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Variable High-Pressure-Processing Sensitivities for Genogroup II Human Noroviruses

Fangfei Lou,[a,b] Erin DiCaprio,[a]* Xinhui Li,[d]* Xianjun Dai,[e] Yuanmei Ma,[a] John Hughes,[c] Haiqiang Chen,[d] David H. Kingsley,[f] Jianrong Li[a]

Department of Veterinary Biosciences, College of Veterinary Medicine,[a] Program in Food Science and Technology,[b] and Department of Molecular Virology, Immunology, and Medical Genetics,[c] The Ohio State University, Columbus, Ohio, USA; Department of Animal and Food Sciences, University of Delaware, Newark, Delaware, USA[d]; College of Life Sciences, China Jiliang University, Hangzhou, People's Republic of China[e]; USDA ARS, Delaware State University, Dover, Delaware, USA[f]

ABSTRACT

Human norovirus (HuNoV) is a leading cause of foodborne diseases worldwide. High-pressure processing (HPP) is one of the most promising nonthermal technologies for the decontamination of viral pathogens in foods. However, the survival of HuNoVs after HPP is poorly understood because these viruses cannot be propagated *in vitro*. In this study, we estimated the survival of different HuNoV strains within genogroup II (GII) after HPP treatment using viral receptor-binding ability as an indicator. Four HuNoV strains (one GII genotype 1 [GII.1] strain, two GII.4 strains, and one GII.6 strain) were treated at high pressures ranging from 200 to 600 MPa. After treatment, the intact viral particles were captured by porcine gastric mucin-conjugated magnetic beads (PGM-MBs) that contained histo-blood group antigens, the functional receptors for HuNoVs. The genomic RNA copies of the captured HuNoVs were quantified by real-time reverse transcriptase PCR (RT-PCR). Two GII.4 HuNoVs had similar sensitivities to HPP. The resistance of HuNoV strains against HPP ranked as follows: GII.1 > GII.6 > GII.4, with GII.4 being the most sensitive. Evaluation of temperature and matrix effects on HPP-mediated inactivation of HuNoV GII.4, GII.1, and GII.6 strains showed that HuNoV was more easily inactivated at lower temperatures and at a neutral pH. In addition, phosphate-buffered saline (PBS) and minimal essential medium (MEM) can provide protective effects against HuNoV inactivation compared to H_2O. Collectively, this study demonstrated that (i) different HuNoV strains within GII exhibited different sensitivities to high pressure, and (ii) HPP is capable of inactivating HuNoV GII strains by optimizing pressure parameters.

IMPORTANCE

Human norovirus (HuNoV) is a leading cause of foodborne disease worldwide. Noroviruses are highly diverse, both antigenically and genetically. Genogroup II (GII) contains the majority of HuNoVs, with GII genotype 4 (GII.4) being the most prevalent. Recently, GII.1 and GII.6 have emerged and caused many outbreaks worldwide. However, the survival of these GII HuNoVs is poorly understood because they are uncultivable *in vitro*. Using a novel receptor-binding assay conjugated with real-time RT-PCR, we found that GII HuNoVs had variable susceptibilities to high-pressure processing (HPP), which is one of the most promising food-processing technologies. The resistance of HuNoV strains to HPP ranked as follows: GII.1 > GII.6 > GII.4. This study highlights the ability of HPP to inactivate HuNoV and the need to optimize processing conditions based on HuNoV strain variability and sample matrix.

Human norovirus (HuNoV) is the most common agent implicated in foodborne gastroenteritis in humans, accounting for 58% of foodborne outbreaks in the United States according to the latest report from the Centers for Disease Control and Prevention (1). HuNoV is responsible for more than 90% of nonbacterial acute gastroenteritis worldwide (2–5). HuNoV belongs to the family *Caliciviridae* under genus *Norovirus*, which is divided into six genogroups numbered genogroup I (GI) to GVI (3). Within a genogroup, noroviruses can be further divided into different genotypes. Three genogroups (GI, GII, and GIV) are known to infect humans. There are ≥19 genotypes assigned to GII noroviruses (6). Currently, the most prevalent HuNoVs belong to genogroup II, genotype 4 (GII.4). In the past 10 years, more than three global HuNoV pandemics have occurred, all of which were due to strains of GII.4 (7, 8). However, GII.1 and GII.6 strains have also contributed to several outbreaks in recent years (8). HuNoV is transmitted through the fecal-oral route or by exposure to aerosolized vomitus via direct person-to-person contact or by contaminated food or water. Foods at risk for HuNoV contamination include fresh produce, seafood, and ready-to-eat foods, which are minimally processed and come into contact with food processors and handlers. Outbreaks are frequently associated with restaurants, hotels, day care centers, schools, nursing homes, cruise ships, swimming pools, hospitals, and military installations because these are crowded locations in which common foods are often consumed (9). Despite the significant economic impact and health burden caused by HuNoVs, research on these viruses has

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Address correspondence to Jianrong Li, li.926@osu.edu.

* Present address: Erin DiCaprio, Department of Food Science and Technology, University of California—Davis, Davis, California, USA; Xinhui Li, Department of Microbiology, University of Wisconsin—La Crosse, La Crosse, Wisconsin, USA.

been seriously hampered because many biological experiments on HuNoVs must rely on a robust cell culture system (3, 5). To date, the survival of HuNoVs treated by food-processing technologies is poorly understood. This is mainly due to the lack of a cell culture system or a small animal model in which to grow the virus *in vitro* or study its pathogenesis *in vivo*. Currently, nucleic acid-based quantification methods (such as real-time reverse transcriptase PCR [RT-PCR]) have been widely used in environmental monitoring, food analysis, and clinical diagnosis of HuNoVs. Although real-time RT-PCR is fast, sensitive, and quantitative, the main disadvantage is that it cannot differentiate the RNA from infectious viruses or noninfectious virus particles.

Nonthermal technologies are becoming increasingly popular because they can be used for decontamination, pasteurization, and sterilization of foods with no significant quality changes, nutrition loss, or use of preservatives (10–14). High-pressure processing (HPP) has come to the forefront as a promising intervention for viral inactivation in foods since it has been reported to effectively inactivate some foodborne viruses (such as hepatitis A virus and rotavirus), prevent internalization of particles, and inactivate both surface-contaminated and internalized particles (11, 13, 15–17). In recent years, HPP has been used to treat high-risk foods for virus contamination, including fruit and vegetable product categories (e.g., salsa, apple sauce, and various fruit blends and purees) and shellfish (e.g., oysters and clams). However, the effectiveness of HPP to inactivate HuNoV, the major foodborne virus, remains poorly understood. Many studies have shown that HPP can effectively inactivate HuNoV surrogates (murine norovirus [MNV-1], feline calicivirus [FCV], and Tulane virus [TV]) in aqueous media and foods at 400 MPa and 4°C for 2 min (13, 18–20). However, the validity of using these surrogates has been recently questioned because HuNoV differs from these surrogates in many aspects, such as clinical manifestations, pathogenesis, and host receptors (21). In fact, significant differences have been observed between surrogates and HuNoVs. Leon et al. (22) found that a pressure level of 600 MPa at 6°C for 5 min inactivated a HuNoV GI.1 strain in oysters using a human volunteer study, although reduced infection in volunteers when HPP was performed at 400 MPa at 6°C was noted, suggesting a reduction in infectivity. Thus, the pressure levels required for HuNoV inactivation appear to be somewhat higher than the levels required for the inactivation of viral surrogates, such as MNV-1, which is inactivated by 400 MPa at 4°C for 2 min (23). Scientifically speaking, human volunteer studies would probably be the ideal *in vivo* assay for the inactivation of HuNoV. However, these studies are not practical due to safety concerns, high costs, and complicated logistics. Therefore, there is an urgent need to develop more practical and convenient strategies to understand the survival of HuNoV after HPP.

One novel approach is to utilize viral receptor-binding activity as an indicator for HuNoV infectivity. Disruption of receptor-binding activity will likely be lethal to the virus, as it is the first step in the virus life cycle. The cellular receptor for HuNoVs is the histo-blood group antigen (HBGA), a carbohydrate moiety that can be cross-linked to the surface of magnetic beads (24). Therefore, receptor-coated beads can be used as a novel method to capture intact viral particles that possess receptor-binding ability. Interestingly, it was found that porcine gastric mucin (PGM) contains HBGAs type A, type H1, and Lewis b and can be used as a source of HuNoV receptors (23). Based on this theory, a PGM magnetic bead (PGM-MB) binding assay was developed to detect intact HuNoV (25, 26). It was found that HuNoVs GI.1 and GII.4 bound efficiently to PGM-MB (26, 27). Previously, we found that HPP disrupts the structure of the norovirus capsid without degradation of viral genomic RNA (13). Thus, intact HuNoV that contains receptor-binding activity will bind to PGM-MB, and only those virus particles with intact enclosed RNA can be quantified by real-time RT-PCR. The combination of the PGM-MB binding capture and the real-time RT-PCR assay is more biologically relevant to estimate viral survival and to detect viruses that are not only intact but also retain receptor-binding activity after HPP treatment.

Using the PGM-MB assay, it was found that HuNoV GI.1 and GII.4 strains can be reduced by 0.4 to 4 $\log_{10}$ by HPP at 300 to 600 MPa (27). In addition, a GI.1 virus was found to be more resistant than a GII.4 virus to HPP. Although these results provided new insights into the survival of HuNoV after HPP, several questions remain. First, the degree to which different norovirus strains within the GII genogroup have different susceptibilities to HPP is not known. This is an important question because norovirus strains are highly diverse, and GII is the major genogroup that causes pandemics. Second, the optimal HPP inactivation conditions and the uniformity of strain responses to those conditions for multiple HuNoVs have not been defined. In recent years, GII.6 and GII.1 have emerged worldwide and have caused many outbreaks (28, 29). Therefore, there is an urgent need to determine whether HPP can effectively inactivate these newly emerged genotypes.

The objective of this study was to estimate the survival of different HuNoV strains within genogroup II by using a combined PGM-MB binding and real-time RT-PCR assay. Using this assay, we identified the optimal processing parameters (temperature, pH, and matrix) to enhance the efficiency of inactivation of HuNoV GII strains by HPP.

MATERIALS AND METHODS

Human norovirus stocks. HuNoV strains GII.1-509, GII.4-5M, GII.4-765 (GII.4-7I), and GII.6-490 were originally isolated from outbreaks of acute gastroenteritis in Ohio. The fecal suspensions were diluted 10-fold in phosphate-buffered saline (PBS) and shaken vigorously at 4°C for 10 min, followed by centrifugation for 10 min at 5,000 × *g*. Finally, fecal suspensions were filtered through 0.45-μm and 0.22-μm filters, aliquoted, and stored at −80°C until use. The major capsid genes (VP1) of HuNoV GII.1-509, GII.4-5M, GII.4-765, and GII.6-490 have been sequenced and can be found in GenBank under accession numbers KC463911, JQ798158, JX126912, and KC464321, respectively. Sequence analysis showed that the VP1 proteins of these HuNoV GII strains share 60.9% to 98.7% identity at the amino acid level. The VP1 proteins of GII.4-5M and GII.4-765 share 98.7% amino acid homology, and they share 62.8 to 63.0% homology with the GII.1-509 VP1 and 60.9 to 61.3% homology with the GII.6-490 VP1. In addition, the VP1 proteins of GII.1-509 and GII.6-490 share 69.0% amino acid homology.

High-pressure treatment of HuNoV strains. The genomic RNA copies of the filtered HuNoV strains GII.1-509, GII.4-5M, GII.4-765, and GII.6-490 were quantified by real-time RT-PCR and were diluted using minimal essential medium (MEM) to reach approximately 6-log RNA copies/ml. Two hundred microliters of each strain of HuNoV was double bagged and double sealed in a sterile polyethylene stomacher pouch (Fisher Scientific International, Ontario, Canada) and then subjected to pressure treatments ranging from 200 to 600 MPa for a 5-min holding time at a 4°C initial temperature. Pressurization of the samples was carried out in a high-pressure unit with temperature control (Model Avure PT-1;

Avure Technologies, Kent, WA) using water as the hydrostatic medium at the University of Delaware. Processing temperatures and pressures were monitored and recorded (DasyTec USA, Bedford, NH). The 5-min holding time in this study did not include the pressure come-up time (about 22 MPa/s) and release time (<4 s). HuNoV survival after processing was estimated by PGM-MB capture followed by real-time RT-PCR assay.

Subsequently, HuNoV GII.4-5M, GII.1-509, and GII.6-490 strains were used to optimize the processing parameters. In order to determine the effect of temperature on HuNoV inactivation, all three strains were pressurized at 200 MPa at different initial temperatures (4°C, 20°C, or 40°C). To determine the effect of pH on the effectiveness of HuNoV inactivation, virus stocks were diluted 10-fold in MEM, and the pH of the medium was artificially adjusted to 4.0, 7.0, or 10.0 using either hydrochloric acid or sodium hydroxide. The samples were treated at 200 MPa and 4°C for 5 min. After processing, the pH was immediately adjusted back to 7. Lastly, to determine the matrix effects on HuNoV, three aqueous mediums of various complexities were tested. MEM is a cell culture medium that contains inorganic salts, amino acids, vitamins, and carbohydrates; PBS contains only inorganic salts; and distilled H_2O has no other components. HuNoV stocks were diluted 10-fold in MEM, PBS, or distilled water prior to 200-MPa HPP at 4°C for 5 min. After pressurization, HuNoV survivors were estimated using a PGM-MB binding assay and real-time RT-PCR.

PGM-MB binding assay. Type III PGM (Sigma, St. Louis, MO) was cross-linked to MagnaBind carboxyl-derivatized beads (Pierce Biotechnology, Rockford, IL) following the manufacturer's recommendations. Briefly, 1 ml of beads was washed 3 times with PBS and separated using a magnetic separation rack (New England BioLabs, Ipswich, MA). Following washing, 1 ml of 10 mg/ml type III PGM in 0.1 M 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer (0.9% NaCl, pH 4.7) and 0.1 ml of 10 mg/ml 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) in MES buffer were added to the beads and incubated for 30 min at room temperature rotating at 8 rpm. After incubation, beads were separated from the PGM solution using the magnetic attracter followed by 3 washes with PBS. The PGM-MBs were finally suspended in 1 ml of PBS containing 0.05% sodium azide and stored at 4°C until use. The level of PGM incorporation was determined.

Before HPP treatment, 100 μl of 10-fold serial dilutions of each virus was first used to bind to 100 μl of PGM-MBs followed by RNA extraction and real-time RT-PCR to ensure that the binding capacity of the beads was not saturated. The optimal concentration of virus that resulted in the best binding efficiency was selected for the inactivation experiments. After HPP at 200 to 600 MPa, 100 μl of untreated or treated viruses was added to 100 μl of PGM-MBs and suspended in 800 μl of PBS followed by incubation for 30 min at room temperature on a LabQuake shaker rotisserie (Thermo Scientific, Waltham, MA) at 8 rpm. The beads were separated, washed 3 times with PBS, and resuspended in 100 μl of PBS for RNA extraction using an RNeasy kit (Qiagen, Valencia, CA). HuNoV-specific RNA levels were determined by real-time RT-PCR.

Reverse transcription and real-time PCR. First-strand cDNA of each viral strain was synthesized by SuperScript III (Invitrogen, Grand Island, NY) using the primer VP1-P1 (5′-TTATAATACACGTCTGCGCCC-3′), which targets the VP1 gene of HuNoV. The VP1 gene was then quantified by real-time PCR using custom TaqMan primers and probes, which were designed to detect the viral capsid VP1 gene of each HuNoV GII strain. Specifically, the forward primer 5′-GGTGCAGCCGGTCTCGTA-3′, reverse primer 5′-CGGCTCAAGTGCCATCGT-3′, and probe 5′-FAM-CAGAGGTCAACAACG-MGB-3′ were used for the GII.1-509 strain, while the forward primer 5′-CACCGCCGGGAAAATCA-3′, reverse primer 5′-GCCTTCAGTTGGGAAATTTGG-3′, and probe 5′-FAM-ATTTGCAGCAGTCCC-NFQ-3′ were used for the GII.4-5M and GII.4-765 strains. The forward primer 5′-TGAATGAAGATGGCGTCGAA-3′, reverse primer 5′-GAGGTTGGCAGCACCATCA-3′, and probe 5′-FAM-ACGCTGCTCCATCG-MGB-3′ were used for the GII.6-490 strain. A standard plasmid for each strain of HuNoV was constructed by inserting the sequence of the entire open reading frame 2 (ORF2) (VP1 gene) into the pGEM-T easy vector (Promega, Madison, WI). The purified plasmid of each strain with a known concentration was then 10-fold serial diluted to generate a standard curve for real-time PCR.

TABLE 1 Binding efficiency of HuNoV GII strains to PGM-MBs

Input (log_{10} RNA copies/ml)	Binding efficiency (%) of HuNoV strain			
	GII.1-509	GII.4-5M	GII.4-765	GII.6-490
6.0	87.7 ± 4.3	94.2 ± 3.5	87.5 ± 3.5	95.1 ± 2.5
7.0	44.3 ± 2.1	18.3 ± 4.3	28.2 ± 4.6	23.5 ± 4.2

Real-time PCR assays were performed on a StepOne real-time PCR machine (Life Technologies, Grand Island, NY). TaqMan fast universal PCR master mix (Life Technologies) was used for all of the reactions following the manufacturer's recommendations. PCR parameters included a holding stage at 95°C for 20 s, followed by 50 cycles of 95°C for 15 s for denaturation, 1 s for annealing, and 60°C for 20 s for extension. Standard curves and StepOne software v2.1 were used to quantify genomic RNA copies. Viral RNAs are expressed as mean log_{10} genomic RNA copies per milliliter ± standard deviation.

Data analysis. All experiments were performed in three independent trials. Statistical analysis of one-way analysis of variance (ANOVA) was performed using Minitab statistical analysis software (Minitab, Inc., State College, PA). A P value of ≤0.05 was considered statistically significant.

Accession number(s). The sequences for the major capsid genes (VP1) of HuNoV GII.1-509, GII.4-5M, GII.4-765, and GII.6-490 can be found in GenBank under accession numbers KC463911, JQ798158, JX126912, and KC464321, respectively.

RESULTS

Determination of the binding efficiency of HuNoV to PGM-MBs. The objective of this study was to compare the pressure sensitivity of different HuNoV strains within genogroup II. Thus, we first determined whether HuNoV strains 509 (GII.1), 5M (GII.4), 765 (GII.4), and 490 (GII.6) could bind to PGM-MBs. In order to determine the binding efficiencies of HuNoV to PGM-MBs and to avoid saturation of the beads, serial 10-fold dilutions of virus in PBS were performed for each HuNoV strain (initial titer of 10^7 genomic RNA copies/ml). Each dilution was subjected to an identical PGM-MB binding assay, and the RNA copies of bound HuNoV were quantified by real-time RT-PCR. As shown in Table 1, all HuNoV strains bound efficiently to the PGM-MBs. At a dilution of 6 log_{10} RNA copies/ml, 88% to 95% of the HuNoVs bound to the PGM-MBs. When 7 log_{10} RNA copies/ml were used, the efficiency of binding to the PGM-MBs was only 18% to 44%. This is probably due to saturation of the binding sites on the PGM-MBs by an overabundance of HuNoV. Subsequently, a concentration of 6 log_{10} RNA copies/ml was selected for all strains used with the HPP inactivation treatments.

Estimation of HuNoV survival after HPP using a combination of a PGM-MB binding assay and real-time RT-PCR. To determine the inactivation kinetics of HuNoV by HPP, 6 log_{10} RNA copies/ml of each HuNoV strain (GII.1, GII.4, and GII.6) were treated at pressure levels ranging from 200 to 600 MPa at 4°C for 5 min. We chose these viral strains because GII.4 is the most common HuNoV strain that causes pandemics in humans and GII.1 and GII.6 recently emerged and caused many outbreaks. After treatment, all samples were subjected to PGM-MB binding, and the bound viral particles were quantified by real-time RT-PCR assay. As shown in Fig. 1, RNA copies of HuNoV bound to

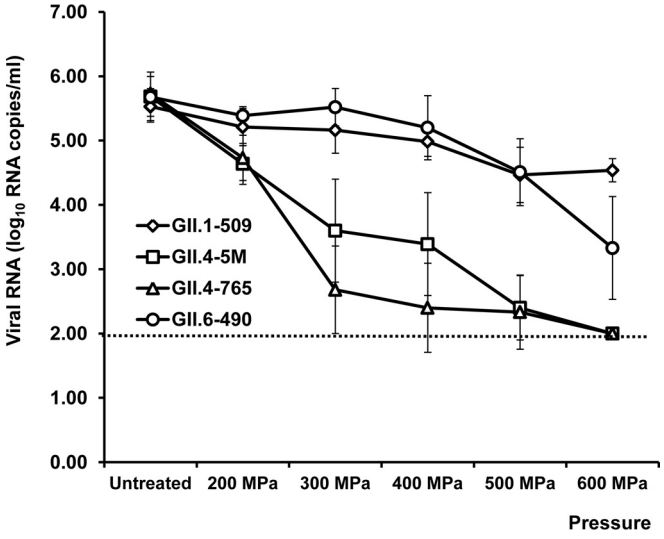

FIG 1 Estimation of survival of HuNoV GII strains after HPP by PGM-MB binding assay. HPP treatments of HuNoVs GII.1-509, GII.4-5M, GII.4-765, and GII.6-490 stocks (10^6 RNA copies/ml) were performed at a range of 200 MPa to 600 MPa at 4°C for 5 min. Untreated and treated HuNoV samples from each strain were incubated with PGM-MBs for 30 min at room temperature followed by real-time RT-PCR. The detection limit was 2 $\log_{10}$ RNA copies/ml.

PGM-MBs decreased as pressure level increased. At a pressure level of 300 MPa, approximately 2.1- and 3.0-log reductions were achieved for GII.4-5M and GII.4-765, respectively. However, only 0.37- and 0.16-log reductions were achieved for GII.1-509 and GII.6-490, respectively. At 500 MPa, 3.29-, 3.40-, 1.06-, and 1.17-log reductions were observed for GII.4-5M, GII.4-765, GII.1-509, and GII.6-490, respectively. The genomic RNA concentrations of GII.4-5M and GII.4-765 were decreased to undetectable levels ($<$2 $\log_{10}$ RNA copies/ml) upon treatment at 600 MPa, suggesting that the receptor-binding ability of human GII.4 strains was disrupted at that pressure. The GII.4-765 strain appeared to be more susceptible than the GII.4-5M strain at pressure ranges of 300 to 400 MPa, although no significant difference was observed between the two strains over the entire pressure range ($P > 0.05$). Interestingly, GII.1 and GII.6 HuNoVs were more stable under pressure in comparison to the two GII.4 strains ($P < 0.05$). At a pressure level of 600 MPa, only 0.99- and 2.35-log reductions were achieved for GII.1 and GII.6, respectively. Thus, the pressure resistance of the tested HuNoV strains can be ranked as follows: GII.1-509 $>$ GII.6-490 $>$ GII.4-5M $>$ GII.4-765 with strain GII.1-509 being the most pressure resistant. Taken together, these results demonstrated that (i) HPP is capable of inactivating HuNoV in a pressure-dependent manner, and (ii) different HuNoV strains can have highly variable sensitivities to HPP.

Effect of temperature on the pressure inactivation of GII.1, GII.4, and GII.6 HuNoV strains. To determine the effect of temperature on the inactivation of HuNoV, the GII.4-5M, GII.1-509, and GII.6-490 strains were treated at 200 MPa at different initial temperatures (4°C, 20°C, or 40°C) for 5 min. After treatment, the samples were subjected to a PGM-MB binding assay, and the amount of HuNoV bound to the beads was quantified by real-time RT-PCR. We chose 200 MPa for pressurization because it resulted in a moderate reduction in GII.4-5M viral genomic RNA (Fig. 1). Therefore, the effect of each processing parameter can be compared.

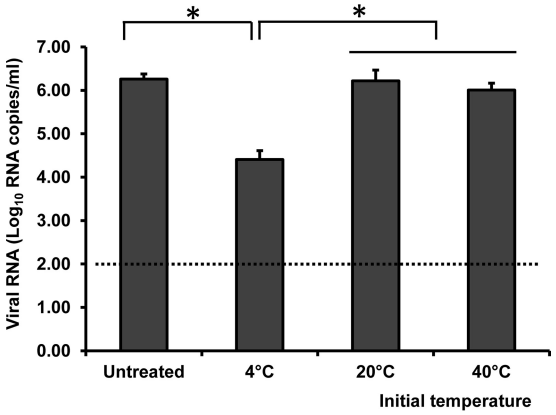

FIG 2 Effect of temperature on the pressure inactivation of the HuNoV GII.4-5M strain. HuNoV stock was processed at a pressure of 200 MPa for 5 min at 4°C, 20°C, or 40°C. Both untreated and treated samples were incubated with PGM-MBs for 30 min at room temperature followed by real-time RT-PCR. Asterisks denote that the groups are significantly different ($P < 0.05$). The dashed line indicates the detection limit (2 $\log_{10}$ RNA copies/ml).

As shown in Fig. 2, temperature played a significant role in the disruption of the binding of HuNoV GII.4-5M to PGM-MBs after HPP treatment. A 1.8-log reduction in viral genomic RNA copies of GII.4-5M was achieved at 200 MPa for 5 min when the initial temperature was 4°C. In contrast, less than a 0.1-log decrease of viral genomic RNA was observed when the initial temperature was increased to 20°C under the same conditions. Similarly, only a 0.26-log reduction was observed at the initial temperature of 40°C. Therefore, the inactivation of the HuNoV GII.4-5M strain was significantly enhanced at 4°C compared to that at 20°C or 40°C ($P < 0.05$).

HuNoV GII.1-509 inactivation was most notable at an initial temperature of 4°C, resulting in an approximate 1-log reduction (Fig. 3). Similarly HuNoV GII.6-490 inactivation was also enhanced at the initial temperature of 4°C, and treatment lead to a

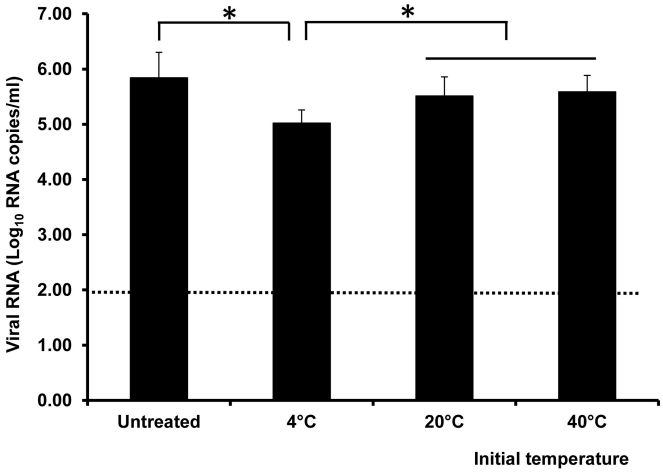

FIG 3 Effect of temperature on the pressure inactivation of the HuNoV GII.1-509 strain. HuNoV stock was processed at a pressure of 200 MPa for 5 min at 4°C, 20°C, or 40°C. Both untreated and treated samples were incubated with PGM-MBs for 30 min at room temperature followed by real-time RT-PCR. Asterisks denote the groups that are significantly different ($P < 0.05$). The dashed line indicates the detection limit (2 $\log_{10}$ RNA copies/ml).

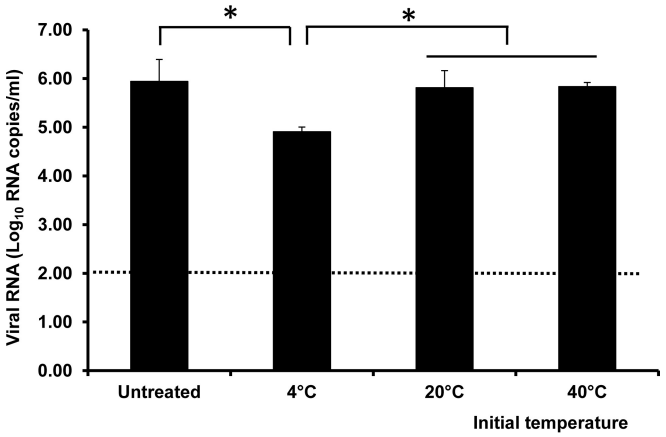

FIG 4 Effect of temperature on the pressure inactivation of the HuNoV GII.6-490 strain. HuNoV stock was processed at a pressure of 200 MPa for 5 min at 4°C, 20°C, or 40°C. Both untreated and treated samples were incubated with PGM-MBs for 30 min at room temperature followed by real-time RT-PCR. Asterisks denote that the groups are significantly different ($P < 0.05$). The dashed line indicates the detection limit (2 $\log_{10}$ RNA copies/ml).

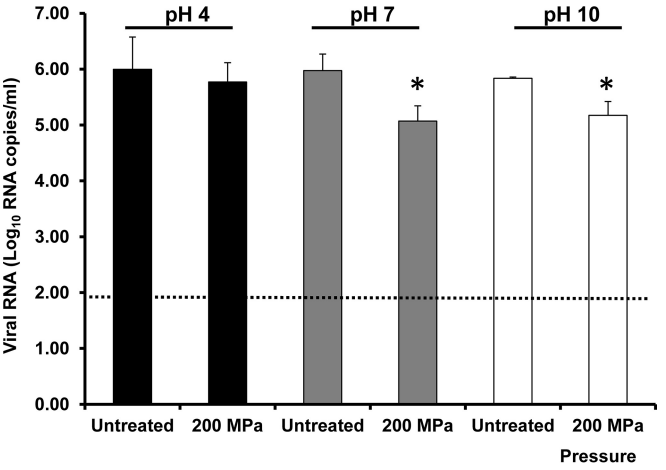

FIG 6 Effect of pH on the pressure inactivation of the HuNoV GII.1-509 strain. HuNoV stock was inoculated into MEM with pH 4.0, 7.0, and 10.0 and treated at 200 MPa for 5 min at 4°C. The surviving viruses were evaluated by a PGM-MB binding assay and real-time RT-PCR. Asterisks indicate that the treated group is significantly different from the untreated control ($P < 0.05$). The dashed line indicates the detection limit (2 $\log_{10}$ RNA copies/ml).

1.2-$\log_{10}$ RNA copy reduction at this treatment parameter (Fig. 4). At initial temperatures of 20°C and 40°C, significantly higher levels of genomic RNA were detected for both HuNoV GII.1-509 and GII.6-490 than at the initial temperature of 4°C (Fig. 3 and 4). Although more stable to HPP treatment compared to GII.4-5M, both HuNoV GII.1-509 and GII.6-490 followed the same trend of a lower initial temperature leading to increases in viral inactivation by HPP.

Effect of pH on the pressure inactivation of GII.1, GII.4, and GII.6 HuNoVs. To determine the influence of pH on HuNoV inactivation by HPP, the pH of the cell culture medium (MEM) was adjusted from 7.0 to 4.0 and 10.0 using hydrogen chloride and sodium hydroxide, respectively. HuNoV GII.4-5M, GII.1-509, or GII.6-490 stocks were then diluted 10-fold in MEM at a pH of 4.0, 7.0, or 10.0. After treatment, the pH of each sample was adjusted back to 7.0 to minimize the impact of pH on the stability of the HuNoV. Figure 5 shows the effect of pH on the inactivation of the HuNoV GII.4-5M strain with or without HPP. Without pressure treatment, the binding affinity of HuNoV to PGM-MBs was not affected by acidic pH ($P > 0.05$). However, suspension of HuNoV at a basic pH (10.0) led to a slight decrease in viral binding as measured by the PGM-MB assay, but this was not significantly different from pH 7.0 ($P > 0.05$). After HPP at 200 MPa and 4°C for 5 min, it is notable that the HuNoV 5M strain was more sensitive to HPP at neutral and basic pH than at acidic pH ($P < 0.05$). A 2.43-log reduction and a 1.77-log reduction in viral genome RNA copies were achieved at pH 7.0 and pH 10.0, respectively, whereas only a 0.58-log reduction was observed at pH 4.0. These results suggest that HuNoV inactivation by HPP was favored at a neutral pH condition.

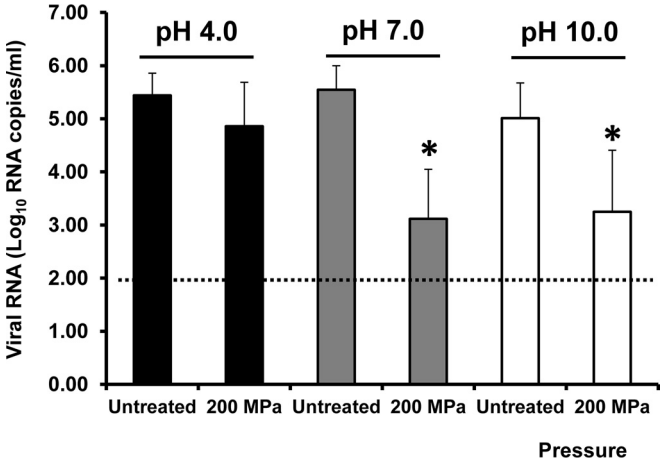

FIG 5 Effect of pH on the pressure inactivation of the HuNoV GII.4-5M strain. HuNoV stock was inoculated into MEM with pH 4.0, 7.0, and 10.0 and treated at 200 MPa for 5 min at 4°C. The surviving viruses were evaluated by a PGM-MB binding assay and real-time RT-PCR. Asterisks denote that the groups are significantly different ($P < 0.05$). The dashed line indicates the detection limit (2 $\log_{10}$ RNA copies/ml).

HuNoV GII.1-509 inactivation by 200 MPa at 4°C for 5 min was also enhanced when at neutral and basic pH compared to that at an acidic pH (Fig. 6). There was a 1.2-log reduction in GII.1-509 RNA detected at pH 7 and a 1.0-log reduction at pH 10 (Fig. 6). Interestingly, there was no reduction in HuNoV GII.1-509 RNA levels following HPP treatment at pH 4 (Fig. 6). HPP inactivation of HuNov GII.6-490 was similar to GII.1-509 under acidic, basic, and neutral conditions (Fig. 7). A 1.3-log reduction was achieved in GII.6-490 RNA levels at pH 7, and a 0.9-log reduction was achieved at pH 10 (Fig. 7). Overall, all strains were more susceptible to HPP inactivation at a neutral or basic pH than at an acidic pH.

Effect of matrix composition on the pressure inactivation of GII.4 HuNoV. To determine whether matrix composition can affect the efficacy of the survival of HuNoV, the GII.4-5M, GII.1-509, and GII.6-490 strains were diluted 10-fold in three aqueous media (MEM, PBS, or H_2O) and treated at 200 MPa and 4°C for 5 min. After treatment, the survival of HuNoV was estimated using a PGM-MB capture assay and a real-time RT-PCR assay. As shown in Fig. 8, variable degrees of reduction were observed for HuNoV GII.4-5M upon HPP treatment. A more than a 4-log RNA copy reduction was reached after a 200-MPa treatment at 4°C for 5 min when HuNoV GII.4-5M was diluted in H_2O. In fact, the RNA level was diminished to an

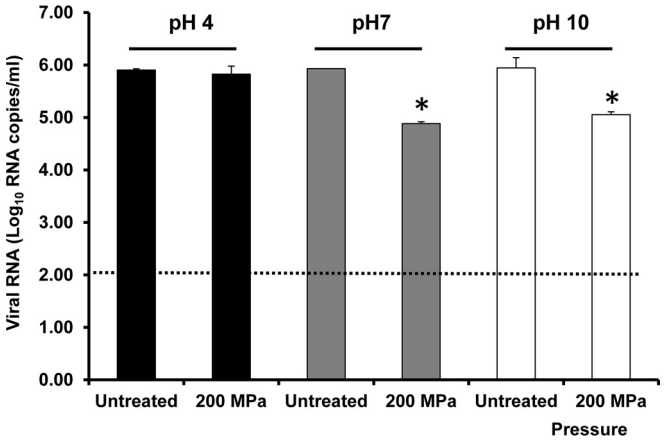

FIG 7 Effect of pH on the pressure inactivation of the HuNoV GII.6-490 strain. HuNoV stock was inoculated into MEM with pH 4.0, 7.0, and 10.0 and treated at 200 MPa for 5 min at 4°C. The surviving viruses were evaluated by a PGM-MB binding assay and real-time RT-PCR. Asterisks indicate that the treated group is significantly different from the untreated control ($P < 0.05$). The dashed line indicates the detection limit (2 $\log_{10}$ RNA copies/ml).

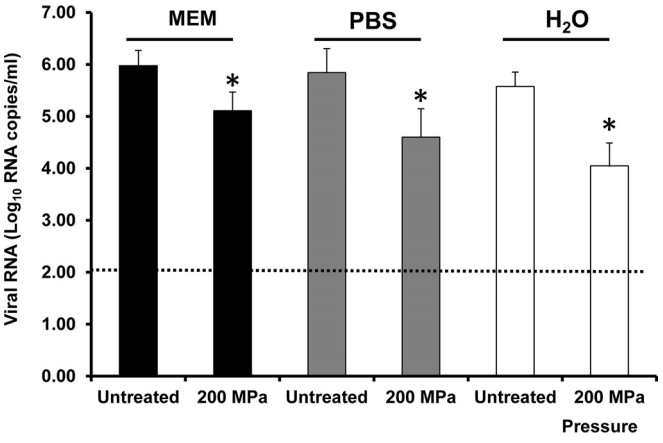

FIG 9 Effect of matrix on the pressure inactivation of the HuNoV GII.1-509 strain. HuNoV GII.1-509 stock was inoculated into MEM, PBS, and H_2O and treated at 200 MPa for 5 min at 4°C. The surviving viruses were evaluated by a PGM-MB binding assay and real-time RT-PCR. Asterisks indicate that the treated group is significantly different from the untreated control ($P < 0.05$). The dashed line indicates the detection limit (2 $\log_{10}$ RNA copies/ml).

undetectable level (<2 $\log_{10}$ RNA copies/ml). However, the same treatment resulted in only 2.7- and 1.7-log reductions when HuNoV GII.4-5M was diluted in PBS and MEM, respectively. These results demonstrated that the inactivation of HuNoV GII.4-5M was significantly enhanced in H_2O ($P < 0.05$).

Under the same treatment conditions, HuNoV GII.1-509 RNA was reduced by 1.6 $\log_{10}$ RNA copies when the virus was suspended in H_2O (Fig. 9). A similar reduction was observed in PBS; however, there was <1-log reduction when the virus was suspended in MEM (Fig. 9). HuNoV GII.6-490 in H_2O had a 2-log reduction in RNA levels following HPP treatment, which was comparable to the viral RNA reduction in PBS (Fig. 10). In MEM, the reduction in HuNoV GII.6-490 was approximately 1 log (Fig. 10). These results also suggest that components in MEM and PBS, such as salts, amino acids, and/or vitamins may reduce HuNoV inactivation by HPP.

DISCUSSION

HuNoVs are highly diverse both antigenically and genetically (2–4). GII strains are reported to be the predominant cause in human clinical cases (1, 7, 8). Within genogroup II, at least 19 genotypes have been defined (6). Thus, it is critical to determine whether different strains within GII have different susceptibilities to HPP. In this study, we directly compared the sensitivities of two GII.4 strains, one GII.1 strain, and one GII.6 strain to HPP using a combination of PGM-MBs and real-time RT-PCR, which detect viral genomic RNAs from intact viral particles containing receptor-binding capability. We found that two GII.4 strains showed similar pressure sensitivities and were significantly more susceptible to HPP than the GII.1 and GII.6 strains. The resistances of the

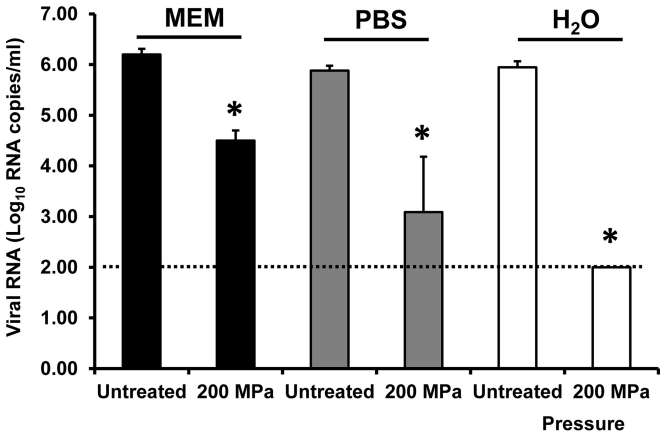

FIG 8 Effect of matrix on the pressure inactivation of the HuNoV GII.4-5M strain. HuNoV GII.4-5M stock was inoculated into MEM, PBS, and H_2O and treated at 200 MPa for 5 min at 4°C. The surviving viruses were evaluated by a PGM-MB binding assay and real-time RT-PCR. Asterisks indicate that the treated group is significantly different from the untreated control ($P < 0.05$). The dashed line indicates the detection limit (2 $\log_{10}$ RNA copies/ml).

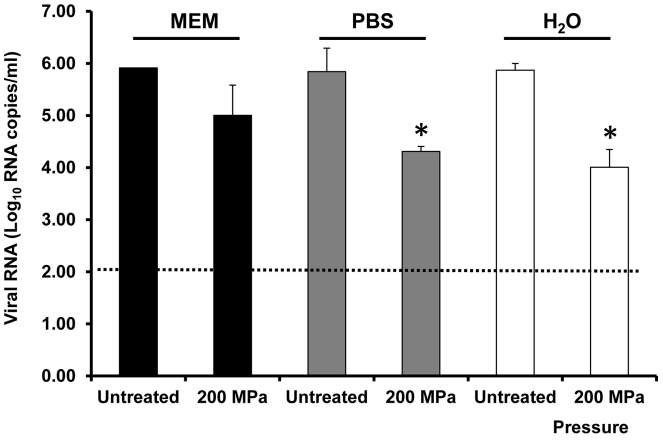

FIG 10 Effect of matrix on the pressure inactivation of the HuNoV GII.6-409 strain. HuNoV GII.6-409 stock was inoculated into MEM, PBS, and H_2O and treated at 200 MPa for 5 min at 4°C. The surviving viruses were evaluated by a PGM-MB binding assay and real-time RT-PCR. Asterisks indicate that the treated group is significantly different from the untreated control ($P < 0.05$). The dashed line indicates the detection limit (2 $\log_{10}$ RNA copies/ml).

HuNoV strains used in this study can be ranked as follows: GII.1 > GII.6 > GII.4. In addition, we optimized the conditions for the inactivation of HuNoV strains and found that HuNoV GII.4, GII.1, and GII.6 were all more easily inactivated at a lower temperature, neutral pH, and when in an H_2O solution.

Combination of PGM-MBs and real-time RT-PCR is an improved method for the estimation of HuNoV survival. Currently, the survival of HuNoV is difficult to assess due to the fact that it cannot be propagated *in vitro*. The traditional methods used to detect the presence of HuNoV are RT-PCR and quantitative real-time RT-PCR. However, a major limitation of these nucleic acid-based methods is that they cannot discriminate between RNAs from infectious viruses and those from noninfectious viruses. In particular, nucleic acid-based methods cannot directly be used for high-pressure inactivation of viruses since HPP does not break covalent bonds and thus does not degrade or damage the viral genome (13). Since the mechanism of viral inactivation by high pressure is the perturbation of the viral capsid and receptor-binding ability, intact viral particles, but not damaged particles, should be able to bind to PGM-MBs. Using this method, Ye et al. (27) found that 600 MPa for 5 min at 6°C was required for reduction below the detection limit (>4.1-log reduction) of the HuNoV GI.1 strain, which seems to be consistent with a recent human volunteer study. Leon et al. (22) reported that HPP at 600 MPa and 6°C for 5 min, but not 5-min 400-MPa treatments (at 6°C or 25°C), completely inactivated 4 logs of the GI.1 HuNoV within seeded oysters. Human volunteers who received oysters treated at 600 MPa did not exhibit symptoms of HuNoV infection, and no viral shedding was detected in subjects' stool or vomitus samples as determined by RT-PCR detection of HuNoV RNA. However, treatment of oysters at 400 MPa (at 6°C or 25°C) for 5 min, a pressure that is generally sufficient to inactivate HuNoV surrogates (FCV and MNV-1), did reduce the frequency of illness among volunteers but was insufficient to prevent all human subjects from HuNoV infection and shedding. These observations suggest that surrogate inactivation by HPP may not mimic HuNoV inactivation accurately. Therefore, the combination of PGM-MBs and real-time RT-PCR is an expedient assay for estimating the survival of HuNoV after HPP treatment. The PGM-MB capture assay offers many additional advantages, such as the capability of concentrating HuNoV particles, removing PCR inhibitors, and increasing the sensitivity of viral detection in complex samples (oysters, lettuce, strawberries, etc.) (21).

Differential pressure resistance of HuNoV strains. One interesting observation during pressure inactivation of viruses is that the sensitivity of viruses to HPP does not necessarily correspond with genetically related taxonomic groups. For example, poliovirus, a picornavirus, is resistant to HPP, with less than a 1-log virus reduction achieved after treatment at 600 MPa for 1 h (30). In contrast, hepatitis A virus (HAV), another picornavirus, was found to be more sensitive to HPP, especially under acidic conditions, with more than a 5-log virus reduction observed after treatment at 400 MPa for 1 min (31). Recently, Li et al. (26) and Ye et al. (27) found that HuNoV strains from two different genogroups (G1.1 and GII.4) showed different sensitivity to HPP using a PGM-MB binding assay. Specifically, a 3.6-log reduction of GII.4 was observed in oyster homogenate at 400 MPa and 6°C for 5 min, whereas only 1.3 logs of GI.1 lost binding ability under the same conditions. In this study, we found that different genotypes of HuNoVs within the same genogroup (GII) had highly variable sensitivities to HPP. Two HuNoV GII.4 strains (5M and 765) were inactivated at 600 MPa and 4°C for 5 min, as evidenced by genomic RNA copies being below the detection limit upon PGM-MB binding followed by real-time RT-PCR. At the same treatment conditions, a 2.4-log reduction of genomic RNA copies was observed for the GII.6 strain. However, the HuNoV GII.1 strain was highly resistant to HPP with only an approximately 1-log reduction achieved at 600 MPa. The mechanism underlying the different sensitivities of different HuNoV strains is not known. This may be attributed to the nature of the virus itself, the size and shape of the virus particle, its high thermodynamic stability, differences in the viral receptor-binding property, or differences in protein structure, amino acid composition, and isoelectric point.

Parameters for inactivation of HuNoV by HPP. To enhance the effectiveness of HPP on the inactivation of HuNoV, it is critical to optimize the processing parameters. Our study addressed the roles of pressure, temperature, pH, and suspending media complexity along with the pressure inactivation of HuNoVs. We showed that the efficiency of HuNoV inactivation was enhanced as the pressure level increased. We also found that a low temperature (4°C) significantly enhanced the pressure inactivation of HuNoV compared to that at higher temperatures (20°C and 40°C). A 200-MPa treatment for 5 min at 4°C inactivated 1.8 logs of GII.4 HuNoV, whereas the same treatment at 20°C and 40°C only achieved a reduction of less than 0.3 log. The same trend was observed for GII.1-509 and GII.6-490. In addition, no significant difference was observed between 20°C and 40°C at a pressure level of 200 MPa. From this study, the phenomenon of being more pressure sensitive at 4°C was similar to what has been observed for other caliciviruses, such as MNV-1 and TV (13, 20). Although generally sensitive to pressure, FCV was found to be the least affected by pressure at room temperature with enhanced inactivation observed at temperatures below or above 20°C (18). Other viruses have exhibited completely different phenotypes with regard to temperature and HPP. For example, the pressure inactivation of HAV was enhanced as temperatures increased above 30°C compared to that at temperatures ranging from 5°C to 30°C (32).

The pH of the medium is an important factor for the pressure inactivation of viruses. In this study, we found that the HuNoV GII.4-5M, GII.1-509, and GII.6-490 strains were more easily inactivated at pH 7 and pH 10 than at pH 4. HuNoV is an enteric virus and is known to be tolerant to stomach acid (4, 5). This pressure tolerance of HuNoV at acidic pH is consistent with previous reports with surrogate viruses. It was previously found that 8.1 logs of MNV-1 were reduced at 350 MPa for 2 min at pH 7.0, whereas only a 6.0-log virus reduction was achieved at pH 4.0 after the same treatment (13). Li et al. (26) also showed that both GI.1 and GII.4 strains were more sensitive to pressure at pH 7.0 than at pH 4.0. Taken altogether, we conclude that HuNoV was more easily inactivated by HPP at a neutral pH than at an acidic pH. This effect of pH during pressure treatment needs to be considered when applying HPP to food products. For example, it may be important to use food products at higher pH values for pressure processing or apply a higher pressure dose to acidic foods to ensure safety.

Finally, we investigated the influence of matrix composition on the pressure treatment of HuNoV. It has been reported that the food matrix, such as carbohydrates, fats, salts, and proteins, can protect viruses from inactivation by HPP (10, 31, 33–35). Consistently, we found that MEM and PBS provided a baroprotective effect against the HPP inactivation of a GII.4 HuNoV compared to

when H_2O was used. More than a 4.0-log reduction was achieved in water at 200 MPa for 5 min at 4°C, whereas only 1.7- and 2.7-log reductions were achieved in MEM and PBS, respectively. The GII.1 and GII.6 strains tested in this study also showed enhancement of inactivation by HPP when suspended in H_2O or PBS compared to that when suspended in MEM. HuNoV was more resistant to HPP in MEM than in PBS, which is probably due to the fact that MEM contains salts, amino acids, vitamins, and sugar. Similar protective effects were also observed in a HuNoV surrogate study. It was reported that FCV infectivity was higher in mussels (2.84 logs) and oysters (2.17 logs) than in seawater (0.58 log) and in medium (1.58 logs) at 250 MPa for 5 min at 20°C (36). For MNV-1, inactivation by HPP was higher in medium (8 logs) than in strawberries (5.8 logs) and strawberry puree (4.7 logs) at 450 MPa for 2 min at 4°C (13). These observations showed that the food matrix confers protection from inactivation of HuNoV surrogates by HPP. Thus, it is important to increase the processing pressure level when treating complex food products in order to achieve high efficiency of viral inactivation.

In summary, we found that (i) different HuNoV strains within genogroup II exhibited different sensitivities to high pressure, and (ii) the HuNoV GII.1, GII.4, and GII.6 strains were more easily inactivated at lower temperature, neutral pH, and in an H_2O solution. Overall, HPP is capable of effectively inactivating HuNoV GII.1, GII.4, and GII.6 strains at commercially acceptable pressures within a short time. Further optimization of HPP parameters is needed to enhance the inactivation of the HuNoV GII.1 strain. This study facilitates the use of HPP to inactivate HuNoV, the major foodborne virus, thereby improving the safety of high-risk foods.

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