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A method for detection of SARS-CoV-2 RNA in healthy human stool: a validation study

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Summary

Background Faecal shedding of SARS-CoV-2 has raised concerns about transmission through faecal microbiota transplantation procedures. Validation parameters of authorised tests for SARS-CoV-2 RNA detection in respiratory samples are described in product labelling, whereas the published methods for SARS-CoV-2 detection from faecal samples have not permitted a robust description of the assay methodology. We aimed to develop and validate a test specifically for detection of SARS-CoV-2 in human stool.

Methods In this validation study, we evaluated performance characteristics of a reverse transcriptase-real-time PCR (RT-rtPCR) test for detection of SARS-CoV-2 in human stool specimens by spiking stool with inactivated SARS-CoV-2 material. A modified version of the US Centers for Disease Control and Prevention RT-rtPCR SARS-CoV-2 test was used for detection of viral RNA. Analytical sensitivity was evaluated in freshly spiked stool by testing two-fold dilutions in replicates of 20. Masked samples were tested by a second laboratory to evaluate interlaboratory reproducibility. Short-term (7-day) stability of viral RNA in stool samples was assessed with four different stool storage buffers (phosphate-buffered saline, Cary-Blair medium, Stool Transport and Recovery [STAR] buffer, and DNA/RNA Shield) kept at –80°C, 4°C, and ambient temperature (approximately 21°C). We also tested clinical stool and anal swab specimens from patients who were SARS-CoV-2 positive by nasopharyngeal testing.

Findings The lower limit of detection of the assay was found to be 3000 viral RNA copies per g of original stool sample, with 100% detection across 20 replicates assessed at this concentration. Analytical sensitivity was diminished by approximately two times after a single freeze-thaw cycle at –80°C. At 100 times the limit of detection, spiked samples were generally stable in all four stool storage buffers tested for up to 7 days, with maximum changes in mean threshold cycle values observed at –80°C storage in Cary-Blair medium (from 29·4 [SD 0·27] at baseline to 30·8 [0·17] at day 7; p<0·0001), at 4°C storage in DNA/RNA Shield (from 28·5 [0·15] to 29·8 [0·09]; p=0·0019), and at ambient temperature in STAR buffer (from 30·4 [0·24] to 32·4 [0·62]; p=0·0083). 30 contrived SARS-CoV-2 samples were tested by a second laboratory and were correctly identified as positive or negative in at least one of two rounds of testing. Additionally, SARS-CoV-2 RNA was detected using this assay in the stool and anal swab specimens of 11 of 23 individuals known to be positive for SARS-CoV-2.

Interpretation This is a sensitive and reproducible assay for detection of SARS-CoV-2 RNA in human stool, with potential uses in faecal microbiota transplantation donor screening, sewage monitoring, and further research into the effects of faecal shedding on the epidemiology of the COVID-19 pandemic.

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Introduction Faecal microbiota transplantation (FMT) is of interest as a treatment for numerous conditions associated with dysbiosis of the gut microbiome, the most well studied of which is the treatment of recurrent Clostridioides difficile infection.1 The screening process for stool donors includes stool and blood testing to minimise the potential for transmission of pathogens of concern.2 Testing recommendations for stool donors are expected to change over time, particularly as new pathogens emerge. In 2020, the US Food and Drug Administration (FDA) issued safety alerts indicating the need for increased donor screening or changes in donor testing methods, including tests for multidrug-resistant organisms, enteropathogenic Escherichia coli, and Shiga toxin-producing E. coli.3,4 The emergence and global spread of COVID-19 and the discovery of SARS-CoV-2 in the stool of infected individuals has led to concerns regarding the possibility of SARS-CoV-2 transmission via FMT.5 In response to early reports of faecal shedding, the FDA issued a safety alert on March 23, 2020 (updated on April 9, 2020), to indicate that there should be no clinical use of FMT products manufactured from stool donated on or after Dec 1, 2019, until additional screening and testing
Research in context

Evidence before this study
On Feb 1, 2021, we searched PubMed for articles published since database inception, in any language, using the terms (“SARS-CoV-2” OR “COVID-19”) AND (“feces” OR “stool”). Our search identified multiple studies that have documented shedding of SARS-CoV-2 RNA in faeces and considered the potential for faecal-oral transmission of the virus. This potential risk led the US Food and Drug Administration (FDA) to issue a safety alert on March 23, 2020 (updated on April 9, 2020), recommending that stool donated after Dec 1, 2019, should be quarantined and not used for manufacture of faecal microbiota transplantation (FMT) products in the USA until sufficient screening procedures could be put in place to mitigate this risk. The addition of the term (“validated” OR “validation”) into our search yielded only 11 results, including many studies related to wastewater testing methods. Two studies assessed stool testing with some reference to validation, but neither study provided full validation according to the current FDA emergency use authorisation guidelines for COVID-19 respiratory diagnostics. We were also unable to find any studies assessing SARS-CoV-2 detection in healthy stool or the effects of storage conditions on the assay performance.

Added value of this study
We report the development and validation of an assay for the detection of SARS-CoV-2 RNA in the stool of healthy individuals. This assay does not rely on proprietary primer and probe sets or specialised testing platforms and was validated using inactivated SARS-CoV-2 spiked into healthy human stool specimens from multiple donors. Additionally, we provide data on viral stability in stool stored at various temperatures and in different transport media and assess the use of the method by testing it on stool and anal swabs from individuals with previous SARS-CoV-2-positive nasopharyngeal tests.

Implications of all the available evidence
We describe a validated SARS-CoV-2 stool assay with potential applications in FMT donor screening protocols, sewage monitoring protocols, and research studies assessing the role of stool shedding and transmission on the epidemiology of COVID-19. With minimal use of proprietary reagents or specialised equipment, this stool testing protocol is easily implemented at other laboratories, potentially improving the availability of other laboratory-developed testing protocols.

Methods

Study design and samples
In this validation study, we evaluated the performance characteristics of a reverse transcriptase real-time PCR (RT-rPCR) test for detection of SARS-CoV-2 in human stool specimens by spiking stool with inactivated SARS-CoV-2.

Heat-inactivated SARS-CoV-2 was used for spike-in experiments to facilitate safe handling. Virus stocks were propagated in vitro under biosafety level 3 conditions (appendix 1 p 1) and harvested supernatants were inactivated in a water bath at 60°C for 1 h. Inactivated stocks were diluted in sterile phosphate-buffered saline (PBS; Gibco, Gaithersburg, MD, USA), aliquoted, and stored at –80°C. Subsequent experiments with this material were done under biosafety level 2 conditions. To minimise degradation from freeze–thaw activity, fresh freezer stocks were used for each experiment. Absolute concentrations of viral genome targets were quantified using droplet digital PCR (appendix 1 p 1).

Initial RT-rPCR protocol development, limit of detection experiments, and subsequent experiments were done using human faecal material from a total of five donors purchased from two separate vendors (Lee Biosolutions, Maryland Heights, MS, USA, and OpenBiome, Somerville, MA, USA). All faecal samples were collected by the vendors with informed consent from healthy donors aged 18–50 years, with no use of antibiotics for at least 30 days before sample donation. To ensure SARS-CoV-2 negativity, only samples collected and frozen by the vendors before January, 2020, were purchased.

Procedures
The protocols for isolation of viral RNA from stool samples and RNA target detection were adapted from previously published work.19 Whole stool was diluted 1:5 (weight per volume) in sterile PBS, homogenised, and clarified by centrifugation at 4000 relative centrifugal force for 20 min at 4°C. 140 µL of clarified supernatant
was used as starting material for RNA extraction using the QIAamp Viral RNA mini kit (Qiagen, Germantown, MD, USA) spin-column protocol, with a final elution volume of 50 µL.

For detection of SARS-CoV-2 viral RNA, extracted RNA was tested via one-step RT-rtPCR using 2019_nCoV RUO testing kits (Integrated DNA Technologies, Coralville, IA, USA) with a modified thermal cycling protocol (appendix 1 p 1–2). The assay uses three sets of sequence-specific primers and fluorescent hydrolysis probes described in the SARS-CoV-2 US Centers for Disease Control and Prevention (CDC) EUA protocol (appendix 1 p 3).14 Two of the primer and probe sets, 2019_nCoV_N1 (N1) and 2019_nCoV_N2 (N2), target conserved regions within the nucleocapsid gene. A third set, targeting the human ribonuclease P (RNase P; RPP30) gene was used as an internal amplification and sample adequacy control. For this testing protocol, amplification of either the N1 or N2 viral targets with a threshold cycle (Ct) of less than 40·0 was considered SARS-CoV-2 positive. Samples were classified as negative when neither N1 and N2 amplified with Ct of less than 40·0 whereas the RNase P gene target was successfully detected with Ct of less than 40·0. Samples in which none of the targets amplified with Ct less than 40·0 were considered invalid.

An approximate range for the expected limit of detection was determined by testing stool specimens spiked with a ten-fold dilution series of heat-inactivated SARS-CoV-2 material. Negative stool slurries from two independent donors were spiked with heat-inactivated SARS-CoV-2 reference material from a two-fold dilution series and tested in replicates of 20 (ten per donor stool), including independent RNA extraction for each replicate. At least one unspiked stool from each donor was tested with each batch of extractions as a negative specimen control, along with no-template controls and positive controls for each reaction. The limit of detection was defined as the lowest concentration at which at least 95% of samples tested positive.

Leftover spiked stool material from these dilutions was stored at –80°C and later tested to investigate the effects of freezing SARS-CoV-2-positive samples at or near the limit of detection. Frozen samples at one and two times the empirical limit of detection were thawed and tested in replicates of eight (four per donor) to determine the effect of freeze–thaw activity on low-copy-number specimens.

Performance evaluation
24 positive specimens and six negative specimens (from a single donor during four different donations) were evaluated in duplicate rounds of testing. Positive specimens were contrived by spiking heat-inactivated SARS-CoV-2 reference material into a negative stool matrix at one, two, five, and ten times the limit of detection (six samples per concentration). Samples were prepared at the FDA (Silver Spring, MD, USA) and shipped overnight on dry ice to Stanford University (Stanford, CA, USA). All samples were analysed in duplicate according to the aforementioned testing protocols. The recipient laboratory was masked to the spike-in status of the specimens. Testing at Stanford University was done on the 7900 HT Fast Realtime PCR system (Applied Biosystems, Foster City, CA, USA).

Additionally, stool and anal swab specimens were obtained from paediatric patients with recent SARS-CoV-2-positive respiratory tests (in the previous 0–13 days). Samples were collected after obtaining verbal assent from patients and written consent from the patients’ parents under protocols approved by the institutional review board of Ann & Robert H Lurie Children’s Hospital (Chicago, IL, USA). RNA was extracted from stool at the Ann & Robert H Lurie Children’s Hospital and shipped overnight on dry ice to the FDA for RT-rtPCR analysis.

The stability of SARS-CoV-2 RNA was tested in stool stored in different buffers and at different temperatures. Four storage media were tested: PBS, Cary-Blair medium (appendix 1 p 2), Roche Diagnostics Stool Transport and Recovery (STAR) buffer (Fisher Scientific, Hampton, NH, USA), and DNA/RNA Shield (Zymo Research, Irvine, CA, USA). Stool slurries in each buffer (1·5 g of stool to 5 mL of buffer) were spiked with approximately 100 times the limit of detection of inactivated SARS-CoV-2. Aliquots of each slurry were stored at –80°C, 4°C, or ambient temperature (approximately 21°C). RNA was extracted from each set of samples immediately (day 0) and on days 1, 2, 3, and 7 of storage at 4°C and ambient temperature, and on day 7 only from samples stored at –80°C. Extracted RNA was stored at –80°C until the end of the experiment, when RT-rtPCR was done in duplicate using only the N1 target because it was more sensitive than the N2 target in previous experiments. This experiment was replicated using independently collected stool samples from the same donor, for a total of two biological replicates.

Data analysis
Mean sample Ct values and SDs were calculated in Microsoft Excel version 1902, and were inclusive of any detected Ct values, regardless of whether they met the 40 Ct cutoff for positive determination. We also calculated 95% CIs for mean Ct values in GraphPad Prism version 8.4.0 (GraphPad Software, La Jolla, CA, USA). Contrived clinical testing agreement rates (simple percentage agreement) and associated Clopper-Pearson 95% CIs were calculated using the Westgard QC online 2×2 contingency calculator tool.15 Quantitative Ct comparisons were plotted and analysed in GraphPad Prism. Fresh and frozen Ct values were compared using a mixed-effects model with Sidak multiple comparison testing, treating fresh or frozen status and storage buffers as the fixed effects and replicate measurements as random effects. Ct values from 4°C and ambient temperature storage conditions were analysed using a mixed-effects model.
model with Geisser-Greenhouse correction and Dunnett’s multiple comparison testing, treating storage conditions and sampling day as fixed variables and replicate measurements as random effects. Clinical Ct values were compared between positive whole stool and anal swab specimen types using a two-way ANOVA for paired N1 and N2 measurements, with no further post-hoc testing. We considered p values of less than 0·05 to be statistically significant, with multiplicity-adjusted p values when appropriate.

Role of the funding source
The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results
We developed a protocol for an RT-rtPCR-based assay for SARS-CoV-2 detection in stool samples and found its limit of detection to be 600 viral RNA copy equivalents per mL of stool matrix (table 1). After accounting for the 1:5 (weight per volume) stool dilution, this is equivalent to 3000 copies per g of whole stool using this extraction protocol. The test positivity rate was 19 (95%) of 20 replicates at a viral concentration of 1200 copies per mL of stool slurry, 20 (100%) of 20 at 600 copies per mL, and 14 (70%) of 20 at 300 copies per mL (table 1). The RNase P target was detected in all 20 replicates tested from each dilution, as well as in all negative specimen control extractions tested. At the limit of detection, N1 targets were positive in 19 (95%) of 20 replicates and N2 targets were positive in 16 (80%) of 20 replicates (table 1). Of note, N2 amplification above baseline fluorescence was detected in all 20 samples, but only 16 met the criteria for positive detection (Ct<40·0). The limit of detection in samples frozen at −80°C was twice that in freshly spiked samples. After one freeze–thaw cycle, samples had a test positivity rate of six (75%) of eight at the limit of detection and of eight (100%) of eight at two times the limit of detection (table 2).

The stability of SARS-CoV-2 RNA was evaluated in different storage buffers and at different temperatures. At baseline (day 0), the mean Ct value of spiked stool suspended in PBS was 28·8 (SD 0·13), in Cary-Blair medium was 29·4 (0·27), in STAR buffer was 30·4 (0·24), and in DNA/RNA Shield was 28·5 (0·15). Comparing samples stored at −80°C for 1 week with their respective baseline values (figure part A), mean Ct values increased significantly to 29·7 (0·14) in PBS (p<0·0001) and to 30·8 (0·17) in Cary-Blair medium (p<0·0001) but did not change significantly in STAR buffer (p>0·99) or DNA/RNA Shield (p>0·99).

Among samples stored at 4°C for up to 1 week, only those stored in DNA/RNA Shield had significant increases in Ct values above baseline, reaching significance on day 2 (p<0·0019) and a maximum mean Ct of 29·8 (SD 0·09) on day 7 (p<0·0001). Comparing those stored in Cary-Blair medium (p<0·0001) and in STAR buffer (p<0·0001) but not in DNA/RNA Shield (p>0·99).

In the interlaboratory assessment, 30 contrived samples were tested at Stanford University. Only one was found to be invalid due to the non-amplification of all three targets after initial testing. Of the 29 valid test results, 22 of 24 spiked samples tested positive, and five of five negative samples tested negative, for an overall correct testing rate of 93·1% (95% CI 78·0–98·1) in 27 of 29 valid results. In a replicate round of testing for all samples, three of the contrived samples returned invalid results. Of the 27 valid replicate test results, 21 of 23 contrived positive samples tested positive and four of four negative samples tested negative, for an overall correct testing rate of 92·6% (76·6–97·9) in 25 of 27 valid results. All false-negative tests were in the concentration range of one to two times the assay limit of detection, within which freezer storage can negatively affect SARS-CoV-2 detection (table 2). Of note, no false positive test results occurred and none of the samples tested were invalid or falsely negative in both rounds of replicate testing.

Table 1: Summary of LOD dilution testing in stool specimens freshly spiked with heat-inactivated SARS-CoV-2

| Viral RNA copies per mL of specimen | Equivalent copies per g of stool | SARS-CoV-2 positive† | N1 positive | Ct† | N2 positive | Ct† | RNase P positive | Ct† |
|-----------------------------------|---------------------------------|----------------------|-------------|-----|-------------|-----|-----------------|-----|
| 1·2×10³ | 6·0×10³ | 19/20 (95%) | 3·1 (1·0) | 16/20 (80%) | 38·5 (1·5) | 20/20 (100%) | 31·9 (0·5) |
| 6·0×10³ | 3·0×10³ | 19/20 (95%) | 34·6 (1·0) | 16/20 (80%) | 38·7 (1·5) | 20/20 (100%) | 32·2 (0·8) |
| 3·0×10³ | 1·5×10³ | 14/20 (70%) | 35·0 (0·5) | 7/20 (35%) | 40·3 (1·2) | 20/20 (100%) | 32·2 (0·8) |

Data are n/N (%) or mean (SD) unless specified otherwise. Ct=threshold cycle. LOD=limit of detection. RNase P=human ribonuclease P gene (RP30). *Positive detection of either the N1 or N2 target with Ct<40·0. †Calculations inclusive of LOD=limit of detection.

Table 2: Summary of LOD dilution testing after one freeze–thaw cycle

| Viral RNA copies per mL of specimen | Equivalent copies per g of stool | SARS-CoV-2 positive† | N1 positive | Ct† | N2 positive | Ct† | RNase P positive | Ct† |
|-----------------------------------|---------------------------------|----------------------|-------------|-----|-------------|-----|-----------------|-----|
| 1·2×10³ | 6·0×10³ | 8/8 (100%) | 8/8 (100%) | 34·6 (1·0) | 38·4 (1·5) | 20/20 (100%) | 31·9 (0·7) |
| 6·0×10³ | 3·0×10³ | 8/8 (100%) | 34·6 (1·0) | 7/20 (88%) | 38·4 (1·5) | 20/20 (100%) | 32·2 (0·8) |
| 3·0×10³ | 1·5×10³ | 4/8 (50%) | 35·0 (0·5) | 6/8 (75%) | 39·6 (1·8) | 20/20 (100%) | 32·2 (0·8) |

Data are n/N (%) or mean (SD) unless specified otherwise. Ct=threshold cycle. LOD=limit of detection. RNase P=human ribonuclease P gene (RP30). *Positive detection of either the N1 or N2 target with Ct<40·0. †Calculations inclusive of LOD=limit of detection.
day 7 (p=0.0007 vs baseline; figure part B). Although mean Ct values of samples stored in PBS (p=0.14) and Cary-Blair medium (p=0.44) at 4°C increased over time, each with a maximum increase at 7 days, these changes were not significant. Mean Ct values for samples stored in STAR buffer at ambient temperature increased significantly compared with baseline, reaching maximum values of 30·4 (0.09) for PBS (p<0.0001) and 32·4 (0.62) for STAR buffer (p=0.0083), both at day 7. Mean Ct values of samples stored in Cary-Blair medium and DNA/RNA Shield increased over time compared with baseline, reaching maximum observed increases at day 7; however, the increases were not significant (figure part C).

A total of 12 whole stool and 26 anal swab specimens were collected from 23 patients with recent SARS-CoV-2-positive nasopharyngeal PCR tests (table 3). The age of patients ranged from 5 months to 20 years at the time of sample collection, with a median age of 9 years (IQR 4·0–14·5). Both N1 and N2 viral RNA targets were detected in 22 clinical samples, including eight stool specimens from six different patients and 14 anal swab specimens from nine patients. No samples tested positive for a single viral target without testing positive for the other. Full details on patients, samples collected, and test results are provided in appendix 2.

Among specimens testing positive, we observed a wide range of Ct values for both the N1 (20·0–38·3) and N2 (21·2–39·6) gene targets. A narrower range was observed for the RNase P target (26·5–38·3) among all specimens. Mean Ct values were 28·7 (SD 4·6) for N1, 31·7 (5·1) for N2, and 31·6 (2·8) for RNase P. There was no significant difference in viral target Ct values between whole stool and anal swab specimen types (two-way ANOVA p=0.78).

**Discussion**

Widely documented faecal shedding of SARS-CoV-2 viral RNA, sometimes in the absence of positive respiratory testing, has led to an interest in assessing viral loads in stool for a range of purposes, from diagnostics to environmental monitoring for rapid outbreak detection.16–19 Additionally, the potential for transmission of SARS-CoV-2 via stool has led to concerns regarding the possible transmission as part of an FMT procedure, particularly with the possibility of asymptomatic shedding in stool.20–23 Therefore, SARS-CoV-2 testing needs to be incorporated into FMT donor screening protocols in the COVID-19 era.24–26 Here, we report the development and validation of a method for the detection of SARS-CoV-2 RNA in healthy human stool. We provide data indicating acceptable storage and transport conditions for material to be tested. Finally, we show the clinical application of this test in detection of SARS-CoV-2 RNA in the stool of infected patients.
individuals. Previous studies assessing methods for SARS-CoV-2 stool testing have either lacked validation complying with FDA EUA requirements or have assessed only the ability of specific commercial test kits to detect virus in cases of disease, with liquid stool samples being assessed for viral RNA.\textsuperscript{2,13,20} Additionally, other published methods for detection of viral RNA in stool were developed or validated with reference materials other than full, intact, inactivated SARS-CoV-2, which is recommended in the latest FDA guidance on COVID-19 diagnostics.\textsuperscript{2,19} By contrast, using accurate reference materials, including human stool from healthy donors and heat-inactivated SARS-CoV-2, we have shown sensitive and reproducible detection of SARS-CoV-2 RNA in stool using methods modified from the CDC RT-rtPCR diagnostic protocol.\textsuperscript{4} Importantly, over the course of all testing, heat-inactivated SARS-CoV-2 was spiked into stool from five separate healthy individuals and no donor-specific signal loss was observed. The reported assay has a low limit of detection (3000 copies per g of stool), which is similar to other molecular tests used for detection of other enteric pathogens.\textsuperscript{21} Further research is needed to understand the actual risk of faecal–oral transmission and the infectious dose of SARS-CoV-2 to fully evaluate the effectiveness of this assay.

The assay was able to detect SARS-CoV-2 RNA in 22 (58%) of 38 stool samples from patients with confirmed SARS-CoV-2 infection by nasopharyngeal PCR test, emphasising the complexity between nasopharyngeal and faecal viral loads. In one meta-analysis of 95 different studies, only 43% of infected patients tested positive for SARS-CoV-2 in stool or anal swab specimens.\textsuperscript{22} Thus, we did not expect all samples tested to find detectable amounts of SARS-CoV-2 RNA. Negative test results for stool samples included in this study could be due to either an absence of faecal shedding in those individuals or shedding at a level below the limit of detection of the assay. This discrepancy between nasopharyngeal and faecal positivity emphasises the limitations of stool testing for COVID-19 diagnostic use. Of note, our samples came primarily from individuals with a positive nasopharyngeal test who were symptomatic. Although testing in asymptomatic SARS-CoV-2-positive individuals would have been ideal, we were unable to obtain samples from this group.

Without access to stool from these individuals, we did an interlaboratory assessment using contrived samples in lieu of a clinical performance study. To prevent bias, the secondary laboratory doing the tests was masked to the status of contrived SARS-CoV-2 positive and negative specimens. Replicate testing results showed the reproducibility of this protocol between users in different laboratories. Although the blinded assessment did yield a small number of false-negative results at low viral spike-in loads, this was not unexpected based on our characterisation of analytical sensitivity in frozen samples at or near the limit of detection. However, replicate testing improved detection sensitivity, because all samples tested correctly in at least one of two rounds of testing. This finding provides support for incorporating replicate testing for the detection of low viral copy numbers in stool testing protocols. More work is needed to determine the expected viral load range in a study population of asymptomatic individuals infected with SARS-CoV-2. Due to these limitations, this evaluation was not sufficiently powered for robust estimation of clinical sensitivity or specificity.

During the early stages of the COVID-19 pandemic, high demand for testing reagents, as well as challenges with cold-chain management, necessitated evaluation of alternate specimen collection media and storage conditions.\textsuperscript{22} With this in mind, we sought to assess the loss of sensitivity observed after storage in some conditions, which represents a potential limitation of this assay. We evaluated the stability of heat-inactivated virus-spiked stool samples, at a moderately high viral copy number, stored in four different buffers and at different temperatures (–80°C, 4°C, or ambient temperature). Detection of SARS-CoV-2 was consistent across all media and storage temperatures. However, variation in detection signal (Ct value) suggests that both stool transport and dilution media and sample storage temperature should be considered when developing protocols for collection, storage, and testing for SARS-CoV-2 RNA in stool. Among the media tested, DNA/RNA Shield performed best overall. Samples stored in DNA/RNA Shield showed improved stability compared with PBS and STAR buffer at ambient temperature and compared with PBS at –80°C. Of note, DNA/RNA Shield performed better at ambient temperature than at 4°C, which is consistent with the manufacturer’s recommendations for use and should be considered when using this product. Cary-Blair medium, a common substrate for stool transport, provided the least protection from freezing but otherwise performed similarly to PBS at 4°C. The results after freezing of spiked stool samples did show potential negative effects on sample stability. Of note, freezer storage of spiked specimens reduced detection in samples with low copy numbers. This effect was also apparent in our reproducibility evaluation in which some frozen specimens with low copy numbers returned false-negative results. Because we were unable to evaluate viral RNA stability in stool from known positive donors, we do

| Specimens positive | All samples | Anal swabs | Stool specimens |
|-------------------|-------------|------------|-----------------|
| Patients positive | 11/23 (48%) | 9/18 (50%) | 6/10 (60%)      |
| N1 Ct             | 28.7 (4.6)  | 29.0 (5.3) | 28.0 (3.4)      |
| N2 Ct             | 31.7 (5.1)  | 31.9 (5.7) | 31.0 (4.4)      |
| RNase P Ct        | 31.6 (2.8)  | 31.9 (2.9) | 30.9 (2.6)      |

Data are n/N (%), or mean (SD). Ct=threshold cycle. RNase P=human ribonuclease P gene (RPP30).
not know whether freezing of raw stool samples would have a similar effect on SARS-CoV-2 RNA detection. Although transmission of SARS-CoV-2 via the fecal–oral route or in FMT remains a topic of debate, evidence of gastrointestinal infection has been reported, including isolation of infectious virus from stool.\textsuperscript{2,3,9} Here, we present a technical validation of methods developed for testing stool for SARS-CoV-2 viral RNA. In contrast to other published testing protocols, we considered detection of either N1 or N2 target sufficient for a SARS-CoV-2 RNA-positive test result, regardless of human RNase P amplification. Although some of our results were consistent with previous reports that the N1 primer and probe set is more sensitive than N2,\textsuperscript{7,2} our data include several valid results which were N2 positive and N1 negative, supporting the continued use of N2 in SARS-CoV-2 testing. We did not investigate the use of alternative SARS-CoV-2 RT-rtPCR primer and probe sets, nor did we explore alternative testing technologies. The data presented here show a validated stool detection method for SARS-CoV-2 RNA that has the potential for use in a variety of applications, including FMT donor screening, sewage monitoring, and clinical research.

Contributors
MPC, JLH, WJM, and PEC designed the study. MPC did assay development, validation, and implementation. MPC, MAF, and PEC analysed the data. MI, NP, PPM, and MAF did secondary site implementation and testing. CZL and TTW generated inactivated virus used in the studies. JR, DBW, TH-S, LNS-P, JC, and WJM acquired and processed clinical samples. MPC, JLH, RLS, WJM, and MAF assisted in manuscript preparation. MPC, JLH, and PEC verified all underlying data in the study. All authors had full access to all the data in the study, reviewed the final manuscript, and had final responsibility for the decision to submit for publication.

Declaration of interests
PEC reports grants from the National Institute of Allergy and Infectious Diseases, US National Institutes of Health, during the conduct of the study. All other authors declare no competing interests.

Data sharing
All data generated in these studies are available upon request made to the corresponding author.

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