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Inactivation of Human Norovirus in Contaminated Oysters and Clams by High Hydrostatic Pressure

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Human norovirus (NoV) is the most frequent causative agent of food-borne disease associated with shellfish consumption. In this study, the effect of high hydrostatic pressure (HHP) on inactivation of NoV was determined. Genogroup I.1 (GI.1) or genogroup II.4 (GII.4) NoV was inoculated into oyster homogenates and treated at 300 to 600 MPa at 25, 6, and 1°C for 5 min. After HHP, samples were treated with RNase and viral particles were extracted with porcine gastric mucin (PGM)-conjugated magnetic beads (PGM-MBs). Viral RNA was then quantified by real-time reverse transcription (RT)-PCR. Since PGM contains histo-blood group-like antigens, which can act as receptors for NoV, deficiency for binding to PGM is an indication of loss of infectivity of NoV. After binding to PGM-MBs, RT-PCR-detectable NoV RNA in oysters was reduced by 0.4 to >4 $\log_{10}$ by HHP at 300 to 600 MPa. The GI.1 NoV was more resistant to HHP than the GII.4 NoV ($P < 0.05$). HHP at lower temperatures significantly enhanced the inactivation of NoV in oysters ($P < 0.05$). Pressure treatment was also conducted for clam homogenates. Treatment at 450 MPa at 1°C achieved a >4 $\log_{10}$ reduction of GI.1 NoV in both oyster and clam homogenates. It is therefore concluded that HHP could be applied as a potential intervention for inactivating NoV in raw shellfish. The method of pretreatment of samples with RNase, extraction of viral particles using PGM-MB binding, and quantification of viral RNA using RT-PCR can be explored as a practical means of distinguishing between infectious and noninfectious NoV.

Human norovirus (NoV), belonging to the family *Caliciviridae*, is the most frequent cause of sporadic and epidemic nonbacterial gastrointestinal disease in the United States. It accounts for 58% of food-borne illnesses, approximately 5.5 million cases each year (1). Shellfish are known as vectors for human pathogens and are a high-risk food for viral outbreaks. Among shellfish, oysters are most frequently involved in NoV outbreaks, presumably because oysters are the most commonly consumed shellfish, and they are usually consumed raw (2). Clams have also been involved in NoV outbreaks (2). In 1993, a large multistate outbreak related to NoV resulted from the consumption of oysters (3). With over 4 million oysters harvested and an attack rate of 62% among oyster eaters, it was estimated that as many as 186,000 people might have become ill (3). As filter feeders, bivalve shellfish can readily accumulate pathogenic microorganisms from surrounding marine and estuarine waters and are thus particularly susceptible to NoV contamination (4). It has been suggested that NoVs can persist in oyster tissues for weeks and cannot be effectively removed during commercial depuration (5–7). Though cooking is the most effective way of eliminating NoV in shellfish; however, this process changes the organoleptic qualities of the shellfish. Alternative processing methods, therefore, are needed to improve the safety of shellfish for human consumption.

High hydrostatic pressure (HHP) has emerged as a promising processing intervention to inactivate pathogenic *Vibrio* spp. and spoilage bacteria in oysters (8–10). This nonthermal process has made a revolutionary change in the oyster-shucking process and has been shown to dramatically improve the appearance, storability, texture, flavor, and yield of treated oysters (11, 12). HHP has also been identified as a potential means of inactivating viral pathogens, such as hepatitis A virus and NoV surrogates, within raw shellfish (13–16). For example, when live oysters, contaminated to high levels by uptake of the NoV surrogate murine norovirus (MNV-1) suspended in seawater, were treated at 400 MPa for 5 min at 5°C, a 4 $\log_{10}$ reduction was observed (15). Arcangeli et al. (17) reported that HHP at 500 MPa for 1 min was effective in inactivating MNV-1, with >4 $\log_{10}$ reductions of the virus in experimentally contaminated live clams. In a recent clinical trial, no NoV infection was observed when NoV-inoculated oyster meats, inoculated through injection, were treated at 600 MPa at 6°C for 5 min and subsequently fed to human subjects (18).

The main difficulty hampering research on NoV is that there is no available *in vitro* cell culture system or small-animal model. Reverse transcription (RT)-PCR is the most common detection method for NoVs because of its rapidity and high sensitivity (19); however, since it only detects the presence of NoV RNA and cannot distinguish between infectious and noninfectious viral particles, the number of infectious viral units in foods that have been treated is potentially overestimated (20–22). Attachment to a receptor on a cell surface, the first step of a viral life cycle, is essential to initiate the infection. Porcine gastric mucin (PGM) has been reported to bind to genogroup I (GI) and genogroup II (GII) NoVs (23, 24). PGM contains mixed type A, type H1, and Lewis b histo-blood group antigens (HBGAs), which are known to function as direct receptors of NoV (24, 25). This binding ability has been exploited as a method to expediently detect different NoVs from foods by means of PGM-conjugated magnetic beads (PGM-

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MBs) (23, 24). After binding and washing, only bound viral particles can be collected and detected by RT-PCR. Recently, Dancho et al. (26) demonstrated that human NoV GI.1 and GII.4 strains were capable of binding to PGM-MBs, whereas thermal, UV, and HHP treatments rendered NoV substantially deficient for binding to PGM-MBs.

The objectives of this study were to (i) determine whether the PGM-MB binding assay could be used to quantify infectious NoV by comparing the results obtained using the binding assay with those reported in the human challenge study (18); (ii) determine whether NoV in two important genogroups, GI and GII, had different pressure sensitivities; and (iii) identify effective HHP parameters for processing of oysters and clams.

MATERIALS AND METHODS

NoV stock. GI.1 8FIIb NoV in a stool sample from patient 34-9 of a human volunteer study (18) and a GII.4 strain (Cincinnati Children's Hospital) in fecal suspension were used in this study. The stool was suspended in sterile deionized water, followed by centrifugation at 4,000 × *g* for 20 min. The supernatants from both the stool and the fecal suspension were passed through a 0.22-μM filter (EMD Millipore, Billerica, MA), aliquoted, and stored at −80°C until use.

Sample preparation and pressure treatments. Live oysters (*Crassostrea virginica*) and clams (*Mercenaria mercenaria*) were purchased from local seafood markets. Twenty oysters and clams were shucked, and the meats and fluids inside the shells were blended for 30 s using a blender (model 36BL23; Waring Commercial, New Hartford, CT). Oyster supernatant was prepared by centrifugation of the oyster homogenates at 3,000 × *g* for 5 min. Before inoculation, the oyster supernatant and oyster and clam homogenates were treated at 600 MPa for 5 min at 6°C to inactivate NoV that could be naturally present in the oysters (18). The HHP-treated oyster supernatant (100 μl) was mixed with 10 μl of GII.4 NoV stock. The HHP-treated oyster homogenates (0.2 g) were mixed with 40 μl of the GII.4 or GI.1 NoV stocks, and the HHP-treated clam homogenates (0.2 g) were mixed with 40 μl of the GI.1 NoV stocks. The inoculated samples were then transferred into sterile plastic pouches (polyethylene; Fisher Scientific, Fair Lawn, NJ). The pouches were double sealed and double bagged. Oyster supernatant samples were treated at 300 to 600 MPa for 5 min at 25 and 1°C; oyster homogenate samples at 300 to 600 MPa for 5 min at 25, 6, and 1°C; and clam homogenate samples at 400 to 500 MPa for 5 min at 1°C. The pressure treatments were conducted using a high-pressure unit (model Avure PT-1; Avure Technologies, Kent, WA) with water as the hydrostatic medium. A circulating bath surrounded the pressure cell to control temperature. The pressure come-up time was approximately 22 MPa/s. The pressure release was <4 s. The pressurization time reported in this study does not include the pressure come-up or release times.

Virus extraction from oyster samples. Oyster supernatant samples were incubated with or without 2 μl (20 U/μl) of RNase (Life Technologies, Grand Island, NY) for 30 min at 37°C and mixed with 1 ml of phosphate-buffered saline (PBS) (pH 7.2) in a 1.5-ml microcentrifuge tube before being subjected to PGM-MB binding assays. Oyster or clam homogenates (0.2 g) were blended with glycine buffer (pH 9.5; 0.1 M glycine, 0.3 M NaCl) at room temperature (~21°C) at a 1:9 ratio to make a 10-fold dilution. The mixture was centrifuged at 15,000 × *g* for 15 min at room temperature. Viral particles were precipitated from the supernatant by an equal volume of 16% polyethylene glycol 8000 (PEG) (Sigma, St. Louis, MO) with 0.525 M NaCl. After a 1-h precipitation on ice, the mixture was centrifuged at 10,000 × *g* for 5 min at room temperature. The pellet was suspended in 2 ml PBS by vigorous vortex mixing and pipetting and incubated with 5 μl (20 U/μl) of RNase (Life Technologies) for 30 min at 37°C. All samples were transferred to 15-ml centrifuge tubes, and the volume was made up to 5 ml with PBS before being subjected to PGM-MB binding assays.

PGM-MB binding assay and viral RNA extraction. PGM-MBs were prepared as described by Tian et al. (23). Briefly, 1 ml of MagnaBind carboxyl-derivatized beads (Thermo Scientific Pierce, Rockford, IL) were washed three times in 1 ml of PBS using a magnetic separation stand (EMD Millipore). Type III PGM (Sigma; 10 mg/ml) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (Thermo Scientific Pierce; 10 mg/ml), both dissolved in10 mg/ml conjugation buffer [0.1 M 2-(4-morpholino)-ethane sulfonic acid, 0.9% NaCl, pH 4.7], were added to the washed beads. The mixture was incubated for 30 min at room temperature on a Labquake shaker rotisserie (Thermo Scientific, Waltham, MA) rotating at approximately 8 rpm to allow the PGM to conjugate to the beads. The beads were separated magnetically from the suspension solution and washed three times in 1 ml of PBS. Finally, the PGM-MBs were resuspended in 1 ml PBS containing 0.05% sodium azide (Sigma) and stored at 4°C until they were used.

PGM-MBs (100 and 800 μl, respectively) were added to the tube containing oyster supernatant and homogenate samples. The tubes were incubated for 15 min at room temperature on a Labquake shaker rotisserie at 8 rpm. A magnetic separation stand was used to separate the PGM-MBs from the liquid. The PGM-MBs were washed three times in 1 ml of PBS and resuspended in 140 μl of PBS in a 1.5-ml microcentrifuge tube. Viral RNA was extracted from PGM-MB suspensions using a QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions.

Real-time RT-PCR. Quantitative real-time RT-PCR was performed on an Applied Biosystems 7900 HT Fast Real-time PCR instrument (Life Technologies). Primers and probes for the individual quantitation of GI.1 and GII.4 NoVs were adapted from previous publications (27–29) with modifications. The primers and probes for GI.1 NoV were QNIF4 (5′ CGCTGGATGCGNTTCCAT 3′) (500 nM), NV1LCR (5′ CCTTAGACGCCATCATCATTTAC 3′) (900 nM), and NVGG1p (5′ 6-6-carboxyfluorescein [FAM]-TGGACAGGAGAYCGCRATCT-6-carboxytetramethylrhodamine [TAMRA] 3′) (100 nM). The primers and probes for GII.4 NoV were QNIF2 (5′ ATGTTCAGRTGGATGAGRTTCTCWGA 3′) (500 nM), COG2R (5′ TCGACGCCATCTTCATTCACA 3′) (900 nM), and QNIFS (5′ 6-FAM-AGCACGTGGGAGGGCGATCG-TAMRA 3′) (250 nM). All primers and probes were purchased from Applied Biosystems (Life Technologies). Each 10-μl reaction mixture contained 2.5 μl of TaqMan Fast Virus 1-Step Master Mix PCR Master Mix (Life Technologies) and 2 μl of extracted RNA. Tenfold serially diluted RNA extracts were used as standards. Based on the manufacturer's recommendation, cycling times and temperatures were 50°C for 5 min for reverse transcription, 95°C for 20 s for initial denaturation, and 45 cycles of 95°C for 3 s and 60°C for 30 s.

Statistical analysis. Three independent trials were conducted for each treatment. $\log_{10}$ reductions of viral RNA were calculated from the differences between untreated and treated samples. Statistical analyses were conducted using JMP (SAS Institute, Cary, NC). Tukey's one-way multiple comparisons were used to determine significant differences among treatments ($P < 0.05$).

RESULTS

Pressure inactivation of GII.4 NoV in oyster supernatant. Prior to inactivation experiments, serial 10-fold dilutions of NoV stock were inoculated into oyster supernatant or homogenate samples, and the virus was extracted with PGM-MBs. The RT-PCR results indicated that the binding capacity of the beads was not saturated and the NoV extracted from oyster samples was proportional to the inoculation level. The extraction recovery rate of GII.4 NoV from oyster supernatant averaged 53% (the ratio of RNA copies of NoV extracted from oyster samples to RNA copies extracted from the same amount of NoV stock as inoculated in oyster samples). The initial RT-PCR-detectable NoV RNA in unpressurized samples was approximately 4 $\log_{10}$ units compared to standard viral

TABLE 1 Inactivation of GII.4 norovirus in oyster supernatant by HHP

HHP (MPa)[a]	Mean $\log_{10}$ reduction of NoV RNA ± SD at initial sample temp[b]: 25°C	6°C
300		
No RNase	1.0 ± 0.3A	
RNase	1.1 ± 0.3aA	3.5 ± 0.4b
400		
No RNase	1.4 ± 0.3A	
RNase	3.3 ± 0.3aB	3.9 ± 0.4a
500		
No RNase	1.7 ± 0.4A	
RNase	3.7 ± 0.5B	>4.0
600		
No RNase	1.7 ± 0.1A	
RNase	>4.0	>4.0

[a] Pressure treatment was performed at 300 to 600 MPa for 5 min.
[b] Data in the same row followed by the same lowercase letter are not significantly different ($P > 0.05$). Data in the same column with the same pressure level followed by the same uppercase letter are not significantly different ($P > 0.05$).

TABLE 2 Inactivation of GI.1 and GII.4 norovirus in oyster homogenates by HHP

HHP (MPa)[a]	Mean $\log_{10}$ reduction of NoV RNA ± SD at initial sample temp[b]: 25°C GI.1	25°C GII.4	6°C GI.1	6°C GII.4
300	0.4 ± 0.2a	1.7 ± 0.1b	0.7 ± 0.1a	2.9 ± 0.1b
400	1.0 ± 0.2a	3.6 ± 0.6b	1.3 ± 0.2a	3.6 ± 0.4b
600	Not done		>4.1	>4.0

[a] Pressure treatment was performed at 300 to 600 MPa for 5 min at initial sample temperatures of 25 and 6°C.
[b] Under the same initial sample temperature, data in the same row followed by the same lowercase letter are not significantly different ($P > 0.05$).

RNA in each reaction mixture. The inoculated oyster supernatant was pressurized at 300 to 600 MPa and treated or not with RNase before being subjected to PGM-MB binding and RT-PCR assay. Without RNase treatment, 1.1 to 1.8 $\log_{10}$ reductions of RT-PCR-detectable NoV RNA were observed after HHP treatments of 300 to 600 MPa at 25°C (Table 1). With RNase treatment, RT-PCR-detectable NoV RNA was substantially reduced in a pressure-dependent manner; an extra ~2 $\log_{10}$ reduction was observed for HHP treatments at 400 to 600 MPa. HHP treatments at 6°C significantly enhanced viral inactivation of HHP-treated NoV in oyster supernatant compared with the HHP treatments at 25°C. For example, treatment with 300 MPa at 6 and 25°C reduced RT-PCR-detectable NoV RNA by 3.5 and 1.1 $\log_{10}$ units, respectively. At pressure levels of 400 to 600 MPa, no significant difference in the reduction of GII.4 NoV RNA was observed. A 400-MPa treatment achieved 3.9 $\log_{10}$ reduction, which was almost as high as the detection limit of 4 $\log_{10}$. Thus, whether pressure treatments at higher levels would have resulted in additional virus inactivation could not be determined.

Pressure inactivation of GI.1 and GII.4 NoV in oyster and clam homogenates. Both the unpressurized samples and the pressurized samples were treated with RNase, bound to PGM-MBs, and quantified by RT-PCR. The extraction recovery rate of NoV from oysters averaged 27%. The initial RT-PCR-detectable NoV RNA in unpressurized samples was approximately 4 $\log_{10}$ units in each reaction mixture for both GI.1 and GII.4 NoVs. In the first stage, HHP inactivations of GI.1 and GII.4 NoVs at 300, 400, and 600 MPa at 25 and 6°C were compared, and the reductions in RT-PCR-detectable NoV RNA from HHP-inactivated NoV bound to PGM-MB are shown in Table 2. For GII.4 NoV, the results were consistent with those obtained from the oyster supernatant trials. HHP treatments of 300, 400, and 600 MPa reduced the RT-PCR-detectable GII.4 NoV RNA by 1.7 to 4.0 $\log_{10}$ units. GI.1 NoV was more resistant to HHP in oysters than the GII.4 strain. For example, treatment of 400 MPa at 25°C reduced RT-PCR-detectable NoV RNA of the GII.4 strain by 3.6 $\log_{10}$ units, while only 1.0 $\log_{10}$ unit was achieved for the GI.1 strain. Similar to the GII.4 strain, treatment at 6°C caused significantly greater reduction of GI.1 at 300 MPa than that at 25°C ($P < 0.05$), but for the 400-MPa treatment, the results for the two temperatures were not significantly different ($P > 0.05$). Treatment at 600 MPa achieved >4.1 $\log_{10}$ reduction of RT-PCR-detectable GI.1 NoV RNA after binding.

In the second phase of the study, only GI.1 NoV was used, since it was more pressure resistant than the GII.4 strain. The goal was to further investigate the temperature effect and identify high-pressure-processing parameters that could achieve a ≥4 $\log_{10}$ reduction of GI.1 NoV. Oyster homogenates inoculated with GI.1 NoV were treated at 400 to 500 MPa at initial sample temperatures of 25, 6, and 1°C (Table 3). The initial sample temperature had a significant effect on pressure inactivation of GI.1 NoV. Pressure sensitivity of the GI.1 strain substantially increased with the decrease of the initial sample temperature (1 > 6 > 25°C). For example, a 0.9, 2.8, and 4.3 $\log_{10}$ reduction was observed for the 450-MPa treatment at 25, 6, and 1°C, respectively, and the difference was statistically significant ($P < 0.05$). With an initial sample temperature of 1°C, pressure levels at ≥450 MPa could achieve ≥4 $\log_{10}$ reductions of RT-PCR-detectable GI.1 NoV RNA after binding. Since an initial sample temperature of 1°C was the most effective for pressure inactivation of GI.1, it was used for the pressure treatment of clam homogenates. The results ($\log_{10}$ reduction of NoV RNA) were as follows: 400 MPa, 2.8 ± 0.1; 450 MPa, 4.1 ± 0.2; 500 MPa, 4.0 ± 0.2. No difference in pressure sensitivity of the GI.1 NoV was observed in clams and oysters when treated at the same pressure level at 1°C ($P > 0.05$).

TABLE 3 Effect of initial sample temperature on pressure inactivation of GI.1 NoV in oyster homogenates

HHP (MPa)[a]	Mean $\log_{10}$ reduction of NoV RNA ± SD at initial sample temp[b]: 25°C	6°C	1°C
400	1.0 ± 0.2aA	1.3 ± 0.2aA	2.9 ± 0.3bA
450	0.9 ± 0.0aA	2.8 ± 0.4bB	4.3 ± 0.5cB
500	2.1 ± 0.4aB	4.0 ± 0.5bC	4.0 ± 0.5bB

[a] Pressure treatment was performed at 400 to 500 MPa for 5 min.
[b] Data in the same row followed by the same lowercase letter are not significantly different ($P > 0.05$). Data in the same column followed by the same uppercase letter are not significantly different ($P > 0.05$).

DISCUSSION

The mechanisms of viral inactivation by HHP are thought to involve the dissociation and/or denaturation of proteins of the virus capsid rather than direct damage to viral nucleic acids. Kingsley et al. (14) found that HHP treatments at 500 MPa for 5 min were sufficient to inactivate >7 $\log_{10}$ PFU/ml of hepatitis A virus but did not disrupt the capsid to such a degree that the genomic RNA was susceptible to RNase A treatment (14). For MNV, a treatment of 400 MPa for 5 min at 0°C abrogates the ability of MNV-1 to bind to its host cell, suggesting loss of receptor-binding function (30). Tang et al. (30) demonstrated that the RNA of MNV-1 was protected from RNase treatment after a 400-MPa treatment, which suggests that the viral capsid remained intact. However, a recent electron microscopy-based study by Lou et al. (31) showed that a 600-MPa treatment was able to destroy MNV-1 capsid integrity and prevent release of viral genomic RNA. In the present study, HHP treatments of 400 to 600 MPa at 25°C caused 2-fold-higher $\log_{10}$ reductions of RT-PCR-detectable RNA in the GII.4 NoV samples with RNase treatment than in those without RNase treatment (Table 1). However, for the 300-MPa treatment, no significant difference in $\log_{10}$ reduction between the treatments was observed, possibly due to the inability of HHP at 300 MPa to disrupt the viral capsid. As the pressure level increased, the virus started to lose its integrity and the viral RNA was no longer protected from degradation by RNase. Therefore, RNase treatments were carried out in the subsequent studies to ensure that any viral RNA released as a result of HPP treatment was not subsequently detected by RT-PCR.

Since real-time RT-PCR only quantifies the total RNA in a virus sample and cannot distinguish between infectious and noninfectious viruses, PGM was used in this study to bind and collect potentially infectious NoV and to exclude inactivated virions with capsids that had been rendered unable to bind to PGM by HHP. Apparently, some inactivated virions with damaged capsids could still bind to PGM, since there were differences in the reductions of RT-PCR-detectable RNA copies between the GII.4 NoV samples with RNase treatment and those without RNase treatment (Table 1). Therefore, the combination of RNase treatment and PGM binding was necessary to exclude as many inactivated virions as possible from being collected and quantified in the subsequent RT-PCR assay.

The clinical trial reported by Leon et al. (18) is the only study where the pressure inactivation profile of GI.1 NoV has been roughly defined. In that study, human subjects were challenged with pressure-treated and untreated oysters inoculated with NoV GI.1 via injection. There was no significant difference between the NoV infection rates of subjects challenged with oysters treated at 400 MPa for 5 min at 25°C (3 out of 5 [60%] subjects infected) and subjects challenged with untreated oysters (7 out of 15 [47%] subjects infected). The same HHP treatment (400 MPa for 5 min) conducted at a lower temperature, 6°C, reduced the infection rate to 21% (3 out of 14 subjects infected). Conversely, none of the 10 subjects challenged with NoV-seeded oysters treated at 600 MPa at 6°C for 5 min became infected with NoV, indicating a >4 $\log_{10}$ reduction of genomic-equivalent copies of NoV. To determine whether the PGM-MB binding method (pretreatment of samples with RNase, extraction of viral particles using PGM-MB binding, and quantification of viral RNA using RT-PCR) used in this study for quantifying infectious NoV worked, we used the same GI.1 strain and pressure treatment conditions that were used by Leon et al. (18) so that our results would be more comparable to theirs. Our results appear to correlate with observations from the human challenge study (Table 2) and indicate that the PGM-MB binding method could potentially reflect the infectivity of NoV after HHP and could be used as a means to quantitate inactivation of NoV.

The results of this study indicate that to achieve substantial inactivation of NoV in oysters (4 $\log_{10}$ reduction), the HHP needs to be ≥400 MPa at 25 or 6°C for 5 min for the GII.4 strain and ≥450 MPa at 1°C or ≥500 MPa at 6°C for 5 min for the GI.1 strain. Our previous study with NoV surrogates, Tulane virus (TV) and MNV-1, found that TV could be effectively inactivated at pressure levels of ≥300 MPa at 4°C and MNV-1 at pressure levels of ≥400 MPa at 4°C in oysters (32). Li et al. (16) showed that a treatment at 400 MPa for 5 min at 0°C reduced MNV-1 in oysters to undetectable levels (>4 $\log_{10}$ PFU/ml). The human challenge study conducted by Leon et al. (18) was an endpoint study in which subjects were either infected or not infected with NoV, and the extent to which NoV was inactivated at 400 MPa for 5 min at 25 or 6°C was not known. Based on the present results and the studies mentioned above, one can reasonably conclude that TV is more sensitive to pressure than the GII.4 and GI.1 NoVs and is not a suitable surrogate for NoV for HHP inactivation studies. MNV-1 may present pressure resistance similar to that of the GII.4 NoV but probably is more sensitive to pressure than the GI.1 NoV. A major limitation in studies using NoV surrogates is a lack of correlation in the inactivation rates of the surrogates and the NoVs. The PGM-MB binding method could be used to obtain direct information on HHP inactivation of NoV and could provide more confidence in the results than using surrogates.

The HHP inactivation of NoV in oysters increased as temperature decreased (1 > 6 > 25°C). This finding is in agreement with a recent study by Li et al. (29), which reported that the efficacy of pressure inactivation of both GI.1 and GII.4 virus in PBS increased with decreasing initial sample temperatures (1 > 4 > 10 > 21 > 35°C). Our previous studies also showed that NoV surrogates, feline calicivirus (FCV), MNV-1, and TV, were significantly more sensitive to HHP at lower temperatures (15, 32, 33). The enhanced pressure inactivation of these viruses is probably also in agreement with the results of the human challenge study (18). Similarly, the NoV GI.1 infection rate in human volunteers was lower with 400-MPa HPP at 6°C than with the same pressure level at 25°C; however, due to the limited sample size, statistical significance of these differences could not be established (18).

One characteristic of shellfish-related outbreaks is their frequent association with multiple virus strains, observed in both infected patients and the involved shellfish. Among reported shellfish-related outbreaks from 1998 to 2009 around the world, contamination by multiple NoV strains was reported in 65% of the outbreaks, with GI and GII NoVs detected in 75% and 92%, respectively, of shellfish samples in outbreak investigations (34). In comparison to their overall frequency, GI NoVs are proportionately more common than GII NoVs in shellfish-related outbreaks. It is suggested that GI NoVs are also more actively and efficiently concentrated in oysters and have greater persistence in oyster tissues than GII strains (7, 35, 36). Among GI NoVs, the most frequently reported genotype is GI.1, and among GII NoVs, the GII.4 genotype is the most frequently reported from shellfish-related outbreak samples worldwide (35). In the present study, the GII.4 strain lost its ability to bind to PGM at lower pressure levels

than the GI.1 strain (Table 2), indicating that the GI.1 strain is probably more resistant to HHP than the GII.4 strain, which could be a more stringent indicator in future HHP studies. Variation in the pressure resistances of different strains of other viruses has been reported by Kingsley et al. (37). In that study, a 5-min HPP treatment at 600 MPa resulted in a 7.6 $\log_{10}$ tissue culture infectious dose (50%) ($TCID_{50}$) reduction of coxsackievirus strain A9 in minimum essential growth medium (MEM) supplemented with 10% fetal bovine serum (FBS), while coxsackievirus strain B5 remained fully infectious after a 5-min treatment at 600 MPa.

Ideally, NoV should be inoculated into live oysters and clams through feeding to simulate the real-life contamination situation. This method of inoculation requires a large amount of virus to be placed in seawater so that a high level of virus uptake by oysters and clams can occur. That is not practical, due to limited NoV stock, since NoV is nonculturable. In the present study, three substrates, oyster supernatant, oyster homogenates, and clam homogenates, were used. It is well documented that the substrates in which a virus is suspended can influence its pressure sensitivity. For example, Murchie et al. (38) demonstrated that both bovine enterovirus and FCV were more pressure resistant when treated in mussels and oysters than when treated in seawater and culture medium. Our results indicated that the substrates did not significantly affect the pressure sensitivity of NoV, possibly due to the similar characteristics of the three substrates. It was found that the oyster and clam contents also bound to PGM-MBs, in addition to NoV. Therefore, small sample sizes of supernatants and homogenates were used to minimize the amount of PGM-MBs added to the samples, since PGM-MBs are very expensive. Using supernatant instead of homogenates as a substrate for the pressure inactivation study provides two benefits. The labor-intensive and time-consuming viral extraction steps can be avoided if supernatant is used. In addition, the cost is reduced, since a smaller amount of PGM-MBs is needed for supernatant than for homogenates.

Commercially, HHP at pressures of ≤300 MPa is used by the shellfish industry to facilitate oyster shucking, extend shelf life, and reduce the numbers of *Vibrio* spp. (10, 12). These pressure levels are unlikely to inactivate NoVs in oysters and clams based on the results of the current and the human volunteer studies (18). Use of a higher pressure level in combination with refrigeration temperature is needed. It is not clear whether the higher pressure levels required to inactivate NoVs are viable for commercial application. In addition, whether HHP at higher pressures could adversely affect the sensory quality of shellfish needs to be investigated.

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