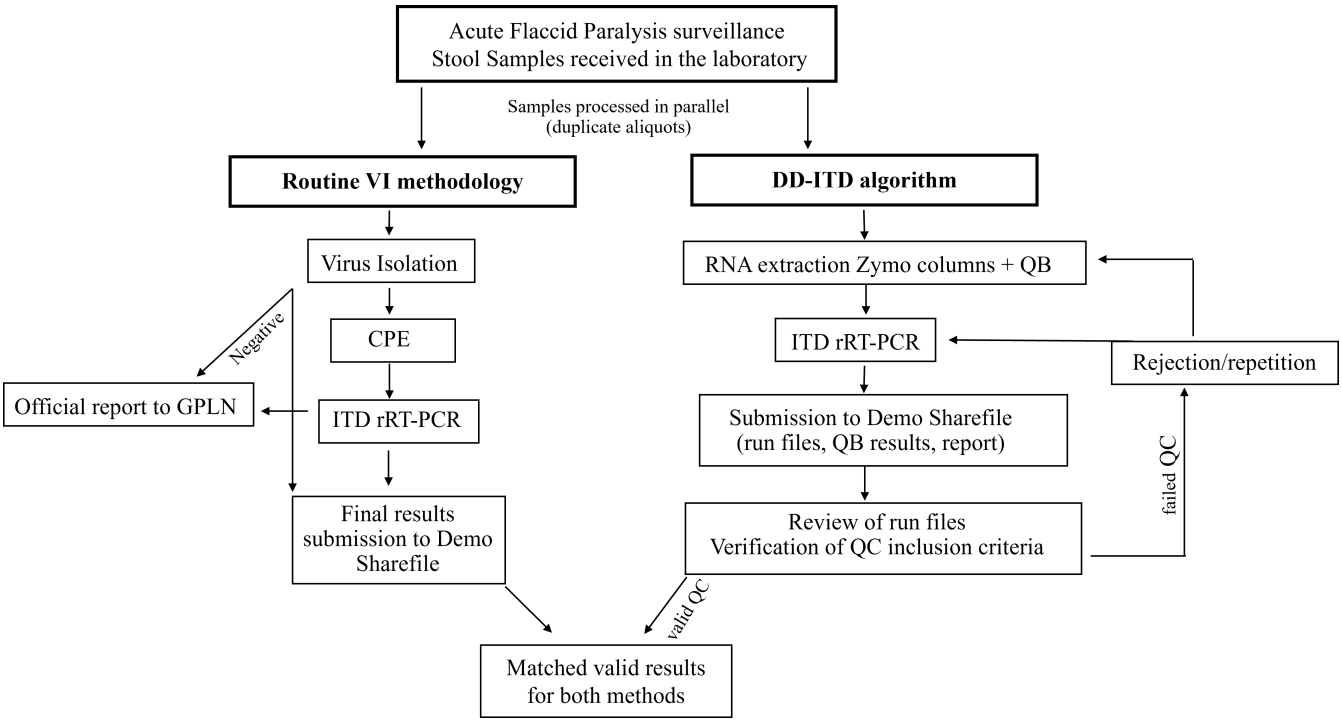

FIG 3 Workflow showing parallel testing VI and DD-ITD for this demo. Sample processing was performed by selected GPLN laboratories. Quality control (QC) was done by the CDC-Polio ITD team, through a review of run files and amplification results, RNA extraction quality assessment, and final results reporting.

the CDC-ITD kits through IRR. The Qβ bacteriophage (Qβ, positive-stranded RNA phage infecting *E. coli*) was included in each RNA extraction as the quality control: 10 µL of Qβ were added to each 800 µL stool suspension before the extraction. A processing control (10 µL Qβ +800 µL of nuclease-free water) was included in each RNA extraction batch. The Qβ target is duplexed in the WPV1/Qβ rRT-PCR assay (13).

Real-time RT-PCR screening for PV

On both VI isolates and RNA extracts, molecular PV screening and typing were achieved through the 6 rRT-PCR ITD assays: a suite of primer/probe assays previously described (13, 15, 19–22) for 10 targets: PanEV (targeting any enterovirus), PanPV (targeting any Poliovirus), PV Type 2 (targeting Poliovirus type 2), Sabin 1, Sabin 2, Sabin 3 (targeting Sabin serotypes 1, 2, and 3), QB (QB bacteriophage), WPV1 (wild PV1), WPV3 (wild PV3), and nOVP2 (targeting novel oral poliovirus vaccine 2). Briefly, 10 µL qScript XLT One-Step RT-qPCR (Quanta Biosciences, Beverly, MA), 1 µL of each primer/probe mix (CDC-ITD kit), 5 µL of extracted NA (template), and 4 µL of ultra-purified water were combined in a total reaction volume of 20 µL. To carry out ITD testing for VI specimens, 1 µL clarified supernatant from CPE-positive specimens was used as the template, and the ultra-purified water volume was adjusted to 9 µL. Cycler conditions were the same for all ITD assays as published before. The CFX96 (Bio-Rad laboratories), the ABI 7500 and QuantStudio 5 (Applied Biosystems by ThermoFisher) were the real-time PCR cyclers accepted for this study.

The ITD rRT-PCR suite of assays was analyzed following a specific algorithm (13, 15, 19, 20). In the context of polio eradication, it is critical that no PV-positive result is missed, thus the ITD testing algorithm is designed to be redundant, meaning that more than one assay is expected to be positive when a sample is positive for PV. This aims to account for variability in a assay limit of detection (LOD), targeting different PV genome regions, possible PV mutations, and variable PV RNA content in testing specimens. Briefly, a sample is considered PV positive when the PanEV and PanPV assays are positive (show amplification of a sigmoidal curve), and a serotype-specific assay (or assays) that show positive amplification would indicate the specific PV serotype (or serotype mixes) for that sample.

DD-ITD-positive results also include *ITD-non typeable* results (i.e., indeterminate, if PanEV and PanPV are positive, but no specific serotype assay is positive) and *ITD-inconclusive* results (i.e., invalid, when either PanEV and/or PanPV were negative, but a specific serotype assay was positive). These results are expected within the ITD method, especially for low RNA concentration, and, per algorithm, samples are considered "presumptive" PV positives and are referred for sequencing confirmation. Sequencing confirmation of DD-ITD results was not part of this demo project. All VI positive results were typeable and confirmed poliovirus-positive according to the GPLN VI algorithm.

The criteria for quality control (QC) for inclusion in subsequent data analysis was determined by Qβ Ct value cut-offs established for each sample (Qβ in each stool sample Ct ≤32) and for each extraction batch (valid process control with Qβ Ct ≤26). The RNA extraction for a specific stool suspension was requested to be repeated if the Qβ did not meet the cut-off for that sample.

Data management, curation, and statistical and visual analyses

To compare both methods with a 95% statistical probability, a power analysis for sample size determination was performed based on the number of poliovirus positives detected through AFP surveillance. GPLN laboratories screen yearly about 220,000 AFP stool samples through virus isolation, having reported an overall positivity rate for poliovirus of 6.8% and 4.3% in 2021 and 2022 respectively (7). The initial target size for this demo project was determined to be between 10,800–16,000 specimens, aiming to detect about 540 VI poliovirus-positive stool specimens.

Each participating laboratory was provided with login credentials to a Sharefile platform managed by CDC, where all DD-ITD results were submitted in batches (sample

size n = 13 + 1 control). Results were gathered in standardized reporting sheets, which included the Ct values for each ITD target (n = 10) and the corresponding PCR run files. The CDC Polio Molecular Diagnostic Development team verified the quality of each result by reviewing the run files for each RT-PCR assay for each sample. When required, submitting GPLN laboratories received specific troubleshooting recommendations and data analysis training. The data QC process entailed repeated RNA extractions, ITD amplification, and resubmission of data until they met QA criteria.

The QB Ct values for each extraction batch and each sample was reviewed to determine the validity of each extraction. If the individual sample results met the QC requirements, Ct values were recorded for each sample, and ITD target. If PCR results did not meet QC standards with valid negative and positive controls for each assay, they were rejected, and repetition was requested.

ITD results were compiled, matched by sample for both VI and DD-ITD methods, organized in a standardized format, and merged in a consolidated database for all submitting laboratories. The R statistical software was used to develop an iterative script used to consolidate and analyze the results, verify identifiers, check for duplicate submissions, and catch reporting inconsistencies. The McNemar's test (23) was used for parallel testing analysis comparing the outcome of the two methods on the same specimens. Cohen's Kappa score was utilized to assess method agreement per laboratory (McHugh, 2012). Visualizations and tables were obtained using the dplyr and ggplot2 packages in R studio/R software (24, 25).

ACKNOWLEDGMENTS

We thank the members of the small working group (Global Polio Laboratory Network regional coordinators and Global specialized laboratories), in particular Dr. Mark Pallansch for continuous guidance and critical feedback during the years of the project development and the implementation stages. We thank GPLN WHO headquarters, especially Dr. Ousmane Diop, Monika Regucka, for facilitating the provision of resources to the laboratories and extensive logistical support. A special recognition to the personnel of the laboratories that provided invaluable results processing the samples with both methodologies. We thank the individuals who were involved at the earlier stages of this project: Stacey Jeffries Miles, Kimberly Wong, Farzana Dola, and Dr. Everardo Vega.

The findings and conclusions in this report are those of the author and do not necessarily represent the views of the Centers for Disease and Prevention (CDC). The use of trade names is for identification only and does not imply endorsement by the CDC or the US government. Ethical considerations. The CDC internal program for research determination deemed that this study is categorized as public health non-research and human subject research did not apply. All specimen identifiers were anonymized by assigning a unique laboratory ID created by the submitting laboratories before sharing data with CDC.

AUTHOR AFFILIATIONS

[1]Division of Viral Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia, USA
[2]Tanaq, Contracting agency to the Division of Viral Diseases, CDC, Atlanta, Georgia, USA
[3]World Health Organization, Global Polio Laboratory Network Headquarters, Geneva, Switzerland
[4]National Institute for Health, Islamabad, Pakistan
[5]National Polio Laboratory, Maiduguri, Nigeria
[6]National Polio Laboratory, Ibadan, Nigeria
[7]Uganda Virus Research Institute, Entebbe, Uganda
[8]National Polio Laboratory, Research Institute for Tropical Medicine, Manila, Philippines
[9]Polio Regional Reference Laboratory, VACSERA, Greater Cairo, Egypt

[10]Noguchi Memorial Institute for Medical Research, Accra, Ghana
[11]Kenya Medical Research Institute, Nairobi, Kenya
[12]National Institute of Communicable Diseases, Johannesburg, South Africa
[13]National Institute of Biomedical Research, Kinshasa, Democratic Republic of Congo
[14]Centre Pasteur du Cameroun, Yaoundé, Cameroon
[15]National Polio Laboratory, Addis Ababa, Ethiopia
[16]World Health Organization, Regional Office for Africa, Brazzaville, Democratic Republic of Congo
[17]World Health Organization, Regional Office for the Eastern Mediterranean, Amman, Jordan
[18]World Health Organization, Regional Office for the Western Pacific, Manila, Philippines

AUTHOR ORCIDs

Paula L. Marcet http://orcid.org/0000-0002-0676-3020
Ashley Deas http://orcid.org/0009-0005-3950-9423
Hong Sun http://orcid.org/0000-0002-1306-8050
Chelsea Harrington http://orcid.org/0000-0003-2879-444X
Shahzad Shaukat http://orcid.org/0000-0003-3502-6940
Salmaan Sharif http://orcid.org/0000-0002-2558-3068
Cara C. Burns http://orcid.org/0000-0003-2582-2476
Nancy Gerloff http://orcid.org/0000-0001-5696-8318

AUTHOR CONTRIBUTIONS

Paula L. Marcet, Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Validation, Visualization, Writing – original draft, Writing – review and editing | Brandon Short, Data curation, Investigation, Validation, Writing – review and editing | Ashley Deas, Data curation, Formal analysis, Investigation, Software, Validation, Visualization, Writing – original draft, Writing – review and editing | Hong Sun, Investigation, Methodology, Writing – review and editing | Chelsea Harrington, Conceptualization, Investigation, Methodology, Software, Writing – review and editing | Shahzad Shaukat, Conceptualization, Funding acquisition, Project administration, Resources, Writing – review and editing | Muhammad Masroor Alam, Investigation, Supervision | Marycelin Baba, Investigation, Supervision | Adedayo Faneye, Investigation, Supervision | Prossy Namuwulya, Investigation, Supervision | Lea Necitas Apostol, Investigation, Supervision | Tamer Elshaarawy, Investigation, Supervision | John Kofi Odoom, Investigation, Supervision | Peter Borus, Investigation, Supervision | Shelina Moonsamy, Investigation, Supervision | Yogolelo Riziki, Investigation, Supervision | Marie Claire Endegue Zanga, Investigation, Supervision | Mesfin Tefera, Investigation, Supervision | Anfumbom K. W. Kfutwah, Project administration, Supervision | Salmaan Sharif, Project administration, Supervision | Varja Grabovac, Project administration, Supervision | Cara C. Burns, Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision, Writing – review and editing | Nancy Gerloff, Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Supervision, Validation, Writing – original draft, Writing – review and editing

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Table S1 (Spectrum02279-24-s0001.pdf). Summary of total samples analyzed throughout the DD-ITD demo project.

REFERENCES

1. GPEI. 1988. Global polio eradication initiative. Available from: https://polioeradication.org
2. Kishore N, Krow-Lucal E, Diop OM, Jorba J, Avagnan T, Grabovac V, Kfutwah AKW, Johnson T, Joshi S, Sangal L, Sharif S, Wahdan A, Tallis GF, Kovacs SD. 2024. Surveillance to track progress toward polio eradication - Worldwide, 2022-2023. MMWR Morb Mortal Wkly Rep 73:278–285. https://doi.org/10.15585/mmwr.mm7313a1
3. Diop OM, Kew OM, de Gourville EM, Pallansch MA. 2017. The global polio laboratory network as a platform for the viral vaccine-preventable and emerging diseases laboratory networks. J Infect Dis 216:S299–S307. https://doi.org/10.1093/infdis/jix092
4. GPEI. 2024. The global polio laboratory network, on global polio eradication initiative. Available from: https://polioeradication.org/polio-today/polio-now/surveillance-indicators/the-global-polio-laboratory-network-gpln
5. WHO. 2004. Polio laboratory manual (WHO/IVB/04.10). 4th ed. World Health Organization, Geneva, Switzerland. https://polioeradication.org/wp-content/uploads/2017/05/Polio_Lab_Manual04.pdf.
6. WHO. 2007. S1. Supplement to the WHO polio laboratory manual: an alternative test algorithm for poliovirus isolation and characterization. World Health Organization, Geneva, Switzerland.
7. WHO. 2015. Reporting and classification of vaccine-derived polioviruses. GPEI guidelines. World Health Organization Geneva, Switzerland.
8. WHO. 2015. GAP III. WHO global action plan to minimize poliovirus facility-associated risk after type-specific eradication of wild polioviruses and sequential cessation of oral polio vaccine use. World Health Organization Geneva, Switzerland.
9. Geiger K, Stehling-Ariza T, Bigouette JP, Bennett SD, Burns CC, Quddus A, Wassilak SGF, Bolu O. 2024. Progress toward poliomyelitis eradication - Worldwide, January 2022-December 2023. MMWR Morb Mortal Wkly Rep 73:441–446. https://doi.org/10.15585/mmwr.mm7319a4
10. Akello JO, Bujaki E, Shaw AG, Khurshid A, Arshad Y, Troman C, Majumdar M, O'Toole Á, Rambaut A, Alam MM, Martin J, Grassly NC. 2023. Comparison of eleven RNA extraction methods for *Poliovirus* direct molecular detection in stool samples. Microbiol Spectr 11:e0425222. https://doi.org/10.1128/spectrum.04252-22
11. Harrington C, Sun H, Jeffries-Miles S, Gerloff N, Mandelbaum M, Pang H, Collins N, Burns CC, Vega E. 2021. Culture-independent detection of *Poliovirus* in stool samples by direct RNA extraction. Microbiol Spectr 9:e0066821. https://doi.org/10.1128/Spectrum.00668-21
12. Ueno MK, Kitamura K, Nishimura Y, Arita M. 2023. Evaluation of direct detection protocols for *Poliovirus* from stool samples of acute flaccid paralysis patients. Viruses 15:2113. https://doi.org/10.3390/v15102113
13. Miles SJ, Harrington C, Sun H, Deas A, Oberste MS, Nix WA, Vega E, Gerloff N. 2024. Validation of improved automated nucleic acid extraction methods for direct detection of *Polioviruses* for global polio eradication. J Virol Methods 326:114914. https://doi.org/10.1016/j.jviromet.2024.114914
14. Kahle D, Wickham H. 2013. ggmap: spatial visualization with ggplot2. R J 5:144. https://doi.org/10.32614/RJ-2013-014
15. Gerloff N, Sun H, Mandelbaum M, Maher C, Nix WA, Zaidi S, Shaukat S, Seakamela L, Nalavade UP, Sharma DK, Oberste MS, Vega E. 2018. Diagnostic assay development for *Poliovirus* eradication. J Clin Microbiol 56:e01624-17. https://doi.org/10.1128/JCM.01624-17
16. de Sousa FDB. 2021. Plastic and its consequences during the COVID-19 pandemic. Environ Sci Pollut Res Int 28:46067–46078. https://doi.org/10.1007/s11356-021-15425-w
17. Thorley BR, Roberts JA. 2016. Isolation and characterization of *Poliovirus* in cell culture systems. Methods Mol Biol 1387:29–53. https://doi.org/10.1007/978-1-4939-3292-4_4
18. Burns CC, Kilpatrick DR, Iber JC, Chen Q, Kew OM. 2016. Molecular properties of poliovirus isolates: nucleotide sequence analysis, typing by PCR and Real-Time RT-PCR, p 177–212. In Martín J (ed), Poliovirus: methods and protocols. Springer, New York, NY.
19. Sun H, Harrington C, Gerloff N, Mandelbaum M, Jeffries-Miles S, Apostol LNG, Valencia M-L, Shaukat S, Angez M, Sharma DK, Nalavade UP, Pawar SD, Pukuta Simbu E, Andriamamonjy S, Razafindratsimandresy R, Vega E. 2021. Validation of a redesigned pan-poliovirus assay and real-time PCR platforms for the global poliovirus laboratory network. PLoS One 16:e0255795. https://doi.org/10.1371/journal.pone.0255795
20. Kilpatrick DR, Yang C-F, Ching K, Vincent A, Iber J, Campagnoli R, Mandelbaum M, De L, Yang S-J, Nix A, Kew OM. 2009. Rapid group-, serotype-, and vaccine strain-specific identification of *poliovirus* isolates by real-time reverse transcription-PCR using degenerate primers and probes containing deoxyinosine residues. J Clin Microbiol 47:1939–1941. https://doi.org/10.1128/JCM.00702-09
21. Kilpatrick DR, Nottay B, Yang CF, Yang SJ, Da Silva E, Peñaranda S, Pallansch M, Kew O. 1998. Serotype-specific identification of polioviruses by PCR using primers containing mixed-base or deoxyinosine residues at positions of codon degeneracy. J Clin Microbiol 36:352–357. https://doi.org/10.1128/JCM.36.2.352-357.1998
22. Kilpatrick DR, Nottay B, Yang CF, Yang SJ, Mulders MN, Holloway BP, Pallansch MA, Kew OM. 1996. Group-specific identification of *polioviruses* by PCR using primers containing mixed-base or deoxyinosine residue at positions of codon degeneracy. J Clin Microbiol 34:2990–2996. https://doi.org/10.1128/jcm.34.12.2990-2996.1996
23. Trajman A, Luiz RR. 2008. McNemar chi2 test revisited: comparing sensitivity and specificity of diagnostic examinations. Scand J Clin Lab Invest 68:77–80. https://doi.org/10.1080/00365510701666031
24. Wickham H. 2016. ggplot2: elegant graphics for data analysis (use R). Springer. Verlag, New York.
25. R_Core_Team. 2022. R: a language and environment for statistical computing. Vienna, Austria R Foundation for Statistical Computing. https://www.R-project.org/.