In vitro time and temperature dependence effects of three molecular weight (53, 421 and approximately1150 kilodaton) chitosans against human noroviral surrogates

A Thesis Presented for the

Master of Science Degree

The University of Tennessee, Knoxville

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May 2013

Acknowledgements

I would like to express my sincere gratitude to my major professor/advisor Dr. Doris H. D‘Souza for giving me an opportunity to work in her lab to complete my M.S. degree. I greatly appreciate her guidance, support, and encouragement throughout my research in her lab. I would like to thank Dr. Michael P. Davidson, Dr. Svetlana Zivanovic and Dr. Melissa Kennedy for serving on my committee, for their assistance, and input during my research. I would like to thank all my lab members for sharing their technical assistance and constructive advice. Last, but not least, I would like to thank my family members and friends for their encouragement throughout my studies. It is to them this work is dedicated.

Abstract

Chitosan, a deacetylated chitin, is known to have a broad spectrum of antimicrobial activity. The objectives of this research were to determine the *in vitro* time and temperature dependent effects of chitosan with different molecular weights (53, 421 and ~1150 kDa) at two concentrations (35 percent and 0.7%) against the human noroviral surrogates, feline calicivirus (FCV-F9) and murine norovirus (MNV-1) at two temperatures (37^{o}C and 18^{o}C) over 2 hours. The virus titers were determined using standard plaque assays and compared to untreated virus controls. The tested chitosans were shown to decrease FCV-F9 titers, but had no observable effects on MNV-1. Chitosan with molecular weight (M.W.) of 53 kDa at 0.35% decreased FCV-F9 titers by ~1.0 log PFU/ml, whereas at 0.7% decreased FCV-F9 titers by ~3.0 log PFU/ml after 2 h at 37^{o}C and by ~1.0 log PFU/ml after both 1 and 2 h at 18^{o}C. Chitosan with M.W. of 421 kDa at 0.35% decreased FCV-F9 titers by ~1.0 log PFU/ml whereas at 0.7% decreased FCV-F9 titers by ~2.0 at 37^{o}C after 2 h. Chitosans with 421 and ~ 1150 kDa at 0.35% and 0.7% decreased FCV-F9 titers by ~1.0 log PFU/ml after 2 h at 18^{o}C. Chitosan with ~ 1150 kDa at 0.35% decreased FCV-F9 titers by ~1.0 log PFU/ml whereas at 0.7% decreased FCV-F9 titers by ~2.0 log PFU/ml at 37^{o}C after 2 h. The results show that, in general, higher temperature and longer incubation cause greater inactivation of FCV-F9, with 53 kDa chitosan showing the greatest activity. Transmission electron microscopy did not show clear evidence of capsid damage of FCV-F9 treated chitosan and chitosan's mode of action could not be clearly elucidated. Overall, the tested chitosans showed minimal effect against MNV-1. Thus, caution must be used in interpreting the data based solely on chitosan's effects against FCV-F9 alone for the use of chitosan as a natural antiviral agent to control foodborne viral infections.

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Chapter 1 – Literature Review

Foodborne Viruses and Inactivation

Diseases of bacterial, viral, or parasitic origin are frequently spread through foods. Each year, foodborne diseases have been known to cause around 9.4 million illnesses and 1,351 deaths in the United States (Scallan et al., 2011). Viruses are estimated to be the responsible for 59% of the total foodborne illnesses, 27% of the hospitalizations, and 12% of the deaths (Scallan et al., 2011). Enteric viruses in water and food are an important cause of mortality in infants and young children in less developed countries (Bernstein, 2009).

Human enteric virus transmission is known to occur through contaminated food or water (by the fecal-oral route), person to person, aerosols, and contact with contaminated surfaces. Human enteric viruses have a low infectious dose and as few as 10 virus particles are capable of causing illness (CDC 2010a). Foods that are associated with transmission include shellfish, fresh produce and ready-to-eat (RTE) foods that do not undergo further processing (Sair et al., 2002a). Infected food handlers have been responsible for contaminating foods with viruses causing outbreaks of foodborne illness (Greig, et al., 2007). Human enteric viruses are environmentally stable, resistant to pH and enzymes of the gastrointestinal tract (Jaykus et al., 2000). Food serves as a vehicle for viral transmission because of the ability of the viruses to withstand many food processing and storage conditions (Sair et al., 2002b). The main viruses associated with foodborne and waterborne diseases include human noroviruses, hepatitis A virus, adenoviruses, Aichi virus (AiV), astroviruses, rotaviruses, sapoviruses, and other enteroviruses (CDC, 2010a; Hirneisen et al., 2010).

Human noroviruses belong to the *Caliciviridae* family, and are non-enveloped, single stranded, positive-sense RNA viruses. Their RNA is about 7.5 kilobytes in length. They are

icosahedral in shape and 27-38 nanometers in size with the RNA enclosed in a capsid (Donaldson et al., 2008; Hyde et al., 2009; Taube et al., 2010). Noroviruses are broadly classified into five genogroups and human norovirus isolates belong to genogroups GI, GII, and GIV. Genotypes GIII infect cattle and genotypes GV have been isolated from mice. GII genotypes, distinct from human strains, infect swine. Members of the genogroup GII are the most common strains and over time evolve into new variants, causing pandemic outbreaks about every 2–4 years such as the genogroup II.4b norovirus (Glass et al. 2009*;* Siebenga et al., 2009).

Human noroviruses usually cause relatively mild gastroenteritis, with vomiting and diarrhea which lasts for 1–3 days. However, there are reports which show serious consequences of norovirus infection, with benign seizures in infants (Chen et al., 2009) and chronic debilitating diarrhea in immunocompromised patients (Roddie et al., 2009). Usually, the incubation period following exposure to norovirus is between 24 and 48 h. The feces of infected individuals may contain 10^6 or more virus particles per gram (Teunis et al., 2008). Susceptibility to norovirus infection requires the involvement of acquired immunity and genetic resistance (Parrino et. al., 1977).Host genotype is an important factor in the development of norovirus infection because it depends on the presence of specific human histo-blood group antigen (HBGA) receptors in the gut of susceptible hosts. An increased susceptibility of Norwalk virus infection was associated to blood group O (Lindesmith et. al., 2003).

Human noroviruses cannot be cultured in the laboratories, which makes it difficult to assess the effectiveness of processing methods and interventions to prevent contamination (Duizer et al., 2004). Hence cultivable surrogates such as feline calicivirus (FCV-F9), murine norovirus (MNV-1), and coliphage MS2, are used to determine infectivity after treatments. Recently, Tulane virus has been also cultivated in the lab and is being investigated as an alternate

surrogate for human noroviruses (Herniesen and Kniel, 2013). A few properties of these surrogates are described below.

Feline calcivirus (FCV-F9) also belongs to the same *Caliciviridae* family as human noroviruses and has been most commonly used as a surrogate for human noroviruses in the past (Bidawid et al., 2000; D'Souza et al., 2006). However, FCV-F9 is a respiratory virus that is more susceptible to environmental conditions, like low pH than human noroviruses and so the suitability of this virus as a human norovirus surrogate remains questionable (Duizer et al., 2004; Perry et al., 2009; Radford et al., 2007; Cannon et al., 2006).

Murine norovirus (MNV-1) is a recently cultivable virus and is more resistant to environmental conditions than FCV-F9. Murine norovirus belongs to the genogroup V within the Norovirus genus and is shed in feces, and hence considered a more suitable surrogate for use under some conditions (such as low pH) (Bae and Schwab, 2008; Cannon et al., 2006; Su et al., 2009; Wobus et al., 2006).

Bacteriophage MS2 is a member of the *Leviviridae* family and like human noroviruses is a positive sense, single stranded RNA virus, about 22 - 29 nm in size with an icosahedral shape (Dawson et al., 2005; Langlet et al., 2007; Toropova et al., 2008). MS2 is used as a surrogate for RNA viruses for environmental studies of water and soil, since it is resistant to environmental conditions (Dawson et al., 2005; Guan et al., 2006; Shin and Sobsey, 2003).

Tulane Virus (TV) was recently isolated from the stool samples of captive juvenile rhesus macaques (*Macaca mulatta*) at the Tulane National Primate Research Center. TV has a typical calcivirus morphology, 36 nm in diameter, and represents a new calicivirus proposed genus "Recovirus" (rhesus enteric CV) (Farkas, et al., 2008). TV contains a complete genome of 6,714 nucleotides plus a poly (A) tail and similar to human noroviruses has three open reading

frames (ORFs) that encode the nonstructural (NS) polyprotein (ORF1), the capsid protein (ORF2), with a molecular mass of 57.9 kDa, as well as a minor structural protein (ORF3), with an isoelectric point (pI) of 10.0 (Farkas, et al., 2008). These researchers could culture TV in a monkey kidney cell line (LLC-MK2) with typical cytopathic effects.

Sapoviruses of the family of *Caliciviridae*, also contain RNA viruses of 7.5 kb in length. They were detected at high concentrations in stool samples of asymptomatic food handlers associated with an outbreak in Japan (Yoshida at al. 2009) along with other enteric viruses and associated with clams (Nakagawa-Okamoto et al., 2009). In 2007, Sapovirus caused a gastroenteritis outbreak affecting 55 college students in Taiwain (Wu et al., 2008). It was believed to be a foodborne illness though no vehicle of transmission of Sapovirus was identified (Wu et al., 2008).

Hepatitis A virus (HAV) is non-enveloped with single-strand positive sense RNA genome about ~7.5 kb in size and belongs to the *Picornaviridae* family. The symptoms are severe that lasts for 4 to 6 weeks including fever, abdominal discomfort followed by jaundice. HAV infection occurs by fecal–oral route and the virus is excreted in feces. Often, HAV outbreaks are a result of waterborne transmission although it has been traced to foods contaminated by food handlers as well as to foods contaminated before distribution. HAV incubation period averages 28 days (4 to 6 weeks) making it difficult to trace the origin/source in many outbreaks. HAV is relatively heat stable, survives more than a month in the environment and over a year in the frozen state (Greening, 2006a).

Thousands of HAV infection incidents are reported annually (2,585 cases in 2008), though the national incidence of HAV has dramatically decreased to 92%, since the introduction of HAV vaccines in 1995(CDC, 2010b). Hepatitis A vaccination is recommended for people at

increased risk for infection or complications from Hepatitis A infection, and for all children at 1 year of age (CDC, 2009). The HAV vaccines consist of two-dose series, with a booster dose administered 6-12 months after the primary immunization (CDC, 2009). In underdeveloped countries, most children are exposed to the HAV virus by age six and have a mild illness or are asymptomatic which induces life-long immunity. With better drinking water and sewage systems, children in more developed countries are not typically exposed to HAV, therefore a high percentage of the population lacks immunity. When people from developed countries travel to underdeveloped countries or consume foods harvested in underdeveloped countries, people may be exposed to HAV and become ill (Payne and Coulombier, 2009).

Adenoviruses belong to the family of *Adenoviridae*, and are icosahedral, non-enveloped viruses with a double-stranded DNA genome. Adenoviruses are often associated with respiratory infections. Adenoviruses have been detected in shellfish, wastewater, and surface waters in many locations. Certain serotypes, 40 and 41, cause gastroenteritis and have been suspected in foodborne infections (Greening, 2006a).

Aichi virus is a non-enveloped single-strand RNA virus of the genus Kobuvirus in the *Picornaviridae* family. The genome of Aichi virus contains 8,280 nucleotides and a poly (A) tail. Aichi virus was recently recognized as a cause of oyster-associated gastroenteritis (Yamashita, 1993). Aichi virus been detected in shellfish and diarrheic stool samples from China, Japan, France, Germany and other countries (Le Guyader, 2008*;* Vilarino 2009; Yang, 2009). There are other vehicles of foodborne infection suspected besides shellfish, but they have not yet been identified.

Astroviruses are non-enveloped positive-sense single-stranded RNA viruses with a genome size of 6.8 to 7.9 kb. Astroviruses cause enteric disease especially in children, elderly,

and immunocompromised persons. At 14 schools in Japan, a large outbreak of diarrhea affecting >4700 people was caused by astrovirus and food provided by a common supplier to the schools was believed to be the means of infection transmission (Oishi, 1994). Astroviruses also are known to cause an important disease in turkeys but there is no evidence that astrovirus is zoonotic (Koci and Schultz-Cherry, 2002).

Rotaviruses belong to the family *Reoviridae.* Rotaviruses contain double-stranded RNA (dsRNA) (Estes and Cohen, 1989), are non-enveloped icosahedral-shaped viruses approximately 75 nm in diameter. Rotaviruses are responsible for a large percentage of cases of diarrhea in children under 5 years of age. In the United States (U.S.) rotaviruses have been the most common cause agent for hospitalization for diarrhea among children. The recently developed rotavirus vaccine for children is expected to decrease morbidity (Bernstein, 2009). In Germany, rotavirus was detected in potato stew served during an outbreak in a sanatorium (Mayr et al., 2009). At a college, in the United States, sandwiches probably contaminated by a cafeteria worker were associated with an outbreak in 2000 (Fletcher at al., 2000).

Parvoviruses are very small, non-enveloped single-DNA viruses of average genome size of 5000 nucleotides long, belong to *Parvoviridae* family, of about 18–26 nm in diameter. Parvoviruses have been associated with human respiratory and foodborne illness traced to contaminated shellfish (Greening, 2006a).

Bocavirus, was first isolated in 2005, is associated with respiratory disease that has also been detected in the digestive tract. Bocavirus spread by the fecal–oral and respiratory routes. Bocavirus are very resistant to heat and many disinfectants (Chow and Esper , 2009*;* Eterpi, 2009).

Poliovirus belongs to the genus of enteroviruses of the family *Picornaviridae.* Polio virus has a single-stranded positive-sense RNA genome of about 7.5 kb nucleotides and the virus is 30 nm in diameter with an icosahedral symmetry. The most frequently reported vehicles implicated in foodborne outbreaks include raw milk, lemonade and cream-filled pastries (Cliver, 2008). Research has found that poliovirus has the ability to survive in the middle of hamburgers cooked to 60ºC (rare) (Sullivan, 1975) and in dry as well as semi-dry fermented sausages (Kantor and Potter, 1975). During 1960s to 1970s in the U.S, poliovirus was detected in market samples of raw meat (Cliver, 1986). With mass immunization campaigns, few human cases of polio now occur except in a few countries where routine polio vaccination of children has not been achieved. Even though polio was eradicated from the United States in 1979, polio is still documented in many developing countries and global eradication initiative programs are in place (de Quadros et al., 1992).

Control Strategies for foodborne viruses.

Interventions for controlling microorganisms in foods include low pH, drying, decreased water activity, thermal inactivation, irradiation, high pressure, ultraviolet light, Pulsed electric fields, and chemical disinfection including chlorine, chlorine dioxide and ozone.

Low pH

Many enteric viruses can tolerate pH values of 3 or 4 and as high as 9 or 10 (FAO/WHO, 2008). At pH 1, HAV was reported to remain infectious after 90 min and at pH 2.7, norovirus in a stool filtrate remained infectious after 3 h (Baert et al., 2009). Foodborne viruses must survive passage through the acidic stomach. At pH 4.5–5.5, little to no reduction in norovirus titers were reported after storage in foods like ketchup and salads (Mormann at al., 2010). Hence, low pH does not help to curb the spread of norovirus illness through food.

Drying/Decreased Water Activity

To limit bacterial growth, sodium chloride as well as drying (where reduction of water activity is obtained by drying or addition of water-soluble substances like sugar or salt) have been used for thousands of years to decrease water activity in food products like meat, fish, vegetables, eggs, and some fruits. Most often drying increases viral resistance to stresses like heat and sanitizers (Terpstra et al., 2007*;* Welch et al., 2006). Salt has shown a protective effect on hepatitis A virus subjected to high pressure in media. HAV titer was reduced by 0.4 log in the presence of 6% NaCl for 1 min at 400 MPa at 50ºC. In the absence of added salt, HAV titer was reduced by 3.9 logs (Kingsley and Chen, 2009*;* Kingsley and Chen, 2008).

Thermal Inactivation

The high temperature used for food preservation is based on the destructive effects of heat on microorganisms. There are two categories commonly used for high-temperature: pasteurization and sterilization. Pasteurization uses high temperature for the destruction of disease-causing microorganisms or for the reduction in the number of spoilage microorganisms. Sterilization uses high temperature to destroy all viable organisms (Ramesh, 1999). Norovirus-like virus particles (VLPs) are highly stable up to 55ºC, at temperatures above 60^{o}C, norovirus VLPs undergo phase transitions and the secondary, tertiary, and quaternary protein level structures are disrupted (Ausar et al., 2006). Poliovirus has the ability to persist in oysters after 30 min of steaming at an internal temperature of 94 ºC (DiGirolamo et al., 1970). Noroviruses are known to be more heat-resistant than poliovirus showing their ability to survive the steaming process (Dolin et al., 1972). Complete HAV inactivation in shellfish meat was achieved after steaming to an internal temperature of 85 to 90 ºC for 1.5 min (Millard et al., 1987). Excessive shellfish heating causes undesirable organoleptic changes like toughening of the meat texture (West et al., 1985). Even

though heating offers some inactivation of foodborne viruses, most of the foods implicated in viral outbreaks are not heat-treated before consumption. Therefore, novel nonthermal technologies such as irradiation, Ultraviolet (UV) Light**,** high-pressure, and PEFs are being studied as alternative means for viral inactivation in foods.

Irradiation

Irradiation may be useful in controlling bacteria, parasites, and insects in foods. High irradiation doses are required for significant reduction of viral titers which may adversely affect sensory properties of foods. Due to their small size viruses are more resistant to irradiation than bacteria, including spore-formers. Hepatitis A virus, poliovirus, and rotavirus in oysters were reported to have D_{10} values of 2.94, 2.0 and 2.4 kilogray (kGy), respectively (Baert et al., 2009*;* Jung et al., 2009). A dose of 3 kGy was needed for 1-log reduction of hepatitis A virus on lettuce or strawberries (Bidawid et al., 2000).

High hydrostatic pressure (HHP)/ High pressure homogenization (HPH)

High hydrostatic pressure (HHP) can be applied to foods to inactivate foodborne microorganisms and prolong the shelf life of food products (Kovac et al., 2010; Guerrero-Beltran et al., 2005; Korhonen et al., 1998). HHP yields higher quality products with unaffected sensory and nutritional attributes (San Martin et al., 2002). Pressure is transmitted uniformly through the pressure-transferring medium and pressure effects are independent of product size and geometry. (Guerrero-Beltran et al., 2005; Knorr, 1993). Generally, vegetative bacteria are the most sensitive to HHP, followed by yeasts and molds, whereas viruses and bacterial spores are reportedly more resistant (Kovac et al., 2010; Patterson et al., 2007). Inactivation of microorganisms is found to be time-dependent and also dependent on the initial number (titer) of microbes. High pressure homogenization (HPH) is another promising novel non-thermal

processing method for fluid foods. HPH is used extensively in the food industry, for creating dairy and food emulsions, to improve texture, flavor, and shelf-life attributes (Diels and Michiels 2006; Dickinson and Stainby, 1988). Gram-negative bacteria are more sensitive to HPH than Gram-positive bacteria, because of the thinner peptidoglycan layer (Kelemen and Sharpe, 1979; Wuytack et al., 2002). HPH has been used to inactivate norovirus surrogates. At pressures of 250 MPa or more, bacteriophage MS2 showed 3.3 log PFU/ml reduction and MNV-1 showed reduction of 0.8 log PFU/ml at 300 MPa (D'Souza et al., 2009).

Ultraviolet (UV) Light

Short wave ultraviolet light (has a wavelength of 180 to 280 nm) primarily damages the RNA and DNA genome of viruses and at very high doses can damage proteins as well. Thymine or uracil dimers are formed in the nucleic acids, which results in mutations. Because only one strand is damaged and the other strand can serve as a template for repair, double-stranded DNA and RNA viruses are more resistant to UV light (Eischeidj et al., 2009*;* Hirneisen et al., 2010). UV light leaves no residues but has limited applications in the food industry because UV light has low penetration power. Also turbidity in liquids decreases the effectiveness of UV light. UV light is used for disinfection of water as well as for inactivating microbes on environmental and food surfaces. For FCV, HAV, PV1, MS2 and phiX174, the decimal inactivation doses (the dose that is required to inactivate 90 percent of the initial population) of UV were 47.85, 36.50, 24.10, 23.04 and 15.48 mW s/cm^2, respectively. The most UV resistant among the tested viruses was FCV (Nuanualsuwan et al., 2002). Inactivation of HAV, FCV, and Aichi virus by UV on lettuce was in the range of 4.5–4.6 logs whereas on strawberries, the range was only 1.9–2.6 logs and the uneven matrix of the strawberry surface protected some virus particles from the UV light (Fino and Kniel, 2008).

Pulsed electric fields (PEF)

For microbial inactivation, PEF uses short bursts of electricity while causing minimal effect on food quality. PEF is used to treat liquid and semiliquid food products including juices, milk, yogurt, soups, and liquid eggs (Ramaswamy et al., 2005). Electric field, wave forms and strengths, distribution of the treatment chamber, temperature, pressure, and time of exposure are the processing variables (Clark, 2006). PEF inactivates bacteria and fungi in field strengths of 35000 V/cm (Clark, 2006). When bacteria are exposed to high electrical field pulses, pores develop in the cell membrane that increases membrane permeability allowing the loss of cellular contents (Clark, 2006). PEF has shown little effect on spores (Clark, 2006). Limited research has been conducted on the effect of PEF on enteric virus inactivation. Rotavirus was resistant to PEF treatment of 20 to 29 kV/cm for 145.6 μs. This ineffectiveness may be due to the protein capsid on enteric viruses compared to the lipid membranes of bacterial cells (Khadre and Yousef, 2002).

Chlorine

In Hamburg, Germany, in 1893, chlorine was first used as a disinfectant in a wastewater treatment plant (Vaughn and Novotny, 1991). Chlorine is the most commonly used sanitizing agent, chlorine gas (Cl2), calcium hypochlorite (CaClO2), and sodium hypochlorite (NaOCl2) are the three forms of chlorine approved for use (Fonseca, 2006). The efficacy of chlorine disinfection relies on the pH of the chlorine solution. When pH values are greater than 8.0, chlorine will be ionized to form the hypochlorite ion and at pH levels below 6.0, the available chlorine is in the form of hypochlorous acid (HOCl) (95%), a more potent disinfectant (Clark et al., 1956; Weidenkopf, 1958). A 10-fold increase in poliovirus inactivation with the HOCl form of chlorine has been reported (Weidenkopf, 1958). Disinfection by chlorine has also been shown to be affected by a variation in temperature. Poliovirus inactivation rates were increased 2-fold

when the temperature was increased from 5 to 25 °C (Scarpino, 1972). In the United States, chlorine is predominantly used for the disinfection of water (Vaughn and Novotny, 1991). Chlorine is used in the processing of fresh produce, either as a spray to reduce soil and microbiota associated with lettuce or as a flume water additive to control cross-contamination for tomatoes, carrots, melons, citrus, bulb vegetables, leafy greens, and potatoes. In a spray, chlorine is commonly used at 50 to 200 mg/L for 2 to 10 s at a temperature range of 12 to 21°C between pH 5 and 7. In flume water, depending on the food product, chlorine is used from 10 to 200 mg/L for 30 s to 5 min at 12 to 21°C between pH 5 and 7 (Burnett, 2008). There was 95%, 98%, and 99% inactivation of poliovirus type 1, phiX174 phage and MS2 phage, respectively, on strawberries after a 2-min water wash with 50 mg/L chlorine at 43°C (Lukasik et al., 2003). FCV was inactivated by approximately 1 log on both strawberries and lettuce after 10 min with 80 mg/L chlorine (Gulati et al., 2001). HAV and MS2 were inactivated by 90% to 99% when 20 mg/L of free chlorine was applied for 5 to 10 min on strawberries, cherry tomatoes, and lettuce leaf pieces (Casteel et al., 2008). Chlorine effectiveness is highly dependent on the organic load, therefore, monitoring and controlling the chlorine level is critical. The formation of chlorine byproducts and the potential for toxic chlorine gas formation are the disadvantages of chlorine. Chlorine also has the ability to form chlorinated byproducts that may be detrimental to the environment and can corrode processing equipment (Burnett, 2008).

Chlorine dioxide (ClO2)

ClO2 is an oxidizing agent extremely soluble in water and up to 5 times more soluble than chlorine. Unlike chlorine, ClO2 does not form trihalomethanes by reacting with ammonia or organic compounds (White, 1978; Schnoor et al., 1979). The oxidation of iron and manganese reducing discoloration of finished water, inability to react with ammonia, and relative pH

stability are advantages of ClO2 disinfection (Aieta and Berg, 1986). The disadvantages are that ClO2 must be generated on site due to its instability and it has the potential to be explosive when concentrated (Beuchat, 1998). ClO2 was found to be twice as effective as chlorine in the inactivation of poliovirus type 1 and bacteriophage in wastewater (Tifft et al., 1977). In the presence of high amounts of organic matter, such as those found in flume processing waters and dump tanks, activity of ClO2 was not found to be substantially reduced (Bernard et al.,1965). ClO2 inactivated poliovirus more efficiently at pH 10.0 when compared to pH 6.0 and this observed pH effect which could be that the high pH increases the sensitivity of the virus to ClO2 attack. It is also possible that the form of ClO2 in alkaline solution (Chlorate anion - ClO^{-}_{3}) is the most active species for viral inactivation (Alvarez and O'Brien, 1982). ClO2 usage with wastewater effluents that are generally more acidic could compromise the greater efficacy of ClO2 at alkaline pH (Vaughn and Novotny, 1991). ClO2 could be used as a spray to remove soil to reduce microbiota associated with lettuce surfaces and its residual concentration is usually 3 mg/L, applied from 30 s to 5 min at a temperature range of 2 to 24 ◦C at a pH range of 6 to 10 (Burnett, 2008). A maximum of 200 mg/L ClO2 is allowed for equipment sanitation, in fruit and vegetable processing in the United States.

Ozone

Ozone gas is a strong oxidizing agent which can effectively kill microorganisms. Non-enveloped viruses, with their protein coats exposed to ozone, undergo protein peroxidation while lipid oxides are formed in enveloped viruses (Murray et al., 2008). Ozone gas is used for surface disinfection (Hudson et al., 2009) and the gas may be bubbled through liquids such as water and juices. Ozonated water has been used to rinse produce and chicken carcasses. Organic compounds present in foods react with ozone, causing a decrease in the amount of available gas

to inactivate microbes. Besides flavors, colors of the food alter when ozone reacts with food components. Ozone reacts more rapidly with organic material as temperature increases, but ozone also decomposes more quickly. At pH 5, ozone is most stable in solution and starts to decompose as pH increases (Hirneisen et al., 2010). Under more acidic conditions, some viruses, including poliovirus, are more resistant to ozone (Roy et al., 1982). At 20°C, 0.25 to 0.38 mg of ozone per liter was required for complete inactivation of hepatitis A virus (Herbold, 1989). For norovirus, a dose of 0.37 mg of ozone/liter could reduce the virus by >3 log within a contact time of 10 seconds (Shin, and Sobsey, 2003).

In addition to the chemicals and processes described above, natural antiviral agents such as chitosan are being researched to control foodborne viruses.

Chitosan

Chitosan is a linear cationic polysaccharide and a deacteylated derivative of chitin which is the major structural biopolymer in crustacean shells, fungi, and insects (Roller and Covill, 1999). Chitosan is present naturally in fungi, zygomycetes (Shahidi et al., 1999). Chitosan is not generally recognized as safe (GRAS) in the United States, although chitosan is approved as a food additive in Japan and Korea (No et al., 2007). Chitosan has been sold as a dietary supplement and as an additive to dog food (Park et al., 2002) and chitosan has been accepted as a bio pesticide (Docket No. EPA-HQOPP- 2007-0566-0019). Chitosan is used to form beads, films, fibers, and gels (Guibal 2004; Sankararamakrishnan et al., 2006; Zivanovic et al. 2008), as a chelator, in waste water management (Shahidi et al., 1999), and as wound dressings in medicine (Koide, 1998). The use of chitosan in the food industry has been explored in various fields, as clarifying agent in juice, as an antimicrobial, as an antioxidant, as a packaging material, and as a thickening agent in beverages (Devlieghere et al., 2004; Shahidi et al., 1999; Xie et al., 2001).

Chitosan Antimicrobial Properties

Chitosan's antibacterial properties have been widely confirmed and investigated (Coma et al., 2003; Dutta et al., 2009; Kong et al., 2008; Liu et al., 2004; Zivanovic et al., 2004). There are several proposed mechanisms of the antimicrobial actions of chitosan. The first proposed antibacterial mechanism of chitosan is due to electrostatic interactions between the chitosan amine group and the negatively charged lipopolysaccharides and proteins of the outer cell causing distortion of the cellular membrane and leakage of intracellular material (Coma et al., 2003; Dutta et al., 2009; Helander et al., 2001; Kong et al., 2008; Liu et al., 2004). In a study, *E. coli* and *S.* Typhimurium were treated with 0.025% of 85% DDA chitosan, which modified the outer membrane of the cell and formed a layer around *E. coli* (Helander et al. 2001). Researchers suggested that chitosan affects Gram-positive and Gram-negative cells differently. They proposed that higher molecular weight chitosan forms a polymer membrane in order to prevent nutrients from entering and leaving the cell of Gram-positive organisms, whereas lower molecular weight chitosan enters the Gram negative cell to bind to electronegative substances, which results in flocculation in the cytoplasm as well as disruption of physiological processes (Zheng and Zhu, 2003). Chitosan is shown to inhibit mRNA as well as protein synthesis by penetrating the cell of the microorganism and binding with DNA (Sudarshan et al., 1992). Another proposed antibacterial mechanism of chitosan is the chelation of essential nutrients required for microbial growth (Dutta et al., 2009). A study has found that chitosan microspheres of 1456 kDa were chelating Mg^{2+} of the *E. coli* outer membrane causing destabilization of the cell (Kong et al., 2008).

Studies have shown the interaction of chitosan with the cell plasma membrane causing leakage of intracellular material (El Ghaouth et al., 1992; Liu et al., 2007). Chitosan and chitosan

derivatives of less than 120 kDa (85% Degree of Deacetylation (DDA)) at 1% concentrations reduced the growth of *Botrytis cinerea* due to a permeability change in the plasma membrane (Badaway et al., 2004). Chitosan hydrochloride of 120 kDa caused disruption of the plasma membrane leading to permeable cells in *Candida albicans*, *C. krusei*, and *C. glabrata* (Seyfarth et al., 2008). Chitosan was found to be effective in either inducing natural plant resistant mechanisms or was able to control fungal growth through fungistatic properties in *B. cinerea* infection in strawberries (Reddy et al., 2000). Chitosan has been shown to cause leakage of amino acids as well as morphological changes due to the accumulation of chitosan in the cell wall of *Rhizopus stolinfer* and *B. cinerea* (El Ghaouth et al., 1992). Chitosan has been shown to be involved in the chelation of Ca^{2+}, Zn^{2+}, and other essential minerals required for fungal growth (Cuero et al., 1991; Roller and covill, 1999). In *Fusarium solani,* chitosan has been shown to interfere with conformation or physical properties of DNA (Hadwiger et al., 1981). At pre-harvest, chitosan was shown to exhibit indirect antiviral activity by penetrating the plant cell to induce resistance (Kulikov et al., 2006). At post-harvest chitosan was shown to cause structural damage to the virus (Kochkina and Chirkov., 2000a) and electron microscopy revealed that chitosan caused loss in viral tail fibers with receptors and viral sheath contraction exposing DNA of phage T2 and 1-97A (Kochkina et al., 2000c). Chitosan is hypothesized to interact with the negative charge of the viral capsids (Kochkina et al., 2000b; Su et al., 2009). Chitosan has been reported to inhibit the lytic infection of bacteriophage T2 and T7 after adsorption into the host by possibly attaching to the viral particles in the cell (Kochkina et al. 1995). Chitosan inhibits viral replication of phages T2 and T7 (Chirkov, 2002; Kochkina and Chirkov, 2000a). Chitosan was found to be efficient at inhibiting phage replication of bacteriophage 1-97A in *B. thuringiensis*, which may be due to the interruption of the intracellular reproduction of the

phages (Kochkina and Chirkov, 2000a). The effectiveness and mechanisms of action of chitosan against foodborne viruses needs to be investigated.

Recently, chitosan was shown to be effective against human noroviral surrogates, where water-soluble chitosan (53 kDa) at 1.4% decreased FCV-F9 titers by ~2.83 log PFU/ml at 37ºC after 3 h with decreasing effects at lower concentrations (Su et al., 2009). These researchers showed that bacteriophage MS2 was reduced by ~1.18 to 1.41 log PFU/ml irrespective of the chitosan (at 53 kDa) concentration used, without any reported effect against MNV-1. Chitosan oligosaccharide showed similar viral reduction trends but to a lesser extent compared to that of water-soluble chitosan at 53 kDa (Su et al., 2009).

The effectiveness of different molecular weight chitosans (53, 222, 307, 421, ~1150 kDa) at 0.7% against feline calicivirus, FCV-F9, murine norovirus, MNV-1, bacteriophages MS2 and phiX174 for 3 h at 37 ºC was determined. Water-soluble chitosan of 53 kDa reduced phiX174, MS2, FCV-F9 and MNV-1 titers by 0.59, 2.44, 3.36, and 0.34 log10 PFU/ml respectively. Molecular weight increase of chitosan corresponded with an increase in antiviral effect for MS2, but did not play a role for the other three tested viral surrogates. Overall, chitosan treatments showed the greatest reduction for FCV-F9, and MS2 followed by phiX174, and with no significant effect on MNV-1(Davis et al., 2012).

Based on literature, the working hypotheses were as follows:

1. Increasing the concentration of chitosan could result in increased inactivation of virus due to the interaction of more available positive charges of chitosan with negatively charged components of the virus capsid.
2. Increasing the temperature and incubation time could result in improved antiviral activity of chitosan.
3. Potentially higher molecular weight of chitosan could result in greater damage to the viral structure than lower molecular weight chitosan resulting in greater viral reduction.
4. The viral surrogates used would mimic the behavior of human noroviruses in the presence of chitosan.

The specific research objectives were:

1) To determine the *in vitro* time and temperature dependence effects of three different molecular weight (53, 421 and ~ 1150 kDa) chitosans against human norovirus surrogates, FCV-F9 and MNV-1 over 2 h at 18^{o}C and 37^{o}C using two concentrations (0.7% and 0.35%).
2) To understand the potential antiviral mechanism of chitosan by
    a) Determining any structural changes to the virus caused by chitosan using Transmission Electron Microscopy.
    b) Determining if chitosan prevents viral host cell entry or prevents replication, using blocking experiments.

CHAPTER II

In vitro time and temperature dependence effects of three molecular weight (53, 421 and ~ 1150 kDa) chitosans against human noroviral surrogates, feline calicivirus (FCV-F9), murine norovirus (MNV-1)

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Running Title: *Time and temperature dependence effects of chitosan on foodborne viruses*

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Key words: Chitosan, human norovirus surrogate, reduction

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Abstract

Chitosan is known to have a broad spectrum of antimicrobial activity. Human noroviruses are the leading cause of non-bacterial gastroenteritis, yet information on the efficacy of chitosan against foodborne viruses including human noroviruses is very limited. The objectives of this research were to determine the *in vitro* time and temperature-dependent effects of chitosan with different molecular weights (53, 421 and ~ 1150 kDa) using two concentrations (0.7% and 0.35%) at two temperatures (37^{o}C and 18^{o}C) over 2 h against the human noroviral surrogates, feline calicivirus (FCV-F9), and murine norovirus (MNV-1). Each treatment run twice in duplicate was neutralized in cell-culture media containing 10% fetal bovine serum before determining viral recovery by plaque assays for comparison to untreated virus controls. Chitosan at 53 kDa with 0.35% decreased FCV-F9 by ~1.0 log PFU/ml after 2 h at 37^{o}C, whereas 0.7% caused a reduction of ~3.0 log PFU/ml after 2 h at 37^{o}C and ~1.0 log PFU/ml after 1 and 2 h at 18^{o}C. Chitosan at 421 kDa and 0.7% and 0.35% at 37^{o}C for 2 h decreased FCV-F9 titers by ~2.0 and ~1.0 log PFU/ml, respectively. Chitosans at 421 and ~ 1150 kDa at both concentrations decreased FCV-F9 titers by ~1.0 log PFU/ml after 2 h at 18^{o}C. At 37^{o}C, the ~1150 kDa chitosan at 0.7% and 0.35% decreased FCV-F9 titers by ~2.0 and ~1.0 log PFU/ml, respectively after 2 h. In general, these results showed that higher chitosan concentration, higher temperature, and longer incubation period have a greater effect, with 53 kDa chitosan showing the greatest effect on FCV-F9 and had no observable effects on MNV-1.

Introduction

Foodborne viruses are recognized as the major causes of foodborne illness responsible for 5.5 of the 9.4 million foodborne cases (59%), 27% of the 55,961 hospitalizations, and 12% of the 1,351 deaths related to food contamination (Scallan et al., 2011; Mead et al., 1999; Patel et al., 2008). Human noroviruses of the *Caliciviridae* family have single-stranded positive-sense RNA genomes of about ~7.5kb in size (Jiang et al.,, 1990; Perry and Wobus, 2010). They are non-enveloped, isocahedral in shape, about 27-38 nm in size, and only need about 10-100 particles to cause infection (Bok et al., 2009; Cheesbrough et al., 2000; Hyde et al., 2009; Taube et al., 2010). They are found to be sensitive to heat treatments and chlorine at concentrations >2mg/l, but have been found to be less susceptible to pH (Cliver et al., 2002; Koopmans et al. 2002).

Viral transmission occurs from contaminated food and water, through the fecal-oral route, through person to food contact, person to person contact, person to environmental surface, contaminated vomit, or aerosolization (Greening, 2006b; Patel et al., 2009; Sair et al., 2002b). Handled and ready to eat foods which include fresh produce, juices, shellfish, salads, and boxed lunches that do not undergo further cooking conditions are considered at-risk foods (Grove et al., 2006; Sair et al., 2002b) Currently cultivable surrogates, such as feline calicivirus (FCV-F9), murine norovirus (MNV-1), and bacteriophage MS2, are used in infectivity assays, since there is no laboratory host cell culture system for human norovirus propagation (Doultree et al., 1999; Duizer et al., 2004).

FCV-F9 differs from the human noroviruses in being transmitted through the nasal, oral, or conjunctival routes causing a respiratory infection as well as in being more susceptible to low pH than enteric viruses (Cannon et al., 2006; Duizer et al., 2004; Perry et al., 2009; Radford et al., 2007). MNV-1, a member of the same *Calicivirdae* family and under the Norovirus genus, is considered a better surrogate system to study the biology and pathogenesis of the human

noroviruses, being more resistant to environmental conditions than FCV-F9 (Cannon et al., 2006; Green et al., 2001; Karst et al., 2003; Wobus et al., 2006). Novel methods to control their spread and prevent disease transmission, including natural alternates are needed.

Chitosan is a linear cationic polysaccharide and a deacetylated derivative of chitin which is the major structural biopolymer in crustacean shells, fungi, and insects (Roller, and Covill, 1999; Shahidi et al., 1999). Chitosan is not generally recognized as safe (GRAS) in the United States, although chitosan is approved as a food additive in Japan and Korea (No et al., 2007). Chitosan has been sold as a dietary supplement and as an additive to dog food (Park et al., 2002) and accepted as a biopesticide in the United States (Docket No. EPA-HQOPP- 2007-0566-0019). Chitosan is used to form beads, films, fibers, and gels (Guibal 2004; Sankararamakrishnan et al., 2006; Zivanovic et al. 2008), as a chelator, in waste water management (Shahidi et al., 1999), and as wound dressings in medicine (Koide, 1998). The use of chitosan in the food industry has been explored in various fields, as clarifying agent in juice, as an antimicrobial, as an antioxidant, as a packaging material, and as a thickening agent in beverages (Devlieghere et al., 2004; Shahidi et al., 1999; Xie et al., 2001).

The antibacterial properties of chitosan have been widely confirmed and investigated (Coma et al., 2003; Dutta et al., 2009; Kong et al., 2008; Liu et al., 2004; Zivanovic et al., 2004). At pre-harvest, chitosan has been shown to exhibit indirect antiviral activity by penetrating the plant cell to induce resistance (Kulikov et al., 2006). At post-harvest chitosan has been shown to cause viral structural damage (Kochkina and Chirkov, 2000a).

Recently, chitosan has been shown to be effective against human noroviral surrogates. Chitosan effect was determined on human enteric viral surrogates, murine norovirus (MNV-1), feline calicivirus (FCV-F9), and MS2 (ssRNA) bacteriophage. Chitosan oligosaccharide lactate

with a molecular weight of <5kDa and water-soluble chitosan with a molecular weight 53kDa at concentrations of 1.4%, 0.7%, and 0.35% were incubated at 37ºC for 3 h. At 0.7% the water-soluble chitosan decreased FCV-F9 titer by ~2.83 log PFU/ml, with decreasing effects at lower concentrations and reduced MS2 at ~7 log PFU/ml by ~1.18 to 1.41- log PFU/ml irrespective of the concentration used. Chitosan treatments at the same concentrations had no effect on MNV-1 at ~7 log PFU/ml. Chitosan oligosaccharide had similar trends but to a lesser extent compared to that of water-soluble chitosan against the viruses. Plaque reduction was observed for FCV-F9 and MS2, but not for MNV-1, when ~5 log PFU/ml were used (Su et al., 2009). Water-soluble chitosan of 53 kDa was reported to reduce phiX174, MS2, FCV-F9 and MNV-1 titers by 0.59, 2.44, 3.36, and 0.34 log PFU/ml respectively. Molecular weight increase of chitosan corresponded with an increase in antiviral effect for MS2, but did not play a role for the other three tested viral surrogates. Overall, chitosan treatments showed the greatest reduction for FCV-F9, and MS2 followed by phiX174, and with no significant effect on MNV-1 (Davis et al., 2012).The specific research objectives were a) To determine the *in vitro* time and temperature dependence effects of three different molecular weight (53, 421 and ~ 1150 kDa) chitosans against human norovirus surrogates, FCV-F9 and MNV-1 over 2 h at 18^{o}C and 37^{o}C using two concentrations (0.7% and 0.35%) and b) to understand the potential antiviral mechanism of chitosan by determining any structural changes to the virus caused by chitosan using Transmission Electron Microscopy and determining if chitosan prevents viral host cell entry or prevents replication, using blocking experiments.

Materials and Methods

Viruses, hosts and cell lines.

FCV-F9 and Crandell Reese Feline Kidney (CRFK) cells were purchased from ATCC (Manassas, VA) and murine norovirus, MNV-1, was provided as a gift by Dr. Skip Virgin (Washington Univ., St Louis, MO). RAW 264.7 cells (Murine Macrophage Leukemia cell line) were obtained from the University of Tennessee at Knoxville.

Virus Propagation and Preparation.

CRFK for FCV-F9 and Raw 264.7 for MNV-1 were incubated at 37°C with 5% CO_2 in Dulbecco‘s modified Eagle‘s medium (DMEM-F12) with 1% penicillin-streptomycin and 10% heat-inactivated fetal bovine serum (FBS). CRFK cells were infected with FCV-F9 and RAW 264.7 cells were infected with MNV-1, incubated at 37°C with 5% CO_2 until > 90% lysis, around ~24 h for FCV-F9 and 4 to 6 days for MNV-1. The viruses were harvested by freeze-thawing, once for FCV-F9 and three times for MNV-1 after lysis, and centrifuged at 5,000 x g for 10 min. Supernatants were filtered through a 0.22 μm membrane filter and 1 ml aliquots stored in vials and frozen at - 80°C until use.

Chitosan preparation and application for viral inactivation.

Preparation of 53 kDa chitosan.

Chitosan with a molecular weight of 53 kDa was purchased from EZ Life Science Co. Ltd (Seoul, South Korea) as previously reported (Su et al., 2009). The chitosan was dissolved in sterile deionized distilled water at concentrations of 1.4% and 0.7% and stored as stock for use.

Preparation of 421 and ~ 1150 kDa chitosans.

421 kDa chitosan (30.3 % DA) from Primex (Iceland) and high molecular weight chitosan (estimated ~ 1150 kDa; 25% DA) from Aldrich (St. Louis, MO) were prepared at 1.4% (w/v) in

1% acetic acid and filtered through 0.22 μM filter. Since previous research (Davis et al., 2010) showed that acetic acid in the concentrations used in this experiment had no effect on the viruses tested, acid controls were not used. Thus, the data are treated as effect of molecular weight of chitosan and not as effect of solvent used.

Chitosan Treatment.

FCV-F9 at a titer of 7 log PFU/ml and MNV-1 at a titer of 6 log PFU/ml were incubated with equal volumes of each chitosan to obtain a final concentration of 0.7% and 0.35% and incubated at 37°C or 18^{o}C for 2 h. Treatments after each time point were stopped and neutralized in DMEM-F12 media containing 10% heat-inactivated FBS, serially diluted with cell culture media containing fetal bovine serum (FBS), and plaque assayed as described below. Each treatment was carried out in duplicate and replicated twice. Untreated controls included incubation of each virus with phosphate buffered saline (1X PBS, HyClone, Logan, Utah) at the two temperatures, followed by serial dilutions and plaque assays to determine viral infectivity.

Infectious Plaque Assays.

FCV-F9 plaque assays were conducted using confluent CRFK cells in 6-well plates as described earlier (Su et al., 2009). Briefly, 0.5ml of the tenfold serial dilutions of untreated or chitosan treated samples in DMEM-F12 containing 2% FBS was inoculated into each well. Viruses were adsorbed for 2 h at 37^{o}C and overlaid with 2 ml of DMEM-F12 containing 0.75% agarose, 2% FBS and 1% penicillin-streptomycin. Plates were incubated for 48 h and overlaid with a second overlay media containing 0.01% neutral red (Sigma). Plaques were counted after incubation at 37^{o}C for 20-24 h.

For MNV-1, the plaque assay described by Wobus et al. (2004) and Su et al. (2009) was followed. Briefly, RAW 264.7 cells were seeded onto 6-well plates and incubated until ~90%

confluency and 0.5 ml of the ten-fold serial dilutions of chitosan treated or untreated viral samples were inoculated into each well after aspiration of media. Viruses were adsorbed for 2.5 h at 37°C and then overlaid with 2 ml of DMEM-F12 containing 0.75% agarose, 10% FBS and 1% penicillin-streptomycin and incubated for 72 h under 5% CO_2. A second overlay media containing 0.02% neutral red (Sigma, St. Louis, MO) was used and plaques were counted after incubation at 37^{o}C in a CO_2 incubator for 3-5 h.

Transmission Electron Microscopy (TEM) of chitosan treated FCV-F9 and MNV-1.

TEM studies were carried out at the UT-Knoxville Advanced Microscopy and Imaging Center with the assistance of Dr. John Dunlap to determine the effect of 53 kDa chitosan on FCV-F9 and MNV-1 titer reduction. Chitosan of 53 kDa was treated with FCV-F9 or MNV-1 including the untreated FCV-F9 in PBS (control), incubated at 37^{o}C or 18^{o}C for 2 h and 10 µl of each sample was applied to a freshly glow discharged, formvar (support film for TEM grids) and carbon coated copper grid. After 1 min, excess sample was removed and the remaining sample was stained with uranyl acetate for 1 min and allowed to dry. The stained viruses were examined in a Hitachi H800 operating at 75 keV. Images were recorded on Kodak 4489 film.

Chitosan blocking and virus infection assay.

The mechanism of action of chitosan (to determine viral adsorption/attachment prevention to host cells by chitosan) on the viral surrogates was determined by first blocking the confluent cell lines of RAW 264.7 or CRFK in 6-well plates with two concentrations (0.7% and 0.35%) of 421 kDa chitosan, incubated at 37ºC or 18^{o}C for 2 h, followed by infection of confluent cell lines with MNV-1 or FCV-F9 for 2 h in a CO_2 incubator, and plaque assay was done as described above. Chitosan effect on viral replication was determined by first infecting the confluent cell lines in 6-well plates (RAW 264.7 or CRFK) with MNV-1 or FCV-F9, for 2 h in a CO_2

incubator, followed by blocking of confluent cell lines with 421 kDa chitosan (0.7% and 0.35%) at 37ºC or 18ºC for 2 h, and plaque assay was done as described above.

Statistical Analysis.

Data from the two replicate treatments were analyzed using ANOVA and Tukey's test on a completely random design (SAS, Cary, NC).

Results and Discussion

Plaque reduction by 53 kDa chitosan. The time dependence of 53 kDa chitosan against foodborne viral surrogates, MNV-1 and FCV-F9 *in vitro* showed that at both temperatures, increasing contact time increased the reduction of FCV-F9 titers (Figure 1), but showed no effect on MNV-1 (Table 1). Chitosan at 0.7% decreased FCV-F9 titers by 0.2, ~ 0.3, ~0.8, ~1.5, and ~3 log PFU/ml at 37ºC after 5, 15, 30 min, 1 h, and 2 h, respectively, having almost negligible reduction within the first 15 min. Decreasing the concentration of chitosan to 0.35% resulted in lower FCV-F9 titer reductions of ~0.6 log PFU/ml after 2 h at 37ºC with insignificant reductions at ≤1 h at 37ºC ($P>0.05$) (Figure 1). These results show that 53 kDa chitosan had a dose dependent effect on FCV-F9 at 37ºC. At 18ºC, 53 kDa chitosan at 0.7% decreased FCV-F9 titers by ~0.5 and ~1 log PFU/ml after 1 and 2 h respectively, with insignificant reductions at ≤30 min at 18ºC ($P>0.05$). Decreasing the concentration of chitosan to 0.35% showed decreased titer reductions for FCV-F9, where only ≤1 log PFU/ml at 18ºC were obtained. In the case of 53 kDa chitosan, higher temperature, higher concentration, and longer contact times had increased effects on FCV-F9 titer reductions. On the other hand, MNV-1 treated with 53 kDa chitosan at concentrations, time and temperatures studied, showed minimal to no reductions in viral titers. The number of MNV-1 plaques recovered was found to be similar to those of the untreated virus controls (Table 1).

Plaque reduction by 421 kDa chitosan.

At both temperatures, 421 kDa chitosan with increased contact time caused the reduction of FCV-F9 titers (Figure 2), but had no effect on MNV-1 titers. Chitosan at 0.7% reduced FCV-F9 by ~ 2 and 1 log PFU/ml at 37ºC and 18^{o}C, respectively, after 2 h. Shorter incubation times of 5, 15, 30 min, and 1 h at 0.7% resulted in FCV-F9 reductions of ~0.4, ~0.4, ~1, and ~1 log PFU/ml at both temperatures. Incubation of the virus with 0.35% 421 kDa chitosan for 5, 15, 30 min, 1 h, and 2 h caused FCV-F9 reductions of 0.2, 0.2, ~ 1, ~ 1, and ~ 1 log PFU/ml, respectively at both temperatures. Thus, for 421 kDa chitosan increasing the temperature or dose did not appear to play a significant role in FCV-F9 titer reduction. However, the effect was time dependent with increasing contact time showing increasing titer reduction at 0.7 % after 2 h at 37ºC. In contrast, MNV-1 treated with 421 kDa chitosan at both concentrations and temperatures showed minimal to no reductions in virus survival compared to untreated controls (Table 1).

Plaque reduction by ~ 1150 kDa.

At both temperatures, ~1150 kDa chitosan with increased contact time enhanced reduction of FCV-F9 titers (Figure 3), but with no significant effect on MNV-1 titers. The chitosan at 0.7% for 2h, showed FCV-F9 reductions of 2 and 1.0 log PFU/ml, respectively at 37ºC and 18^{o}C. Incubation of the virus with 0.7% of ~1150 kDa chitosan for 5, 15, 30 min, and 1 h showed FCV-F9 reductions of ~0.4, ~ 0.8, ~1, and ~1 log PFU/ml, respectively at both temperatures. Incubation of the virus with 0.35% ~1150 chitosan for 5, 15, 30 min, 1 h, and 2 h showed FCV-F9 titer reductions of ~0.3, ~ 0.6, ~1, ~1, and 1 log PFU/ml respectively, at both temperatures. Thus, for ~1150 kDa chitosan, increased chitosan concentration and temperature did not appear to play a significant role ($p>0.05$), but increasing contact time at 37ºC had an effect at 0.7% after 2 h. Similar to 53 and 421kDa chitosans, MNV-1 treated with ~1150 kDa chitosan at both

concentrations and temperatures studied showed no reductions in viral titers and the number of MNV-1 plaques recovered were almost the same as untreated virus controls (Table 1).

The results of this study are in agreement with the previous findings that MNV-1 is more resistant than FCV-F9 to inactivation treatments and to chitosan treatment. It has been reported that in the environment, MNV-1 is more persistent and stable than FCV-F9 in waters at 25ºC (Bae et al., 2008), at basic and acidic pHs (Cannon et al., 2006), in the presence of 1-5% trisodium phosphate (TSP), 10% bleach and 1-2% glutaraldehyde (D'Souza and Su, 2010), and in the presence of a hand sanitizer which has a polyquaternium polymer and organic acid (Macinga et al.,2008).

Bean plant resistance to bean mild mosaic virus increased as the molecular weight of chitosan increased from 3-50 kDa (Chirkov et al., 1998). In contrast, studies have shown that bean plant resistance to bean mild mosaic virus decreased as the molecular weight of chitosan increased from 1.2-40.4 kDa (Kulikov et al., 2006). Recently, Su et al. (2009) studied the direct effects of chitosan of different molecular weights on human noroviruses surrogates and demonstrated that 53 kDa chitosan reduced ~5 log PFU/ml viral titers of bacteriophage MS2 and FCV-F9 (1.70 and 4.21 log PFU/ml, respectively) more efficiently when compared to 5 kDa chitosan (0.98 and 1.41 log PFU/ml, respectively). Davis et al., (2012) studied the effectiveness of different molecular weight chitosans (53, 222, 307, 421, ~1150 kDa) against FCV-F9, MNV-1, MS2 and phiX174. and found that water-soluble chitosan of 53 kDa reduced phiX174, MS2, FCV-F9 and MNV-1 titers by 0.59, 2.44, 3.36, and 0.34 log10 PFU/ml respectively. Molecular weight increase of chitosan corresponded with an increase in antiviral effect for MS2, but did not play a role for the other three tested viral surrogates.

In this study, the chitosans with the molecular weight of 421 and ~ 1150 kDa were found to be less effective in decreasing the infectivity of FCV-F9 than 53 kDa chitosan. Chitosans with 421 and ~1150 kDa at 0.7% decreased FCV-F9 titers by ~2 and ~1 log PFU/ml after 2 h at 37^{o}C and 18^{o}C, respectively, while 0.35% for both chitosans decreased FCV-F9 by ~1 log PFU/ml after 2 h at both temperatures.

In some earlier reports, the antiviral activity has been shown to be dependent on the concentration of chitosan. When bean plants were sprayed with chitosan concentrations ranging 0.00001-0.1% for 15 min before inoculation with alfalfa mosaic virus increasing inhibition of the virus with increasing concentration was found with complete inhibition at 0.01% (Prospieszny et al. 1991). An increase in infection inhibition from less than 50% to 100% against bacteriophage T2 and T7 was obtained when concentration of chitosan, chitosan acetate, and chitosan hydrochloride was increased from 0.00005 to 0.01% (Kochkina and Chirkov, 2000a).

Recently our laboratory showed that an increase in concentration from 0.175, 0.35, to 0.7% of 53 kDa caused a statistically significant increase in the reduction of initial titer of ~10^{7} (7- log) FCV-F9, which was 1.09, 2.09, 2.83 log PFU/ml, respectively, and 0.44, 0.99, 1.44 log PFU/ml, respectively, by 5 kDa chitosan (Su et al., 2009). A similar trend was shown by 53 kDa and < 5 kDa chitosan against 5-log PFU/ml FCV-F9. Chitosan of 53kDa at 0.175% was shown to cause a slightly higher reduction (0.32 log PFU/ml) of MNV-1 compared to 0.7% chitosan of 53 kDa (0.04 log PFU/ml). The same trend was seen for 53 kDa and < 5 kDa chitosan as concentration decreased against ~7 log PFU/ml MS2 and MNV-1. An increase or decrease in concentration of chitosan did not affect some viruses (Su et al., 2009). In this study, at both temperatures, increasing contact time enhanced the reduction of FCV-F9 with regard to 53, 421 and ~1150 kDa chitosans. The 53 kDa chitosan at 0.7% decreased FCV-F9 titers by ~3 and ~1

log PFU/ml after 2 h at 37^{o}C and 18^{o}C, respectively while 0.35% resulted in decreases of ~0.6 and 0.03 log PFU/ml after 2 h at 37^{o}C and 18^{o}C, respectively.

Transmission Electron Microscopy of chitosan treated FCV-F9 and MNV-1.

Chitosan of 53 kDa treated with FCV-F9 or MNV-1 including untreated FCV-F9 in PBS (control) for 2 h at 37^{o}C and 18^{o}C were examined by Transmission Electron Microscopy (TEM) (Figure 4 A and B). TEM results with chitosan did not help us to obtain a clear evidence of structural damage to the viral capsids. Extensive work on the interaction of chitosan with these surrogates is needed to obtain insights into the mechanism of action.

Chitosan action in blocking and virus infection assay.

To obtain insights into the mechanism of action by chitosan on the tested viral surrogates, blocking experiments were carried out. Inhibition of viral adsorption / attachment to host cells by chitosan was studied by first blocking of confluent host cell lines with different concentrations (0.7% and 0.35%) of 421 kDa chitosan, at 37ºC and 18^{o}C for 2 h, followed by virus infection for 2 h. Inhibition of viral replication by chitosan was studied by infecting the confluent cell lines with each virus, followed by blocking of confluent cell lines with 421 kDa chitosan at both concentrations and temperatures for 2 h. Blocking the cell lines with chitosan or viral infection of cell lines with 421 kDa chitosan did not show any observable reduction in FCV-F9 or MNV-1 titers. The results obtained show that the chitosan did not have any significant effect in blocking viral binding to the host cells or at the stage of viral replication. This leads us to deduce that chitosan may interact with the virus capsid, causing alterations or damage to the structure to prevent infection. Therefore, extensive work on the interaction of chitosan with these surrogates is needed to obtain insights into the mechanism of action.

Studies have shown that chitosan exhibited indirect antiviral activity by penetrating the plant cell to induce resistance (Kulikov et al., 2006). Chitosan has been shown to interact with the negative charge of the viral capsids (Kochkina et al., 2000c; Su et al., 2009), cause viral inactivation due to structural damage (Kochkina et al., 2000c), interferes with a step in the viral replication (Chirkov, 2002; Kochkina et al., 2000c) and inhibits infection by phage (Kochkina et al., 1995). FCV has pI of 4.9 (Su et al., 2010) with a negative charge at pH 6.0, which is the pH of the mixture of chitosan and viruses used in these studies. The pI information for MNV-1 is not yet available. This suggests that the charge effect of chitosan could play a significant role on the inactivation of norovirus (NoV) surrogates. Currently, there is limited literature on the effect of chitosan on foodborne viruses (Su et al., 2009; Davis et al., 2012).

Future research should focus on the effect of chitosan against other human enteric viruses such as hepatitis A Virus or rotaviruses, or Tulane virus as a human norovirus surrogate to explore the antiviral activity of chitosan against foodborne viruses. Many researchers have evaluated the application of chitosan coatings as well as films for its antimicrobial properties and to extend product shelf-life in food products like bread, produce, and meat (No et al., 2007). Based on this, the direct application of chitosan films or coatings on produce commodities to control foodborne viruses should be evaluated in the future.

Conclusion

Antiviral effect of chitosan is dependent on chitosan concentration, contact time, temperature, and target viruses.Chitosan with three molecular weights (53, 421 and ~ 1150 kDa) decreased FCV-F9 titres, with no observable effects on MNV-1. Chitosans with 53 kDa had a greatest effect on decreasing FCV-F9 titers.Chitosan of higher molecular weight and higher concentration with longer contact times on MNV-1 may be required to cause decrease in viral titers.

CHAPTER III

Conclusions

The tested chitosans with three molecular weights (53, 421 and ~ 1150 kDa) were shown to decrease the infectivity of FCV-F9 with no observable effects on MNV-1. The antiviral properties of chitosan depend on the type of virus. Overall, FCV-F9 was most susceptible than MNV-1. When FCV-F9 and MNV-1 were compared, chitosan did not show any significant effects in reducing titers of MNV-1.

These results indicate that the effect of chitosan in reducing viral plaque infectivity is dependent on the temperature and time. In case of 53 kDa chitosan, at higher temperature of 37ºC, 0.35% had increased effects after 2 h and 0.7 % had increased effect after 30 min whereas at 18 ºC, 0.7% had increased effect after 1 h on FCV-F9 titer reductions. For 421 kDa chitosan increasing the temperature and time did not appear to play a significant role in FCV-F9 titer reduction from 30 min to 2 h, but increasing contact time at 37ºC had an effect after 2 h at 0.7% concentration. For ~1150 kDa chitosan, increased temperature and time did not appear to play a significant role from 15 min to 2 h, but increasing contact time at 37ºC had an effect after 2 h at 0.7% concentration.

The inactivation of FCVF9 was not molecular weight dependent while MNV-1 was not affected by chitosan. Chitosan with three molecular weights (53, 421 and ~ 1150 kDa) decreased FCV-F9 titers. Overall, the chitosans with the molecular weight of 421 and ~ 1150 kDa were found to be less effective in decreasing the infectivity of FCV-F9 than 53 kDa chitosan.

Chitosan concentration play significant role in the antiviral activity. The 53 kDa chitosan at 0.7% decreased FCV-F9 titers by ~3 and ~1 log PFU/ml after 2 h at 37^{o}C and 18^{o}C, respectively while 0.35% resulted in decreases of ~0.6 and ~0.03 log PFU/ml after 2 h at 37^{o}C

and 18^{o}C, respectively. Chitosans with 421 and ~1150 kDa at 0.7% decreased FCV-F9 titers by ~2 and ~1 log PFU/ml after 2 h at 37^{o}C and 18^{o}C, respectively, while 0.35% for both chitosans decreased FCV-F9 by ~1 log PFU/ml after 2 h at both temperatures. For 421 and ~1150 kDa chitosan, increased chitosan concentration did not appear to play a significant role but increasing contact time at 37^{o}C had an effect after 2 h at 0.7% concentration.

To obtain insights into the mechanism of action by chitosan on the tested viral surrogate, the mechanism of chitosan action was evaluated. TEM results with 53 kDa chitosan did not help us to obtain a clear evidence of structural damage to the viral capsids. Blocking the cell lines with chitosan or viral infection of cell lines with 421 kDa chitosan did not show any observable reduction in FCV-F9 or MNV-1 titers. The results obtained show that the chitosan did not have any significant effect in blocking viral binding to the host cells or at the stage of viral replication. This leads us to deduce that chitosan may interact with the virus capsid, causing alterations or damage to the structure to prevent infection. Therefore, extensive work on the interaction of chitosan with the tested surrogates is needed.

Overall, these results indicate that the effect of chitosan in reducing viral plaque infectivity is dependent on the target viruses, the temperature, time of contact, and concentration of chitosan. The chitosans with three molecular weights (53, 421 and ~ 1150 kDa) were shown to decrease the infectivity of FCV-F9, having no observable effects on MNV-1. The chitosans with the molecular weight of 421 and ~ 1150 kDa were found to be less effective in decreasing the infectivity of FCV-F9 than 53 kDa chitosan. Thus, 53 kDa chitosan has the most antiviral effect against FCV-F9 among the three tested chitosans. For MNV-1, the chitosans with two concentrations (0.7% and 0.35%) and three molecular weights (MW 53, 421 and ~ 1150 kDa) did not have significant effects in plaque reduction and MNV-1 was quite resistant to treatment

using the concentrations, time, temperature and different molecular weights of chitosan studied. For MNV-1, higher molecular weight chitosan, higher concentrations and longer incubation times may be necessary to cause reduction in viral titers.

Future research should focus on the effect of chitosan against other human enteric viruses such as hepatitis A Virus or rotaviruses, or Tulane virus as a human norovirus surrogate to explore the antiviral activity of chitosan against foodborne viruses. Also the direct application of chitosan films or coatings on produce commodities to control foodborne viruses should be evaluated. Chitosan has antiviral activity against only one of the tested HNoV surrogates. Thus, caution must be used in interpreting the data based solely on chitosan's effects against FCV-F9 for the use of chitosan as a natural antiviral agent to control foodborne viral infections.

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Appendix

Table 1: The effect of chitosans (53, 421 and ~1150 kDa) on the recovery of MNV-1 titers at 37°C and 18°C over time up to 2 h from a starting titer of ~ 6 log PFU/ml.

Recovery of MNV-1 titers (PFU/ml)					
Temperature	Concentration	Time	53 kDa	421 kDa	~ 1150 kDa
18°C	0.35%	0			0.02
		5	0.25	- 0.05	
		15	0.13	0.11	
		30	0.16	0.05	
		60	0.26	0.02	0
		120	0.71	- 0.13	0.09
	0.70%	0			0.04
		5	0.56	- 0.06	
		15	0.31	0.01	
		30	0.14	0.14	
		60	0.28	0.06	0.13
		120	0.71	- 0.06	0.07
37°C	0.35%	0			0.02
		5	0.19	- 0.03	
		15	0.26	- 0.01	
		30	0.38	- 0.20	
		60	0.14	0.05	0.05
		120	0.38	- 0.13	0.06
	0.70%	0			0.05
		5	0.66	0.11	
		15	0.3	- 0.04	
		30	0.77	0.15	
		60	0.39	- 0.02	0.13
		120	0.76	- 0.28	0.03

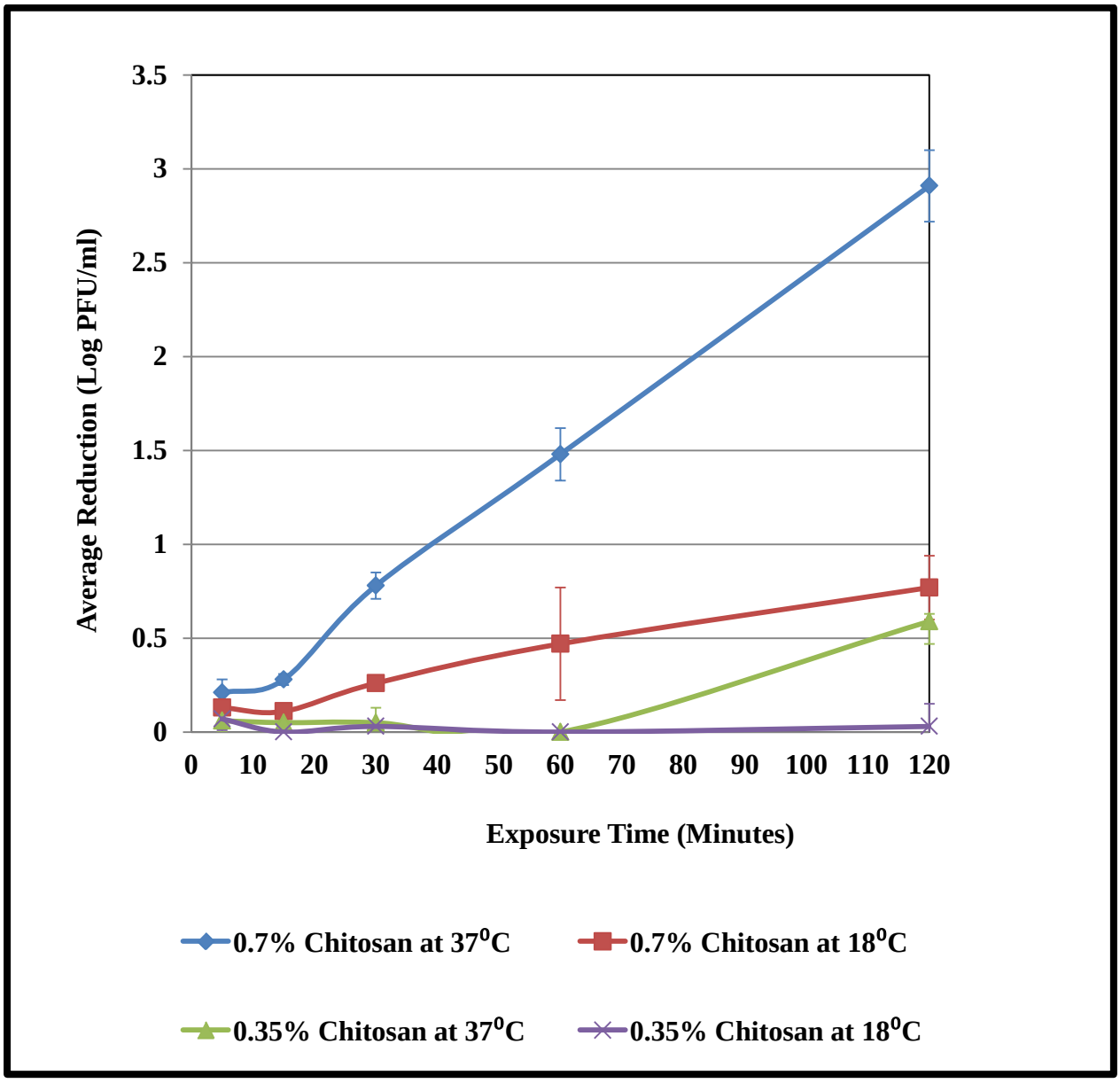


Figure 1. Effect of 53 kDa chitosan at 0.7% and 0.35% on the reduction of FCV-F9 titers at 37°C and 18°C over time up to 2 h from a starting titer of ~ 7 log PFU/ml using infectivity plaque assays. Each experiment was run in duplicate and results from the treatments and controls were analyzed statistically.

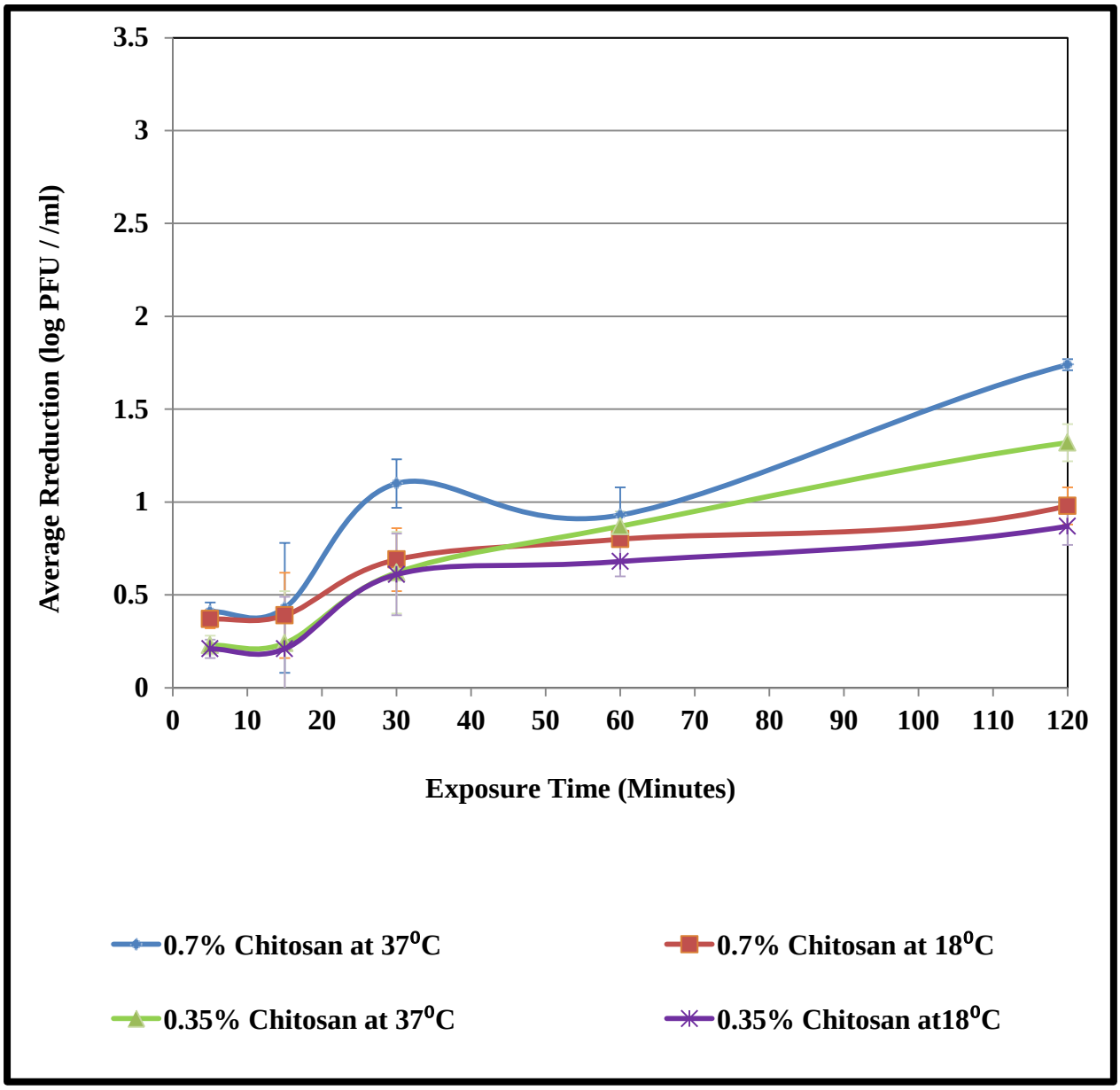


Figure 2. Effect of 421 kDa chitosan at 0.7% and 0.35% on the reduction of FCV-F9 titers at 37^{o}C and 18^{o}C over time up to 2 h from a starting titer of ~ 7 log PFU/ml using infectivity plaque assays. Each experiment was run in duplicate and results from the treatments and controls were analysed statistically.

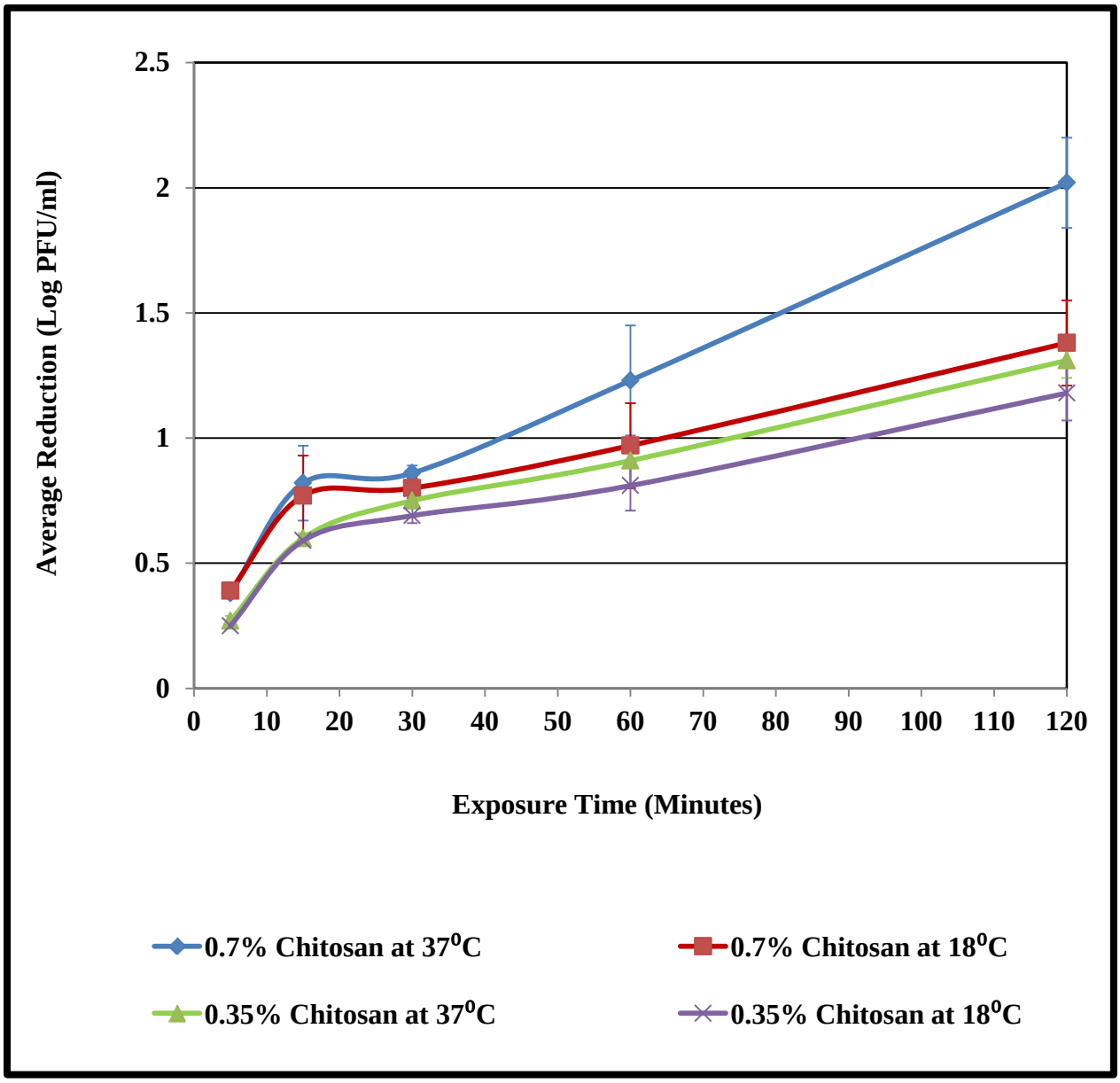


Figure 3. The effect of ~1150 kDa chitosan 0.7% and 0.35% on the reduction of FCV-F9 titers at 37°C and 18°C over time up to 2 h from a starting titer of ~ 7 log PFU/ml using infectivity plaque assays. Each experiment was run in duplicate and results from the treatments and controls were analysed statistically

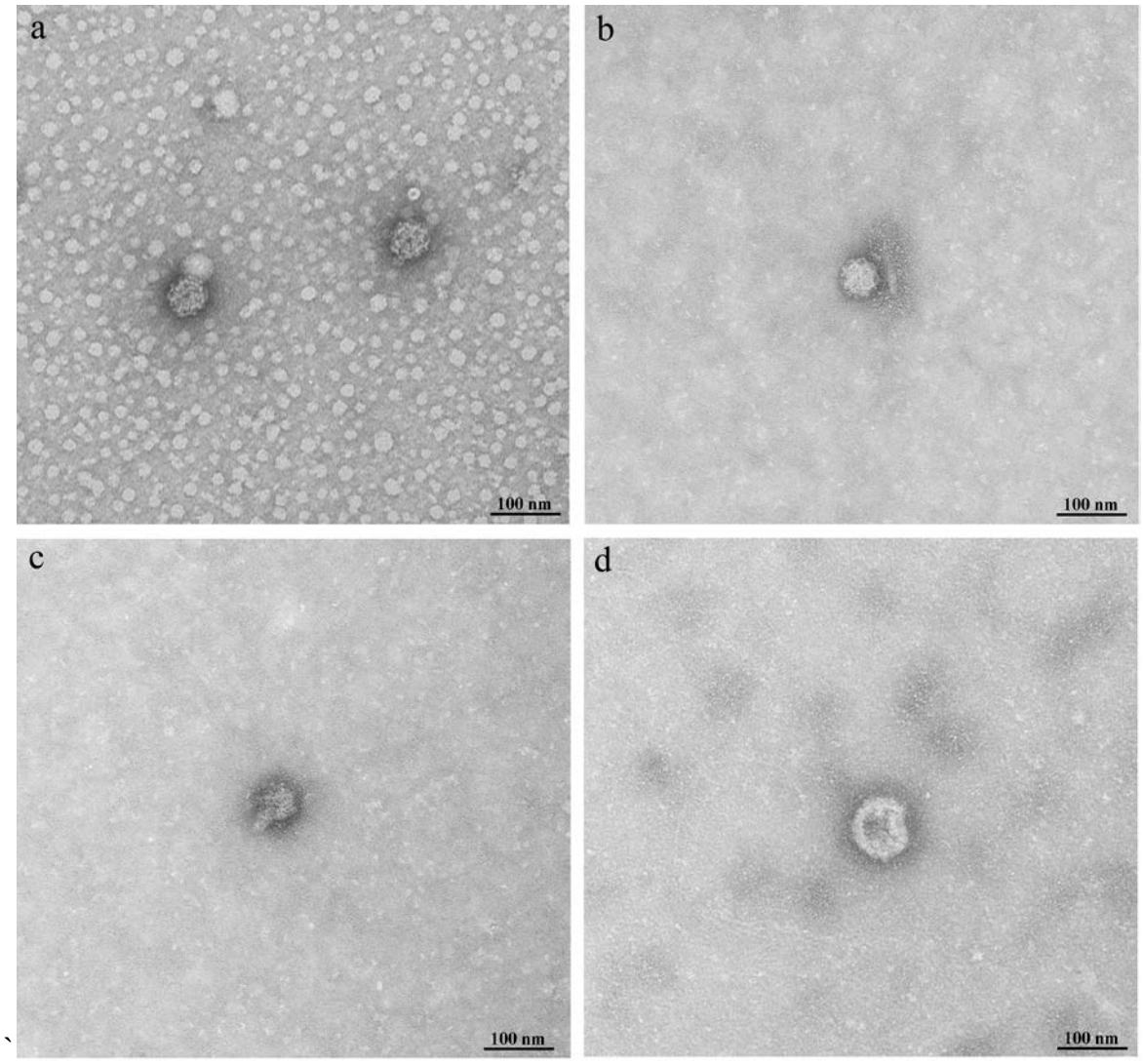


Figure 4A: TEM images of FCV-F9 stained with uranyl acetate after 2 h incubation with PBS at 37°C (Control) (a), 0.7% 53kDa chitosan at 18^{o}C (b and c), and 0.7% 53kDa chitosan at 37^{o}C (d).

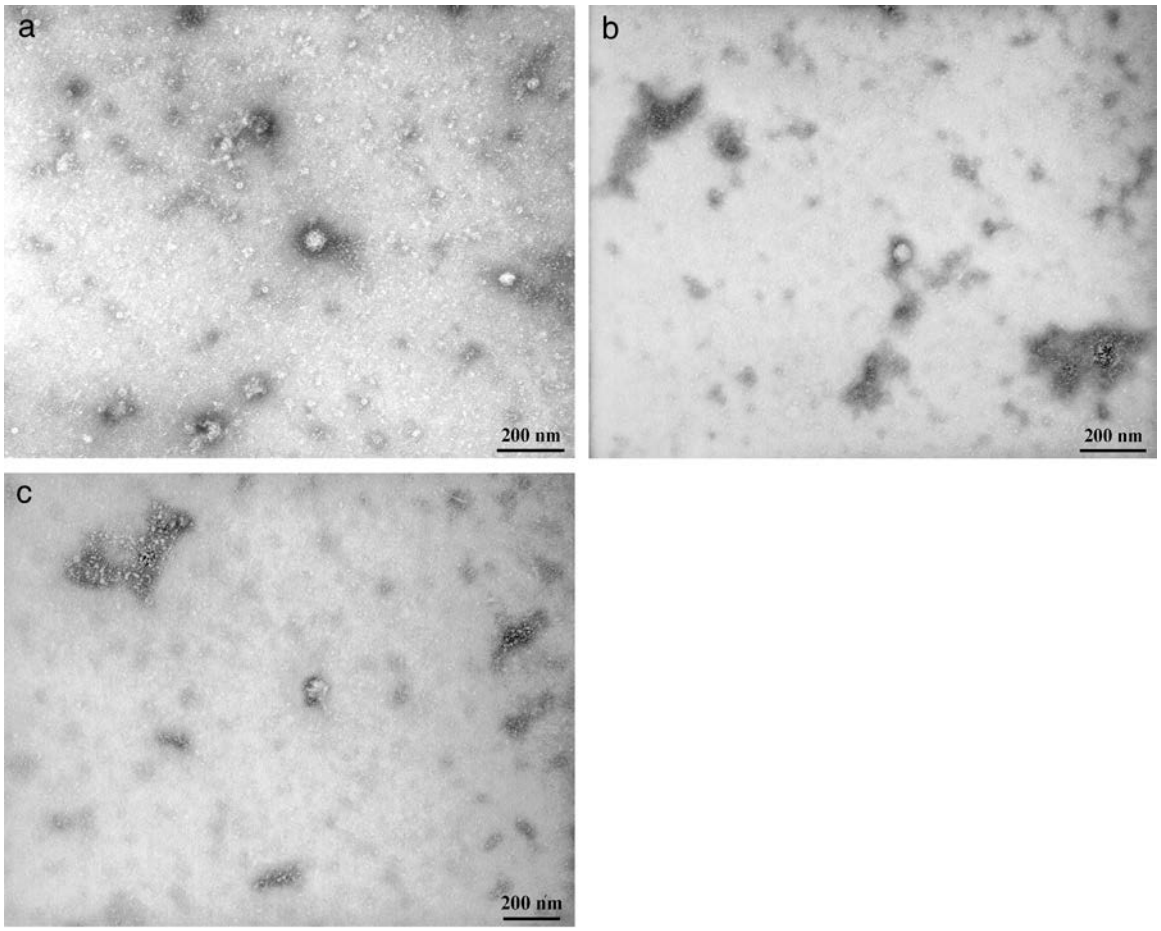


Figure 4B: TEM images of MNV-1 stained with uranyl acetate after 2 h incubation with PBS at 37°C (Control) (a), 0.7% 53kDa chitosan at 18^{o}C (b), and 0.7% 53kDa chitosan at 37ºC

Vita

Radha Ganapathy was born on November 8, in Tamil Nadu, India. She grew up in Tamil Nadu, India. She continued her education in the United States, at the University of Tennessee, Knoxville where she earned a Master of Science degree in Microbiology at the Department of Microbiology. She joined the department of Food Science and Technology at the University of Tennessee, Knoxville in January 2010. She conducted research under Dr. Doris H. D‘Souza’s supervision. She is currently a candidate for a Master of Science degree in food science and technology.