DR. LEENA MAUNULA (Orcid ID : 0000-0002-0841-5353)

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Performance of pre-RT-qPCR treatments to discriminate infectious human rotaviruses and noroviruses from heat-inactivated viruses: Applications of PMA/PMAxx, benzonase and RNase

Satu Oristo, Hye-Jeong Lee, and Leena Maunula

Department of Food Hygiene and Environmental Health, Faculty of Veterinary Medicine, University of Helsinki, Agnes Sjöbergin katu 2, 00790 Helsinki, Finland

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Corresponding author:

Satu Oristo

P.O. Box 66

00014 University of Helsinki

Helsinki, Finland

satu.oristo@helsinki.fi

Tel. +35850 4486 618

Abstract

Aims: Detection/Quantitation of RNA viruses is mostly done by reverse transcriptase (RT)-(q)PCR, but it does not distinguish between infectious and non-infectious viruses. Our aim was to test, how different pretreatments before RT-qPCR could eliminate positivity originated from external nucleic acids or genomes of damaged particles.

Methods and Results: Heat-inactivated (80 °C for 10 min) rotavirus Wa strain and fecal samples containing rotavirus or norovirus were treated with PMA/PMAxx, benzonase or crude extract RNase prior to RT-qPCR. PMA/PMAxx pretreatments were not consistently efficient for RV, although they seemed to work to some extent for heat-inactivated norovirus. Benzonase and RNase provided consistently 2.2 – 2.8 $\log_{10}$ reductions in the titer of fecal rotavirus.

Conclusions: All pretreatments need to be further validated for each virus separately, taking into account sample matrix and inactivation conditions. Although none of the pretreatments could completely render inactivated viruses undetectable, RNase worked most consistently for both rota- and norovirus.

Significance and Impact of the Study: This study sheds light on capacity of the most common pre-RT-qPCR treatments to eliminate damaged, non-infectious rotaviruses and noroviruses after thermal treatment. To our knowledge, this is the first time, when benzonase has been used in this context.

Keywords

Viruses, Detection, Environmental Health, Enzymes, Thermal processes

Introduction

Human rotavirus (RV) and norovirus (NoV) are the most important viruses causing gastrointestinal illness worldwide. Despite effective vaccines have greatly decreased RV mortality, ≥ 200 000 children still die annually because of RV infection (Tate and Parashar 2014); Tate *et al.* 2016). Majority of these deaths occur in the developing countries, where contaminated drinking water is an important source of infection. For NoV, it has been estimated, that annual morbidity was 677 billion in 2010, and it caused over 200 000 deaths (Pires *et al.* 2015).

RV has a segmented double-stranded RNA genome that is shielded by an inner core (core protein VP2), an inner capsid (VP6) and an outer capsid (protein VP7 and a spike protein VP4; Azevedo *et al.* 2014). NoV is quite different from RV, since it has a non-segmented, single-stranded RNA genome, and its virion is formed of a major capsid protein VP1 and a minor structural protein VP2 (Tan and Jiang, 2014).

Capsid damage with subsequent damage of the genome appears to be the most common reason for rendering viruses non-infectious (Cliver 2009). An undamaged capsid is crucial for viruses for entering the host cell, but it also protects the viral genome, e.g. from nucleases. Another important mechanism for rendering viruses non-infectious is a direct damage to the genome, which can happen, for example, when the virus is subjected to ultraviolet light (UV).

Infected persons shed immense amounts (up to 10^{10}) of these viruses into feces, which is further discharged to sewage. Survival of the viruses in surface or ground water depends on the virus in question; rotavirus and norovirus can survive infectious for weeks to months (Bae and Schwab 2007; Espinosa *et al.* 2008). Nowadays, the detection of viruses is usually made by (reverse-transcription)-polymerase chain reaction, RT-PCR. The downside of this method is, when

compared to cell culture methods, that it does not provide any information on whether the genome equivalents are associated to infectious viral particles or not. The infectivity of the detected viral particles is, however, important to know when assessing the risk of fecally contaminated water to consumers.

The abilities of different enzymatic pretreatments for removing damaged, non-infectious viral particles from samples containing viruses prior to RT-PCR have been tested widely (Nuanualsuwan and Cliver 2002; Lamhoujeb *et al.* 2008; Topping *et al.* 2009; Diez-Valcarce *et al.* 2011; Li *et al.* 2012; Escudero-Abarca *et al.* 2014; Rönnqvist *et al.* 2014; Manuel *et al.* 2017; Monteiro and Santos 2017). Proteinases have been used to break down partially damaged viral capsids, and RNases to digest free nucleic acids from damaged viral particles. In addition to enzymatic treatments, also chemicals, such as intercalating dyes propidium monoazide (PMA) and ethidium monoazide (EMA), as well as surfactants, have been used to get the same end result. (Parshionikar *et al.* 2010; Kim *et al.* 2012; Sánchez *et al.* 2012; Coudray-Meunier *et al.* 2013; Elizaquivel *et al.* 2014; Escudero-Abarca *et al.* 2014; Karim *et al.* 2015; Leifels *et al.* 2015; Monteiro and Santos 2017). PMA acts by covalently binding to nucleic acids upon photoactivation and prevents amplification, while surfactants may help PMA to penetrate mildly damaged viral capsids (Coudray-Meunier *et al.* 2013). Other methods, such as various binding assays and large fragment amplification, have been applied for discriminating intact particles or genomes from the damaged ones (Li *et al.* 2011; Ceuppens *et al.* 2014). Based on the literature, it seems that the effect of a pretreatment is highly dependable on the virus in question, sample matrix and of the process that has been used for viral inactivation, be it heat, chlorination, UV-irradiation, or something else.

The purpose of this study was to evaluate the abilities of PMA, PMAxx (a dye structurally related to PMA, but with different chemical properties), benzonase and RNase pretreatments to reduce the quantified RT-qPCR genome copy (gc) numbers of thermally-inactivated RV and NoV. Benzonase is a nuclease that degrades both single- and double-stranded RNA and DNA (Miller *et al.* 1994). To our knowledge, it has not been used in this context before. As model viruses, we used cell culture-propagated RV strain Wa, and two fecal samples containing either RV or NoV.

Materials and methods

Preparation of the virus stocks

MA104 cells were grown in Eagle's Minimum Essential Medium (EMEM; Sigma-Aldrich Co., Saint Louis, MO, USA), containing 10% heat-inactivated foetal bovine serum (FBS; Thermo Fischer Scientific Inc., Waltham, MA, USA), 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) solution (Sigma-Aldrich Co.) and 1 % glutamine-penicillin-streptomycin (Sigma-Aldrich Co.) The cells were incubated at 37°C in an atmosphere containing 5% CO_2 until they reached 80% confluency. The cells were infected with rotavirus strain Wa G1P[8] (kindly gifted by Professor Lennart Svensson at the Linköping University, Linköping, Sweden) according to the protocol described by Arnold *et al.* (2009). Incubation at 37°C was continued for 2 – 3 days, and the cells were checked for cytopathic effect (CPE). The viruses were released by freeze-thawing and centrifuged at 4,500 g for 10 min. The supernatant was divided into aliquots and stored at -70°C. Infectivity of the virus stock was measured by the 50% tissue culture infectious dose method ($TCID_{50}/ml^{-1}$). In brief, 1.0 x 10^3 cells were seeded into each well in a 24-well plate, and four parallel wells

were infected with 200 µl of each 10-fold serial dilution of trypsin-activated rotavirus Wa. The Wa stock contained approximately 3 x 10^5 $TCID_{50}/ml^{-1}$.

Also, two 10% human (anonymous) fecal suspensions, one containing a high number of RV G1P[8] particles by electron microscopy (EM; from here on referred as F_RV), and the other containing a high genome load of NoV GI.Pb-GI.6 (from here on referred as F_NoV; GenBank accession no. for ORF1 identical to KT943508 and for ORF2 to KT943509; Oristo *et al.* 2017), were prepared in sterile 1 x phosphate buffered saline (PBS; Sigma-Aldrich Co.). The fecal suspensions were thoroughly mixed by vortexing, and incubated at 6°C for 24 – 72 hours, before clarification by centrifugation at 10,000 g for 10 min. The supernatants were stored in aliquots at -70°C.

To obtain optimal concentrations for the RT-qPCR experiments, the Wa stock and the fecal suspensions F_RV and F_NoV were further diluted in PBS. The working dilutions were freshly prepared for each experiment. The RT-qPCR concentrations of these diluted Wa, F_RV and F_NoV aliquots were determined to be 1.7 x 10^8 (gc) ml^{-1} (corresponding to approximately 3 x 10^3 $TCID_{50}$ ml^{-1}, 7.6 x 10^8 gc ml^{-1}, and 3.2 x 10^8 gc ml^{-1}, respectively, by the RT-qPCR methods described below). The corresponding concentrations in 1 µl of extracted RNA were 2.8 x 10^5 gc for Wa, 1.3 10^6 for F_RV, and 5.3 x 10^5 for F_NoV.

Thermal inactivation of the model viruses

To determine the time for complete inactivation of Wa, virus aliquots were thermally treated at 80 °C for 1 – 30 min in a dry bath, and the $TCID_{50}$ concentration was determined as described above. In addition, the stage of the heat-inactivated (80°C

for 10 min) samples containing F_RV particles were examined by EM. Viruses were identified on grids negatively stained with 1% potassium phosphotungstate, pH 6.5.

For PMA and nuclease treatment studies, we inactivated the viral aliquots in a dry bath set at 80 °C for 10 min, after which they were placed on ice immediately. Virus samples without and with heat-inactivation are referred as “infectious” and “heat-inactivated” viruses, respectively.

PMA/PMAxx pretreatments

Based on our preliminary tests, we decided to use a final concentration of 100 µmol l^{-1} PMA/PMAxx (Biotium, Hayward, CA, USA). PMA/PMAxx in sterile H_2O at 2mM was stored in aliquots at -20°C, protected from light. As a first step, we tested the ability of PMA/PMAxx to bind to free nucleic acids by treating 100 µl aliquots of extracted Wa RNA with µmol l^{-1} of PMA/PMAxx. PMA/PMAxx treated samples underwent incubation in the dark for 60 min at 6°C, and photoactivation (performed with Biotium-Lite LED Photolysis device (Biotium) for 15 min at room temperature.

Half of the PMA/PMAxx-treated RNA samples were further purified with the QiaQuick PCR Purification kit (QIAGEN), according the manufacturer’s instructions, and half was not purified further. Adequate controls treated with µmol l^{-1} PMA/PMAxx, and either subjected to photoactivation or not, were included in the experiments.

PMA/PMAxx treatment for infectious or heat-inactivated Wa, F_RV and F_NoV samples was done accordingly by adding the dye into 100 µl aliquots of virus. Virus containing samples were frozen (-20°C) until subjected to RNA extraction. All experiments with the infectious and heat-inactivated viruses were done at least thrice. With PMA/PMAxx, we used the 1.5 ml DNA LoBind Eppendorf tubes (Sigma-Aldrich Co.).

Nuclease pretreatment

We first wanted to ensure that benzonase (Sigma-Aldrich, St. Louis, MO, USA) degrades nucleic acids efficiently. Benzonase treatment was otherwise performed similarly to PMA treatment, but with 100 U of benzonase per sample, and incubation at 37°C for 1 h. Treatment of the 100 µl aliquots of infectious and heat-inactivated Wa, F_RV and F_NoV samples was performed similarly prior to RNA extraction.

Pretreatment of the viral aliquots with a crude mixture of RNases from bovine pancreas (Roche Diagnostics, Mannheim, Germany) was performed as described by Rönnqvist *et al.* (2014), with minor modifications. Briefly, 4 U of RNase was added per 100 µl of diluted Wa, F_RV or F_NoV aliquots, and the mixtures were then incubated for 30 min at 37 °C. 80 U of RiboLock RNase inhibitor (Promega, Madison, USA) was added after the incubation to stop the reaction.

The control samples were always treated the same way as the nuclease-treated samples, but without addition of nuclease. We also tested, if the heating of F_NoV samples at 37 °C for 30 or 60 min would decrease the viral titer in comparison with the samples kept at 6°C (PMA treatment) for the similar amount of time. All experiments with the infectious and heat-inactivated viruses were done at least thrice, except for the infectious Wa samples treated with benzonase, which was done twice.

RNA extraction and detection of Wa and F_RV by RT-qPCR

RNA of the Wa and F_RV samples was extracted with the E.Z.N.A Viral RNA kit (Omega Bio-tek Inc., Georgia, USA). The extractions were performed according to the manufacturer's instructions, except that the volume of each sample was adjusted

to 140 µl with PBS prior to the extraction, and the samples were eluted in 60 µl using the elution buffer provided by the extraction kit's manufacturer. The RNA samples were stored at -20°C until further use.

Expand reverse-transcriptase enzyme (25 U) (Roche Diagnostics GmbH, Mannheim, Germany) was used to produce cDNA of RV RNA. The 10µl-mix was otherwise prepared as described by Kantala *et al.* (2009), but with 1.0 µmol l^{-1} of rotavirus-specific reverse primer Z_RV_R (Table 1) and 3 µl of denatured (97°C, 5 min) RV RNA. The RT was performed at 42°C for 60 min. One PCR reaction mix (25 µl) consisted of 10 µl 2x Rotor-Gene SYBR Green PCR Master Mix (QIAGEN), 1.0 µmol l^{-1} of each reverse (Z_RV_R) and forward (Z_RV_F) primers (Table 1), H_2O, and 5 µl of template cDNA. Amplification was performed with the Rotor-Gene Q thermal cycler (QIAGEN) in the following conditions: initial activation at 95°C for 5 min, followed by 45 cycles of 95 °C for 5 s and 60 °C for 10 s.

RNA extraction and detection of F_NoV by RT-qPCR

RNA of all the F_NoV samples was extracted with the E.Z.N.A Viral RNA kit (Omega Bio-tek Inc.), as explained above. Detection of F_NoV by RT-qPCR was performed in 20 µl-volume with the QuantiTect Probe RT-PCR kit (QIAGEN), 0.9 µmol l^{-1} of NVLCR1 reverse primer, 0.9 µmol l^{-1} of QNIF4 forward primer, and 0.3 µmol l^{-1} of TM9 probe (Table 1). Amplification was performed with the Rotor-Gene Q (QIAGEN) thermal cycler in the following conditions: RT at 55°C for 60 min and initial activation at 95°C for 15 min, followed by 45 cycles of 95 °C for 15 s, 60 °C for 60 s and 65 °C for 60 s.

Digital droplet PCR and standard curves

Digital droplet PCR (ddPCR) was used for determining the genome copy numbers of the standard samples (Wa and F_NoV). Detection of viruses was performed in 22 µl-volume reactions with the One-Step RT-ddPCR Advanced Kit for Probes (Bio-Rad, CA, USA), 1.0 µmol l^{-1} of each (virus-specific) forward and reverse primers, 0.2 µmol l^{-1} of probe (Table 1), and 5 µl of template RNA. Droplets were generated with Automated Droplet Generator (Bio-Rad), and the samples were then subjected to amplification in the following conditions: RT at 50°C for 60 min and initial activation at 95°C for 10 min, followed by 43 cycles of 95°C for 30 s and 60°C for 60 s. Deactivation was done at 98°C for 10 min. Results were analysed using the QX200™ Droplet Digital™ PCR System (Bio-Rad). Standard curves for the RT-qPCR protocols were prepared from 10-fold serial dilutions of the standard samples. The RT-qPCR titers for the samples were obtained by plotting their C_t values against their corresponding standard curves.

Statistical and mathematical analysis

GraphPad Software (https://www.graphpad.com/quickcalcs/ttest1/) was used for the statistical pairwise t-test analyses of the results. P-values of <0.05 were considered statistically significant. When calculating $\log_{10}$-reductions, our principle was to compare every inactivated sample with the control (non-treated or treated), that gave the lowest titer. Thus, RV was compared with controls that were not heat-inactivated, but treated with PMA/PMAxx or nucleases. NoV was compared with controls that were heat-inactivated, but not treated with PMA/PMAxx or nucleases.

Results

Thermal inactivation of the model viruses

RV Wa was completely inactivated after heat treatment at 80°C for 5 min, when determined by the $TCID_{50}$ method (data not shown). The heat-inactivated (80°C, 10 min) fecal sample F_RV contained only destroyed remnants of viral particles in EM (data not shown). The effect of the thermal inactivation on the RT-qPCR titers of Wa and F_RV (-0.07, and -0.26 log_{10} reductions, respectively) were negligible. In contrast, the titer of F_NoV decreased significantly, by 2.35 log_{10} gc ($P < 0.05$; data not shown). The absolute titers are presented in table 2.

Since the genome of F_NoV in our sample seemed to be heat-labile at 80°C, we tested if incubation at 37°C (the temperature used during nuclease treatments), would also alter its titer. However, no statistically different results were obtained between the samples incubated at 37°C and 6C° (the temperature used during PMA treatment) for 30 or 60 min ($P > 0.05$; data not shown). Thus, identical pretreatment conditions were applied for RV and F-NoV.

Effects of PMA/PMAxx treatments on the RT-qPCR titers of infectious and inactivated viruses

Unbound PMA effectively inhibited the detection of free RV Wa nucleic acids by RT-qPCR ($P < 0.05$; data not shown). This effect was avoided by purification of the Wa RNA with the QiaQuick PCR Purification kit. Without photoactivation, PMA reduced the titer of the extracted, purified Wa RNA only negligibly (0.03 log_{10} gc; $P > 0.05$), but with photoactivation, the reduction was 1.31 log_{10} gc ($P < 0.05$; data not shown).

Accordingly, when infectious or heat-inactivated RV were treated with PMA or PMAxx, but not subjected to photoactivation, their titers did not differ significantly from the controls (-0.23 – 0.09 log_{10} gc; P >0.05; data not shown).

Unexpectedly, however, PMA treatment with photoactivation reduced the titers of infectious (no thermal treatment) Wa and F_RV by 0.73 and 1.65 log_{10} gc, respectively (P <0.05; Table 2, Fig. 1A, columns below x-axis). The same applied for PMAxx, with reductions of 1.63 and 2.02, respectively (P <0.05; Table 2, Fig. 1B). So, to verify the efficiency of PMA/PMAxx, we compared the results of the heat-inactivated PMA/PMAxx-treated samples with the infectious, non-heat-inactivated PMA/PMAxx-treated controls (see the methods section for additional information). The log_{10} reductions of the heat-inactivated, PMA or PMAxx-treated Wa were modest, 0.18 and 0.21 log_{10} gc, respectively (P values for both <0.05; Fig. 1A and 1B; columns on or above x-axis). PMA treatment hardly caused any titer-reduction for heat-inactivated F_RV (P = 0.5; Fig. 1A). However, the performance of PMAxx was better (0.86 log_{10} gc reduction; P <0.05; Fig.1B).

Promisingly, and in contrast to the above-mentioned results with infectious RV and PMA/PMAxx, application of PMA or PMAxx into infectious F_NoV did not affect its titer (-0.05 to 0.02 log_{10} gc; P >0.05; Table 2), but thermal treatment did. So, we compared the heat-inactivated F_NoV that was PMA/PMAxx-treated with the heat-inactivated non-PMA/PMAxx-treated counterparts (the controls that gave the lowest value). In addition to the F_NoV genome load decrease of 2.35 log_{10} by heat alone, pretreatment with PMA or PMAxx still resulted in further reductions of 0.79 and 0.84 log_{10} gc, respectively (P values for both <0.05; Table 2, Fig. 1A and 1B).

Effects of nuclease treatments on the RT-qPCR titers of infectious and inactivated viruses

Treatment of the extracted, non-purified or purified RV Wa RNA with 100 U of benzonase could render it non-detectable by RT-qPCR (4.48 - 4.72 $\log_{10}$ gc reduction; data not shown). When applied to the infectious Wa, benzonase pretreatment resulted in an unwanted decrease in the titer (0.76 $\log_{10}$ gc; $P < 0.05$; Table 2, Fig. 1C), but, contrarily, did not decrease the titers of infectious F_RV and F_NoV (0.15 and 0.00 $\log_{10}$ gc, respectively ($P > 0.05$; Table 2, Fig. 1C). Benzonase pretreatment reduced the titer of heat-inactivated Wa only by 0.38 $\log_{10}$ ($P > 0.05$), but that of F_RV by 2.17 $\log_{10}$ gc ($P < 0.05$) – the latter being the second-best value in the study – when compared to their infectious controls treated with benzonase (Fig. 1C). Benzonase pretreatment of the heat-inactivated F_NoV did not cause any further titer reduction (Fig. 1C).

The titers of the infectious Wa, F_RV and F_NoV were somewhat reduced by the RNase treatment (0.88, 0.51 and 0.46 $\log_{10}$ gc, respectively; P values for all <0.05; Table 2, Fig. 1D). RNase worked well for the inactivated samples, since after heat-treatment, the titers of Wa and F_RV were reduced by 1.66 and 2.77 $\log_{10}$ gc (P values for both <0.05), respectively, in comparison with their infectious controls treated with RNase (Fig. 1D). RNase treatment of the heat-inactivated F_NoV resulted in a further 0.80 $\log_{10}$-reduction, when compared to the inactivated F_NoV, not treated with RNase (P value <0.05; Fig. 1D).

Discussion

In this study, we evaluated the performance of different pretreatments in eliminating false-positive RT-qPCR signals originating from heat-inactivated viruses, since

reliable methods are urgently needed, especially for non-cultivable viruses (Ceuppens *et al.* 2014; Fuster *et al.* 2016). None of the pretreatments worked perfectly, since they were not able to render the inactivated virus samples negative in RT-qPCR. PMA/PMAxx pretreatments seemed to work to some extent for heat-inactivated norovirus. Benzonase pretreatment worked well for F_RV (2.2 $\log_{10}$ gc reduction) and moderately for Wa, but not at all for NoV. The most promising of these pretreatments was with crude mixture of RNase that had some effect for both RV and NoV, and that gave the highest titer-reduction (almost 3 $\log_{10}$ for heat-inactivated F_RV) of our study.

We observed a difference in the thermal resistance at 80°C between dsRNA genome of RV and ssRNA genome of NoV GI (GI.6). Contrary to RV, a statistically significant (appr. 2-log) reduction in the F_NoV gc titer was noticed after heat treatment using RT-qPCR without any pretreatment. This finding is somewhat surprising, since other studies have reported zero to very minor decreases in the NoV genome after heating them at temperatures of 72-95°C for 5-15 min (Li *et al.* 2012; Escudero-Abarca *et al.* 2014; Karim *et al.* 2015; Monteiro and Santos 2017). One exception is the study of Parshionikar *et al.* (2010) that shows an 11-fold decrease in Norwalk concentration after treatment at 37°C for 2 weeks by RT-qPCR alone. These discrepancies might be partly explained by that the other studies used more concentrated viruses during heating, while we wanted to perform our NoV tests in approximately the same concentration as the tests with RV, which was our primary target virus in this study. Individual qualitative and quantitative contents of fecal suspensions might have an effect as well.

PMA treatment prior to RT-qPCR has been shown to be relatively reliable in discriminating infectious viruses from inactivated ones when hepatitis A viruses

(Sánchez *et al.* 2012) and polioviruses (Parshionikar *et al.* 2010; Karim *et al.* 2015) have been treated. The results of NoV treatments with PMA are, however, controversial. Escudero-Abarca *et al.* (2014) determined the D value, which indicates the time required to destroy 90% of the microbe population for PMA-treated Snow Mountain virus (SMV; NoV GII) to be only 3.1 min at 80°C, suggesting good performance of PMA treatment for NoV.

On the other hand, Parshionikar *et al.* (2010) reported that PMA treatment (100 µmol l^{-1}) of heat-inactivated (72°C, about 5 min) NoV (10^3 gc ml^{-1}) could render it non-detectable by RT-PCR, although not with RT-qPCR. Karim *et al.* (2015) also reported the inefficiency of PMA to render NoV negative in RT-qPCR. Our results that were somewhat hampered by degradation of NoV genome during heat-inactivation were in line with Karim *et al.* (2015), because PMA caused only less than a $\log_{10}$ reduction for F_NoV. PMAxx was found to function slightly better than PMA, but it was still not able to prevent all false-positive RT-qPCR signals.

In our experiments, PMA and especially PMAxx treatment resulted in the genome titer reduction of the cell-grown Wa sample (0.73 $\log_{10}$ and 1.63 $\log_{10}$ gc, respectively), and also Coudray-Meunier *et al*. (2013) reported a reduction of 0.52 $\log_{10}$ for RV with PMA. Cell-grown crude rotavirus samples contain always immature, non-infectious particles that have lost their outer capsids as well as viral components together with intact virions, as evidenced by EM (Settembre et al 2011). This could partially explain the reduction, since by PMA/PMAxx can enter partially damaged virus particles. To our knowledge, it has not been studied, whether the trypsin treatment of RV (that cleaves spike protein VP4 to subunits VP5* and VP8*), which is necessary for virus infectivity in cell culture, affects the accessibility of PMA into RV particles. More studies are needed to sort out these issues.

Coudray-Meunier et al. (2013) reported that PMA treatment of the heat-inactivated RV Wa (68 or 80°C, 10 min) caused a 1.45 $\log_{10}$ reduction and Leifels *et al.* (2015) found that the PMA treatment reduced the titer of RV by 1.19 $\log_{10}$ (65°C, 10 min). In our hands, PMA/PMAxx treatment did not much affect the titer of the RV treated at 80°C for 10 min (maximum decrease of 0.22 $\log_{10}$ gc). The discrepancies between the results of our and the abovementioned studies may be explained by the differences in the virus preparation, or in the methods used. However, the conclusion in both these studies was that viral targets influence the effectiveness and viruses have different inactivation mechanisms, which statements are in agreement with our results.

To our knowledge, benzonase has not been used as an alternative for RNase before, so we have no reference to our results. Under the same thermal inactivation conditions, nearly similar titer reductions (2.17 and 2.77 $\log_{10}$ gc, respectively) were observed for fecal RV when it was treated with either benzonase or RNase prior to RT-qPCR. However, benzonase had little to no effect for the inactivated Wa and F_NoV. It is possible that there was something in the sample matrix of the cell-cultured Wa that hindered the ability of benzonase to degrade free nucleic acids. While NoV results were obscured by the above-mentioned tremendous NoV genome degradation by heat alone, more research is clearly needed to reveal the applicability of benzonase pretreatment, when virus is inactivated with different processes (heat, UV, chlorine). However, by adjusting the inactivation conditions, and both benzonase and virus concentrations, this pretreatment may finally prove to be very practical.

In our hands, RNase pretreatment performed well for RV (1.66 – 2.77 $\log_{10}$ reductions for Wa and F_RV, respectively), when all three pretreatments were compared. To our knowledge, we do not have published studies to compare our

results with RNase-treated RV. In a study of Coudray-Meunier *et al.* (2013) different pretreatments (PMA/EMA and surfactants) were used to develop a method to differentiate between infectious and inactivated RV particles by RT-qPCR. Given the importance of RV as a cause of waterborne outbreaks, RNase method should be further validated for RV, because it could prove to be very practical in situations, where water or food is suspected to be contaminated by feces.

RNase pretreatment worked to some extent for NoV, causing a similar $\log_{10}$ reduction as PMA/PMAxx pretreatments (0.80 $\log_{10}$ gc) without affecting infectious, non-heat-inactivated virus. Our NoV results are in line with those of several studies that have assessed the usability of RNase pretreatments to measure survival or persistence of NoV (Topping *et al.* 2009; Li *et al.* 2012; Escudero-Abarca *et al.* 2014; Rönnqvist *et al.* 2014; Manuel *et al.* 2017). For example, Li *et al.* (2012) reported a decrease of 1.15 $\log_{10}$ gc after heat-treatment of NoV GII.4 at 85°C for 2 min using RNAse ONE. RNAse ONE treatment was also successfully used in the study of Escudero-Abarca *et al.* (2014) that determined the D value for the RNase treated SMV to be 3.9 min at 80°C. In contrast, in the study of Monteiro and Santos (2017), RNase A caused practically no decrease in the titer of heat-inactivated NoV GII.4. The discrepancies between these studies may result from numerous issues, e.g. the use of different NoV genotypes, virus concentrations, thermal treatments, type of RNase, and/or sample matrices.

Almost all pre-RT-qPCR treatments seemed to be more effective in differentiating infectious from inactivated RV particles in the fecal sample, than in the cell-culture media. Indeed, it is important that pretreatments would work reliably for viruses in fecal matrix, since sewage of human origin is a common source for NoV and RV contamination, e.g. in surface waters (Lodder *et al.* 2005; Kiulia *et al.* 2010). In

conclusion, this study gives us more understanding about the performance of pretreatments in distinguishing infectious RV and NoV. However, numerous data gaps have still to be filled, before the optimal pretreatments for particular viruses in various matrices, and after certain inactivation processes, can be established.

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The authors declare no conflict of interest.

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Figure legend

Figure 1. The $\log_{10}$ genome copy reductions of rotaviruses (cell-cultured Wa strain and fecal F_RV) and fecal norovirus (F_NoV) pretreated with PMA (A), PMAxx (B),

benzonase (C) or RNase (D) with or without heat-inactivation (80˚C, 10 min). Infectious, pretreated rotavirus (RV) was compared with infectious RV □, with heat-inactivated RV ▫ or with heat-inactivated, pretreated RV ■. In case of norovirus, heat-inactivated, pretreated F_NoV was compared with infectious F_NoV □, infectious, pretreated F_NoV ▫ or with heat-inactivated, pretreated F_NoV ■. (See methods for details).

Table 1. The primers and probes used in the RT-qPCR assays in this study

Target virus	Oligonucleotide	Sequence and label (5' → 3')	Nucleotide position	Amplicon length (bp)§	Reference
RV Wa and F_RV*	Z_RV_F	ACCATCTACACATGACCCTCTATGAG	963 – 988 †	87	Modified from Zeng et al. 2008
	Z_RV_R	GGTCACATAACGCCCCTATAGC	1028 – 1049†		Zeng et al. 2008
F_NoV	QNIF4	CGCTGGATGCGNTTCCAT	5291 – 5308 ‡	86	da Silva et al. 2007
	NVLCR1	CCTTAGACGCCATCATCATTTAC	5354 – 5376 ‡		Svraka et al. 2007
	TM9	FAM-TGGACAGGAGATCGC-MGB-NFQ	5345–5359‡		Hoehne & Schreier 2006

* RV Wa: Cell-cultured rotavirus Wa strain; F_RV: RV-positive fecal sample, genotype GI.P[8], NoV GI: Norovirus genogroup I, fecal sample positive for NoV GI.Pb – GI.6.

† Positions based on Human rotavirus NSP3 (NS34) mRNA region (GenBank accession no. X81436)

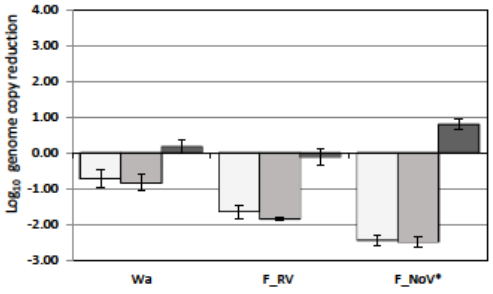
‡ Positions based on complete NoV GI genome (GenBank accession no. M87661)

§ Bp: base pairs

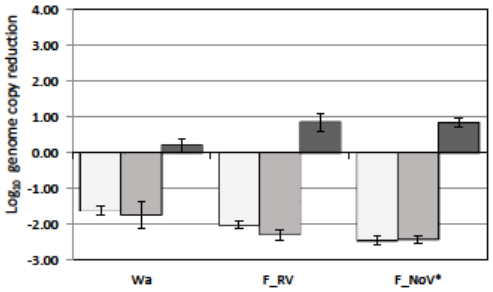
Table 2. The log_{10} genome copy (gc) titers of cell-cultured rotavirus strain Wa, rotavirus G1P[8] positive fecal sample (F_RV), and norovirus GI.Pb-GI.6 positive fecal sample (F_NoV), with or without heat-inactivation (80˚C, 10 min) and pretreatment with either PMA (100 µmol l^{-1}), PMAxx (100 µmol l^{-1}), benzonase (100 U) or RNase (4 U), as calculated with RT-qPCR. (Mean ± standard deviation).

Pretreatment	Virus	Infectious (Log_{10} gc)	Pretreated, infectious (Log_{10} gc)	Heat- inactivated (Log_{10} gc)	Pretreated, heat-inactivated (Log_{10} gc)
PMA	Wa	5.38 ± 0.16	4.65 ± 0.10	5.49 ± 0.10	4.47 ± 0.05
	F_RV	6.09 ± 0.06	4.44 ± 0.24	6.30 ± 0.22	4.56 ± 0.24
	F_NoV	5.82 ± 0.39	5.87 ± 0.43	3.38 ± 0.21	2.59 ± 0.10
PMAxx	Wa	5.38 ± 0.16	3.75 ± 0.11	5.49 ± 0.10	3.54 ± 0.07
	F_RV	6.16 ± 0.09	4.14 ± 0.47	6.43 ± 0.35	3.28 ± 0.41
	F_NoV	5.82 ± 0.39	5.80 ± 0.46	3.38 ± 0.21	2.54 ± 0.10
Benzonase	Wa	5.44 ± 0.21/ 5.24 ± 0.08*	4.48 ± 0.00	5.44 ± 0.12	4.29 ± 0.21
	F_RV	5.98 ± 0.22/ 5.86 ± 0.21*	5.71 ± 0.28	6.19 ± 0.46	3.26 ± 0.40
	F_NoV	5.40 ± 0.12	5.39 ± 0.13	3.10 ± 0.13	3.16 ± 0.08
RNase	Wa	5.42 ± 0.19/ 5.27 ± 0.08*	4.39 ± 0.08	5.46 ± 0.11	2.67 ± 0.12
	F_RV	5.98 ± 0.22/ 5.86 ± 0.22*	5.35 ± 0.19	6.06 ± 0.30	2.52 ± 0.39
	F_NoV	5.33 ± 0.04	4.87 ± 0.30	3.10 ± 0.16	2.30 ± 0.36

* The average log_{10} genome copy (gc) titers of controls that were included in the RT-qPCR tests of the pretreated infectious controls.



A.



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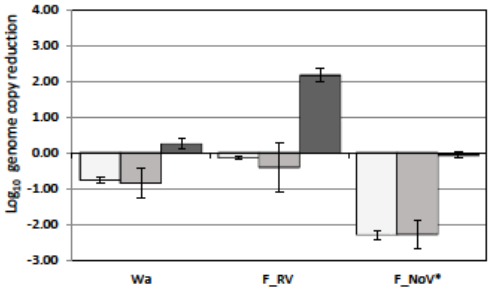

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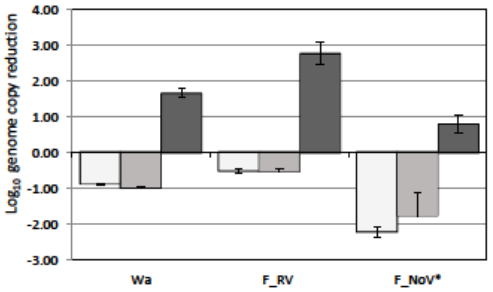

D.