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Short communication

Thermal inactivation of human norovirus on spinach using propidium or ethidium monoazide combined with real-time quantitative reverse transcription-polymerase chain reaction

Myeong-In Jeong, Shin Young Park, Sang-Do Ha*

School of Food Science and Technology, Chung-Ang University, 4726 Seodong-daero, Ansung, Kyunggido, 456-756, Republic of Korea

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ABSTRACT

The present study investigated the inactivation effects of thermal treatment against human norovirus (HuNoV) in suspension and on spinach by using real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR), ethidium monoazide combined with qRT-PCR (EMA/qRT-PCR), and propidium monoazide combined with qRT-PCR (PMA/qRT-PCR). Total titers of non-dye treated, EMA-treated, or PMA-treated HuNoV in suspension were significantly ($P < 0.05$) reduced to 0.22–0.77, 0.42–2.42, and 0.54–2.96 $\log_{10}$ copy number/μL, respectively, after thermal exposure at 65–85 °C for 1 min. HuNoV titers on spinach were significantly ($P < 0.05$) reduced to 0.27–1.01, 0.34–2.39, and 0.82–2.59 $\log_{10}$ copy number/μL in qRT-PCR, EMA/qRT-PCR, and PMA/qRT-PCR, respectively, after treatment at 65–85 °C for 2 min. Non-dye treated HuNoV on spinach exhibited a decrease of <1.5 $\log_{10}$ copy number/μL, whereas EMA-treated or PMA-treated HuNoV on spinach was not detected after treatment at 95 °C for 2 min. Specifically, HuNoV with two dye treatments exhibited a greater decrease compared with non-dye treatment at all heating temperatures in suspension, as well as on spinach. The difference in reduction values between non-dye treatment and dye-pretreatments increased gradually along with the stepwise increase in temperature. Based on these results, qRT-PCR combined with EMA or PMA could be regarded as a more useful method as compared with qRT-PCR alone to discriminate the viability of thermally treated HuNoV. Furthermore, despite a similar decreasing trend observed following both dye-pretreatments, HuNoV titer decreased slightly more with PMA treatment than with EMA treatment under all thermal conditions.

1. Introduction

Human norovirus (HuNoV) is recognized as a serious viral agent and the main cause of acute gastroenteritis diseases worldwide (Lai, Wang, Wu, Hung, & Jiang, 2013; Noda, Fukuda, & Nishio, 2008; Siebenga et al., 2009). According to the National Outbreak Reporting System in the United States, approximately 20 million cases of HuNoV infection occur annually (Hall, Wikswo, Pringle, Gould, & Parashar, 2014). In Korea from 2002 to 2015, HuNoV illnesses accounted for 15% of total food-poisoning outbreaks aggregated by the Korean poisoning statistics system (Korea Ministry of Food and Drug Safety, 2014). HuNoV consists of a single-stranded RNA (~7.6 kb) that includes three open reading frames (ORFs) (Atmar & Esters, 2006). HuNoV belongs to the *Caliciviridae* family, and approximately 10–100 virions can infect a person and cause gastroenteritis symptoms, such as diarrhea, vomiting, and abdominal pain (Lindesmith et al., 2003). HuNoV transmission occurs mostly through person-to-person or fecal-oral route (Noda et al., 2008), and infection via oral routes may be related to intake of food and water contaminated with fecal components (Koopmans & Duizer, 2004).

Agricultural products can be contaminated by viral pathogens at any step from pre-harvest to post-harvest, and produce contaminated with viruses can cause gastroenteritis outbreaks. Based on previous data concerning food-borne outbreaks, vegetables and fruits are causative agents of infectious food poisoning (Berger et al., 2010; Heaton & Jones, 2008). Specifically, HuNoV-related illness occurs via lettuce, perilla leaves, spinach, tomatoes,

* Corresponding author. Dept. of Food Science and Technology, Chung-Ang University, 4726 Seodong-daero, Ansung, Gyunggido, 456-756, Republic of Korea.
E-mail address: sangdoha@cau.ac.kr (S.-D. Ha).

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strawberries, and raspberries (Butot, Putallaz, Amoroso, & Sánchez, 2009; Lynch, Tauxe, & Hedberg, 2009), with 60% of food-borne diseases associated with intake of HuNoV-contaminated green-leaf vegetables in the United States from 1973 to 2006 (Herman, Ayers, & Lynch, 2008). Among green-leaf vegetables, spinach (*Spinacia oleracea*) belongs to the dicotyledon group of flowering plants and is often consumed as a raw food in salad after simply washing with water and without additional disinfection processing at home or in restaurants in Europe and the USA. In Korea, spinach is mostly cooked by blanching at 95 °C–100 °C for 4 min (Kim, Koo, Paik, Lyu, & Lee, 2001), followed by seasoning with soy sauce and other condiments for "*muchim*" (vegetables, dried fish, and seaweed seasoned with various condiments as a side dish).

An obstacle in HuNoV research concerns the inability of the virus to be cultivated *in vitro*. Therefore, HuNoV-contaminated food and water is detected by using reverse transcription-polymerase chain reaction (RT-PCR) or real-time quantitative RT-PCR (qRT-PCR) (El-Senousy, Costafreda, Pintó, & Bosch, 2013; Mattison & Bidawid, 2009). However, neither of these PCR forms can be used to analyze HuNoV survival, because they detect both non-infectious and infectious forms of the virus. These forms of PCR require additional molecular methods to evaluate HuNoV survival. Propidium monoazide (PMA) and ethidium monoazide (EMA) are nucleic acid-intercalating agents applied as a pretreatment prior to qRT-PCR in order to distinguish between live and dead cells. The azide compounds can penetrate damaged viral capsids and then covalently link to viral nucleic acids under visible-light exposure, thereby inhibiting qRT-PCR amplification. In previous studies, PMA- or EMA-pretreatment combined with PCR or qRT-PCR has been shown successful differentiating the infectivity of bacterial pathogens, including *Escherichia coli* (Elizaquível, Gabaldón, & Aznar, 2011), *Campylobacter jejuni* (Josefsen et al., 2010), and *Listeria monocytogenes* (Pan & Breidt, 2007). Additionally, PMA or EMA techniques have selectively detected infectious hepatitis virus (HAV), poliovirus, adenovirus, coxackievirus, and echovirus (Coudray-Meunier, Fraisse, Martin-Latil, Guillier, & Perelle, 2013; Parshionikar, Laseke, & Fout, 2010; Sangsanont, Katayama, Kurisu, & Furumai, 2014; Sánchez, Elizaquível, & Aznar, 2012), whereas experiments using these molecular techniques are currently in progress on norovirus surrogates and HuNoV. Although recently PMA combined with qRT-PCR was demonstrated as a suitable method to identify infectious from non-infectious murine norovirus (MNV) following thermal treatment (Lee et al., 2015), this study did not target HuNoVs directly and was not applied to HuNoV on foods.

Therefore, present study aimed to evaluate the inhibitory efficacy of heat (65–85 °C) against HuNoV by applying PMA- or EMA-pretreatment combined with qRT-PCR (PMA/qRT-PCR or EMA/qRT-PCR). Additionally, we examined the virucidal effects of heat treatment (65–95 °C) against HuNoV on spinach.

2. Materials and methods

2.1. Virus stock and preparation

Norovirus GII.4 human stool (140599) was suspended in phosphate buffered saline (PBS) to obtain a 50% fecal sample. The presence of GII.4 HuNoV was identified by qRT-PCR and sequence analysis. The stool samples were diluted in Dulbecco's minimum essential medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) and serially filtered through 5-, 1.2-, 0.8-, and 0.45-μm Minisart filters (Sigma-Aldrich). The viral suspension was stored at −80 °C until use.

2.2. Thermal treatment of HuNoV in suspension

HuNoV stock samples in 500 μL aliquots (approximately 7 $\log_{10}$ copy number/μL) were added to 1.5-mL tubes, followed by heating for 1 min in a water bath set at 65, 75, or 85 °C. The exposure time was initiated when the internal temperature reached the target temperature, with temperature-acquisition times to 65 °C and 85 °C at 0.4 min and 2 min for the HuNoV suspension, respectively. Subsequently, the heated samples were transferred to ice for 20 min. One HuNoV aliquot was not heat-treated and placed on ice immediately for use as a control.

2.3. Thermal treatment of HuNoV on spinach

The spinach sample was purchased from a local market in Anseong, Korea, and the spinach leaves were separated and washed with flowing tap water to remove dirt and dust. Leaves whose veins are more than 8 cm long with no wound were selected about two dozen to process 2 independent trials using 3 replicate samples at four different temperatures (65, 75, 85, and 95 °C). All leaves were rinsed in deionized water, dried in a laminar flow food for 10 min, and then exposed to a 260 nm ultraviolet lamp (Sankyo Ultraviolet Co., Ltd.; Seoul, Korea) for 2 min. The samples were cut into 5.0 × 5.0 cm (2 ± 0.05 g) pieces using sterile stainless-steel scissors, transferred to sterile petri-dishes, and 1500 μL of virus suspension (approximately 7 $\log_{10}$ copy number/μL) was inoculated onto the surface. In order to attach the virus to the surface, samples were placed on a clean bench for 1 h, followed by transfer of the samples into 50-mL conical tubes containing 18 mL PBS and heating for 2 min in a water bath at 65, 75, 85, and 95 °C. The exposure time was started when the internal temperature reached the target temperature, with temperature-acquisition times of 2.2 min and 5.4 min at 65 °C and 95 °C for spinach, respectively. The heated samples were immediately cooled on ice for 20 min, and viral recovery was performed as previously described by Son et al. (2014), with slight modifications. Briefly, each tube was vortexed for 3 min and shaken at 300 rpm for 1 h using a shaking incubator (Vision Scientific Co., Seoul, Korea). After centrifugation at 10,000 × *g* for 30 min at 4 °C, the supernatant was consecutively filtered through 5-, 1.2-, 0.8-, and 0.45-μm Minisart filters (Sigma-Aldrich). Viruses were concentrated to a volume of 750 μL with Vivaspin 20 filters (Sartorius Stedim, Aubagne, France) by centrifugation at 10,000 × *g* for 5–15 min. The viral concentrate was stored at −80 °C until use.

2.4. Dye treatment and viral quantification

2.4.1. PMA- or EMA-dye treatment

For dye treatment, non-heated and heated viral samples (500 μL) were immediately mixed with 250 μM PMA (Biotium, Hayward, CA, USA) or 25 μM EMA (GenIUL; Terrassa, Barcelona, Spain) and incubated in a dark room at room temperature for 10 min to allow dye penetration. Samples were then exposed to a 40 W LED light (Dynebio, Seongnam, Gyeonggi-do, Korea) at 460 nm wavelength at room temperature for 15 min in order to photoactivate both dyes. To determine whether the dye treatments interfered with viral detection, the control was not treated with either PMA or EMA without the exposure to LED light.

2.4.2. Extraction and quantification of viral RNA

Viral RNA was extracted using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer instructions. For reverse transcription, 1 μL RNA was mixed with 1× PCR buffer, 10 mM dNTP, 25 U MuLV reverse transcriptase, 10 U RNase inhibitor, and 25 μM Oligo d(T), and RNase-free water was added to a 10 μL final volume. The RNA mixture was activated at 42 °C for 15 min,