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Importance of a heat snap in RT-PCR quantification of rotavirus double-stranded RNA in wastewater

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Quantification of copies of double stranded RNA using RT-PCR methods may require denaturation of the double stranded structure using an initial high temperature incubation followed by rapid cooling, herein called "heat snap". Papers in the literature that report rotavirus RNA concentrations in fecal and environmental samples do not consistently report the use of such a "heat snap". In this study, we quantified rotavirus RNA in diverse environmental samples (wastewater solids, wastewater, and drainage samples) using digital RT-PCR methods with and without a heatsnap. Concentrations were higher in samples by a factor of 125 when a heat snap was applied. This was consistent across sample types, and across laboratories and PCR instrumentation. We recommend a heat snap be used when enumerating double stranded RNA from rotavirus and other double stranded RNA viruses in environmental samples.

Water impact

Rotavirus is one of many health relevant double stranded RNA (dsRNA) viruses that is important in water systems. This study presents original research to improve measurement precision of RNA from dsRNA viruses. We show that an initial high temperature and cooling step is needed (a heat snap) to accurately measure rotavirus RNA in wastewater samples. As application of molecular methods to measure pathogens in water becomes more and more common place, it is essential to include a heat snap for measurement of dsRNA viruses.

Introduction

Enumeration of copies of genomic RNA that is natively double stranded is uncommon in molecular biology as dsRNA genomes are amongst the rarest found in viruses; an estimated 3% (430 of 16 215) of known viruses have dsRNA genomes.[1] When using a quantitative RT-PCR approach to quantify dsRNA, an initial high temperature incubation step may be needed to denature dsRNA to ensure the RNA template is available for the reverse transcription (RT) enzyme to synthesize cDNA. This heating step is typically accomplished by heating the reaction at 95–100 °C for 5 min, and then cooling on ice or at 4 °C prior[2] to RT. Herein we refer to this as a "heat snap". The heat snap step is sometimes conducted on the extracted dsRNA alone,[3] and sometimes on the dsRNA combined with primers and dNTP.[4]

Despite the published use of the heat snap to denature dsRNA prior to RT, we find that it is not universally implemented in studies quantifying rotavirus from clinical or environmental samples. In 2024, Awere-Duodu and Donkor[5] published a systematic review of prevalence rates of rotavirus detection in diverse water environments. The review identified 75 publications that reported detection or quantification of rotavirus in drinking water, ground water, sewage, and surface waters, of which 74 used PCR-based detection of RNA. Amongst these 74 publications, only 17 (23%) reported the steps for the heat snap directly in the methods. An additional 10 (14%) did not include the heat snap steps in the methods, but referenced prior studies that describe clearly the heat snap. The remaining 47 (64%) did not describe a heat snap step or include a reference to a prior study or protocol describing a heat snap step, suggesting the authors did not include or were not aware of this step (compiled data provided at Stanford Digital Repository: **https://doi.org/10.25740/rk700qq6039**). It should be noted

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that we did not further address data quality of these papers (*e.g.*, whether they followed MIQE[6] guidelines). In many of the studies that did not include a heat snap, reverse transcription kits were referenced that do not include guidance on denaturing dsRNA prior to reverse transcription. Given the substantial diversity in sample types, locations, and extraction and detection methods, we did not attempt to determine the impact of heat snap on prevalence of rotavirus amongst these studies.

We recently published a systematic review of the literature to identify studies that report concentrations of rotavirus RNA in human excretions (for example, feces, sputum, urine).[7] This set of compiled papers, which were evaluated by us for data quality as part of that study, offered a convenient set to examine whether or not researchers report using a heat snap during rotavirus dsRNA quantification from clinical samples. We identified 25 papers that report rotavirus RNA in excretions as measured using quantitative RT-PCR methods, these papers all happened to be focused on RNA quantification in stool. Of these papers, ten[8–17] did not report using a heat snap, twelve[18–28] did report using a heat snap, and three[29–31] did not provide cycling conditions or clear references to the cycling conditions and therefore it was not possible to discern whether they used a heat snap. It is not necessarily appropriate to compare measurements obtained in the different studies because they each report concentrations of rotavirus RNA in excretions from individuals experiencing different stages and severities of rotavirus infections when RNA concentrations might be higher or lower due to various biological factors. Despite this limitation, we found that rotavirus RNA concentrations reported in papers describing using a heat snap had significantly higher $\log_{10}$ concentrations than those that did not ($\log_{10}$-mean of samples with heat snap = 6.9, $\log_{10}$-mean without heat snap = 6.3 $\log_{10}$ copies per g; *t*-test, $t = 4.428$, dof = 718, $p < 10^{-4}$, compiled data provided at the Stanford Digital Repository: **https://doi.org/10.25740/rk700qq6039**). These findings suggest not using a heat snap may underestimate the concentration of a dsRNA target, especially if the dsRNA is not already denatured in the media where it is being quantified.

Wastewater measurements of infectious disease biomarkers can allow public health professionals and infectious disease researchers to infer information on the occurrence of diseases in contributing populations.[32] As rotavirus is one of the most important etiologies of diarrheal disease and can cause significant morbidity and mortality in children,[33] it is a potentially important target for wastewater surveillance. Rotavirus RNA has been enumerated in wastewater matrices previously,[34–40] but none of the studies describe using a heat snap in their methods. Rotavirus RNA in wastewater may be encapsulated in damaged or intact viral capsids, or exist external to a capsid, and it may be fragmented, or denatured. If the dsRNA is not denatured in wastewater, then the use of a heat snap may be needed for sensitive and accurate rotavirus RNA quantification.

The goal of the present study was to determine whether inclusion of a heat snap affects quantification of rotavirus RNA in wastewater using digital RT-PCR. The work was carried out in two different laboratories using different instruments.

Methods

Quantification of rotavirus RNA in wastewater solids

Wastewater solids were collected from 31 wastewater treatment plants located in 13 states across the United States (Table 1) between 29 April and 27 June 2025. Several plants provided more than one sample yielding a total of $n = 38$ samples. Samples were provided to Stanford University (Stanford, California, USA) for the WastewaterSCAN project. The methods for wastewater solids sample collection, nucleic-acid extraction are outlined in detail by Boehm *et al.*[41] and in published protocols[42,43] and not repeated herein. Nucleic-acids were stored at −80 °C for approximately 7 days prior to analysis. Samples were thawed at 4 °C and immediately used as template in droplet digital one-step RT-PCR reactions using rotavirus primers and probes described previously.[44] The assay was run in multiplex with previously described assays for parvovirus B19,[45] adenovirus group F,[44] and measles[46] RNA using the probe mixing approach (Table 2). The droplet generator, thermocycler, and droplet readers were purchased from Biorad, as described previously.[41] Two different cycling conditions were used using the exact same reaction composition including the same template. One set of conditions did not include a heat snap and are reported by Boehm *et al.*[41] The other set of conditions included an initial denaturation step where the reaction was heated to 99 °C for 5 min followed by cooling at 4 °C for 5 min. These conditions are subsequently referred to as conditions without and with a heat snap, respectively. Nucleic-acid templates were run neat in 6 replicate wells and results from the replicate wells were merged for post processing following methods previously described.[41] A total

Table 1 Locations (city, state) where wastewater solids were collected in the United States. Number of samples per sit is 1 per site except for Kansas City, KS ($n = 6$), Merced, CA ($n = 2$), and Turlock, CA ($n = 2$)

Akron, OH	Red Wing, MN
Clinton, IA	Rochester, MN
Davis, CA	Sacramento, CA
Essex junction, VT	San Jose, CA
Gilroy, CA	Santa Cruz, CA
Harrison, AR	San Mateo, CA
Jackson, MI	San Francisco
Jenison, MI	Stafford, VA
Kansas City, KS	Sunnyvale, CA
Mankato, MN	Sunnyvale, TX
Merced, CA	Turlock, CA
Napa, CA	Union Beach, NJ
Oceanside	Wheaton, IL
Orange County, FL	Wheeling, WV
Palo Alto	Wichita Falls, TX
	Youngstown, OH

Table 2 Sequences for PCR primers and probes used in this study. For assays marked with an asterisk, primers and probes were purchased from Integrated DNA Technologies (Coralville, IA, USA) and those probes contained fluorescent molecules and quenchers (5′ HEX, FAM, Cy5, ROX, and/or ATTO950/ZEN/3′ IBFQ); FAM, 6-fluorescein amidite; HEX, hexachloro-fluorescein; Cy5, cyanine-5; Cy5.5, cyanine5.5; ROX, carboxyrhodamine; ZEN, a proprietary internal quencher from Integrated DNA Technologies (Coralville, IA, USA); and IBFQ, Iowa Black FQ. Primers and probes for other assays were purchased from Microsynth AG (Balgach, Switzerland) and contained one of the fluorescent molecules listed above and a black hole quencher (BHQ-1 or BHQ-2)

Target	Sequences (5′-3′)	
Rotavirus[47]	Forward	GGCTTTTAAAGCGTCTCAGT
	Reverse	AATYTATAGCTATCRTTCTCYARATG
	Probe	HEX/CCATGGCTGAGCTAGCTTGCTT/BHQ-1
Norovirus GII[48]	Forward	ATGTTYAGRTGGATGAGATTCTC
	Reverse	TCGACGCCATCTTCATTCAC
	Probe	Cy5/TGAGCACGTGGGAGGGCGATCGC/BHQ-2
*Parvovirus B19 (ref. 26)	Forward	CCACTATGAAAACTGGGCAATA
	Reverse	GCTGCTTTCACTGAGTTCTTCA
	Probe	ROX/AATGCAGATGCCCTCCACCCAG/ZEN/3′ IBFQ
*Measles[46]	Forward	AGGATGAGGCGGACCARTACTT
	Reverse	CRATATCTGAGATTTCCTTGTTCTC
	Probe	FAM/CATGATGATCCAAGTAGTAGTGA/ZEN/3′ IBFQ
*Rotavirus[49]	Forward	CAGTGGTTGATGCTCAAGATGGA
	Reverse	TCATTGTAATCATATTGAATACCCA
	Probe	ATTO590/ACAACTGCAGCTTCAAAAGAAGWGT/ZEN/3′ IBFQ
*HAdV[44]	Forward	CCTCCTGTGTTACGCCAGA
	Reverse	CAGGCTGAAGTASGTATCGG
	Probe	ROX/ATTO590/CTCGATGATGCCGCAATGGT/ZEN/3′ IBFQ

of six 96-well PCR plates were run (3 with and 3 without a heat snap). Each plate included 1 well with PCR positive controls, 2 to 4 wells with extraction negative controls, and 2 to 3 wells with no template PCR controls, as described elsewhere.[41] Concentrations are provided in units of copies per gram dry weight and errors are provided as standard deviations. In order for a sample to be scored as positive, it had to have at least three positive droplets across the replicate wells.[41] The lowest detectable concentration, calculated as the concentration for a sample with 3 positive droplets, was approximately 1000 copies per g dry weight solids. Additional details about the methods that follow dMIQE guidelines[6] can be found elsewhere.[41]

Quantification of rotavirus RNA in raw wastewater and drain samples

Raw wastewater and drainage samples (potentially containing spilled wastewater or fecal material) were collected from ten drainage channels and two wastewater treatment plants in Kampala, Uganda, from 17 and 28 March 2025. Sampling occurred over ten working days at each site, yielding a total of n = 119 samples (99 drainage samples and 20 wastewater samples). Each grab sample consisted of 50 mL, treated with $MgCl_2$ to achieve a final concentration of 25 mM. Samples were vacuum-filtered using S-Pak® membrane filters (mixed cellulose esters, pore size 0.45 μm, diameter 47 mm; Merck, Cat. No. HAWG047S6). The nucleic acids were extracted from the sample using AllPrep PowerFecal Pro DNA/RNA Kit (Qiagen, Cat. No. 80254). Filters were subsequently torn into small pieces with sterile forceps and transferred into the PowerBead Pro Tube, followed by the manufacturer's protocol. To remove potential PCR inhibitors, the 100 μL extracted nucleic acid was purified using the Zymo OneStep PCR inhibitor removal kit (Zymo Research, Cat. No. D6030). There were no extraction replicates. Extraction blanks were included with each day of sampling, prepared using 50 mL of nuclease-free water and processed in parallel following the same procedure. Purified extracts were 3× diluted with nuclease-free water to minimize potential inhibition and stored at −80 °C until shipment on dry ice to the Eawag Laboratory (Dübendorf, Switzerland). Upon arrival, extracts were stored at −80 °C for 83 to 106 days prior to analysis; differential storage was required for logistical reasons as all samples could not be run at once. Quantification of rotavirus RNA was performed using a one-step digital RT-PCR assay on the naica® PCR system (Stilla Technologies, France). Samples were run in duplicate. The assay employed a duplex primer/probe set for simultaneous detection of rotavirus (HEX-labeled) and norovirus GII RNA (Cy5-labeled).

Each RT-dPCR reaction consisted of a total pre-reaction volume of 27 μL, including 5.4 μL of RNA template and 21.6 μL of mastermix. The mastermix contained qScript XLT One-Step RT-qPCR ToughMix (2×) (Quantabio, USA, Cat. No. 95132), 0.5 μM of forward and reverse primer, 0.2 μM of each probe, 0.05 μM of fluorescein sodium salt (VWR, Cat. No. 0681-100G), and RNase-free water. The sequences of the rotavirus and norovirus GII primers and probes are provided in Table 2.

As with the solid samples above, two different cycling conditions were used using the exact same reaction composition including the same template. One set of conditions did not include a heat snap and the other set included an initial denaturation step where the template was heated to 95 °C for 5 min followed by cooling on ice

for 5 min and then centrifuged briefly before being added to the reaction. The reaction mixture (reaction volume 25 μL) was loaded onto Sapphire chips (Stilla Technologies) were partitioned into droplets of an average 0.519 nl using a Geode system (Stilla Technologies; 12 min at 40 °C), followed by thermocycling with the following conditions: reverse transcription (50 °C for 1 h), enzyme activation (95 °C for 5 min), and 45 cycles consisting of denaturation (95 °C for 15 s) and annealing/extension (54 °C for 1 min). After the reaction, the chips were scanned using a Naica Prism3 Crystal Reader v.4.10.0.3 (Stilla Technologies). Droplet counts and fluorescence signals across three channels (blue, green, and red) were analyzed using Crystal Miner software v.4.10.0.3 (Stilla Technologies) based on manual thresholding using lines in one dimension. As a quality control measure, samples with <15 000 droplets generated were considered invalid. Across all samples, an average 25 305 droplets were generated, with standard deviation of 2515. Absolute concentration of rotavirus RNA (cp μL_{dPCR}^{-1}) was calculated automatically by the software using Poisson distribution analysis of positive droplets, and the unit was converted into cp mL^{-1} considering the volumes of sample (V_{sample}), reaction ($V_{reaction}$), extract elution ($V_{elution}$), and PCR template ($V_{template}$) and dilution factor: gc mL^{-1} = gc μL_{dPCR}^{-1} × ($V_{reaction}$ × $V_{elution}/V_{template}$) × Dilution factor ÷ V_{sample}. Samples were run in technical duplicates, along with a positive control and a no-template control (NTC). Results were expressed as copies per milliliter (cp mL^{-1}), with errors calculated as the standard deviation between duplicates. The lowest detectable concentration was 8 cp ml^{-1}, the concentration corresponding to three positive droplets; samples with less than 3 positive droplets were considered non-detect.

Ethics approval

The protocols for collecting and processing samples from Uganda were approved by the Vector Control Division Research Ethics Committee in Uganda (approval no. VCDR-2024-65) and the Eawag Ethical Review Committee.

Statistics

The ratio of RNA measured with and without a heat snap was calculated; the ratio was found to be log-normally distributed using a Shapiro–Wilks test (p = 0.18). A t-test using the $\log_{10}$-transformed data was used to test the null hypothesis that that ratio for solids is the same as for the liquid (wastewater and drainage) samples, and a p value of 0.05 is used to assess significance. A linear regression was used to assess the relationship between measurements made with and without a heat snap. All analyses were performed using Rstudio (version 1.4.1106) with R (version 4.0.5). All measurements, as well as dMIQE checklists,[6] are deposited in the Stanford Digital Repository (**https://doi.org/10.25740/rk700qq6039**).

Results

All negative and positive controls were negative and positive, respectively indicating that assay performance was acceptable. For the work flow for the wastewater solids, the negative controls all contained 0 positive droplets with the exception of 1 NTC had 1 positive rotavirus droplet (out of 37 975 total droplets) indicating the limit of blank was 0 to 333 cp g^{-1} dry weight. For the workflow for the liquid samples, the negative controls all contained 0 positive droplets, indicating the limit of blank was 0 cp ml^{-1}.

Twenty-three of 99 drain samples, and 3 of 38 wastewater solids samples were negative for rotavirus RNA using no heat snap, yet had detectable RNA when using a heat snap. All raw wastewater samples had detectable RNA using both approaches. These results suggest using the heat snap provides higher sensitivity than not using a heat snap.

Samples for which rotavirus RNA was detected using both with and without a heat snap are considered in the following quantitative analysis (n = 131). The concentrations of rotavirus RNA measured using a heat snap were higher than those measurements without a heat snap in both wastewater solids and liquids. The median ratio was 123 (interquartile range 82 to 196, n = 131). Ratios were not different for liquids *versus* solids (t-test on $\log_{10}$-transformed

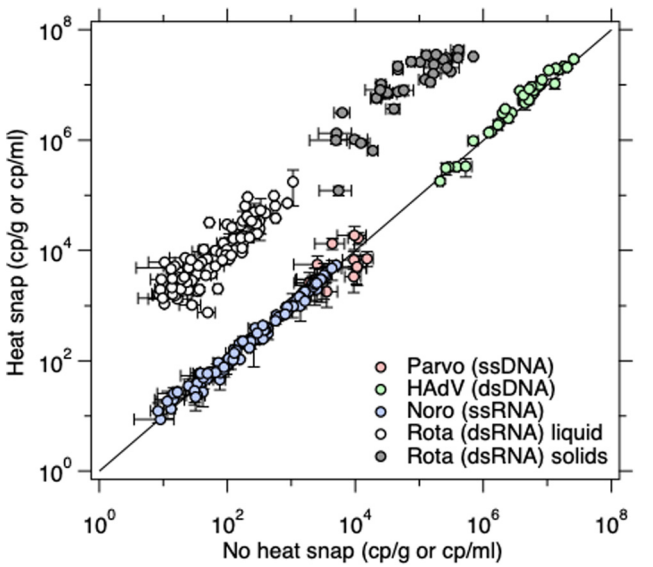

Fig. 1 Concentrations of viral nucleic-acids measured with (y-axis) and without (x-axis) a heat snap. Error bars are standard deviations; both y and x error bars are shown and if they cannot be seen then they are smaller than the symbol. The error bar for one norovirus GII measurement is omitted from the plot since it suggested the lower bound was less than 0 and could not be displayed (41 ± 45 cp ml^{-1}, see data at Stanford Digital Repository). Liquid refers to raw wastewater or drainage samples. Only samples where both measurements were above the lowest detectable concentration are shown. The line represents the 1 : 1 line. Parvo is parvovirus, HAdV is human adenovirus, Noro is norovirus GII, Rota is rotavirus; the configuration of their genome is provided next to the name in the legend. Note that results for measles are not shown because all samples were non-detect.

ratios, t = 1.323, dof = 50.302, p = 0.19). The relationship between $\log_{10}$-transformed concentrations measured with and without a heat snap (C_{HS} and C_{NHS}, respectively) was linear with a slope of 1.0 ± 0.02, and intercept of 2.1 ± 0.05 (errors represent standard deviation, r = 0.98, $p < 0.05$), suggesting a power-law relationship: $C_{HS} = 10^{2.1}C_{NHS}^{1}$. Results suggest that measurements made with a heat snap are approximately 125 times higher than those made without a heat snap.

Concentrations of all other targets run with and without a heat snap fell on a one-to-one line (Fig. 1 shows all samples where both cycling conditions yielded a positive result). Measles RNA was not detected in any sample so those results are not included in the figure. These other targets included viral genomes of all compositions including single stranded RNA (ssRNA), ssDNA, and double standard DNA (dsDNA). These results suggest the heat snap does not affect quantification of other types of viral nucleic-acids in these matrices.

Discussion

Rotavirus RNA was detected consistently in samples when a heat snap was used, suggesting the heat snap increased sensitivity. In addition, higher concentrations were measured using a heat snap. This suggests that rotavirus RNA concentrations in samples based on methods that do not include a heat snap are underestimated. At least a portion of the dsRNA rotavirus genome present in wastewater solids, wastewater, and wastewater impacted drainage is not denatured endogenously. Therefore a denaturing step is needed to more accurately characterize rotavirus RNA concentrations in these matrices. Quantification of other viral nucleic-acids in other genome configurations (ssRNA, dsDNA, ssDNA) was not affected by the heat snap. Further work may be warranted to test the heat snap in other matrices with other assays.

The linear relationship between the rotavirus measurements with and without a heat snap is striking. We note that the relationship was conserved despite samples that were collected from diverse locations and times, and represent different matrices, and that the samples were processed in different laboratories using different extraction methods and quantified using different digital PCR methods. The goal of this study was not to evaluate the effects of these different steps on quantification of rotavirus RNA, there was no sample sharing or splitting in the study. The linear relationship suggests that the proportion of rotavirus RNA accessible to the reverse transcription without heat snap relative to the proportion accessible after heat snap is conserved. In our analyses, the ratio is 1 : 125, suggesting that an average 0.8% of the rotavirus RNA is quantifiable without a heat snap. The conservation of the linear relationship in our samples also suggests that measurements made without a heat snap may be corrected to approximate those with a heat snap by multiplying by 125, though the applicability of this correction factor to other sample matrices beyond those tested here or using alternative extraction methods is uncertain.

A heat snap is likely needed prior to reverse transcription when dsRNA is not denatured endogenously in a sample or otherwise during extraction. Although the work here highlights the impact of heat snap on quantifying rotavirus, there are a number of other important human and animal dsRNA pathogens, including other members of the *Sedoreoviridae* family (*i.e.*, Bluetongue virus and Colorado tick fever virus) and members of the *Birnaviridae* family (*i.e.*, infectious bursal disease virus). Failure to denature dsRNA prior to reverse transcription will likely influence other studies beyond PCR-based quantification assays, such as metagenomic sequencing; dsRNA viruses may also be underrepresented in metagenomic data sets. It is unclear under what conditions a heat snap is needed, but given the results reported herein, we recommend that a heat snap be used when quantifying dsRNA from environmental matrices. We further recommend manufacturers of molecular biological products including RT highlight the need to denature dsRNA prior to RT.

Conflicts of interest

BS and DD are employees of Verily Life Sciences, LLC.

Data availability

Datasets for this manuscript can be found in the Stanford Digital Repository (**https://doi.org/10.25740/rk700qq6039**).

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