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Thermal inactivation kinetic modeling of human norovirus surrogates in blue mussel (*Mytilus edulis*) homogenate

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ABSTRACT

Control of seafood-associated norovirus outbreaks has become an important priority for public health authorities. Due to the absence of human norovirus infectivity assays, cultivable surrogates such as feline calicivirus (FCV-F9) and murine norovirus (MNV-1) have been used to begin to understand their thermal inactivation behavior. In this study, the effect of thermal treatment on inactivation of human norovirus surrogates in blue mussels was investigated at 50, 56, 60, 65, and 72 °C for various times (0–6 min). The results obtained were analyzed using the Weibull and first-order models. The Theil error splitting method was used for model comparison. This method splits the error in the predicted data into fixed and random error. This method was applied to select satisfactory models for determination of thermal inactivation of norovirus surrogates and kinetic modeling. The *D*-values calculated from the first-order model (50–72 °C) were in the range of 0.07 to 5.20 min for FCV-F9 and 0.18 to 20.19 min for MNV-1. Using the Weibull model, the $t_{D=1}$ for FCV-F9 and MNV-1 to destroy 1 log ($D = 1$) at the same temperatures were in the range of 0.08 to 4.03 min and 0.15 to 19.80 min, respectively. The *z*-values determined for MNV-1 were 9.91 ± 0.71 °C ($R^2 = 0.95$) using the Weibull model and 11.62 ± 0.59 °C ($R^2 = 0.93$) for the first-order model. For FCV-F9 the *z*-values were 12.38 ± 0.68 °C ($R^2 = 0.94$) and 11.39 ± 0.41 °C ($R^2 = 0.97$) for the Weibull and first-order models, respectively. The Theil method revealed that the Weibull model was satisfactory to represent thermal inactivation data of norovirus surrogates and that the model chosen for calculation of thermal inactivation parameters is important. Knowledge of the thermal inactivation kinetics of norovirus surrogates will allow development of processes that produce safer shellfish products and improve consumer safety.

1. Introduction

Human norovirus outbreaks associated with consumption of seafood are an important public health problem which are well documented and recognized internationally (Cliver, 1988). Iwatoma et al. (2010) investigated the epidemiology of seafood-associated infections in the United States from 1973 to 2006, and found that human norovirus was the third most commonly reported pathogen associated with seafood and the most common viral agent, causing 77.5% of outbreaks of viral illness. They also stated that 21.3% of seafood associated outbreaks, including those associated with bivalve mollusks (85%), fish (12.5%), and crustaceans (2.5%), were caused by viruses. It has been shown that bivalve mollusks such as clams, cockles, mussels, and oysters, are especially prone to virus transmission and they present an elevated hazard because they are filter feeders. The exposure to human fecal contamination in their growing environment results in retention and concentration of any microorganism present, including viruses. Afzal and Minor (1994) stated that while bacteria are excreted quickly from bivalve mollusks, viruses are known to be persistent and, as a result, can be concentrated by mussels when present in their environment. Although effective methods for the bacterial depuration of contaminated mussels exist, these methods are not as effective for enteric viruses (de Medici et al., 2001). Also, as viruses do not multiply in food or in the environment, typical methods used to control bacterial growth in food products appear to be relatively ineffective against viruses (Jaykus, 2000). Thus, the presence of foodborne enteric viruses in bivalve mollusks constitutes a potential health risk for consumers and is an important concern for health authorities (Hewitt and Greening, 2004).

Current monitoring practices for mussel safety rely on bacteriological criteria that are not suitable for application to viruses. In general, mussels are prepared by cooking but they are often heated just until the shells open which is usually achieved at temperatures under 70 °C for 47 ± 5 s (Baert et al., 2007). It is evident that shell opening is not indicative of whether the product has reached the recommended internal temperature, and a minimum heating period is required to ensure that adequate temperature parameters are reached independently of whether the shells are opened (Hewitt and Greening, 2006), thus it is

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not sufficient for shellfish viral decontamination (Croci et al., 1999). The investigation of thermal inactivation characteristics and generation of precise thermal process data is required. Since there is no specific Federal regulation covering the minimum time–temperature combinations for inactivating virus contaminated mussels, establishment of proper thermal processes for inactivating human norovirus in foods would seem to be essential for protecting public health.

Despite its importance in public health, there is little information on norovirus thermal inactivation characteristics because the virus is nonculturable in the laboratory and infectivity can only be assessed using human dose (feeding studies) experiments. Cultivable surrogates such as feline calicivirus (FCV-F9) and murine norovirus (MNV-1) have been used as human norovirus surrogates in survival studies (Hewitt et al., 2009).

In the current literature, seafood such as cockles (Millard et al., 1987), mussels (Croci et al., 1999; De Medici et al., 2001; Baert et al., 2006; Johne et al., 2011), green shell mussels (Hewitt and Greening, 2006), marinated mussels (Hewitt and Greening, 2004), spiked molluscs (Croci et al., 2012), and soft shell clams (Sow et al., 2011) have been commonly used in studies involving detection, heat inactivation depuration, survival, persistence, and accumulation of human norovirus, hepatitis A, feline calicivirus, murine norovirus, and rotavirus. However, there are limited studies (Hewitt and Greening, 2006; Sow et al., 2011) involving thermal inactivation of human norovirus and/or surrogates in mussels. Thus, to our knowledge, there are limited thermal inactivation data for human norovirus surrogates in shellfish, and there is also no information on the thermal inactivation kinetics or models used to describe thermal inactivation. Some recent studies have evaluated different models to describe thermal inactivation kinetics of human norovirus surrogates in stool suspension (Tuladhar et al., 2012), and buffer (Dulbecco's modified eagle's medium supplemented with 10% fetal bovine serum) (Seo et al., 2012; Bozkurt et al., 2013). These studies revealed that the Weibull model was statistically superior in describing the thermal inactivation kinetics of norovirus surrogates than the first-order model.

Another method for analyzing and comparing different models was proposed by Henri Theil (Theil et al., 1966) and uses splitting of residual error into random and fixed sources. It was used by Harte et al. (2009) in determining the best model for inactivation of *Escherichia coli* in various heating media. In this study, the Theil error splitting method is used as a tool for analyzing and determining the best model to describe thermal inactivation behavior of norovirus surrogates in blue mussels.

The objectives of this study were to (i) determine thermal inactivation behavior of murine norovirus (MNV-1) and feline calicivirus (FCV-F9) in blue mussels, (ii) compare first-order and Weibull models for describing the data, and (iii) to evaluate model efficiency using the Theil method.

2. Material and methods

2.1. Viruses and cell lines

murine norovirus (MNV-1) was obtained from Dr. Skip Virgin (Washington Univ., St Louis, MO) and its host RAW 264.7 cells were obtained from the University of Tennessee, Knoxville. feline calicivirus (FCV-F9) and its host cells (Crandell Reese Feline Kidney, (CRFK)) were obtained from ATCC (Manassas, VA).

2.2. Propagation of viruses

FCV-F9 and MNV-1 stocks were prepared by inoculating FCV-F9 or MNV-1 onto confluent CRFK or RAW 264.7 cells, respectively in 175 cm^2 flasks and incubating at 37 °C and 5% CO_2 until >90% cell lysis was observed. The methods followed for the propagation of the viruses were as described in detail by Su et al. (2010).

2.3. Inoculation of mussel

Fresh blue mussel (*Mytilus edulis*) samples were purchased from a local seafood market. The blue mussels were reportedly harvested from the North Atlantic Coast during the winter season. The fresh mussel samples were shucked and homogenized using a Waring blender (Model 1063, Waring Commercial, USA) at a maximum speed. Five ml of each virus stock with initial titers of 8.06 ± 1.24 log PFU/ml for FCV-F9 and 7.14 ± 1.12 log PFU/ml for MNV-1 were individually added to 25 g of mussel sample in a sterile beaker and held at 4 °C for 24 h.

2.4. Thermal treatment

Heat treatment was carried out in a circulating water bath (Haake model V26, Karlsruhe, Germany) at selected temperatures (50–72 °C ± 0.1 °C) at different times (0–6 min) in 2 ml vial glass tubes. Sterilized vials (2 ml) were carefully filled with inoculated homogenized mussels by using sterile pipettes in a biosafety cabinet. The filled vials were surface washed in 70% ethanol before immersion in a thermostatically controlled water bath. Water bath temperature was confirmed with a mercury-in-glass (MIG) thermometer (Fisher Scientific, Pittsburgh, PA) and by placing type-T thermocouples (Omega Engineering, Inc., Stamford, CT) in the geometric center of the water bath. Another thermocouple probe was placed at the geometric center of a vial through the vial lid and in contact with the mussel sample to monitor the internal temperature. The thermocouples were connected to MMS3000-T6V4 type portable data recorder (Commtest Ins., New Zealand) to monitor temperature. Samples were heated at 50, 56, 60, 65, and 72 °C for different treatment times (0–6 min). The treatment time began (and was recorded) when the target internal temperature reached the designated temperature. The come up times for each temperature were 98, 104, 140, 158, 172 s for 50, 56, 60, 65, and 72 °C, respectively. Triplicate tubes were used for each time and temperature point. After the thermal treatment, sample vials were immediately cooled in an ice water bath for 15 min to stop further thermal inactivation. The vial contents were collected in a sterile beaker using a sterile pipette. The remaining contents of the vials were washed with sterile autoclaved elution buffer (described below) by using sterile pipettes to flush out the entire sample and the virus extraction protocol was followed. Unheated virus suspensions from mussels were used as controls and enumerated.

2.5. Virus extraction

The method for virus extraction was performed as described in Baert et al. (2006) with some modifications. Inoculated and thermally treated mussels were washed with 12.5 ml of elution buffer (1:6 ratio) containing 0.05 M glycine (which inhibits adsorption of negatively charged virus to the food surface in addition to blocking nonspecific binding) and 0.15 M NaCl (to assist precipitation), at pH 9.0 to allow the detachment of virus particles from the food matrix in the presence of an alkaline environment. After addition of the elution buffer to the thermally treated blue mussel samples, the pH was then adjusted to 9.0 using 10 M NaOH. Samples in the sterile beaker were kept shaking on a platform (120 rpm) for 20 min at 4 °C. Samples were centrifuged at 10,000 ×*g* for 15 min at 4 °C. The pH of the supernatant was adjusted to 7.2 to 7.4 using 6 N HCl followed by addition of polyethylene glycol (PEG) 6000 (to precipitate viruses and proteins in the sample) and NaCl to obtain a final concentration of 6% PEG (w/v) and 0.3 M NaCl (while the mechanism is not completely understood, these mask charges and hydrophilic residues on the virus surface facilitating their precipitation or "falling out" of solution). These samples were placed on a shaking platform (120 rpm) overnight at 4 °C and then centrifuged at 10.000 ×*g* for 30 min at 4 °C. The supernatant was discarded and the pellet dissolved in 2 ml phosphate buffered saline (PBS, pH 7.2) and