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Sensitive and Specific Quantitative Detection of Rotavirus A by One-Step Real-Time Reverse Transcription-PCR Assay without Antecedent Double-Stranded-RNA Denaturation

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A real-time quantitative reverse transcription-PCR (qRT-PCR) assay using the recombinant thermostable *Thermus thermophilus* (r*Tth*) enzyme was developed to detect and quantify rotavirus A (RVA). By using r*Tth* polymerase, significant improvement was achieved over the existing real-time RT-PCR assays, which require denaturation of the RVA double-stranded RNA (dsRNA) prior to assay setup. Using a dsRNA transcript for segment 7, which encodes the assay target NSP3 gene, the limit of detection for the improved assay was calculated to be approximately 1 genome copy per reaction. The NSP3 qRT-PCR assay was validated using a panel of 1,906 stool samples, 23 reference RVA strains, and 14 nontarget enteric virus samples. The assay detected a diverse number of RVA genotypes and did not detect other enteric viruses, demonstrating analytical sensitivity and specificity for RVA in testing stool samples. A XenoRNA internal process control was introduced and detected in a multiplexed qRT-PCR format. Because it does not require an antecedent dsRNA denaturation step, this assay reduces the possibility of sample cross-contamination and requires less hands-on time than other published qRT-PCR protocols for RVA detection.

Group A rotaviruses (RVA) are estimated to cause 453,000 deaths among infants and young children each year (1). The genome of RVA, members of the *Reoviridae* family, consists of 11 double-stranded RNA (dsRNA) segments which encode six viral structural proteins (VP1 to VP4, VP6, and VP7) and six nonstructural proteins (NSP1 to NSP6) (2). Surrounding the dsRNA are three concentric protein layers, namely, a central core (VP2), a middle protein layer (VP6), and an outer capsid composed of VP7 and VP4 proteins, while the viral RNA-dependent RNA polymerase (VP1) and the RNA capping enzyme (VP3) are packaged within the core shell (3). Traditionally, viral classification has been based on the serological characteristics and sequence diversity of the outer capsid proteins, VP7 (glycosylated, G-type) and VP4 (protease sensitive, P-type) (4). Although 27 G genotypes and 35 P genotypes have been identified to date (5), 5 strains (G1P[8], G2P[4], G3P[8], G4P[8], and G9P[8]) are associated with 80% to 90% of the global RVA disease burden (6).

Numerous techniques have been developed to detect RVA in stool samples. These techniques include, but are not limited to, virus isolation in cell culture, electron microscopy (EM), enzyme immunoassays (EIA), coupled reverse transcription and PCR amplification (RT-PCR), and real-time quantitative RT-PCRs (qRT-PCRs) (2, 7–19). The molecular techniques are more rapid than the cell culture-based techniques and are more sensitive than EM or EIA. The threshold for detection of RVA by EM is approximately 10^7 viral particles/ml of stool (20). EIAs are 10 to 100 times more sensitive than EM assays, but the sensitivity and specificity of EIAs are more variable (21–23). A number of studies using RT-PCR assays targeting the RVA VP4, VP6, and VP7 gene segments have shown increases of 15% to 27% in the rate of RVA detection in comparison with EIAs (24–27). RT-PCR assays employ either one-step or two-step reverse transcription followed by an amplification approach to achieve increased sensitivity for the detection of RVA, and all require denaturation of the dsRNA before it is added to the RT-PCR mixture (7–9). qRT-PCR assays offer several advantages over traditional RT-PCR, including increased sensitivity, higher throughput, and faster turnaround time as well as quantification of viral loads. Several qRT-PCR assays have been developed for detection of RVA targeting VP2 (28),VP4 (29, 30), VP6 (17, 19, 31–33), VP7 (18, 30), NSP3 (13–16, 34), and NSP4 (35) genes. Due to the genetic diversity of VP4 and VP6 gene segments, the NSP3 gene, encoded by genomic segment 7, has been shown to be the best target for detection of a wide variety of RVA genotypes (13–16, 34, 36). Multiple qRT-PCR assays have targeted a highly conserved region near the 3′ end of the NSP3 gene (14–16, 34).

Most molecular assays for the detection of RVA described to date require antecedent denaturation of the RVA dsRNA before it is introduced into the RT or RT-PCR mixture. The denaturation of dsRNA is accomplished typically by heating for 5 min at 95 to 97°C and is necessary to separate the dsRNA strands prior to the reverse transcription step. Omission of the denaturation step decreases the efficiency of reverse transcription and subsequent amplification of the dsRNA (14). The heat denaturation step must be performed on the dsRNA before it is added to the RT or RT-PCR mixture since heat denaturation of the dsRNA postaddition to the master mix inactivates thermolabile reverse transcriptase enzymes. Avian myeloblastosis virus and Moloney murine leukemia

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virus reverse transcriptase enzymes, both native and recombinant versions lacking RNase H activity, are enzymatically inactive at temperatures greater than 70°C (37).

The *Thermus thermophilus* DNA polymerase (deoxynucleoside triphosphate [DNA deoxynucleotidyltransferase]; EC 2.7.7.7) is a 94-kDa thermostable DNA polymerase that possesses 5′-to-3′ DNA polymerase activity and 3′-to-5′ reverse transcriptase activity in the presence of Mn^{2+} ions and has a half-life of approximately 20 min at 95°C (38, 39). Recombinant *T. thermophilus* (r*Tth*) DNA polymerase has been used for RT-PCR detection of single-stranded RNA viruses such as "Norwalk-like" viruses (40), hepatitis A virus (41), hepatitis C virus (42), foot-and-mouth disease virus (43), simian immunodeficiency virus (39), and Japanese encephalitis virus (44).

In previous studies, standard curves for determination of RVA load using qRT-PCR were generated using native viral RNA, plasmid constructs, cDNA, or single-stranded RNA transcripts (13–17, 19, 28, 29, 33). No previous studies describing RVA qRT-PCR assays have used an artificial dsRNA similar to an actual genomic segment.

In this study, we developed a bona fide one-tube, one-step qRT-PCR assay for detection of the dsRNA RVA NSP3 gene using r*Tth* polymerase and validated the assay using an extensive sample panel. In addition, we have made the assay quantitative through incorporation of a dsRNA positive-control transcript (artificial segment 7) for determining the viral load in samples and have evaluated the introduction of an internal process control detected in multiplex qRT-PCR format.

MATERIALS AND METHODS

Stool samples and RNA extraction. Stool samples ($n = 1{,}906$) from surveillance studies, vaccine effectiveness studies, and outbreak investigations in the United States and from international surveillance studies (45–47) were tested in this study. All specimens were collected between 1998 and 2012 and were analyzed previously by EIA (Premier Rotaclone rotavirus detection kit; Meridian Diagnostics, Inc., Cincinnati OH) and heminested genotyping RT-PCR (47). Of the 1,906 samples, 1,295 previously tested negative for RVA by both EIA and RT-PCR and the remaining samples were determined to be positive for RVA by EIA or RT-PCR or both methods. All samples were deidentified and could not be traced back to patient or hospital case identifiers. Ten percent suspensions of each sample were prepared in phosphate-buffered saline (PBS), and RNA was extracted using an automated KingFisher extraction system (Thermo Electron Corporation, Vantaa, Finland), a MagNA Pure 96 instrument (Roche Applied Science, Indianapolis, IN), or a MagNA Pure Compact instrument (Roche Applied Science, Indianapolis, IN) in accordance with the manufacturers' protocols. RNA extracts were stored at −80°C until qRT-PCR testing.

Reference virus strains. Twenty-three reference RVA strains, previously propagated in MA104 cells, were extracted as described for stool samples except that undiluted stocks were used. The strains used were as follows: Wa (G1P[8]), DS-1 (G2P[4]), ST3 (G4P[6]), P (G3P[8]), US1205 (G9P[6]), 1076 (G2P[6]), 116E (G9P[11]), 69M (G8P[10]), AU-1 (G3P[9]), B223 (G10P[11]), CC425 (G3P[9]), F123 (G14P[12]), HOCHI (G4P[8]), K8 (G1P[9]), L26 (G12P[4]), M37 (G1P[6]), NCDV (G6P[1]), OSU (G5P[7]), Ro1845 (G3P[3]), S2 (G2P[4]), Se584 (G4P[9]), WI61 (G9P[8]), and WC3 (G6P[5]). RNA extracts were stored at −80°C until qRT-PCR testing. Twelve non-RVA gastroenteritis virus strains, including norovirus groups GI (GI.3C and GI.2) and GII (GII.6C and GII.4 New Orleans), sapovirus (GI.2, GII.2, and GIV), astrovirus (Ty3, Ty7, and Ty8), and adenovirus (types 40 and 41), were extracted using the KingFisher extraction system, and nucleic acid extracts were stored at −80°C until qRT-PCR testing.

Generation of a dsRNA standard. A positive-control RNA template was generated from the RVA NSP3 gene (genomic segment 7) of laboratory strain Wa (G1P[8]) RNA. The dsRNA was denatured at 95°C for 5 min and then subjected to RT-PCR. The transcription template was prepared using a Qiagen One-Step kit (Qiagen, Inc., Valencia CA) according to the manufacturer's instructions with the following segment 7-specific primers with T7 promoters at each 5′ end: T7S7.2-F (5′-TAA TAC GAC TCA CTA TAG GGA GAT TTA ATG CTT TTC AIT GGT TGA TGC-3′) and T7S7-R (5′-TAA TAC GAC TCA CTA TAG GGA GAG GTC ACA TAA CGC CC TAT A-3′). The cycling parameters were as follows: 60°C for 30 min; 95°C for 15 min; 30 cycles of 94°C for 30 s, 65°C for 30 s, and 72°C for 45 s; 72°C for 7 min; and a 4°C hold. The RT-PCR was analyzed on a 1% agarose gel, and the specific band was purified using a QIAquick gel extraction kit (Qiagen, Inc., Valencia, CA). The purified product was then used in a single T7 transcription reaction to synthesize dsRNA using a MEGAscript RNA interference (RNAi) kit (Ambion, Austin, TX) following the manufacturer's protocol. dsRNA transcript quality was visualized by electrophoresis on a 1% nondenaturing agarose gel, and full-length transcript was extracted from the gel by using a Zymoclean gel RNA recovery kit (Zymo Research Corp., Irvine, CA). The concentration of the transcript was determined by measuring absorbance at 260 nm using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE), followed by calculation of the dsRNA transcript molarity.

qRT-PCR assay. The qRT-PCR assay was performed by using a GeneAmp EZ r*Tth* RNA PCR kit (Applied Biosystems, Inc., Foster City, CA). Each 25-μl reaction mixture contained 7.5 μl of nuclease-free water, 1× EZ buffer, 300 nM (each) deoxynucleoside triphosphates (dNTP), 2.5 mM $Mn(OAc)_2$, 0.1 U r*Tth* polymerase, 500 nM (each) forward and reverse primer, 150 nM probe, and 5 μl of undenatured RNA extract. The previously published forward primer NVP3-FDeg (5′-ACC ATC TWC ACR TRA CCC TC-3′), reverse primer NVP3-R1 (5′-GGT CAC ATA ACG CCC CTA TA-3′), and probe NVP3-Probe (5′-A 6-carboxyfluorescein [FAM] TG AGC ACA ATA GTT black hole quencher 1 [BHQ1] AAA AGC TAA CAC TGT CAA-3′) were used (14). After adding the reaction mixture and undenatured RNA to MicroAmp Fast Optical 96-well reaction plates (Applied Biosystems, Inc., Foster City, CA), template denaturation, RT, and PCR amplification were carried out on an ABI 7500 Fast real-time PCR system (Applied Biosystems, Inc., Foster City, CA) in standard mode. Thermocycling conditions consisted of a 5-min hold at 95°C for dsRNA template denaturation, 30-min hold at 50°C for RT, 1 min at 95°C, and 45 cycles of 15 s at 95°C and 1 min at 60°C. A test result was considered positive if a sigmoidal amplification curve crossed the threshold before 45 cycles and all positive and negative control reactions gave expected results. In order to establish the limit of detection (LOD) and efficiency of the assay, 10-fold dilutions of the transcript were prepared in water treated with diethyl pyrocarbonate (DEPC) (Ambion, Austin TX) and containing 100 μg/ml of carrier yeast RNA (Bio-Rad Laboratories, Hercules, CA) and dilutions from 10^{-4} to 10^{-10} were tested using the assay as described above. Assay efficiency $[10^{(-1/\text{slope})}]$ was calculated from the slope of the standard curve, which was generated by plotting the log copy number versus the cycle threshold (C_T) value. Copy numbers were calculated by using the following equation:

$$\text{copy number (molecules/}\mu\text{L)} = [\text{concentration (ng/}\mu\text{L)} \times 6.022 \times 10^{23} \text{ (molecules/mol)}]/[\text{length of amplicon} \times 640 \text{ (g/mol)} \times 10^{9} \text{ (ng/g)}].$$

Internal process control and multiplexed qRT-PCR. To introduce an internal process control into the NSP3 qRT-PCR assay, 10^5 copies of XenoRNA (Life Technologies Corp., Grand Island, NY) were spiked into a 50-μl volume of 10% stool suspension prepared in PBS and extracted on a KingFisher extraction system. The extracted RNA was analyzed using the NSP3 qRT-PCR assay with a GeneAmp EZ r*Tth* RNA PCR kit in singleplex using the previously described NSP3 assay and the multiplexed NSP3-Xeno qRT-PCR assay described here. Each 25-μl reaction mixture contained 6.75 μl of nuclease-free water, 1× EZ buffer, 300 nM (each)

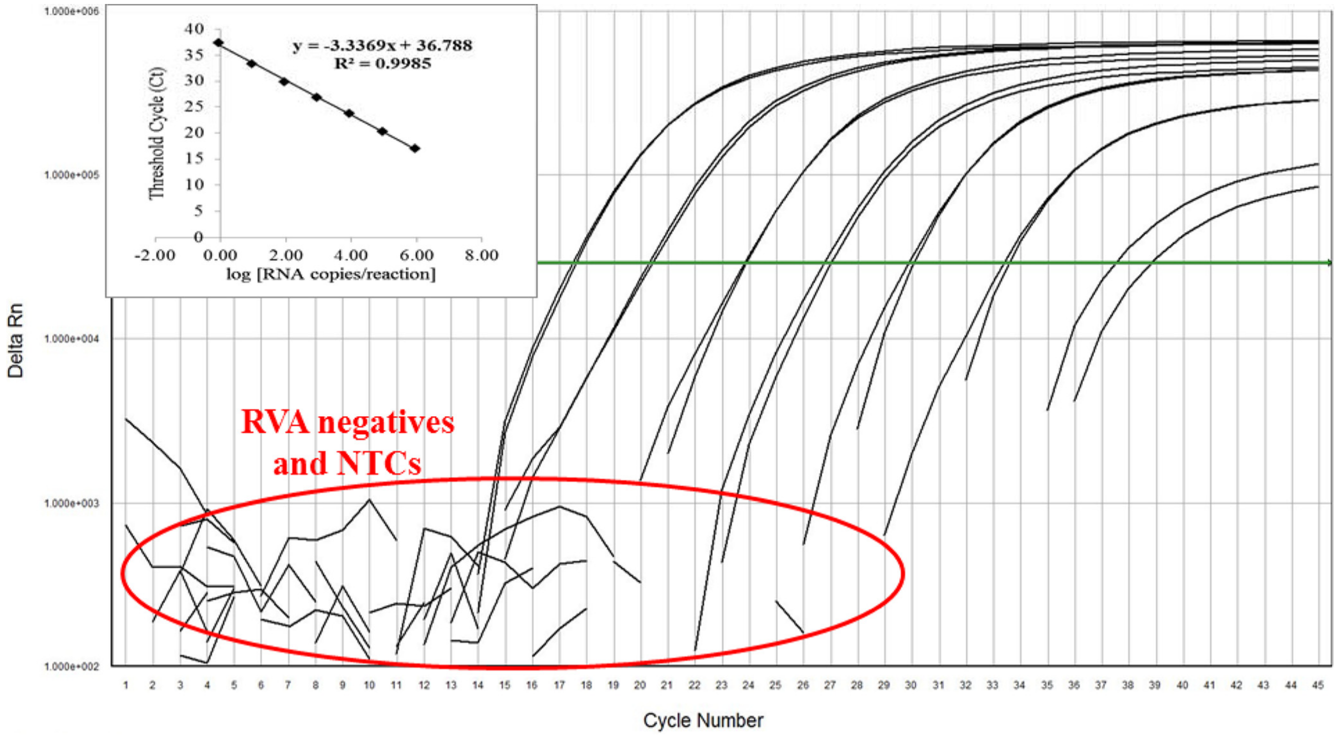

FIG 1 Amplification curves of 10-fold dilutions of RVA strain Wa dsRNA transcript and the linear relationship between threshold cycle (Ct) and log transcript copy number per reaction (insert). Serial 10-fold dilutions of strain Wa dsRNA transcript were tested using the NSP3 qRT-PCR assay. The graph showing the C_T value versus the log copy number was fitted with a regression line, and the slope for calculation of efficiency was obtained from the regression line. Dilutions of dsRNA transcript ranging from 10^{-4} to 10^{-10}, corresponding to 9.0×10^5 and 0.9 copies per reaction, respectively, are shown. The efficiency of the assay was calculated to be 99.38%. The *y* axis unit represents relative fluorescence, and Delta Rn refers to the fluorescence emission of a reaction minus the background fluorescence measured from early cycles of the real-time PCR run before a logarithmic increase in fluorescence. The fluorescent signals from RVA-negative samples and no-template controls (NTCs) are indicated by the red circle.

dNTP, 2.5 mM $Mn(OAc)_2$, 0.1 U r*Tth* polymerase, 500 nM each forward and reverse NSP3 primer, 150 nM NSP3 probe labeled with FAM, 400 nM (each) forward and reverse XenoRNA primers, 100 nM XenoRNA probe labeled with Texas Red, and 5 μl of undenatured RNA. The thermocycling conditions and real-time systems described above were used for this experiment except that plate readings were done for both the FAM and ROX-Texas Red channels. The efficiency of each assay was calculated as described previously.

RESULTS

qRT-PCR assay efficiency. To determine the efficiency of the new assay, a 10-fold dilution series of the dsRNA transcript was prepared and used as a standard in assay runs. Each template and non-template control (NTC) was tested in duplicate. Analysis of the calibration data indicated that the range of the quantitative analysis was from 9.0×10^5 to 0.9 copies per reaction, corresponding to C_T values of 17.0 to 37.4, respectively, and approximately 3.3 cycles were recorded between the C_T values of consecutive 10-fold template dilutions of the dsRNA standard (Fig. 1). The plot of C_T values versus log transcript copy numbers indicated a linear correlation with a R^2 value of 0.9985, and the efficiency of the assay was calculated to be 99.38% (Fig. 1). The limit of detection (LOD), which was detected 90.1% of the time (20 of 22 replicates), was calculated to be 0.9 copies per reaction (Fig. 1). The detection frequency of 9.0 copies was 100%, and dilutions of dsRNA transcript at 0.09 copies were never detected.

qRT-PCR assay sensitivity. A total of 1,906 stool samples were extracted and subjected to analysis using the NSP3 qRT-PCR assay (Table 1). Compared to the EIA and RT-PCR assays, this assay has a sensitivity of 100% (Table 1). Increases of 17% and 10% in the detection rate were recorded for the NSP3 qRT-PCR assay compared to EIA and RT-PCR, respectively. The majority of EIA-positive samples contained more than 9.6×10^4 NSP3 gene (segment 7) copies per reaction, and over 96% had more than 100 NSP3 gene copies (Table 2). Four EIA-positive, RT-PCR-negative samples were detected by the NSP3 qRT-PCR assay (Table 2). Of the EIA-negative, RT-PCR-positive samples, more than half con-

TABLE 1 Detection of rotavirus using real-time RT-PCR compared to EIA and conventional nested RT-PCR methods

	No. of samples with indicated qRT-PCR result				
Assay and result	Positive	Negative	Total	Sensitivity (%)	Specificity (%)
EIA					
Positive	439	0	443		
Negative	317	1,070	1,387		
Total	756	1,074	1,830	99	77
Genotyping RT-PCR					
Positive	597	0	597		
Negative	183	1,109	1,292		
Total	780	1,109	1,889	100	86

TABLE 2 Quantitative distribution of NSP3 gene copies as determined by qRT-PCR assay in samples from gastroenteritis cases based upon their EIA and RT-PCR testing results

EIA and RT-PCR results	No. of samples	No. of qRT-PCR positives	Copy no. per reaction[a]				
			$8.9 \times 10^7 \le n < 9.6 \times 10^4$	$9.6 \times 10^4 \le n < 100$	$100 \le n < 3.4$	$3.4 \le n < 1$	≤ 1
EIA$^+$, RT-PCR$^+$	467	467	323	128	11	5	0
EIA$^+$, RT-PCR$^-$	4	4	0	2	0	2	0
EIA$^-$, RT-PCR$^+$	140	140	10	54	47	20	9
EIA$^-$, RT-PCR$^-$	1,295	180	4	30	60	56	30
Total	1,906	791	337	214	118	83	39

[a] *n*, copy number per reaction.

tained 100 or fewer NSP3 gene copies per reaction and approximately 20% had fewer than 3.4 copies (Table 2). The NSP3 qRT-PCR assay detected RVA in 180 samples that were negative by EIA and RT-PCR and could not be genotyped. More than 80% of these samples contained 9.6×10^4 or fewer NSP3 gene copies per reaction, 48% contained fewer than 100 copies, and 17% contained 1 copy or less than 1 copy (Table 2).

RVA strains represented in the stool samples tested included monotypic genotypes G1P[8], G2P[4], G3P[8], G4P[8], G9P[6], G9P[8], G12P[6], G12P[8], G1P[4], G1P[6], G2P[6], G2P[8], G3P[4], G3P[6], G3P[9], G4P[6], G8P[4], G9P[4], and G12P[4] as well as mixed genotypes G1,2P[4,8], G1,2P[4], G1,2P[8], G1,3P[8], G1,9P[8], G1P[4,8], G2,3P[4], G2,3P[8], G2,9P[4], G2,9P[8], G2P[4,6], G2P[4,8], G3,4P[8], G3,9P[8], G4,12P[6], G9P[4,8], and G14P[24] (Table 3). The NSP3 qRT-PCR assay was able to detect all RVA genotypes present in the stool samples (Table 3) as well as the 23 reference rotavirus strains (data not shown). For all genotypes, the highest proportion of samples contained more than 9.6×10^4 copies per reaction (Table 3).

Assay specificity. Compared to EIA and RT-PCR for detection of RVA, the NSP3 qRT-PCR assay had specificities of 78% and 86%, respectively (Table 1). Tested using nucleic acid extracts for 12 non-RVA gastroenteritis viruses, no fluorescent signal was generated with any of the nontarget viruses (data not shown).

Precision. To determine precision for this assay, three operators tested 8 samples in triplicate on different days (Table 4). All 9 replicates for 5 positive and 3 negative RVA samples were positive and negative, respectively; therefore, the precision of the assay was calculated to be 100%.

Detection of the internal process control. Seven 10-fold dilutions of an RVA-positive stool and an RVA-negative stool were spiked with XenoRNA and then extracted. The RNA extracts were then tested using the NSP3 qRT-PCR in singleplex format as well as a multiplexed NSP3 and XenoRNA assay. The efficiencies of the NSP3 assay were calculated to be 94.25% and 94.09% for the assay performed in singleplex and multiplex, respectively (Fig. 2). Thus, the presence of XenoRNA in stool samples had no effect on NSP3 qRT-PCR amplification and did not interfere with RVA dsRNA detection and quantitation. No NSP3 amplification was observed for RVA-negative stools containing XenoRNA extract in either the singleplex or multiplex qRT-PCR assay. We have also found that the addition of 2×10^5 copies of XenoRNA to stool specimen suspensions prior to extraction does not interfere with extraction or detection of RVA RNA; C_T values were unchanged when duplicate specimen dilution series were tested with and without the addition of XenoRNA (data not shown).

DISCUSSION

A rapid, highly sensitive, and highly specific one-step qRT-PCR assay which eliminates the need for the antecedent dsRNA denaturation has been developed and extensively validated for the detection of RVA, as well as for quantification of viral load in stool samples, using dsRNA transcript as a standard. To the best of our knowledge, this is the first bona fide one-step RVA molecular

TABLE 3 Distribution of NSP3 qRT-PCR assay C_T values in samples from gastroenteritis cases based on genotype

Genotype	No. of samples	No. of qRT-PCR positives	Copy no. per reaction[a]				
			$8.9 \times 10^7 \le n < 9.6 \times 10^4$	$9.6 \times 10^4 \le n < 100$	$100 \le n < 3.4$	$3.4 \le n < 1$	≤ 1
G1P[8]	91	91	30	26	19	10	6
G2P[4]	104	104	53	33	13	5	0
G3P[8]	125	125	79	37	6	2	1
G4P[8]	22	22	12	8	2	0	0
G9P[6]	19	19	15	4	0	0	0
G9P[8]	60	60	44	10	4	2	0
G12P[6]	11	11	6	4	0	1	0
G12P[8]	55	55	39	15	0	1	0
Other strains[b]	120	120	54	47	13	4	2
Total	607	607	332	184	57	25	9

[a] *n*, copy number per reaction.

[b] Other strains: G1P[4], G1P[6], G1,2P[4,8], G1,2P[4], G1,2P[8], G1,3P[8], G1,9P[8], G1P[4,8], G2P[6], G2P[8], G2,3P[4], G2,3P[8], G2,9P[4], G2,9P[8], G2P[4,6], G2P[4,8], G3P[4], G3P[6], G3P[9], G3,4P[8], G3,9P[8], G4P[6], G4,12P[6], G8P[4], G9P[4], G9P[4,8], G12P[4], and G14P[24].

TABLE 4 Precision measurement data for the NSP3 qRT-PCR assay

	Copy no. per reaction for sample:							
Operator	1	2	3	4	5	6	7	8
1	3.5×10^5	7.5×10^5	ND[a]	ND	6.7×10^5	9.4×10^5	2.1×10^5	ND
	3.5×10^5	7.1×10^5	ND	ND	6.4×10^5	9.2×10^5	2.5×10^5	ND
	3.4×10^5	7.1×10^5	ND	ND	6.3×10^5	9.1×10^5	2.3×10^5	ND
2	3.6×10^5	6.5×10^5	ND	ND	7.1×10^5	9.2×10^5	1.8×10^5	ND
	3.6×10^5	6.5×10^5	ND	ND	7.0×10^5	9.6×10^5	2.2×10^5	ND
	3.4×10^5	6.6×10^5	ND	ND	6.3×10^5	1.0×10^5	2.7×10^5	ND
3	3.3×10^5	7.5×10^5	ND	ND	7.2×10^5	1.2×10^5	2.3×10^5	ND
	3.7×10^5	3.4×10^5	ND	ND	7.4×10^5	1.0×10^5	2.5×10^5	ND
	2.4×10^5	7.3×10^5	ND	ND	6.8×10^5	9.3×10^5	2.4×10^5	ND
Avg	3.4×10^5	6.6×10^5	ND	ND	6.8×10^5	9.8×10^5	2.3×10^5	ND
SD	4.1×10^4	1.3×10^5	ND	ND	4.1×10^4	8.2×10^4	2.4×10^4	ND

[a] ND, not detected.

detection assay in which the dsRNA denaturation, RT, and PCR are carried out with uninterupted thermal cycling. Most previously published RNA detection assays for RVA required a denaturation step prior to RNA addition to the RT or RT-PCR mixture (13, 14, 17, 28, 29, 31–33, 35). Typically, RVA dsRNA and oligonucleotides are mixed in a reaction vessel and heated for 5 min at 95 to 97°C followed by 1 min incubation on ice. The RT or RT-PCR mixture is prepared in a separate tube and then combined with the mixture of denatured RNA and oligonucleotides. We have found that omission of the dsRNA denaturation step when performing the NSP3 qRT-PCR assay results in a 9-to-10-cycle increase in C_T values, corresponding to a 3-log reduction in analytic sensitivity (data not shown). The advantages of performing a denaturation and an RT-PCR with uninterrupted thermocycling include decreased manipulation of samples, a reduced time to results, and a decreased possibility of sample cross-contamination and false-positive results. These advantages are particularly evident when high-throughput testing is performed.

This is the first RVA qRT-PCR assay to use an artificial quantitative standard that simulates an actual RVA genomic segment. Previously, the standard curves for quantification of RVA were generated using a variety of standards—native viral RNA, plasmids, cDNA, and ssRNA transcripts. Using the various standards, reported LODs of RVA qRT-PCR assays ranged from an estimated 44 copies of genomic RNA per reaction (14) to approximately 1 cDNA target copy per reaction (29). Ward et al. (36) determined the LOD of 4 published qRT-PCR assays (13, 16–18) to be 2.5 copies per reaction using plasmid standards. The improved NSP3 assay developed in this study has an LOD of approximately 1 segment 7 or NSP3 gene copy per reaction and thus has analytical sensitivity equal to that of the most sensitive previously described qRT-PCR assays. Previous studies have shown that each RVA virion contains equimolar amounts of each of the 11 segments; however, the number of each of those segments per virion has not been determined (48, 49). Production of native viral RNA is labor-intensive—it requires growth of RVA in culture and possible requirements for titration of stocks and virus purification by ultracentrifugation. Also, the use of native RVA RNA does not permit determination of the precise target copy number; it only provides estimates based upon the quantity of RNA tested and RVA genome size (14). The use of plasmid DNA, a molecule which differs greatly in structure from an RVA genomic RNA segment, does not incorporate a control for the RT step during the qRT-PCR. Additionally, the plasmids are very stable macromolecules which can increase the potential for sample and laboratory contamination when used as standards for qRT-PCR. Similarly, ssRNA transcripts and cDNA differ from dsRNA in size, molecular weight, and structural complexity. The artificial dsRNA transcript developed in this study has the same structural characteristics of a natural dsRNA segment, can be produced in large quantities through *in vitro* transcription, and can be easily quantitated. Therefore, we believe that the artificial dsRNA transcript is the best positive control for generating the standard curves in order to determine RVA loads in samples and for determining and comparing assay performance. We propose that the artificial dsRNA transcript be adopted as the universal standard for RVA qRT-PCR assays.

The sensitivity and specificity of our NSP3 qRT-PCR assay were determined to be 100% and 78%, respectively, when EIA was used as the gold-standard assay and 100% and 86%, respectively, when RT-PCR was used as the gold-standard assay. The superior sensitivity and reduced specificity of the NSP3 qRT-PCR assay can be attributed to the use of less-sensitive gold-standard assays, which introduces reference-test bias (50). Previous versions of this NSP3 qRT-PCR assay were found to be 3 to 4 logs more sensitive than the Premier Rotaclone EIA (14) and 100-fold more sensitive than conventional heminested RT-PCR (15).

The region of NSP3 amplified as described in this and previous reports (13–16, 34) is an optimal target because it is highly conserved compared to regions of other genome segments, including VP4, VP6, and VP7, used to develop other qRT-PCR assays (10, 15, 17, 33, 36, 51). Choosing a highly conserved region is important because RVA genomes continue to evolve, and amplifying a target with low variability and broad strain detection is essential for preserving the analytical performance of the assay. Even though we demonstrated this assay to be highly specific and sensitive, some rarer human RVA genotypes (for example, G6P[9] or G10P[14]) (52) were not tested due to our inability to acquire such genotypes. However, a high degree of sequence similarity

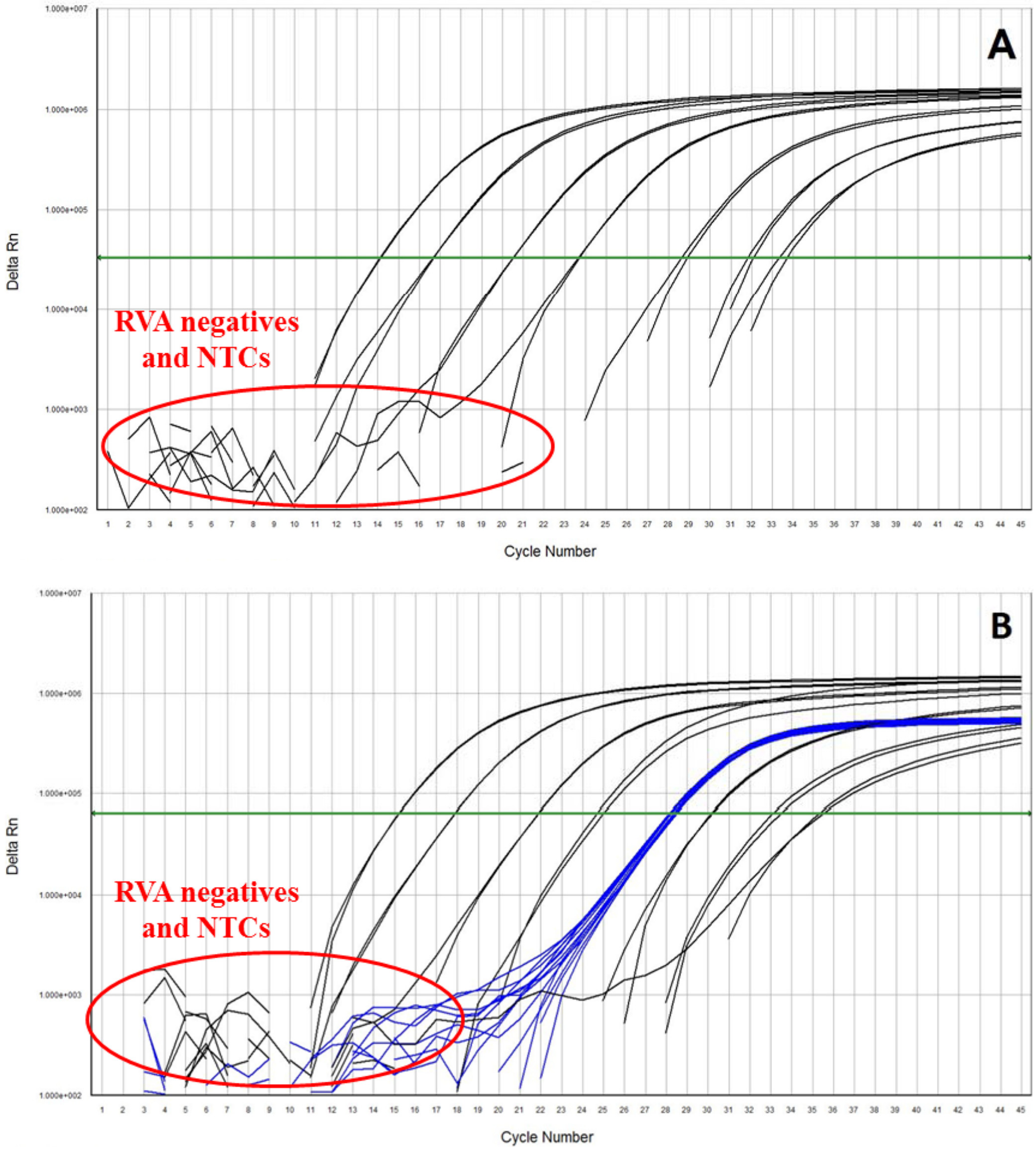

FIG 2 Amplification curves of 10-fold dilutions of RVA-positive stool sample spiked with XenoRNA process control obtained by singleplex NSP3 qRT-PCR assay (A) and multiplexed NSP3-Xeno qRT-PCR assay (B). Black amplification curves represent RVA amplification with the NSP3 qRT-PCR assay, and blue amplification curves represent XenoRNA amplification with the Xeno assay. Seven 10-fold dilutions of a rotavirus-positive sample were spiked with 10^5 copies of XenoRNA process control and extracted using the KingFisher extraction system. Extracted RNA for each dilution was tested by both an NSP3 qRT-PCR assay in singleplex and a multiplexed NSP3(FAM)-Xeno(Texas Red) qRT-PCR assay. The graph showing the C_T value versus the sample dilution was fitted with a regression line, and the slope for efficiency calculation was obtained from the line. Efficiencies of the singleplex and multiplex assays were calculated to be 94.25% and 94.09%, respectively. The *y* axis unit represents relative fluorescence, and Delta Rn refers to the fluorescence emission of a reaction minus the background fluorescence measured from early cycles of the real-time PCR run before a logarithmic increase in fluorescence. The fluorescent signals from RVA-negative samples and NTCs are indicated by the red circles.

noted previously among all genotypes, rare and common, in the NSP3 region targeted in this report (14, 15, 34) suggests that it has a high probability of success in the detection of rare genotypes.

A limitation of this study is that a small number of samples were tested in the multiplex assay with the internal process control. The proprietary internal control RNA and its detection assay were made available after we had finished assay validation. We plan to do future testing of RVA samples in multiplex with an internal process control assay and obtain performance data for the multiplexed assay. Also, we used multiple nucleic acid extraction systems, so in some cases the quantitative data are not directly comparable due to possible extraction method bias. An additional limitation is that we did not have a confirmatory assay for the 180 qRT-PCR-positive samples that were negative by EIA and RT-PCR. All replicates for these samples, however, yielded reproducible positive results when tested by qRT-PCR, suggesting that these detections were not artifactual. The NSP3 qRT-PCR assay itself has some limitations. Some qRT-PCR assays (14) incorporate a "no-reverse transcriptase" control to detect potential amplicon contamination in the laboratory. Since the r*Tth* enzyme used in this assay possesses both reverse transcriptase and DNA polymerase activities, this type of control cannot be incorporated. Users of the assay must carefully monitor "no-template control" reactions for possible amplicon contamination. An issue common to almost all qRT-PCR assays for RVA is that the increased sensitivity of the assay compared to techniques such as EIA increases the likelihood that positive results would be obtained in cases when another gastroenteritis agent, i.e., norovirus, adenovirus, *Escherichia coli*, etc., is actually the causative agent of disease, particularly when RVA is detected at lower copy numbers. RVA is shed typically at very high levels in the feces of RVA gastroenteritis cases (53, 54). Thus, testing for other agents of gastroenteritis should be performed, particularly when viral load is less than 100 NSP3 gene copies, since our study found that less than 6% of all EIA and/or RT-PCR RVA-positive specimens fell in this range and RVA detections in these instances may be incidental.

In conclusion, the qRT-PCR assay described in this report was shown to be highly sensitive and specific for a broad spectrum of RVA genotypes, with several advantages over previously published qRT-PCR assays. Most notably, this is the first bona fide one-step molecular assay for detection of RVA in stool specimens. Additionally, this qRT-PCR assay may be very useful for testing other clinical (i.e., serum, cerebrospinal fluid [CSF]) or environmental samples. Finally, the assay in combination with the dsRNA transcript is a true quantitative tool for determination of RVA load in stool samples.

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