International Journal of Food Microbiology 266 (2018) 1–7

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

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

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ARTICLE INFO

Keywords:
Intercalating dyes
Viability PCR
Norovirus
Shellfish
Sewage
RT-qPCR

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 > 3 log. Additionally, this pretreatment reduced the signal of thermally-inactivated HuNoV GI and GII between 1 and 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.

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

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https://doi.org/10.1016/j.ijfoodmicro.2017.11.011
Received 1 August 2017; Received in revised form 13 November 2017; Accepted 14 November 2017
Available online 15 November 2017

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 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 (Randazzo et al., in press; 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 °C in aliquots.

The cytopathogenic strain MC0 of mengovirus (courtesy of Prof. Albert Bosch, University of Barcelona) was propagated and assayed in HeLa cells. Semi-purified viruses were harvested by three freeze-thaw cycles of infected cells followed by centrifugation at 660 × *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 (Pinto et al., 1994).

2.2. Sewage and shellfish samples

Sewage samples collected from the municipal wastewater treatment plant of Quart (Valencia) from September to December 2016. The plant treats the flow from seven municipalities accounting for 164,171 equivalent inhabitants at a projected flow of 60,000 m^3/day. Sewage samples were concentrated by ultracentrifugation as described by Rodriguez-Diaz et al. (2009). Briefly, 35 ml of sewage was centrifuged at 140,000 × *g* for 2 h 30 min at 4 °C. Then the pellet was eluted by incubating on ice for 30 min with 5 ml of 0.25 N glycine buffer (pH 9.5). The solution was neutralized by adding 5 ml of 2 × PBS. 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 added in the 35 ml of sewage as a process control virus to monitor extraction efficiency following the ISO 15216:2017 guidelines (Comite Europeen de Normalisation, 2017).

Oysters samples were collected from January to March 2013 (4 samples) and on February 2014 (2 samples) and were processed as described in the ISO 15216-1:2017 (Comite Europeen de Normalisation, 2017). Mengovirus was added as an extraction efficiency control to each dissected tissue (2 g) before homogenization according the ISO 15216:2017 guidelines (Comite Europeen de Normalisation, 2017).

2.3. Performance of PMAxx pretreatment in artificially contaminated shellfish

Mussels (*Mytilus galloprovincialis*), oysters (*Crassostrea gigas*) and cockles (*Cerastoderma edule*) were purchased at a local market and tested for HuNoV GI and GII contamination 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 3000*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.

2.4. 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)