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ORIGINAL PAPER

Assessment of the Applicability of Capsid-Integrity Assays for Detecting Infectious Norovirus Inactivated by Heat or UV Irradiation

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Abstract

Human noroviruses are the leading cause of viral gastroenteritis. In the absence of a practical culture technique for routine analysis of infectious noroviruses, several methods have been developed to discriminate between infectious and non-infectious viruses by removing non-viable viruses prior to analysis by RT-qPCR. In this study, two such methods (RNase and porcine gastric mucin) which were designed to remove viruses with compromised capsids (and therefore assumed to be non-viable), were assessed for their ability to quantify viable F-specific RNA bacteriophage (FRNAP) and human norovirus following inactivation by UV-C or heat. It was found that while both methods could remove a proportion of non-viable viruses, a large proportion of non-viable virus remained to be detected by RT-qPCR, leading to overestimations of the viable population. A model was then developed to determine the proportion of RT-qPCR detectable RNA from non-viable viruses that must be removed by such methods to reduce overestimation to acceptable levels. In most cases, nearly all non-viable virus must be removed to reduce the log overestimation of viability to within levels that might be considered acceptable (e.g. below 0.5 $\log_{10}$). This model could be applied when developing alternative pre-treatment methods to determine how well they should perform to be comparable to established infectivity assays.

Keywords Norovirus · Infectivity · Capsid integrity · Porcine gastric mucin · Rnase · Coliphage · GA

Introduction

Human noroviruses (HuNoV) are the leading cause of viral gastroenteritis and are estimated to cause 219,000 deaths and cost 60.3 billion US dollars annually worldwide (Bartsch et al. 2016). Release of human faecal waste containing norovirus into the environment can lead to subsequent infections as a result of direct exposure to the water or by consumption of food that has come into contact with contaminated water. In particular, growth of bivalve molluscan shellfish (BMS) in such waters often leads to accumulation of the virus within BMS, which can then cause infections in humans when consumed (Hassard et al. 2017; Lowther 2018). Detection and quantification of HuNoV is most commonly performed using RT-PCR-based methods, but RT-PCR is unable to discriminate between infectious and non-infectious viruses. Detection by RT-PCR alone is, therefore, likely to misrepresent the level of infection risk that a sample poses. Despite recent advances in norovirus culture methods (Ettayebi 2017; Zou 2017) there still remains no robust method that allows HuNoV to be routinely cultured to determine the level of infectivity in contaminated food and environmental samples. To overcome this problem of non-culturability, several methodologies (collectively termed capsid integrity methods) have been proposed that combine RT-PCR with a pre-treatment step to discriminate between viruses with intact capsids and viruses without intact capsids (which are therefore presumed to be non-infectious). Examples of such methods include pre-treatment with enzymes such as RNases (Topping et al. 2009) or a combination of enzymes such as proteinase K and RNase (Nuanualsuwan and Cliver 2002; Yang and Griffiths 2014) to remove free RNA and RNA

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from damaged virus particles, enrichment of intact viruses using binding assays (Dancho et al. 2012; Langlet et al. 2015), and the use of intercalating dyes such as propidium monoazide (Randazzo et al. 2016, 2018) or chelating platinum compounds (Fraisse et al. 2018) to render free RNA and RNA from damaged virus particles unavailable to RT-PCR.

In this study, the ability of two capsid integrity methods to measure HuNoV viability was assessed. The GA strain of F-specific RNA bacteriophage (FRNAP) was used to demonstrate that the level of virucidal treatment of the samples was similar to those used in waste water treatment. GA, the type strain of FRNAP genogroup II (FRNAP-II) was selected as a surrogate due to its demonstrated applicability for determining human enteric viral pathogen risk in environmental samples (Doré et al. 2000; Hartard et al. 2016, 2018; Cho et al. 2018; Dias et al. 2018) and its ease of culture.

The capsid integrity assays evaluated in this study were an RNase-based method adapted from the study by Topping et al. (Topping et al. 2009), and a porcine gastric mucin magnetic bead (PGM-MB)-based assay (Farkas et al. 2018). UV levels for inactivation were selected based on their similarity to the levels of inactivation commonly achieved by UV inactivation in waste water treatment works in the UK. Such levels would best reflect assay performance in water and BMS samples affected by waste water effluents. The UK Environment Agency specifies a target 4.4 log reduction of enteroviruses in waste water that impacts bathing water (Environment Agency 2011). Much of this reduction is achieved at earlier steps in waste water treatment than UV disinfection; this stage usually accounts for between a 1 and 3 log reduction in infectious enteric virus load (Campos et al. 2016). Similar levels of GA inactivation were also achieved in this study by heating at 72 °C to allow direct comparison between the efficacy of the treatments between heat- and UV-treated viruses.

Methods

GA Culture and Plaque Assay

A stock of the GA strain of FRNAP was kindly supplied by Dr Stephanie Friedman (US EPA, FL) and was propagated according to Annex C of ISO 10705-1 (ISO 1995). GA broth culture was mixed with a final concentration of 5% glycerol (w/v) and stored in aliquots at − 80 °C. Aliquots of GA were thawed at 4 °C and diluted in 0.1% peptone water as required before use. Enumeration of viable GA in stock cultures and samples was carried out by double agar overlay plaque assay according to ISO 10705-1 using the WG49 strain of *Salmonella typhimurium* (NCTC 12484) as the host.

Inactivation of Viruses

An anonymised stool sample from a patient infected with HuNoV genotype GII.4 was provided by Public Health England. The sample was diluted tenfold with PBS and centrifuged at 3000×*g* for 10 min. The supernatant was confirmed to be HuNoV genogroup II positive and FRNAP-II negative by RT-qPCR and plaque assay as described below. The supernatant was spiked with GA and diluted with 0.1% peptone water (Oxoid, UK) and glycerol (Sigma-Aldrich, UK) to a final concentration of approximately 10^8 HuNoV GII genome copies/ml, 10^6 GA plaque forming units (PFU)/ml and 5% glycerol. This sample, herein referred to as virus mixture (VM), was aliquoted and stored at − 80 °C until used.

Inactivation by Heat

The system used to inactivate viruses by heat was designed to replicate as closely as possible, that used in the Araud et al. (2016) study of inactivation of HAV, Tulane virus, Human rota virus and murine norovirus. Heating viruses to pasteurising temperature (72 °C) has been used by previous studies investigating viral inactivation by heat (Wigginton et al. 2012; Araud et al. 2016) and is cool enough to allow for control over the degree of inactivation achieved. Soda glass melting point capillary tubes (Fisher Scientific, UK, 1.8 mm outer diameter, 0.28 mm wall thickness, 100 mm length open ended) were filled with 80 µl of VM. The tubes were sealed at each end with Cristaseal (Hawksley, UK), Parafilm (Bemis, WI, USA) and PVC adhesive tape (3 M, UK). The capillary tubes were placed into a recirculating water bath in triplicate (Grant Instruments, UK) at 72 °C for 3, 10 and 20 s to achieve approximate 1, 2 and 3 log reductions in GA infectivity. Additional capillary tubes were treated for 300 s at 72 °C to inactivate all viable viruses (> 5.5 log reduction in infectivity). After the capillary tubes were heat-treated, they were immediately plunged into ice-water to stop further inactivation by heat. The treated viral suspensions were removed from the capillary tubes and diluted tenfold with 0.1% peptone water (Oxoid, UK). These were then immediately subjected to GA enumeration by plaque assay, or quantification by RT-qPCR, following RNA extraction with or without capsid integrity pre-treatments as described below.

Inactivation by UV

The absorbance of VM at 254 nm was measured as 1.384 on a SpectraMax M2E spectrophotometer (Molecular Devices, CA, USA). Inactivation by UV was carried out in triplicate

using a CX-2000 UV crosslinker (UVP, UK) with low pressure mercury lamps emitting almost monochromatic light with a wavelength of 254 nm. UV-C doses of 38, 96 and 155 mJ/cm^2 (as measured by a UVX Radiometer and UVX-25 sensor (Analytik Jena, Germany)) were applied to 2 ml of VM in 30 mm diameter wells of polystyrene cell culture dishes (Corning, NY, USA) to achieve a 1, 2 and 3 log reduction of GA. Additional subsamples were treated with a dose of 500 mJ/cm^2 to inactivate all viable viruses (> 5.5 log reduction in infectivity). Following UV treatment, samples were diluted tenfold with 0.1% peptone water (Oxoid, UK). These were then subjected to GA enumeration by plaque assay, or quantification by RT-qPCR, following RNA extraction with or without capsid integrity pre-treatments as described below.

Capsid Integrity Pre-treatments

Following inactivation by UV or heat, a subset of samples of VM were subjected to capsid integrity pre-treatment with either porcine gastric mucin-conjugated magnetic beads (PGM-MBs) or RNase. The treatment with PGM-MBs followed the method used by Farkas et al. (2018). Briefly, porcine gastric mucin (PGM, Sigma-Aldrich, UK) was covalently bonded to 0.5 ml of MagnaBind Carboxyl Derivatised Beads (Thermo Fisher Scientific, USA) using the two step EDAC (1-ethyl- 3-(3-dimethylaminopropyl) carbodiimide hydrochloride), sulfo-NHS (N-hydroxysulfosuccinimide) method described by Farkas et al. (2018). PGM-MB treatment was performed by adding 500 µl of sample to a suspension of 450 µl PBS and 50 µl of PGM-MBs. This mixture was agitated gently for 30 min at room temperature to make PGM-MB/RNA complexes. The PGM-MB/RNA complexes were separated from suspension on a magnetic stand and washed three times and suspended in 500 µl PBS. The RNA was unbound from PGM-MBs by heating to 95 °C for 5 min and separated on a magnetic stand.

Treatment with RNase was carried out as described previously by Topping et al. (Topping et al. 2009). Briefly, 60 µl of RNase buffer containing 50 U RNase ONE (Promega, UK) was added to each 500 µl sample and incubated at 37 °C for 15 min. For both pre-treatment methods an untreated sub-sample of VM that had not been subjected to any virucidal treatment was used as a no-inactivation control.

RNA Extraction

All RNA extractions were carried out using the Nuclisens magnetic bead extraction reagents (BioMerieux, France) according to the manufacturer's recommendations, with a sample volume of 500 µl and an elution volume of 100 µl.

RT-qPCR

Following RNA extraction, all samples were analysed in triplicate using the RNA Ultrasense one-step RT-qPCR system (ThermoFisher Scientific, UK) to quantify GA and HuNoV GII. For GA detection, each 25 µl reaction was made up of 0.5 µM forward primer, 0.9 µM reverse primer, 0.25 µM probe, 1 × reaction mix, ROX reference dye, 1 × enzyme mix and 5 µl sample RNA using the FRNAP-II primer and probe set (for detection of GA and related strains) developed by Wolf et al. (2008). For HuNoV GII detection, each 25 µl reaction was made up of 1 µM forward primer, 1.8 µM reverse primer, 2 µM probe, 1 × Reaction Mix, ROX reference dye, 1 × enzyme mix and 5 µl sample RNA using the HuNoV genogroup II primer and probe set combination used in Lowther et al. (2017). Amplification and detection were carried out on a Stratagene Mx3005P qPCR machine (Agilent Technologies, CA, USA) using the following program: 60 min at 55 °C, 5 min at 95 °C, 45 cycles of 15 s at 95 °C, 60 s at 60 °C, 60 s at 65 °C.

The concentration of GA or HuNoV detected by RT-qPCR were determined by comparing *C*q values to a standard curve generated using five tenfold serial dilutions of dsDNA standards containing 10^5 copies/µl for each RT-qPCR plate as described by Lowther et al. (2017).

Data Analysis

The average concentrations of GA and HuNoV in the non-virucidal treated controls were calculated for each of the viral detection methods used (plaque culture, RT-qPCR, PGM-MB RT-qPCR and RNase RT-qPCR). The log reduction in detected virus (ΔV) was then calculated as by Park et al. (2011).

All statistical analyses were performed using the Minitab software (Minitab 17.1.0, Minitab Inc.).

To investigate how effective the RNase treatment was for excluding non-viable GA from RT-qPCR, the percentage of RT-qPCR signal that was from viable GA was calculated using

Equation 1. The proportion of PCR-detectable, non-viable GA in an inactivated sample that was removed by RNase pre-treatment (V_E) was calculated using Eq. 2.

Equation 1: the proportion of RT-qPCR signal that is from viable virus (V_V) where ΔV_P is log reduction in virus as detected by RT-qPCR and ΔV_C is log reduction in virus as detected by GA plaque assay.

$$V_V = 10^{\Delta V_P - \Delta V_C} \quad (1)$$

Equation 2: the proportion of PCR detectable but non-viable virus excluded by pre-treatments (V_E) where T_{Rn} is the total PCR-detectable virus after inactivation,T_{Rt} is the total

PCR-detectable virus after inactivation and capsid integrity pre-treatment and T_C is the total viable virus after inactivation.

$$V_E = \frac{T_{Rn} - T_{Rt}}{T_{Rn} - T_C} \quad (2)$$

Viable Population Overestimate Model

A theoretical model was designed to determine the degree to which viable virus populations are overestimated if < 100% of RNA from non-viable viruses is removed by capsid integrity pre-treatment methods. The use of the model is independent of the differing ways in which different viral species respond to pre-treatments or virucidal treatments, and as it is theoretical applies equally to culturable and non-culturable viruses. Equation 3 summarises this model that was developed to determine the "overestimation ratio" (O), given only two theoretically known parameters:Equation 3: The predicted overestimation ratio (O) of remaining viable virus in sample as measured by RT-qPCR following pre-treatment by a capsid integrity-based method, where V_E is the proportion of PCR detectable but non-viable virus excluded by pre-treatments and V_{Vn} is the proportion of RT-qPCR signal that is from viable virus before pre-treatment.

$$O = \frac{1 - V_E}{V_{Vn}} + V_E \quad (3)$$

- The proportion of viable virus in a sample before pre-treatment (V_{Vn}) and
- The proportion of non-viable virus removed by the pre-treatment (V_E)

For a sample where:

- Total PCR-detectable virus in the absence of pre-treatment = B
- Total PCR-detectable virus after pre-treatment = C
- PCR-detectable, non-viable virus after pre-treatment = Y
- Viable virus = D

Expressed in terms only of B, V_{Vn} and V_E

- $D = V_{Vn}B$
- $Y = (B - D)(1 - V_E) = (B - V_{Vn}B)(1 - V_E)$
- $C = Y + D = (B - V_{Vn}B)(1 - V_E) + V_{Vn}B$

In its simplest form $O = \frac{C}{D}$, therefore:

$$O = \frac{(B - V_{Vn}B)(1 - V_E) + V_{Vn}B}{V_{Vn}B}$$

$$O = \frac{B - V_{Vn}B - V_EB + V_EV_{Vn}B + V_{Vn}B}{V_{Vn}B}$$

$$O = \frac{1 - V_{Vn} - V_E + V_EV_{Vn} + V_{Vn}}{V_{Vn}}$$

$$O = \frac{1 - V_E + V_EV_{Vn}}{V_{Vn}}$$

$$O = \frac{1 - V_E}{V_{Vn}} + V_E$$

Results

Differences in Detectable Virus Levels (Δ*V*) Between Analysis Methods

The change in detectable virus levels (ΔV) in heat- or UV-treated VM compared to VM where no virucidal treatments were applied was determined using four different methods as outlined in Fig. 1. For GA only, ΔV was determined using plaque assay, to provide a direct measure of virus inactivation. Indirect measures of inactivation were determined as follows: for both GA and HuNoV, ΔV was determined by RT-qPCR on RNA extracts from subsamples subjected to a capsid integrity pre-treatment with RNase (RNase RT-qPCR) and subsamples where no capsid integrity pre-treatment was applied). For HuNoV only, ΔV was additionally determined by RT-qPCR on RNA extracts from subsamples

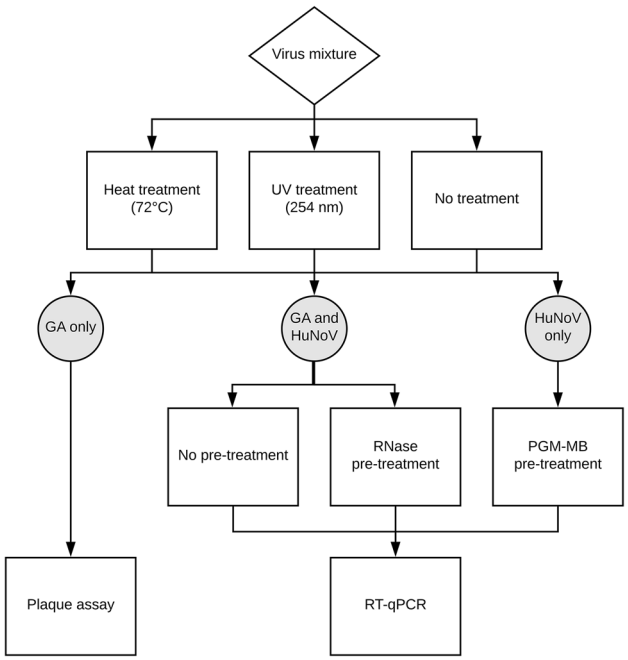

Fig. 1 Flow diagram outlining the experimental design to determine the effectiveness of capsid integrity methods to measure infectious HuNoV and GA

subjected to a capsid integrity pre-treatment with PGM-MBs (PGM-MB RT-qPCR). T-tests showed that ΔV, as determined using GA culture, was significantly higher than ΔV as determined for any of the PCR-based methods, at all levels of treatment for heat and UV for both HuNoV and GA ($p = 0.011$ to < 0.001). Figure 2 summarises the differences between expected ΔV (as measured by GA culture) and observed ΔV by the PCR-based methods. One-way ANOVA tests showed significant differences between RT-qPCR assay types at 20 s and 300 s at 72 °C ($p = 0.002$ and $p < 0.001$ respectively). PGM-MB RT-qPCR had significantly higher ΔV for HuNoV than other RT-qPCR assays for 20 s treatment and significantly lower ΔV for HuNoV than other RT-qPCR assays for 300 s treatment according to Tukey post hoc tests. In samples treated with UV-C, no significant difference was found between ΔV for RT-qPCR assays for HuNoV at 38, 96 or 155 mJ/cm^2 ($p = 0.848$, 0.110 and 0.479 respectively), but at 500 mJ/cm^2, RT-qPCR had significantly lower ΔV for HuNoV than PGM RT-qPCR or RNase RT-qPCR ($p = 0.016$).

T tests were used to compare the mean ΔV for GA between RT-qPCR and RNase RT-qPCR. All datasets were found to be normally distributed according to Anderson–Darling tests. For heat-treated samples, there was no significant difference in ΔV detected by RT-qPCR and RNase RT-qPCR for GA until 300 s of treatment at 72 °C, when RNase RT-qPCR had significantly higher ΔV for GA than RT-qPCR ($p < 0.001$). Similarly, for UV-treated samples, there were no significant differences in ΔV for GA between RT-qPCR and RNase RT-qPCR below 500 mJ/cm^2 at which point RNase RT-qPCR had significantly higher ΔV for GA than RT-qPCR ($p = 0.008$).

Measurement of Pre-treatment Effectiveness

The effectiveness of the pre-treatments was assessed using Eqs. 1 and 2 as follows. However, given the standard deviations in ΔV (as shown in Fig. 2), it should be noted that there was uncertainty in the exact values calculated.

The percentage of RT-qPCR signal that was attributable to viable viruses was calculated using Eq. 1 (Table 1). RNase RT-qPCR performed slightly better at detecting viable GA than RT-qPCR with no pre-treatment, but the increase in percentage of detected viable GA was only significant ($p < 0.05$) in samples inactivated for 3 s at 72 °C. RNase RT-qPCR was no better at detecting viable GA than RT-qPCR with no pre-treatment following inactivation by UV.

Further to this, the percentage of PCR detectable but non-viable GA that was removed by RNase pre-treatment was calculated using Eq. 2 (Table 2). Only samples inactivated at

Table 1 Percentage of RT-qPCR signal that was attributable to viable GA viruses

	RT-qPCR (%)	RNase RT-qPCR (%)
Time at 72 °C (s)		
3	13	18
10	44	50
20	13	19
UV dose (mJ/cm^2)		
38	11	20
96	1	2
155	< 1	< 1

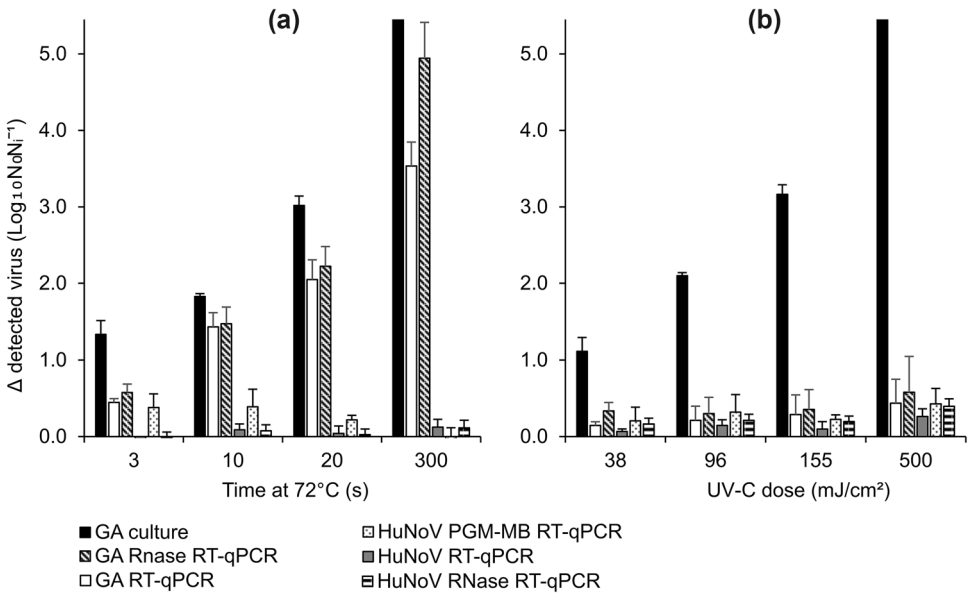

Fig. 2 Change in detected virus (ΔV) by RT-qPCR, PGM-MB RT-qPCR, RNase RT-qPCR to detect HuNoV or GA, compared with the change in GA infectivity by plaque assay following treatment at 72 °C (**a**) or UV-C (**b**). PGM-MB RT-qPCR was not performed for GA. Error bars are standard deviation (n = 6)

Table 2 Percentage of PCR-detectable non-viable GA virus that was removed by capsid integrity-based pre-PCR treatments

	RNase RT-qPCR (%)
Time at 72 °C (s)	
3	28
10	7
20	27
300	96[a]
UV dose (mJ/cm^2)	
38	28
96	12
155	13
500	27[a]

[a]Assuming 100% inactivation

72 °C for 300 s and pre-treated with RNase before RT-qPCR had > 95% of the non-viable virus removed.

Viable Population Overestimate Model

To determine the required level of performance of capsid integrity-based pre-treatments such as RNase and PGM-MBs, a model was developed to determine the level of overestimation of viable levels of a virus following pre-treatments that do not remove 100% of non-viable viral RNA. This model was applied to theoretical populations where 50, 10, 1, 0.1 and 0.01% of the PCR-detectable material derives from viable virus. In inactivated virus populations that are 100% viable before inactivation, these levels are equivalent to ΔV of 0.3, 1, 2, 3 and 4, respectively. The model showed that as the proportion of viable virus decreases, a much greater proportion of the non-viable PCR-detectable population needs to be removed by pre-treatments to reduce overestimation of viable virus levels to levels that might be considered acceptable (e.g. 0.5 log10, Fig. 3).

Discussion

Human norovirus (HuNoV) is the most common cause of viral gastroenteritis worldwide but it cannot be routinely cultured. In this study we have evaluated the efficacy of two capsid integrity-based methods for determining the infectivity of HuNoV after treatment with heat or UV-C radiation. The level of inactivation achieved by heat or UV-C, and therefore, its comparability to inactivation commonly achieved in UV-treated wastewater, was measured by culturing GA.

Application of three RT-qPCR methodologies (including two methods for inferring capsid integrity), to faecal samples in which GA was inactivated by approximately 1, 2, 3 and > 5.5 log units showed that RT-qPCR with or without a pre-treatment step greatly underestimated inactivation of viruses. However, for GA, when the RT-qPCR-based methods were applied to heat-treated samples, the results were more aligned to culture results than for RT-qPCR methods applied to UV-treated GA. A study by Wigginton et al. (2012) indicated that the MS2 genome was not affected when the virus was inactivated at 72 °C and that reductions in viral infectivity were most likely to be due to degradation of the capsid. MS2 and GA are closely related and are both in the Leviviridae family of viruses. The effect of heat on GA detection by RT-qPCR in this study was, therefore, surprising. The cause of this is unclear, but it may suggest that the region of the GA genome targeted by the PCR is more

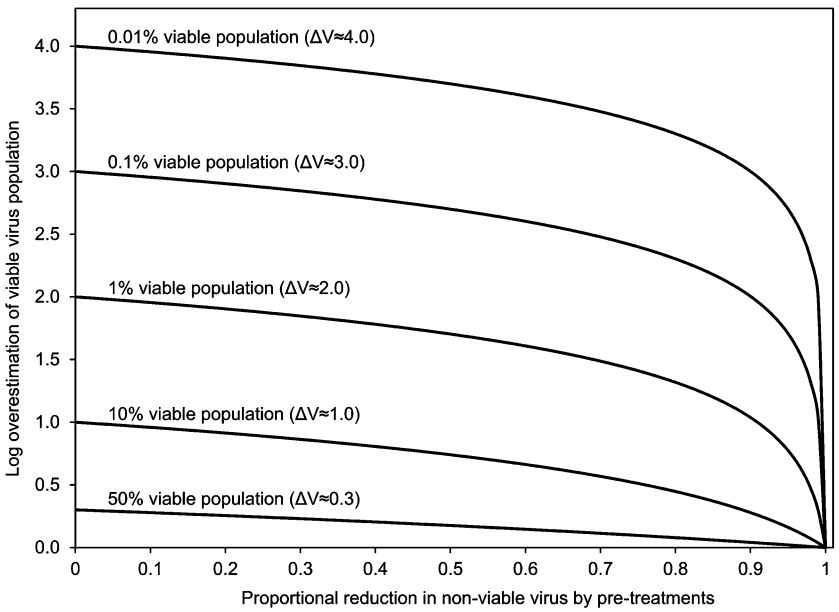

Fig. 3 A hypothetical model to determine the overestimation of viable virus in samples where < 100% of non-viable viruses are removed by capsid-integrity methods prior to RT-qPCR

susceptible to heat degradation than that of MS2. The PCR primers and probe used for quantifying GA in this study targeted the genomic region which codes for the coat protein (position 1778–1889) and where the genome directly interacts with the capsid (Wolf et al. 2008). It is, therefore, possible that this close interaction with the capsid made the target for RT-qPCR more susceptible to degradation. However, the homologous region of the MS2 genome was found by Wigginton et al. to remain stable following heat treatment. This may suggest that closely related GA and MS2 have dissimilarities in their inactivation kinetics, which may have implications on the use of general FRNAP as indicators of faecal viral risk and warrants further investigation. Wigginton et al. also found that the main mechanism on UV inactivation of MS2 was at the genomic level, while having minimal impact on the capsid. The lack of significant removal of non-viable GA by RNase in UV-treated samples may, therefore, be accounted for by a minimal effect on capsid integrity, thereby reducing the effectiveness of RNase treatment to removal genomes of non-viable viruses.

While both PGM-MB and RNase pre-treatments reduced HuNoV RNA detection by RT-qPCR significantly after at least one virucidal level, neither method reduced detection to the levels that might be expected given the inactivation demonstrated by GA culture. FRNAP which include GA have been proposed as environmental surrogates for viral infectivity risk (Doré, Henshilwood and Lees 2000; Hartard et al. 2016, 2018; Cho et al. 2018; Dias et al. 2018). While it is not possible to draw direct comparisons of GA and HuNoV infectivity from GA culture alone, studies by Park et al. (2011) and Weng et al. (2018) showed that FRNAP are less susceptible to inactivation by UV than culturable Caliciviruses (which includes noroviruses) and so indicate that GA is likely to act as a conservative surrogate for noroviruses. Until similar comparisons have been made between culturable HuNoV and other noroviruses, it is difficult to determine the exact relationship between FRNAP and HuNoV infectivity. However, based on the currently known patterns of inactivation of culturable noroviruses, if a sample containing both GA and HuNoV is treated with UV, then it may be expected that the level of HuNoV inactivation would be greater than that measured for GA. A similar decrease should then be reflected in HuNoV detected by capsid integrity-based assays. This was not found to be the case in this study.

RNase treatment was found to increase the percentage of RT-qPCR signal that was attributable to viable virus in some cases for GA. However, for the most part, it did not significantly increase the detectable level of viable viruses and where this was significant, the increases were slight. Calculation of the proportion of non-viable virus that was removed by pre-treatment showed that RNase did indeed remove some of the RNA from non-viable viruses. While this shows that pre-treatments can be somewhat effective, the percentage of non-viable virus removed was much too low for the pre-treatments to reflect the true level of viral inactivation as predicted by GA culture. To investigate the implications of this for the future design and improvement of pre-treatment methods, a model was developed to predict the overestimation of viable virus from the proportional removal of non-viable virus RNA using pre-treatments. As the proportion of viable virus in a sample decreases (i.e. by increased virucidal treatment), the overestimation of viable virus greatly increases if < 100% of non-viable RNA is removed by the pre-treatment. This means that in order for capsid integrity-based methods to satisfactorily reflect the true level of viable virus in a sample, they generally need to be close to 100% efficient. This has not been shown to be the case in this study where the efficiency of removal of non-viable GA by the pre-treatments investigated was generally < 30%. The model's primary function is to demonstrate the limitations of the pre-treatment approach in theory (as shown in Fig. 3), however, it could also be applied as a benchmarking tool in the future. For example, if a laboratory were to decide that a 0.5 $\log_{10}$ overestimation of viable levels was acceptable given a sample where 1% of PCR-detectable virus is viable, then application of Eq. 3 shows that any pre-treatment method must remove 97.8% of non-viable virus to meet this acceptability criteria. Although the effectiveness of a pre-treatment method can only be determined for a culturable virus, where it might be assumed direct assessment of viable virus by culture may be preferable to indirect measurement of viability using a pre-treatment method, there are occasions where use of a PCR-based viability method with pre-treatments may be favoured. For example, in situations where rapid detection of viable virus is desirable such as point-of-care diagnosis or outbreak investigations, the use of pre-treatment techniques to rapidly detect viable viruses may be very beneficial (Deshmukh et al. 2016; Kozel and Burnham-marusich 2017). Additionally, in the case of human noroviruses, for which culture-based assays exist, but are not currently applicable to routine analyses, pre-treatment techniques are likely to remain an attractive alternative.

This study has shown that capsid integrity methods such as RNase and PGM-MB treatment prior to RT-qPCR are not capable of accurately determining the levels of infectious HuNoV. It can be noted that other studies have come to similar conclusions (Pecson et al. 2009; Li et al. 2012; Rönnqvist et al. 2014). While these methods may serve to indicate that some proportion of the HuNoV or GA population is non-infectious, it is not possible to determine what that proportion is using these methods alone. Such methods are therefore not suited to routine analysis of samples for statutory monitoring programmes, where a knowledge of the level of infectivity may help to improve the management of resources. The use of genome integrity to infer

virus infectivity has been shown previously to underestimate actual viral inactivation (Pecson et al. 2009) and so indirect measurements of viral infectivity by PCR appears to be universally un-representative at present. While newer technologies such as platinum compounds may provide improvements in non-viable viral RNA removal before PCR detection compared with PGM-MBs and RNase (Fraisse et al. 2018), the performance still remains lower than required for reliable, quantifiable discrimination between viable and non-viable viruses.

It is, therefore, recommended that while PCR pre-treatment assays cannot remove close to 100% of non-viable viruses and the culture of HuNoV remains impractical for routine analysis, future studies for determining the infectivity risk of HuNoV focus on the use of surrogates and culturable faecal indicator viruses. An example of the former is culturable Caliciviruses which can be spiked into samples for laboratory-based assessments, and examples of the latter are culturable faeces associated viruses such as somatic or FRNAP for naturally contaminated samples. The value of such assays would be greatly improved if comparisons of surrogate and indicator susceptibility to inactivation were carried out in parallel to that of HuNoV using the newly available culture methodology (Ettayebi et al. 2017; Zou et al. 2017) to establish how infectivity and inactivation of surrogates and indicators relate to the same factors for HuNoV under different conditions.

However, in some cases a culture-based assay may not be the most practical option and so future research into alternative methods is critical. This study provides a caution that such methods will require significant validation before they are implemented, and also provides a benchmarking model by which to assess that validation process.

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