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Thermal inactivation of human norovirus surrogates in oyster homogenate

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ABSTRACT

Human norovirus (HNV) is the most frequent causative agent of foodborne diseases in the US. Raw and undercooked oysters are commonly associated with outbreaks caused by HNV. Many guidelines recommend that shucked oysters be boiled for at least 3 min, but it is not clear this thermal treatment can inactivate HNV. The objective of this research was to evaluate whether this recommendation was sufficient to inactivate two HNV surrogates, murine norovirus (MNV-1) and Tulane virus (TV) in oyster homogenate as well as to determine their thermal inactivation kinetics. Inoculated oyster homogenate was heated in boiling water and circulating water bath at 49 to 67 °C for different time durations. After 3 min of boiling, both MNV-1 and TV titers decreased to below the detection limits. First-order model and Weibull model were used to describe thermal inactivation kinetics. TD = 1 values from Weibull mode are used as an analog to D values in first-order model. The D values of MNV-1 and TD = 1 values ranged from 28.17 to 0.88 min and 26.64 to 0.78 min at 49 to 67 °C, respectively. The D values of TV and TD = 1 values ranged from 18.18 to 1.56 min and 19.35 to 1.56 min at 49 to 63 °C, respectively. The kinetics demonstrated that at temperatures > 58 °C, TV was much more heat sensitive than MNV-1. As the temperature increased over 58 °C, the inactivation of both viruses occurred at a faster rate. Boiling treatment for 3 min as recommended by FDA for cooking shucked oysters, inactivated MNV-1 and TV in oyster homogenate below detection limit. One minute heating of TV at 63 °C or MNV-1 at 67 °C in contaminated oyster homogenate reduced the viral titers below the detection limits. Our research identified effective combinations of time and temperature to inactivate two HNV surrogate viruses, and thus provides insights on thermal processing to reduce the risk of foodborne viral illness outbreaks associated with consumption of oysters.

1. Introduction

In the United States, human norovirus (HNV) is the most common cause of acute gastroenteritis, leading to 19–21 million illnesses, 56,000–71,000 hospitalizations and 570–800 deaths each year (Hall et al., 2013). It is considered the most frequent causative agent causing more than half of foodborne diseases in the US cases each year (Scallan et al., 2011). Norovirus causes acute gastrointestinal infection with common symptoms include diarrhea, vomiting, nausea, abdominal cramping, chills, headache, dehydration and a high-grade fever (Li et al., 2013). It is highly contagious because only a few particles have the potential to cause infection (Donaldson et al., 2008). It can be spread by contaminated food or water, through person to person contact and via cross contamination surfaces (Hall et al., 2013). Raw and undercooked oysters are commonly involved in outbreaks caused by HNV; they are filter feeders and concentrate and retain viruses derived from the environment (Lees, 2000). From 1998 to 2015, 5362 foodborne outbreaks in the US were caused by norovirus, in 86 outbreaks of which oyster is the vehicle (Foodborne Outbreak Online Database (FOOD Tool), 2016). In 2016, approximately 75 people in the outer Cape Cod area in Massachusetts developed norovirus-like illness after eating raw shellfish (NoroCore, 2016). In 2002, over 100 people in Italy became ill after consuming norovirus contaminated oyster (Le Guyader et al., 2006).

It has been demonstrated that HNV can interact specifically with carbohydrate structures in the bivalve digestive organ (Le Guyader et al., 2000). Viruses cannot multiply in food or in the environment, but they can persist for several days or weeks without loss of infectivity (Seitz et al., 2011). Typical methods used to prevent bacterial growth in food products may not be effective against viruses (Jaykus, 2000). Therefore, the potential presence of HNV in oysters poses a serious health threat to consumers and is an important concern for health authorities (Hewitt and Greening, 2004). It is then essential to understand whether current seafood handling and processing can mitigate HNV survival and persistence in oysters.

Recently, Dr. Mary Estes and her research team have successfully

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grown human norovirus in enterocytes in stem cell–derived, non-transformed human intestinal enteroid monolayer cultures with bile (Ettayebi et al., 2016). However, before the research, the main difficulty that hampers research of HNV is there is no in vitro cell culture system or small animal model. As a result, most of research relies on viral surrogates, including feline calicivirus (FCV) and murine norovirus (MNV-1). FCV is structurally different from HNV and it is a respiratory virus and very sensitive to low pH (2.0 to 4.0) (Cannon et al., 2006; Li et al., 2012). MNV-1 has been shown to be more similar to HNV immunologically, biochemically, genetically, and molecularly. And it belongs to genus norovirus and is also resistant to acid and heat, and highly stable and persistent in the environment (Cannon et al., 2006; Li et al., 2012). However, clinical symptoms of gastroenteritis caused by MNV-1, which present as hepatitis, pneumonia, and inflammation of nervous systems, are quite different from that caused by HNV (Karst et al., 2003). More importantly, MNV-1 uses sialic acid as a functional receptor whereas HNV uses HBGA as receptors (Wobus et al., 2006; Tan and Jiang, 2010). Previous research on thermal inactivation showed that FCV and MNV-1 behaved similarly when heated at 63 °C (Cannon et al., 2006). It was reported that Tulane virus (TV), a calicivirus isolated from stools of rhesus macaques, represents a new genus, *Recovirus* (Farkas et al., 2008). TV can be cultivated in rhesus monkey kidney cells (LLC-MK2) and is close to HNV based on its genomic sequence (Farkas et al., 2010). More importantly, like HNV, it recognizes the type A and B HBGAs (Farkas et al., 2010). Therefore, it has the potential for use as a surrogate of HNV. In terms of thermal resistance, TV in culture medium is more heat sensitive than MNV-1 at 50 to 60 °C (Hirneisen and Kniel, 2013). However, there is no report of the stability of TV when heated in seafood matrix such as oysters.

Thermal processing is one of the most effective methods to reduce viruses in any food product. Cooking oysters thoroughly will impact organoleptic characteristics and can toughen oyster meat, which make them undesirable for consumers. Light cooking may be acceptable to some consumers, but might be insufficient to kill all enteric viruses, since most of the viruses are inside the shellfish and would not be subjected to sufficient heat for their total inactivation (Richards et al., 2010). It has been suggested that an internal temperature of 90 °C for at least 90 s is a virucidal treatment. FDA suggests that seafood is cooked to an internal temperature of 63 °C (145 °F) for 15 s (FDA, 2009), which ensures that food-borne bacteria is destroyed. Consumers, without thermometers, can rely on shells to open to determine the doneness of shellfish. However, this practice may be insufficient to reach the virucidal treatment. Previous studies on steaming mussel showed that the mean internal temperature was 83 °C when all 50 mussels were tested (Hewitt and Greening, 2006). It is also recommended by many guidelines that shucked oyster is simmered or boiled for at least 3 min (Villalba et al., 2008; Hicks, 2010). Since there is no specific regulation covering the minimum time–temperature combination for inactivating virus in contaminated oysters, establishment of proper thermal processes for inactivating HNV in a high risk food such as oysters would be essential for protecting public health.

The specific objectives of this study were to (1) determine thermal inactivation behavior of murine norovirus (MNV-1) and Tulane virus (TV) in oyster homogenate (2) test 3 min of boiling water heating efficacy on inactivation of both viral surrogates in oyster homogenate and (3) compare first-order and Weibull models to understand the kinetics of thermal inactivation behavior of two viral surrogates.

2. Material and methods

2.1. Viruses and cell lines

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 with 10% heat-inactivated fetal bovine serum (FBS) (Life Technologies) at 37 °C under a 5% CO_2 atmosphere. MK2-LLC cells were cultured in M199 medium (Mediatech, Manassas, VA) with 10% heat-inactivated FBS (Life Technologies) and penicillin G (100 U/ml) and streptomycin (100 μg/ml) at 37 °C under a 5% CO_2 atmosphere. To prepare 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. The 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. Virus was stored at −80 °C until use.

2.2. Oyster sample preparation and inoculation

Samples of 25-g oyster meats were homogenized with a homogenizer (OMNI international, Kennesaw, GA, 30144) for 1 min, then 2 ml of homogenate were added into a 15-ml conical centrifuge tube (Falcon). The homogenate was inoculated with 0.3 ml of virus (5–6 $\log_{10}$ PFU/ml), vortexed thoroughly and stored at 4 °C overnight.

2.3. Temperature – time profile of boiling water treatment and thermal treatment

A thermocouple was placed in the center of the uninoculated homogenate in a 15-ml centrifuge tube. Then the tube was immersed in the boiling water for 5 min. The thermocouples were connected to a portable data recorder (HH506RA, multilogger thermometer, Omega Engineering) to record the temperature. The water temperature was also monitored. As for the different thermal treatments, the samples were heated in a circulating water bath at 49, 54, 58, 63, and 67 °C for up to 5 min. The center temperature and water temperature were recorded every 2 s. The temperature-time profiles were tested 4 times. At each time two replicates were conducted.

2.4. Boiling water treatment of MNV-1 and TV in oyster homogenate

The tubes containing the inoculated oyster homogenate were immersed in boiling water. A thermocouple was placed in the boiling water to monitor the temperature. The samples were treated for 2, 3, and 4 min. After boiling water treatments, the tubes were cooled immediately in an ice-water bath for 10 min. Extraction and plaque assay were conducted for enumeration of viral survivors.

2.5. Thermal treatment of MNV-1 and TV in oyster homogenate

The inoculated oyster homogenate samples were added into centrifuge tubes and heated at 49, 54, 58, 63, and 67 °C in a circulating water bath for different treatment times (0 to 60 min). Duplicates were tested at each time. A thermocouple was placed in the water to monitor the water temperature. After the thermal treatment, the tubes were cooled immediately in an ice-water bath for 10 min. Extraction and plaque assay were conducted for enumeration of viral survivors.

2.6. Extraction MNV-1 and TV from oyster homogenate

MNV-1 and TV survivors were extracted from oyster homogenate using the procedures described by Ye et al. (2014) with slight modification. A volume of 18 ml glycine buffer (pH 9.5; 0.1 M glycine, 0.3 M NaCl) was added into 2 ml homogenate and mixed well at room temperature (21 °C). The mixture was centrifuged at 10,000 g for 15 min at 4 °C. An equal volume (20 ml) of 16% polyethylene glycol 8000 (PEG) with 0.525 M NaCl was added to the supernatant to precipitate viral particles. After a 1 h precipitation on ice, the mixture was centrifuged at 10,000 g for 10 min at 4 °C. The pellet was suspended in 2 ml PBS (pH 7.2), and then samples were stored at −80 °C until quantified by