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Evaluation of viability PCR performance for assessing norovirus infectivity in fresh-cut vegetables and irrigation water

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ABSTRACT

Norovirus (NoV) detection in food and water is mainly carried out by quantitative RT-PCR (RT-qPCR). The inability to differentiate between infectious and inactivated viruses and the resulting overestimation of viral targets is considered a major disadvantage of RT-qPCR. Initially, conventional photoactivatable dyes (i.e. propidium monoazide, PMA and ethidium monoazide, EMA) and newly developed ones (i.e. PMAxx and PEMAX) were evaluated for the discrimination between infectious and thermally inactivated NoV genogroup I (GI) and II (GII) suspensions. Results showed that PMAxx was the best photoactivatable dye to assess NoV infectivity. This procedure was further optimized in artificially inoculated lettuce. Pretreatment with 50 μM PMAxx and 0.5% Triton X-100 (Triton) for 10 min reduced the signal of thermally inactivated NoV by ca. 1.8 logs for both genogroups in lettuce concentrates. Additionally, this pretreatment reduced the signal of thermally inactivated NoV GI between 1.4 and 1.9 logs in spinach and romaine and lamb's lettuces and by >2 logs for NoV GII in romaine and lamb's lettuce samples. Moreover this pretreatment was satisfactorily applied to naturally-contaminated water samples with NoV GI and GII. Based on the obtained results this pretreatment has the potential to be integrated in routine diagnoses to improve the interpretation of positive NoV results obtained by RT-qPCR.

1. Introduction

Gastroenteritis caused by human noroviruses (NoVs) is the leading cause of acute viral gastroenteritis throughout the world and is mainly transmitted via the fecal-oral route. In 2013, the CDC identified viruses as the causative agent of 36% of illnesses due to food consumption in outbreaks with a single confirmed etiologic agent. NoVs were the most common cause, being responsible for 154 outbreaks, while *Salmonella* was next, accounting for 149 (34%) outbreaks (CDC, 2015). Within the European Union, *Salmonella* remained the most commonly confirmed causative agent in the foodborne outbreaks reported (22.5%), followed by NoV which accounted for 18% (EFSA and ECDC, 2015).

The current knowledge of NoV has been hampered by the lack of a cultivation system for their *in vitro* propagation. Recently the use of B lymphocytes combined with the presence of HBGA-expressing enteric bacteria showed the effective growth of a GII.4-Sydney NoV strain isolated from a stool sample (Jones et al., 2014). However, until issues are resolved regarding cell-culture method complexity, cost effectiveness, and validity for the detection of a broad spectrum of NoV genotypes, infectivity is not yet a useful method for detecting NoV in water and food samples. Thus, current methodologies for the detection of NoV naturally present in water and foods are based on molecular techniques (reviewed by Bosch et al., 2011).

Despite advances in the development of standardized molecular techniques, for example the technical specification norm for NoV and hepatitis A virus (HAV) detection in foodstuffs (ISO/TS 15216), the food and environmental virology field still presents many difficulties at the analytical level. For instance, molecular detection methods still require approaches to better assess the infectivity of the samples (reviewed by Knight et al., 2013). In this sense, the use of photoactivatable dyes has received special attention due to its compatibility with RT-qPCR assays, and the potential to be used in food and food processing facilities (reviewed by Elizaquível et al., 2014). The use of photoactivatable dyes on enteric viruses was first introduced by Parshionikar in 2010 by applying a propidium monoazide (PMA) pretreatment (Parshionikar et al., 2010). Theoretically, these photoactivatable dyes cannot enter intact capsids but are able to penetrate destroyed or damaged capsids. Once penetrated, the photoactivatable dye intercalates covalently into RNA/DNA after exposure to strong visible light, interfering with PCR and RT-PCR amplification.

Until now, photoactivatable dyes combined with qPCR (viability PCR) have successfully been applied to discriminate between infectious and thermally-inactivated poliovirus, coxsackievirus, echovirus, HAV and murine norovirus suspensions (Lee et al., 2015; Kim et al., 2011;

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Parshionikar et al., 2010; Sánchez et al., 2012a). However, reports on the application of this procedure in environmental and food samples are somewhat limited. To date, only Parshionikar et al. (2010) and Moreno et al. (2015) have successfully applied PMA pretreatment in water and food samples for infectious poliovirus and HAV detection.

For NoV, the performance of viability PCR is still under discussion. Karim et al. (2015) reported that PMA-RT-PCR and PMA-RT-qPCR could not differentiate selectively between infectious and thermally (5 min at 72 °C), chlorine (0.5 mg/l) or UV light (187 m J/cm2) treated NoV suspensions while Parshionikar et al. (2010) reported that PMA-RT-PCR was able to discriminate between infectious and thermally-treated NoV (1 min at 72 °C) suspensions. Additionally, Escudero-Abarca et al. (2014) have recently reported that a SYBR Green PMA-RT-qPCR assay, but not a Taqman RT-qPCR, distinguished between infectious and thermally-treated NoV GI when applied to a monodispersed NoV suspension.

The purpose of this work was to further evaluate the potential of photoactivatable dyes to discern between infectious and thermally-inactivated NoV suspensions using two NoV genogroups and the RT-qPCR assays proposed in the framework of the ISO/TS 15216, and to assess its applicability in water and food samples.

2. Materials and methods

2.1. NoV samples

NoV genogroup I genotype 4 (GI·*P*4) and a genogroup II genotype 4 (GII.4 variant Den Haag 2006b) from stool specimens of patients with gastroenteritis (kindly provided by Dr. Javier Buesa, University of Valencia, Spain) were used as NoV reference material. NoV stool samples were suspended (10%, wt/vol) in phosphate-buffered saline (PBS) containing 2 M $NaNO_3$, 1% beef extract, and 0.1% Triton X-100 (Sigma-Aldrich) (pH 7.2) and pelleted at 1000 ×g for 5 min. The supernatant was stored at −80 °C in aliquots.

2.2. Optimization of photoactivatable dye treatments on NoV suspensions

PMA and ethidium monoazide reagents (EMA; GenIUL) were dissolved in 20% dimethylsulfoxide (DMSO) at 20 mM while PEMAX™ (a double dye technology developed by GenIUL) and PMAxx™ (a new and improved version of PMA developed by Biotinum) reagents were dissolved in water at 4 mM. All reagents were stored at −20 °C protected from light. Photoactivatable dyes were added to 3–4 log PCRU of infectious and thermally-treated NoV suspensions (99 °C for 5 min) diluted in PBS, PMA enhancer for gram-negative bacteria buffer 1× (buffer designed to improve PMA discrimination developed by Biotium), standard buffer 1× or reaction buffer plus 1× (buffers for combining with PMA or EMA developed by GenIUL) to obtain a final concentration of 50 μM (PMA, PEMAX and PMAxx) or 20 μM (EMA). After the addition of the photoactivatable dye, incubation in the dark at room temperature was performed for 10 min at 150 rpm to allow reagent penetration, unless otherwise indicated. Immediately, samples were exposed to light for 15 min using a photo-activation system (Led-Active Blue, GenIUL). After photoactivatable dye pretreatments, RNA was extracted using the NucleoSpin® RNA virus kit (Macherey-Nagel GmbH & Co.) according to the manufacturer's instructions. Three types of controls were always included in the experiments; infectious viruses treated with photoactivatable dyes and infectious and thermally-inactivated viruses without photoactivatable dye treatment. Experiments were carried out in triplicate. In the present study, all experiments that include photoactivatable dyes were performed in DNA LoBind 1.5 ml tubes (Eppendorf) to avoid photoactivatable dye interaction with the plastic surface of the tubes.

2.3. Detection and quantification of viral RNA

The set of primers and probes used in this study are targeted to the junction of open reading frame 1 and 2 (ORF1/ORF2) of NoVs (ISO/TS 15216, 2013). RNA samples were analyzed in duplicate by RT-qPCR using the RNA UltraSense One-Step quantitative RT-PCR system (Invitrogen SA) and the LightCycler 480 instrument (Roche Diagnostics). For each RT-qPCR, serial dilutions of standard curve were run in quintuplicates and the numbers of PCRU were calculated.

2.4. Performance of photoactivatable dye treatments to discriminate infectious from thermally-inactivated NoV in vegetable samples

Initial experiments were performed with romaine lettuce (*Lactuca sativa*) obtained from a local supplier that was used to prepare lettuce concentrates as previously described (Sánchez et al., 2012b). Briefly, lettuce was washed with Buffered Peptone Water (BPW) using the Pulsifier equipment (Microgen Bioproducts) and concentrated by polyethylene glycol (PEG) precipitation. The pellet was immediately resuspended in 500 μl of PBS. Aliquots of 100 μl of lettuce concentrate were inoculated with 3–4 log PCRU of infectious or thermally-inactivated (99 °C for 5 min) NoV GI or NoV GII suspensions. Thereafter, samples were added to 50 μM PMAxx (based on results from Section 2.2) with or without 0.5% Triton and incubated in the dark at room temperature for 10 or 30 min at 150 rpm. Finally, samples were exposed to light for 15 min using a photoactivation system (Led-Active Blue). After photo-induced cross-linking, samples were pretreated with the Plant RNA Isolation Aid product (Ambion) to remove plant PCR inhibitors such as polyphenolics and polysaccharides (Sánchez et al., 2012b). For this purpose, 100 μl of the concentrated sample was mixed with 25 μl of the Plant RNA Isolation Aid 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. All the experiments were performed in triplicate.

In the second part of the study, spinach (*Spinacia oleracea*) and romaine and lamb's lettuce (*Valerianella locusta*) concentrates were prepared as described above. One-hundred microliters aliquots of vegetable concentrates were inoculated with 10^2 or 10^3 PCRU of thermally-inactivated (99 °C for 5 min) NoV GI or NoV GII suspensions and added to 50 μM PMAxx and 0.5% Triton. Photoactivation, RNA extraction and RT-qPCR were performed as described above. All the experiments were performed in triplicate.

2.5. Naturally contaminated irrigation water samples

Three types of irrigation water obtained from an experimental growing field located in Murcia (Spain) were used: tertiary treatment effluent from the urban wastewater treatment plant of Roldán-Balsicas (tertiary), secondary treatment effluent from the same treatment plant (secondary), and surface water from an irrigation community (surface). Secondary treatment consisted in activated sludge systems followed by coagulation-flocculation. Tertiary treatment effluent was obtained after the secondary reclaimed water was sand-filtered followed by UV disinfection. Recovery of NoV from water was performed as described by Helmi et al. (2011). Briefly, $MgCl_2$ was added to 200 ml of water samples to a concentration of 0.05 M, adjusting the pH to 3.5. Then water samples were filtered through 0.45 μm cellulose nitrate filters (Sartorius). Filters were then transferred to sterile tubes and 5 ml of elution buffer (1% beef extract, 3% Tween-80 and 0.5 M NaCl) were added and pH adjusted to 9.5. Tubes were shaken for 1 min in a vortex, kept for 4 min in an ultrasonic bath and shaken again in a horizontal orbital shaker at 250 rpm for 10 min and then, pH was adjusted to 7. Samples were kept at −70 °C until analysis. Thereafter, 100 μl of concentrated samples were added with 50 μM PMAxx and 0.5% Triton