Inactivation of Selected Non-enveloped and Enveloped Viruses by High Pressure Processing: Effectiveness, Mechanism, and Potential Applications

Thesis

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By

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Abstract

Viruses are the leading cause of foodborne illness worldwide (67%). Specifically, human norovirus (HuNoV) is the major foodborne virus that accounts for more than 90% of acute nonbacterial gastroenteritis in humans. Fresh produce is often at high risk for norovirus contamination because it can be easily contaminated at both pre-harvest and post-harvest stages and it undergoes minimal or no processing. The increasing produce-associated outbreaks suggest that there is an urgent need to develop novel interventions to eliminate foodborne enteric viruses in fresh produce. High pressure processing (HPP), a non-thermal processing technology, effectively inactives bacteria, fungi, and molds in foods. HPP may provide a new approach to reduce the virus load in fresh produce and related products. In the present study, we systematically investigated the effectiveness of HPP on inactivating norovirus in aqueous medium, lettuce, strawberry, and fruit puree using murine norovirus (MNV-1) as a surrogate for noncultivable HuNoV. Approximately 5 log virus reduction was observed in all food items upon treatment at 400 MPa for 2 min at 4°C, thus demonstrating that HPP is highly effective in reducing the MNV-1 load in fresh produce. Moreover, our results showed that pressure, pH, temperature, and the food matrix affected the virus survival. MNV-1 was more effectively inactivated at 4°C than at 20°C in both medium and fresh produce. MNV-1 was also found to be more sensitive to high pressure at neutral conditions (pH 7.0) than at acidic conditions (pH 4.0). In addition, the overall impact of HPP on the quality of fresh
produce is minimal; however this impact varied with the type of food and the pressure level to a certain extent. Taken together, these findings support the notion that HPP is a promising intervention to eliminate and minimize the norovirus risk in fresh produce and related products while the organoleptic and nutritional properties of these foods are affected to the minimal extent.

To further evaluate the potential of HPP in inactivating viruses, we continued to investigate the effectiveness of HPP on the inactivation of human rotavirus (HRV), vesicular stomatitis virus (VSV), and avian metapneumovirus (aMPV). HRV represents a major foodborne virus other than HuNoV and is non-enveloped; VSV and aMPV are enveloped and their virion structures are strikingly different from those of MNV-1, HuNoV, and HRV. We found that HPP was capable of efficiently inactivating all tested viruses at optimal conditions, although the pressure susceptibilities varied considerably among these viruses. Under the pressure of 350 MPa at 4°C for 2 min, 1.1-log, 3.9-log, and 5.0-log virus reductions were achieved for VSV, HRV, and aMPV, respectively. Additionally, both VSV and aMPV were more susceptible to HPP at higher temperature and lower pH. In contrast, HRV was more easily inactivated at higher pH and inactivation appeared to be independent of temperature. These results suggest that the presence of an envelope, temperature, and pH contribute to the susceptibility to HPP independently for each virus.

We also explored the mechanisms underlying HPP-induced inactivation using MNV-1, HRV, and VSV as models. We found that the damage to the virion structure by
disrupting the viral envelope and/or the viral capsid, not the degradation of viral genomic RNA, was the primary mechanism of HPP-induced inactivation of all these viruses. Notably, even when the virion structures were completely damaged upon HPP, the major immunogenic proteins (such as MNV-1 capsid protein and VSV glycoprotein) remained antigenic, implying that HPP-inactivated viruses could be used as candidate for inactivated vaccines.

In summary, our results demonstrate that HPP is able to effectively inactivate most common viral agents in aqueous medium and fresh produce by disrupting the virion structure, viral capsid, and envelope whereas the pressure sensitivities and optimal conditions required for efficient inactivation vary considerably. Thus, HPP has the potential to be a novel intervention strategy for eliminating viral contaminants in high risk foods as well as a promising technique to generate effective vaccines.
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It is my pleasure to thank all the people who made this thesis possible.

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ABBREVIATIONS

aMPV - Avian metapneumovirus
APV - Avian pneumovirus
CDC – Centers for Disease Control and Prevention
CPE – Cytopathic effect
DMEM – Dulbecco’s modified eagle medium
ELISA – Enzyme-linked immunosorbant assay
EM – Electron Microscopy
FBS – Fetal bovine serum
FCV – Feline calicivirus
EDTA – Ethylenediamine tetraacetic acid
BHK-21 – A baby hamster kidney cell line
FCV - Feline calicivirus
FDA – Food and Drug Administration
HAV – Hepatitis A virus
HBGA – Histo-blood group antigen
HCMV - human cytomegalovirus
HIUS - High intensity ultrasound
HPP – High pressure processing
HRV – Human rotavirus
HSV-1 - herpes simplex virus type 1
HuNoV – Human norovirus
MA-104 - Rhesus monkey kidney cell line
MEM – Minimal essential medium
MNV-1 – Murine norovirus
MOI – Multiplicity of infection
MPa - Mega Pascals
NIAID – National institute of Allergy and Infectious Diseases
NoV - Norovirus
ORF – Open reading frame
PCR – Polymerase chain reaction
PBS – Phosphate buffered saline
PFU – Plaque forming units
PGM - porcine gastric mucin
PGM-MB - porcine gastric mucin conjugated to magnetic beads
RAG2 - Recombination-activating gene 2
RAW 264.7 – A mouse macrophage cell line
RdRp – RNA-dependent RNA polymerase
RNA – Ribonucleic acid
RT-PCR – Reserve transcriptase-polymerase chain reaction
SDS-PAGE – Sodium dodecyl sulfate- polyacrylamide gel electrophoresis
STAT-1 - Signal transducer and activator of transcription 1
TEM- Transmission electron microscopy
TMV – Tobacco mosaic virus
TRTV - Turkey rhinotracheitis virus
Vero – An african green monkey kidney cell line
VLP – Virus-like particle
VSV – Vescicular Stomatitis virus
CHAPTER 1  
LITERATURE REVIEW

1.1. Introduction to foodborne illness

Foodborne illnesses are defined as the diseases caused by agents that enter the body through the ingestion of contaminated food (WHO). Due to frequent international travel and global food trade, foodborne illnesses are becoming an extremely critical public health issue worldwide (Koopmans et al., 2002). Food contamination not only threatens human health, it also imposes an enormous social and economic burden on communities. For example, it is estimated that the foodborne disease caused by the major food-associated pathogens alone can cost up to 35 billion dollars annually (1997) in medical charges and lost productivity in the United States (WHO).

Foodborne illnesses can be caused by three major contaminants: (i) biological contaminants including bacteria, viruses, parasites, fungi, and biological toxins; (ii) chemical contaminants including toxic metals, pesticides, cleaning products, sanitizers, and equipment lubricants; and (iii) physical contaminants including any foreign object that is accidentally introduced into food or a naturally occurring object, such as a bone in a filet, that poses a physical hazard. Of these contaminants, biological hazards usually cause epidemic and even pandemic foodborne diseases. A comprehensive statistical analysis of outbreak data published by Mead et al. (1999) showed that there are
approximately 76 million cases of foodborne illness, resulting in 323,914 hospitalizations and 5,194 deaths annually in the United States alone. Viruses, bacteria, and parasites account for 67%, 30%, and 2.6% of foodborne illnesses, respectively. According to the most recent statistical outbreak report, the CDC claims that the annual cases of foodborne illnesses in the United States has decreased to 48 million, with viruses still being the leading causative agent, accounting for more than 60% of these illnesses (Fox et al., 2011).

1.2. Introduction to food- and water-borne viruses

A foodborne virus is defined as any virus that may be transmitted by food and is able to cause illness via the fecal-oral route. Similarly, a waterborne virus is usually transmitted by contaminated water. These food- and water-borne viruses usually attack the human digestive system, and are subsequently shed and dispersed by stool and vomit. The most common food- and water-borne viruses include human norovirus (HuNoV), sapovirus, astrovirus, rotavirus, adenovirus, poliovirus, enterovirus 71, hepatitis A, and hepatitis E viruses. The properties and major symptoms of these viruses are summarized in Table 1. Among these foodborne viruses, HuNoV has long been considered the most prominent virus associated with foodborne illness. HuNoV causes more than 95% of non-bacterial acute gastroenteritis. Approximately 23 million people suffer from norovirus-associated gastroenteritis each year in the United States (CDC, 2010). The total number of norovirus outbreaks may even be underestimated due to the large number of unreported cases and the lack of reliable detection methods.
Table 1. The properties and major symptoms of the most common food- and water-borne viruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>Family</th>
<th>Genus</th>
<th>Genetic material</th>
<th>Envelope</th>
<th>Transmission mode</th>
<th>Major symptom</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human norovirus</td>
<td>Caliciviridae</td>
<td>Norovirus</td>
<td>Single-strand Positive-sense-RNA</td>
<td>No</td>
<td>Food, water</td>
<td>gastroenteritis</td>
</tr>
<tr>
<td>Human sapovirus</td>
<td>Caliciviridae</td>
<td>Sapovirus</td>
<td>Single-strand Positive-sense-RNA</td>
<td>No</td>
<td>Food, water</td>
<td>gastroenteritis</td>
</tr>
<tr>
<td>Astrovirus</td>
<td>Astroviridae</td>
<td>Astrovirus</td>
<td>Single-strand Positive-sense-RNA</td>
<td>No</td>
<td>Food, water</td>
<td>gastroenteritis</td>
</tr>
<tr>
<td>Rotavirus</td>
<td>Reoviridae</td>
<td>Rotavirus</td>
<td>Double-strand RNA</td>
<td>No</td>
<td>Food, water</td>
<td>gastroenteritis</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>Adenoviridae</td>
<td>Mastadenovirus</td>
<td>Double-strand DNA</td>
<td>No</td>
<td>Water, food</td>
<td>gastroenteritis</td>
</tr>
<tr>
<td>Hepatitis A virus</td>
<td>Picornaviridae</td>
<td>Hepatovirus</td>
<td>Single-strand Positive-sense-RNA</td>
<td>No</td>
<td>Food, water</td>
<td>hepatitis</td>
</tr>
<tr>
<td>Hepatitis E virus</td>
<td>Hepeviridae</td>
<td>Hepevirus</td>
<td>Single-strand Positive-sense-RNA</td>
<td>No</td>
<td>Water, food</td>
<td>hepatitis</td>
</tr>
<tr>
<td>Poliovirus</td>
<td>Picornaviridae</td>
<td>Enterovirus</td>
<td>Single-strand Positive-sense-RNA</td>
<td>No</td>
<td>Water</td>
<td>poliomyelitis</td>
</tr>
<tr>
<td>Enterovirus 71</td>
<td>Picornaviridae</td>
<td>Enterovirus</td>
<td>Single-strand Positive-sense-RNA</td>
<td>No</td>
<td>Water, food</td>
<td>neurological diseases</td>
</tr>
<tr>
<td>Aichivirus</td>
<td>Picornaviridae</td>
<td>Kobuvirus</td>
<td>Single-strand Positive-sense-RNA</td>
<td>No</td>
<td>Water, food</td>
<td>gastroenteritis</td>
</tr>
</tbody>
</table>
Unlike bacteria, viruses are strict intracellular parasites and are only able to replicate inside a living host. Therefore, viruses will never amplify in food or water during transportation, processing, and storage (Vasikcova et al., 2005; Koopmans et al., 2002). Importantly, all known food- and water-borne viruses are non-enveloped viruses and extremely stable in food, water, and the environment. They can survive on human hands, dried human and animal feces, kitchen surfaces, floors, carpets, and even hospital lockers for a long time period (Green, 2011; Bidawid et al., 2004). Only a few virus particles are necessary to cause illness and a large amount of virus particles can be shed in the stool of an infected person (up to $10^{11}$) leading to rapid spread of the disease (Rzezutka and Cook, 2004). Most food-and water-borne viruses are much more resistant to heat, acidic pH, and disinfection than bacteria, thus the current procedures to prevent bacterial infections in food processing, preservation, and storage may not be fully effective against viral pathogens (Koopmans and Duzier, 2004; Rzezutka and Cook, 2004). Unfortunately, it is impossible for consumers and food handlers to notice the viral contamination in food or water because the contaminated products look, smell, and even taste normal (Koopmans and Duzier, 2002). Furthermore, it has been technically challenging to detect viruses in foods because of the complicated food matrix. The molecular methods commonly used to detect viruses are difficult to apply to viruses found in foods, and methodologies for detection of foodborne viruses are lacking.

In order to develop novel processing technologies to effectively control viral hazards in food and water, we conducted the present study with five objectives: (1) to perform a comprehensive review of the viruses used in this study, including two non-enveloped viruses (human norovirus and rotavirus) and two enveloped viruses (vesicular
stomatitis virus and avian metapneumovirus); (2) to perform a comprehensive review of virus inactivation by non-thermal processing techniques with an emphasis on high pressure processing (HPP); (3) to investigate the effectiveness of HPP on inactivating a human norovirus surrogate in aqueous medium and fresh produce; (4) to compare the sensitivity of non-enveloped and enveloped viruses to HPP; (5) to explore the mechanism of virus inactivation by HPP.

1.3. The discovery of human norovirus

The first documented outbreak of human norovirus (HuNoV) occurred in Norwalk, Ohio, USA, in 1968 (Kapikian et al. 1972). It was reported that more than 50% of the students and teachers at Bronson Elementary School developed acute gastroenteritis with the symptoms involving nausea and vomiting. Moreover, 32% of home contacts of the students and staff also developed the same symptoms. In 1972, Kapikian et al. first determined the etiology of this disease. Immune electron microscopy analysis of human stool samples identified a large number of small-round structured viral particles ranging from 27-38 nm in diameter and the virus was termed Norwalk virus. The name of this virus was changed to norovirus after being identified in numerous outbreaks with similar symptoms occurring on cruise ships and in many other settings. In 2002, the name norovirus (Norovirus for the genus) was approved by the International Committee on Taxonomy of Viruses. In addition, some other names used to refer to norovirus are Norwalk virus, food poisoning virus, small round-structured virus, and Snow Mountain virus (Appleton, 1987). The common names of the illness caused by
noroviruses include winter vomiting disease, acute non-bacterial gastroenteritis, viral gastroenteritis, and stomach flu.

1.4. Clinical features and epidemiology of human norovirus

When a person becomes infected with norovirus, the virus begins to replicate within the small intestine. The principal symptom is acute gastroenteritis that develops between 24 and 48 hours after exposure. HuNoV infected individuals usually develop symptoms including watery diarrhea, vomiting, nausea, abdominal cramping, chills, headache, dehydration, and low-grade fever in some cases. For healthy individuals, the illness associated with HuNoV is considered as self-limiting, with symptoms normally persisting for 2-3 days (Koopmans and Duizer, 2004). However, parenteral fluid therapy and even hospitalization are needed for some severe cases (Kaplan et al., 1982; Koopmans et al., 2002). Although HuNoV infection is debilitating and unpleasant, the clinical manifestation is usually a mild disease with an average mortality rate of only 0.003% (CDC, 2011). Out of the 23 million annual cases of norovirus infection, there are about 300 cases that are fatal, most of which occur in infants, children, the elderly, the malnourished, or the immunocompromised (Goodgame, 2006; Carter, 2005).

HuNoV is shed in the stools and vomit of the infected persons throughout the infection, starting from the incubation period and lasting even after full clinical recovery. The duration of viral shedding is increased in the immunocompromised, the elderly, or children (Rockx et al. 2002). It has been estimated that the stool of an individual with an active norovirus infection may shed up to 100 billion virus particles per gram of feces
Norovirus infections are highly contagious, and only a few particles (≤ 10) are sufficient to cause the disease (Teunis et al., 2008; Koopmans and Duizer, 2004). As a result, the disease has a high rate of transmission through contact. Outbreaks typically occur in places where a large amount of people are in close contact with each other, such as restaurants, cruise ships, schools, swimming pools, hospitals, nursing homes, daycare centers, hotels, prisons, military installations, and even sport stadiums (Goodgame, 2006, Rockx et al., 2002). As shown in Figure 1, of the reported cases of norovirus-associated gastroenteritis monitored by the CDC from 1994-2006, 234 cases (35.4%) were associated with long-term care facilities, 205 cases (31.1%) were associated with restaurants or social events, 135 cases (20.5%) were associated with vacations including cruises, and 86 cases (13.0%) were associated with schools or the community (CDC, 2011).
1.5. Transmission routes of human norovirus

HuNoV is usually transmitted through contaminated food, water, and fomites, or directly transmitted from person to person (Fig. 2). For example, it was reported that out of eleven outbreaks in New York State, the suspected modes of transmission were seven outbreaks as person-to-person, two as foodborne, one as waterborne, and one as unknown (Hedberg, 1993). The fecal-oral route is the most common transmission mode for HuNoV. Transmission through infectious vomit or feces either by direct contamination (through hand and/or mouth contact) or by indirect contamination (such as by

![Figure 1. Number of norovirus outbreaks confirmed by CDC by setting and genotype in United States from 1994 to 2006](image-url)
aerosolization) may also account for the rapid and excessive transmission of the virus in closed settings (Widdowson et al. 2005). The infectious virus particles can be aerosolized by a toilet flush after vomiting or diarrhea. Subsequently, infection may occur from eating near an episode of vomiting even if cleaned up. HuNoV is highly contiguous and extremely stable in the environment. The virus remains viable for several months after shedding (Becker et al. 2000; Teunis et al. 2008, Koopmans and Duizer, 2004). Recent human volunteer studies and mathematical modeling showed the average probability of infection for a single norovirus particle to be close to 0.5 (Teunis et al. 2008). More importantly, there is no long term immunity to norovirus; therefore, repeated infections may occur throughout life. Therefore, HuNoV and other caliciviruses are classified as category B priority biodefense agent by National Institute of Allergy and Infectious Diseases (NIAID).
Figure 2. Diagram of transmission of food- and water-borne viruses.

1.6. Classification and genetic diversity of human norovirus

Table 2. Classification of family *Caliciviridae*

<table>
<thead>
<tr>
<th>Genus</th>
<th>Specific strains</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Norovirus</em></td>
<td>Norwalk, Southampton, Desert shield, Chiba, BS5 kidneys, etc. (GI) Hawaiii, Lordsdale, Camberwel, U201, Alphatron, etc. (GII) Bovine enteric calicivirus, Murine norovirus, Swine norovirus, etc.</td>
</tr>
<tr>
<td><em>Sapovirus</em></td>
<td>Sapporo, Manchester, Houston, Parkville, etc. Porcine enteric sapovirus</td>
</tr>
<tr>
<td><em>Vesivirus</em></td>
<td>Feline calicivirus, Urbana, F9, Japanese F4, Vesicular exanthema of swine virus, etc. Bovine calicivirus, Primate calicivirus, San Miguel sea lion virus, etc.</td>
</tr>
<tr>
<td><em>Lagovirus</em></td>
<td>Rabbit hemorrhagic disease virus</td>
</tr>
<tr>
<td><em>Recovirus</em></td>
<td>Tulane virus</td>
</tr>
</tbody>
</table>
Noroviruses belong to the family *Caliciviridae* under the genus of *Norovirus*. As shown in Table 2, the *Caliciviridae* include the other five genera: *Norovirus* (e.g. HuNoV, murine norovirus), *Vesivirus* (e.g. feline calicivirus, Vesicular exanthema of swine virus), *Lagovirus* (e.g. Rabbit hemorrhagic disease virus), *Recovirus* (e.g. Tulane virus), and *Sapovirus* (e.g. Sapporo virus). Caliciviruses are non-enveloped, single-stranded positive-sense RNA viruses. The outer shell of the virus particle is the highly stable protein capsid which exhibits icosahedral symmetry and protects the genomic RNA. Unfortunately, many of caliciviruses are not cultivable or lack a suitable small animal model. Many caliciviruses cause gastroenteritis in humans and animals. However, some caliciviruses also cause other diseases in animals. For instance, feline calicivirus causes respiratory tract infections in cats; murine norovirus causes systemic infections in mice; rabbit haemorrhagic disease virus causes haemorrhagic disease in rabbit (Karst et al., 2003; Doultree et al., 1999).

Based on the diversity of viral capsid protein, noroviruses can be classified into five genogroups (GI, GII, GIII, GIV, and GV) and each genogroup can be further divided into different genotypes (Fig. 3). For an example, the most prevalent genogroup of norovirus, GII, contains 19 genotypes (Gospodarek and Zalas-Wiecek, 2009). Within genogroup II, genotype 4 (GII.4) is the most prevalent HuNoV and is responsible for the majority of outbreaks of gastroenteritis worldwide. The prototype norovirus strain, Norwalk virus, belongs to genogroup I and genotype 1, and is designated as GI.1. It is known that genogroups I, II and IV infect human, whilst genogroup III (e.g. bovine norovirus) is found in cows, and genogroup V (e.g. murine norovirus) has recently been isolated in mice (Gospodarek and Zalas-Wiecek, 2009). To date, there is no evidence to
support that noroviruses are zoonotic. Humans who have been infected by animal strains have not been reported, suggesting that noroviruses are species specific. Although, HuNoV strains have been found in cattle and swine by RT-PCR (Mattison et al., 2007), it is possible that these products were contaminated by an external source during processing and the animals themselves were not infected with HuNoV.

Figure 3. Major genogroups of norovirus.
1.7. Molecular biology of human norovirus

1.7.1. Human norovirus genome

As shown in Fig. 4, the genome of HuNoV is approximately 7.5-7.7 kb and composed of three open reading frames (ORFs) (Jiang et al., 1993). The first ORF (ORF1) is located in the first two-thirds of the full-length genome and encodes for a ~200kDa nonstructural polyprotein, which can be proteolytically cleaved into six non-structural proteins including N-terminal protein (designated p48 for Norwalk virus), NTPase, “3A-like protein” (designated p22 for Norwalk virus), VPg, viral protease (3CL\textsuperscript{pro}), and RNA-dependent RNA polymerase (RdRp) (Green, 2007). These non-structural proteins play many essential roles in virus life cycle (Table 3). The second ORF (ORF2) is approximately 1.8kb in length and encodes the 57 kDa major capsid protein VP1 while ORF3 (~0.6 kb in length) encodes the 22 kDa minor structural capsid protein VP2 (Hardy, 2005).

Figure 4. Genome of human norovirus (adapted from Donaldson 2010).
Table 3. Function of norovirus proteins

<table>
<thead>
<tr>
<th>Proteins name</th>
<th>Function(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P48</td>
<td>Inhibits trafficking of host protein to the cell surface</td>
</tr>
<tr>
<td></td>
<td>Involved in the formation of replication complex</td>
</tr>
<tr>
<td>NTPase</td>
<td>Has NTPase activity</td>
</tr>
<tr>
<td>P22</td>
<td>Inhibits secretion of host proteins</td>
</tr>
<tr>
<td></td>
<td>Involved in the formation of replication complex</td>
</tr>
<tr>
<td>VPG</td>
<td>Primes viral RNA replication following its uridylylation</td>
</tr>
<tr>
<td></td>
<td>Recruits host translation initiation factors</td>
</tr>
<tr>
<td>3C</td>
<td>Functions as protease and cleaves the ORF1 polyprotein</td>
</tr>
<tr>
<td>RdRp</td>
<td>Replicates the viral genomic RNA</td>
</tr>
<tr>
<td></td>
<td>Generates uridylylated VPG</td>
</tr>
<tr>
<td>VP1</td>
<td>Major capsid protein</td>
</tr>
<tr>
<td>VP2</td>
<td>Minor capsid protein, increases expression of VP1 and stabilizes particles</td>
</tr>
</tbody>
</table>

1.7.2. The major capsid protein VP1 of human norovirus

The major capsid protein VP1 normally ranges from 530-555 amino acids in length, and has an approximate molecular weight of 58-60 kDa. VP1 forms the outer shell of virus particle that protects viral genomic RNA. VP1 plays a number of essential roles in virus life cycle. VP1 protein binds to its functional receptor, the histo-blood group antigen (HBGA) on the host cells that mediates virus entry. VP1 protein determines the antigenicity and strain specificity (Prasad et al., 1999). In fact, norovirus genogroups and genotypes are classified based on the diversity of VP1 protein. VP1 protein is the host protective antigen that is responsible for eliciting neutralizing antibody, cellular, and mucosal immunities (Bertolotti-Ciarlet et al., 2003). In addition, VP1 may play many other roles in the virus life cycle such as uncoating, assembly, and exit (Hardy, 2005). Jiang et al. (1992) first found that expression of VP1 alone in *E.coli*. 
resulted in the assembly of HuNoV virus-like particles (VLPs). Indeed, these VLPs can also be efficiently produced in other expression systems such as insect cells, mammalian cells, and yeast (Ma and Li, 2011). These VLPs don’t contain the viral genetic material (RNA), but are structurally and antigenically similar to native virions. Thus, VLPs are important tools to study the structural, immunological, and biochemical properties of HuNoV.

Figure 5. (A) The morphologies of human norovirus and (B) human norovirus virus-like particle under electron microscope.

Figure 6. The structure of norovirus virion.
1.7.3. The minor capsid protein of VP2 of human norovirus

VP2 is the minor capsid protein composed of 208-268 amino acids with a molecular weight of about 29kDa. VP2 is not necessary for virus-like particle assembly but is essential for the production of infectious virus (Hardy, 2005). VP2 is a basic protein which has an isoelectric point of >10, suggesting that it may be involved in RNA binding and play a role in RNA packaging (Hardy, 2005). Another report showed that the presence of VP2 resulted in an increased expression of VP1, indicating that VP2 regulates the synthesis of VP1 protein (Bertolotti-Ciarlet et al., 2003). Moreover, it is believed that VP2 stabilizes VP1 and protects VP1 from disassembly and protease degradation (Bertolotti-Ciarlet et al., 2003). The proposed model for the role of VP2 in stabilizing VP1 is shown in Fig. 7.

![Proposed model for the mechanism of the VP2 stabilizing effect on VP1 protein](image)

Figure 7. Proposed model for the mechanism of the VP2 stabilizing effect on VP1 protein (Adapted from Bertolotti-Ciarlet et al. 2003).
1.8. Proposed life cycle of human norovirus

The life cycle of HuNoV is poorly understood because it cannot be grown in cell culture. Most of our understanding of the life cycle of HuNoV comes from the studies of surrogates such as VLPs, murine norovirus, and feline calicivirus. The proposed HuNoV life cycle is summarized as following (Fig.8):

(1) Attachment: All viruses must recognize their cellular receptors for attachment. HuNoV utilizes HBGAs as functional receptors in a strain-dependent manner. This is mediated by the P domain of the VP1 protein. Mutations in the P domain specifically abolished receptor binding activity.

(2) Penetration: Presumably, viruses enter the host cells by receptor-mediated endocytosis after attachment. However, it was reported that entry of murine norovirus does not require clathrin- and caveolin-mediated endocytosis. In addition, phagocytosis or macropinocytosis are not required for MNV-1 uptake. Interestingly, MNV-1 requires host cholesterol and dynamin II for entry (Perry and Wobus, 2010).

(3) Uncoating: After entering the host cell, the viral capsid is released from the virion and the viral genomic RNA is delivered into the cytoplasm of the host cell.

(4) Replication and gene expression: Like most RNA viruses, norovirus replicates in the cytoplasm. The viral RNA dependent RNA polymerase (RdRp) is responsible for genome replication and uridylylation of VPg. Briefly, the positive-sense RNA genome is recognized as a template for the RdRp to synthesize negative-sense RNA which in turn acts as a template for the synthesis of positive-sense RNA
genome. During replication, norovirus produces subgenomic RNA which only contains the VP1 and VP2 genes. The VPg is covalently linked to the 5’-end of genome which recruits host translation initiation factors to synthesize viral proteins. The three ORFs are translated into a large polyprotein, VP1, and VP2. Subsequently, the polyprotein is cleaved into six non-structural proteins.

(5) Assembly: The newly synthesized viral capsid proteins and genomic RNAs are packaged into new virions.

(6) Release: The mature virions escape from the cell by lysing the cells.

Figure 8. Proposed life-cycle of norovirus. The cycle is from infection of a cell by a single virus to release of thousands of offspring.
1.9. Human norovirus receptors and host susceptibility

Viruses must bind to cellular receptors to initiate an infection. It was found that HuNoV utilizes histo-blood group antigens (HBGAs) as its functional receptors. HBGAs are complex carbohydrates present on the surface of erythrocytes as well as the intestinal, genitourinary, and respiratory epithelia (Ravn and Dabelsteen, 2000). They also exist as free oligosaccharides in saliva, blood, milk, and contents of the intestine (Tan and Jiang, 2005). There are three major families of HBGAs, namely Lewis, ABO, and secretor, and the recognition of HBGAs by noroviruses is strain specific. The norovirus-receptor interaction has been extensively studied. It was found that the viral capsid protein VP1 interacts with HBGAs. Specifically, it has been demonstrated that the P domain of VP1 protein is the primary site of receptor recognition and the amino acid residues in P domain are responsible for the specificity of receptor binding (Tan and Jiang, 2005, Tan et al. 2008).

The susceptibility of an individual to HuNoV is dependent on the type of human HBGA receptors. In a human volunteer study, it was found that individuals with an O blood type were easily infected by some norovirus strains, while those with a B blood type had the lowest risk of infection (Hutson et al. 2004). Volunteers with a B blood type lacked the matched viral receptors on their intestinal epithelium, thus they never became infected with certain norovirus strains following challenge. Researchers also found that some individuals with a high level of antibodies against norovirus were even more susceptible to norovirus challenge than those with no or lower levels of antibodies (Parrino et al., 1977). These studies suggest receptor specificity play an important role in
host susceptibility although acquired immunity is also involved in resistance to norovirus infection.

1.10. Major challenges for human norovirus research

Unfortunately, the research on HuNoV has been severely hampered. This is due to the fact that HuNoV cannot be grown in cell culture and lacks of a small animal model for infection. Currently, many aspects of HuNoV including molecular biology, gene expression, replication, pathogenesis, and immunology are poorly understood. In the food safety respect, the stability and susceptibility of HuNoV to food processing technologies are not understood. Therefore, the development of proper surrogates is necessary for HuNoV studies.

Although numerous efforts have been devoted to cultivate HuNoV, the establishment of an in vitro cultivation system for HuNoV has failed thus far. Duizer et al. (2004) systematically evaluated a variety of cell lines and laboratory methods to cultivate HuNoV. A in vitro cell culture system that mimics the intestinal epithelium using gastric cells, duodenal cells, and small intestinal enterocyte-like cells was created. However, none of the cell culture combinations were successful in cultivating HuNoV. HuNoV also did not replicate in macrophages or dendritic cells which support the replication of murine norovirus (Lay et al., 2010). In 2007, Straub et al. first reported that HuNoV can infect and replicate in a physiologically relevant 3-dimensional (3-D), organoid model of human small intestinal epithelium. The cytopathic effect and norovirus RNA were detected at each of the five cell passages for HuNoV genogroup I and II.
These results are very encouraging; however, the robustness of virus replication and the amount of newly synthesized viruses have been questioned (Chan et al., 2007). In response to this question, Straub et al. (2007) claimed that norovirus titer did increase in their culture system. However, the magnitude and time course of these increases is dependent on both virus strain and multiplicity of infection. Recently, Leung et al. (2010) reported an ex vivo culture system to cultivate HuNoV using freshly collected human duodenal tissues that mimics the primary site of viral replication in vivo. They found that viral genomic RNA levels in cell-free culture supernatants increased over time as measured by real time RT-PCR. Notably, in situ hybridization of viral RNA and immunohistochemical staining of VP1 as well as newly synthesized viral protease revealed that HuNoV displays a marked tropism for glandular epithelial cells. It appears that ex vivo culture can support key stages of complete HuNoV replication, ranging from virus adsorption and internalization to viral RNA replication and protein synthesis. While these findings are promising, how robust these cultivation systems are remains to be determined.

1.11. Human norovirus surrogates for food safety research

Research on HuNoV has to rely on suitable surrogates since HuNoV is noncultivatable. To date, HuNoV virus-like particles (VLPs), feline calicivirus (FCV), murine norovirus (MNV), canine calicivirus (CaCV), bacteriophage MS2, and poliovirus have been used as HuNoV surrogates (Bae and Schwab, 2008). However, FCV and MNV are by far the most commonly used surrogates.
1.11.1. Human norovirus virus-like particles (VLPs)

HuNoV VLPs are antigenically and structurally similar to native virions. These VLPs are not infectious because they don’t contain viral genomic RNA. There are two major advantages for using VLPs as a surrogate. First, VLPs can be efficiently and easily produced by expression of VP1 gene in insect cells and mammalian cells. Second, VLPs possess all the authentic properties of HuNoV such as antigenic sites, structure, and receptor binding activities. Therefore, the VLPs can be an important alternative tool to study the epidemiological, immunological, structural, and biochemical properties of HuNoV. It would be lethal to the virus if receptor binding activity was damaged. Hence, the receptor binding of VLPs can be used as an indicator for virus survival. Alternatively, the damage of VLPs can be examined by electron microscope and SDS-PAGE. Feng et al. (2011) demonstrated that gamma irradiation disrupted the structure of VLPs and degraded VP1 protein. More importantly, the authors found out that the capsid of HuNoV has a similar stability compared to MNV-1 after exposure to gamma irradiation (Feng et al. 2011). VLPs can also be used as a surrogate for studying the interactions (namely, attachment, penetration, internalization, or dissemination) of norovirus with high risk foods such as fresh produce and seafood.

1.11.2. Feline calicivirus

Feline calicivirus (FCV) is one of the major causes of feline respiratory disease. The virus was first discovered in the 1950s. Clinical symptoms of FCV infection include sneezing, nasal discharge, fever, salivation, lethargy, and loss of appetite. Unlike HuNoV,
FCV does not cause gastroenteritis in cats or infect humans (Doultree et al., 1999). FCV belongs to the genus *Vesivirus* within the family of *Caliciviridae*. In last two decades, FCV has been widely used as a surrogate for HuNoV because of its genetic relatedness to HuNoV. Unfortunately, recent studies found that FCV has distinct biochemical properties as compared to HuNoV. For example, FCV is not an enteric virus. Furthermore, it was found that FCV is susceptible to low pH and elevated temperature (Cannon et al., 2006). Therefore, it appears that FCV is not an ideal surrogate for HuNoV.

![Figure 9](image.jpg)

**Figure 9.** Feline calicivirus (FCV) (adapted from CDC) and murine norovirus (MNV) (adapted from Lou et al., 2011) under TEM.

### 1.11.3. Murine norovirus

Murine norovirus (MNV) was first isolated from severely immunocompromised mice that were deficient in recombination-activating gene 2 (RAG2) and signal transducer and activator of transcription 1 (STAT-1) (RAG2/STAT1<sup>−/−</sup> mice) in 2003
(Karst et al., 2003). This virus causes systemic infection and lethal disease in mice deficient in components of the innate immune response but no clinical symptoms in immunocompetent mice (Hsu et al., 2007). It is now believed that MNV is an effective enteric pathogen since it can be isolated from brain, liver, spleen, blood, lung, and intestine of infected mice. Most importantly, a number of new MNV strains have been identified which are found in the feces of laboratory mice. These studies indicate that MNV is capable of transmitting via the fecal-oral and, possibly, respiratory routes, as its human counterparts do. However, MNV does not cause vomiting and diarrhea which are the characteristic clinical manifestations of HuNoV infections (Karst et al., 2003).

In 2004, Wobus et al. (2004) developed a cell-culture based system for MNV. This was the first successful in vitro cultivation of a norovirus, as attempts with HuNoV have all failed. Thus far, the MNV is the only norovirus that replicates in cell culture and small animals among all the noroviruses that have been identified in humans, cattle, swine, and mice (Karst et al., 2003; Wobus et al., 2004; Liu et al., 1999). Therefore, the MNV model system provides the first opportunity to gain insight into the norovirus biology and pathogenesis in vitro and in vivo, particularly the relationship between the mechanisms of norovirus growth in a cell culture system and pathogenesis in a natural host. It also generates questions with regard to the relationship between noroviruses and cells of the hematopoietic lineage and the role of innate immunity in the control of norovirus infection (Wobus et al., 2006). In addition, laboratory mice are a relatively inexpensive and versatile model for the analysis of viral pathogenesis.

Since its discovery, MNV has been widely used as a surrogate for HuNoV due to its distinct advantages. The most important characteristics of MNV for the analysis of the
biology and pathogenesis of HuNoV are the biological and molecular properties that
MNV shares with its human counterpart in general (Wobus et al., 2006). Specifically, the
biochemical features they share include a similar shape (icosahedral), particle size (28-35nm in diameter), and buoyant density (1.36± 0.04 g/cm$^3$) (Green et al., 2001; Karst et al., 2003). As shown in Fig 9B and 5A, MNV and HuNoV are morphologically similar under the electron microscope. Another advantage is that MNV has a similar structure as HuNoV. The surfaces of both MNV and HuNoV possess a highly stable viral major protein VP1 that surrounds and protects the viral genomic material, as well as a minor capsid protein (VP2) that stabilizes VP1. Importantly, MNV is genetically related to HuNoV (Fig. 10). Both viruses belong to genus *Norovirus* and share a similar genome size and gene organization. Similar to HuNoV, the analysis of the MNV-1 genome identified three open reading frames (ORF). ORF1 of MNV encodes the nonstructural proteins such as NTPase, protease, 3A-like protein, and RNA dependent RNA polymerase. ORF2 of MNV encodes a 58.9-kDa major capsid protein (VP1) that can self-assemble into virus-like particles when expressed in a baculovirus expression system. ORF3 of MNV encodes a putative 22.1-kDa basic protein which is known as minor capsid protein (VP2). Finally, MNV can be persistent as HuNoV in the environment since it is also highly resistant to environmental stress such as acid and heat (Cannon et al., 2006; Wobus et al., 2006).
However, MNV differs in several aspects from HuNoV such as viral receptor binding, pathogenesis, and immunity. MNV uses sialic acid as a functional receptor and has tissue tropism for macrophages and dendritic cells (Wobus et al., 2006). By comparison, HuNoV uses HBGAs as cellular receptors for infection and infects intestinal cells \textit{in vivo} (Tan and Jiang, 2005; Tan et al., 2008). Additionally, MNV does not cause the clinical symptoms of gastroenteritis that the human counterpart does, such as projectile vomiting and/or explosive, watery diarrhea. It is reported that a murine model is not able to recapitulate norovirus-induced vomiting, since mice lack an emetic reflex (Wobus et al., 2006).

Cannon et al. (2006) directly compared the viabilities of MNV and FCV and found MNV to be a more suitable surrogate for HuNoV. The stabilities of both viruses against
pH, organic solvents, heat, and surface persistence in wet and dry conditions have been investigated and compared. It was found that MNV was stable within the entire pH range from 2 to 10. However, FCV significantly lost infectivity at pH extremes (pH ≤ 3 and pH ≥ 9). Evidence indicated that FCV was more stable than MNV at 56ºC but they both exhibited similar stability at both 63ºC and 72ºC. The long-term survival of these two viruses was also investigated by suspending the viruses in a fecal matrix and inoculating them onto stainless steel coupons. It was shown that MNV was more persistent than FCV at room temperature (Cannon et al., 2006). Taken together, these studies suggest that MNV is currently the most relevant and promising surrogate for HuNoV.

1.12. Norovirus and high-risk foods

1.12.1. Fresh produce

In modern society, the consumption of fruits and vegetables has increased as individuals strive for a healthier diet. However, fresh produce is considered a major vehicle of norovirus contamination because it normally undergoes little to no processing before consumption. Contamination may occur at any step from pre-harvest to post-harvest stages (Everis, 2004). Also, fruits and vegetables are often eaten without peeling (Carter, 2005). More importantly, they are often traded around the world which can facilitate the virus dissemination globally. As is shown in Fig. 11, norovirus was responsible for more than 40% of outbreaks in fresh produce from 1998-2005 in the United States (DeWaal and Bhuiya, 2007). These outbreaks of norovirus frequently occurred in fresh produce including lettuce, tomatoes, melons, green onions, strawberries,
raspberries, blueberries, peppers, and fresh cut fruits (DeWaal and Bhuiya, 2007). As a consequence, fresh produce related products, such as mixed fruits, mixed vegetables, puree, salad, and salsa are also high risk foods for norovirus contamination (CDC, 2010; Le Guyader et al., 2004). Fresh produce can be easily contaminated by the use of contaminated water for irrigation, fertilizer, or washing. Contamination may also occur through the handling of the food during harvesting, processing, or distribution by infected workers.

Figure 11. Major pathogens in produce related outbreaks (Adapted from DeWaal and Bhuiya, 2007).
Currently, the detailed mechanism of viral contamination in fresh produce is poorly understood. There are some evidence supports that noroviruses not only attach tightly in fresh produce but also become internalized and disseminated to other portion of fresh produce. As for attachment of HuNoV to produce, one possible mechanism is the presence of carbohydrate moieties which resemble the HBGAs, the functional receptors for HuNoV attachment in host tissues. Carbohydrate moieties, such as glucose and glycan, are highly abundant in vegetables and fruits. Recently Gandhi et al. (2010) found that human norovirus VLPs attached to the surface of romaine lettuce and were distributed primarily on the leaf veins. Moreover, extracts of romaine lettuce leaves, cilantro, iceberg lettuce, spinach, and celery also bound to VLPs. However, their results further suggested that it was non-HBGA molecules in romaine extract that bind VLPs by a binding site(s). More research is needed to determine whether receptors are indeed involved in attaching of HuNoV to fresh produce.

Bacterial pathogens are internalized and disseminated in fresh produce via stomata or wounds in the cuticula. It would be easier for viruses to be internalized into plants due to the fact that the size of virus is much smaller than bacteria (Chancellor et al., 2006). Even though the internal contamination level is low compared with external contamination, it may still pose an equivalent threat to food safety because the internal viruses cannot be removed by peeling or washing (Carter, 2005). The feasibility of internalization of human enteric viruses by plants depends on the ability of plants to uptake their own viral pathogens from soil and water. It is likely that HuNoV could also be taken into the plant through the roots and/or leaves since they may be present in sewage-contaminated soil or water. Unfortunately, only limited studies have been
conducted thus far to investigate norovirus internalization in produce. Urbanucci et al. (2009) performed the first norovirus internalization in plants. The roots of lettuce, growing either in hydroponic culture or in soil, were exposed to both canine calicivirus (CaCV) and a HuNoV genogroup 2. No matter the roots were intact or damaged, the internalization of CaCV occurred, although with a low frequency of positive results, in both seedling and mature plants in either hydroponic or soil cultures. Interestingly, internalization of HuNoV was not detected even when the plants were exposed to a high concentration of HuNoV. These results suggested that viral contamination of lettuce plants via roots can occur, but may not be an important transmission route for norovirus internalization. On the other hand, Wei et al. (2010) reported that another human norovirus surrogate, MNV, can be internalized via roots in romaine lettuce (Lactuca sativa). Genomic RNA of MNV was detected in some lettuce leaf samples when the lettuce roots being inoculated with MNV. However, infectious MNV can only be found in lettuce samples challenged with high virus inoculums but not in lettuce grown with low level inoculums. Thus, it can be concluded that norovirus can be potentially internalized via plant roots and disseminated over the plant, although the detailed mechanism is still unclear. It should also be noted that these studies were done under very simplistic conditions and laboratory settings, and therefore, these results may or may not represent what would actually happen in the field.
1.12.2. Seafood

Filter-feeding molluscan shellfish (including cockles, mussels, and oysters) are another major group of high risk food for norovirus contamination. High numbers of norovirus particles can be detected in shellfish and oyster tissues (Burkhardt and Calci, 2000; Croci et al., 2006). It is believed that the level of viruses may become 100-1000 fold concentrated within shellfish in the surrounding water (Carter, 2006). In fact, the virus accumulation by shellfish is not virus-specific and thus any virus (including norovirus, hepatitis A virus, poilo, and so on) might act synergistically and induce worse disease. Worldwide, a large number of outbreaks were associated with the consumption of raw or undercooked shellfish and the trade in shellfish also enables the long-distance transmission.

The percentage of HuNoV positive oysters depends on the location of coast where oysters were grown, seasonality, and the sensitivity of detection methods. A study from France showed that HuNoV positive rate in oyster samples is about 23% (Le Guyader et al., 2000). However, mussel samples, collected in areas routinely impacted by human sewage, were more highly contaminated by HuNoV (35%) (Le Guyader et al., 2000). A study in the United Kingdom showed that the percentage of norovirus positive oysters in two locations was 77% and 95% from October to March. However, a lower positive rate (32% and 48%) was found from April to September. Companies producing oysters have often implemented recalls because of norovirus contamination and this causes an enormous economic impact (Dore et al., 2010).
Interestingly, it was found that HuNoV VLPs and native virions bound to the mid gut, the main and secondary ducts of the digestive diverticula, and tubules of oyster by HBGA-like molecules, similar to what is seen in humans (Le Guyader et al., 2006). Binding of HuNoV VLPs to oyster digestive cells were specifically inhibited by human saliva and antibodies to carbohydrates and lectins. Furthermore, amino acids substitutions in the receptor binding site of VLPs inhibited the binding of these mutant VLPs to oyster tissues. These results provided compelling evidence that HBGA-like molecules were involved in bioaccumulation of HuNoV in oysters. It was also shown that VLPs derived from different HuNoV genogroups exhibited different binding affinity to oyster tissues (Maalouf et al., 2010). In a study to determine the rate of bioaccumulation of norovirus by oysters, three different strains of HuNoV (GI.1, GII.3, and GII.4) were compared (Maalouf et al., 2011). It was found that strain differences in combination with seasonality impact the norovirus bioaccumulation in oysters. The GI.1 strain was the most efficiently concentrated strain and the bioaccumulation levels in the digestive tissue were significantly higher in winter (Jan-Mar). However, the GII.4 strain was very poorly bioaccumulated and low level of virus was recovered in almost all tissues without seasonal influence.

1.12.3. Handled foods

Any food items that are frequently contacted by food handlers are at a risk for norovirus contamination. Typical examples of these foods are the ready-to-eat foods such as deli sandwiches, finger foods, dips, and bakery items. Many of these foods can easily
become contaminated at any point from harvesting to serving due to the poor hygiene of food handlers. The virus can then be isolated from people who have become ill after consumption of the contaminated food items. Sometimes an outbreak may be traced to a food handler who harbors norovirus. It has also been demonstrated that approximately 30% of norovirus infections are asymptomatic, but in fact these infected individuals may actively be shedding the virus while appearing healthy (CDC, 2011). These asymptomatic carriers can still shed the virus and pass norovirus to other people or to foods that they handle. To improve food safety, food handlers should aware the importance of proper hygiene in controlling food-borne infection and strictly follow food service as well as food handling regulations.

1.13. Challenges for the detection of human norovirus in high risk foods

Because HuNoV does not grow in cell culture, a cell-based detection assay is not feasible. Detection of HuNoV usually relies on electron microscopy, antigen-based assays, and molecular biology techniques.

Transmission electron microscopy (TEM) is usually used for the detection of HuNoV from clinical specimens (such as stool and vomitus). TEM is a relatively insensitive method and requires a vast number of virus particles ($\geq 10^4$ particles per ml) present in samples. Moreover, TEM needs skilled personnel and expensive equipment. Another often used detection method for HuNoV is enzyme linked immunosorbant assays (ELISA), which is based on the reaction between the norovirus capsid protein and a specific antibody. ELISA is generally easy to perform, quick, and cost-effective, but its
sensitivity needs to be improved (de Bruin et al., 2006). Currently, the most widely used detection method for HuNoV is reverse transcription polymerase chain reaction (RT-PCR) or real-time RT-PCR. These methods can be highly sensitive and specific. Unfortunately, the PCR-based methods cannot distinguish the infectious viruses from the inactivated viruses. Thus, a combination of antigen and PCR-based detection methods may be more valid for HuNoV detection.

Detection of norovirus in implicated foods is often difficult because of the complexity of the food matrix, the low level of virus in the food, and the genetic diversity of the virus. In addition, there are many RNases, RT, and PCR inhibitors in the food matrix that may interfere with viral RNA extraction and the following RT-PCR. Given the fact that only a few particles can cause illness, the specificity, sensitivity, and accuracy must be improved for RT-PCR based methods. The aim is to identify a technique that can concentrate the viruses from the complicated food matrix. Harrington et al., (2004) developed a magnetic bead-virus capture method in which streptavidin-precoated magnetic beads were coated with specific synthetic, biotinylated histo-blood group antigen. These beads specifically captured the norovirus particle since the norovirus-receptor interaction is highly specific. This method could be valuable for concentrating, purifying, or diagnosing noroviruses in clinical or environmental samples. Cannon and Vinjé (2008) further optimized the magnetic-bead-based histo-blood group antigen assay and showed that this assay was able to recover 30 to 300 genomic copies of norovirus in environmental waters. Similarly, porcine gastric mucin (PGM) derived histo-blood group antigen was conjugated to magnetic beads (PGM-MB). All GI and 85% of
GII noroviruses tested could be captured and concentrated by PGM-MB method (Tian et al., 2010). The binding occurred rapidly and was enhanced at low pH (Tian et al., 2010).

1.14. Vaccines against human norovirus

No FDA approved vaccine is currently available for HuNoV. Vaccination is believed to be the most effective strategy to protect humans from infectious diseases. There is urgent need to develop an effective vaccine against HuNoV due to the fact that it causes significant health, economic, and emotional burdens to humans. Since VLPs are structurally and antigenically similar to native virions, most HuNoV vaccine studies have focused on VLPs. To date, HuNoV VLPs have been successfully expressed in *E. coli*, yeast, insect cells, mammalian cell lines, tobacco, and potatoes. Mice immunized with these VLPs orally or intranasally induced variable humoral, mucosal, and cellular immunities. However, no protection data is available because mice cannot be used as an animal model for challenge assay. Using gnotobiotic pig model, Souza and Menira (2007) found that VLPs-based HuNoV vaccine provided protection to the challenge of homologous GII.4 HuNoV strain. In 1999, Ball and colleagues performed the first clinical study to demonstrate that baculovirus-expressed HuNoV VLPs were safe and immunogenic in humans when administered orally. Tacket et al. (2000) performed a human volunteer study of transgenic potato-based VLP vaccine, showing that 19 of 20 human volunteers developed an immune response. In the US, LigoCyte Pharmaceuticals Inc. performed human clinical trials for the VLP-based vaccine. Volunteers that received the dry powder VLP vaccine reduced the risk of illness by 47% after exposure to HuNoV.
There were significant reductions in clinical norovirus illness, infection, and severity of illness in individuals who received vaccine compared to those who received the placebo (LigoCyte Pharmaceuticals Inc., 2010). This data suggests that a VLP-based vaccine is a promising vaccine candidate against HuNoV.

1.15. Antiviral drugs against human norovirus

No antiviral drug is currently available for HuNoV. For healthy individuals, norovirus-associated illness is usually self-limited, and no treatment is needed. However, effective anti-viral drugs may be necessary and beneficial for some severe cases. In fact, there is a high mortality rate due to HuNoV infection reported in infants and young children in some developing countries. Since the virus cannot grow in vitro, approaches to screen potential antiviral drugs are very limited. Feng and Jiang (2007) developed a saliva-based receptor binding assay to screen a compound library for inhibition of norovirus VLPs binding to HBGA receptors. Among 5,000 compounds screened, 153 revealed inhibitory activities against VLPs binding to the A antigen, and 14 of the 153 compounds revealed strong inhibition, with a 50% effective concentration less than 15 µM. Using a similar assay, Zhang et al. (2011) screened 50 Chinese herbal medicines and identified that two medicines (Chinese Gall and Pomegranate) were highly effective in blocking norovirus-receptor binding. More interestingly, Tannic acid was identified in these medicines as a strong inhibitor of the binding of norovirus P particles to both A and B saliva with a half maximal inhibitory concentration (IC50) of 0.1 µM. From these studies there is promise that antiviral drugs targeting the first step of viral infection, the
virus-receptor binding, may be developed. To date, no clinical data is available to evaluate the effectiveness of these compounds against norovirus infection.

1.16. Rotavirus

1.16.1. Epidemiology of rotavirus

First discovered in Australia by Ruth Bishop in 1973, rotaviruses are now recognized as the major cause of acute dehydrating diarrhea in infants and young children, accounting for approximately 114 million cases of gastroenteritis and 600,000 deaths in children of less than 5-years-old worldwide each year (Parashar et al., 2003). In US, it has been reported that 95% of children are infected by rotavirus by the age of 5. The highest incidence is among the children at 3 to 35 months of age. The most prevalent symptoms of rotavirus infection include abdominal pain, vomiting, fever and watery diarrhea. The diarrhea can last 3-8 days and can cause mild to severe dehydration, potentially leading to death if untreated. Adults and older children can also become infected but with mild symptoms, sometimes even without any symptom. Like other foodborne viruses, rotavirus is highly contagious and 10-100 virus particles are sufficient to cause the disease (Graham et al., 1987).

The transmission of rotavirus is primarily through the fecal-oral route. After entering through the mouth, the virus can replicate fast in the epithelium of the small intestine, resulting in a large amount of infectious viral particles that are shed in the diarrheal stool (Dennehy, 2000). Rotavirus can also be transmitted by other modes, such as fecally contaminated food and water and respiratory droplets from sneeze or cough. It
has been shown that outbreaks of rotavirus diarrhea are usually caused directly or indirectly by contaminated water. Therefore, shellfishes growing in contaminated water are at high risk for rotavirus transmission (Le Guyader et al., 2000). It is also worth noting that rotavirus is highly stable in the environment; thus, its transmission can occur by contact with contaminated objects or surfaces.

**Figure 12. The morphology of intact double-shelled rotavirus particles under electron microscope (Adapted from CDC)**

### 1.16.2. Molecular and structural biology of rotavirus

Rotaviruses form one genus of the family *Reoviridae*. These viral pathogens are very diverse and can be classified serologically into seven distinct groups (A-F). Only groups A, B, and C infect humans. Specifically, group A rotavirus is the most common species to cause infection in children (Kirkwood, 2010).
A common morphologic feature shared by members of the rotavirus genus is that mature rotavirus particles are non-enveloped and exhibit icosahedral symmetry with 70-75 nm in diameter. Intact virus particles resemble a wheel with short spokes and a well-defined rim under electron microscope (Fig. 12) (Anderson and Weber, 2004). The name rotavirus (from the Latin rota, meaning wheel) was virtually derived from such characteristic. Each rotavirus virion has a triple-layer protein capsid and a core containing genetic materials. The genome of rotavirus consists of 11 segments of double-stranded RNA ranging in size from 667 (segment 11) to 3,302 base pairs (segment 1), with the total genome containing about 18,522 base pairs (Table 4) (Estes and Cohen, 1989). There are 12 proteins encoded by the genome of rotavirus, including six virus structural proteins (VP1, 2, 3, 4, 6, 7) and six non-structural proteins (NSP1-6) (Graff et al., 2002). As presented below, these proteins have distinct functions and play pivotal roles in the life cycle of rotavirus.

VP1 is the RNA-dependent RNA polymerase and produces mRNA transcripts for viral protein synthesis and produces copies of the viral RNA segments in the infected cells. VP2 forms the innermost core layer of the virion and associates with VP1 and VP3. VP3 is a guanylyl transferase and functions in the capping of viral RNA transcripts. VP6 forms the middle capsid layer and it is an important antigenic determinant which can be used to determine the rotavirus species. VP4 forms spikes that extend from the surface of the virus and contributes to attachment and virulence. As a major component of the outer surface of the virion, VP7 is a glycoprotein determining the G serotype of the strain and, along with VP4, is involved in immunity to infection (Pesavento et al., 2006; Golantsova et al., 2004; Graham et al., 2003).
Table 4. Functions of rotavirus gene products (adapted from Murray, 2005)

<table>
<thead>
<tr>
<th>RNA Segment (Gene)</th>
<th>Size (base pairs)</th>
<th>Protein</th>
<th>Molecular weight kDa</th>
<th>Location</th>
<th>Copies per particle</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3302</td>
<td>VP1</td>
<td>125</td>
<td>At the vertices of the core</td>
<td>&lt;25</td>
<td>RNA-dependent RNA polymerase</td>
</tr>
<tr>
<td>2</td>
<td>2690</td>
<td>VP2</td>
<td>102</td>
<td>Forms inner shell of the core</td>
<td>120</td>
<td>Stimulates viral RNA replicase</td>
</tr>
<tr>
<td>3</td>
<td>2591</td>
<td>VP3</td>
<td>88</td>
<td>At the vertices of the core</td>
<td>&lt;25</td>
<td>Guanylyl transferase mRNA capping enzyme</td>
</tr>
<tr>
<td>4</td>
<td>2362</td>
<td>VP4</td>
<td>87</td>
<td>Surface spike</td>
<td>120</td>
<td>Cell attachment, virulence</td>
</tr>
<tr>
<td>5</td>
<td>1611</td>
<td>NSP1</td>
<td>59</td>
<td>Nonstructural</td>
<td>0</td>
<td>5 RNA binding</td>
</tr>
<tr>
<td>6</td>
<td>1366</td>
<td>VP6</td>
<td>45</td>
<td>Inner Capsid</td>
<td>780</td>
<td>Structural and species-specific antigen</td>
</tr>
<tr>
<td>7</td>
<td>1104</td>
<td>NSP3</td>
<td>37</td>
<td>Nonstructural</td>
<td>0</td>
<td>Enhances viral mRNA activity and shut-offs cellular protein synthesis</td>
</tr>
<tr>
<td>8</td>
<td>1059</td>
<td>NSP2</td>
<td>35</td>
<td>Nonstructural</td>
<td>0</td>
<td>NTPase involved in RNA packaging</td>
</tr>
<tr>
<td>9</td>
<td>1062</td>
<td>VP1(^1) VP1(^2)</td>
<td>38 and 34</td>
<td>Surface</td>
<td>780</td>
<td>Structural and neutralisation antigen</td>
</tr>
<tr>
<td>10</td>
<td>751</td>
<td>NSP4</td>
<td>20</td>
<td>Nonstructural</td>
<td>0</td>
<td>Enterotoxin</td>
</tr>
<tr>
<td>11</td>
<td>667</td>
<td>NSP5 NSP6</td>
<td>22</td>
<td>Nonstructural</td>
<td>0</td>
<td>ssRNA and dsRNA binding modulator of NSP2</td>
</tr>
</tbody>
</table>
1.16.3. Diagnosis, treatment and prevention for rotavirus

Rotavirus infection is conventionally diagnosed by rapid antigen detection of rotavirus in stool specimens using ELISA, immunochromatographic assays, or latex agglutination (LA) which is only for group A rotavirus. Other techniques include electron microscopy (EM), cell culturing, and reverse transcription-polymerase chain reaction (RT-PCR). Of all these techniques, RT-PCR is able to detect and identify all species and
serotypes of human rotavirus (Fischer and Gentsch, 2004); however, only group A rotaviruses can grow in monkey kidney cells thus being detected in cell culture-based assays. Some ELISA-based kits are commercially available to detect all serotypes of rotavirus A with high sensitivity and specificity.

Treatments for rotavirus infection are supportive aimed at maintenance of hydration (Diggle, 2007). Severe effects of dehydration, even death, can occur to children if untreated (Alam and Ashraf, 2003). Based on the degrees of severity of infection, treatments are usually oral rehydration or intravenous rehydration.

It is believed that good hygiene may help but is not enough to prevent the prevalence of rotavirus infection. Moreover, the first infection usually does not lead to permanent immunity, thus reinfection can occur at any age even though subsequent infections are generally less severe. Therefore, the primary public health intervention for rotavirus is vaccination (Bernstein, 2009). Currently, two different rotavirus vaccines, RotaTeq® (RV5) and Rotarix® (RV1), are licensed for use in infants in the United States, both of which are designed to prevent group A rotaviruses (Dennehy, 2008).

1.17. Vesicular stomatitis virus

Vesicular stomatitis virus (VSV) is an enveloped virus that belongs to the Rhabdoviridae family. It is known that there are two serotypes of VSV in the US, namely VSV-New Jersey (VSV-NJ) and VSV-Indiana (VSV-IN). As shown in Fig. 14B, the VSV virions are bullet-shaped particles under electron microscope. It commonly infects cattle, horses, and pigs and causes an acute disease, including symptoms such as vesicles
and ulcers around the mouth, in the teats and coronary bands, as well as fever and excessive salivation (Letchworth et al., 1999). The disease is rarely fatal but will result in huge loss of milk production and weight gain. In addition, this virus has also been shown to infect sheep, goats, llamas and humans who exposed to the infected animals. VSV infections in humans usually induce flu-like symptom or even being asymptomatic.

The genome of VSV is a non-segmented negative-sense RNA that encodes five structural proteins: glycoprotein (G), large polymerase protein (L), phosphoprotein (P), matrix protein (M) and nucleocapsid (N) protein (Fig. 14A). The viral genomic RNA is completely encapsidated by N protein to form an RNase-resistant N-RNA complex that acts as a template for the RNA dependent RNA polymerase (RdRp) during RNA synthesis (Abraham and Banerjee, 1976; Ball and White, 1976; Ball, 1977; Li et al., 2005). L and P proteins are the major components of RdRP that catalyzes the replication of viral genomic RNA and the transcription of viral mRNA (Gaudier et al., 2002). Furthermore, this viral RdRp is tightly bound to N-RNA which results in the formation of the ribonucleoprotein (RNP) complex. Moreover, this RNP complex is further surrounded by the M protein and lipid bilayer envelope. The G protein is anchored in the envelope that plays an essential role in viral entry. The robust growth of VSV in cell culture systems makes it a good virus model for research purpose.
Avian metapneumovirus (aMPV), also named as avian pneumovirus (APV) or turkey rhinotracheitis virus (TRTV), is classified in the *Metapneumovirus* genus within *Paramyxoviridae* family. Since the first isolation of aMPV in South Africa in 1978, the virus has become prevalent worldwide. Based on antigenicity and genetic diversity, four subtypes of aMPV, designated A, B, C, and D, have been defined (Broor and Bharaj, 2007; Alvarez et al; 2003). Subtypes A, B, and D are found mainly in Europe and Asia, while subtype C is prevalent in the US. This virus is the primary causative agent of severe rhinotracheitis in turkeys and chickens. The symptoms of the respiratory disease include cough, nasal and ocular discharge, swelling of infraorbital sinuses, tracheal rales, and foamy conjunctivitis. In fact, aMPV also targets at the reproductive tract. In laying and breeding birds, there is a sharp drop in egg production, along with mild respiratory
tract illness (Senne et al., 1998). The virus was transmitted to US in 1996 and caused significant economic losses in the poultry industry.

Avian metapneumovirus is an enveloped virus with a non-segmented negative-strand RNA genome (Cecchinato et al., 2010). The genome, with approximately 13 kb in length, consists of eight viral genes which encode nine proteins (Easton, 2004). The virions are typically spherical (diameters of 100 to 500 nm), with some degree of pleomorphism, and filamentous particles of up to 400 nm in length also can be seen (Fig. 15B). As shown in Fig. 15A, aMPV possesses three virus glycoproteins, the attachment (G), fusion (F), and small hydrophobic (SH) proteins that are anchored on the surface of the lipid envelop. The F and G proteins, which are responsible for virus fusion and attachment, interact with the viral matrix (M) protein layer on the internal surface of the envelope. The linear genomic RNA is tightly encapsidated by the nucleoprotein (N) protein that associated with the phosphoprotein (P) and large (L) polymerase protein to form a helical ribonucleoprotein (RNP) complex. This RNP complex serves as template for viral mRNA transcription as well as genome replication (Conzelmann et al., 1998).
1.19. Non-thermal Food Processing Technology

An effective food processing technology is key in eliminating food borne viruses in high risk foods. Although the use of thermal energy has long been recognized as a process to ensure food safety and prolong shelf life, heating can cause undesirable changes in organoleptic (e.g. texture, color) and nutritional qualities (e.g. vitamin, enzyme, and protein degradation) in products (Rastogi et al., 2007; Grove et al., 2006).

Currently, consumers are increasingly demanding high quality products with natural tastes and flavors, and as well a fresh appearance. At the same time, consumers are looking for natural products without the addition of chemical additives and preservatives. Thus, the development of novel and reliable food preservation technologies is necessary to satisfy consumer demands without compromising safety. Non-thermal
processes, such as high pressure processing, irradiation, ultraviolet light, ultrasound, and pulsed-electric fields are of interest since they cause minimal sensory changes to a product compared to thermal processing while inactivating microorganisms (Grove et al., 2006; Lou et al., 2011; Rastogi et al., 2007; Baert et al., 2009).

The choice of food processing technology is dependent on the type of food, the effectiveness of the technique, and the location of the pathogens in food. For example, thermal processing technologies (such as heating, boiling, and microwaving) are not practical for fresh produce. For internalized viruses, technologies (such as UV and cold plasma) that lack the ability of penetration are also not effective. Some technologies (such as pulsed-electric field) are only suitable for liquid foods. For seafood, thermal and non-thermal processing technologies may be feasible. Other technologies, including gamma irradiation, X-Ray, electron beam (E-beam), may be highly effective for bacterial pathogens, but not for foodborne viruses.

1.20. High pressure processing

1.20.1 Brief history and benefits

High pressure processing (HPP) (also called high hydrostatic pressure, HHP, or ultra high-pressure processing, UHP) was first discovered able to kill microorganisms and preserve foods in 1899 in the USA. It was demonstrated that pressurization at 658 MPa held for 10 min destroys microorganisms in milk, fruit juice, meat, and fruits (Hite, 1899). However, research was not continued and the technology was not commercialized
until the late 1980s in Japan due to the difficulties in manufacturing high-pressure equipment and the lack of packaging materials (Rastogi et al., 2007). The first high pressure processed products were sold in Japan in 1990, and included jams, fruit jellies, sauces, and fruit juices. Currently, a variety of pressure-processed foods, including fresh bivalve shellfish, yogurt, jams, fruit smoothies, avocado products, chopped onions, and ready-to-eat meat products are commercially available in many countries (Baert et al., 2009; Calci et al., 2005; Chen et al., 2006; Grove et al., 2008). This technology is especially beneficial for heat sensitive products. Interestingly, HPP also confers additional benefits such as facilitating the shucking of oysters (Hsu et al., 2010). A survey of consumers conducted in three European countries showed that high-pressure processed foods are majorly accepted (Butz, 2003). Depending on the operation parameters and scale, the cost of high pressure treatment is typically in the range of $0.05-$0.50 per liter or per kilogram of food products (Thakur, 1998; Balasubramaniam, 2003).

HPP is recognized as the most promising non-thermal preservation and pasteurization technology that ensures food safety, food quality, and extends shelf-life by eliminating pathogenic and spoilage microorganisms. The primary advantage of HPP is it has minimal effects on the organoleptic and nutritional properties of foods because the pressure acts instantaneously and uniformly throughout foods regardless of size, shape, and geometry. Additionally, processing is conducted at either ambient temperature or lower temperatures (Balny et al., 2002; Chen and Hoover, 2003; Chen et al., 2006; Grove et al., 2006; Kingsley and Chen, 2009). It is known that covalent bonds are unaffected by HPP and hydrogen bond formation is favored under pressure, thus foods usually do not
undergo significant chemical transformations during pressurization. HPP is also believed to be a reliable tool to inactivate both surface and internalized pathogens. In addition to food preservation, HPP can also be used to develop novel products by altering the structural, textural, or functional properties of foods (Rastogi et al., 2007).

1.20.2. How high pressure processing works

Generally, in operation, food samples need to be pre-packaged in flexible containers and loaded in a pressure vessel filled with a pressure-transmitting fluid (water or oil). Once the vessel is filled and sealed, pressure is generated by pumping additional fluids into the vessel. This pressure is applied uniformly throughout the product (Fig. 16). The vessel volume increases under pressure due to expansion. HPP is an energy-efficient process because it requires no additional energy once the desired pressure is reached (Farr, 1990). Once the process is completed, the pressure relief valve opens to allow the compressed fluid to return to atmospheric pressure. The material of the package must also be flexible, because foods decrease in volume under pressure and expand in volume during decompression. Suitable packaging materials include glass containers, pouches, plastic bottles, polyvinyl alcohol films, and ethylene-vinyl alcohol copolymers. Of note, although HPP is a non-thermal process, a moderate temperature increase will occur due to the compression of water and food under pressure (Cheftel, 1995).
HPP equipment is commercially available. In the US, Avure Technologies (Kent, WA) offers many HPP units, ranging from small sizes (2 L capacity) to commercial sizes (215 L capacity). For example, the 215-L HPP equipment has the capacity to process about 10 million pounds of food per year. The small scale HPP units are ideal for laboratory research. In fact, all the experiments in our study were conducted in a PT-1 Research System (Avure Technologies, Kent, WA), which is a small-scale high-pressure testing system intended for laboratory research (Fig. 17).

Figure 16. Diagram of the pressure chamber in HPP unit
Figure 17. Appearance of PT-1 Research System (A) and PT-1 Research System main unit cart front view
As shown in Fig. 17A, the system consists of two cart-mounted components. The main mechanical cart (Fig. 17B) contains a pressure generator with a maximum operating pressure of 700 MPa, a 3 cubic inch (87” ID x 4.25“L) pressure chamber, and a temperature controlled liquid bath. The Type K stainless steel jacketed thermocouples with pressure fittings are available for measuring the temperature inside the pressure chamber. The other component is an electronic cart containing the PC-based data acquisition and control system, which is able to record time, pressure, chamber internal temperature, chamber external temperature, and liquid bath temperature.

1.20.3. Effectiveness of HPP in inactivating non-viral pathogens

High pressure inactivation of bacterial pathogens has been extensively studied. A number of studies have shown that HPP effectively inactivates bacterial pathogens and spores at optimized conditions. In general, bacterial vegetative cells, yeasts, molds can be inactivated under pressure between 200 to 700 MPa by disrupting permeability of cellular membranes (Chen and Hoover, 2003; Chen et al., 2006; Rastogi et al., 2007). For instance, a pressure of 375 MPa at 20°C held for 15 min caused a 6-log reduction of *Listeria monocytogenes* NCTC 2433. Furthermore, only a 1 min holding time is sufficient to achieve a 4-log reduction of *Escherichia coli O157* in orange juice at 550 MPa and 20°C (San Martin et al., 2002). However, bacterial spores are found to be highly pressure resistant because pressure above 1200 MPa is needed to inactivate them (Rastogi et al., 2007).
1.20.4. Effectiveness of HPP in inactivation of viral pathogens

The effectiveness of HPP on virus inactivation is poorly understood. Limited work has been conducted on the inactivation of viruses, especially foodborne viruses. In 1929, tobacco mosaic virus (TMV), a plant virus, became the first model to investigate viral sensitivity to HPP. It was found that TMV is highly resistant to HPP (Giddings et al., 1929). A pressure of 800 MPa held for 45 min was required to completely inactivate purified TMV (Basset et al. 1938). Fortunately, follow-up research showed HPP is capable of efficiently inactivating many human and animal viruses (Grove et al., 2006). Generally speaking, effective viral inactivation (≥ 5-log reduction) resulted from a pressure of ≤ 600 MPa was reported for a variety of non-enveloped food- and water-borne viruses, including human norovirus surrogates (FCV and MNV-1), HAV, HRV, and coxsackievirus A9 (Lou et al., 2011; Buckow et al., 2008; Kingsley et al., 2006; Grove et al., 2006). For example, it was reported that more than 5-log virus reductions were observed for MNV-1 and FCV at 300 MPa at 20°C for 5 min, and 400 MPa at 20°C for 2 min, respectively (Lou et al., 2011; Kingsley et al., 2002; Muchie et al., 2007). An approximate 7-log virus reduction was achieved for HAV under 450 MPa at 22°C for 5 min (Kingsley et al., 2002). However, several water-borne viruses are highly resistant to HPP. The infectivity of Aichivirus and coxsackievirus B5 remained unaffected after 600 MPa-treatment at ambient temperature for 5 min (Kingsley et al., 2004). Moreover, less than 1 log virus reduction was observed for poliovirus at pressure level of 600 MPa held for 1 h (Wilkinson et al., 2001). Therefore, it seems that different non-enveloped viruses have strikingly different susceptibilities to high pressure. It is worth noting that both coxsackievirus and poliovirus belong to the genus *Enterovirus* within the *Picornaviridae*
family. It seems there is no consensus sensitivity for viruses even if they are in the same genus whose members usually share similarities in virion structure, replication, and gene expression strategy. The factors that govern viral sensitivity in the presence of high pressure are unknown. It is possibly due to the differences in viral protein sequences and structures.

Compared to non-enveloped viruses, pressurization of enveloped viruses is less understood. Pressurization of enveloped viruses, such as avian influenza virus, herpes simplex virus type 1 (HSV-1), and human cytomegalovirus (HCMV) has been reported. It was found that avian influenza virus (H7N7) can be efficiently inactivated under 550 MPa at 15°C held for 90 s. HSV-1 and HCMV were inactivated by approximately 7 and 4 logs, respectively, under pressure of more than 400 MPa for 10 min at 25°C (Nakagami et al., 1992; Isbarn et al., 2007). To date, no study has been performed to compare the sensitivities of non-enveloped and enveloped viruses by HPP.

1.20.5. Factors that affect the effectiveness of virus inactivation by HPP

To effectively inactivate pathogens, it is critical to optimize the conditions for pressure treatment. The effectiveness of HPP is influenced by many factors such as processing parameters (pressure, temperature, and holding time) and non-processing parameters (the nature of the virus, food matrix, and pH of foods) (Chen et al., 2005; Grove et al., 2006; Grove et al., 2008; Lou et al., 2011).
1.20.5.1. The effects of processing parameters

First, virus inactivation depends on processing pressure and holding time. Generally, virus inactivation will be more prominent when the pressure is increased. Similarly, more virus reduction will be achieved if the holding time is increased. However, it is reported virus inactivation relies more on treatment pressure than holding time. For instance, a 5-log reduction of simian immunodeficiency virus (SIV) was reached at 250 MPa for 1 h, while the same reduction at 200 MPa and 150 MPa were required for 3 h and 10 h holding time, respectively (Jurkiewicz et al., 1995; Grove et al., 2006). The inactivation kinetics of FCV has also been determined. As shown in Fig.18, the inactivation curves of FCV as a function of treatment time showed a pronounced tailing, particularly at 200 MPa, indicating that longer holding time did not significantly further enhance inactivation. For instance, 20 min treatment at 200 MPa at room temperature reduced the titer of FCV by 2.8 logs; however, extending the holding time to 72 min only achieved an additional 0.9 log of virus reduction (Chen et al., 2005).
Secondly, treatment temperature may have a dramatic impact on virus inactivation. The responses of various viruses to temperature during HPP have been evaluated. As for non-enveloped viruses, previous work has shown that HAV is more sensitive to HPP at room temperature than at lower temperature; in contrast, pressure inactivation of MNV-1 is favored at refrigerated temperature (4°C) (Kingsley et al., 2007; Lou et al., 2011). FCV was found to be minimally affected by pressure at room temperature and inactivation was enhanced at either below or above 20°C (Kingsley and Chen, 2008). Avian influenza virus H7N7, an enveloped virus, was found to be more sensitive to HPP at higher temperature, which may be due to the heat sensitivity of the lipid envelope (Isbarn et al., 2007). These observations suggest that temperature may play completely opposite roles for virus inactivation. Overall, viruses have different susceptibilities to temperature after HPP, which may result from different sensitivities of viral proteins to thermal energy.

Figure 18. Inactivation curves of feline calicivirus at 21°C in cell culture medium at 200 and 250 MPa (Adapted from Chen et al., 2005).
1.20.5.2. The effects of non-processing parameters

It is necessary to investigate the role of pH in virus inactivation since many food products are acidic. Similar to the effect of the temperature, pH may play completely opposite roles in inactivating different viruses. To date, the role of pH in inactivation of a few non-enveloped viruses has been reported. It was shown that MNV-1, FCV, and TMV were easily inactivated at neutral pH than at acidic pH (Chen et al., 2005; Lou et al., 2011). For MNV-1, 8.1 logs of virus titer was 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 at the same pressure level and holding time (Lou et al., 2011). For FCV, the titer was reduced by 4.1 logs at 250 MPa for 1 min at 20° C at pH 6.0, whereas the titer was only reduced by 0.5 log at pH 4.0 (Kingsley and Chen, 2008). In sharp contrast, inactivation of HAV was favored at an acidic pH rather than at a neutral pH. For example, approximately 3 additional logs of HAV was reduced at pH 3.0 compared to pH 7.0, at 400 MPa for 1 min at 20° (Kinsley et al., 2009). The mechanism by which pH plays such a distinct role in inactivating different non-enveloped viruses is unknown. It is likely that the susceptibility of virus to pH is dependent on the nature of the viral capsid protein. To date, no study has been performed to examine the role of pH in inactivating enveloped viruses.

The composition of a medium or food matrix is another important factor that affects viral inactivation during pressure treatment. It has been reported that carbohydrates, fats, salts, proteins, ions, and other food constituents can protect viruses from inactivation (Baert et al., 2009; Balny et al., 2002; Gross et al., 1994; Kingsley et al., 2009). For example, HAV and FCV became more difficult to inactivate when the aqueous solution was supplemented with sodium chloride. Perhaps, salts can stabilize
viral capsid proteins which protect viruses from inactivation (Chen et al., 2005; Kingsley and Chen, 2009; Kingsley et al., 2007; Kingsley et al., 2002). In addition, Lou et al. (2011) found that there was almost an 8-log MNV-1 reduction in aqueous medium under 450 MPa at 4°C, while only 5.8- and 4.7-log reductions were reached in strawberries and strawberry puree, respectively. Clearly, the distinct composition of strawberry protected MNV-1 from inactivation. Moreover, FCV was more easily inactivated in aqueous medium than in mussels and oysters (Murchie et al., 2007). These observations showed that food matrix consistently provides protection. Thus, it is necessary to optimize HPP parameters for each food since the efficiency of viral inactivation in different foods will vary.

1.20.6. Mechanism of virus inactivation by high pressure

Virus particles consist of several pieces: the genetic material made from either DNA or RNA, the protein capsid surrounding the genetic material, and in some cases a lipid bilayer envelope that surrounds the protein capsid. Thus, potential mechanisms of virus inactivation may include (i) damaging the viral genetic materials; (2) disrupting the structure and functions of viral protein coats; and (3) disrupting the viral envelope. Lou et al. (2011) performed a systematic study on the mechanism of norovirus inactivation by HPP. It was demonstrated that the disruption of virion capsid structure is the primary mechanism of norovirus inactivation. Normally, MNV-1 is a small round structured virion. After pressure treatment, the virion structure was completely disrupted and a large amount of protein debris was visualized by electron microscopy. However, major proteins of MNV-1 were not degraded and still reacted with the antibody, suggesting the
primary and secondary structures of viral proteins remained intact, although the qua
ternary and tertiary structures of viral capsid proteins were completely distorted (Lou et al., 2011). Another study showed that HPP affected the receptor binding ability of the MNV-1 capsid (Tang et al., 2010). Disruption of the viral capsid structure would impair its functions, including attachment, receptor binding, and virus entry, which would lead to the loss of viral infectivity. However, HPP does not degrade viral genomic RNA (Tang et al., 2010, Lou et al., 2011). This is consistent with the fact that HPP usually does not break down covalent bonds. Mechanism of inactivation of enveloped viruses by HPP has not been reported.

1.20.7. Other potential applications of HPP

Besides the application of HPP as an effective tool to achieve inactivation of microorganisms, a number of other potential applications of HPP have been reported. A recent study showed that HPP successfully prevents MNV-1 infection in vivo. The immunodeficient (STAT-1(-/-)) female mice fed with HPP-treated MNV-1-seeded oyster extracts did not have any weight loss or hepatocellular necrosis and lymphoid depletion in the spleen; virus was also not present within the liver, spleen, and brain, but mice given non-HPP-treated MNV-1 experienced typical symptoms and virus was recovered from multiple organs (Gogal et al., 2011). In the medical field, it was found that HPP can efficiently block the outgrowth of tumor cells from tumor-afflicted bone and cartilage segments, while leaving their biomechanical and key biological properties unimpaired. HPP technology is now in preclinical testing with the aim of being an alternative
technique of sterilizing resected tissue segments to kill pathological microorganisms and tumor cells to allow autologous reimplantation (Diehl et al., 2008). On the other hand, several studies have showed the possibility of HPP to be an effective approach to produce antiviral vaccines. In 1992, Silva et al. (1992) found that a pressure of 260 MPa held for 12 h effectively inactivated vesicular stomatitis virus (VSV) without loss of immunogenicity. Ishimaru et al. (2004) also demonstrated that HPP, combined with subzero temperatures and sub-denaturing urea concentrations, inactivated foot-and-mouth disease virus while preserving its ability to elicit neutralizing antibody production in rabbits. These results suggest that HPP may be a good technique to prepare inactivated vaccine

1.21. Effectiveness of other nonthermal techniques on virus inactivation

1.21.1. Irradiation

Food irradiation (gamma irradiation and E-beam) has been shown to be an effective non-thermal processing technology to inactivate bacteria, fungi, insects, and pests in foods without posing health risks (Farkas, 1998). Food irradiation technologies include gamma irradiation, E-beam, and X-ray. However, viruses are thought to be more resistant to irradiation than bacteria and fungi due to their small sizes and stable structures. Unfortunately, studies demonstrated that food irradiation is not effective in inactivating MNV-1 at the dosage approved by the FDA to inactivate bacteria in fresh iceberg lettuce and spinach (up to 4.0 kGy). Only 1.7- to 2.4-log MNV-1 reduction was observed in fresh produce (strawberries, romaine lettuce, and spinach) by gamma
irradiation at the dosage of 5.6 kGy (Feng et al., 2011). Furthermore, Sanglay et al. (2011) found that less than 1 log MNV-1 reduction was observed in either cabbage or strawberries after 4 kGy of E-beam irradiation. It seems that irradiation is not a practical technology to eliminate viruses from foods at FDA approved dosages. Additionally, comprehensive mechanistic insight into the inactivation of MNV-1 and VSV by gamma irradiation has been reported. It was demonstrated that gamma irradiation disrupted virion structure and viral capsid proteins, and also degraded viral proteins and genetic material (Feng et al., 2011). Overall, it is believed that an additional hurdle in processing is required in combination with food irradiation to ensure food safety.

1.21.2. Ultraviolet (UV) light

UV irradiation, with wavelengths between 250-260nm, has a germicidal effect on a wide range of microorganisms including bacteria, viruses, protozoa, mycelial fungi, yeasts and algae (Fino et al., 2008; Roberts and Hope, 1995). MNV-1 and FCV were shown to be susceptible to UV. The titers of MNV-1 and FCV were reduced by approximately 3 logs at a dosage of 26 mJ/cm² under 254-nm UV light (Tree et al., 2005). A 5-log inactivation of FCV is achieved at a dose of 50 mW s/cm² under 253.7 nm UV light (Fino et al., 2008). UV light represents an effective alternative or complement to conventional sterilization techniques to eliminate viruses. Unfortunately, the major disadvantage of UV technology is that it lacks the ability to penetrate into foods and other objectives. For example, UV is not able to inactivate viruses that have been internalized in fresh produce. UV cannot be used for shellfish because pathogens are protected by the shells and tissues. Thus, the application of UV technology in food industry is limited. As
a cost-saving and easy-to-use technology, the practical applications of UV light also include surface disinfection, decontamination in restaurants or food processing pilot plants, liquid sterilization in aquaculture or pharmaceutical industries (Fino et al., 2008; Jean et al., 2011).

1.21.3. Ultrasound

High intensity ultrasound (HIUS), with frequency between 20 kHz–2MHz and power between 100–500W/cm², can be used as a food processing tool for microorganisms and enzyme inactivation, bio-component separation, and etcetera. Su et al. (2010) investigated the effectiveness of HIUS in eliminating human norovirus surrogates, MS2, FCV-F9, and MNV-1. In this study, an ultrasound probe was directly placed into virus suspensions to avoid dissipating any ultrasound energy. When MS2, FCV-F9, and MNV-1 were suspended in PBS, a 4-log reduction was found after 10-, 5-, and 30-min exposure to ultrasound, respectively. However, the inactivation effect was significantly reduced when the viruses were suspended in orange juice, indicating that the food matrix protected the viruses from inactivation. This result also suggests that ultrasound may not be sufficient to inactivate human norovirus surrogates in foods by 5 log reductions to meet the FDA standard. Therefore, the combination of ultrasound with other techniques such as heat, pressure, or antimicrobials should be used to ensure food safety (Su et al., 2010).
CHAPTER 2

INACTIVATION OF A HUMAN NOROVIRUS SURROGATE BY HIGH-PRESSURE PROCESSING: EFFECTIVENESS, MECHANISM, AND POTENTIAL APPLICATION IN THE FRESH PRODUCE INDUSTRY

2.1. Abstract

Fresh produce is often a high-risk food for norovirus contamination because it can become contaminated at both preharvest and postharvest stages and it undergoes minimal or no processing. Currently, there is no effective method to eliminate the viruses from fresh produce. This study systematically investigated the effectiveness of high-pressure processing (HPP) on inactivating murine norovirus (MNV-1), a surrogate for human norovirus, in aqueous medium and fresh produce. We demonstrated that MNV-1 was effectively inactivated by HPP. More than a 5-log-PFU/g reduction was achieved in all tested fresh produce when it was pressurized at 400 MPa for 2 min at 4°C. We found that pressure, pH, temperature, and food matrix affected the virus survival in foods. MNV-1 was more effectively inactivated at 4°C than at 20°C in both medium and fresh produce. MNV-1 was also more sensitive to HPP at neutral pH than at acidic pH. We further demonstrated that disruption of viral capsid structure, but not degradation of viral genomic RNA, is the primary mechanism of virus inactivation by HPP. However, HPP
does not degrade viral capsid protein, and the pressurized capsid protein was still antigenic. Overall, HPP had a variable effect on the sensorial quality of fresh produce, depending on the pressure level and type of product. Taken together, HPP effectively inactivated a human norovirus surrogate in fresh produce with a minimal impact on food quality and thus can provide a novel intervention for processing fruits intended for frozen storage and related products such as purees, sauces, and juices.

2.2. Introduction

Viruses account for more than 67% of food-borne illnesses worldwide (Larson et al., 1994; Mormann et al., 2010). Human norovirus (NoV) is the major food-borne virus that causes gastroenteritis outbreaks in humans. The symptoms often involve projectile vomiting, diarrhea, nausea, and low-grade fever (Adler et al., 1969, Estes et al., 2006, Koopmans and Duizer, 2004). It is estimated that more than 90% of outbreaks of acute nonbacterial gastroenteritis are caused by human NoV, but this percentage may be underestimated due to the large number of asymptomatic NoV infections and lack of proper detection methods (Estes et al., 2006; Koopmans and Duizer, 2004; Mead, 1999). Human NoVs are transmitted primarily through the fecal-oral route, either by direct person-to-person spread or by fecally contaminated food or water. Human NoV is highly contagious, and only a few particles are thought to be sufficient to cause infection (Duizer, 2004; Estes, 2006; Koopmans and Duizer, 2004). Outbreaks frequently occur in restaurants, hotels, day care centers, schools, nursing homes, cruise ships, swimming pools, hospitals, and military installations (Adler and Zickl, 1969; Cliver, 1997; Estes et
al., 2006; Larson et al., 1994; Tuan Zainazor et al., 2010). However, research on human NoVs has been hampered by the lack of a suitable cell culture system or a small animal model (Estes et al., 2006; Koopmans and Duizer, 2004). Therefore, studies of human NoV must rely on proper surrogates such as murine norovirus (MNV) and feline calicivirus (FCV) (Cannon 2006; Karst et al., 2003; Thurston-Enriquez et al., 2005; Wilkinson et al., 2001). A recent study demonstrated that MNV was a more suitable surrogate than FCV (Cannon et al., 2006). For these reasons, human NoV and other caliciviruses have been classified as category B priority biodefense pathogens by the National Institute of Allergy and Infectious Diseases.

Fresh produce is often at risk of NoV contamination because it undergoes minimal or no processing and can become contaminated during preharvest and postharvest stages. Contamination by NoV may occur at the farm level by fecal contamination in irrigation water, soil, animals, or fertilizer and by infected workers during harvesting, processing, handling, and freezing (Allwood et al., 2004; Baert et al., 2008; Butot et al., 2007; Heaton and Jones, 2008; Seymour and Appleton, 2001; Steele and Odemeru, 2004; Tuan Zainazor, 2010). According to a recent compilation of U.S. outbreak data from 1998 to 2005, fresh produce has become dominant as a vehicle in food-borne virus outbreaks (Doyle and Erickson, 2008; Heaton and Jones, 2008; Seymour and Appleton, 2001; Sivapalasingam et al., 2004). Disease surveillance shows that NoV is the top causative agent for fresh produce outbreaks (40%), followed by Salmonella species (18%), Escherichia coli O157:H7 (8%), Clostridium species (6%) and hepatitis A virus (HAV) (4%) (Doyle and Erickson, 2008). Fresh-produce-related outbreaks of NoVs have been reported in lettuce, salad, fruit salad, tomato, carrot, melon, strawberry, raspberry, orange
juice, fresh-cut fruits, spring onion, and other fresh produce (Allwood, 2004; Baert et al., 2008, Beuchat, 1996; Cotterelle et al., 2005; Falkenhorst et al., 2005; Lynch et al., 2009). The increasing produce-associated outbreaks suggest that there is an urgent and critical need to develop a novel intervention method to inactivate NoVs in fresh produce.

Unfortunately, none of the decontamination methods investigated thus far have been shown to effectively control human NoV in fresh produce (Butot et al., 2009; Cliver, 2007; Doyle and Erickson, 2008; Duizer et al., 2004; Koopmans and Duizer, 2004; Mormann et al., 2010). Sanitizers are not effective in the removal of food-borne viruses from fresh produce. Most commonly used sanitizers such as chlorine, hydrogen peroxide, peracetic acid, and sodium bicarbonate reduced human NoV surrogates in vegetables only by 1 to 1.5 logs (Butot et al., 2008; Duizer et al., 2004; Seymour and Appleton, 2001). More importantly, recent evidence suggests that human NoV may be internalized in the leaves of vegetables and disseminated to other portions of the plants (Rawsthorne et al., 2009; Urbanucci et al., 2009; Wei et al., 2010). Sanitizers may be effective in reducing the viruses on the surface but not those internalized into vegetables and fruits. Although thermal processing could efficiently inactivate viruses, it is impractical for fresh produce, since high temperatures would adversely affect the food quality and degrade vitamins and other nutritional components. Perhaps nonthermal processing technologies may provide alternative methods to improve the safety of vegetables and fruits due to the fact that these technologies have a minimal impact on organoleptic and nutritional properties (Balny et al., 2002; Chen and Hoover, 2003; Grove et al., 2006). However, the survival of food-borne viruses and the mechanism of virus inactivation by nonthermal processing technologies are poorly understood.
High-pressure processing (HPP) provides a unique advantage because pressure acts uniformly throughout a food regardless of size, shape, and geometry. By destroying pathogenic and spoilage organisms without breaking the covalent bonds, HPP has minimal effects on the taste, flavor, texture, appearance, and nutritional value of food (Balny et al., 2002; Chen and Hoover, 2003; Chen et al., 2006; Grove et al., 2006; Kingsley et al., 2005; Kingsley et al., 2009). The technology also satisfies consumers' demand for minimal processing without the use of preservatives. High pressure has been shown to effectively inactivate bacterial pathogens and spores (Chen and Hoover, 2003; Chen et al., 2006; He et al., 2002). However, limited work on the inactivation of food-borne viruses by HPP has been reported. It has been shown that both processing parameters (pressure, temperature, and time) and nonprocessing parameters (pH value and salt concentration) affect the inactivation of FCV (Chen et al., 2005; Grove et al., 2006; Grove et al., 2008). It was shown that the inactivation of FCV was favored when HPP was performed at refrigeration temperature rather than at room temperature (Chen et al., 2005). In sharp contrast, Kingsley and Chen's groups showed that the inactivation of HAV in solutions was enhanced at a higher temperature (Calci et al., 2005; Kingsley et al., 2005; Kingsley and Chen, 2009; Kingsley et al., 2002; Kingsley et al., 2007). These findings suggest that different viruses have different susceptibilities to and optimal parameters for HPP. Previously, the effect of temperature on pressure inactivation of MNV-1 in oyster tissue was reported by Kingsley et al. (Kingsley et al., 2007). However, other optimal parameters (such as pH, combination of pH and temperature, and different food matrix) for the inactivation of MNV-1 by HPP have not been reported. Also, the molecular mechanism involved in virus inactivation is still poorly understood. Although
one recent study suggests that HPP affects virus binding to host cells (Tang et al., 2010),
the effect of HPP on the structure of the virion and viral capsid protein was not
understood. In addition, no study has been performed to inactivate MNV-1 in fresh
produce and related products such as purees and juices.

In the present study, we performed a systematic study on the inactivation of
human NoV using MNV-1 as a surrogate. Specifically, we determined the effect of pH,
temperature, pressure, and food matrix on the survival of MNV-1 by HPP. We
determined the mechanism of virus inactivation by HPP. Furthermore, we demonstrated
that HPP may be a novel intervention method to eliminate viruses from foods and may be
a promising technology to process fruits intended for frozen storage and fruit-related
products (such as puree, sauce, and juice).

2.3. Materials and Methods

2.3.1. Virus and cell culture

Murine norovirus strain MNV-1 was kindly provided by Herbert W. Virgin IV,
Washington University School of Medicine (Karst et al., 2003; Wobus et al., 2006).
Working stocks of MNV-1 were propagated in confluent monolayers of murine
macrophage cell line RAW 264.7 (ATCC, Manassas, VA). The cells were cultured in
high-glucose Dulbecco's modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA)
supplemented with 10% fetal bovine serum (FBS) (Invitrogen), at 37°C under a 5% CO₂
atmosphere. Confluent RAW 264.7 cells were infected with MNV-1 at a multiplicity of
infection (MOI) of 0.01. After 1 h of incubation at 37°C, 15 ml of serum-free DMEM was added. Virus was harvested after 2 days postinoculation by freeze-thawing three times.

2.3.2. MNV-1 plaque assay

RAW 264.7 cells were seeded into six-well plates (Corning Life Sciences, Wilkes-Barre, PA) at a density of 2 x 10^6 cells per well. After 24 h of incubation, cell monolayers were infected with 400 µl of a 10-fold dilution series of the virus and the plates were incubated for 1 h at 37°C. The cells were overlaid with 2.5 ml of Eagle minimum essential medium (MEM) containing 1% agarose, 2% FBS, 1% sodium bicarbonate, 0.1 mg of kanamycin/ml, 0.05 mg of gentamicin/ml, 15 mM HEPES (pH 7.7), and 2 mM L-glutamine (Invitrogen). After incubation at 37°C and 5% CO₂ for 2 days, the plates were fixed in 10% formaldehyde and the plaques were visualized by staining with crystal violet.

2.3.3. Purification of murine norovirus

Large stocks of MNV-1 were generated by inoculation of 8 to 10 confluent T150 flask RAW 264.7 cells at an MOI of 0.01 in a volume of 3 ml of DMEM. At 1 h postadsorption, 15 ml of DMEM with 2% FBS was added to the cultures, and infected cells were incubated at 37°C for 48 h. When extensive cytopathic effect (CPE) was observed, cell culture fluids were harvested and subjected to three freeze-thaw cycles to release virus particles. The purification of MNV-1 was performed using the method
described by Katpally et al. (2008) with minor modifications. The virus suspension was centrifuged in a Sorvall SS-34 rotor (Kendro Laboratory Products, Germany) at 8,000 x g for 15 min to remove cellular debris. The supernatant was then digested with DNase I (10 µg/ml) and MgCl₂ (5 mM) at room temperature. After 1 h of incubation, 10 mM EDTA and 1% lauryl sarcosine were added to stop nuclease activity. Virus was concentrated by centrifugation at 82,000 x g for 6 h at 4°C in a Ty 50.2 rotor (Beckman Coulter, Fullerton, CA). The pellet was resuspended in phosphate-buffered saline (PBS) and further purified by centrifugation at 175,000 x g for 6 h at 4°C through a sucrose gradient (7.5 to 45%) in an SW55 Ti rotor (Beckman). The final virus-containing pellets were resuspended in 100 µl PBS. The virus titer was determined by plaque assay on RAW 264.7 cells. Viral protein was measured by Bradford reagent (Sigma Chemical Co., St. Louis, MO).

**2.3.4. High-pressure treatment of MNV-1 in aqueous cell culture medium**

The pH values of DMEM were adjusted to 4.0 and 7.0 by citric acid because it is often used as a food additive to lower the pH of food products. One-milliliter aliquots of MNV-1 stock (10⁸ PFU/ml) in DMEM were placed in sterile polyethylene stomacher pouches (Fisher Scientific International, Ontario, Canada) and heat sealed using an Impulse sealer (American International Electric Co., Whittier, CA). The samples were treated at 200, 250, 300, 350, 400, and 450 MPa for 2 min at 4 or 20°C using a pressure unit with temperature control (model Avure PT-1; Avure Technologies, Kent, WA). All temperatures reported in this study were initial sample temperatures. The pressure come-up rate was approximately 22 MPa/s. The pressure release was almost immediate (<4 s).
Pressurization time did not include the pressure come-up or release time. After processing, the bags were removed and cooled in an ice bath, and the surviving viruses in the samples were quantified by viral plaque assay.

2.3.5. High-pressure treatment of MNV-1 in fresh produce

Fresh iceberg lettuce and strawberries were purchased from a local supermarket. Strawberries were homogenized into purees. Fresh lettuce and strawberries were cut into pieces. All fresh produce was placed into sterile polyethylene pouches. The MNV-1 cocktail was inoculated into each bag to achieve a final inoculation level of approximately $10^7$ PFU/g. The contents in each bag were mixed thoroughly in a biosafety cabinet for 1 h to allow for virus attachment, binding, and internalization, and then the bags were vacuum sealed. All samples were pressurized at 350, 400, and 450 MPa at 4 or 20°C for 2 min. After processing, the bags were removed and cooled in an ice bath, and the surviving virus was eluted and quantified by plaque assay as described above. In addition, uninoculated blueberries, raspberries, strawberries, strawberry puree, and lettuce were pressurized at 350 and 600 MPa in 4°C for 2 min. After processing, these samples were subjected to sensory evaluation and visual assessment.

2.3.6. High-pressure treatment of MNV-1 in fruit purees

Using strawberry puree as a model food matrix, the effect of pH on virus inactivation was determined. The natural pH of strawberry puree is around 3.5. The pH of
strawberry puree was adjusted to 2.5, 4.5, 5.5, and 6.5 using either citric acid or sodium hydroxide. The samples with pH values of 2.5 to 6.5 were inoculated with MNV-1 to a final inoculation level of approximately $10^6$ PFU/g and subjected to HPP for 2 min at 400 MPa and 4°C. The surviving virus was determined by plaque assay. In addition, to determine the role of food matrix in virus inactivation, a number of other fruit and vegetable purees and juices with different natural pH values were also used in pressure treatment. These purees included lemon (pH 2.5), strawberry (pH 3.5), tomato (pH 4.5), watermelon (pH 5.3), and carrot (pH 5.8) in addition to carrot juice (pH 6.3). Two grams of each food sample was placed in a stomacher pouch, and the MNV-1 cocktail was inoculated to achieve a final inoculation level of approximately $10^7$ PFU/g. The samples were pressurized at 400 MPa and 450 MPa for 2 min at 4°C, and the surviving viruses were determined by plaque assay.

2.3.7. Transmission electron microscopy

Negative-staining electron microscopy of purified virions was performed to determine whether HPP damages the virus particles. Sixty microliters of highly purified MNV-1 suspension was treated by HPP at 350, 500, and 600 MPa at 4°C for 2 min. A viral plaque assay was conducted to confirm the inactivation of virus. Aliquots (20 µl) of either HPP-treated or untreated samples were fixed in copper grids (Electron Microscopy Sciences, Hatfield, PA) and negatively stained with 1% ammonium molybdate. Virus particles were visualized by an FEI Tecnai G2 Spirit transmission electron microscope (TEM) at 80 kV at the Microscopy and Imaging Facility at the Ohio State University.
Images were captured on a MegaView III side-mounted charge-coupled-device (CCD) camera (Soft Imaging System, Lakewood, CO), and figures were processed using Adobe Photoshop software (Adobe Systems, San Jose, CA).

2.3.8. RT-PCR

Viral RNA was extracted from MNV-1 suspensions using an RNeasy minikit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Reverse transcription-PCR (RT-PCR) was performed using a One Step RT-PCR kit (Qiagen). Two primers (5'-ATGAGGATGAGTGATGGCGC-3' and 5'- TTATTTGTGACATTCCGCC-3') were designed to target the viral capsid VP1 gene. The amplified products were analyzed by 1% agarose gel electrophoresis.

2.3.9. Analysis of viral proteins by SDS-PAGE

Two micrograms of highly purified MNV-1 suspensions (either HPP treated at 600 MPa at 4°C for 2 min or untreated) was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Samples were boiled for 5 min in loading buffer containing 1% SDS, 2.5% β-mercaptoethanol, 6.25 mM Tris-HCl (pH 6.8), and 5% glycerol and loaded onto a 12% polyacrylamide gel. Viral proteins were visualized by Coomassie blue staining.
2.3.10. Western blotting

Two micrograms of highly purified MNV-1 suspensions (either HPP treated at 600 MPa at 4°C for 2 min or untreated) was separated by 15% SDS-PAGE and transferred to a Hybond ECL nitrocellulose membrane (Amersham) in a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad). The blot was probed with rabbit anti-MNV VP1 polyclonal antibody (a generous gift from H. W. Virgin IV) at a dilution of 1:10,000 in blocking buffer (5% skim milk), followed by horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology) at a dilution of 1:20,000. The blot was developed with SuperSignal West Pico chemiluminescent substrate (Thermo Scientific) and exposed to Kodak BioMax MR film (Kodak).

2.3.11. Statistical analysis

All experiments were carried out in triplicate. Virus survival was expressed as mean log titer ± standard deviation. Statistical analysis was performed by one-way multiple comparisons using SPSS 8.0 statistical analysis software (SPSS Inc., Chicago, IL). A P value of <0.05 was considered statistically significant.

2.4. Results

1. Pressure inactivation of MNV-1 in aqueous medium. In order to determine the effectiveness of the inactivation of MNV-1 by HPP, we first aimed to investigate the survival of MNV-1 in aqueous cell culture medium after pressurization. DMEM was
inoculated with MNV-1 and was treated at different pressures, pHs, and temperatures. The kinetics of MNV-1 inactivation by HPP is shown in Fig. 19. Clearly, pressure magnitude, pH value, and treatment temperature affected the effectiveness of virus pressure inactivation. As expected, MNV-1 was efficiently inactivated at higher pressure levels. At each pressure level, it became easier for MNV-1 to be inactivated at 4°C than at 20°C ($P < 0.05$). For instance, an 8.1-log virus reduction was observed at 4°C when the sample was treated under 350 MPa at pH 7.0. However, only 4.1-log virus reductions were achieved at 20°C under the same treatment condition. To determine the effect of pH on pressure inactivation of virus, the pH of DMEM was adjusted to 4.0 using citric acid. MNV-1 was more efficiently inactivated in a neutral environment (pH 7.0) than in an acidic environment (pH 4.0) at both 4 and 20°C throughout the pressure range. For example, at pH 7.0, the virus was almost completely killed (>8-log reductions) by 350 MPa of pressure at 4°C, whereas only a 6.0-log virus reduction was observed at pH 4.0 when other conditions were the same. Taken together, these results clearly demonstrated that the effectiveness of virus inactivation in the medium was significantly affected by pressure, pH, and temperature. It can be concluded that the optimal conditions for virus inactivation are a minimum pressure level of 350 MPa, pH 7.0, and a temperature of 4°C in cell culture medium. These results also suggested that HPP may be a promising technology to inactivate viruses in foods.
2. Pressure inactivation of MNV-1 in fresh produce. Fresh vegetables and fruits were chosen as the food matrices, since they are the major vehicles to transmit NoVs (Allwood et al., 2004; Baert et al., 2008; Beuchat, 1996; Butot et al., 2008; Cotterelle et al., 2005; Falkenhorst et al., 2005; Lynch et al., 2009; Pönkä et al., 1999). Available evidence suggests that food-borne viruses can attach tightly and be internalized...
in fresh vegetables and fruits (Rawsthorne et al., 2009; Urbanucci et al., 2009; Wei et al., 2010). The internalized viruses cannot be removed by traditional washing and sanitization strategies. To determine whether HPP can effectively inactivate MNV-1 in fresh produce, fresh lettuce, fresh-cut strawberries, and strawberry puree were inoculated with MNV-1 to a final concentration of approximately $10^7$ PFU/g and then subjected to HPP at pressures ranging from 350 to 450 MPa at either 4 or 20°C for 2 min. These pressures were chosen since MNV-1 can effectively be inactivated in the medium under these conditions. As shown in Fig. 20, significant inactivation of MNV-1 was observed in all three fresh produce types. At 4°C and 350 MPa, 2.4-, 2.2-, and 2.4-log virus reductions were achieved in fresh lettuce, fresh-cut strawberry, and strawberry puree, respectively. When the pressure was increased to 400 and 450 MPa, virus inactivation was significantly improved. At 450 MPa and 4°C, 4.7- to 7.0-log virus reductions were achieved in all samples. Statistics analysis showed there was no significant difference in the efficiency of virus inactivation across the three types of fresh produce ($P > 0.05$). However, MNV-1 was more easily inactivated in aqueous medium than fresh produce ($P < 0.05$). At 350 MPa of pressure, 6.0- and 8.1-log reductions were observed in aqueous medium at pH 4.0 and pH 7.0, respectively (Fig. 19). These results suggested that some components (as yet undefined) of fresh produce may provide a protective effect for MNV-1 inactivation.

The inactivation efficiency was also dependent on the treatment temperature in fresh produce samples. MNV-1 was significantly more sensitive to HPP at 4°C than at 20°C in fresh produce ($P < 0.05$) across the pressure spectrum, which is consistent with the results for aqueous medium. For example, at 4°C and 450 MPa, approximately 4.7-
7.0-log virus reductions were found in all three products. This was significantly more efficient than a higher temperature (20°C), which achieved only 4.1- to 4.9-log reductions under the same pressure. Taken together, these results demonstrated that MNV-1 can be more effectively killed at a subambient temperature of 4°C when subjected to a moderate pressure level of 450 MPa.

Figure 20. Effectiveness of MNV-1 inactivation in DMEM, strawberry puree, and fresh-cut strawberries and lettuces by HPP. Fresh-cut iceberg lettuce and strawberries and strawberry puree were inoculated with MNV-1 to a final inoculation level of approximately 10⁷ PFU/g and pressure treated at 350 MPa, 400 MPa, and 450 MPa at either 4°C or 20°C for 2 min. The surviving viruses were determined by plaque assay. Log virus reduction = log (titers of untreated samples) – log (titers of pressurized samples). Data are the means of three replicates. Error bars represent ±1 standard deviation.
3. Effect of pH on pressure inactivation of MNV-1 in strawberry puree. The pH of fruits and vegetables typically ranges from 2.5 to 6.5. Our results showed that MNV-1 was more sensitive to HPP at higher pH in cell culture medium (Fig. 19). To further investigate the role of pH on virus inactivation in fruits and related products, strawberry puree was selected as the model food matrix for HPP. The natural pH of strawberry puree is about 3.5. The pH values of strawberry puree were artificially adjusted from 2.5 to 6.5 using either citric acid or sodium hydroxide, and the samples were subjected to HPP for 2 min at 400 MPa and 4°C. Without pressure treatment, MNV-1 was highly stable in strawberry purees within the pH values ranging from 2.5 to 6.5. Exposure of MNV-1 under an acidic condition for 24 h did not reduce the viral titer (data not shown). As shown in Fig. 21, MNV-1 was easily inactivated at higher pH in strawberry puree samples, which is consistent with the previous observation from the aqueous medium. A 4.8-log virus reduction was achieved at pH 6.5; however, only a 2.8-log virus reduction was reached at pH 2.5. Taken together, these results demonstrated that MNV-1 was more sensitive to HPP at higher pH in both strawberry and aqueous medium.
4. Effectiveness of MNV-1 inactivation by HPP in different purees. It is necessary to determine the effectiveness of MNV-1 inactivation in different purees, since they have different pH values and the food matrix may play a role in virus inactivation. Therefore, the MNV-1 survival was determined in a number of purees and juices at two pressures (350 and 400 MPa) at 4°C for 2 min. These food samples included lemon (pH 2.5), tomato (pH 4.5), strawberry (pH 3.5), watermelon (pH 5.3), carrot (pH 5.8), and...
carrot juice (pH 6.3). With the exception of lemon puree, the effectiveness of virus inactivation appeared to be correlated with the natural pH in foods (Fig. 22). The higher the pH of the food, the greater the log reduction of the virus. At 350 MPa, 4.3- and 4.7-log virus reductions were achieved in carrot puree (pH 5.8) and carrot juice (pH 6.3), respectively, while only 3.4 logs of reduction were observed in strawberry puree (pH 3.5) ($P < 0.05$). At 400 MPa, 6.8-log PFU/ml of virus was inactivated in carrot puree, which is significantly higher than the result for strawberry puree (5.4-log reduction) ($P < 0.05$). Surprisingly, a 5.4-log virus reduction was achieved in lemon puree at 350 MPa even at extremely low pH (2.5) (Fig. 22). It should be noted that the viral titer reduction (5.4 log) in lemon puree was higher than that of strawberry puree in which the pH was artificially modified to 2.5, after the same pressure processing (Fig. 21). These results provided strong evidence that the food matrix plays an important role in protecting virus from inactivation. Collectively, the results suggested that both pH and food matrix affected MNV-1 inactivation in purees.
Figure 22. Pressure inactivation of MNV-1 in different purees. Purees were inoculated with MNV-1 to a final inoculation level of approximately $10^7$ PFU/g and pressure treated at either 350 MPa or 400 MPa at 4°C for 2 min. The purees used include lemon (pH 2.5), strawberry (pH 3.5), tomato (pH 4.5), watermelon (pH 5.3), carrot (pH 5.8), and carrot juice (pH 6.3). High-pressure treatments of inoculated purees were performed at either 350 MPa or 400 MPa at 4°C for 2 min. The surviving viruses were determined by plaque assay. Data are the means of three replicates. Error bars represent ±1 standard deviation.

5. Visual assessment of pressure-treated fresh produce. Fresh strawberries, raspberries, blueberries, lettuce, and strawberry puree were selected as models to evaluate the possible change in quality attributes of foods after HPP. Samples were treated at 350 MPa and 4°C for 2 min. Visual examination showed that processed products retained the freshness and the original color (Fig. 23a). Strawberries displayed a slight color change. Strawberries had partially lost the opacity of the inner white tissue, appearing more
translucent. In addition, the color of their seeds changed from green to tan. The influence of treatment on the food texture was product dependent. For example, blueberries, raspberries, and strawberries underwent a minor textural change, with slight softening of the tissue. In the case of lettuce, although the crunchiness and crispiness were mostly preserved after pressure treatment, the leaves had partially lost their opacity, acquiring a more glassy or translucent appearance. Thus, HPP may affect the quality of leafy greens. There were no perceived changes in the appearance of strawberry purees after processing, but they appeared to have a lighter consistency and thickness than the untreated purees. These observations suggested that HPP had a minimal impact on the quality of the tested fresh produce (except lettuce) but that different foods may have different responses to processing. At 600 MPa, HPP did not significantly affect the color, freshness, and texture of blueberries and strawberry puree although lettuce, strawberries, and raspberries underwent considerable textural loss due to pressure softening of the tissue (Fig. 23b).
Figure 23. Visual assessment of fresh produce processed by HPP. Raspberries (A), blueberries (B), fresh-cut strawberries (C), lettuce (D), and strawberry puree (E) were used as models to evaluate the effect of HPP on food quality. (a) Samples were processed at 350 MPa at 4°C for 2 min. (b) Samples were processed at 600 MPa at 4°C for 2 min.
6. **HPP disrupts virus particles.** To gain insight into the mechanism of virus inactivation by HPP, highly purified MNV-1 was treated at 350, 500, and 600 MPa at 4°C for 2 min. At 350 and 500 MPa, there were approximately 1-log virus survivors. Under these conditions, a mixture of inactivated and intact viruses may be observed. We chose a pressure of 600 MPa since MNV-1 was completely killed under this condition, thus allowing us to precisely determine the mechanism of virus inactivation by HPP. The virus particles were visualized by electron microscopy. As shown in Fig. 24A, unpressurized MNV-1 particles were small round-structured virions approximately 30 to 38 nm in diameter. At a pressure of 350 MPa, we found a large amount of irregularly shaped particles with sizes ranging from 5 to 30 nm (Fig. 24B). In addition, we saw some empty particles with sizes of approximately 10 to 15 nm in diameter. These particles still retained a round-structured shape but were smaller. Most likely, the viral genomic RNA was spilled out by HPP, which resulted in empty particles. At a pressure of 500 MPa, we also saw a large amount of irregularly shaped particles, protein debris, and some small empty particles (Fig. 24C). At 600 MPa, only small protein debris, but very few empty particles, was observed (Fig. 24D). In all three pressure treatments, we failed to observe any small round-structured virions with a size of 30 to 38 nm (Fig. 24B to D). Because electron microscopy (EM) analysis requires a high number of particles (usually $10^4$ to $10^6$ per ml), it is not surprising that we failed to find any intact virus particles since the majority of viruses were killed at 350 MPa. Therefore, this result demonstrated that the structure of the virion and the capsid was gradually disrupted when pressure was increased.
Figure 24. HPP disrupts virus particles. Purified MNV-1 was completely inactivated at 350, 500, and 600 MPa at 4°C for 2 min. Treated and untreated virus particles were negatively stained with 1% ammonium molybdate and visualized by a transmission electron microscope. (A) Untreated MNV-1 virion. (B) MNV-1 particles treated with 350 MPa. (C) MNV-1 particles treated with 500 MPa. (D) MNV-1 particles treated with 600 MPa.

7. HPP disrupts the structure of the viral capsid but not the primary and secondary structures of the VP1 protein. Norovirus is a nonenveloped positive-sense RNA virus. The virus particle is composed of extremely stable capsid proteins that protect
the viral genetic material, RNA. It was known that MNV possesses two capsid proteins, the major capsid protein (VP1) and the minor capsid protein (VP2) (Bertolotti-Ciarlet ET AL., 2003; Jiang et al., 1992). VP1 plays many essential roles in viral infection, including attachment, entry, packaging of mature virion, and release, whereas VP2 protein plays a role in stabilizing the VP1 capsid structure by preventing the virus particle from degradation and disassembly (Bertolotti-Ciarlet ET AL., 2003; Jiang et al., 1992). To investigate whether HPP treatment destroyed the viral capsids, equal amounts of viral proteins (before and after HPP) were analyzed by SDS-PAGE. As shown in Fig. 25A, we observed a 58-kDa VP1 protein band in the untreated sample in a 10% SDS-PAGE gel. Surprisingly, we detected an equivalent amount of VP1 protein from 350-, 500-, and 600-MPa-pressure-treated samples, demonstrating that the viral major capsid protein was not degraded by HPP. To visualize the minor capsid protein (VP2), we analyzed the virus samples in a 12% SDS-PAGE gel. As expected, two protein bands (VP1 and VP2) with molecular sizes of approximately 58 and 18 kDa, respectively, were visualized (Fig. 25B, lane 2). Neither of the viral capsid proteins (VP1 and VP2) was degraded (Fig. 25B, lane 3) after 600-MPa pressure treatment despite the fact that viruses were completely inactivated and virus structure was completely disrupted under this condition (Fig. 24D). These results suggested that HPP treatment may not affect the primary and secondary protein structures of the viral capsid proteins but damages the tertiary and quaternary structures of the viral capsids, which results in the disruption of the virion.

To determine whether pressurized viral proteins were still antigenic, we performed Western blot analysis using a polyclonal antibody against the VP1 protein. Briefly, total viral proteins before and after pressure (600 MPa for 2 min) treatment were analyzed by
SDS-PAGE, followed by Western blotting. As shown in Fig. 25C, similar amounts of VP1 from untreated and treated samples were detected by Western blotting. This result confirmed that pressure-treated VP1 still reacted with antibody despite the complete disruption of the three-dimensional structure of VP1.

Figure 25. HPP does not degrade viral capsid proteins. (A) Visualization of MNV-1 capsid protein by 12% SDS-PAGE. The purified MNV-1 was pressurized at 350, 500, and 600 MPa at 4°C for 2 min. Total viral proteins were analyzed by 12% SDS-PAGE followed by Coomassie staining. VP1, major capsid protein. (B) Visualization of MNV-1 capsid protein by 15% SDS-PAGE. The purified MNV-1 was pressurized at 600 MPa at 4°C for 2 min. Total viral proteins were analyzed by 15% SDS-PAGE followed by Coomassie staining. VP2, minor capsid protein. (C) Western blot analysis of VP1 protein. Identical samples from panel B were separated by SDS-PAGE and subjected to Western blotting using rabbit anti-MNV VP1 polyclonal antibody.
8. HPP does not degrade viral genomic RNA. It would be lethal to the virus if the viral genome was damaged. Therefore, RT-PCR was performed to determine whether HPP damages the viral RNA. Two primers were designed to amplify the VP1 gene from viral genomic RNA. Since viral genomic RNA was extremely sensitive to environmental RNase, all experiments were done under RNase-free conditions. As expected, a strong 1.5-kb DNA band (VP1 gene) was observed in RNA extracted from untreated viruses (Fig. 26A, lane 2). Similar amounts of the VP1 gene were detected in RNA extracted from viruses treated with 350, 500, and 600 MPa (Fig. 26A, lanes 2 to 4). This result suggests that viral genomic RNA was not degraded by HPP, at least under the conditions of 600 MPa for 2 min. To further confirm this result, we first extracted the viral genomic RNA from the virus and then subjected it to 350-, 500-, and 600-MPa treatment. As shown in Fig. 26B, similar amounts of VP1 were amplified from each sample, suggesting that viral genomic RNA was not degraded by HPP. Next, we purified some virus samples for which the final elution buffer (PBS) was not treated with diethyl pyrocarbonate (DEPC), a reagent that inactivates the RNase. Presumably, these virus samples were contaminated by environmental RNase. All other remaining steps were done under RNase-free conditions. These viruses were treated by three pressure levels, and viral RNA was extracted, followed by RT-PCR. As shown in Fig. 26C, only a weak VP1 band was observed at a pressure level of 350 MPa, and no VP1 gene was detected at a pressure level of 500 or 600 MPa (Fig. 26C). This result suggests that genomic RNA was liberated when the capsid was disrupted by HPP, which in turn was degraded by endogenous RNase. For virus-contaminated foods and environmental samples, we may not be able to detect any viral RNA genome after viruses are completely inactivated by HPP, since
many endogenous RNases exist in foods and the environment. Finally, we also treated the VP1 gene (double-stranded DNA) with three pressure levels. As shown in Fig. 26D, HPP does not degrade the VP1 gene. Altogether, these experiments demonstrated HPP that does not degrade viral nucleic acids.

Figure 26. HPP does not degrade viral genomic RNA. (A) Detection of VP1 gene from pressure-treated MNV by RT-PCR. Viral genomic RNA was extracted from either HPP-treated or untreated MNV-1. The VP1 gene of MNV-1 was amplified by one-step RT-PCR. (B) Detection of VP1 gene from pressure-treated viral genomic RNA by RT-PCR. Viral genomic RNA was extracted from MNV, and an equal amount of RNA was treated by HPP followed by RT-PCR. (C) Detection of VP1 gene from pressure-treated MNV contaminated by environmental RNases. MNV was purified and eluted in PBS buffer (not treated by DEPC). All other remaining steps were done under RNase-free conditions. These viruses were treated with three pressure levels, and viral RNA was extracted; this process was followed by RT-PCR. (D) Direct treatment of VP1 gene by HPP. The VP1 gene was amplified by RT-PCR, and an equal amount of purified VP1 gene was treated by HPP.
2.5. Discussion

Fresh produce is one of the high-risk foods for NoV contamination. Available evidence suggests that NoV becomes internalized and disseminated in fresh produce (Rawsthorne et al., 2009; Wei et al., 2010). None of the decontamination methods investigated thus far has been shown to effectively inactivate NoV in fresh produce (Cliver, 1997; Doyle and Erickson, 2008; Duizer et al., 2004; Koopmans and Duizer, 2004; Mormann et al., 2010). In this study, we systematically investigated the survival of a human NoV surrogate (MNV-1) by HPP. We found that MNV-1 was efficiently inactivated at optimized HPP parameters with minimal impact on the quality of certain fruits or fruit products. We further provided new mechanistic insight into virus inactivation by HPP. Our results suggest that HPP may be a novel intervention technology appropriate for processing select fruits and vegetables intended for frozen storage as well as related products such as purees, sauce, and juices.

**Susceptibility of MNV-1 to high pressure and the optimal inactivation condition.** Pressure, holding time, temperature, pH, and food matrix affect the effectiveness of virus inactivation by HPP. Generally speaking, the higher the pressure and the longer the holding time, the more effective inactivation becomes. However, consumer perception of food safety depends not only on microbial safety but also on food quality. Based on our results, MNV-1 can be effectively inactivated by HPP. The optimal conditions for inactivation of MNV-1 are refrigeration temperature and neutral pH with a treatment pressure of 450 MPa and a holding time of 2 min. Under this condition, 7.4-log
and 4.7- to 7.0-log virus reductions were achieved in DMEM and fresh produce, respectively, without significantly altering the physical quality of the food samples.

It should be noted that the susceptibilities of different viruses to HPP were significantly different. For instance, FCV can be efficiently inactivated (5-log reductions) at 300 MPa at ambient temperature for 3 min (Chen et al., 2005; Grove et al., 2008). For rotavirus, it has been reported that an 8-log virus reduction was achieved at 300 MPa and 25°C for 2 min in cell culture medium (Khadre et al., 2002). HAV can achieve more than 6 logs of reduction at 450 MPa at ambient temperature for 5 min in cell culture medium (Kingsley and Chen, 2009; Kingsley et al., 2002). Surprisingly, no virus reduction was observed for poliovirus or Aichi virus even at 600 MPa at ambient temperature for 5 min in cell culture medium (Kingsley et al., 2004; Wilkinson et al., 2001). Therefore, human NoV surrogates (such as MNV-1 and FCV) and the two cultivable food-borne viruses (HAV and rotavirus) can be efficiently inactivated by HPP between 500 and 600 MPa. Under this pressure range, bacterial vegetative cells, yeasts, and molds were also killed (Chen and Hoover, 2003; Chen, 2006), while bacterial spores can still survive above 1,000 MPa (Chen et al., 2005; Chen et al., 2005; Gross and Jaenicke, 1994). Taken together, these results suggest that HPP is capable of inactivating most common bacterial and viral agents with a minimal impact on the quality of certain food products.

**Effect of food matrix on virus inactivation.** The food matrix and type of substrate have a dramatic effect on the survival of pathogens during pressure treatment. Carbohydrates, proteins, ions, lipids, and other food constituents can offer a protective effect (Baert et al., 2009; Balny et al., 2002; Gross and Jaenicke, 1994). Under 450 MPa
at pH 4.0 and 4°C, there were almost 8-log virus reductions in DMEM, while only 5.8- and 4.7-log reductions were observed in strawberries and strawberry puree, respectively. In another example, we found that MNV-1 was more sensitive to HPP at higher pH in aqueous medium than in all food samples. However, lemon puree is a special case. Specifically, a large virus reduction was detected in lemon puree at 350 MPa even at an extremely low pH (2.5). In contrast, MNV-1 was more difficult to inactivate in strawberry puree when the pH was adjusted to 2.5 (Fig. 21). Perhaps food constituents of lemon puree confer lesser protection on MNV-1 than those of other types of purees. Another possibility is that some food components in lemon may directly inactivate MNV-1. These results demonstrated that the food matrix plays an important role in virus inactivation by HPP. It has been reported that sucrose, Ca$^{2+}$, and other cations protect bacteria from inactivation (Baert et al., 2009; Chen et al., 2006). Recently, Kingsley and Chen reported that a supplement of sodium chloride in liquid solution protected HAV and FCV from high-pressure inactivation (Chen et al., 2005; Kingsley and Chen, 2009; Kingsley et al., 2007). It is possible that salts may stabilize viral capsid proteins at high pressures.

**Effect of temperature on virus inactivation.** In both aqueous medium and food samples, we found that MNV-1 was more susceptible to HPP at a refrigerated temperature of 4°C than at an ambient temperature of 20°C. Under 350 MPa at pH 7.0, an 8.1-log virus reduction was observed at 4°C in aqueous medium. However, only a 4.1-log virus reduction was achieved at 20°C under the same pressure and pH. Under 450 MPa at 4°C, approximately 5- to 7-log virus reductions were found in all fresh produce, but only 4- to 5-log reductions were observed at 20°C (Fig. 20). Similar phenomena were
observed for FCV (Chen et al., 2005; Grove et al., 2005; Grove et al., 2008). Perhaps low temperature may promote the exposure of nonpolar side chains to water and increase the density of water molecules in a protein solvation cage to maximize protein denaturation. In contrast, HAV, a picornavirus, is more resistant to HPP at a lower temperature than at room temperature (Calci et al., 2005; Kingsley and Chen, 2009; Kinsley et al., 2002). Thus, different viruses may have different responses to temperature during HPP.

**Effect of pH on virus inactivation.** Our results demonstrated that pH is a critical parameter that influences the survival of MNV-1 after HPP. Specifically, we found that MNV-1 was more efficiently inactivated in a neutral pH environment than an acidic environment either at 4°C or at 20°C across the pressure range studied. In aqueous medium, MNV-1 was completely killed (8.1-log reductions) by 350 MPa of pressure at 4°C and pH 7.0, whereas only a 6.0-log virus reduction was observed at pH 4.0. In the same food matrix (strawberry puree), a 4.8-log virus reduction was achieved at pH 6.5; however, only a 2.8-log virus reduction was observed at pH 2.5. For different purees, a 4.3-log virus reduction was achieved in carrot puree (pH 5.8) at 350 MPa, whereas only a 3.4-log reduction was observed in strawberry puree (pH 3.5). This finding will be helpful to determine an optimal pressure to treat food products with different pH values. Our observation is consistent with the previous reports for FCV (Chen et al., 2005) and tobacco mosaic virus (Bonafe et al., 1998; Santos et al., 2004), which were also more resistant to HPP at lower pH. In contrast, a significantly enhanced inactivation of HAV was found in acidic environments (Kingsley et al., 2005; Kingsley and Chen, 2009; Kingsley et al., 2002). It is unclear why pH has an opposite effect on pressure inactivation
for different viruses. It is likely that the nature of viral capsid protein determines the susceptibility of virus to pH.

**Mechanism of virus inactivation by HPP.** Our study also provided a mechanistic insight into virus inactivation by HPP. For bacterial pathogens, it was reported that HPP denatured bacterial proteins and lipids, destroyed the integrity of bacterial membranes, inhibited key enzymes, and disrupted transcription and translation and cellular functions responsible for survival and binary fission (Baert et al., 2009; Chen and Hoover, 2003; Chen et al., 2006). However, the mechanism of virus inactivation by HPP is poorly understood. Although it has been suggested that virus inactivation by HPP may include the damage of viral proteins (Calci et al., 2005; Chen et al., 2005; Kingsley and Chen, 2009; Kingsley et al., 2002; Oliveira et al., 1999), the detailed mechanism of inactivation has not been experimentally demonstrated.

HPP affects only noncovalent bonds, such as ionic, hydrophobic, and hydrogen bonds (Buckow et al., 2008; Gross and Jaenicke, 1994; Heremans and Smeller, 1998), which suggests that primary and secondary protein structures remain intact. However, HPP may alter quaternary and tertiary structures and/or conformation of a protein that results in a misfolding or unfolding. In our study, we found that the two structural proteins of MNV-1 (VP1 and VP2) were not degraded by HPP from a 350- to 600-MPa pressure range (Fig. 25A and B), although MNV-1 was completely inactivated at 600 MPa for 2 min. However, the structure of the virion and viral capsid was completely destroyed, as shown by EM analysis (Fig. 24B). It is worth noting that pressure-treated VP1 protein still reacted with antibody, suggesting that VP1 was antigenic. This evidence
supports the hypothesis that the primary and secondary structures of viral proteins remained intact but that the quaternary and tertiary structures of viral proteins were damaged. It has been known that expression of VP1 capsid protein resulted in virus-like particles that are morphologically and structurally similar to native virions (Jiang et al., 1992). The minor capsid protein stabilizes the structure of VP1 (Bertolotti-Ciarlet et al., 2003). HPP may have affected the proper folding of VP1 and VP2, which in turn destroyed the three-dimensional structure of the proteins and thus disrupted the small round-structured virions. Most recently, it has been shown that HPP affected the receptor binding of the MNV-1 capsid, which led to the loss of viral infectivity (Tang et al., 2010). However, our study clearly demonstrated that the damage of the viral capsid structure is the primary mechanism of virus inactivation. Disruption of the viral capsid would likely impair the functions of capsid such as attachment, receptor binding, and virus entry.

It is known that HPP usually does not affect covalent bonds. Thus, it may not break down viral genomic RNA. Previously, Tang et al. (2010) showed that there was a significant concentration of the VP1 gene detected from HPP-treated MNV-1. This is not surprising, since they reported a 3-log virus survival post-HPP treatment. Sánchez et al. used a quantitative RT-PCR (RT-qPCR) method to evaluate the survival of MNV and human NoV genogroup II.4 and showed that the RNA of human NoV was more resistant to HPP (up to 500 MPa) than the RNA of MNV (Sánchez et al., in press). However, it is not clear whether their experiments were performed under RNase-free conditions. In contrast, our RT-PCR was performed using RNA samples from the completely inactivated viruses after 600 MPa for 2 min. There were equivalent amounts of VP1 gene amplified from untreated and treated viruses. A similar result was obtained when viral
genomic RNA was directly treated by HPP. Thus, these results demonstrated that the viral genomic RNA was not degraded by HPP at a pressure range from 350 to 600 MPa for 2 min. Taken together, these results suggest that the primary mechanism of MNV-1 inactivation by HPP is the damage of viral capsid structure but not genomic RNA.

**HPP may be a novel intervention to enhance the safety of fresh produce.** A number of commercial HPP applications have been successful, including raw bivalve shellfish, yogurt, avocado products, chopped onions, and ready-to-eat meat products (Baert et al., 2009; Calci et al., 2005; Chen et al., 2006; Grove et al., 2008; He et al., 2002). Our results suggest that HPP may be a practical processing intervention for the control of food-borne viruses in fresh-produce-related products, such as purees, sauces, and juices, as well as fruits intended for frozen storage. With the exception of lettuce, HPP at 350 MPa did not affect the sensory qualities of the tested fresh produce such as appearance, color, and aroma; a more severe textural loss was observed in lettuce, raspberries, and strawberries at 600 MPa. In the last two decades, there have been many NoV outbreaks associated with frozen fruits, including blueberries, raspberries, strawberries, and grapes (Allwood et al., 2004; Baert et al., 2008; Beuchat, 1996; Butot et al., 2008; Cotterelle et al., 2005; Falkenhorst et al., 2005; Le Guyader et al., 2004). These fruits are important ingredients for salads, cakes, purees, juices, smoothies, creams, ice creams, and other related products. Furthermore, viruses survive longer during frozen and chilled storage (D'Souza et al., 2006; Rzezutka et al., 2004). Measures used to control bacterial levels, such as decreasing temperature and lowering water activity, have no effect on viruses (Baert et al., 2008; Butot et al., 2008; Estes et al., 2006; Koopmans and Duizer, 2004). No reduction of MNV-1 was observed in frozen onions and spinach even
after 6 months of freezing (Baert et al., 2008). Since HPP can efficiently inactivate both surface and internalized viruses, application of HPP in the fresh produce industry will significantly improve the safety of fresh produce.

In summary, our study demonstrated that human NoV surrogate MNV-1 is effectively inactivated by HPP under optimum processing conditions of moderate pressure level, refrigeration temperature, and neutral pH. HPP may be a promising intervention to eliminate and minimize the NoV risk in fresh produce and related products, with a minimal impact on food quality.
CHAPTER 3

COMPARISON OF THE SENSITIVITIES OF ENVELOPED AND NON-ENVELOPED VIRUSES TO HIGH PRESSURE PROCESSING:
A NO CLEAR-CUT RELATIONSHIP

3.1. Abstract

High pressure processing (HPP) is a non-thermal technology that has been shown to effectively inactivate a wide range of microorganisms. However, the effectiveness of HPP on inactivation of viral pathogens is relatively less understood. In this study, we systematically investigated the effects of HPP parameters (pressure, time, temperature, and pH) on the inactivation of a non-enveloped virus (human rotavirus, HRV) and two enveloped viruses (Vesicular stomatitis virus, VSV; and avian metapneumovirus, aMPV). We demonstrated HPP can efficiently inactivate all tested viruses at optimal conditions, although the pressure susceptibilities and the roles of temperature and pH varied among these viruses regardless if a viral envelope was present. We found that VSV was much more stable than most foodborne viruses, whereas aMPV was highly susceptible to HPP. Under the pressure of 350 MPa at 4°C held for 2 min, 1.1-log, 3.9-log, and 5.0-log virus reductions were achieved for VSV, HRV, and aMPV, respectively. Both VSV and aMPV were more susceptible to HPP at higher temperature and lower pH. In contrast, HRV was more easily inactivated at higher pH and temperature did not have a significant impact on
its inactivation. Furthermore, we demonstrated virion structure damage caused by disrupting the viral envelope and/or the viral capsid is the primary mechanism underlying HPP-induced viral inactivation. In addition, VSV glycoprotein remained antigenic although VSV was completely inactivated. Taken together, our findings suggest that HPP is a promising technology to eliminate most viral contaminants in high risk foods and provides a novel approach in preparation of inactivated vaccines.

3.2. Introduction

High pressure processing (HPP) is a non-thermal pasteurization technology that uses pressure instead of thermal energy to inactivate harmful pathogens. In the food industry, HPP has been used to inactivate foodborne pathogens (such as bacteria, viruses, fungi, protozoa and prions) and proteins (such as enzymes, allergens, and toxins) (Chen and Hoover, 2003; Chen et al., 2006; He et al., 2002; Rastogi et al., 2007). The primary advantage of HPP is that it only has minimal effect on the orgranelleptic and nutritional properties of foods, since the pressure acts instantaneously and uniformly throughout foods to achieve homogenous treatment (Balny et al., 2002; Chen et al., 2006; Grove et al., 2006; Kingsley et al., 2005). In modern society, consumers are increasingly demanding food products that are minimally processed and free of preservatives (Rastogi et al, 1994). Thus, HPP is becoming a widely used non-thermal processing technology that can ensure food safety, food quality, and shelf-life extension.

Despite the extensive research on the inactivation of bacterial pathogens by HPP, the inactivation of viruses by HPP is less understood. For food safety purposes, HPP studies have been focused on food- and water-borne viruses, including human norovirus,
rotavirus, sapovirus, astrovirus, adenovirus, poliovirus, enterovirus 71, hepatitis A, and hepatitis E viruses, all of which are non-enveloped viruses (Carter, 2005). It is known that these viruses are highly stable in food, water, and other fomites. Interestingly, the susceptibilities of food- and water-borne viruses are highly diverse when treated with HPP despite their high similarities in virion structure, protein composition, and genetic material. Poliovirus, a picornavirus, is extremely resistant to HPP, with less than 1-log virus reduction achieved after treatment at 600 MPa for 1 h (Wilkinson et al., 2001). Conversely, hepatitis A virus (HAV), another picornavirus, is quite sensitive to HPP, with more than 5-log virus reduction observed after treatment at 350 MPa for 2 min (Kingsley et al., 2002). Additionally, virus inactivation is dependent upon both processing parameters (pressure, temperature, and time) and non-processing parameters (pH, salt, water activity, and food matrix composition) (Chen et al., 2005; Grove et al., 2006; Grove et al., 2008). The effect of some parameters on viral survival is predictable. For example, virus inactivation is enhanced when pressure and holding time are increased, and food matrices can protect virus particles from inactivation (Lou et al., 2011; Kingsley and Chen, 2008). Of these parameters, temperature and pH may play variable or completely opposite roles in inactivating viruses. Murine norovirus (MNV-1), a surrogate for uncultivable human norovirus, was more efficiently inactivated at a cooler temperature (4°C) than at ambient temperature (20°C) (Lou et al., 2011). In contrast, inactivation of HAV was favored at ambient temperature (Kingsley and Chen, 2009). Moreover, MNV-1 is more sensitive to HPP at neutral pH than at acidic pH, whereas inactivation of HAV is favored at acidic pH (Lou et al, 2011; Kingsley and Chen, 2009).
To date, the mechanism by which temperature and pH govern the sensitivity of viruses to HPP is yet to be elucidated.

In comparison with non-enveloped viruses, the sensitivity of enveloped viruses to HPP is less understood. Although most enveloped viruses are not foodborne, a better understanding of the inactivation of enveloped viruses would help to identify the optimal processing parameters, which can facilitate many other important applications of HPP such as preparation of inactivated vaccines. To date, there are only a few studies on high pressure inactivation of enveloped viruses. For example, the titers of herpes simplex virus type 1 (HSV-1) and human cytomegalovirus (HCMV) were reduced by more than 7 and 4 logs treated at 300 MPa at 25 °C for 10 min, respectively (Nakagami et al., 1992). Additionally, a pressure of 260 MPa held for 12 h at 20 °C reduced 4 logs of vesicular stomatitis virus (VSV) (Silva et al., 1992). However, no study has been reported that directly compared the sensitivities of non-enveloped and enveloped viruses to HPP. Furthermore, the role of temperature and pH regarding the inactivation of enveloped viruses has not been investigated.

In this study, we systematically investigated the effects of HPP parameters (pressure, time, temperature, and pH) on the inactivation of a non-enveloped virus (human rotavirus, HRV) and two enveloped viruses (Vesicular stomatitis virus, VSV; and avian metapneumovirus, aMPV) in aqueous media. We chose VSV and aMPV because both belong to non-segmented negative-sense RNA viruses and share many common characteristics in terms of virion structure, replication, and gene expression strategy. Surprisingly, we found that VSV was much more stable than most foodborne viruses in
the presence of pressurization, whereas aMPV was highly susceptible to HPP. Both VSV and aMPV were more sensitive to HPP at higher temperature and lower pH. In contrast, HRV was more sensitive to HPP at higher pH, and temperature did not have a significant impact on its inactivation. These results suggest that the virus susceptibility to HPP is independent of the presence of an envelope, and temperature and pH play distinct roles in virus inactivation.

3.3. Materials and Methods

3.3.1. Viruses and cell lines

Human rotavirus (HRV) strain WA (ATCC, Manassas, VA.) was propagated in Rhesus monkey kidney cells (MA-104), which were grown in Eagle’s MEM (ATCC) supplemented with 10% non-heat inactivated fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA). Confluent monolayers of MA-104 cells were washed with serum-free MEM and infected by HRV at a multiplicity of infection (MOI) of 1. After 1 h incubation at 37°C under a 5% CO₂ atmosphere, 15 ml of Eagle’s MEM supplemented with 2% FBS and 6 µg/ml of trypsin (Invitrogen) was added. Virus was harvested after 48 h post inoculation by three freeze-thaw cycles followed by low speed centrifugation at 1,500 × g for 15 min. Vesicular stomatitis virus (VSV) Indiana strain was a generous gift from Dr. Sean Whelan at Harvard Medical School and avian metapneumovirus (aMPV) subtype C Minnesota strain was kindly provided by Dr. Mo Saif at The Ohio State University. VSV and aMPV were propagated in BHK cells and Vero-E6 cells, respectively. Briefly, both cells were cultured in high-glucose Dulbecco’s modified Eagle medium (DMEM)
(Invitrogen) supplemented with 10% FBS. Confluent cells were infected with VSV and aMPV at MOI of 0.01 and 1, respectively. Viruses were harvested when extensive cytopathic effect was observed.

3.3.2. Viral plaque assay

Viral plaque assays were performed as described by Lou et al. (2011) with minor modifications. HRV plaque assay was performed in MA-104 cells. VSV and aMPV plaque assay were conducted in Vero-E6 cells. Briefly, cells were seeded into six-well plates (BD, Franklin Lakes, NJ) at a density of 2 x 10^6 cells per well. After 24 h of incubation, cell monolayers were infected with 400 µl of a 10-fold dilution series of each virus, and the plates were incubated for 1 h at 37°C, with agitation every 10 min. The cells were overlaid with 2.5 ml of Eagle minimum essential medium (MEM) containing 1% agarose, 2% FBS, 1% sodium bicarbonate, 0.1 mg of kanamycin/ml, 0.05 mg of gentamicin/ml, 15 mM HEPES (pH 7.7), and 2 mM L-glutamine. Additionally, HRV and aMPV plaque assays were performed in the presence of 2.5 µg/ml trypsin and 1 µg/ml actinomycin-D, respectively. Incubation periods for HRV, VSV, and aMPV were 3, 2, and 6 days, respectively. The plates were fixed in 10% formaldehyde, and the plaques were visualized by staining with 0.05% (wt/vol) crystal violet.

3.3.3. Purification of VSV and HRV

Purification of VSV was performed as described previously (Li et al., 2005). Briefly, large stocks of VSV were generated by an infection of 10 confluent T150 flasks of BHK cells. After 24 h post-infection, the cell lysate containing the virus and cell debris
was harvested by centrifugation at 1,500 \times g for 30 min. Virus was then concentrated by ultracentrifugation in a Ty50.2 rotor (Beckman Coulter, Fullerton, CA) at 40,000 \times g for 90 min at 4°C. The pellet was resuspended in 300 μl of NTE buffer (100 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 7.4) and further purified through a 10% sucrose (w/v) NTE cushion by centrifugation for 1 h at 150,000 \times g at 4°C in an SW50.1 rotor (Beckman). The final VSV-containing pellet was resuspended in 300 μl of NTE buffer for overnight. The virus titer and protein content were measured by the plaque assay and Bradford reagent (Sigma Chemical Co.), respectively.

Purification of HRV was performed as described by Chen et al. (1992). Briefly, a large stock of HRV was grown in 8 flasks of confluent MA-104 cells as described above. Virus was harvested by three freeze-thaw cycles, followed by low-speed centrifugation (1,500 \times g) for 30 min. The supernatant was purified by ultracentrifugation through a 40% (w/v) sucrose cushion for 5 h at 82,000 \times g at 4°C in Ty50.2 rotor (Beckman), followed by CsCl isopycnic gradient (1.37 g/ml) ultracentrifugation (115,000 \times g, 18 h, 4°C in an SW50.1 rotor). The virus-containing band was collected and suspended into TNC buffer (0.05 M Tris-HCl, 0.15 M NaCl, 15mM CaCl$_2$, pH 6.5). After centrifugation at 68,000 \times g for 2 h, the final HRV-containing pellet was resuspended in 300 μl of NTE buffer overnight.

### 3.3.4. Pressure inactivation of viruses

One ml aliquots of each virus (10$^6$ PFU/ml of HRV and aMPV; 10$^8$ PFU/ml of VSV) in cell culture medium were packaged in sterile polyethylene stomacher pouches
(Fisher Scientific International, Ontario, Canada) and double sealed in a larger pouch to avoid leakage using an Impulse sealer (American International Electric, Whittier, CA).

Pressurization of samples was carried out in a high-pressure unit with temperature control (Model Avure PT-1, Avure Technologies, Kent, WA) using water as hydrostatic medium. The virus samples were treated at a range of pressure levels from 200 MPa to 550 MPa at different temperatures (4°C and 20°C) at various holding times (from 2 min to 10 min). The holding time for all the HPP treatments reported in this study did not include the pressure come-up time (approximately 22 MPa/s) and release time (<4 s). All the temperatures referred here were the initial sample temperatures. The temperature and pressure during processing were monitored and recorded (DASYTEC USA, Bedford, NH). The surviving viruses after processing were quantified by viral plaque assay and the inactivation kinetics for each virus was determined.

To determine the effect of pH on viral inactivation by HPP, DMEM was artificially adjusted to pH 4.0 and pH 7.0 using citric acid and then filtered by a 0.22 μm membrane filter. The final virus concentration in the pH-adjusted medium was approximately 10^6 PFU/ml. For HRV and aMPV, samples were pressurized at 250 MPa and 300 MPa at 4°C. For VSV, samples were pressurized at 450 MPa and 550 Mpa at 20°C. The holding time for all treatments was 2 min. After pressurization, virus survivors were quantified by plaque assay.
3.3.5. Transmission electron microscopy

Purified VSV suspensions were treated at 550 MPa and 4°C for 8 min and 10 min, while HRV was fully inactivated upon 450 MPa at 4°C for 2 min. Twenty µl of either HPP treated or untreated samples were fixed in copper grids (Electron Microscopy Sciences, Inc.), and negatively stained with 1% ammonium molybdate. The VSV and HRV particles were visualized by FEI Tecnai G2 Spirit Transmission Electron Microscope (TEM) operated at 80 kV at Microscopy and Imaging Facility at The Ohio State University. Images were captured by a MegaView III side-mounted CCD camera (Soft Imaging System, Lakewood, CO).

3.3.6. Analysis of viral proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The proteins of VSV and HRV before and after HPP treatment were analyzed by SDS-PAGE. Four µl of each virus suspension was diluted five-fold in SDS-PAGE loading buffer containing 1% SDS, 2.5% β-mercaptoethanol, 6.25 mM Tris-HCl (pH 6.8) and 5% glycerol, and boiled for 5 min. Viral proteins were separated on 12% polyacrylamide gel, followed by Coomassie blue staining.

3.3.7. Western Blotting

VSV virus proteins were separated by 12% SDS-PAGE as described above and further transferred onto a Hybond ECL nitrocellulose membrane (Amersham, Piscataway, NJ) in a Trans-Blot Semi-Dry electrophoretic transfer cell (Bio-Rad, Hercules, CA). The blots were blocked in 5% skim milk in PBST (phosphate-buffered...
saline supplemented with 0.02% Tween) and incubated with mouse monoclonal anti-VSV glycoprotein (G) antibody (Sigma-Aldrich) at a dilution of 1:5,000 in blocking buffer. Afterwards, the blot was incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG secondary antibody (1:100,000 dilution). After being washed in PBST for three times (15 min per time), the blots were developed with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Pittsburg, PA) and exposed to Kodak BioMax MR film (Kodak, Rochester, NY).

3.3.8. Reverse transcription polymerase chain reaction (RT-PCR)

Purified VSV was treated at 550 MPa and 20°C for 8 min and 10 min, respectively. The samples were digested with 10 unit of RNase at 37°C for 1 h. Viral genomic RNA was extracted from 100 μL of VSV suspensions (either HPP treated or untreated) using an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. RT-PCR was performed using a One Step RT-PCR kit (Qiagen). Two primers (5’-ATGTCTGTACAGTCAAGAG-3’ and 5’-TCATTTGCTCATTCTGAC-3’) were designed to amplify VSV N gene. The PCR products were analyzed on 1% agarose gel electrophoresis.

3.3.9. Statistical analysis

All experiments were performed in triplicate. Virus titer was expressed as mean log titer ± standard deviation. Statistical analysis was conducted using two-sided Student’s t test. A P value of <0.05 was considered statistically significant.
3.4. Results

1. The effects of temperature and pH on pressure inactivation of HRV.

Eagle’s MEM medium was inoculated with HRV at the final concentration of $10^6$ PFU/ml and then processed under different pressures (200-450 MPa) and temperatures (4°C and 20°C) for 2 min. As shown in Fig. 2A, similar HRV inactivation kinetics was observed at both temperatures. The survival curve exhibited a concave downward slope, followed by a flat tail. As the pressure increased, the rate of inactivation became more efficient. HRV was rapidly inactivated at the pressure range of 200-300 MPa and remained at low titer (1-2 log) at the pressure range of 300-400 MPa. Approximately, a 5-log virus reduction was observed under 400 MPa at either 4 or 20°C. Viruses were completely inactivated when the pressure increased to 450 MPa. Interestingly, temperature did not significantly affect the effectiveness of HRV inactivation throughout the tested pressure range, ($P > 0.05$) although higher temperature (20°C) slightly enhanced HRV inactivation when the pressure level was above 250 MPa. These results demonstrate that (i) HRV can be effectively inactivated by HPP and (ii) temperature plays a minor role in the inactivation of HRV.

The pH value is a critical parameter that affects the effectiveness of pathogen inactivation by HPP. Thus, we evaluated the survival of HRV at pH 4.0 and pH 7.0 under two different pressures (250 and 300 MPa) at 4°C. As shown in Fig. 2B, HRV was more easily inactivated at neutral pH than at acidic pH during HPP under both tested pressure levels tested ($P < 0.05$). At 250 MPa, 3.4- and 1.3- log virus reductions were observed at pH 7.0 and pH 4.0, respectively. Similarly, at 300 MPa, 4.1-log PFU/ml of HRV lost...
infectivity at pH 7.0, which was significantly higher than at pH 4.0 (1.9 logs). Overall, HRV was more sensitive to HPP at higher pH in aqueous medium.

Figure 27. Effect of temperature and pH on the pressure inactivation of HRV in aqueous medium. (A) HRV stock (10⁶ PFU/ml) was processed under pressure ranging from 200 MPa to 250 MPa for 2 min at either 4°C or 20°C. (B) HRV was inoculated into cell culture medium with pH 4.0 and pH 7.0 to a final concentration of approximately 10⁵ PFU/ml and pressure treated at 250 MPa and 300 MPa for 2 min at 4°C. The surviving viruses were determined by plaque assay. Data are the means of three replicates. Error bars represent ± standard deviation.

2. The effects of temperature and pH on pressure inactivation of VSV. To date, most HPP studies have been focused on non-enveloped viruses such as MNV-1,
FCV, and HAV. However, the sensitivity of enveloped viruses to HPP is poorly understood. Generally, enveloped viruses are less stable than non-enveloped viruses due to the fact that the lipid bilayer envelope is more sensitive to environmental stress than a protein capsid (Silva et al., 1992). To compare the stability of enveloped and non-enveloped viruses to HPP, we evaluated the efficacy of HPP in inactivating VSV, an enveloped virus. Fig. 28 represents the survival of VSV after high pressure treatment. Surprisingly, VSV was much more resistant to HPP than non-enveloped viruses reported previously. For example, only 1.1-1.5-log virus reductions were achieved for VSV at the pressure range of 350 to 550 MPa for 2 min at 4°C (Fig. 28A). In contrast, similar treatment conditions under 400 MPa brought about 7, 6, and 5 logs virus reductions for MNV-1, HAV and HRV, respectively (Lou et al., 2011; Kingsley and Chen, 2009). We also evaluated the effect of the temperature on inactivation of VSV by HPP. As shown in Fig. 28A, VSV was more easily inactivated at 20°C than at 4°C ($P < 0.05$). At 550 MPa held for 2 min, 1.4- and 3.4-log reductions of VSV were observed at 4°C and 20°C, respectively. Since VSV is highly resistant to HPP, we increased the HPP holding time to improve the efficacy of VSV inactivation. As shown in Fig. 28B, virus inactivation was enhanced when the holding time was increased. Specifically, approximately 5-log reduction of VSV was achieved upon treatment at 550 MPa for 4 min. VSV was completely inactivated when HPP holding time increased to 10 min. Taken together, these results demonstrate that (i) VSV, an enveloped virus, is much more resistant to HPP than most foodborne viruses which are non-enveloped and (ii) VSV is more easily inactivated at 20°C than 4°C.
Subsequently, we determined the role of pH in VSV inactivation by HPP. Briefly, VSV was inoculated into DMEM at pH 4.0 and pH 7.0, and subjected to HPP at two different pressures (450 and 550 MPa). At 450 MPa, there is no significant difference on VSV survival between pH 4.0 and pH 7.0 (P>0.05). However, at 550 MPa, VSV was more easily inactivated at pH 4.0 (5.7-log reduction) than at pH 7.0 (4.0-log reduction) (Fig. 28C). It appears that VSV is more sensitive to HPP at acidic pH than at neutral pH. However, we also noticed VSV was unstable in an acidic environment. 1-log virus reduction was observed when the unpressurized VSV control was incubated in DMEM at pH 4.0 for 24 h at 4°C. Therefore, the instability of VSV in acidic environments may be magnified in the presence of high pressure.
The effects of temperature and pH on pressure inactivation of aMPV. To further investigate the impact of HPP on enveloped viruses, the inactivation kinetics of aMPV, another enveloped virus, was determined. In contrast to VSV, aMPV is quite sensitive to HPP (Fig. 29). Approximately 5.1-log virus reduction was achieved upon treatment at 350 MPa at 4°C for 2 min (Fig. 29A), whereas the same treatment only reduced VSV by 1.1 logs (Fig. 28A). Furthermore, inactivation of aMPV was significantly enhanced at 20°C in comparison to 4°C within the pressure range of 200
MPa to 350 MPa ($P < 0.05$). After treatment at 300 MPa, a 4.8-log reduction was observed at 20°C; however, the corresponding reduction at 4°C was about 3.5 logs (Fig. 29A). In combination with our observations with VSV, these results suggest that different enveloped viruses have different susceptibilities to HPP. However, it is also true that both VSV and aMPV were easily inactivated at higher temperatures. Overall, the behavior and responses of different viruses to HPP appear to be independent of the presence of envelopes.

The effect of pH on inactivation of aMPV was also determined. As shown in Fig. 29B, aMPV is much more sensitive at pH 4.0 than at pH 7.0 under both 250 MPa and 300 MPa. At the pressure of 300 MPa, aMPV in acidic medium was completely inactivated; however, there were still 3.5 logs of virus survival after the same treatment at pH 7.0. Moreover, aMPV is considerably stable in an acidic environment. For untreated control samples, there is no significant reduction of virus titer at pH 4.0 during the experimental time period. Taken together, these data support the notion that aMPV is more easily inactivated at acidic condition by HPP than at neutral pH.
4. HPP damages virus particles. To determine the mechanism of virus inactivation by HPP, we analyzed the pressurized virus particles by electron microscopy (EM). Briefly, highly purified VSV samples (10^{10} PFU/ml) were treated under 550 MPa at 20\degree C for 8 min and 10 min separately. While approximately 1 log of VSV remained infectious after 8 min-pressurization, VSV was completely eliminated after 10 min treatment. Similarly, purified HRV (10^8 PFU/ml) was completely inactivated after treatment at 450 MPa at 4\degree C for 2 min. The samples were negatively stained with
ammonium molybdate and the virus particles were observed by EM. In the absence of pressurization, the VSV virion is a bullet-shaped particle that is approximately 70 nm in diameter and 140 nm in length (Fig. 30A). After treated by HPP for 8 min, the morphology of VSV particles was severely distorted. Most virions were round rather than bullet-shaped. The viral envelope was disrupted and became ambiguous (Fig. 30B). As expected, more severe damage was observed when the treatment time increased to 10 min. Indeed, we failed to find any intact VSV virion after 10 min treatment (Fig. 30C). The viral envelopes were lost and the viral nucleocapsid-genomic RNA (N-RNA) complex spilled out of the damaged particles. In addition, a large number of debris was observed. These observations indicated that the VSV structure and envelope were completely disrupted by HPP.

**Figure 30. HPP disrupts VSV particles.** Purified VSV was treated at 550 MPa at 20°C for 8 min and 10 min separately. Treated and untreated virus particles were negatively stained with 1% ammonium molybdate and visualized by TEM.
The geometry of intact rotavirus is wheel-shaped with a diameter of approximately 70 nm (Fig. 31A). After HPP, no intact wheel-like particles were observed. Instead, we found a large number of broken particles mixed in with debris (Fig. 31B). The outer capsid was dissociated and virus particles were disrupted by HPP. Additionally, some empty particles were observed, indicating that the inner core containing genomic RNA spilled out of the particles due to HPP. Taken together, HPP disrupted virus particles which resulted in virus inactivation.

**Figure 31. HPP disrupts HRV particles.** Purified HRV was completely inactivated at 450 MPa at 4°C for 2min. HPP-treated and untreated samples were negatively stained by 1% ammonium molybdate and visualized by TEM.

5. **The effect of HPP on viral proteins.** It is generally accepted that HPP alters quaternary and tertiary structures, but not primary and secondary structures, of proteins (Rastogi et al., 2007). To determine whether HPP disrupted viral structural proteins, we
analyzed the pressurized viruses by SDS-PAGE. VSV possesses five structural proteins. The viral genomic RNA is completely encapsidated by nucleocapsid protein (N) that forms the N-RNA complex. The large polymerase protein (L) and phosphoprotein (P) are tightly associated with N-RNA complex resulting in the formation of the RNP complex. The RNP complex is further surrounded by a matrix (M) protein and the viral envelope. The viral glycoprotein (G) is anchored in the envelope. As shown in Fig. 32A, all five structural proteins, L, G, P, N and M, were visualized by SDS-PAGE. The abundance of each viral protein was similar in all untreated and treated and samples (P>0.05), suggesting that even though HPP completely disrupted the virions, the primary or even the secondary structure of viral proteins remained intact upon pressurization. The viral proteins were subjected to Western blot using a monoclonal antibody against VSV G protein. Comparable amounts of VSV G protein were detected in untreated and treated samples, indicating that G protein remained antigenic upon HPP (Fig. 32B). Of note, a light band corresponding to a protein smaller than intact G protein was also detected in the sample treated for 10 min, which may result from partial degradation of G protein after high levels of pressure at a longer holding time.
Similarly, we analyzed the effect of HPP on HRV structural proteins. The rotavirus virion is composed of three concentric layers of proteins and 11 segments of double-stranded RNA. The outermost layer of the virion is comprised of two proteins, VP4 and VP7. VP4 forms dimeric spikes that project from the surface of the virion, which is constituted by VP7. The middle layer is comprised of VP6. The innermost layer

Figure 32. HPP does not degrade the viral proteins of VSV. (A) Visualization of VSV capsid proteins by 12% SDS-PAGE followed by Coomassie staining. The purified VSV was treated at 550 MPa and 20°C for 8 min and 10 min. (B) The viral proteins of untreated and HPP-treated VSV samples were separated by SDS-PAGE and subjected to Western blotting using mouse monoclonal anti-VSV glycoprotein.
is composed of three proteins, VP1, VP2 and VP3. As shown in Fig. 33, all six structural proteins were observed in untreated HRV, although VP3 and VP4 were difficult to separate on a 12% polyacrylamide gel due to their similar molecular weights. However, HRV VP1 was significantly diminished after 450MPa pressure treatment. Decreased amounts of VP2, VP3, and VP4 were also found in the HPP-inactivated virus samples. In contrast, the abundance of VP7 remained unchanged after HPP. Apparently, HPP leads to considerable reduction in the abundance of some viral proteins.

![Figure 33. Effect of HPP viral proteins of HRV.](image)

The purified HRV was pressurized at 450 MPa at 4°C for 2min. The six capsid proteins of untreated and treated HRV were analyzed by 12% SDS-PAGE followed by Coomassie staining.
6. **HPP does not degrade the viral genomic RNA.** Previously, we reported that HPP did not degrade viral genomic RNA of MNV-1 (Lou et al., 2011). However, disruption of the capsid of MNV-1 rendered viral genomic RNA sensitive to RNase degradation. VSV genomic RNA is strikingly different from that of MNV-1. The RNA genome of MNV-1 is positive-sense and naked. In contrast, the negative-sense RNA genome of VSV is completely encapsidated by the N protein, forming the N-RNA complex. To determine whether VSV genomic RNA was released from N protein, we performed an RNase degradation assay. Briefly, the untreated and pressurized VSV were digested by 10 Units of RNase, followed by RNA extraction and RT-PCR to amplify VSV N gene. The VSV genomic RNA would be degraded by RNase if it has been separated from N protein. As shown in Fig. 34, a 1.3-kb band responding to VSV N gene was present in all untreated and treated samples regardless of the presence of RNase. The intensities of such bands were essentially identical, indicating that HPP was not able to separate VSV genomic RNA from N protein although VSV was completely inactivated at 600MPa for 10 min at 20°C. This result also suggests that VSV N protein is highly resistant to HPP.
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Figure 34. HPP does not degrade viral genomic RNA of VSV. The purified VSV was treated at 550 MPa and 20°C for 8 min and 10 min. Unpressurized and pressurized samples were digested by RNase and followed by RNA extraction. N gene was detected by one-step RT-PCR.

3.5. Discussion

Since the late 1980’s, HPP has been emerging as a supplement to conventional thermal processing for eliminating microbial load and a viable substitution to chemical preservatives in the food industry (Rastogi et al., 2007). While most HPP studies have focused on inactivating foodborne vegetative bacteria, yeast, moulds, and worms,
inactivating viruses by HPP has been understudied (Knorr, 1995; Rastogi et al., 2007; Grove et a., 2006). Recently, a number of studies have shown that HPP is capable to significantly reduce the load of foodborne viruses, but the pressure resistance of these viruses varies considerably. In order to better understand factors contributing to HPP-induced virus inactivation, we systematically investigated and compared the barosensitivities of one non-enveloped virus (HRV) and two enveloped viruses (VSV and aMPV) under different pressure, temperature and pH conditions. Overall, all three viruses were efficiently inactivated at optimal processing conditions, and such optimal conditions were specific for each virus, indicating the pressure sensitivity and the impacts of temperature and pH on HPP-induced inactivation varied accordingly to viruses. We also demonstrated that the disruption of virion structure by disrupting the viral envelope and/or damaging the viral capsid was the major mechanism underlying HPP-induced inactivation of all these viruses. In combination with previous studies in our laboratory as well as in other groups, these results strongly support that HPP represents a universal and practical strategy to control the health threat caused by viruses.

**Susceptibility of enveloped and non-enveloped viruses to HPP.** Most enveloped viruses are dependent on their envelopes for their infectivity (Sun and Whittaker, 2003). Thus, it is generally accepted that an enveloped virus is less stable than a non-enveloped virus since the lipid envelope is susceptible to dryness, pH changes, heat, organic solvents, disinfectants, and many other stresses (Sands et al., 1979; Maillard, 2001). In this study, we directly compared the pressure inactivation profiles of enveloped (VSV and aMPV) and non-enveloped (HRV) viruses. We demonstrated that
these viruses showed different sensitivities in response to HPP regardless of the presence of a viral envelope. While VSV is much more stable than either HRV or aMPV after HPP, the pressure resistance of aMPV is substantially lower than that of HRV. For example, under treatment of 350 MPa at 4°C for 2 min, 1.1-log, 3.9-log, and 5.0-log virus reductions were achieved for VSV, HRV, and aMPV, respectively. Apparently, VSV exhibited a remarkably high resistance to HPP, which is consistent with previous findings showing that it took up to 12 h to achieve a 4-log reduction of VSV under 260 MPa at 20°C (Silva et al., 1992). VSV and aMPV belong to the family *Rhabdoviridae* and *Paramyxoviridae*, respectively, in the order of *Mononegavirales* (Silva et al., 1992; Broor and Bharaj, 2007). Even though they share considerable resemblance in virion structure and other biological properties, the question remains with regards to the structural basis underlying their distinct responses to HPP. Of note, pressurization of a few other enveloped viruses, such as avian influenza virus, HSV-1, and HCMV has also been reported. While treatment under 550 MPa at 15°C for 90 s was able to efficiently inactivate avian influenza virus (H7N7), the titers of HSV-1 and HCMV were reduced by approximately 7 and 4 logs, respectively, upon HPP at more than 400 MPa for 10 min at 25°C (Nakagami et al., 1992; Isbarn et al., 2007). Taken together, these results indicate that the susceptibility to pressure varies among enveloped viruses, and VSV is most stable among tested viruses.

Similarly, non-enveloped viruses also exhibited diverse sensitivities to HPP. Effective viral inactivation (at least 5-log reduction) resulted from pressure treatment (≤ 600 MPa) was reported for a variety of non-enveloped viruses, including human norovirus surrogates (feline calicivirus and murine norovirus), HAV, HRV, and
coxsackievirus A9 (Lou et al., Kingsley et al., 2002; Khadre and Yousef, 2002; Buckow et al., 2008; Cannon et al., 2006; Kingsley et al., 2004). In contrast, the infectivity of Aichivirus and coxsackievirus B5 remained unaffected after 600 MPa-treatment at ambient temperature for 5 min (Kingsley et al., 2004). Moreover, poliovirus is highly resistant to HPP as evidenced by the fact that less than 1-log virus reduction achieved under 600 MPa for 1 h (Wilkinson et al., 2001). Notably, both coxsackievirus and poliovirus belong to the genus Enterovirus within the family Picornaviridae, but their sensitivities to high pressure are significantly different from each other.

Collectively, the pressure susceptibilities vary substantially among viruses regardless of the presence of a viral envelope. There is no consensus regarding virus resistance to HPP as judged by the size of the particles or the similarities in virion structure and other biological properties. Also, the sensitivity may not be comparable among viruses within the same genus, family, and/or order. The specific determinants for the sensitivity of a virus to HPP remain to be further elucidated. Perhaps, the sensitivity of a non-enveloped virus may be related to the three dimensional structure of the viral capsid; thus, differences in the primary amino acid sequences of viral capsid proteins could have a bearing on its resistance to pressure. For enveloped virus, the sensitivity may depend on the stability of the envelope and surface glycoproteins. A virus would lose its infectivity when glycoproteins are disrupted or disassociated from the envelope. Hence, enveloped viruses could have different stabilities under pressure since the lipid bilayer of each virus is unique in composition as well as in interactions with inner core and/or surface glycoproteins.
**Effect of temperature on pressure inactivation of viruses.** Temperature is one of the key factors that affect the efficiency of virus inactivation by HPP (Chen et al., 2005; Kingsley and Chen, 2009; Oliveira et al., 1999; Lou et al., 2011). Since all viruses become susceptible to inactivation if the temperature reaches a certain point (56°C), we selected two temperatures (4 and 20°C) at which viruses are relatively stable. This allows us to determine the effect of pressure on viral survival and not thermal energy itself. We found that enveloped viruses, VSV and aMPV, were more easily inactivated at higher a temperature across the entire pressure spectrum. For example, about 4.9 logs of aMPV were inactivated under 300 MPa at 20°C for 2 min, whereas only a 3.5-log virus reduction was observed after the same processing at 4°C. For VSV, an additional 2-log virus reduction was achieved upon a 2 min-treatment of 550 MPa at 20°C than 4°C. Presumably, the higher susceptibility to pressure at higher temperature may be ascribed to the heat sensitivity of the viral envelope even though further studies are needed to determine whether this phenomenon is observed with other enveloped viruses.

The role of temperature in HPP-induced inactivation of non-enveloped virus appears to be complicated. In the case of HRV, we found that temperature did not significantly influence the effectiveness of inactivation throughout the entire pressure range. In comparison, previous studies in our laboratory showed that MNV-1 is significantly favored at refrigerated temperature (4°C). An 8-log reduction of MNV-1 was observed at 400 MPa at 4°C for 2 min, whereas the same treatment at 20°C only brought about a 3-log reduction (Lou et al., 2011). Similarly, a 4-min treatment of FCV under 200MPa at -10°C led to a 5.0-log reduction while the corresponding reduction at 20°C was only 0.3 logs (Chen et al., 2005). Conversely, inactivation of HAV appears to
be favored at ambient temperature (20°C). Treatment of HAV under 400MPa for 1 min at
-10°C and 20°C reduced the titers by 1.0 and 2.5 logs, respectively (Kingsley et al.,
2006). These observations suggest temperature plays distinct roles in HPP-inactivation of
non-enveloped viruses through unknown mechanisms.

Nonetheless, pathogen inactivation is enhanced after pressurization in
combination with thermal energy (usually above 50°C), a processing procedure termed
“temperature-assisted HPP”. For example, HPP (400 MPa) at 50°C for 1 min reduced the
titer of HAV by 4.7 logs, whereas similar treatments at -10 and 20°C only gave 1- and
2.5-log reductions, respectively. Moreover, treatment of FCV at 200MPa at 20 and 50°C
led to 0.3- and 4.0-log reductions, respectively. Similar enhancement has been observed
with bacteria and spores upon temperature-assisted HPP (Kingsley et al., 2006; Chen et
al., 2005; Rastogi et al., 2007; Grove et al., 2006). Therefore, combination of pressure
and temperature would provide an alternative strategy to inactivate highly resistant
pathogens.

**Effect of pH on pressure inactivation of viruses.** pH is a critical non-processing
parameter. Like temperature, pH may have a completely opposite effect on virus
inactivation by HPP. Here we demonstrated HRV was more sensitive to HPP at neutral
pH than acidic pH (pH 4.0). After treatment at 250 MPa for 2 min, about 3.4 logs of HRV
lost its infectivity at pH 7.0, which was significantly higher than at pH 4.0 (1.3 logs). Our
previous study also showed that inactivation of MNV-1 was substantially enhanced at
neutral pH. For example, an 8-log virus reduction was observed at 400 MPa at pH 7.0 for
2 min, whereas only 5 logs of MNV-1 were inactivated at pH 4.0 under the same
treatment (Lou et al., 2011). It was also found that inactivation of FCV and tobacco mosaic virus favored neutral pH (Santos et al., 2004; Kingsley and Chen, 2008). In sharp contrast, inactivation of HAV was significantly enhanced at pH 4.0. Treatment of HAV at 400 MPa at pH 4.0 and 7.0 for 2 min resulted in 7.0- and 4.0-log virus reductions, respectively (Kingsley and Chen, 2009). Mechanisms by which pH modulates virus inactivation by HPP are yet to be determined. It is possible that pH affects the stability of the capsid proteins of non-enveloped viruses, and thus contributing to HPP-induced inactivation. In the presence of pressure, a virus could be more easily inactivated if its capsid proteins undergo unfavorable structural changes at a specific pH. This notion is supported by a study demonstrating that rotavirus spike protein VP4 underwent an irreversible conformational change at elevated pH, and rotavirus with altered VP4 lost its infectivity (Pesavento et al., 2006).

As for VSV and aMPV, our results showed that their inactivation by HPP was favored at acidic pH. For instance, 4.6 logs of VSV lost its infectivity at pH 4.0 under 550 MPa, whereas only a 2.9-log reduction was observed at pH 7.0. The effect of pH on aMPV inactivation was more dramatic. 6.0- and 2.2-log virus reductions were achieved at pH 4.0 and pH 7.0, respectively, at 300 MPa for 2 min. It is well known that the viral envelope is highly susceptible to acidic pH, which may contribute to the enhancement in HPP-induced inactivation of enveloped viruses at acidic pH. It is also likely that surface glycoproteins are more easily disassociated from the envelope and/or more easily undergo unfavorable structural changes at acidic pH, thus resulting in loss of viral infectivity.
Mechanism of virus inactivation by HPP. Our previous studies on MNV-1 showed that the disruption of viral capsid structure, but not the degradation of genomic RNA, was the primary mechanism underlying HPP-induced inactivation (Lou et al., 2011). To determine whether this is a universal mechanism, we extended our study to VSV and HRV whose virion structures are strikingly different compared to MNV-1. For VSV, we found that the morphology of its virion was severely altered, the structure and envelope were completely disrupted, and the RNP complex spilled out from particles after HPP. For HRV, the outermost layer of the virion was disrupted and the inner core was released from the particles. For both VSV and HRV, large amount of debris were observed in the HPP-inactivated viruses. These observations further support the notion that the mechanism underlying HPP-induced virus inactivation is the disruption of the virion structure by breaking the viral envelope and/or damaging the viral capsid. Because of the limitation of EM, we are unable to determine whether viral glycoproteins dissociated from the envelope or underwent irreversible conformational changes upon HPP. Putatively, any damage to viral glycoproteins could impair virus attachment and entry, which in turn results in the loss of infectivity. Indeed, as discussed below, our results do not rule out the possibility that HPP destabilizes viral glycoproteins, and thus contributing or partially contributing to HPP-induced viral inactivation.

Due to its mechanic nature, HPP generally does not cause significant changes in the primary and secondary structures of a protein (Buckow et al., 2008; Gross and Jaenicke, 1994; Heremans and Smeller, 1998; Lou et al., 2011). Consistent with this, the abundance of all VSV structural proteins was not significantly altered despite the fact that virion structure was completely disrupted. However, the situation with HRV is somehow
different. After HPP, the amount of structural protein VP7 remained unchanged; the abundance of other structural proteins, namely, VP1, VP2, VP3, and VP4 was reduced to a certain extent, indicating that HRV structural proteins have distinct stabilities under pressure. It may be possible that that some proteins are degraded when the pressure reaches a point beyond the endurance of some covalent bonds. It is also important to note that HPP inactivates viruses but preserves its immunogenic properties. As shown in Figure 32B, even though the VSV virion was completely damaged, its G protein was still recognized by anti-G protein monoclonal antibody, indicating that HPP-treated G protein remained antigenic. From this perspective, HPP-induced inactivation of viruses may provide a novel approach in vaccine preparation.

In conclusion, HPP is capable of efficiently inactivating most common viral agents although different viruses have varying pressure susceptibilities and require different conditions for efficient inactivation. Moreover, disruption of the virion structure, viral capsid, and envelope, but not the degradation of viral genomic RNA, is the primary mechanism of viral inactivation by HPP. Overall, HPP is a promising technology to eliminate viral contaminants in high risk water and foods. More importantly, HPP tends to be a promising method to generate effective vaccines since it inactivates viruses without losing immunogens.
CHAPTER 4

CONCLUSION

First, this study demonstrated that high pressure processing (HPP) is capable of effectively inactivating murine norovirus (MNV-1), the most relevant surrogate for human norovirus, in both aqueous and food systems under optimized processing condition. Specifically, under pressure treatment of 400 MPa at 4°C held for 2 min, 8-log virus reduction of MNV-1 was observed in aqueous medium and at least 5-log virus inactivation was achieved in all the tested fresh produce. Pressure inactivation of MNV-1 was favored at refrigeration temperature and at neutral pH.

Second, this study demonstrated that HPP may be a feasible technology for processing fresh produce, particularly fruits intended for frozen storage and related products such as purees, sauces, and juices. At pressure level less than 500 MPa, the overall impact of HPP on the quality of fresh produce is minimal, although it varied with the type of produce to an extent.

Third, this study is the first report to directly compare the susceptibilities of enveloped and non-enveloped viruses treated by HPP. Our results demonstrated that HPP effectively inactivated both enveloped and non-enveloped viruses at optimized processing conditions. However, the pressure susceptibilities and the roles of temperature
and pH on HPP-induced inactivation varied considerably among different viruses regardless of the presence of a viral envelope. There is no consensus regarding virus resistance to HPP as judged by the size of the particles or the similarities in virion structure and other biological properties.

Fourth, this study systematically explored the mechanisms of pressure inactivation of both enveloped and non-enveloped viruses. We found the disruption of virion structure by damaging the viral envelope and/or the viral capsid, but not the degradation of viral genetic material, was the primary mechanism underlying HPP-induced virus inactivation. In general, HPP disrupted the quaternary and tertiary structures, but not the primary and secondary structures, of the viral capsid proteins. The pressurized viral proteins remained antigenic although the viruses were completely inactivated.

Overall, these results strongly support that (i) HPP is a novel intervention to effectively inactivate foodborne viruses in high risk foods with minimal alteration on food quality; (ii) HPP is capable of effectively eliminating other health threatening viral pathogens; and (iii) HPP tends to be a promising strategy to generate effective vaccines since it inactivates viruses without degrading immunogens.
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