International Journal of Food Microbiology 167 (2013) 138–143

Contents lists available at ScienceDirect

International Journal of Food Microbiology

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

The influence of temperature, pH, and water immersion on the high hydrostatic pressure inactivation of GI.1 and GII.4 human noroviruses ☆

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ARTICLE INFO

Article history:
Received 4 June 2013
Received in revised form 27 August 2013
Accepted 29 August 2013
Available online 6 September 2013

Keywords:
Human norovirus
Hydrostatic pressure
Blueberries
Temperature
pH
PGM binding assay

ABSTRACT

Detection of human norovirus (HuNoV) usually relies on molecular biology techniques, such as qRT-PCR. Since histo-blood group antigens (HBGAs) are the functional receptors for HuNoV, HuNoV can bind to porcine gastric mucin (PGM), which contains HBGA-like antigens. In this study, PGM-conjugated magnetic beads were used to collect and quantify potentially infectious HuNoV strains GI.1 and GII.4 treated by high hydrostatic pressure (HHP). Both GI.1 and GII.4 strains used in this study showed increasing pressure sensitivity as judged by loss of PGM binding with decreasing temperature over a range of 1 to 35 °C. Both GI.1 and GII.4 were more resistant to pressure at pH 4 than at neutral pH. Because GI.1 was significantly more resistant to pressure than GII.4, it was used to evaluate HuNoV pressure inactivation in blueberries. GI.1 on dry blueberries was very resistant to pressure while immersion of blueberries in water during pressure treatments substantially enhanced the inactivation. For example, a 2 min-600 MPa treatment of dry blueberries at 1 and 21 °C resulted in <1-log reductions while a 2.7-log reduction of GI.1 was achieved by a treatment at 500 MPa for 2 min at 1 °C when blueberries were immersed in water. In total, this novel study provides unique information for designing pressure processing parameters (pressure, temperature, and time) and product formulations (such as pH) to inactivate HuNoV in high-risk foods such as berries.

1. Introduction

Human norovirus (HuNoV) causes the majority of foodborne illness and is primarily transmitted through the fecal–oral route (Li et al., 2012; Scallan et al., 2011). Foods consumed raw such as berries, have frequently been involved in HuNoV outbreaks because they can be contaminated by irrigation water, liquid fertilizer and pesticide application, or by human harvesters and food handlers (Carter, 2005; Cotterelle et al., 2005; Falkenhorst et al., 2005; Le Guyader et al., 2004; Potera, 2013; Rodriguez-Lazaro et al., 2012). The main difficulty hindering research of HuNoV inactivation method research has been the lack of suitable cell culture systems or practical small animal models (Duizer et al., 2004; Herbst-Kralovetz et al., 2013; Li et al., 2012). Therefore, HuNoV detection mostly depends on molecular methods, such as RT-PCR. However, RT-PCR only detects the presence of HuNoV RNA, and does not provide information about whether the virus from which the RNA was derived was infectious or inactivated. What complicates this issue is the fact that in many cases, viruses inactivated by capsid damage can retain virion integrity, protecting viral RNA from enzymatic degradation by environmentally-ubiquitous RNAses (Diez-Valcarce et al., 2011; Kingsley, 2013).

The obligatory first step for any virus infection is binding to the appropriate cell receptor. The HuNoV receptors are histo-blood group antigens (HBGAs) in the human intestinal tract (Marionneau et al., 2002). Subsequent work by Tian and coworkers showed that norovirus and norovirus-like particles can bind to porcine gastric mucin (PGM) because porcine mucins are chemically- and antigenically-similar to human histo-blood group antigens (Tian et al., 2007, 2008, 2010). Of particular note, it was shown that all GI strains tested, and 85% of GII HuNoVs tested, bound to PGM (Tian et al., 2010). When the porcine gastric mucin was conjugated to magnetic beads (PGM-MB), HuNoV could be extracted and purified from complex food matrices such as fresh produce, salad, and sewage, for subsequent quantification using qRT-PCR (Pan et al., 2012; Tian et al., 2008, 2011, 2012). The utility of the PGM-MB binding followed by qRT-PCR assay for discriminating potentially infectious human norovirus was further demonstrated by Dancho et al. (2012) who showed that thermal, UV, and high hydrostatic pressure (HHP) treatments resulted in loss of PGM-MB binding consistent with inactivation of the virus by damage to its capsid.

HHP is a non-thermal processing technique that has been successfully applied in the food industry for different food products, such as oysters, guacamole, fruit jams, ready-to-eat meats, salsa, and orange juice. Previously, most studies evaluating the potential for HHP to

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0168-1605/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.ijfoodmicro.2013.08.020

inactivate noroviruses relied on surrogates such as feline calicivirus (FCV), murine norovirus (MNV), and Tulane virus (TV) (Kingsley, 2013; Kovač et al., 2010, 2012; Li et al., 2013). Until recently, direct evaluation of HuNoV inactivation by HPP has been restricted due to lack of practical assays to assess HuNoV inactivation. One study used human volunteers for direct assessment of HuNoV inactivation, using the GI.1 8FIIb Norwalk strain (Leon et al., 2011). The volunteer study evaluated HHP's potential to inactivate 4-log RT-PCR units within oysters showing that a 5 min-600 MPa treatment at room temperature did protect human volunteers, but a 5 min-400 MPa treatment at room temperature was not protective. A 5-min-400 MPa treatment performed at 6 °C suggested that cooler temperatures might enhance HuNoV inactivation since most volunteers were protected by this treatment.

Recent application of the PGM-MB binding assay to HHP-treated norovirus confirmed that HHP inactivated virus does not bind to PGMs. In Dancho et al. (2012), a GI.1 strain 8FIIa in DMEM plus 10% FBS was treated from 300 to 600 MPa at 5 °C for 5 min. When GI.1 was treated at 400–600 MPa, additional 2–3 log reductions of qRT-PCR detectable RNA with prior PGB-MB binding were noticed compared to qRT-PCR detectable RNA without prior binding, suggesting that PGM-MB binding probably could extract infectious virus particles for subsequent qRT-PCR quantification.

Based on research with HuNoV surrogates FCV, MNV-1, TV and other viruses (Chen et al., 2005; Kingsley, 2013; Li et al., 2013; Lou et al., 2011), the temperature at which HPP is performed, the food matrix pH, and the presence of water would be predicted to affect HuNoV inactivation by HPP. Given the nascent availability of the PGM-MB binding assay, it is now possible to confirm and characterize these effects, as well as to determine if different HuNoV strains responded differently to pressure. The objectives of this study were to investigate the effects of temperature and pH on HHP inactivation of HuNoVs GI.1 and GII.4 strains and the effect of the presence of water on HHP inactivation of GI.1 on blueberries.

2. Materials and methods

2.1. HuNoV stock preparation

Fecal suspensions containing a HuNoV GII.4 strain was generously provided by Dr. Xi Jiang at Cincinnati Children's Hospital Medical Center. GI.1 8FIIb norovirus sample was from patient #34-9 of a human volunteer study (Leon et al., 2011). Fecal suspensions were centrifuged at 4000 ×*g* for 20 min, filtered through a 0.22-μm filter, aliquoted, and stored at −80 °C until use.

2.2. HHP treatment of GI.1 and GII.4 at different temperatures

HuNoV GII.4 and GI.1 stocks were diluted using phosphate-buffered saline (PBS, pH 7.2, KeraFAST, Boston, MA). Each virus (150 μl) was double-bagged and double-sealed in sterile polyethylene stomacher pouches (Seward, Port Saint Lucie, FL). GII.4 samples were pressurized at 200–350 MPa and GI.1 samples at 400–550 MPa using an Avure PT-1 pressure unit (Avure Technologies, Kent, WA) with temperature control and with water as a hydrostatic medium. Pressure treatments were conducted at initial sample temperatures of 1, 4, 10, 21, and 35 °C for 2 min. The pressure come-up rate was approximately 22 MPa/s and pressure release time was <4 s. The pressurization times reported did not include the pressure come-up or release times. Control or un-treated samples were prepared the same way as the pressure-treated samples. Negative controls contained all the reagents without the viruses. For each virus, a titration curve of ten-fold serially-diluted virus was produced along with other samples to determine the detection limit and to ensure that the PGM-MBs used in following experiments were not saturated. After pressure treatments, 100 μl of each sample was mixed with 800 μl PBS and incubated with 40 μg of RNase A (Life Technologies, Carlsbad, CA) at 37 °C for 30 min. Viruses were then assayed using the Binding-PCR Method as described in sections of 2.6 and 2.7.

2.3. HHP treatment of GI.1 and GII.4 at different pH levels

Cell culture medium DMEM (Life Technologies) with 10% FBS (Life Technologies) was adjusted to pH 4 using ~12 M HCl and sterilized by filtration through 0.22 μm filter (EMD Millipore, Billerica, MA). GII.4 and GI.1 stocks were diluted 20 and 15 times respectively using cell culture media adjusted to pH 4 as well as the original media (pH 7.1–7.4). Negative controls contained all the reagents without virus. GII.4 samples were pressurized at 300 and 350 MPa and GI.1 samples at 500 and 550 MPa. For each virus, a titration curve of ten-fold serially-diluted virus was produced along with other samples. Pressure treatments were conducted at 21 °C for 2 min. After pressure treatments, 100 μl of viruses were mixed with 800 μl PBS and incubated with 40 μg of RNase A at 37 °C for 30 min. Viruses were then assayed using the PGM-MB binding method.

2.4. HHP treatment of GI.1 on blueberries

Blueberry samples were prepared as described previously with slight modifications (Li et al., 2013). After blueberry samples were treated under ultraviolet (UV) light inside a biosafety cabinet for 10 min to eliminate background microorganisms, each blueberry was spot-inoculated with 100 μl of GI.1 virus stock and dried for 1 h. One blueberry was either placed into an empty sterile polyethylene pouch (dry state) or a pouch containing 5 ml of sterile water (wet state). The wet-state samples were pressurized at 400–600 MPa and the dry-state samples at 600 MPa. Pressure treatments were conducted at 1 or 21 °C for 2 min. Standard curves correlating the −log (dilution level of GI.1) and the log (qRT-PCR Units) (RTUs) were also prepared for this part of the study. The RTUs were obtained directly from the qRT-PCR results. Since each blueberry was considered as a unit and for later experiments, treated samples were compared with untreated samples to calculate reductions, RTUs were used directly for standard curves. To establish the curves, the GI.1 virus stock was serially-diluted in 10-fold increments (10^{-1}, 10^{-2}, and10^{-3} for the dry state; 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} for the wet state). The serially diluted virus solutions were inoculated on blueberries, air dried for 1 h and packaged under dry and wet state conditions.

For wet state samples (pressure-treated samples, negative controls and samples for standard curves), each blueberry along with the 5 ml of water in each pouch was placed into a 50 ml sterile centrifuge tube. For the dry state samples (pressure-treated samples, negative controls and samples for standard curves), each blueberry in each pouch was placed into a 50 ml sterile centrifuge tube and 5 ml of sterile water was used to rinse the inside of the pouch and transferred to the centrifuge tube. To release virus from blueberries, each tube containing the berry was vortex for 5 times and each pulse lasted for 5 s on a Vortex-Genie 2 vortexer (Scientific industries, Bohemia, NY) with speed set at "4"-"5". Liquid was then transferred to 15 ml tubes, buffered to 1× PBS using 10× PBS (KeraFAST) and incubated with 100 μg of RNase A at 37 °C for 30 min. Viruses were then assayed using the PGM-MB binding method.

2.5. Preparation of PGM-MB

PGM-MBs were prepared as described by Dancho et al. (2012) and Tian et al. (2008). Briefly, MagnaBind carboxyl-derivatized beads (Thermo Scientific, Rockford, IL) were washed 3 times using 1 ml PBS for each wash and a bead attractor (EMD Millipore) was used to separate the beads. One milliliter of 10 mg/ml type III mucin from porcine stomach (Sigma, St. Louis, MO) and 0.1 ml of 10 mg/ml 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC), both in conjugation buffer (0.1 M MES (2-(N-morpholino) ethanesulfonic