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Pressure inactivation of Tulane virus, a candidate surrogate for human norovirus and its potential application in food industry

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ABSTRACT

Human norovirus (HuNoV) is the leading causative agent for foodborne disease. Currently, studies of HuNoV usually rely on surrogates such as murine norovirus (MNV) due to the lack of a suitable cell culture system and a small animal model for HuNoV. Tulane virus (TV), a monkey calicivirus, is a cultivable enteric calicivirus that not only recognizes the same receptors as HuNoV, but is also genetically closely related to HuNoV. In this study, we determined the pH stability of TV and MNV-1, as well as the effect of high hydrostatic pressure (HHP) on inactivating both viruses in aqueous media, blueberries and oysters. We demonstrated that both TV and MNV-1 were very stable under an acidic environment. They were more resistant to pressure at an acidic environment than at neutral pH. Pressure treatment of 600 MPa for 2 min at different temperatures (4, 21 and 35 °C) barely caused any reduction of TV, as well as MNV-1, on un-wetted (dry) blueberries. However, both TV and MNV-1 on blueberries were successfully inactivated by a pressure of ≤400 MPa when blueberries were immersed in phosphate-buffered saline during HHP. Pressure inactivation of both TV and MNV-1 in blueberries and oysters increased as sample temperature decreased in the order of 4 > 21 > 35 °C. TV was more sensitive to pressure than MNV-1 for the three matrices tested, culture media, blueberries and oysters. This study provides important information on the use of TV as a surrogate for HuNoV study. Results obtained from this study lay a foundation for designing effective HHP treatments for inactivation of HuNoV in high-risk foods such as berries and oysters.

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1. Introduction

Human Norovirus (HuNoV), belonging to family *Caliciviridae*, is the leading cause of foodborne illnesses with an estimated 5.5 million (58%) cases each year in the United States (Scallan et al., 2011). It is mainly transmitted through fecal–oral route, by person-to-person contact or by consuming contaminated foods and water (Lou et al., 2011a). Fruits and vegetables can be contaminated at the pre-harvest stage through contact with fecally contaminated irrigation water or organic-based fertilizer in the field (Carter, 2005; Rodríguez-Lázaro et al., 2012). For shellfish, polluted growing/harvesting water is a potential source of viral contamination (Lees, 2000). Therefore, fresh produce including berries and shellfish have been involved with many HuNoV outbreaks (Falkenhorst et al., 2005; Lees, 2000; Ponka et al., 1999). In 1993, a large multistate outbreak of gastroenteritis related to HuNoV by consumption of oysters occurred (Dowell et al., 1995). With over 4 million oysters harvested and an attack rate of 62% among oyster eaters, the authors estimated that as many as 186,000 people might have become ill (Dowell et al., 1995). Symptoms of HuNoV infection involve diarrhea, vomiting, nausea, abdominal cramping, chills, headache, dehydration, and a high-grade fever (Li et al., 2012). HuNoV is highly contagious because only a few particles can cause infection (Koopmans and Duizer, 2004; Li et al., 2012; Teunis et al., 2008). The main difficulty hindering research of HuNoV is that there is no available *in vitro* cell culture system or small animal model so that studies have to rely on surrogate viruses.

Feline calicivirus (FCV) and murine norovirus (MNV) have been widely used as such surrogates because they are cultivable calicivirus (Cannon et al., 2006; Li et al., 2012; Scipioni et al., 2008). Even though FCV belongs to the genus *Vesivirus* within the same family as HuNoV, it has different biochemical properties compared to HuNoV (Cannon et al., 2006; Li et al., 2012). Unlike FCV, MNV not only belongs to genus norovirus and has similar biochemical features compared with HuNoV, but is also resistant to acid and heat, and highly stable and persistent in the environment (Cannon et al., 2006; Li et al., 2012). In addition, MNV has similar genome size and gene organization as HuNoV (Karst et al., 2003; Li et al., 2012; Wobus et al., 2006). Therefore, MNV gradually replaced FCV as the main surrogate for HuNoV studies. However, MNV is still different from HuNoV in its pathogenesis and infection of MNV in murine does not cause the same symptoms as HuNoV (Karst et al., 2003; Li et al., 2012; Wobus et al., 2006). More importantly, MNV uses sialic acid as a functional receptor while HuNoV uses histo-blood group antigens (HBGAs) as receptors (Marionneau et al., 2002; Taube et al., 2009). Recently,

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HuNoV virus-like particle (VLP) was also proposed as a potential surrogate for HuNoV because VLPs have similar antigenicity and morphology as HuNoV (Lou et al., 2012). However, since VLPs do not contain viral genetic materials, the reliability of using them as a surrogate for HuNoV is compromised, especially in some treatments targeting the genome of HuNoV for inactivation. Farkas et al. (2008) reported the discovery of Tulane virus (TV), a monkey calicivirus isolated at the Tulane National Primate Research Center, and later demonstrated that TV was close to HuNoV based on its genomic sequence (Farkas et al., 2010). TV can be cultured in monkey kidney cells (LLC-MK2 cells). More importantly, similar to HuNoV, it recognizes the type A and B HBGAs (Farkas et al., 2010). One recent study demonstrates that TV and HuNoV behaved similarly when they internalized and disseminated in hydroponically grown Romaine lettuce (Dicaprio et al., 2012). Therefore, with its characteristics and closeness with HuNoV, TV could potentially serve as a surrogate for HuNoV. So far, there is no report of the stability of TV to pH, heat, disinfectant and food-processing technologies including high hydrostatic pressure (HHP).

The commercial application of HHP in the food industry has increased considerably in the last decade due to its advantages of inactivating foodborne pathogens with minimal impacts on the taste, texture, appearance and nutritional value of food (Li et al., 2012; Lou et al., 2011a, 2011b). Guacamole, fruit jams, jellies, ready-to-eat meats, salsa, orange juice and oysters are among some of the commercial food applications of HHP. During the last three decades, research on HHP has been mainly focused on bacterial inactivation, while studies on virus inactivation by HHP are relatively limited (Kovač et al., 2010). The efficacy of HHP on virus inactivation is closely associated with pressure level, treatment time, treatment temperature and pH of substrates (Kingsley et al., 2006; Kingsley and Chen, 2009; Lou et al., 2011a, 2011b; Wilkinson et al., 2001). Increasing the pressure level and treatment time would generally enhance virus inactivation, whereas the effect of treatment temperature and pH cannot be generalized and seems to depend on the individual virus itself (Kingsley et al., 2006; Kingsley and Chen, 2009; Lou et al., 2011a, 2011b). Human rotavirus was found to be more sensitive to pressure at neutral than acidic condition while temperature (4 and 20 °C) did not have a significant impact on its inactivation (Lou et al., 2011b). MNV was reported to be more sensitive to pressure at neutral than acidic condition and at a colder temperature (Lou et al., 2011a). Kovač et al. (2012a) also reported that human adenoviruses-2 was more resistant to pressure at acidic pH. Hepatitis A virus was reported to be more sensitive to pressure at a higher temperature and at acidic condition (Kingsley et al., 2006; Kingsley and Chen, 2009). Human rotavirus was more sensitive to pressure at higher pH and temperature did not have a significant impact on its inactivation (Lou et al., 2011b).

The overall objective of this study was to investigate the effect of HHP on TV inactivation by comparing it with MNV-1. Specifically we determined the pH stability of TV and MNV-1 and the effects of pH, presence of water during pressure treatment of food matrices and treatment temperature on pressure inactivation of TV and MNV-1. Effectiveness of HHP inactivation was evaluated in aqueous media as well as food matrices such as blueberries, and oysters.

2. Materials and methods

2.1. Virus and cell lines

A MNV strain (MNV-1) and murine macrophage cell line RAW 264.7 were generously provided by Dr. Jianrong Li at the Ohio State University. Tulane virus and monkey kidney cell line LLC-MK2 were generously provided by Dr. Xi Jiang at Cincinnati Children's Hospital Medical Center. MNV-1 and TV were propagated in RAW 264.7 and LLC-MK2 respectively. Raw 264.7 cells were cultured in high-glucose Dulbecco's modified Eagle medium (DMEM) (Life Technologies, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Life Technologies). MK2-LLC cells were cultured in M199 medium (Mediatech, Manassas, VA) supplemented with 10% heat-inactivated FBS (Life Technologies) and penicillin G (100 U/ml) and streptomycin (100 μg/ml). Both cells were cultured at 37 °C under a 5% CO_2 atmosphere. To grow MNV-1 stock, confluent RAW 264.1 cells were infected with MNV-1 at a multiplicity of infection (MOI) of 1. After 1 h of incubation at 37 °C under a 5% CO_2 atmosphere, 25 ml of DMEM supplemented with 2% FBS was added. MNV-1 was harvested 2 days after post-inoculation by three freeze–thawing cycles and centrifugation. Virus was stored at −80 °C until use. Same procedures were followed to grow TV except that cells were infected with TV at a MOI of 0.1 and 25 ml of M199 supplemented with 10% FBS was used after 1 h incubation period.

2.2. Viral plaque assay

MNV-1 and TV were quantified by plaque assay following published procedures with slight modifications (Farkas et al., 2008; Lou et al., 2011a). For MNV-1, RAW 264.7 cells were seeded into six-well plates (Becton, Dickinson and Company, Franklin Lakes, NJ) at a density of 2×10^6 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 and 5% CO_2 with gentle agitation every 10 min. Cells were overlaid with 2.5 ml of Eagle minimum essential medium (MEM) containing 0.5% agarose, 5% FBS, 0.12% sodium bicarbonate, penicillin G (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (0.25 μg/ml), 25 mM HEPES (pH 7.7), and 2 mM L-glutamine (Life Technologies). Plates were then incubated at 37 °C and 5% CO_2 for 2 days, and fixed in 3.7% formaldehyde (Fisher Scientific, Pittsburg, PA) and the plaques were visualized by staining with 0.05% (w/v in 10% ethanol) crystal violet. TV plaque assay was slightly different from MNV-1, LLC-MK2 cells were seeded to six-well plates at a density of 4×10^5 cells per well. Cells were overlaid with 0.5 volume of M199 medium, 10% fetal bovine serum, penicillin G (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (0.25 μg/ml), and 0.5% agarose. Plates were incubated at 37 °C and 5% CO_2 for 4 days before being fixed with formaldehyde.

2.3. pH stability study

Culture media containing FBS used for RAW 264.7 and LLC-MK2 cells were adjusted to pH 2, 3, 4, 7 and 10 using ~12 M HCl or 5 M NaOH and sterilized by filtration through 0.22 μm filter (EMD Millipore, Billerica, MA). One volume of TV (~10^6 PFU/ml) or MNV-1 (~10^8 PFU/ml) virus stocks was mixed with 9 volumes of corresponding cell culture media with the aforementioned pH values, as well as original media (pH 7.1–7.4) used as control. Solutions were incubated at room temperature (25 °C) for 1 h and were serially diluted in plain M199 (for TV) and DMEM (for MNV-1). Diluted virus solutions were used for plaque assay to determine titers for viruses.

2.4. HHP treatment of TV and MNV-1 in aqueous cell culture media

Culture media with 10% FBS for RAW 264.7 and LLC-MK2 cells were adjusted to pH 4 using HCl and sterilized by filtration. One volume of TV (~10^6 PFU/ml) or MNV (~10^8 PFU/ml) virus stocks were mixed with 9 volumes of corresponding cell culture media adjusted to pH 4 as well as the original media. Each virus solution (2 ml) was double bagged and double sealed in sterile polyethylene stomacher pouches (Seward, Port Saint Lucie, FL). TV samples were pressurized at 300 and 350 MPa while MNV-1 samples were pressurized at 350 and 400 MPa using a pressure unit with temperature control and with water as a hydrostatic medium (model Avure PT-1; Avure Technologies, Kent, WA). The experiments were conducted at 21 °C for 2 min. The pressure come-up rate was approximately 22 MPa/s and pressure release time was <4 s. Pressurization time reported in

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