Kinetics of Poliovirus Shedding following Oral Vaccination as Measured by Quantitative Reverse Transcription-PCR versus Culture

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Amid polio eradication efforts, detection of oral polio vaccine (OPV) virus in stool samples can provide information about rates of mucosal immunity and allow estimation of the poliovirus reservoir. We developed a multiplex one-step quantitative reverse transcription-PCR (qRT-PCR) assay for detection of OPV Sabin strains 1, 2, and 3 directly in stool samples with an external control to normalize samples for viral quantity and compared its performance with that of viral culture. We applied the assay to samples from infants in Dhaka, Bangladesh, after the administration of trivalent OPV (tOPV) at weeks 14 and 52 of life (on days 0 [pre-OPV], +4, +11, +18, and +25 relative to vaccination). When 1,350 stool samples were tested, the sensitivity and specificity of the quantitative PCR (qPCR) assay were 89 and 91% compared with culture. A quantitative relationship between culture⁺/qPCR⁺ and culture⁻/qPCR⁺ stool samples was observed. The kinetics of shedding revealed by qPCR and culture were similar. qPCR quantitative cutoffs based on the day +11 or +18 stool samples could be used to identify the culture-positive shedders, as well as the long-duration or high-frequency shedders. Interestingly, qPCR revealed that a small minority (7%) of infants contributed the vast majority (93 to 100%) of the total estimated viral excretion across all subtypes at each time point. This qPCR assay for OPV can simply and quantitatively detect all three Sabin strains directly in stool samples to approximate shedding both qualitatively and quantitatively.

Both immunity to paralytic poliomyelitis and reduction of transmission of wild-type and vaccine-derived polioviruses are critical in polio eradication efforts (1, 2). Oral polio vaccine (OPV) is widely utilized in low-income settings for ease of delivery, low cost, and potential for mucosal immunity. OPV immunogenicity is imperfect (3), however, and shedding of vaccine strains further complicates the poliovirus reservoir. Measurement of poliovirus in stool samples can thus be useful in evaluating mucosal immunogenicity (4), in estimating the transmission intensities of vaccine-derived virus, and for surveillance after polio eradication (5).

Poliovirus culture is still considered the gold standard procedure but requires up to two 7-day passages in two cell lines, followed by molecular or antigenic methods to differentiate and type the poliovirus isolates (6). This process is time-consuming and greatly diminishes the number of stool samples that can be surveyed. Furthermore, subcultured isolates are not always representative of the viruses present in the original stool sample and results are usually not quantitated by titer determination (7, 8). PCR methods are therefore attractive in terms of cost, time, the ability to subtype, and quantitation (9–12). PCR for poliovirus has been found to be severalfold more sensitive than poliovirus isolation from cell culture (13) and has also been found to be more sensitive with cerebrospinal fluid, throat swabs, or stool samples (14–17). However, studies of quantitative PCR (qPCR) versus culture have rarely been applied comparatively to cohorts after OPV administration.

In this study, we sought to develop a one-step serotype-specific reverse transcription (RT)-PCR assay that could provide quantitative information on OPV excretion for all three Sabin strains directly in stool samples. We included an external control to normalize quantities for extraction and amplification efficiency and

then measured its performance at two OPV administration time points against culture in a cohort of Bangladeshi infants receiving trivalent OPV (tOPV).

MATERIALS AND METHODS

Study design. This study was part of an oral vaccine immunogenicity study that followed infants from birth to 24 months of age in the Mirpur region of Dhaka, Bangladesh, from November 2010 to August 2012. tOPV was administered at weeks 6, 10, 14, and 52 (as part of the study, at week 39, half of the infants received inactivated polio vaccine [IPV] and the other half received tOPV). We evaluated stool specimens from 88 infants (46 male and 42 female) after the week 14 vaccination and from 182 infants (98 male and 84 female) after the week 52 vaccination because we had stool samples collected and tested by both methods on all days, i.e., 0, +4, +11, +18, and +25. Stool samples were collected at the study clinic or during home visits and delivered to the laboratory within 6 h while

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maintaining a cold chain. Stool samples were sent to the Bangladesh National Polio and Measles Laboratory for poliovirus culture and stored at -70°C until further testing. Viral cultures were performed according to the methodologies of the 4th edition of the World Health Organization polio laboratory manual (6). Briefly, two stool sample aliquots each were inoculated onto the L20B and RD cell lines. If the cultures were positive for poliovirus on L20B cells, then they were subcultured onto RD cells and vice versa for confirmation of positivity. A subset of stool sample cultures ($n = 87$) was characterized for Sabin type by singleplex real-time PCR using Sabin-specific primers (9, 18). This study was approved by the institutional ethics committees at the University of Virginia, the University of Vermont, and the International Centre for Diarrhoeal Disease Research, Bangladesh.

RNA extraction from stool samples. RNA was extracted from stool samples with the QIAamp Viral RNA kit (Qiagen, Gaithersburg, MD) or the QuickGene RNA Tissue kit II (Kurabo, Osaka, Japan) on the Fujifilm Quickgene-810 (Fujifilm, Tokyo, Japan) with slight modifications as described previously (19). Briefly, 50- to 100-mg aliquots of stool samples were added to 250 μl of phosphate-buffered saline, vortexed for 1 min, and then centrifuged at $4,000 \times g$ for 20 min. Lysis buffer (LRT) was spiked with 5 μl of 2 M dithiothreitol and 1 μl of bacteriophage MS2 (approximately 2×10^6 copies, ATCC 15597B1; American Type Culture Collection, Manassas, VA) per stool sample to serve as an extraction and amplification control. A 146- μl volume of solubilization buffer (SRT) was added, mixed, and incubated for 10 min, and then 250 μl of 70% ethanol solution was added. The extraction volume was 50 μl , and RNA was stored at -70°C with 50 μl of RNA Storage Solution (Ambion AM700; Life Technologies, Grand Island, NY).

Multiplex real-time RT-PCR. The serotype-specific primers and probes for the VP1 region used in this multiplex assay were adopted from Kilpatrick et al. (9). Sabin 1 (S1) and Sabin 2 (S2) probes were minor-groove-binding TaqMan probes (Life Technologies, Grand Island, NY) with the VIC and 6-carboxyfluorescein fluorophores, respectively. The Sabin 3 (S3) probe was labeled with Texas Red-X NHS Ester and black hole quencher 2 (Integrated DNA Technologies, Coralville, IA). The external control MS2 primers and probe (Quasar 670 and black hole quencher 3; Biosearch Technologies, Petaluma, CA) were described previously (19). The PCR included the SensiFAST Probe No-ROX One-Step kit (Bioline USA Inc., Taunton, MA) in a 20- μl total volume containing 0.4 μl of each 20 μM primer and 0.2 μl of each 10 μM probe, 10 μl of $2 \times$ One-Step Reaction mixture, 0.2 μl of reverse transcriptase, 0.4 μl of Ribosafe RNase inhibitor, 1.4 μl of nuclease-free water, and 4 μl of template RNA. The cycling conditions were 15 min at 50°C , 1 min at 95°C , and 40 cycles of 10 s at 95°C and 30 s at 60°C on the CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA) using the CFX Manager software.

Analytical sample preparation for validation of PCR assay. *In vitro*-transcribed (IVT) RNAs were generated by cloning target amplicons with the TOPO TA cloning kit (Life Technologies, Grand Island, NY) and isolating plasmids from colonies (Qiagen, Gaithersburg, MD). Linearizing DNA was generated by PCR with the TOPO TA cloning kit and cleaned with the QIAquick PCR purification kit (Qiagen, Gaithersburg, MD). IVT RNAs for S1, S2, and S3 were generated with the AmpliScribe T7 high-yield transcription kit (Epicentre Technologies Corp., Chicago, IL) and purified with the RNeasy MinElute Cleanup kit (Qiagen, Gaithersburg, MD). Concentrations were measured with a spectrophotometer (NanoDrop 2000; Thermo Scientific, Wilmington, DE), and serial dilutions were used to spike the LRT lysis buffer and stool sample supernatants that were previously tested and negative for vaccine poliovirus.

Calculation of normalized target copy numbers. A standard curve for the external control MS2 phage was generated by performing 10-fold serial dilutions in triplicate. Four microliters of undiluted material contained 8×10^4 copies (copy number C_{input} , 100% efficiency). Using the standard curve and the cycle threshold (C_T) for the external control from

the PCR, we estimated the corresponding copy number, C_{MS2} . Efficiency (E) of MS2 extraction and amplification was calculated as $E = C_{\text{MS2}}/C_{\text{input}}$. Standard curves for S1, S2, and S3 were generated by testing 10-fold serial dilutions of IVT RNA for the three serotypes in triplicate per dilution in a similar manner. Sample viral copy numbers (V) were estimated by using the standard curve and the C_T for the target from the PCR. The viral copy number, V , was then normalized to account for PCR extraction and amplification efficiency and calculated as the normalized viral copy number ($V_{\text{normalized}} = V/E$).

Sanger sequencing. To sequence discrepant samples, we performed PCR in singleplex. The PCR amplicon was cloned using the TOPO TA cloning kit for sequencing (Invitrogen, Grand Island, NY) according to the manufacturer's protocol. Plasmids were purified by using QIAprep Spin Miniprep (Qiagen, Gaithersburg, MD), quantified with the NanoDrop 2000 (Thermo Scientific, Wilmington, DE), and sequenced (GENEWIZ, Frederick, MD).

Statistical analysis. All statistical analyses (receiver operating characteristic [ROC], Mann-Whitney, linear regression, and chi-square tests) were performed by using SPSS Statistical Software (IBM). Results were reported as means $\pm$ standard errors unless indicated otherwise. $P < 0.05$ was considered statistically significant. All P values were two tailed.

RESULTS

Analytical performance of real-time RT-PCR assay. In order to evaluate the performance of the multiplex quantitative RT-PCR (qRT-PCR), we compared it to the singleplex assays. Serial dilutions of each IVT RNA for the serotypes showed that the limit of detection (defined as the lowest dilution where 10/10 samples were detected) of the multiplex assay was the same for S1 (1.02 copies/4 μl of input IVT RNA) and S2 (1.45 copies/4 μl input IVT RNA) with a C_T increase of approximately 1 for the multiplex reactions. The limit of detection of S3 was 1.05 copies/reaction for the singleplex and 10.5 copies/reaction for the multiplex. These concentrations corresponded to a C_T of 36 for S1 and 37 for S2 and S3; thus, these C_T cutoffs were applied. The repeatability and reproducibility of the assay were both 100% (10/10 replicates and 10/10 extractions at 10^4 and 10^{10} copies), and the linearity was $R^2 = 0.99$ to 1.00. All probes were specific for each of the targets with no cross-reaction detected among Sabin poliovirus and non-poliovirus enterovirus strains. We evaluated several commercial enzymes (Life Technologies AGPATH-ID one-step RT-PCR mix, Qiagen OneStep RT-PCR kit, Quanta qScript XLT One-Step RT-qPCR ToughMix, and TaKaRa PrimeScript One Step RT-PCR kit ver. 2) and utilized the Bioline SensiFAST Probe No-ROX One-Step kit (Bioline USA Inc., Taunton, MA) because of consistent performance (data not shown).

Performance of qRT-PCR with clinical samples. We then evaluated clinical specimens. We examined stool samples collected from infants after their third OPV vaccination at 14 weeks of age and after a subsequent OPV administration at 52 weeks of age. At each time point, stool samples were collected on days 0 (pre-OPV), +4, +11, +18, and +25. In total, there were 88 infants with culture data at each time point at the 14-week OPV and 182 infants at the 52-week OPV, of whom 44 were the same infants. Of the total of 1,350 samples ($270 \text{ infants} \times 5 \text{ time points}$), the sensitivity and specificity of the qRT-PCR versus culture were 89 and 91%, respectively (Table 1). That is, the qRT-PCR detected 129 (89%) of 145 culture-positive specimens and detected an additional 108 of 1,205 culture-negative specimens as well. As we have seen with other intestinal infections (20), the qPCR C_T , either raw or normalized to the MS2 external control to yield the number of copies per gram of stool sample, displayed a quantitative relationship with culture (i.e., qPCR, $1.3 \times 10^{10} \pm 5.6 \times 10^{10}$ copies/g

TABLE 1 Performance of multiplex qRT-PCR assay versus viral culture for detection of Sabin strains in stool specimens

PCR result	No. of samples		% Sensitivity (95% CI)	% Specificity (95% CI)
	Culture ⁺	Culture [−]		
Positive	129 ^a	108 ^b	89 (82–93)	91 (89–93)
Negative	16	1,097		

^a $C_T = 27.7 \pm 0.4$; $1.3 \times 10^{10} \pm 5.6 \times 10^{10}$ copies/g (Mann-Whitney $P < 0.001$).
^b $C_T = 31.4 \pm 0.3$; $1.4 \times 10^8 \pm 5.0 \times 10^8$ copies/g (Mann-Whitney $P < 0.001$).

in culture-positive specimens versus $1.4 \times 10^8 \pm 5.0 \times 10^8$ in culture-negative specimens, $P < 0.001$, Table 1). Figure 1 illustrates this quantitative relationship at both the week 14 and week 52 OPV administrations ($P < 0.05$). We examined 28 of the qRT-PCR-positive/culture-negative specimens by sequencing and confirmed that all of the amplicons were true S1, S2, or S3 sequences. On the subset of the positive cultures that were subtyped for S1 to S3 ($n = 87$), the subtype-specific stool sample qRT-PCR result revealed a sensitivity and specificity of 67 and 72% for S1, 80 and 74% for S2, and 83 and 56% for S3.

Interpretation of qRT-PCR on clinical samples. We then sought to evaluate the shedding kinetics revealed by the two methodologies (Fig. 2). The overall shapes of culture and qRT-PCR positivity were similar at both the week 14 and 52 time points. The highest rate of shedding was observed at day +4 at both time points with both modalities. PCR was notably more positive than culture at the tail end (day +25) of the week 14 immunization. A similar phenomenon was observed at the tail of the week 10 OPV administration (i.e., day 0 of week 14) yet diminished with the week 52 dose. qRT-PCR yielded subtype data on each specimen and showed that shedding of S1 (5.1% of all specimens) and S3 (6.4%) was more common than that of S2 (1.9%) and that mixed infections were present in 6.5% of the

samples ($n = 14$ S1 and S2, $n = 23$ S1 and S3, $n = 21$ S1 to S3, and $n = 12$ S2 and S3). The subtyping information obtained by culture supported the lower rate of S2 versus S1 and S3 ($n = 15$ versus 55 and 30, respectively).

We then examined the duration and intensity of shedding in infants. The duration of shedding is typically defined as the child’s last date of positive culture (in this study, day +4, +11, +18, or +25). By categorizing infants on this basis, we found that the qRT-PCR viral copy numbers were generally higher in those with longer durations of shedding (Fig. 3). However, shedding was highly sporadic (e.g., of the 34 infants that were culture positive at any of the time points after week 14, 13 shed sporadically, with similar findings by qPCR; see Table S1 in the supplemental material). Given this sporadic excretion, one could instead evaluate the frequency on the basis of whether virus was culturable at one, two, three, or all four of the time points. Similar to the duration curves, across this semiquantitative 1+ to 4+ spectrum, we saw an expected increase in the qRT-PCR viral copy number (data not shown). For either the culture-defined duration or frequency curves, the day 11 or 18 post-OPV stool sample qPCR appeared particularly informative for separating the culture-positive shedders. Although the numbers of shedders in these groups were often small, which limited statistical analyses, by ROC analysis, an S1-to-S3 qRT-PCR viral copy number exceeding 8.4×10^5 /g of stool sample at day +11 or +18 would optimally identify shedders qualitatively (yes/no), and those with viral copy number exceeding 8.2×10^6 /g at day 18 would likely be long-duration (e.g., day 18 or 25 day shedders) or high-frequency shedders.

Finally we sought to estimate an infant’s contribution to the overall burden of excretion in the cohort. To do so, we calculated each individual’s serotype-specific shedding index (SI) on the basis of a modified calculation of the excretion index (21), whereby

FIG 1 Temporal comparison of viral copy numbers determined by qRT-PCR assay and culture results. qRT-PCR assay results were normalized to the external control to yield a copy number. For mixed S1-to-S3 specimens, the highest viral copy number per specimen is shown. The median viral copy numbers in the culture-positive and culture-negative samples are shown as black horizontal bars. *, $P < 0.05$. nd = not detected.

FIG 2 Kinetics of tOPV excretion determined by qRT-PCR and culture. Stool samples were assayed by qRT-PCR and culture for OPV as described in Materials and Methods in 88 infants at week 14 and 182 infants at week 52 after OPV administration. Percentages of infants with stool specimens positive by qRT-PCR or culture (y-axis) at the relevant time points are shown, with day 0 = pre-OPV and days 4, 11, 18, and 25 after OPV. S1, S2, S3, and mixed Sabin results are shown for PCR (see color key on the right) but were not routinely available by culture. Values above culture bars reflect the sensitivity of PCR versus culture at these time points.

the SI was defined as the product of shedding duration (last day of shedding by PCR) and quantity (average number of copies per gram). The subset of 44 infants who had measurements at both weeks 14 and 52 is shown (Table 2); 19 and 18 of them had viral

excretion measurable by qPCR at any time point postvaccination at weeks 14 and 52, respectively. The top 3 shedders (3/44 or 7%) accounted for 93, 99, and 93% of the total measured S1, S2, and S3 excretion at week 14 and for 100, 96, and 98% at week 52.

FIG 3 Duration of shedding as a function of the viral copy number determined by qRT-PCR. Duration was defined by the date of the last positive culture. The average qRT-PCR-determined viral copy number $\pm$ the standard deviation is shown for each group. n = numbers of children at 14 and 52 weeks, respectively. The asterisk indicates that shedding infants generally had higher viral copy numbers than nonshedders, with statistically significant differences by Mann-Whitney test as follows: a, day 25, 18, 11, and 4 shedders versus nonshedders; b, day 25, 18, 11, and 4 shedders versus nonshedders and day 25 versus day 4 shedders; c, day 25 and 18 shedders versus nonshedders and day 18 versus day 11 and 4 shedders; d, day 25 and 18 shedders versus nonshedders and day 25 versus day 18 and 4 day shedders; e, day 25, 18, and 4 shedders versus nonshedders and day 11 versus day 4 shedders; f, day 25, 18, 11, and 4 shedders versus nonshedders, day 18 versus day 4 shedders, and day 11 versus day 4 shedders; g, day 18 shedders versus nonshedders and day 18 versus day 11 and 4 shedders. nd, not detected.

TABLE 2 Individual child SIs^a by serotype

Wk, serotype, and child or children	SI (% total)
14	
S1	
1047	3.63×10^{10} (50)
1107	2.87×10^{10} (39)
1106	2.77×10^9 (4)
Other 41	5.23×10^9 (7)
All (<i>n</i> = 44)	7.30×10^{10} (100)
S2	
1107	1.94×10^{11} (95)
1257	9.00×10^9 (4)
1285	8.87×10^8 (0.4)
Other 41	2.31×10^8 (0.1)
All (<i>n</i> = 44)	2.04×10^{11} (100)
S3	
1107	4.20×10^{10} (39)
1268	3.83×10^{10} (36)
1079	1.97×10^{10} (18)
Other 41	7.73×10^9 (7)
All (<i>n</i> = 44)	1.08×10^{11} (100)
52	
S1	
1020	1.12×10^{12} (58)
1039	6.53×10^{11} (34)
1246	1.44×10^{11} (8)
Other 41	5.04×10^9 (0.3)
All (<i>n</i> = 44)	1.92×10^{12} (100)
S2	
1285	2.46×10^{11} (90)
1223	1.16×10^{10} (4)
1045	6.88×10^9 (2)
Other 41	1.02×10^{10} (4)
All (<i>n</i> = 44)	2.75×10^{11} (100)
S3	
1285	6.61×10^{11} (93)
1070	2.97×10^{10} (4)
1132	7.34×10^9 (1)
Other 41	9.61×10^9 (1)
All (<i>n</i> = 44)	7.07×10^{11} (100)

^a SI = duration of shedding $\times$ mean number of copies/g determined by qPCR. Data for the top three shedding children are shown for illustration purposes.

DISCUSSION

In this work, we developed an OPV-specific qRT-PCR assay and examined its performance with sequential stool samples in an urban Bangladeshi setting of OPV-immunized infants. The analytic metrics of the assay performed well versus the WHO culture method, with $\sim 90\%$ sensitivity and specificity. We expected this performance, as it is similar to that of a host of other intestinal infections where molecular results are compared to culture results (20). As has been seen before (17), there was significantly more detection by qRT-PCR than by culture (17.6% of the stool samples were qRT-PCR positive, and 10.7% were culture positive), with significantly higher qRT-PCR quantities in culture-positive than culture-negative samples.

Poliovirus shedding is traditionally measured by culture, in

terms of both the proportions of children who are culture positive and the relevant durations (22). Measuring durations of shedding is particularly onerous because first culture must be performed and second it must be done with several sequential stool samples. Furthermore, we found that shedding by culture is so sporadic that in the absence of daily measurements or measurement of every stool sample, any duration information is inherently precarious. Additionally, without subtype and sequence information, it is impossible to determine whether sporadic shedding represents intermittent shedding post-OPV or new environmental acquisition.

Our qRT-PCR measurements in this cohort seem to mirror previous findings based on culture, with similar curves of poliovirus positivity early after OPV administration and a qRT-PCR viral copy number 1 to 2 logs higher than the 50% tissue culture infective dose. We demonstrate the principle that a qRT-PCR viral copy number cutoff (e.g., exceeding 8.4×10^5 copies/g of stool) could reasonably predict culture-positive shedders and a higher cutoff could further approximate shedding duration or frequency. The precision of these cutoffs is not certain and needs to be evaluated in other settings and in other cohorts with greater power. Yet the use of a qRT-PCR cutoff should assuage concerns that qRT-PCR overdetects insignificant infections or dead virus. One could also use qRT-PCR positivity with an analytical C_T cutoff of ~ 36 to 37 as a screen to determine which stool samples to culture for further testing. With these samples, one would have found a day that 237 of 1,350 stool samples were qRT-PCR positive, this subset could be cultured, and such a strategy would miss only 16 cultures (11% of all positives) and greatly reduce stool sample culture demands.

It may be particularly important to identify long-duration or high-frequency shedders since they appear to contribute disproportionately to the OPV reservoir. Indeed, we found by qRT-PCR that a small minority of infants contributed disproportionately to the total measured excretion. An individual SI from the qPCR data allowed the identification of high-risk shedders such as child 1107, who shed 39, 95, and 39% of the total S1, S2, and S3 burden of the cohort, respectively. This infant level quantitation is extremely difficult to obtain by culture in field studies, since most studies of OPV excretion measure the proportion of a population that is culture positive after vaccination and rarely determine the viral titer of the stool sample to identify the dilution that infects a cell line (2). Surprisingly, the infants with the highest SI at the week 14 OPV were different from those with the highest SI at the week 52 OPV. Further examination of risk factors for the shedding phenotype is under way; however, preliminarily, it does not appear that seronegativity correlates with this high-level shedding in that all of the top 3 52-week high shedders were seropositive (data not shown).

There were limitations to this work. This study was an intensive look at the week 14 and 52 OPV immunizations in an urban neighborhood of Dhaka. However, since OPV administration time points vary with a country's immunization practices, since the propensity to shed virus depends on environmental factors, and since shedding declines with subsequent OPV doses, the multiplex qRT-PCR assay's performance and empirical qRT-PCR cutoffs are not generalizable to all settings. As for the assay, several poliovirus qPCR assays have been described in the literature (16); however, ours has some differences. The assay described by Laassri et al. uses gel or SYBR green for detection and does not include an

external control, which we found useful for quantitation (23). We developed our assay on the basis of S1, S2, and S3 primers and probes developed by the CDC because these have a track record of use and therefore this assay can be compared to previous studies.

In sum, we believe that the careful use of qRT-PCR to monitor shedding will be an important contribution toward the goal of monitoring OPV and vaccine-derived poliovirus during eradication efforts.

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