Improving efficiency of viability-qPCR for selective
detection of infectious HAV in food and water samples

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Running title: Detection of infectious HAV

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Abstract
Aim: To improve the efficacy of intercalating dyes to distinguishing between infectious and inactivated hepatitis A virus (HAV) in food.

Methods and results: Different intercalating dyes were evaluated for the discrimination between infectious and thermally-inactivated HAV suspensions combining with the RT-qPCR proposed in the ISO 15216. Among them, PMAxx was the best dye in removing the RT-qPCR signal from inactivated HAV. Applied to lettuce and spinach, PMAxx-Triton pretreatment resulted in complete removal of the RT-qPCR signal from inactivated HAV. Likewise, this study demonstrates that this pretreatment is suitable for the discrimination of inactivated HAV in shellfish without further sample dilution. In mussels and oysters, the developed viability RT-qPCR method reduced the signal of inactivated HAV between 1.7 and 2.2 logs at high inoculation level, and signal was completely removed at low inoculation level.

Conclusions: This study showed that the use of PMAxx is an important improvement to assess HAV infectivity by RT-qPCR. It was shown that PMAxx-Triton pretreatment is suitable for the analysis of infectious HAV in complex food samples such as vegetables and shellfish.

Significance and impact of the study: The PMAxx-Triton pretreatment can be easily incorporated to the ISO norm for infectious virus detection.

Keywords: Hepatitis A virus, viability PCR, food, water.
Hepatitis A virus (HAV) is a small (27 to 32 nm), non-enveloped, and positive-sense single-stranded RNA virus which is excreted in feces at levels from $10^6$ to $10^{11}$ viruses per gram (Costafreda et al. 2006). Consequently, hepatitis A infection generally occurs through the fecal–oral route either by direct contact with an HAV-infected person, ingestion of contaminated water or food, or in a lesser extent by contact with contaminated fomites (Hollinger and Emerson 2001). As a result of the increasing number of hepatitis A outbreaks associated with imported foods in high-income countries (reviewed by Sánchez 2015), HAV has been considered as a re-emerging foodborne public health threat (Sprenger 2014). Moreover, the World Health Organization (WHO) has recently estimated that there are 14 million cases and 28,000 deaths of foodborne hepatitis A worldwide every year (WHO 2015). Therefore, there is a great demand for rapid, specific, sensitive, accurate and standardized procedures for HAV detection in foods. Recently a standardized RT-qPCR-based procedure has been developed for norovirus and HAV detection in some food matrices (ISO 15216-1:2017). Like any other technologies, PCR-based methods have limitations, such as discrimination of infectious and inactivated viruses that may overestimate the presence of infectious foodborne viruses. To circumvent this limitation, monoazide intercalating dyes such as ethidium monoazide (EMA) and propidium monoazide (PMA) have been applied to pretreat samples before RNA extraction. Both intercalating dyes contain a photo-inducible azide group that covalently binds to available nucleic acids of inactivated viruses after a photoactivation step, thus preventing further amplification by RT-qPCR (Elizaquível et al. 2014). PMA pretreatment was first reported to effectively exclude the RT-PCR signal of thermally-inactivated poliovirus, echovirus and coxsackievirus (Parshionikar et al. 2010). Later, this approach was adapted by other researchers to discriminate between infectious and inactivated enteric viruses in water, manure, and food samples by viability PCR (Leifels et al. 2015; Moreno et al. 2015; Fongaro et al. 2016; Fuster et al. 2016; Prevost et al. 2016; Randazzo et al. 2016). Apart from heat treatment, performance of intercalating dyes has also been evaluated upon inactivation by high-pressure processing (Sánchez et al. 2012a), UV exposure (Sangsanont et al. 2014; Leifels et al. 2015; 2016; Prevost...
et al. 2016), chlorine (Leifels et al. 2015; Fuster et al. 2016; Prevost et al. 2016), and epigallocatechin gallate (Falcó et al. 2017).

For HAV, only conventional intercalating dyes (i.e. PMA and EMA) have been evaluated for assessing HAV infectivity, with PMA being the more preferably used intercalating dye for the detection of infectious HAV (Sánchez et al. 2012a; Moreno et al. 2015; Fuster et al. 2016) compared with EMA (Coudray-Meunier et al. 2013). In a previous study, our group reported that PMA pretreatment combined Triton was quite effective to discriminate between infectious and thermally-inactivated HAV in vegetable samples, but not in shellfish concentrates (Moreno et al. 2015). Therefore, the main goal of this study was to further explore the potential of new intercalating dyes to differentiate between infectious and thermally-inactivated HAV suspensions using the RT-qPCR assay proposed in the framework of the ISO 15216-1, and to assess its applicability in vegetables and shellfish. Additionally, sewage water samples were analyzed to evaluate the performance of intercalating dyes in naturally contaminated samples.

Materials and Methods

Virus propagation and cell lines

The cytopathogenic HM-175 18f strain of HAV (ATCC VR-1402) and the strain MC of mengovirus (courtesy of Prof. Albert Bosch, University of Barcelona) was propagated and assayed in confluent FRhK-4 and HeLa cells, respectively. Semi-purified viruses were harvested by three freeze-thaw cycles of infected cells followed by centrifugation at $660 \times g$ for 30 min to remove cell debris. Infectious viruses were enumerated by determining the 50% tissue culture infectious dose (TCID$_{50}$) with eight wells per dilution and 20 μl of inoculum per well using the Spearman-Karber method (Pintó et al. 1994).

Evaluation of intercalating dyes

Stock solution of PMA (GenIUL) was reconstituted with 2.6 M dimethylsulfoxide at 20 mM, and PMAxx$^\text{TM}$ (Biotium) and PEMAX$^\text{TM}$ (GenIUL) were dissolved in water at 4 mM. All
reagents were stored at −20°C protected from light. Initially, intercalating dyes at 50 µM was added to 3-4 log PCRU of infectious and thermally-inactivated HAV suspensions (99 ºC for 5 min) diluted in PBS or water in DNA LoBind 1.5 ml tubes (Eppendorf). Then, samples were 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 min using a photo-activation system (Led-Active Blue, GenIUL). After intercalating dye pretreatments, RNA was extracted using the NucleoSpin® RNA virus kit (Macherey-Nagel GmbH & Co.) according to the manufacturer's instructions. Experiments were carried out in triplicate.

HAV quantification

The set of primers (FW: TCACCGCCGTTTGCTTAG and REV: GGAGAGCCCTGG AAGAAAG) and probe (CCTGAACCTGACGAATTAA) used in this study were previously validated (Costafreda et al. 2006). Probe was labelled at the 5’-end with 6-carboxyfluorescein (FAM) and at the 3’-end with MGBNFQ (minor groove binder/non-fluorescent quencher). HAV genome copies were quantified in duplicate by one-step RT-qPCR assay using the RNA UltraSense One-Step quantititative system (Invitrogen SA) with a half-scale modification of the manufacturer’s protocol and the LightCycler 480 instrument (Roche Diagnostics) following the standardized ISO procedure (ISO 15216-1:2017). 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. The standard curve was generated by serial end-point dilution, amplifying 10-fold dilutions of the HAV stock by RT-qPCR in quintuplicates. The crossing points (Cp) obtained from the assay of each dilution were used to plot a standard curve by assigning a value of 1 RT-PCR unit (PCRU) to the highest dilution showing a positive crossing point value and progressively 10-fold-higher values to the lower dilutions.

Performance of PMAxx pretreatment in thermally treated HAV

HAV suspensions in PBS pH 7.2 (3-4 log PCRU) were incubated at 60 ºC, 72 ºC and 95 ºC for 15 min in a thermal block to achieve different degrees of viral inactivation. An aliquot of HAV
suspension was kept at room temperature during heat treatment and used as a control. After thermal treatment, heat-treated and control samples were further subject to infectivity assay on FRhk-4 cells, RT-qPCR and PMAxx-RT-qPCR as described above. Experiments were performed in triplicate.

Performance of intercalating dye treatments to discriminate infectious from thermally-inactivated HAV in food samples

Romaine lettuce (*Lactuca sativa*) and spinach (*Spinacia oleracea*) concentrates were prepared as previously described by Sánchez et al. (2012b). Briefly, 10 g of vegetables were washed with 90 mL of buffered peptone water in a sterile plastic bag with lateral filter (BagPage S 400, Interscience) using the Pulsifier equipment (Microgen Bioproducts) for 15 s. The mixture was taken from the filter side to remove particulate debris, and the resulting filtrate was supplemented with a final concentration of 12.5 mM PEG 8000 and 0.3 M NaCl. After gentle shaking for 1 h at 4 °C, samples were centrifuged for 30 min at 10,000×g at 4 °C. Then the pellet was resuspended with 500 μL of PBS.

Mussels (*Mytilus galloprovincialis*) and oysters (*Crassostrea gigas*) concentrates were prepared treating 2 g of digestive glands with proteinase K as described in the ISO 15216-1. Finally, 100 μl of vegetable concentrates or shellfish supernatants were inoculated with 3-4 log TCID₅₀ of infectious or thermally-inactivated (99°C for 5 min) HAV suspensions. Thereafter, samples were added to 50 μM PMAxx (based on results from evaluation of intercalating dyes section) and 7.7 mM Triton 100-X (Fisher-Scientific), incubated in the dark at room temperature for 10 min at 150 rpm and photoactivated as described above. After photoactivation, 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 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.
Sewage samples collected from the municipal wastewater treatment plant of Quart (Valencia) in September-December 2016. This treatment plant is receiving wastewater from seven municipalities with about 164,000 inhabitants, and has an average flow of 60,000 m$^3$/day. Samples were concentrated by ultracentrifugation as described by Rodríguez-Díaz et al. (2009). Briefly, 35 ml of sewage was centrifuged at 140,000 × g using an SW28 rotor for 2 h 30 min at 4°C. Then pellet was eluted by incubating on ice for 30 min with 5 ml of 0.25 M glycine buffer (pH 9.5). The solution was neutralized by adding 5 ml of 2× phosphate-buffered saline. The suspended solids were removed by centrifugation (12,000 × g for 15 min), and viruses were finally recovered by centrifugation at 229,600 × g for 1 h at 4°C in an 70Ti rotor. Viral particles were resuspended in 500 µl of 1× PBS. Mengovirus was included in the 35 ml of sewage as process control virus to monitor extraction efficiency following the ISO 15216 guidelines. Subsequently, 100 µl of concentrated sewage water samples were added with 50 µM PMAxx and 7.7 mM Triton (PMAxx-Triton). Photoactivation, plant RNA Isolation Aid treatment, RNA extraction and RT-qPCR were performed as described above. As a control, 100 µl of concentrated samples were processed without performing the PMAxx-Triton pretreatment.

**Statistical analyses**

Results from three replicates of the treatments and controls were statistically analyzed using ANOVA with STATISTICA software version 10 (StatSoft Inc., Tulsa, OK, USA) and Tukey’s test on a completely randomized design. A P value <0.05 was deemed significant.

**3. Results**

*Evaluation of intercalating dyes and buffers on HAV suspensions*

In order to establish the optimal intercalating dye conditions for the discrimination of infectious and thermally-inactivated viruses, HAV suspensions were completely inactivated by incubation at 99 °C for 5 min, and samples were then treated with three different commercial intercalating
dyes for 10 min and activated using a continuous photoactivation system. Results showed that the three intercalating dyes rendered statistically significant (p< 0.05) signal reductions of thermally-inactivated HAV suspensions, however PMAxx provided significantly higher differences between untreated and thermally-inactivated viruses (p<0.05) (Table 1). Performance of PMAxx was further evaluated using water and PBS as reaction buffers. The use of PBS improved PMAxx efficiency since it complete eliminated the RT-qPCR signal of thermally-inactivated HAV (Table 2).

**Performance of PMAxx pretreatment to monitor heat treatments**

In addition, the ability of PMAxx to monitor HAV inactivation after heating was evaluated by RT-qPCR, with or without PMAxx pretreatment. Figure 1 shows that quantification using RT-qPCR remained constant at the different temperatures applied for thermal inactivation of HAV suspensions. The thermal treatment produced a higher degree of inactivation as estimated by the infectivity assay, being inactivated by 3.00, 3.75 and >5.05 logs, respectively after treatments at 60, 72 and 95 °C, while the decrease of HAV titers after PMAxx pretreatment was 1.32, 3.03 and >3.18 logs reductions, respectively (Figure 1).

**Efficiency of intercalating dye treatment on different types of food matrices.**

To validate the improved efficiency of the PMAxx pretreatment to detect infectious HAV in food, thermally-inactivated HAV suspensions were inoculated at $6 \times 10^4$ and $6 \times 10^3$ PCRU and treated with 50 μM PMAxx with 7.7 mM Triton. Table 3 shows that PMAxx–Triton pretreatment completely prevented RT-qPCR detection of thermally-inactivated HAV in lettuce and spinach samples at low and high inoculation levels.

Similarly, the PMAxx-Triton pretreatment was applied to discriminate thermally-inactivated HAV in shellfish, specifically in mussels and oysters. The PMAxx-Triton pretreatment completely reduced the RT-qPCR signal of inactivated HAV when inoculated at $2 \times 10^3$ PCRU, while this pretreatment reduced by 1.69 and 2.17 logs the signal in mussels and oysters inoculated at $2 \times 10^4$ PCRU, respectively (Table 3).
Application of PMAxx-Triton pretreatment in sewage waters

Additionally, to evaluate the performance of PMAxx pretreatment in naturally contaminated samples, sewage water samples were analyzed in parallel with and without PMAxx-Triton pretreatment, and HAV quantification was adjusted with the mengovirus recovery rate. According to ISO 15216:2017, the extraction efficiencies were calculated as >1% for all the sample and thus results considered valid. As shown in Table 4, PMAxx-Triton pretreatment did not modify the levels of HAV quantification.

4. Discussion

Consumption of produce (soft fruits and leafy greens) and shellfish are commonly attributed to hepatitis A outbreaks (Sánchez 2015). Despite the development of an ISO method for HAV detection in different food matrices, one of the main challenges for the food industry is the need to better understand the relationship between RT-qPCR signals and infectivity of wild-type HAV strains. In this sense, the use of intercalating dyes (i.e. PMA and EMA) has been shown as an innovative and promising technology to selectively detect infectious HAV by RT-qPCR (Sánchez et al. 2012a; Coudray-Meunier et al. 2015; Moreno et al. 2015; Fuster et al. 2016). In this study, we first compared the performance of a conventional intercalating dye (i.e. PMA) and newly developed ones (i.e. PMAxx and PEMAX). When applied on suspensions, all intercalating dyes statistically (p<0.05) decreased the RT-qPCR signal of thermally-inactivated HAV suspensions, with PMAxx being the most effective. These results are in line with the results previously reported for norovirus GI and GII, whereas PMAxx was the most efficient intercalating dye (Randazzo et al. 2016). Moreover, performance of PMAxx treatment was better in PBS rather than in water. This finding is important in case of working with complex samples, e.g. manure, sewage, and shellfish, which may have to be diluted to improve the performance of intercalating dye treatments (Moreno et al. 2015; Leifels et al. 2016).

Moreover, in this study we investigated the correlation of PMAxx-RT-qPCR with the infectious titer of HAV after inactivation by heat treatment at 60 ºC, 72 ºC and 95 ºC for 15 min. Our
results suggest that PMAxx pretreatment could be effectively used to assess the infectivity of HAV at 72 °C and 95 °C. However, our results at 60 °C indicated that although HAV lost 3 logs of infectivity as shown by TCID₅₀ assays, their capsids did not allow the penetration of PMAxx in most of the capsids because only 1.3 logs of virus reduction were 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. Nevertheless, these findings are better than the ones reported by the pretreatment with PMA (Fuster et al. 2016) and clearly demonstrate that the viability RT-qPCR with PMAxx pretreatment is more reliable than the RT-qPCR alone to assess HAV infectivity.

In order to adapt the use of PMAxx to the routine analysis of HAV in food samples, particularly for leafy greens and shellfish, the PMAxx pretreatment was combined with 7.7 mM Triton as recommended by Moreno et al. (2015) and Randazzo et al. (2016). Doing that, our results showed that in lettuce and spinach samples, the PMAxx–Triton pretreatment completely prevented RT-qPCR detection of thermally-inactivated HAV also when inoculated at high concentration. In a previous study, PMA-Triton pretreatment applied to fresh vegetables was only able to partially reduce the signal of thermally-inactivated HAV, indicating that the use of PMAxx is an important improvement (Moreno et al. 2015).

Moreover, this study shows that PMAxx-Triton pretreatment cannot completely prevent RT-qPCR amplification from thermally-inactivated HAV in shellfish samples, leading to an overestimation of infectious HAV. Nevertheless the use of PMAxx highly helped on the improvement of the performance of these pretreatments, since in previous studies complex samples such as shellfish and sewage were diluted in order to reduce the high turbidity of the sample and allow light penetration (Moreno et al. 2015; Leifels et al. 2016). Such dilution limits the utility of RT-qPCR in naturally contaminated samples, where viruses are in low numbers and will most likely not be detected. Supplementary approaches to improve PMAxx-Triton efficiency in shellfish samples include modifications in the application of the intercalating dyes (e.g. temperature or time incubation, concentration of the intercalating dye, repeated
intercalating dye exposure) or modification of the photoactivation step (e.g. incubation time, agitation, temperature).

Despite the fact that the PMAxx-Triton pretreatment is a step forward to better interpret quantification of HAV in food samples, still this pretreatment faces some challenges that need to be addressed. One of the most evident challenges is the fact that this pretreatment has only been evaluated in thermally-inactivated HAV in vegetables and shellfish samples. As different inactivation processes can be applied to these food products (e.g. UV light, high-pressure processing, sanitation with chlorine or natural compounds, etc.), this pretreatment must be evaluated for each treatment separately. For instance, HAV treatment with epigallocatechin gallate, a natural compound, only affected the infectivity of HAV, while no effects were observed in the genome copy numbers after PMAxx-Triton pretreatment (Falcó et al., 2017).

Pretreatment using intercalating dyes in naturally contaminated water samples proved to reduce false positive RT-qPCR signals for several enteric viruses (Fuster et al. 2016; Leifels et al. 2016; Prevost et al. 2016; Randazzo et al. 2016). Therefore, we further evaluated the PMAxx-Triton pretreatment in sewage water samples. Our results indicate that HAV genomes quantified in sewage corresponded to infectious HAV. These results are consistent with those reported by Fuster and collaborators (2016), who also reported not significant differences in HAV titers from sewage samples pretreated with PMA. Moreover, the levels of HAV (around $10^4$-$10^5$ genome copies per liter) in sewage were similar to the previously reported (Fuster et al. 2016).

In conclusion, we showed that PMAxx-Triton pretreatment is suitable for the analysis of infectious HAV in food and water samples. Interestingly, in our study we used the RT-qPCR assays proposed in the ISO 15216-1 standard. Likewise, PMAxx has recently been described as the most effective intercalating dye for assessing norovirus infectivity (Randazzo et al. 2016) using this ISO standard. Therefore, the PMAxx pretreatment can be easily incorporated to the ISO procedure to allow detection of infectious HAV and norovirus.

Acknowledgements
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Conflict of Interest

We have no conflict of interest to declare
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Table 1. Quantification by RT-qPCR of infectious and thermally-inactivated HAV suspensions, at two concentration levels, after intercalating dye treatment.

<table>
<thead>
<tr>
<th>HAV concentration</th>
<th>2 × 10^4 PCRU</th>
<th>2 × 10^3 PCRU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercalating dye (50 µM)</td>
<td>Quantification (log PCRU)^1</td>
<td>Reduction^2</td>
</tr>
<tr>
<td>Infectious</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>4.10 ± 0.12^a</td>
<td>0.13</td>
</tr>
<tr>
<td>PMAxx</td>
<td>3.97 ± 0.46^a</td>
<td></td>
</tr>
<tr>
<td>Inactivated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>4.12 ± 0.00^a</td>
<td>1.22</td>
</tr>
<tr>
<td>PMA</td>
<td>2.90 ± 0.02^b</td>
<td>2.35</td>
</tr>
<tr>
<td>PMAxx</td>
<td>1.77 ± 0.44^c</td>
<td></td>
</tr>
<tr>
<td>PEMAX</td>
<td>2.73 ± 0.22^b</td>
<td>1.39</td>
</tr>
</tbody>
</table>

^1 Results are mean values of three replicates, and HAV titers were obtained by RT-qPCR using a HAV standard curve based on PCRU.

^2 Reduction in titers obtained between inactivated viruses before and after intercalating dye treatment.

^3 Mean values with different letters in the same column denote significant differences between treatments (P < 0.05).

*only one replicate was positive
Table 2. Quantification of thermally-inactivated HAV suspensions in different buffers using PMAxx pretreatment and RT-qPCR.

<table>
<thead>
<tr>
<th>PMAxx</th>
<th>HAV quantification (log PCRU)(^1)</th>
<th>Reduction(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>- 4.40 ± 0.00(^{a})</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+ 1.85 ± 0.85(^{b})</td>
<td>2.55</td>
</tr>
<tr>
<td>PBS</td>
<td>- 4.35 ± 0.06(^{a})</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+ ND</td>
<td>&gt;4.35</td>
</tr>
</tbody>
</table>

\(^1\)Results are mean values of three replicates, and HAV titers were obtained by RT-qPCR using a HAV standard curve based on PCRU.

\(^2\)Reduction in titers obtained between inactivated viruses before and after PMAxx treatment.

\(^3\)Values with different letters in the same column denote significant differences between treatments (P < 0.05).

ND, not detected.
Table 3. Quantification of thermally-inactivated HAV suspensions inoculated in food concentrates by PMAxx-Triton pretreatment and RT-qPCR

Levels of HAV in food concentrates\(^1\)
(PCRU/100 µl)

<table>
<thead>
<tr>
<th>PMAxx-Triton</th>
<th>Quantification(^2) (log PCRU)</th>
<th>Reduction(^3)</th>
<th>Quantification(^2) (log PCRU)</th>
<th>Reduction(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lettuce</td>
<td>-</td>
<td>4.05 ± 0.19(^a)</td>
<td>&gt;4.05</td>
<td>2.68 ± 0.28(^a)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>ND(^b)</td>
<td>&gt;2.68</td>
<td>ND(^b)</td>
</tr>
<tr>
<td>Spinach</td>
<td>-</td>
<td>3.81 ± 0.32(^a)</td>
<td>&gt;3.81</td>
<td>2.92 ± 0.25(^a)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>ND(^b)</td>
<td>&gt;2.92</td>
<td>ND(^b)</td>
</tr>
<tr>
<td>Mussels</td>
<td>-</td>
<td>4.46 ± 0.13(^a)</td>
<td>&gt;3.96</td>
<td>3.96 ± 0.03(^a)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>2.77 ± 0.23(^b)</td>
<td>1.69</td>
<td>ND(^b)</td>
</tr>
<tr>
<td>Oysters</td>
<td>-</td>
<td>4.14 ± 0.14(^a)</td>
<td>&gt;4.05</td>
<td>3.02 ± 0.09(^a)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1.97 ± 0.08(^b)</td>
<td>2.17</td>
<td>ND(^b)</td>
</tr>
</tbody>
</table>

\(^1\)Results are mean values from three replicates, and HAV titers were obtained by RT-qPCR using a HAV standard curve based on PCRU.\n\(^2\)Values with different letters in the same column and same matrix denote significant differences between treatments (P < 0.05).\n\(^3\)Reduction in titers between thermally inactivated viruses before and after pretreatment.\nND, not detected.
Table 4. Quantification of HAV present in sewage water samples by RT-qPCR with and without PMAxx-Triton pretreatment

<table>
<thead>
<tr>
<th>Sewage sample</th>
<th>Mengovirus recovery (%)</th>
<th>RT-qPCR (log PCRU/L)</th>
<th>PMAxx-Triton RT-qPCR (log PCRU/L)</th>
<th>Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>1.47</td>
<td>5.55 ± 0.43</td>
<td>5.05 ± 0.12</td>
<td>0.5</td>
</tr>
<tr>
<td>R4</td>
<td>4.37</td>
<td>4.53 ± 0.36</td>
<td>4.52 ± 0.22</td>
<td>0.01</td>
</tr>
<tr>
<td>R10</td>
<td>8.76</td>
<td>4.17 ± 0.46</td>
<td>4.00 ± 0.11</td>
<td>0.17</td>
</tr>
<tr>
<td>R11</td>
<td>3.80</td>
<td>4.55 ± 0.32</td>
<td>4.52 ± 0.21</td>
<td>0.03</td>
</tr>
</tbody>
</table>