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Evaluation of an Alternative Recombinant Thermostable *Thermus thermophilus* (r*Tth*)-Based Real-Time Reverse Transcription-PCR Kit for Detection of Rotavirus A

Eric M. Katz,[a] Rashi Gautam,[a] Michael D. Bowen[b]

IHRC Inc., Atlanta, Georgia, USA[a]; Division of Viral Diseases, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia, USA[b]

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A previously published one-step real-time quantitative reverse transcription-PCR (qRT-PCR) targeting the group A rotavirus (RVA) NSP3 gene is a sensitive technique for detection of RVA (1). This assay used the GeneAmp EZ recombinant thermostable *Thermus thermophilus* (r*Tth*) RNA PCR kit (Applied Biosystems, Inc., Foster City, CA, USA), which was discontinued in July 2014. Thus, a replacement kit was needed to perform the assay.

Here, we report evaluation of a one-step RT-PCR master mix kit (EMD Millipore Corporation, Billerica, MA, USA) for detection of the RVA NSP3 gene. RNA extraction with an MS2 bacteriophage RNA (ZeptoMetrix, Buffalo, NY, USA) internal process control (IPC) (1–3) was conducted as described previously. Each 25-μl reaction mixture contained 7.75 μl nuclease-free water, 12.5 μl 2× one-step RT-PCR master mix (r*Tth* DNA polymerase, antibody, buffer, deoxynucleotides), 1.25 μl 50 mM manganese(II) acetate [$Mn(OAc)_2$], the NSP3 (400 nM) and MS2 (400 nM) forward and reverse oligonucleotide primers and probes (NSP3-FAM, MS2-Texas Red, with the probes at 100 nM), and 2 μl of undenatured RNA extract. NSP3 and MS2 primer/probe sequences were published previously (1–4). Assay modifications for use of the EMD Millipore kit were as follows: NSP3 primer and probe concentrations were reduced to 400 nM and 100 nM, respectively; thermal cycling conditions were changed to 40 cycles of 15 s at 95°C and 1 min at 60°C; and the assay was run on an Applied Biosystems 7500 Fast real-time PCR system in fast mode instead of standard mode. Establishment of the limit of detection and efficiency of the EMD Millipore-based assay was as described previously (1), except that the calculation to determine the double-stranded RNA (dsRNA) copy number per reaction was amended to use RNA template volume rather than the ratio of RNA template volume to overall reaction volume (see Fig. S1 in the supplemental material).

Amplification curves of 10-fold dilutions of RVA strain Wa dsRNA transcript spiked with MS2 RNA showed that the dynamic range of the EMD Millipore-based assay spanned 6.5×10^6 to 6.5 copies/reaction, with threshold cycle (C_T) values ranging from 16.6 to 37.6. Approximately 3.3 cycles separated the C_T values of each dilution, with an R^2 value of 0.9991 and an assay efficiency of 94.69% (Fig. 1). The MS2 IPC was detected across the dilution series and in the NSP3-negative controls, and no amplified product was detected in the no-template controls (NTCs) (Fig. 1). Using the previously published calculations, the dynamic range for the GeneAmp-based assay spanned 9.0×10^5 copies to 0.9 copy per reaction, with C_T values ranging from 17.0 to 37.4, an R^2 value of 0.9985, and an assay efficiency of 99.38% (1). The change in assay efficiency using the EMD Millipore-based assay may be attributed to reduced primer/probe concentrations,

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Address correspondence to Michael D. Bowen, mkb6@cdc.gov.

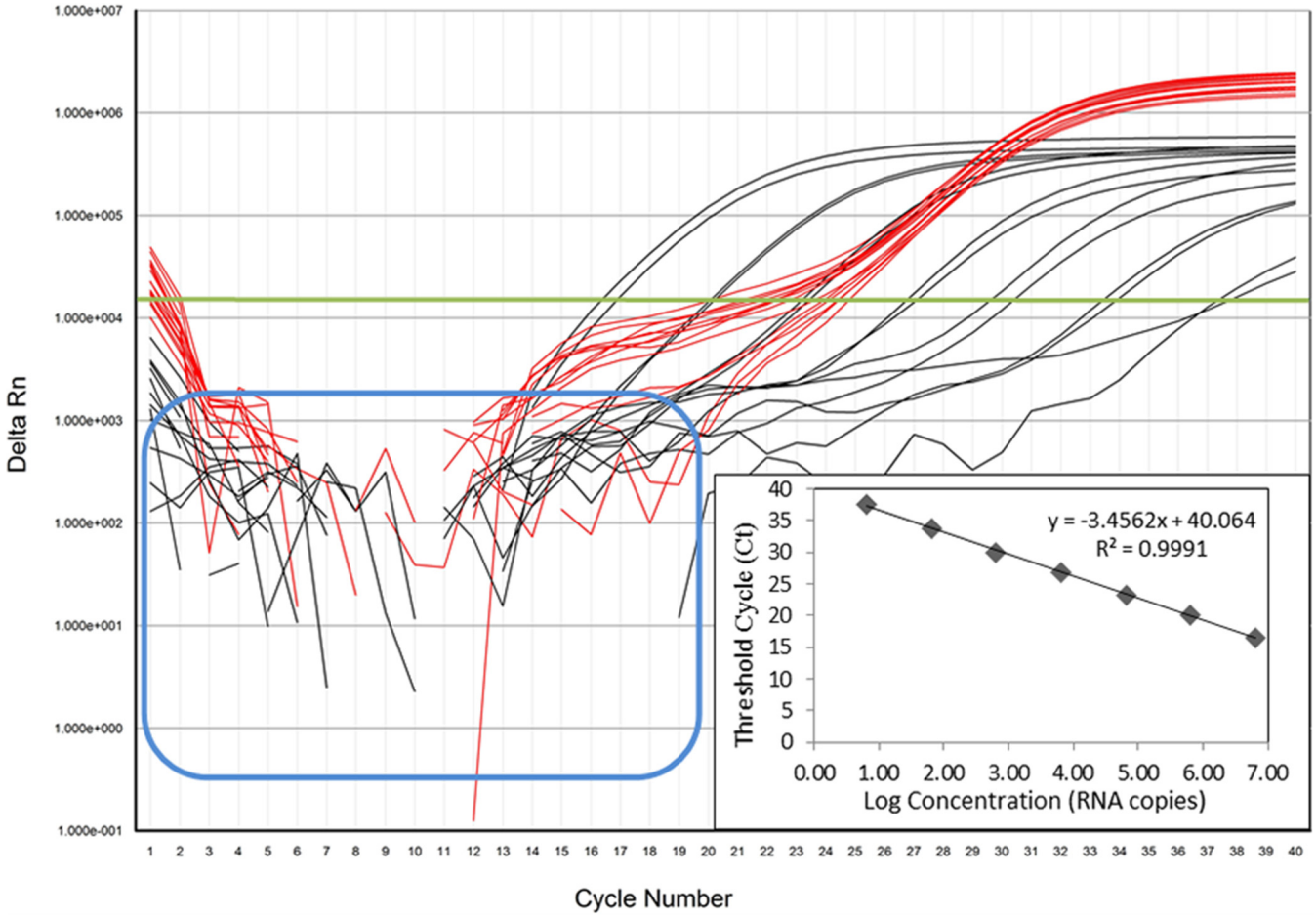

FIG 1 Amplification curves of 10-fold dilutions of the RVA strain Wa dsRNA transcript spiked with MS2 bacteriophage RNA (from 6.5×10^6 to 6.5 copies per reaction), obtained with the EMD Millipore NSP3 qRT-PCR assay. Using the threshold for delta Rn (the normalized reporter value [Rn] of the reaction minus the Rn of the baseline signal) (green line), black curves show the 10-fold dilutions of the NSP3 gene transcript and red curves show MS2 IPC amplification. 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. The fluorescent signals from RVA-negative samples and no-template controls are indicated by the blue outline.

running the assay in fast mode instead of standard mode, and/or differing polymerase efficiencies between kits.

The EMD Millipore-based assay was compared to the GeneAmp-based assay using previously frozen clinical stool samples ($n = 111$; 50 samples were positive and 61 negative as determined by previous testing for RVA antigen and/or RNA) run in duplicate to measure agreement (5) between the two kits (222 total qRT-PCRs) (Tables S1 and S2). Positive percent agreement (PPA), negative percent agreement (NPA), and overall percent agreement (OPA) of the EMD Millipore-based assay were 100.00%, 83.05%, and 90.99% (5), respectively, when the GeneAmp-based assay was used as the gold-standard assay (Table S2). Poor NPA can be attributed to the use of a less-sensitive gold-standard assay, introducing test bias (1). Increased NSP3 gene detection by the EMD Millipore-based assay can be attributed to differences in enzyme activity, which were confirmed through discrepant analysis. Three of seven samples, where both replicates showed NSP3 qRT-PCR amplification using the EMD Millipore-based assay but were not detected using the GeneAmp-based assay (Tables S1 and S2), were confirmed as RVA positive using a nested RT-PCR assay for detection of RVA (6). Comparatively, the EMD Millipore-based assay showed significantly lower C_T values (*t* test [paired], $P < 0.0001$; the average difference was 0.815) (Table S1), along with an increase of 9.01% in RVA detection (Tables S1 to S2). A qualitative assessment of precision for the EMD Millipore-based assay in which three operators tested 20 samples (10 positive samples and 10

negative samples, as determined by previous testing for RVA RNA) in triplicate on different days showed that all 90 replicates for the RVA-negative samples were negative and that 86 of 90 replicates for the RVA-positive samples were positive. The four false-negative results for detection of RVA were for low-titer samples likely just under the established limit of detection. The precision of the EMD Millipore-based assay was calculated to be 97.78%. Despite differences from analysis of assay standards and the NPA observed through parallel-kit comparison, evaluation of the EMD Millipore one-step master mix kit suggests that it is a suitable alternative for the GeneAmp EZ r*Tth* RNA PCR kit used in the original rotavirus NSP3 gene qRT-PCR assay.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JCM.00126-17.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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