Optimization of PMAxx pretreatment to distinguish between human norovirus with intact and altered capsids in shellfish and sewage samples

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Abstract

Shellfish contamination by human noroviruses (HuNoVs) is a serious health and economic problem. Recently an ISO procedure based on RT-qPCR for the quantitative detection of HuNoVs in shellfish has been issued, but these procedures cannot discriminate between inactivated and potentially infectious viruses. The aim of the present study was to optimize a pretreatment using PMAxx to better discriminate between intact and heat-treated HuNoVs in shellfish and sewage. To this end, the optimal conditions (30 min incubation with 100 µM of PMAxx and 0.5% of Triton, and double photoactivation) were applied to mussels, oysters and cockles artificially inoculated with thermally-inactivated (99°C for 5 min) HuNoV GI and GII. This pretreatment reduced the signal of thermally-inactivated HuNoV GI in cockles and HuNoV GII in mussels by more than 3 log. Additionally, this pretreatment reduced the signal of thermally-inactivated HuNoV GI and GII between 1-1.5 log in oysters. Thermal inactivation of HuNoV GI and GII in PBS, sewage and bioaccumulated oysters was also evaluated by the PMAxx-Triton pretreatment. Results showed significant differences between reductions observed in the control and PMAxx-treated samples in PBS following treatment at 72 and 95 °C for 15 min. In sewage, the RT-qPCR signal of HuNoV GI was completely removed by the PMAxx pretreatment after heating at 72 and 95 °C, while the RT-qPCR signal for HuNoV GII was completely eliminated only at 95 °C.

Finally, the PMAxx-Triton pretreatment was applied to naturally contaminated sewage and oysters, resulting in most of the HuNoV genomes quantified in sewage and oyster samples (12 out of 17) corresponding to undamaged capsids. Although this procedure may still overestimate infectivity, the PMAxx-Triton pretreatment represents a step forward to better interpret the quantification of intact HuNoVs in complex matrices, such as sewage and shellfish, and it could certainly be included in the procedures based on RT-qPCR.

Keywords: Intercalating dyes, viability PCR, Norovirus, Shellfish, Sewage, RT-qPCR

1. Introduction

Diarrheal diseases caused by human noroviruses (HuNoV) are one of the most common illnesses resulting from consumption of contaminated food (WHO, 2015). In the USA, foodborne transmission is estimated to account for 23% of HuNoV outbreaks (Hall et al., 2014). In the European Union, crustaceans, shellfish and mollusks were the most commonly implicated food vehicles (27.8% of HuNoV outbreaks in 2015), followed by other foods (19.4%) (EFSA and ECDC, 2016). Also in Europe, the consumption of oysters contaminated with HuNoVs cause 11,800 illnesses per year in the UK (Hassard et al. (2017). In the United States, the annual economic burden due to HuNoV infections and attributed to shellfish contamination is estimated to be US$184 million (Batz et al., 2011).

Most shellfish-borne outbreaks caused by HuNoVs have been associated with the consumption of raw or under-cooked shellfish, especially oysters (Iritani et al., 2014; Lunestad et al., 2016), usually harvested from waters affected by the discharge of treated and untreated sewage (Campos and Lees, 2014). In addition, the scientific community agrees on the inadequacy of commercial shellfish depuration processes for HuNoVs (Le Mennec et al., 2017; McLeod et al., 2017) that could be explained by the presence of oyster ligands which specifically bind HuNoVs (Le Guyader et al., 2012).

The development of rapid, specific, sensitive, and standardized procedures for HuNoV detection in shellfish is of great interest. Recently a standardized RT-qPCR based procedure has been issued for HuNoV genogroup I (GI) and GII in several food matrices, including shellfish, berries and vegetables (Comite Europeen de Normalisation, 2017). RT-qPCR methods detect the viral RNA of both infectious and inactivated HuNoVs, potentially overestimating the amount of infectious viruses (Butot et al., 2009; Hewitt and Greening, 2006; Sanchez et al., 2011). To overcome this limitation, different strategies have been evaluated to predict infectivity using PCR-based methods, such as: (i) pretreatment with nucleases and/or proteolytic enzymes prior to nucleic acid extraction, eliminating the signal of nucleic acids belonging to damaged or inactivated viruses (Lamhoujeb et al., 2008; Nowak et al., 2011), (ii) using the HuNoV ability to bind porcine gastric mucin (PGM) (Tan and Jiang, 2005; Tang et al., 2010)

which allows for selective recovery of potentially infectious HuNoVs in foods (Dancho et al., 2012), and (iii) pretreatment with nucleic acid intercalating dyes, such as ethidium monoazide (EMA) or propidium monoazide (PMA) (reviewed by Elizaquivel et al., 2014; Escudero-Abarca et al., 2014; Parshionikar et al., 2010). This latter approach is based on the ability of intercalating dyes (e.g. PMA or EMA) to penetrate only damaged or altered capsids and intercalate covalently into a viral genome after exposure to strong visible light, thus interfering with PCR amplification. Interestingly, this approach proved to reduce RT-qPCR signals for damaged enteric viruses in naturally contaminated water samples (Blanco, Guix, Fuster, Fuentes, Bartolomé, et al., 2017; Falco et al., 2017; Fuster et al., 2016; Leifels et al., 2015; Prevost et al., 2016; Randazzo et al., 2016a). Moreover, Moreno et al. (2015) applied PMA pretreatments to detect infectious hepatitis A virus (HAV) in vegetable and shellfish samples. PMAxx combined with RT-qPCR has been reported to be a very efficient intercalating dye for assessing viral infectivity discriminating between HAV and HuNoV with intact and altered capsids in vegetables and irrigation waters (Falco et al., 2017; Randazzo et al., 2016a). In the present work, we optimize the PMAxx pretreatment to better discriminate between intact and heat-treated HuNoVs in shellfish and sewage. Additionally, naturally contaminated sewage and shellfish samples were analyzed to evaluate the performance of the PMAxx pretreatment.

2. Materials and Methods

2.1. Viral strains

Fecal samples containing HuNoV genogroup I genotype 4 (GI·P4) and genogroup II genotype 4 (GII.4 variant Den Haag 2006b) were kindly provided by Dr. Buesa, University of Valencia, Spain. Fecal samples containing HuNoV GI.1 and GII.3 were used for bioaccumulation experiments. Stool samples were suspended (10%, wt/vol) in phosphate-buffered saline (PBS) containing 2 M $NaNO_3$ (Panreac), 1% beef extract (Conda), and 0.1% Triton X-100 (Fisher Scientific) (pH 7.2), vortexed and centrifuged at 1000 × g for 5 min. The supernatant was stored at - 80 ºC in aliquots.

110 The cytopathogenic strain MC0 of mengovirus (courtesy of Prof. Albert Bosch, University of
111 Barcelona) was propagated and assayed in HeLa cells. Semi-purified viruses were harvested by
112 three freeze-thaw cycles of infected cells followed by centrifugation at 660 × g for 30 min to
113 remove cell debris. Infectious viruses were enumerated by determining the 50% tissue culture
114 infectious dose ($TCID_{50}$) with eight wells per dilution and 20 µl of inoculum per well using the
115 Spearman-Karber method (Pinto et al., 1994).

116 **2.3. Sewage and shellfish samples**

117 Sewage samples collected from the municipal wastewater treatment plant of Quart (Valencia)
118 from September to December 2016. The plant treats the flow from seven municipalities
119 accounting for 164,171 equivalent inhabitants at a projected flow of 60,000 m^3/day. Sewage
120 samples were concentrated by ultracentrifugation as described by Rodriguez-Diaz et al. (2009).
121 Briefly, 35 ml of sewage was centrifuged at 140,000 × *g* for 2 h 30 min at 4 °C. Then the pellet
122 was eluted by incubating on ice for 30 min with 5 ml of 0.25 N glycine buffer (pH 9.5). The
123 solution was neutralized by adding 5 ml of 2× PBS. The suspended solids were removed by
124 centrifugation (12,000 × *g* for 15 min), and viruses were finally recovered by centrifugation at
125 229,600 × *g* for 1 h at 4°C in an 70Ti rotor. Viral particles were resuspended in 500 µl of 1×
126 PBS. Mengovirus was added in the 35 ml of sewage as a process control virus to monitor
127 extraction efficiency following the ISO 15216:2017 guidelines (Comite Europeen de
128 Normalisation, 2017).
129 Oysters samples were collected from January to March 2013 (4 samples) and on February 2014
130 (2 samples) and were processed as described in the ISO 15216-1:2017 (Comite Europeen de
131 Normalisation, 2017). Mengovirus was added as an extraction efficiency control to each
132 dissected tissue (2 g) before homogenization according the ISO 15216:2017 guidelines (Comite
133 Europeen de Normalisation, 2017)

134 **2.4. Performance of PMAxx pretreatment in artificially contaminated shellfish**

135 Mussels (*Mytilus galloprovincialis*), oysters (*Crassostrea gigas*) and cockles (*Cerastoderma*
136 *edule*) were purchased at a local market and tested for HuNoV GI and GII contamination
137 following the ISO 15216-1:2017 procedure (Comite Europeen de Normalisation, 2017).

Shellfish concentrates were initially prepared as described in the ISO 15216-1:2017. Briefly, 2 g of digestive tissues were transferred to a tube containing 2 ml of proteinase K solution (30 U/mg). This mixture was incubated at 37 °C with shaking for 60 min, followed by incubation at 60 °C for 15 min. Then a centrifugation at 3,000 g for 5 min was performed. Due to the presence of inhibitors an additional centrifugation at 8000 × g for 20 min was included at the end of the procedure. Moreover, RNase inhibitor (40 U, Roche Diagnostics) was added to 100 µl of shellfish supernatants. Shellfish supernatants were inoculated with two different concentrations (ca. 3 and 4 log RT-PCRU per 100 µl of shellfish supernatant) of thermally-inactivated (99 °C for 5 min) HuNoV GI and GII suspensions. Then, samples were aliquoted into 100 µl and added to 100 µM PMAxx and 0.5% Triton 100-X (Fisher-Scientific). Samples treated with PMAxx were incubated in the dark at room temperature for 30 min at 150 rpm and immediately exposed to 2 cycles of 15-min photoactivation using a photo-activation system (Led-Active Blue, GenIUL) with a dark incubation of 15 min between photoactivations. Finally, 100 µl of the sample were mixed with 25 µl of the Plant RNA Isolation Aid (Ambion) and 600 µl of lysis buffer from the NucleoSpin® RNA virus kit (Macherey-Nagel GmbH & Co.) and subjected to pulse-vortexing for 1 min. Afterwards, the homogenate was centrifuged for 5 min at 10,000 × g to remove the debris. The supernatant was subsequently processed using the NucleoSpin® RNA virus kit according to the manufacturer's instructions. Positive control samples (shellfish supernatants inoculated with thermally-inactivated HuNoV GI and GII suspensions) without PMAxx treatment were used to calculate the viral reduction titer. Efficacy of the PMAxx pretreatment was estimated by comparing the number of genome copies of thermally-inactivated HuNoV without the PMAxx pretreatment in a specific shellfish matrix and on the PMAxx-pretreated samples.

2.5. Virus quantification

We performed a standardized one-step TaqMan RT-qPCR using the RNA UltraSense One-Step quantitative system (Invitrogen SA) with a half-scale modification of the manufacturer's protocol. The LightCycler 480 instrument (Roche Diagnostics) was used to determine the

number of genome copies of HuNoV GI, GII and Mengovirus according the ISO 15216-1:2017 (Comite Europeen de Normalisation, 2017). Undiluted sample RNA and ten-fold diluted RNA (to check for RT-qPCR inhibition) were analyzed in duplicate. A standard curve for HuNoV GI, GII and mengovirus were generated by amplifying 10-fold dilutions of viral suspensions by RT-qPCR in quintuplicates, and the numbers of PCRU were calculated. Amplification was performed for 1 cycle of 55 °C for 1 h, 1 cycle of 95 °C for 5 min, and 45 cycles of 95 °C for 15 s, 60 °C for 1 min and 65 °C for 1 min.

2.6. Bioaccumulation experiments

Live oysters (*Crassostrea gigas*) were purchased at local producer and then transfer to the IFREMER facilities. There, oysters were immediately rinsed and transferred into large seawater aquariums equipped with constant aeration. After 24 hours of immersion at the designated temperature adjusted to the season (8-13 °C), oysters were individually checked and only live oysters showing filtration activity were included in the experiments. Before all bioaccumulation experiments, oysters were tested for HuNoV GI and GII contamination following the ISO 15216-1:2017 procedure. For bioaccumulation, one aquarium was filled with 20 L of seawater and seeded with a mixture of HuNoV GI.1 and GII.3 suspensions. Forty five oysters were added to each aquarium, yielding a ratio of 5 liters of water per kilogram of oysters (including the shell weight) as previously described (Drouaz et al., 2015). At 24 hours post seeding the oysters were collected and immediately dissected and frozen.

2.7. Thermal treatment of HuNoV GI and GII in PBS, sewage and bioaccumulated oysters

HuNoV suspensions in PBS pH 7.2 (3-4 log PCRU), a concentrated HuNoV-positive sewage sample and supernatant from bioaccumulated oysters supernatant were incubated at 60, 72 and 95 °C for 15 min in a thermal block to achieve different degrees of viral inactivation. An aliquot of each sample was kept at room temperature during heat treatment and used as a control sample. Then, an aliquot of control and heat-treated samples were further subjected to PMAxx pretreatment. For HuNoV suspensions in PBS and sewage, samples were added to 50 µM PMAxx and 0.5% Triton, and then incubated in the dark at room temperature for 10 min in a shaker at 150 rpm and immediately exposed to continuous LED light (464 to 476 nm) for 15

194 min using a photo-activation system (Randazzo et al., 2016a). For bioaccumulated oyster
195 samples, the pretreatment consisted of 100 µM PMAxx and 0.5% Triton together with a double
196 photoactivation as detailed above. Heat-treated samples without PMAxx treatment were used to
197 calculate the viral reduction titer. After intercalating dye pretreatments, RNA was extracted
198 using the NucleoSpin® RNA virus kit (according to the manufacturer's instructions) and with
199 plant RNA Isolation Aid treatment in case of sewage and shellfish samples (as detailed above).
200

201 **2.8. Analysis of naturally contaminated sewage and oyster samples**

202 One-hundred µl aliquots of concentrated sewage samples were added with 50 µM PMAxx and
203 0.5% Triton (PMAxx-Triton). Photoactivation, plant RNA Isolation Aid treatment, RNA
204 extraction and RT-qPCR were performed as described above. Three different controls were
205 included: i) 100 µl of concentrated samples were processed without performing the PMAxx-
206 Triton pretreatment; ii) 100 µl of concentrated samples were heated at 99 ºC for 5 min and
207 analyzed by RT-qPCR; iii) 100 µl of concentrated samples were heated at 99 ºC for 5 min and
208 analyzed by PMAxx-Triton-RT-qPCR
209 For shellfish, 100 µl of shellfish supernatant were added with 100 µM PMAxx and 0.5% of
210 Triton, incubated for 30 min and photoactivated twice with an additional dark incubation of 15
211 min between photoactivations. RNA extraction and RT-qPCR were performed as described
212 above. Three different controls were included: i) 100 µl of shellfish supernatant were processed
213 without performing the PMAxx-Triton pretreatment; ii) 100 µl of shellfish supernatant were
214 heated at 99 ºC for 5 min and analyzed by RT-qPCR; iii) 100 µl of of shellfish supernatant were
215 heated at 99 ºC for 5 min and analyzed by PMAxx-Triton-RT-qPCR. The efficiency of the
216 procedure was calculated by comparing the detected and added mengovirus genomes.
217

218 **2.7. Statistical analyses**

219 Results from at least four replicates were statistically analyzed using ANOVA with
220 STATISTICA software version 10 (StatSoft Inc., Tulsa, OK, USA) and Tukey's test on a
221 completely randomized design. A P value <0.05 was deemed significant.

3. Results and Discussion

3.1. Efficiency of the PMAxx pretreatment on artificially inoculated shellfish

Last year, the European Commission launched a monitoring program to estimate the European prevalence of HuNoV contamination in oysters collected from representative monitoring points at production areas and dispatch centers using the ISO 15216-1 (European Food Safety, 2016). However, the number of virus genome copies detected by RT-qPCR does not always correlate with the number of infectious virus particles (Butot et al., 2008; Hewitt and Greening, 2004), and it is important to consider that the infectious risk associated with the levels of HuNoVs in oysters, as determined by RT-qPCR, may be overestimated. Therefore, the use of strategies to remove the RNA from inactivated viruses will improve the risk associated with water and food samples (Blanco, Guix, Fuster, Fuentes, Bartolome, et al., 2017; Randazzo et al., 2016b) .

In a previous study, our group demonstrated that a pretreatment of 50 µM PMAxx combined with 0.5% of Triton was the most efficient pretreatment to discriminate between intact and thermally-inactivated HuNoV GI and GII, in different types of vegetables (Randazzo et al., 2016a). When this procedure was initially applied in shellfish samples, performance of the pretreatment had very limited effect on HuNoV titers due to the complexity of the matrix (data not shown) and the pretreatment has been modified (i.e., increased concentration of PMAxx, 30 min of incubation time and double photoactivation). To validate the improved efficiency of this pretreatment to detect potentially infectious HuNoVs in shellfish, two concentrations of thermally-inactivated HuNoV GI and GII suspensions were artificially inoculated in shellfish, specifically mussels, oysters and cockles. Table 1 shows that the PMAxx–Triton pretreatment completely prevented RT-qPCR detection of thermally-inactivated HuNoV GII in mussels at high and low inoculation levels, while for HuNoV GI this pretreatment only reduced the RT-qPCR signal by approximately 1 log. In oysters, a similar pattern was observed for HuNoV GI and GII, being that the signal was completely removed for thermally-inactivated HuNoV GII at low inoculation levels. A completely different behavior was reported in common cockles, where the RT-qPCR signal was removed for thermally-inactivated HuNoV GI and not for HuNoV GII. An explanation for these differences could be the presence of different compounds, depending

on shellfish species, season or place of harvesting, which may facilitate or interfere with the performance of the PMAxx pretreatment.

3.2. Performance of the PMAxx-Triton pretreatment to monitor heat treatments

In order to assess the ability of PMAxx to monitor HuNoV inactivation after heating, initially HuNoV suspensions in PBS were treated at 60, 72 and 95 °C for 15 min and immediately evaluated by RT-qPCR and PMAxx-Triton-RT-qPCR. As previously reported (Escudero-Abarca et al., 2014; Li et al., 2017), HuNoV RNA levels in PBS remained constant regardless of temperature treatment when evaluated by RT-qPCR (Table 2 and 3). When amplification was preceded by a PMAxx-Triton pretreatment, the RT-qPCR signal from HuNoV GI in PBS was easier to remove than the signal of HuNoV GII. Recently, Ettayebi et al. (2016) reported that heating at 60°C for 15 min resulted in complete inactivation of HuNoV GII.4 and GII.3 following evaluation by culture in human intestinal enteroids. When a treatment at 60 °C for 15 min was evaluated by PMAxx-Triton RT-qPCR a relative high reduction of RNA levels (approximately 2.8 log) was observed for HuNoV GI (Table 2), while no reduction was detected for HuNoV GII. For HuNoV GI, there was no further reduction after the PMAxx pretreatment, regardless of holding temperature, while for HuNoV GII at 72 and 95 °C, the RNA copy number decreased by approximately 1.5 log. These results are in line with recent findings where a PMA pretreatment (250 μM) of heat-treated (85 °C for 1 min) HuNoV GII.4 suspensions reduced the RT-qPCR signal by 1.6 log (Jeong et al., 2017).

The results at 60°C for HuNoV GII indicated that infectivity might be lost (Ettayebi et al., 2016), but their capsids did not allow the penetration of PMAxx because no reduction of RNA levels was observed. These discrepancies may be due to the fact that heat treatment at 60 °C may lead to changes in capsid conformation and, in turn, a loss of infectivity without compromising capsid integrity. These results are consistent with discrepancies found for HAV when applying the same thermal treatments and comparing the PMAxx pretreatment and infectivity by cell-culture (Falco et al., 2017), since HAV infectivity was reduced by 3.00, 3.75

277 and >5.05 logs $TCID_{50}$, after treatments at 60, 72 and 95 °C, while the decrease of HAV titers
278 after the PMAxx pretreatment was 1.32, 3.03 and >3.18 logs, respectively.
279 In addition, a naturally contaminated sewage sample positive with HuNoV GI and GII and a
280 bioaccumulated oyster supernatant were also treated at 60, 72 and 95 °C for 15 min. While the
281 HuNoV RNA levels in the sewage remained unchanged when evaluated by RT-qPCR,
282 regardless of temperature treatment applied, almost one log of reduction was observed in
283 bioaccumulated oysters after heating at 95 °C (Table 3). A plausible explanation for the
284 decrease of RNA levels after heating oyster supernatants would be the action of RNases, despite
285 the presence of an RNase inhibitor in the samples.
286 Additionally, the RT-qPCR signal of HuNoV GI was completely removed by the PMAxx
287 pretreatment after heating sewage samples at 72 and 95 °C (Table 2), while the PMAxx
288 pretreatment for HuNoV GII completely eliminated the RT-qPCR signal only at 95 °C (Table
289 3). In bioaccumulated oysters, the PMAxx-Triton pretreatment was partially effective only in
290 oysters treated at 95 °C, with one log reduction of the RT-qPCR signal. Differences observed in
291 the performance of the PMAxx pretreatment between PBS, sewage and oysters are most likely
292 due to matrix; however, strain variability, in terms of thermal resistance and capsid constrains,
293 may also play an important role (Butot et al., 2009; da Silva et al., 2007).
294 Cooking procedures applied to shellfish in which an internal temperature reaches at least 90 °C
295 for 1.5 min are considered adequate treatments to eliminate viral infectivity (Codex
296 Alimentarius, 2012). Assuming that treatments at 95 °C for 15 min completely inactivate
297 HuNoVs (Ettayebi et al., 2016), these results showed that the PMAxx-Triton pretreatment
298 cannot completely prevent RT-qPCR amplification from thermally-inactivated HuNoVs in
299 oysters, leading to an overestimation of potential infectious HuNoV. Nevertheless, the
300 incorporation of the PMAxx pretreatment improves the discrimination between intact and
301 altered HuNoV capsids.
302 **3.3. Application of the PMAxx-Triton pretreatment in naturally contaminated samples**
303 In order to evaluate the performance of the PMAxx-Triton pretreatment in naturally
304 contaminated samples, sewage samples and oysters were analyzed with and without the

PMAxx-Triton pretreatment. In parallel, naturally contaminated samples were heated at 99 °C for 5 min to assess the performance of the PMAxx-Triton pretreatment individually. Mengovirus recovery ranged from 4.37-13.3 %, and thus the results were considered valid (Comite Europeen de Normalisation, 2017). For sewage, the PMAxx-Triton pretreatment did not modify the levels of HuNoV quantification, indicating that most likely HuNoV genomes quantified in sewage corresponded to undamaged viruses (Table 4). As a control, the PMAxx-Triton pretreatment completely removed the RT-qPCR signal of heat-treated sewage samples naturally contaminated with HuNoV (Table 4).

Currently, a pretreatment using intercalating dyes has only been applied in environmental waters for assessing enteric virus infectivity (Blanco, Guix, Fuster, Fuentes, Bartolomé, et al., 2017; Fuster et al., 2016; Leifels et al., 2016; Prevost et al., 2016; Randazzo et al., 2016a; Randazzo et al., 2017). For the first time, this study evaluated the potential of PMAxx-Triton pretreatment to discriminate between intact and altered HuNoV particles in naturally contaminated shellfish samples. Quantification of HuNoV GI and GII was performed in naturally contaminated oysters with and without the PMAxx-Triton pretreatment in parallel. Mengovirus recovery in oysters was calculated as >1% for all the samples and then, the results were considered valid (Comite Europeen de Normalisation, 2017). As shown in Table 5, no significant differences were reported after the PMAxx-Triton pretreatment in oysters. Since the ISO procedure includes a proteinase K treatment to release viruses from shellfish tissues, the impact of this broadly reactive protease on norovirus capsid cannot be excluded. In order to confirm that the PMAxx-Triton pretreatment was able to reduce the signal of inactivated viruses in oyster concentrates, aliquot samples were heated for 5 min at 99 °C and quantified in parallel with and without the PMAxx-Triton pretreatment. As expected, heat treatment resulted in a reduction of HuNoV titers in all samples (Table 5), although in some of them the PMAxx-Triton-RT-qPCR signal was not completely removed, indicating the need for including this control for complex matrices. Moreover, as reported for bioaccumulated oysters, heat treatment reduced the detection of HuNoVs by RT-qPCR.

Despite the fact that the PMAxx-Triton pretreatment is a step forward to better interpret quantification of HuNoVs, this pretreatment still faces some challenges that need to be addressed. One of them is the fact that the PMAxx-Triton pretreatment has only been evaluated after thermal treatments. Viruses in water and food products may be exposed to different inactivation mechanisms, such us UV light, high pressure or the presence of antiviral compounds. Therefore, this pretreatment must be evaluated for each inactivation process separately. For instance, it was recently shown that inactivation of HuNoV GI and GII with epigallocatechin gallate, a natural compound, was not discriminated by the PMAxx-Triton pretreatment (Falco et al., 2017).

In conclusion, without having a robust method of cell-culture for assessing norovirus infectivity in water and food, RT-qPCR procedures are still the gold standard for HuNoV detection. Our results suggest that a PMAxx-Triton-RT-qPCR assay may still underestimate HuNoV inactivation by heat treatments in shellfish, specifically in oysters. However, adopting a PMAxx-Triton-RT-qPCR procedure in routine monitoring will allow for more accurate quantification of potentially infectious HuNoVs in water and shellfish samples, constituting a useful tool for future risk assessment studies.

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358 Table 1. Quantification of thermally-inactivated (99 °C for 5 min) HuNoV GI and GII
359 suspensions inoculated in shellfish concentrates after PMAxx-Triton pretreatment and
360 RT-qPCR

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			Levels of NoV in shellfish concentrates[a] (log RT-PCRU/100 µl)			
		PMAxx + Triton	**High[b]**	**R[c]**	**Low**	**R**
Mussels	HuNoV GI	-	4.75 ± 0.20A		3.39 ± 0.07A	
		+	3.37 ± 0.06B	1.38	2.31 ± 0.06B	1.08
	HuNoV GII	-	3.51 ± 0.22A		3.08 ± 0.09A	
		+	ND	>3.51	ND	>3.08
Oysters	HuNoV GI	-	4.11 ± 0.13A		3.17 ± 0.23A	
		+	2.86 ± 0.32B	1.25	2.73B *	>0.44
	HuNoV GII	-	2.82 ± 0.08A		1.86 ± 0.16A	
		+	1.69 ± 0.22B **	>1.13	ND	>1.86
Common cockles	HuNoV GI	-	4.31 ± 0.12A		3.08 ± 0.18A	
		+	ND	>4.31	ND	>3.08
	HuNoV GII	-	2.96 ± 0.09A		2.17 ± 0.12A	
		+	2.14 ± 0.26B	0.82	1.76 ± 0.18B	0.41

363

364 [a]Results are mean values from four replicates, and HuNoV titers were obtained by RT-qPCR
365 using a HuNoV standard curve based on RT-PCRU.
366 [b]Values with different letters in the same column and same matrix denote significant differences
367 between treatments ($P < 0.05$).
368 [c]Reduction in titers between thermally inactivated viruses before and after pretreatment.

369 *, one positive replicate out of four; **, two positive replicates out of four.
370

371 ND: Not detected

372 Table 2. Performance of PMAxx-Triton pretreatment to discriminate between intact and
373 thermally-inactivated HuNoV GI (60, 72 and 95 ºC for 15 min) in PBS, sewage and
374 bioaccumulated oysters

375

HuNoV GI [a,b] (log RT-PCRU/100 µl)	**PMAxx + Triton**	**PBS**		**Sewage**		**Bioaccumulated oysters**	
		Mean ± std	**R**	**Mean ± std**	**R**	**Mean ± std**	**R**
Intact	-	5.29 ± 0.42A		3.44 ± 0.15A		5.60 ± 0.23A	
	+	4.95 ± 0.43A	0.34	3.09 ±0.22A	0.35	5.54 ± 0.11A	0.06
Treated at 60 °C	-	5.27 ± 0.10A		3.46 ± 0.13A		5.02 ± 0.07A	
	+	2.43 ± 0.37B	2.84	3.22 ± 0.31A	0.24	4.86 ± 0.08A	0.16
Treated at 72 °C	-	5.42 ± 0.11A		3.40 ± 0.12A		4.84 ± 0.56A	
	+	2.97 ± 0.15B	2.45	ND	>3.40	4.76 ± 0.16A	0.08
Treated at 95 °C	-	5.71 ± 0.35A		3.21 ± 0.23A		4.84 ± 0.11A	
	+	3.22 ± 0.49B	2.49	ND	>3.21	3.99 ± 0.18A	0.85

376 R, reduction in titers obtained between samples before and after PMAxx-Triton
377 pretreatment.

378 [a]Results are mean values from four replicates, and HuNoV titers were obtained by RT-qPCR
379 using a HuNoV standard curve based on RT-PCRU.
380 [b]Values with different letters in the same column denote significant differences between
381 treatments ($P < 0.05$).
382 ND: Not detected

383

384 Table 3. Performance of PMAxx pretreatment to discriminate between potentially intact
385 and thermally-inactivated HuNoV GII (60, 72 and 95 ºC for 15 min) in PBS, sewage
386 and bioaccumulated oysters.

HuNoV GII [a,b] (log RT-PCRU/100 µl)	**PMAxx + Triton**	**PBS**		**Sewage**		**Bioaccumulated oysters**	
		Mean ± std	**R**	**Mean ± std**	**R**	**Mean ± std**	**R**
Intact	-	3.78 ± 0.24A		3.52 ± 0.05A		5.04 ± 0.06A	
	+	3.67 ± 0.11A	0.11	3.42 ± 0.11A	0.10	4.97 ± 0.12A	0.07
Treated at 60 °C	-	3.76 ± 0.36A		3.11 ± 0.86A		4.97 ± 0.04A	
	+	3.63 ± 0.40A	0.13	3.10 ± 0.18A	0.01	4.78 ± 0.03AB	0.19
Treated at 72 °C	-	3.11 ± 0.81A		3.40 ± 0.14A		4.53 ± 0.06B	
	+	1.41 ± 0.63C	1.70	2.61 ± 0.15B	0.79	3.75 ± 0.29C *	0.78
Treated at 95 °C	-	3.85 ± 0.87A		3.15 ± 0.21A		3.93 ± 0.17C	
	+	2.29 ± 0.35B	1.56	ND	>3.15	2.86 ± 0.04D *	1.07

387 *, two positive replicates out of four.
388 R, reduction in titers obtained between samples before and after PMAxx-Triton
389 pretreatment.
390 [a]Results are mean values from four replicates, and HuNoV titers were obtained by RT-qPCR
391 using a HuNoV standard curve based on RT-PCRU.
392 [b]Values with different letters in the same column denote significant differences between
393 treatments ($P < 0.05$).
394

395 ND: Not detected

Table 4. Quantification of HuNoV GI and GII present in sewage by RT-qPCR and PMAxx-Triton RT-qPCR.

Sample	Treatment	PMAxx + Triton	NoV GI [a,b] (log RT-PCRU/100 µl)		NoV GII [a,b] (log RT-PCRU/100 µl)	
			Mean ± SD	R	Mean ± SD	R
R4	untreated	-	ND		2.81 ± 0.12A	
		+	ND		1.22B*	>1.66
	5 min a 99 ºC	-	ND		2.16 ± 0.64AB	
		+	ND		ND	>2.16
R8	untreated	-	2.60 A		2.75 ± 0.37A	
		+	<LOQ	>2.60	2.36 ± 0.09A	0.39
	5 min a 99 ºC	-	<LOQ		2.14A	
		+	<LOQ		ND	>2.14
R10	untreated	-	2.41 ± 0.29 A		2.75 ± 0.02A	
		+	ND	>2.41	1.59 ± 0.29B	1.16
	5 min a 99 ºC	-	<LOQ		2.93 ± 0.03A	
		+	ND		ND	>2.93
R12	untreated	-	3.44 ± 0.15A		3.52 ± 0.05A	
		+	3.09 ±0.22A	0.35	3.42 ± 0.11AB	0.10
	5 min a 99 ºC	-	3.21 ± 0.23A		3.15 ± 0.21B	
		+	ND	>3.21	ND	>3.15
R19	untreated	-	2.23 ± 0.23A		2.16 ± 0.17A	
		+	2.27 ± 0.35A	0.04	1.84 ± 0.64A	0.32
	5 min a 99 ºC	-	ND		1.91 ± 0.26A	
		+	ND		ND	>1.91

R, reduction in titers obtained between samples before and after PMAxx-Triton pretreatment.
*, one positive replicate out of four
ND: Not detected
LOQ, positive sample below the quantification limit

[a]Results are mean values from four replicates, and HuNoV titers were obtained by RT-qPCR using a HuNoV standard curve based on RT-PCRU.
[b]Values with different letters in the same column and same sample denote significant differences between treatments ($P < 0.05$).

Table 5. Quantification of HuNoV GI and GII present in naturally contaminated oysters by RT-qPCR and PMAxx-Triton RT-qPCR.

Sample	Treatment	PMAxx + Triton	NoV GI [a,b] (log RT-PCRU/100 µl)		NoV GII [a,b] (log RT-PCRU/100 µl)	
			Mean ± SD	R	Mean ± SD	R
13/11	untreated	-	3.43 ± 0.21A		2.77 ± 0.06A	
		+	3.48 ± 0.18A	0	2.67 ± 0.16A	0.10
	5 min a 99 ºC	-	2.99 ± 0.26A		1.93 ± 0.19B	
		+	ND	>2.99	ND	>1.93
13/018	untreated	-	3.46 ± 0.08A		1.61 ± 0.23A	
		+	3.10 ± 0.10A	0.36	1.58 ± 0.20A	0.02
	5 min a 99 ºC	-	3.07 ± 0.30A		1.40A *	
		+	2.02B *	1.05	ND	
14/22	untreated	-	2.88 ± 0.34A		2.03 ± 0.13A	
		+	2.55A *	>0.33	1.91 ± 0.12A	0.11
	5 min a 99 ºC	-	2.02A *		1.35 ± 0.30B	
		+	ND	>2.02	ND	>1.35
14/24	untreated	-	2.58 ± 0.51A		2.42 ± 0.58A	
		+	2.97 ± 0.09A	0	2.23 ± 0.51A	0.19
	5 min a 99 ºC	-	2.33 ± 0.44A		1.66 ± 0.29A	
		+	ND	>2.33	1.42 ± 0.02A **	
13/030	untreated	-	2.67 ± 0.05A		1.85 ± 0.10A	
		+	2.57A *	0.10	1.13B *	0.72
	5 min a 99 ºC	-	2.38 ± 0.10 B		ND	
		+	2.58A *		ND	

*, one positive replicate out of four; **, two positive replicates out of four.
R, reduction in titers obtained between samples before and after viability pretreatment.
ND, Not Detected

[a]Results are mean values from four replicates, and NoV titers were obtained by RT-qPCR using a NoV standard curve based on RT-PCRU.
[b]Values with different letters in the same column and same matrix denote significant differences between treatments ($P < 0.05$).

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